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**Applications of innovative technologies
for the cellular and molecular analysis of a non-model organism,
the bottlenose dolphin (*Tursiops truncatus*),
in a changing and challenging marine environment**

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Applications of innovative technologies for the cellular and molecular analysis of a non-model organism, the bottlenose dolphin (*Tursiops truncatus*), in a changing and challenging marine environment

English abstract

The bottlenose dolphin, *Tursiops truncatus,* is a mammal completely adapted to marine life. As long-lived, long-term residents of bays, sounds, and estuaries, high trophic feeder with large blubber stores, acting as depots for anthropogenic toxins, dolphins can be used as important indicators of the health of the marine ecosystems. Dolphins consume the same food and share the coastal environment with humans thus serving also as effective sentinels for public health problems.

In this dissertation, the latest new technologies in cell and molecular biology are applied to the study of bottlenose dolphin biology and health. Included in the new technologies presented are the most used (microarray) and the most current (RNA sequencing, RNA-seq) transcriptomic approaches, and novel technologies in stem cell research for subsequent *in vitro* testing.

In the first investigation, an *ex vivo* assay was applied to gene expression microarrays. Small slices of bioptic skin from the bottlenose dolphin were cultured and exposed to different concentrations of perfluorooctanoic acid (PFOA) and bisphenol A (BPA), to assess the variation in global gene expression induced by two contaminants of emerging concerns (CECs). Transcriptomic changes were analyzed using a species-specific microarray. The skin transcriptome hold information on contaminant exposure, potentially predictive of long-term effects on dolphin health. Moreover, the differentially expressed genes have shown to be a good resource for identification of contaminant-specific biomarkers of exposure.

Although microarrays technology continues to advance generating high throughput transcriptomics, it can only be employed to detect known sequences, and can't be used to discover novel RNA forms and variants. The transcriptomics dramatic expansion observed in the past few years is mainly due to the developments in RNA-seq. In the second investigation presented, we performed longitudinal analysis of healthy managed bottlenose dolphins across seasons to establish baseline data for blood transcriptome analysis using RNA-seq. Dolphin genes showed less seasonal variability than that reported in humans, but the majority of significant genes identified were shared. Besides the seasonal component to

changes in blood gene expression, associations of gene co-expression modules with age, gender or hematological parameters were also found.

The last study presents the development of a protocol to isolate and characterize stem cells for the creation of a cell bank for dolphin's therapeutic use and of an unlimited source of cells for research and biomedical applications. The protocol is developed to be a less-invasive alternative of current mesenchymal stem cells isolation methods (such as the isolation of stem cells from adipose tissue) and it is focused on the placental tissue and the umbilical cord. Quantitative metrics were used to confirm the morphology of the cells, and gene and protein markers of stemness were also examined. The mesenchymal stem cells characterized were viable, with a proper karyotype, and responded positively to the verification tests employed.

The application of the latest technologies to non-model organisms research, such as in the dissertation here presented, reveled a significant contribution for a deeper understanding of the biology and health of the dolphin, establishing its relevance for future studies of the impact of environmental challenges on the marine inhabitants.

Applicazioni di tecnologie innovative per lo studio molecolare e cellulare di un organismo non-modello, il delfino tursiope, *Tursiops truncatus*, in un ambiente marino in cambiamento

Italian abstract

Il tursiope, *Tursiops truncatus*, è un mammifero completamente adattato alla vita in mare. E' un animale longevo, predatore di apice, ha un'attitudine costiera e residente, e inoltre presenta uno deposito di grasso sottocutaneo, in cui si accumulano facilmente tossine di origine antropica. Queste caratteristiche lo rendono un ideale indicatore dello stato di salute dell'ecosistema marino. La sua condivisione con l'uomo degli ambienti costieri marini, e del nutrimento che ne deriva, ne fanno anche un ottimo organismo sentinella per problemi di salute pubblica.

In questa tesi è presentata l'applicazione delle più innovative tecnologie nel campo della biologia cellulare e molecolare allo studio della biologia e dello stato di salute del tursiope. Tra le nuove tecnologie presentate ci sono sia gli ultimi approcci di trascrittomica, come microarray e RNA-sequencing (RNA-seq), sia le ultime novità nel campo delle colture cellulari, come l'utilizzo di cellule staminali.

Il primo studio descritto presenta l'accoppiamento di un saggio *ex vivo* ad analisi di espressione genica, utilizzando un microarray specie-specifico. Fettine di tessuto cutaneo di tursiope sono state messe in cultura e trattate con diverse concentrazioni di bisfenolo A (BPA) e di acido perfluoroottanoico (PFOA), al fine di analizzare variazioni dell'espressione genica indotta da questi due contaminanti, appartenenti della categoria dei contaminanti emergenti. Il trascrittoma della pelle ha dimostrato di essere informativo di esposizioni a contaminanti e potenzialmente predittivo dello stato di salute del tursiope. Inoltre, i geni differenzialmente espressi identificati tramite questa analisi hanno mostrato essere una buona fonte da cui selezionare biomarcatori per la valutazione dell'esposizione a contaminanti antropici.

Sebbene la tecnologia dei microarray continui ad avanzare, generando trascrittomi a sempre più alta risoluzione, essa può essere impiegata solo per analizzare sequenze già note, tralasciando nuove informazioni su RNA e loro varianti. Negli ultimi anni la trascrittomica ha subito un notevole incremento, principalmente grazie all'impiego di *next generation sequencing* in RNA-seq. Il secondo studio affrontato in questa dissertazione ha utilizzato RNA-seq per un'analisi longitudinale, attraverso le quattro stagioni, di un gruppo

di delfini monitorati mensilmente e considerati in ottime condizioni di salute, al fine di generare dati di riferimento per analisi trascrittomiche di sangue del tursiope. I geni espressi nelle quattro stagioni sono, per la maggior parte, gli stessi evidenziati da studi analoghi sull'uomo, se pur in numero notevolmente inferiore. Oltre alla variabilità introdotta dalla stagionalità, sono stati evidenziati moduli di co-espressione genica associati con età, genere e valori ematologici degli animali.

L'ultima sperimentazione presentata descrive la messa a punto di un protocollo sviluppato per isolare e caratterizzare cellule staminali mesenchimali con un duplice scopo di creare una banca cellulare per uso terapeutico per il tursiope stesso, e di avere una fonte illimitata di cellule per ricerca ed applicazioni biomediche. Il protocollo prevede l'utilizzo del tessuto placentale e del cordone ombelicale e offre un'alternativa meno invasiva al metodo attuale di isolamento delle cellule staminali mesenchimali da tessuto adiposo. Le cellule staminali mesenchimali isolate si mantengono stabili nelle condizioni di coltura testate, con cariotipo specie-specifico, e rispondono positivamente ad ogni test di verifica di identità e comportamento.

L'applicazione di tecnologie di avanguardia allo studio di un organismo non-modello, come nel caso del tursiope evidenziato in questa tesi, ha mostrato un sostanziale e approfondito contributo alla comprensione della biologia e dello stato di salute del tursiope, stabilendo la sua rilevanza in studi futuri incentrati sugli effetti dei cambiamenti ambientali sugli abitanti dell'ecosistema marino costiero.

Table of contents

List of figures

Abbreviations

AD-SCs	Adipose-Derived Stem Cells
ADIRF	Adipogenesis Regulatory Factor
AHR	Aryl Hydrocarbon Receptor
ALAS2	Delta-Aminolevulinate Synthase 2
ASCs	Adult Stem Cells
BCAP31	B-Cell Receptor-Associated Protein 31
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
BPA	Bisphenol A
CD	Cluster Of Differentiation
CDC42	Cell Division Control Protein 42 Homolog
CECs	Contaminants of Emerging Concerns
CM	Cryopreserving Medium
DDT	Dichlorodiphenyltrichloroethane
DUC-MSCs	Dolphin Umbilical Cord Mesenchymal Stem Cells
EDs	Endocrine Disruptors
ESCs	Embryonic Stem Cell
FKBP8	FK506 Binding Protein 8
FPKM	Fragments Per Kilobase Million
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GO	Gene Ontology
HABs	Harmful Algal Blooms
HBA	Hemoglobin Subunit Alpha
HBB	Hemoglobin Subunit Beta
HBE	Hemoglobin E
HBM	Hemoglobin Subunit Mu

iPSCs	Induced Pluripotent Stem Cells
MSCs	Mesenchymal Stem Cells
MTSS1	Metastasis Suppressor 1
NANOG	Nanog Homeobox
OCT4	Octamer-Binding Transcription Factor 4
PAH	Polycyclic and Planar Aromatic Hydrocarbons
PBDE's	Polybrominated Diphenyl Ethers
PCBs	Polychlorinated Biphenyls
PFOA	Perfluorooctanoic Acid
PHAH	Polycyclic and Planar Aromatic Hydrocarbons
PL	Dolphin Placenta
POPs	Persistent Organic Pollutants
REX1	Zinc Finger Protein 42
RGS2	Regulator Of G-Protein Signaling 2
RPL13	Ribosomal Protein L13
SOX2	Sex Determining Region Y-Box 2
UC	Dolphin Umbilical Cord
UME	Unusual Mortality Event
YAWHZ	Tyrosine 3-Monooxygenase/Tryptophan 5- Monooxygenase Activation Protein Zeta
ZFX	Finger X-Chromosomal
ZFY	Zinc Finger Y-Chromosomal

1. General Introduction

1.1. The bottlenose dolphin

1.1.1. Description

The bottlenose dolphin, *Tursiops truncatus*, is one of the most well-known species of marine mammals since ancient world. Dolphins have been protagonists of Greek and Roman legends; Byzantine and Arab, Chinese and European explorers had tales of dolphins rescuing sailors or ships in trouble. Depiction of dolphins can be found in sculptures, mosaics, engravings and painted pottery. In the modern era, dolphins are the most abundant marine mammals in marine parks worldwide, they are described in publications for scientific and non-scientific audience and their popularity increases even more through movies and TV show.

In the genus *Tursiops* are nested two separated species: the common bottlenose dolphin, or simply bottlenose dolphin, *T. truncatus*, and the smaller Indo-pacific bottlenose dolphin, *T. aduncus*, both with a well-defined beak about 8 cm long. *T. aduncus* has a proportionately longer rostrum than *T. truncatus* and develops ventral spotting at time of sexual maturity (Wells and Scott, 2002). Due to geographical variation, the taxonomy of bottlenose dolphin is often confusing, and it is likely that additional species will be recognized in the future. *T. truncatus* range in length from 2.5 m to 4 m, with the males usually longer than females, and weight from 150 kg to 650 kg. Their size varies with the habitat: dolphins in cooler pelagic waters tend to be bigger than those in the shallow waters (except in the Pacific Ocean). They have a total of five fins which include: the dorsal fin, located on the top side of the dolphin, fluke fins on the back bottom part of the tail and help dolphins dive or leap out of the water, and the pectoral fins are located on either side of the dolphins body. Bottlenose dolphin have a moderately curved dorsal fin and shade of grey coloration varying among populations, darker on the dorsal fin and lighter in the belly, with a demarcation between the melon and short rostrum (Figure 1.1).

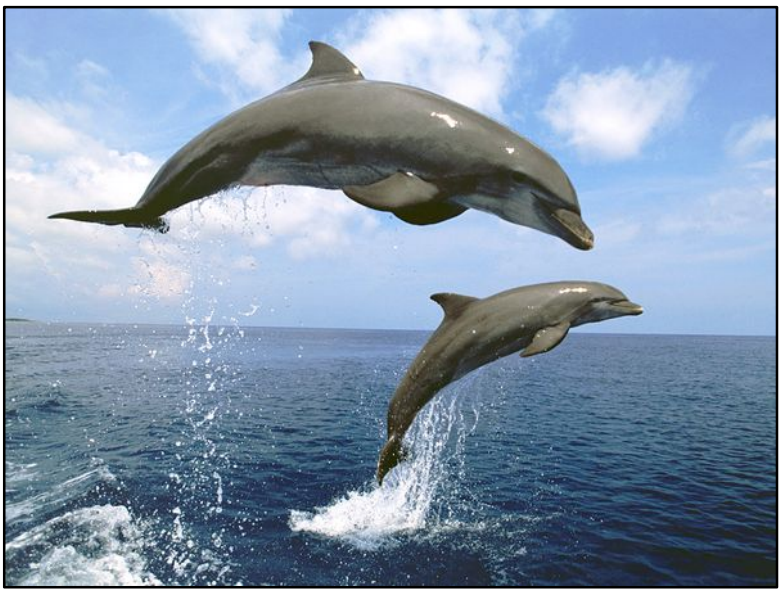

Figure 1.1. The bottlenose dolphin, *Tursiops truncatus.*
Bottlenose dolphins have a well-defined beak, a moderately curved dorsal fin and shade of grey coloration, darker on the dorsal fin and lighter in the belly, with a demarcation between the melon and short rostrum. (Credit: David Tipling, National Geographic, Getty Images).

Bottlenose dolphins can live for more than 40 years, with a generation time of 20 years (Hammond et al., 2012). Sexual maturity varies by population and ranges from 5 -13 years for females and 9 - 14 years for males. Gestation period in usually 12 months, and birth can occur any time of the year, with peaks in the summer. Single calf usually is about 1 m long and weights a maximum of 30 kg.

Bottlenose dolphins are found in temperate and tropical water around the world with a surface temperature ranging from 10 °C to 35 °C and a worldwide distribution ranging from latitudes of 45 °N to 45 °S (Figure 1.2). They are distributed in a wide variety of habitats: coastal, continental shelf waters, lagoons and archipelagos, deep waters of the continental slope (Bearzi et al., 2009). The species is widespread and abundant, and classified as species of 'Least Concern' from the International Union for Conservation of Nature (IUCN; http://www.iucnredlist.org) although threats operating on local populations have been observed and described (Hammond et al., 2012). The bottlenose dolphin has been afforded special protected status under Annex II of the European Union's Habitats Directive (http://ec.europa.eu/environment/nature/legislation/habitatsdirective/index_en.htm)

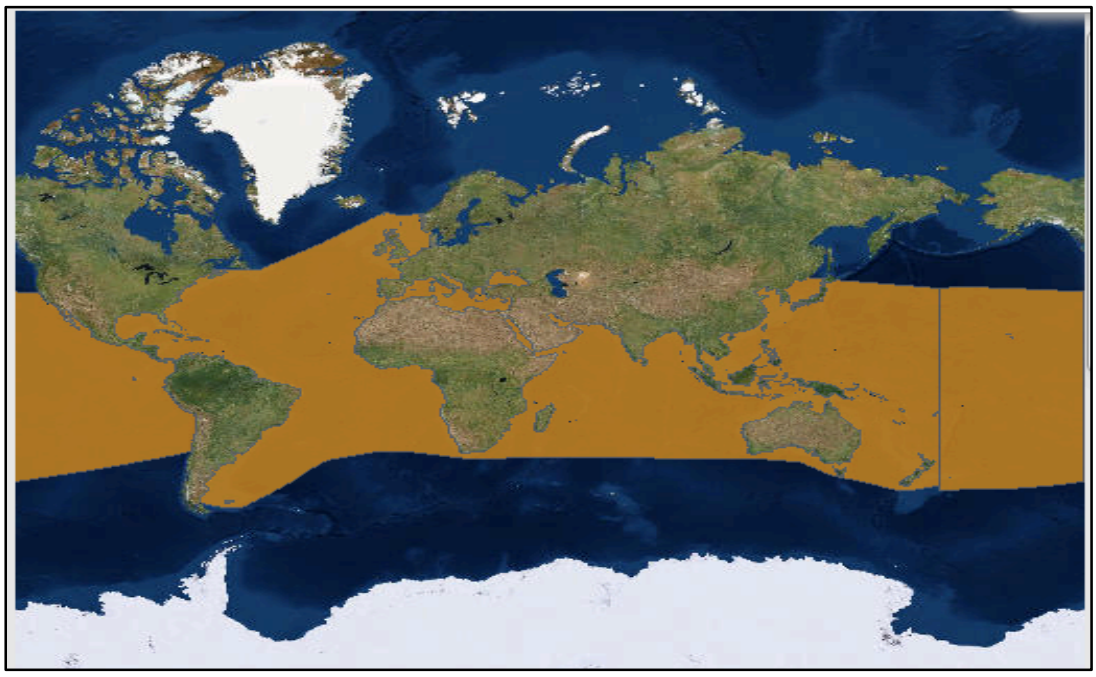

Figure 1.2. Geographical distribution of *T. truncatus.*
Bottlenose dolphins are distributed worldwide through tropical and temperate inshore, coastal, shelf, and oceanic waters. They generally do not range pole-ward of 45 °except in northern Europe (as far as the Faroe Islands 62 °N 7 °W) and to southern New Zealand. The species is rare in the Baltic Sea (where it may be considered an extralimital species) and is vagrant to Newfoundland and Norway. (From: International Union for Conservation of Nature (IUCN) 2012. The IUCN Red List of Threatened Species. Version 2016-3).

Bottlenose dolphins can produce three categories of sound, for communication and feeding: echolocation clicks (40.130 kHz of frequency; Au, 1980), whistles (including signature one) and burst-pulse sounds. Dolphin echolocation clicks are amongst the loudest sounds made by marine animals. The process of echolocation is used to send out sound waves; when those sounds hit an object, it bounces back vibrations to the dolphins. This allows them to identify where objects are located and gives them information about the shape and size (Figure 1.3).

They feed on a large variety of fish and squid "endemic" to their habitat, foraging individually and cooperatively, and visual means as well as active echolocation has been implicated in prey capture. Recent evidence based on the examination of stomach contents has suggested that bottlenose dolphins also likely use passive listening for the purpose of detecting and possibly orienting to “soniferous” fish (Gannon et al., 2005).

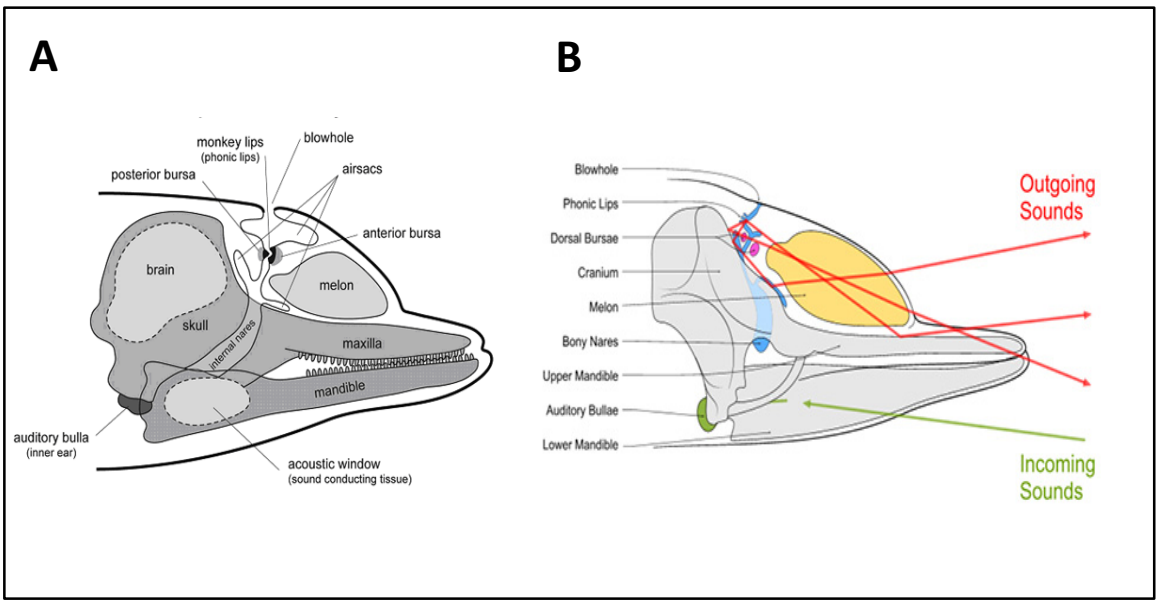

Figure 1.3. Schematic illustration of a dolphin's head anatomy and its correlation with sound production.
A Dolphins use air sacs located near the blowhole and nasal sacs located behind the melon to make whistle and click noises which are then projected from the front of the dolphin; **B** they receive their auditory signals through the lower jaw, which is hollow, allowing the sounds to reverberate and easily transmit to the ear. From the rear end of the jaw, sounds are transmitted to the inner ear. Sound can also be received laterally through fatty lobes that surround the ear called the auditory bullae. The brain of toothed whales can interpret directionality of the echo of the sound they transmit by direction using the directionality of the organs receiving the sounds. Bottlenose dolphins can interpret individual clicks at up to around 600 clicks per second.

Bottlenose dolphins are commonly found in groups of 2 to 15 individuals even though offshore groups may be of several hundred individuals. The size of their groups varies with biogeographic region, prey, human activities and other factors. In some areas, they are mixed with other cetaceans, but stable associations are uncommon. Group compositions tend to be dynamic, with sex, age and reproductive condition, and relative and affiliation histories seem to be the most important aggregating factors.

Bottlenose dolphins have predators, such as killer whales and sharks, depending on the geographical region of distribution. In the Mediterranean Sea, predation is a minor cause of death (Bearzi et al., 2009). There is, in fact, a well-known history of intentional killing, conducted at least until the middle 1960s, with a decline of 50 % over the past 50 years in the northern Adriatic Sea as a consequence of exterminations campaigns to reduce competition for fish. A similar trend is known for the northwest Mediterranean Sea (Bearzi et al., 2004; Bearzi, 2006). Currently, in the Mediterranean basin, deaths are mostly correlated to incidental catch in

commercial and recreational hook-and-line, fishing gear. Incidental capture during fishing activities is a worldwide concern given the potentially drastic effects that it can have on marine mammal populations: Chinese fisheries reach several hundred per year (Guang et al., 1999); in the Black Sea, the increase of incidental mortality is correlated to the rapid expansion of illegal, unreported and unregulated fishing (Birkun 2006). A recent study in southern Brazil showed that fishing-related mortality are unsustainable for the small resident population of bottlenose dolphins that inhabits the Patos Lagoon Estuary, and that this population may be declining (Fruet et al., 2012).

Bottlenose dolphins, especially in coastal areas are exposed to a wide variety of threats: reduced prey viability caused by environmental degradation and overfishing, disturbance caused by boat traffic, habitat loss and degradation, biotoxins, and anthropogenic noise and pollutants, such as xenobiotic contaminants. Dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) found in animal blood are correlated with a decline in the immune system function (Lahvis et al., 1995). Chemical contaminants tend to bioaccumulate and biomagnify in the aquatic food web, making the aquatic mammals such as the cetaceans, which are at the highest trophic level, potentially “at risk”. Dolphins are also subject to the stress posed by biotoxins (e.g., brevetoxins, ciguatoxin/maitotoxin, saxitoxins, domoic acid and okadaic acid) produced by harmful algal blooms (HABs), which are periodically experienced by coastal waters around the world and have been increasing over the last quarter century (Scholin et al., 2000; Flewelling et al., 2005; Van Dolah, 2005). For example, between August 1999 and February 2000, 120 bottlenose dolphins stranded in the Gulf of Mexico, Florida and 2 peaks of stranding coincided with *Karenia brevis* blooms (Van Dolah, 2005). Histopathological analysis showed significant upper respiratory tract lesions but the highest concentrations were found in the stomach contents, followed by liver and kidney suggesting that the dolphins obtained the toxin via the food chain, rather than by inhalation (Van Dolah, 2005).

According to the National Oceanic and Atmospheric Administration (NOAA) reports, bottlenose dolphins have been stranding at elevated rates since July 2013 along the Atlantic coast of the USA; this event has been declared an Unusual Mortality Event (UME). An unusual mortality event (UME) is defined under the Marine Mammal

Protection Act as: "*a stranding that is unexpected; involves a significant die-off of any marine mammal population; and demands immediate response*." The marine mammal UME program was established in 1991. From 1991 to the present, there have been 62 formally recognized UMEs in the U.S., involving a variety of species and dozens to hundreds of individual marine mammals per event. Causes of UMEs include infections, biotoxins, human interactions, and malnutrition (Figure 1.4).

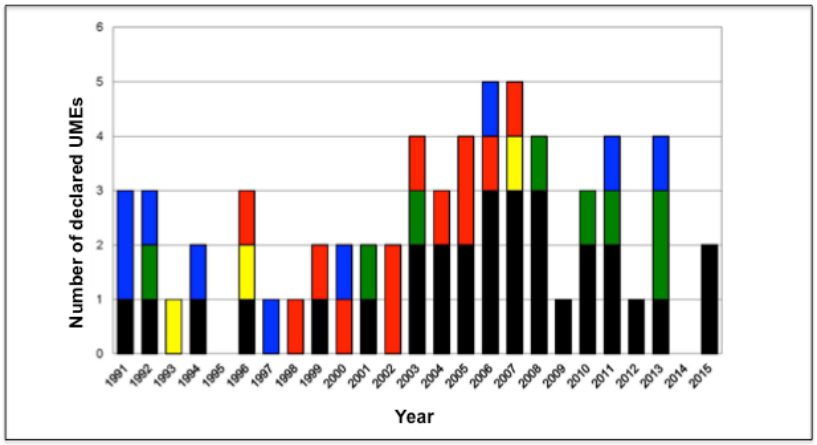

Figure 1.4. Marine mammal unusal mortality events 1991-2015 in the US coasts. Number of declared events per year, by cause (total=62). Yellow, human interactions; green, ecological factors; red, biotoxins; blue, infectious disease; black, undetermined/pending. (Modified from NOAA Fisheries, http://www.nmfs.noaa.gov/).

The extent of the environmental impact can be a dramatic event such as the death of a dolphin, to the death of hundreds of them (UME) but it can also be less evident, affecting their health, immune system and reproductive function.

1.1.2. Evolution

The bottlenose dolphin (*T. truncatus*) belongs to the Mammalian order Cetacea, sub-order Odontoceti, family Delphinidae. In the Cetacea, two major suborders are recognized: Mysticeti (baleen whales) with 14 species, and Odontoceti (toothed whales) with 76 species. Cetacea are nested with the Artiodactyla forming a single clade named Cetartiodactyla. The Archaeoceti, also called “the ancient whales”, represents the ancestors of the two modern sub-orders. It is a paraphyletic group of

primitive cetaceans that lived from the early Eocene to the late Oligocene (55 to 23 million years ago, MYA), including the earliest cetacean radiation, the initial amphibious stages in cetacean evolution. Fossil records of representatives of this group have been found in Egypt, Nigeria, India, Pakistan, and in the United States of America and show a wide range of morphologies that prompted their classification into several families such as the Pakicetidae (early Eocene), Protocetidae (middle Eocene) and Basilosauridae (middle to late Eocene). Modern whales possess newly derived characters compared to the Archaeoceti's with the most noticeable being the association of the bones in the skull in response to the migration of the nasal openings to the top of the skull (telescoping) (Figure 1.5). The modern whale skull has premaxillary and maxillary bones that have migrated far posteriorly and presently form most of the skull roof resulting in a long rostrum or beak and dorsal nasal openings. The occipital bone forms the back of the skull and the nasal, frontal, and parietal bones are inserted between the other bones (Berta 2015) (Figure 1.5).

The bottlenose dolphins are in the family of Delphinidae in the suborder of the toothed whales, the Odontoceti. The Odontoceti split in four lineages: sperm whales, beaked whales, dolphins and porpoises. Living odontocetes generate high frequency sound used for echolocation thanks to the facial muscle complex and nasal apparatus. Correlate to their ability to echolocate is the presence of melon, mass of adipose tissue on the top on the skull, and cranial and facial asymmetry (with the right side larger and more developed than left side) (Figure 1.3).

The oldest reported fossil record of Delphinidae is from 11 MYA while molecular studies support dolphin divergence approximately 9 - 10 MYA (Vilstrup et al., 2011). Explosive radiation of dolphins in Italy is described during Pliocene and most recently during the Pleistocene (Bianucci et al., 2013). First *Tursiops*' fossils are relatively recent, about 5 MYA (Barnes 2012).

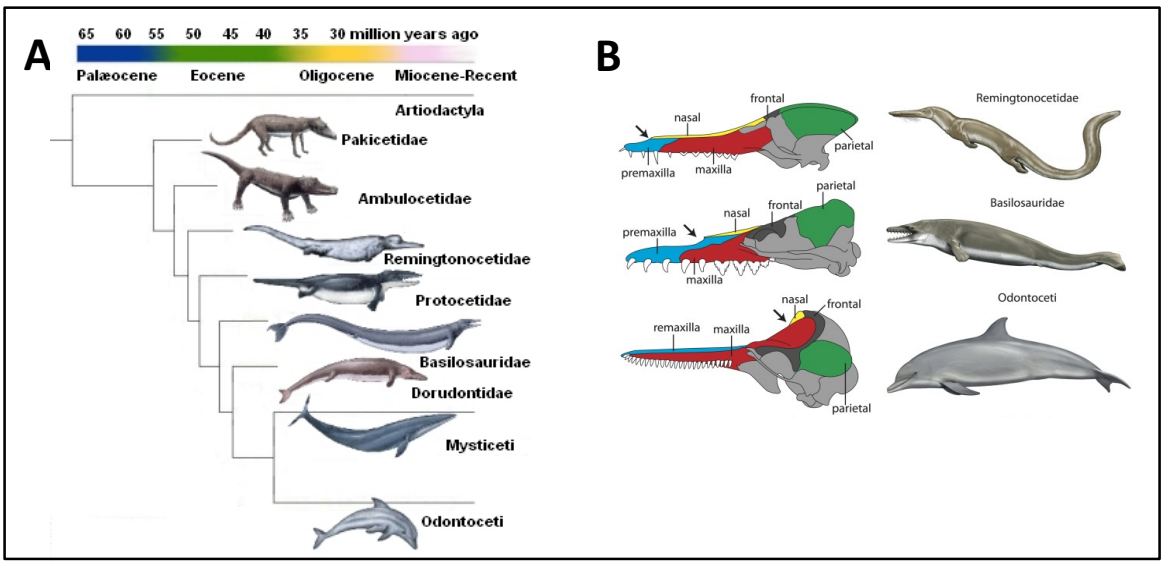

Figure 1.5. Relations of early whales (archaeocetes) to artiodactyls with the two extant groups, odontoceti and mysticeti.
A The archaeocetes are a paraphyletic group of primitive cetaceans that include the earliest, terrestrial 'whales'. **B** Cranial telescoping and posterior migration of bony nares in extinct and extant cetaceans in early Eocene archaeocete (Remingtonocetidae), middle Eocene archaeocete (Basilosauridae) and recent odontocete (*T. truncatus*). (Modified from Berta et al., 2015).

1.1.3. Adaptation to the marine environment

Cetaceans represent one of the most fascinating evolutionary transitions within vertebrates: they are entirely pelagic, but they are still mammals. In order to live in the oceans, they have changed many physiological processes as well as some physical characteristics.

a) Swimming: dolphins have developed a fusiform and hydrodynamic body that reduces friction, and their appendices are modified for maximal propulsion and minimal drag. Wave riding and surface swimming allow them to increase travelling speed while reducing metabolic cost (Williams, 2001).
b) Respiration: they breathe through a single blowhole (nasal opening) on the dorsal surface of their head consisting of a hole and a muscular flap. The muscular flap (muscle sphincter) is closed during muscle relaxation and opens during contraction. The larynx is composed of two elongate cartilages providing a more direct connection between nose and trachea. The trachea is short and broad, consisting of several cartilaginous rings that are interconnected with each other. Dolphins hold their breath while underwater and begin to exhale

when they reach the surface; they are conscious breathers. During each respiration, a dolphin exchanges 80 % or more of its lung air (humans exchange only 17 %) in about 0.3 seconds (exhaling and inhaling) (Ridgway, 1972; Bryden et al., 1986). A bottlenose dolphin average respiratory rate is about two to three breaths per minute (Ridgway, 1972) but, if necessary, it has the ability to remain submerged several minutes.

c) Diving: most bottlenose dolphins regularly dive to depths of 3 to 45 m for 8 to 10 minutes but they are capable of diving much deeper. Under experimental conditions, the deepest trained dive is 547 m (Ridgway. 1990). While diving, dolphins have a slower heartbeat and blood is shunted away from tissues tolerant of low oxygen levels toward the heart, lungs, and brain, where oxygen is needed. An increased total body O_2 store has long been considered an essential factor in the breath-hold capacity of diving mammals that don't have constant access to air. Oxygen is stored in the muscle, which has a content of myoglobin (an oxygen-binding protein) that is 3 to 10 times higher than in land mammals. The oxygen is also stored in the blood and in the lung. O_2 capacity in blood can be boosted increasing the number of red blood cell, with the result of increased blood volume, cells volumes, hemoglobin concentration and myoglobin content (Kooyman et al., 1998). Dolphins, as other marine mammals, have a high tolerance to lactic acid and carbon dioxide and their muscle can work anaerobically while they dive holding their breath. Dolphin can tolerate tremendous atmospheric pressure at great depths. Air spaces are minimized; lungs and ribs are collapsible and nitrogen absorption is limited: this can limit decompression sickness.

d) Thermoregulation: dolphins have a large fat storage under their skin, namely blubber. Blubber makes an excellent insulator but also acts as energy reserve and enhances buoyancy. Dolphin's core temperature is about 37 °C and there is a heat gradient throughout the blubber to the skin (Ridgway, 1972). Their circulatory system adjusts to conserve or dissipate body heat and maintain body temperature; veins surround arteries in the flippers, flukes, and dorsal fin so that some heat from the blood traveling through the arteries is transferred to the venous blood rather than the environment (Figure 1.6). This countercurrent heat exchange aids dolphins in conserving body heat. Moreover, when they dive, blood is shunted away from the surface of the body and this decrease in

circulation helps to conserve body heat (Ridgway 1972). Marine mammals need to limit blood flow during diving, while swimming requires an increase of blood volumes: they have the finest regulation of blood circulation (Hokkanen, 1990).

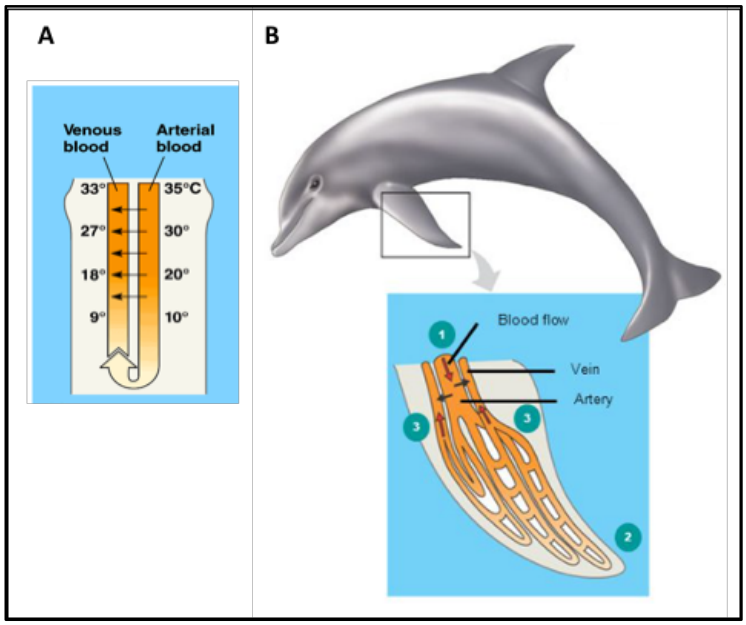

Figure 1.6. Heat exchange in dolphin flippers, flukes and dorsal fin.
A General pattern of a countercurrent system; **B** simplified vascular exchange network in bottlenose dolphin flipper. (Modified from Berta et al., 2006).

e) Sleep: in order to maintain their conscious breathing functioning during sleep, the bottlenose dolphin shuts down only half of its brain, along with the opposite eye while the other half of the brain stays awake at a low level of alertness (predators, obstacles, etc…). After approximately two hours, the animal will reverse this process, resting the active side of the brain and awaking the rested half. Bottlenose dolphins, based on electroencephalogram (EEG) readings, spend an average of 33.4 % of their day asleep (Ridgway 1990; Mukhametov et al., 1988). Young dolphins rest, eat and sleep while their mother swims, towing them along. The mother will also sleep on the move because calves are not born with enough body fat or blubber to float easily.

1.1.4. Biological characteristics and biomedical applications

Dolphins have evolved unique adaptations, which can give valuable insights into human biomedical conditions. A deeper knowledge of the dolphin biology and physiology correlated to its health status can contribute to a better understanding of unresolved underlying mechanisms in human disease.

Dolphin lung and human respiratory distress syndrome

Managing hydrostatic pressure and nitrogen gas can be challenging for all mammals, humans included. Absorption of nitrogen increase with increasing of depth, resulting in higher dissolved gas tensions that reach equilibrium with partial pressure of N_2 in lungs. In humans, using pressurized air during their dives, this issue is well known. Nitrogen gas emboli can be a primary event of decompression sickness. Some studies suggest that marine mammals dive routinely without apparent injuries due to physiological, anatomical and behavioral adaptations. Their large body mass, decreased relative lung size, increased blood volume and myoglobin concentration, as well as the peripheral vasoconstriction, the bradycardia and the lung collapse they adopt during diving, are adaptations shared by all marine mammal divers (Hooker et al., 2012). However, diving comes with a cost for marine mammals too. Recently, diving injuries were detected in marine mammals. Lesions concomitant with gas emboli have been described in beaked whales stranded after military exercises deploying sonar. It has been suggested that the decompression sickness was due to the effects of intense sound that may have caused behavioral change in the dive profiles (Fernandez et al., 2005). Acute and chronic gas bubble lesions have been described also in cetaceans stranded in the UK coast (Jepson et al., 2005). Sperm whale skeletal lesions have been described as osteonecrosis-type surface lesions, probably formed by the repetitive production of gas emboli (Moore et al., 2004). Bubbles have been observed also in marine mammals trapped in fishing nets (Moore et al., 2009; Hooker et al., 2012). Even if they may present lesions, dolphins survive deep, prolonged and frequent dives. They can bear dives that deprive vital organs of oxygen that for humans would be lethal. During dives, they restrict blood flow to their kidneys, liver, heart and lungs to shunt more oxygen to the brain. When they resurface, oxygenated blood flow is restored to those organs without the organs suffering damage. It is not understood what mechanisms

or adaptations exist in the lungs that prevent damage of alveolar cells from hypoxia experienced during deep dives. In addition, the initial expired air or “blow” upon resurfacing also is accompanied by copious amounts of pulmonary surfactant, but what triggers such production and how the lung re-expands so rapidly without damage to the lung remains unknown. Hypoxia occurs when there is an imbalance between the delivery and demand of oxygen in tissues and this can cause damage in a number of organs. Interestingly, hypoxia of the lung can occasionally be considered a “natural” condition, as it happens during fetal development as well as in marine mammals during deep diving events. During fetal lung development *in utero* surfactant production is essential for successful lung inflation and function upon birth. Abnormal surfactant is in fact the major problem that is associated with premature births and the development of respiratory distress syndrome (RDS). Based on observations that hypoxia appears to have a role in the surfactant production associated with lung development and function upon birth, the surfactant production and hypoxia response observed in marine mammals after deep dives could be used as model for the development of new therapeutics to treat and prevent airway disease associated with disruption of surfactant production.

Dolphin wound healing for human regenerative medicine

In human, the healing of tissue wounds is often associated with infection and results in scars. Zasloff, a professor of immunology at the Georgetown University, observed the clinical course of the recovery of two dolphins showing shark’s bites about 30 cm in length and 3 cm in depth (including the blubber layer and the underlying muscle). He reported that, during the first day post-injury, blubber from surrounding tissues had already migrated over the open wound surface. On the second day, newly generated tissues were described, which would gradually fill the wound from its base, restoring the original volume (Zasloff et al., 2011). The wound healed completely in 4 weeks and without any infections, which is remarkable considering the analogies between their immune system and that of terrestrial mammals (Mancia et al., 2007, Beineke et al., 2010). Zasloff suggested a role of the components of the dolphin blubber during the healing process. The composition of dolphin’s blubber is in fact different from other marine mammals: the isovaleric acid is higher in concentration, being 2 - 5 % of total fatty acid in bottlenose dolphin, while there is no detection in several species of whales (Koopman et al., 2003). The

isovaleric acid accumulates in the blubber and doesn't get burned for fuel during times of starvation; moreover, it seems to have antimicrobial activity (Hayashida et al., 2008). Zasloff suggests that the isovaleric acid may be released from the blubber after a serious injury to protect the surrounding tissue from infection. Stem cells present in the blubber could have a role in this remarkable wound healing process. Advances in the comprehension of the mechanisms controlling the healing process could lead to the improvement in the control of wound healing in human and terrestrial mammals leading to new therapies: regenerative medicine is an emerging field, with numerous open trials in veterinary and human field.

Dolphin metabolism for human diabetes

People affected by metabolic syndrome can develop not only type II diabetes, but also cardiovascular disease and the possibilities to have strokes (https://www.cdc.gov/). Like human, bottlenose dolphin can develop metabolic syndrome, characterized by elevated insulin, glucose, triglycerides and ferritin (Venn-Watson et al., 2011 and 2015). Fatty liver disease has been found both in wild and dolphin under human care, suggesting that dolphins are susceptible to metabolic disorders. Venn-Watson group at the US National Marine Mammal Foundation compared two different populations of dolphins: the group from the US Navy Marine Mammal Program (MMP), kept under human care, and a wild group from Sarasota bay, FL (USA). They observed that the MMP's dolphins have higher annual survival and lower mortality rates compared to Sarasota's wild dolphins. A deep study into these animals' diet as a possible risk factor for longer life and metabolic disease, highlighted the potential benefits of C17:0, the margaric or heptadecanoic acid, a saturated fatty acid also present in bovine milk fat. High levels of C17:0 on erythrocyte membranes or plasma phospholipids, have been identified as protective factors against development of metabolic syndrome, type 2 diabetes, and associated inflammation (Venn-Watson et al., 2015). Understanding the potential benefits of C17:0 and the reason why dolphins have a diabetes-like metabolism could aid research of metabolic syndrome and diabetes affecting human populations.

1.2. The bottlenose dolphin in the marine ecosystem

The health decline of the marine ecosystem can be mostly related to anthropogenic impacts such as overfishing, coastal habitat destruction, deep sea mining, oil and gas exploration, and it is also strictly correlated to industrial application and the release of chemical contaminants and pollutants. The marine environment receives a lot of inputs of dangerous substances both from direct discharge and atmospheric deposit. All these substances released in the environment can directly affect organism living in habitat exposed and can cause metabolic disorder, opportunistic infections and population changes in growth, reproduction and survival. Oceans and seas can facilitate the distribution of contaminants, which tend to be stable and lipophilic, such as heavy metals and organochlorine chemicals (e.g. PCBs and chlorinated pesticides, like DDT). Moreover, runoff from urban, industrial and agricultural activities bioaccumulate up the food chain, with the greatest concentrations in animals at the highest trophic levels, such as dolphins (Figure 1.7). Previous studies have shown that dolphins can accumulate anthropogenic contaminants such as organohalogens and heavy metal contaminants (Wells et al., 1994; Houde et al., 2006; Fair et al., 2007; Stavros et al., 2008).

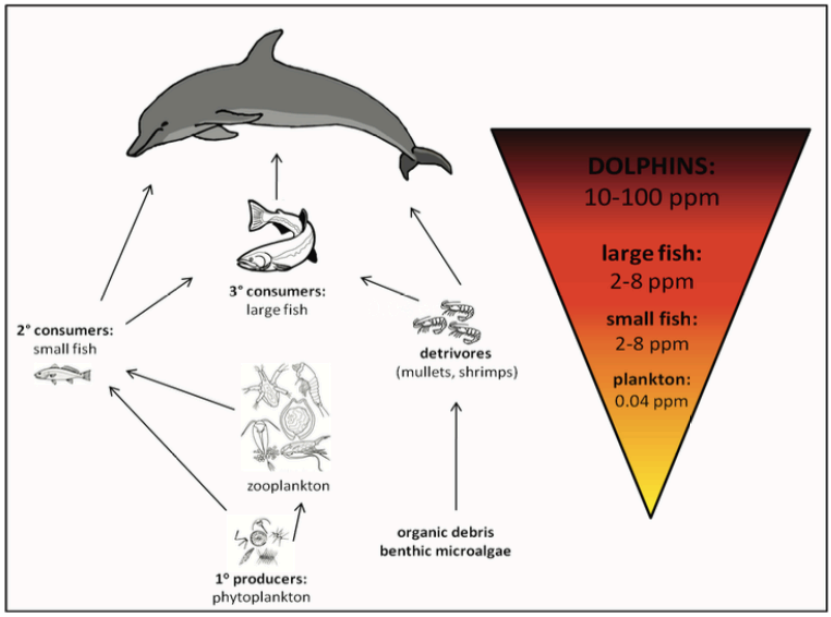

Figure 1.7. Biomagnification in the food chain.
Runoff from urban, industrial and agricultural activities have the greatest concentration in animals at the highest trophic level such as the dolphin. An example of biomagnifications of persistent contaminants (e.g. PCBs) is showed in the pyramid on the right.

1.2.1. Contaminants of emerging concern

“Contaminants of emerging concern” (CECs) or simply “emerging contaminants” are “naturally occurring, manufactured or manmade chemicals or materials which have now been discovered or are suspected present in various environmental compartments and whose toxicity or persistence are likely to significantly alter the metabolism of a living being” (Sauve et al., 2014). CECs are increasingly being detected in surface water, they can be represented by substances that we are beginning to suspect could cause harm: chemicals, pharmaceuticals and personal care products, as well as their metabolites and transformations, nanomaterials, plastics and their derivative and illicit drugs. They may be new substances, or they may have been around for a long time but only recently have been found in the environment. CECs are important because the risk they pose to human health and the environment is not yet fully understood.

Several of these chemical products are classified as endocrine disruptors compounds (EDCs), because they can alter the normal hormone functions affecting an organism’s reproduction, development and metabolism (Casals-Casas and Desvergne, 2011; https://www.epa.gov/wqc/contaminants-emerging-concern-including-pharmaceuticals-and-personal-care-products; Noguera-Oviedo et al., 2016). ED chemicals are found in low doses in thousands of products and almost all plastic items and the most commonly detected in people include DDT, PCBs, bisphenol A (BPA), polybrominated diphenyl ethers (PBDE's), and a variety of phthalates. Some cetaceans have high levels of substances suspected to be EDCs suggesting that current levels of environmental contamination by persistent organochlorines in the wild can cause an imbalance in sex hormones and subsequent reproductive abnormalities (Marsili et al., 1998; Fossi et al., 2013). EDCs range across all continents and oceans but in some geographic areas, such as the Mediterranean Sea, they are potentially more threatening than in others. This basin has limited exchange of water with the Atlantic Ocean, and is surrounded by some of the most heavily populated and industrialized countries in the world. For example, the concentrations of PCBs and DDT in the blubber of striped dolphins living in the Mediterranean are much higher than in specimens of the same species

living in oceans (Fossi et al., 2007). This suggests that Mediterranean top predator species are potentially “at risk” due to EDCs contamination.

1.2.2. Emerging diseases in dolphin

In the past decades, new pathologies and diseases have been described in marine mammals, involving immunologic dysfunctions and very complex pathogenesis (Bossart et al., 2007) and including various papillomaviruses (Rehtanz et al., 2009), dolphin poxvirus (Bracht et al., 2006) and other viral infections (Van Bressem et al., 1999), lobomycosis (Kiszka et al., 2009; Murdoch et al., 2008), toxoplasmosis (Dubey et al., 2009)and various neoplastic diseases (urogenital cancer, lingual papillomas, squamous cell carcinomas and genital papillomas) that may be direct or indirect consequences of pathological infections (Renner et al., 1999; Sanchez et al., 2002; Ewing et al., 2003). In some cases, they are caused by new species-specific pathologic agents, like the dolphin papillomavirus, the etiologic agent of malignant and benign tumors (Rehtanz et al., 2009). Malignant transformations of papilloma lesions have been reported in *T. truncatus* (Bossart et al., 2005), and several orogenital papillomas have been described in many species of free-ranging cetaceans (Bossart, 2007) (Figure 1.8). The virus of papilloma is sexually transmitted and is associated with immunologic dysfunction, thus increasing risk of opportunistic pathogens (Stanley, 2010; Crosbie et al., 2013; Lucs et al., 2015;).

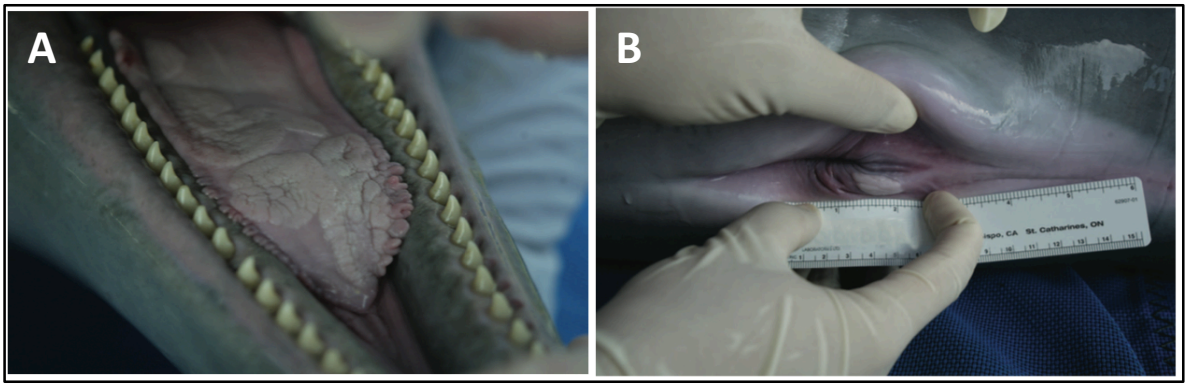

Figure 1.8. Papillomavirus infection in free-ranging bottlenose dolphin.
A Tumor in the tongue of a bottlenose dolphin associated to papilloma virus infection. **B** Genital lesions typical of papilloma virus infection in female dolphin. Bossart *et al.*, 2007.

In some cases, they are caused by agents pathogenic to man as well. An example is toxoplasmosis infections, indicative of water contaminations with *Toxoplasma gondii* oocysts (Dubey et al., 2005). *T. gondii* oocystis is the common way of infections and the oocysts are highly resilient, remaining infective for many months (Mead et al., 1999). Toxoplamosis is a parasitic infection targeting warm-blooded animals: in adult humans does not cause serious illness, but in pregnant women and children can cause many developmental problems (Hill et al., 2002). Free-range marine mammals have been diagnosed with infections worldwide, in adult humans, sheep, goats, pigs, dolphins, and in pregnant female and fetus, congenital toxoplasmosis was also reported (Resendes et al., 2002; Jardine and Dubey, 2005 and 2009). Coastal pollution by sewage from human and pets has been suggested as route of infection (Lindsay et al., 1997; Miller et al., 2002; Hill et al., 2002). Dolphin can serve as sentinel for detection of the parasite's transmission in the marine environment (Conrad et al., 2005). Lombomycosis is a chronic mycotic disease, spreading probably easily in the presence of immunosuppressive co-factor (Rotstein et al., 2009). The etiologic agent is *Lacazia loboi,* a yeast-like organism that is found abundantly within lesions, causing cutaneous nodules that grow in the years. Dolphin and human have the same lesions with the only difference that dolphin's can be more extended (Figure 1.9). In humans, lombomycosis is endemic in rural regions, especially in South and Central America (Talhari et al., 2008). Lombomycosis is referred as a zoonotic disease (e.g. a disease that normally exists in animals but that can infect humans) because it affects only human and delphinidae, but evidence that it can be transferred directly is weak.

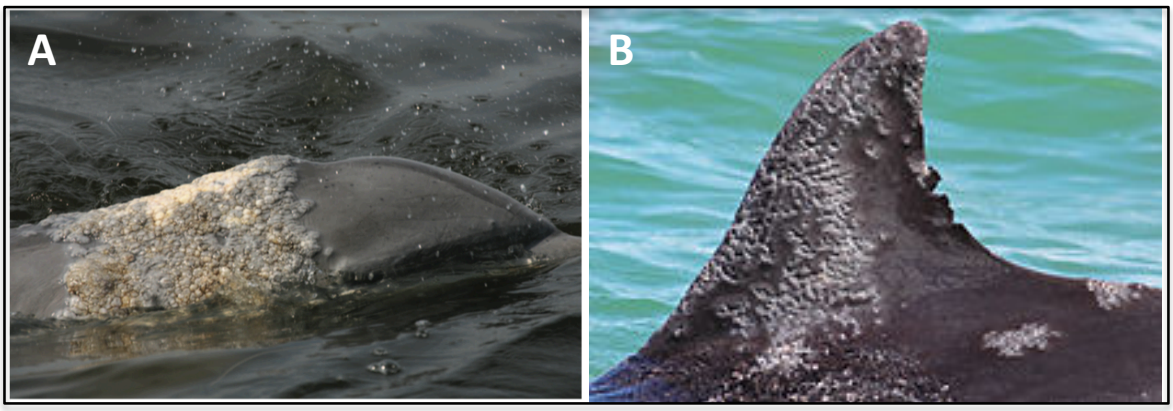

Figure 1.9. Lobomycosis skin lesion in free-ranging bottlenose dolphin.
Dorsal **A** and fin **B** skin lesions associated with the pathogenic fungus *Lacazia loboi* infection in dolphins off the coast of Florida, USA (Bossart 2007).

In recent years, contact between marine mammals and human is increasing, due to the business of marine parks, rehabilitation and research facilities, as well as recreational activities in the marine environment, increasing the exposure of humans to animals and transmission of diseases, when present. Seventy-five percent of know human pathogens are zoonotic (H5N1, H1N1 avian influenza, Nipah virus to cite few) and caused by environmental, ecological and epidemiological factors (Waltzek et al., 2012). Anthropogenic stressor, habitat degradation, pollution may increase the prevalence of infections in marine mammals (Van Bressem et al., 2009); climate change increasing water temperature can modify vectors and reservoir species, changing also the way pathogen and host interact. These changes can be facilitating emergence of new pathogens, affecting the geographical distribution of some viruses, bacteria and protozoa and the all the species, including dolphins, sharing the same marine environment (Van Bressem et al., 2009).

1.2.3. The dolphin, sentinel organism for the marine ecosystem

Marine mammals have been proposed as sentinel organisms for the health of the marine environment because most of the species, including dolphins, present characteristics that can be informative of the status of the marine ecosystem in which they live. Because of their long lifespan, their residential behavior on coastal waters, their feeding at a high trophic level and their extensive fat stores, they can serve as accumulation beds for anthropogenic toxins like organohalogen compounds, heavy metals and persistent organic pollutants, POPs (Stavros et al., 2008; Santos-Neto et al., 2014; Zhu et al., 2014; Lavandier et al., 2015; Durante et al., 2016; Damseaux et al., 2017) (Figure 1.7). They live their entire lives in the aquatic environment where they are directly and constantly exposed to a variety of pathogens and other stressors of natural and anthropogenic origin. Cases of lung disease, immune suppression and impaired thyroid function have been previously described (Schwacke et al., 2012; Mancia et al., 2014). An increase in mortality in defined populations has also been correlated to massive oil spill (Schwacke et al.,

2014; Balmer et al., 2015; Venn-Watson et al., 2015; Lane et al., 2015; White et al., 2016). The first to propose the use of marine mammals as environmental sentinels was Holden, in 1972. In 1998, the Marine Mammal Commission identified the California sea lion (*Zalophus californianus*), the harbor seal (*Phoca vitulina*), the beluga whale (*Delphinapterus leucas*) and the bottlenose dolphin (*Tursiops truncatus*) as model species for investigation into the effects of environmental contaminants on marine mammals.

Many diseases described in bottlenose dolphins are similar to humans. We are mammals, we consume the same food and we share the same coastal environment. Dolphin resembles the outcome of a chronic exposure to coastal anthropogenic stressors and contaminants. Much of the diseases that we are observing are a complex interaction among anthropogenic and natural factors, immunological and infectious diseases. Using the dolphin as sentinel of the marine environment status can not only aid in evaluating the marine ecosystem status, but also help us to better understand the effect of these trends on human health.

1.3. Methods to study bottlenose dolphin's health

1.3.1. Bottlenose dolphin sampling

The assessment of wild dolphins' health status is necessary for a scientific management of the ecosystem. In the US, the National Marine Fisheries Service (NMFS) recently started issuing research contracts for health assessment projects by which it became feasible to capture free-ranged dolphins, followed by release after sampling. Health assessments studies typically rely on physical examination procedures and can include the collection of samples. Skin and blubber biopsies are used for contaminants and toxins investigations, blood samples are useful for immunology, hormones, contaminants and bacteria analysis, urine samples for protein and glucose concentration, feces for bacteria presence, and saliva and skin samples also can be analyzed for the presence of contaminants and bacteria (Hogg et al., 2005; Goldstein et al., 2006; Green et al., 2007). Wild dolphin sampling is accomplished with capture-release studies, where animals are captured in the wild by a group of biologists and veterinarians, and after physical examination and tissue

sampling, released in the wild. Another good source of information on wild dolphin's health status comes from necropsy of stranded animals (Inskeep et al., 1990). However, the animal stranded is often deceased for a long time, thus limiting the quality and quantity of samples to use in any kind of analysis. Another approach is to study dolphins in captivity, which allows veterinarians and biologists to collect valuable information on the physiology of diving, sleeping and social behavior and to monitor their health status (Shirai et al., 1998; Kastelein et al., 2002; Pellisso et al., 2008; Sitt et al., 2008). However, due to limitations in specimen acquisition, limited relevance when compared to the animal in their natural habitat and restrictions on human intervention (e.g. as set forth by the Marine Mammal Protection Act in the US or by The Ministry of the Environment and Protection of Land and Sea of Italy), conclusions drawn from dolphin research on captive animals should be treated with caution. The study of free-ranging animals in their native environments is more informative than the examination of stranded animals or captive animals. Currently, marine scientists monitor coastal and offshore (in less degree) populations of dolphins using 1) photo-identification together with tagging and tracking using Time-Depth-Recorder (TDR), radio transmitters and GPS locators that allow continuous monitoring of the tagged animal (Mazzoil et al., 2008; Bearzi et al., 2009; Van Bressem et al., 2009), skin and blubber biopsy sampling (Wilson et al., 2007; Fossi et al., 2008).

1.3.2. Traditional methods

Many groups are investigating the dolphin immune system applying laboratory methods widely used for other species; for example, blood samples for hematology are collected from the dolphin fluke and used for complete blood count and morphologic analysis using automated analyzers (St. Aubin et al., 2011). This has been very important to generate baseline data over time for species-specific hematology (Goldstein et al., 2006). For the identification of pathogens, such as the *T. gondii,* the dolphin blood serum can be screened using a wide variety of laboratory techniques, such as the enzyme-linked immunosorbent assay (ELISA), the Western Blot, the modified agglutination test (MAT), the indirect fluorescent antibody test (IFAT) (Dubey et al., 2005). To detect and quantify contaminant levels,

such as PBDEs and PCBs in blubber samples gas-chromatograph mass-spectrometer is widely used (Yordy et al., 2010). The impact of stress during capture-release health assessment events is believed to be an important factor to be included in the analysis of the results: some investigators report increased level of corticosteroids in dolphin correlated to capture-release studies (Ortiz et al., 2000). Transcriptomic change in the gene expression profiles from blood samples taken before and after capture-release examination, showed a clear response to stress with the down-regulation of genes involved in the immune response and the up-regulation of genes connected to energy generation, due to glucocorticoids increase (Mancia et al., 2008). The establishment of baseline data including the influence of capture-release procedure is critical to the identification of the direct effects of environmental stressor on the health status wild dolphins (Fair et al., 2014).

1.3.3. Molecular methods

The recent development of high-throughput technologies, computational methods, together with their reduced costs, has enabled the examination of biological systems in remarkable detail. We can now monitor thousands of molecules simultaneously instead of just the few components analyzed with the traditional methods, thus generating a huge amount of data to document the real-time details of any biological system. The expression of hundreds to thousands of genes, proteins, or metabolites can be assessed simultaneously, thanks to the "–omics" disciplines applied to the high-throughput technologies. The advances in the field of genomics, proteomics and transcriptomics, enabled contributions in biology and ecology also of less-known non-model organisms. The potential of genomics or proteomics techniques in marine environmental science and especially in environmental risk assessment, is recognized by many researchers and official organizations (Postlethwait et al., 2004; Bozinovic et al., 2011; Veldhoen et al., 2012).

The dolphin genome was first assembled by the Human Genome Sequencing center, Baylor College of Medicine. Dolphin has been chosen as one of 24 animals whose genome has been sequenced to 2x coverage as part of the comparative

genomic annotation effort seeking the identification of functional elements that are conserved across mammals, a project funded by the National Human Genome Research Institute (Lindblad et al., 2011). Recently, the dolphin 2x genome has been greatly implemented thanks to the assembly of new high-throughput sequences data (Foote et al., 2015). A novel bottlenose dolphin genome with 100x coverage is about to be released.

Transcriptomic analyses aid discovery of novel genes and the connection of molecular and physiological responses to a large scale of stimuli (ecological, anthropogenic). The approach used can vary from the gene expression microarrays, to the more recent RNA-sequencing (RNA-seq) method. With gene expression microarrays, thousands of genes are analyzed simultaneously in any given sample, thus obtaining a lot of information about the physiological systems and the impact of environmental challenges. In 2007, a dolphin cDNA microarray containing 1395 unigenes was first developed (Mancia et al., 2007). The microarray was useful mostly for stress response and immune function studies (Mancia et al., 2008 and 2010). In 2014, a much more comprehensive system, a species-specific oligo microarray, containing 24418 unigene sequences from cDNA libraries of 7 different tissues from bottlenose dolphin was generated (Mancia et al., 2014): the microarray was used to screen wild animal blood transcriptomes and was effective in the differentiation of populations of dolphins inhabiting different geographic locations as well as in the study of the effects of environmental contaminants on dolphin health (Mancia et al., 2014 and 2015). Dolphins inhabiting the coastal waters of Georgia in the USA, known to be heavily contaminated by Aroclor 1268, an uncommon polychlorinated (PCB) mixture, were studied and genes involved in xenobiotic metabolism, development/differentiation and oncogenic pathways were found to be differentially expressed (Mancia et al., 2015). The same microarray was applied to screen also a different tissue, first applied to cell cultures from dolphin skin to study the vitamin D3 pathway, then to biopsy of wild dolphin's skin to the study of the impact of environmental stressors on the skin transcriptome. Gene expression was greatly impacted by season, with one-third of all the genes on the array varying between winter and summer, highlighting the need of creating baseline for natural variability for a better investigation of the effects of stressor (Van Dolah et al., 2015).

Despite the ability to interrogate the expression of thousands of genes, the microarray can still lack information, due to the incorrect abundance of some of the transcripts. The most recent RNA-seq method, in which the cDNA made from the RNA sample is directly sequenced through high-throughput DNA sequencing, provides a less biased evaluation of the transcriptome. RNA-seq analyses are very sensitive and offer the advantages of detecting all the unique sequences and of quantifying levels of RNAs expressed at a very low level. Most importantly, the reduced cost of RNA-seq provides an efficient approach to generate sequences for functional genomics analyses in non-model organism with unsequenced genomes using *de novo* assembly procedure (Gui et al., 2013).

Gui et al., (2013) characterized the leucocyte transcriptome of the Indo-Pacific humpbacked dolphin, identifying genetic markers and genes involved in immune system response and adaptive evolution. Foote et al., in 2015, using *de novo* assembled genomes of Killer whale, walrus and manatee were able to investigate convergent evolution at genomic level, found a high level of convergence along terrestrial sister taxa (dog, elephant and cow) to the marine mammals, highlighting parallel molecular changes in genes evolving under positive selection (Foote et al., 2015). To date, genomes from 16 species of marine mammals have been sequenced and are currently being analyzed (https://sites.google.com/site/marinemammalgenomics/time-tracker).

Another approach to the study of physiological events under normal or diseased condition is the use of cell lines for *in vitro* experiments. Investigators have been trying to develop dolphin cell lines for a long time (Carvan et al., 1994) but despite the effort and the great applicability of cell lines as tools to study the mechanisms of dolphin biological processes, only a few such lines are available. This is due in part to the protected status of the animal and/or the stringent federal permits needed to deal with the samples.

Previous studies have shown interesting applications of marine mammal cell lines to study infection, such as hepatitis A and herpesvirus (Cecil et al., 1970; Kadoi et al., 1992; Dotzauer et al., 1994) but also to study the effect of chemical contaminants including mercury or perfluorinated compounds (Pfeiffer et al., 2000; Wang et al., 2001a, 2001b; Hu et al., 2002). The susceptibility of dolphins to chemicals and toxins may be very different from what has been previously described for human

(for example, for bottlenose dolphin the minimal body burden of methylmercury that produces mild symptoms is 2 mg/kg, which is seven times the human threshold for symptoms) (Rawson et al., 1995). In addition, the response to a specific stressor may vary dramatically as a function of a specific cell type (for example, epithelial vs. endothelial, or kidney vs. liver). Middlebrooks and colleagues cultured endothelial cells from kidney and lung of *T. truncatus* to test the response to specific stress stimuli (Middlebrooks et al., 1999; Garrick et al., 2006). Epidermal cell cultures as tools for the *in vitro* evaluation of environmental stressors on bottlenose dolphin skin were established and successfully immortalized skin (Yu et al., 2005). Primary skin cells were isolated and cultured from skin samples collected from wild bottlenose dolphins to test the expression of a selected panel of 16 genes, following the exposure of methylmercury and perfluorooctane (Mollenhauer et al., 2009). Skin cell cultures were successfully obtained also on *Stenella coeruleoalba, Grampus griseus, Balaenoptera physalus* and *Delphinus delphis* (Marsili et al., 2008).

In order to understand the biology of an organism it is necessary to identify biomarkers of the healthy and diseased state, which requires having sufficient genomic information and an understanding of the relationship between genes and proteins in the cellular context. The combination of the -omic technologies is basic to today's molecular biology research. In the study of a protected species, like dolphins, these approaches that are capable of sensitive and real-time pictures of environmental impacts of stressors are going to be even more powerful and essential.

2. Gene expression microarray: assessment of the impact of contaminants of emerging concern on bottlenose dolphin skin transcriptome.

2.1. Introduction

In order to describe the health status of one ecosystem and its inhabitants is necessary to have sensitive indicators of the level of exposure to biological, chemical and physical contaminants. Elevated concentrations of contaminants in the environment can impact on human and wildlife health status, showing a wide variety of responses, such as endocrine disruptions, decreased reproductive parameters, teratogenic and genotoxic effects, immunosuppression and other immune-system impairments, that can lead to diseased conditions. Sensitive measurements of an organism contaminant exposure level can be obtained using gene expression microarrays, considered valuable biosensors thanks to their principal characteristic of giving a snapshot of the transcriptomic profile of a specific tissue in a defined time.

Recently, the attention is focused on contaminants of emerging concerns (CECs), which are increasingly being detected in surface water. There is concern that these compounds, used heavily in the past in industry and agriculture, may have an impact on aquatic life because many of them act as so-called endocrine disruptor (EDs). A compound considered a CEC with an ED function is represented by the bisphenol A (BPA), a ubiquitous monomer used in the manufacture of polycarbonate plastics (Casals-Casas and Desvergne, 2011). It has been shown that BPA leaches out of plastic products, such as food containers, toys and fax paper (Casals-Casas and Desvergne, 2011) and high monomer concentrations have been identified in human and animal samples (McLachlan et al., 2001). BPA has diverse endocrine effects on mammalian and non-mammalian systems because it can bind the aryl hydrocarbon receptor (*AHR*), promoting both antagonist and agonist activities (Kharrazian et al., 2014). In human, high urinary concentrations are associated with an increased occurrence of cardiovascular disease, diabetes and liver enzyme abnormalities (Lang et al., 2008), and there are evidence linking BPA exposure to adverse effects in perinatal and childhood (Rochester et al., 2013).

Another man-made compound belonging to the CECs category is the acid perfluorooctanoic acid, or PFOA. PFOA has the potential to be a health concern because it can stay in the environment and in the human body for long periods of time. In fact, the chemical structure characteristic of perfluorinated compounds gives them thermal stability and the ability to repel both water and oil, making them very useful in a variety of industrial products that resist sticking, heat, water, stains, and grease like stain-resistant carpet, water-repellent clothes, paper and cardboard packaging, ski wax, and fire-fighting foam. PFOA can be bioaccumulated and once absorbed does not undergo biotransformation, distributing mainly in the plasma and liver, and, to a less degree, in the kidney and lungs (Kudo et al., 2003). Several studies describe detection and biomagnification of PFOA on wildlife, marine mammals included (Lau et al., 2007). PFOA is listed as an ED compound, because it can seriously impact phospholipid metabolism, reduce serum cholesterol (Peng et al., 2013), decrease testosterone and increase estradiol concentrations (Shi et al., 2007).

In this work the effects of BPA and PFOA exposure on bottlenose dolphin gene expression were analyzed using a species-specific microarray. In the first part of the study, an *ex vivo* assay was performed on bioptic skin tissue from a stranded bottlenose dolphin cultured and treated for 24h with different concentration of BPA and PFOA. The analysis of the genes in the cellular context under controlled condition aimed at the identification of differentially expressed genes and at the identification of biomarkers of CECs exposure.

This approach could be informative not only of the impact of CECs on dolphins (and marine mammals in general) gene expression and on the implications at the organism level of the molecular mechanisms that are affected, but also on the impact of threats posed to the marine ecosystem, including the unsustainable fishing, the tourism and development, the intense shipping, the climate change and the pollution. In regard to threats, this approach has a practical outcome due to the development of real-time tools, such as the expression of gene markers or biomarkers, that can be used for rapid detection of contaminant exposure.

In the second part of this study, in fact, we tested 4 putative biomarkers of BPA and PFOA exposure selected from the group of differentially expressed genes. The selected biomarkers were put to test using skin samples collected from *T. truncatus*

and *Stenella coeruleoalba* collected from stranding events and skin samples collected from free-ranging marine mammals, *S. coeruleoalba*, *Grampus griseus*, and *Balaenoptera physalus*.

2.2. Materials and methods

2.2.1. Marine mammals skin samples

The bottlenose dolphin skin sample for *ex vivo* assay was obtained 4 hr after the death of a juvenile female stranded on Tyrrhenian shores in Italy in October 2011. No relevant pathology, parasites and lesions were detected at post mortem examination and the cause of death is unknown. A total of 34 skin samples for biomarkers testing were collected between 2014 and 2016 from stranded *T. truncatus* and *S. coeruleoalba* along the Italian coasts, thanks to the collaboration with the Italian regional institutes for animal protection namely *Istituto ZooProfilattico* (IZS) specifically from the Italian regions of Sicily, IZSS, from Lombardia and Emilia-Romagna, IZSLER, and from Piemonte, Liguria and Valle D'Aosta, IZSPLVA. Three more skin biopsies from free-ranging marine mammals, *S. coeruleoalba* (Sc01PB), *Grampus griseus* (Gg01PB), and *Balaenoptera physalus* (Bp01PB) were collected during the scientific cruise "Plastic Buster 2014" in the Italian marine protected area *Santuario Pelagos for Mediterranean Marine Mammals* located in the Ligurian basin of the Mediterranean Sea (Prot. Num: 0017889/PM). Wild samples were collected thanks to the collaboration with Prof. MC Fossi and her group at the University of Siena and the ISPRA *(Istituto Superiore per la Protezione e la Ricerca Ambientale).* Samples were collected by dart biopsy and immediately stored in RNAlater (Qiagen, Hilden, Germany). Sample IDs used were labeled as follow: Sc17IZSS; Sc19IZSS; Sc20IZSS; Sc07IZSS; Sc26IZSS; Sc32IZSS; Sc34IZSLER; Sc01LER; Gg01PB; Sc01PB; Sc06IZSPLVA; Bp01PB and Tt01LT.

For all skin samples, total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany), and cDNA was obtained using iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) both according to manufacturer's instructions.

For sex determination of unknown samples, PCR for zinc finger Y-chromosomal (*ZFY*) and zinc finger X-chromosomal (*ZFX*) was performed in a total volume of 25 µl containing 1.25 µl of each primer at 0.5 µM, 20 ng cDNA and 12.5 µl of 2X DreamTaq Green PCR Master Mix 2 (Thermo Fisher Scientific, Massachusetts, USA), run as follows: 1 cycle of 95 °C for 3 minutes, 30 cycles of 95 °C for 30 seconds, 51 °C for 30 seconds, 72 °C for 30 seconds, 1 final cycle of 72 °C for 5

minutes. Primers for sex determination of free-ranging marine mammals were obtained from Bérubé et al., 1996 and the sequences are: forward primer, ZFX0582F, ATAGGTCTGCAGACTCTTCTA; reverse primers ZFX0923R, AGAATATGGCGACTTAGAACG and ZXY00767r, TTTGTGTGAACTGAAATTACA. Amplification products were visualized after electrophoresis on 2 % agarose gel for 20 minutes at 110 Volt.

2.2.2. *Ex vivo* assay

The organotypic cultures for the exposure experiments were carried out in 5 ml culture tube using 2 mm-thick slices spanning the epidermis and dermis cut from skin samples of the stranded specimen immediately after collection (Godard et al., 2004). A total of six slices, three for each contaminant, were separately incubated for 24 h at room temperature (24 – 28 °C) in cell culture media (Fossi et al., 2006). The cell culture media for the BPA experiment contained: 1) the vehicle, 0.01% ethanol; 2) 0.1 µg /ml BPA; 3) 1 µg /ml BPA. The cell culture media for the PFOA experiment contained: 1) the vehicle, 0.1% methanol; 2) 0.1 µg /ml PFOA; 3) 1 µg/ml PFOA. The concentrations used were selected using the ecological hazard and health advisory values reported by the US Environment Protection Agency (EPA) and based on worldwide analysis and reports (https://www.epa.gov/sites/production/files/2015-09/documents/bpa_action_plan.pdf;https://www.epa.gov/sites/production/files/2016-01/documents/pfcs_action_plan1230_09.pdf). Immediately after incubation, slices were homogenized using a Tissue Lyser (Qiagen, Hilden, Germany) and RNA was extracted using the Aurum™ Total Fatty and Fibrous Tissue kit (Bio-Rad, Hercules, California, USA) following manufacturer's instructions.

2.2.3. Dolphin microarray hybridization and gene expression analysis

The microarray used was a species-specific, custom 4x44K Agilent oligo array representing 24,418 unigene sequences (Mancia et al., 2015). All RNA labeling and microarray hybridizations were performed according to the manufacturer's

instructions for the One-Color Microarray-Based Gene Expression (Agilent Technologies, Santa Clara, CA). The microarray build and hybridization data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO accession numbers GSM1712791- GSM1712796 (www.ncbi.nlm.nih.gov/geo/).

Microarray results were analyzed using GeneSpring GX v.12 software (Agilent). Data transformation was applied to set all the negative raw values at 1. Data were normalized using quantile normalization. A filter on low gene expression was used to keep only the probes expressed in at least one sample. Then BPA- and PFOA-treated samples were analyzed compared with samples incubated in cell media containing the specific vehicles.

Differentially expressed genes were selected as having a 1.5 fold expression difference (geometrical mean) between the groups of interest and a statistically significant p-value (< 0.05) at moderated t-test statistic, followed by the application of the Benjamini and Hoechberg correction for false positives reduction.

2.2.4. Quantitative real time PCR: validation of microarray data

The expression of 4 genes, selected from the genes showing the highest levels of differential expression in the analysis, was quantified in all the treated samples using quantitative real time PCR (qPCR). The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) genes were used as internal controls for relative quantitation.

Relative mRNA expression levels were determined through qPCR analysis on CFX Connect (Biorad, Hercules, California, USA) with specific primers designed and optimized for efficiency and specifity by running standard curves. qPCR efficiencies were calculated using the equation from Dhar et al., (2009). One microgram of total RNA for each treated sample was reverse transcribed using iScript Select cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. Optimized qPCR parameters for each gene were determined using diluted (1:10) cDNA reverse

transcribed from 1 µg of total RNA using SsoFast™ EvaGreen® Supermix (Bio-Rad) in a total volume of 10 µl of a reaction mix containing 10 ng cDNA, 0.3 µM of each primer, 2X Evagreen enzyme and DNase-free sterile water. qPCR reactions were run as follows: 1 cycle of 98 °C for 30 minutes, 49 cycles of 95 °C for 5 minutes, 60 °C for 10 minutes; melting curve 65 °C - 95 °C: increment 0.5 °C every 5 minutes. Each reaction was run in triplicate, together with a triplicate of no-template control. The average Ct values were normalized to the values of the housekeeping genes *GAPDH* and *YWHAZ*. Comparative Ct method of analysis ($2^{-\Delta\Delta ct}$) was used to determined changes of expression between control and treated samples on CFX connect manager software 3.1 (Bio-Rad). Two-tailed, un-paired one-way ANOVA and Tukey post hoc test were performed used GraphPad Prism 5 (https://www.graphpad.com/scientific-software/prism/).

Primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). The sequences of the primers were: GAPDH-F: CGACCACTTTGTCAAGCTCA; GAPDH-R: CGGAGGACCTCTCTCTTCCT. YWHAZ-F: AGACGGAAGGTGCTGAGAAA; YWAHZ-R: TTTCTTGTCGTCACCAGCAG. BCAP31-F: GGTCGTACTCCTTGGTCAGG; BCAP31-R: GCCGTCAACAAGCAAAAACT. CDC42-F: AGTTGCTGGCCTTCTGAATC; CDC42-R: TCCAAGAGAGAAGGAATACATGC. MTSS1-F: AACCCACTTCAAGAGCAGATG; MTSS1-R: AGCTGGTTGGCCACTTTCT. AHR-F: AAGTCCATCCCAGGTGACAG; AHR-R: GCAAGTTCAGGCCTTCTCTG. RGS-F: AAGCCAAACCAGCAAGAAAA; RGS-R CAAATGCTTCTTCTGACCACAGC. ADIRF-F: TCCAACGTCGCTACGGTTAT; ADIRF-R: GGTCTCATCCACCACTTGCT.

*(For detailed methods on the microarray experiment, please see **PAPER I** in Appendix)*

2.3. Results and discussion

2.3.1 Gene expression profiles of BPA-treated skin biopsies

BPA treatment of organotypic cultures altered expression of 1146 genes of which 449 were upregulated and 697 downregulated in both conditions (fold > 1.5 and p value < 0.05) (Figure 2.1). Based on Gene Ontology (GO) analyses, the differentially expressed genes annotated are involved in metabolic and cellular process (81.4 %), immune response (18.2 %), developmental process (43.3 %), cellular component organization and biogenesis (45.3 %) and cellular signaling (32.8 %) (Figure 2.2). Because of its estrogenic activity, BPA can impact immune signaling pathways in many ways, activating T- and B-lymphocytes and dendritic cells, stimulating TNF-a production by macrophages, and leading to chronic activation of antigen-presenting cells (Kharrazian, 2014). We found many downregulated genes in the 'immune system process' category, like alpha 2 macroglobulin precursor or class I histocompatibility alpha chain. The activation of the genes involved in 'response to stress' GO category after BPA exposure can be linked to a generalized response of the cells to an emergency condition where the cellular pathways involved in the malfunctioning of proteins are interrupted by the change in regulation of key genes, and defected cells keep dividing, avoiding physiological apoptosis. Estrogenic effects of BPA can impact adipogenesis and body weight, through complex interactions with the estrogen, thyroid hormone and glucocorticoid receptors, causing metabolic disturbances (Casals-Casas and Desvergne, 2011; Wang et al., 2012). We found that BPA may affect pathways involved in fat (blubber) differentiation, down regulating several genes involved in lipid metabolism such as the adipogenesis regulatory factor (down, 1.80 fold).

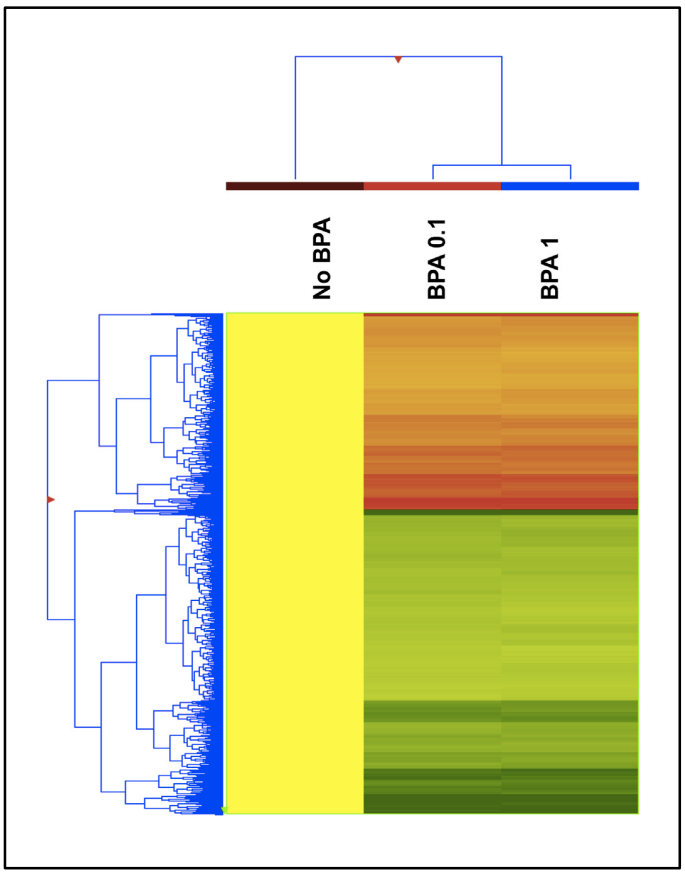

Figure 2.1. BPA-treated clustering of microarray.
Hierarchical clustering of microarray data showing the differentially expressed genes of dolphin skin organotypic cultures treated with different concentrations of BPA (0.1 and 1 µg/ml). The dendrogram on the left depicts the 1146 genes differentially expressed (fold change > 1.5 and p-value < 0.05). Red: up-regulated genes, green: down-regulated genes.

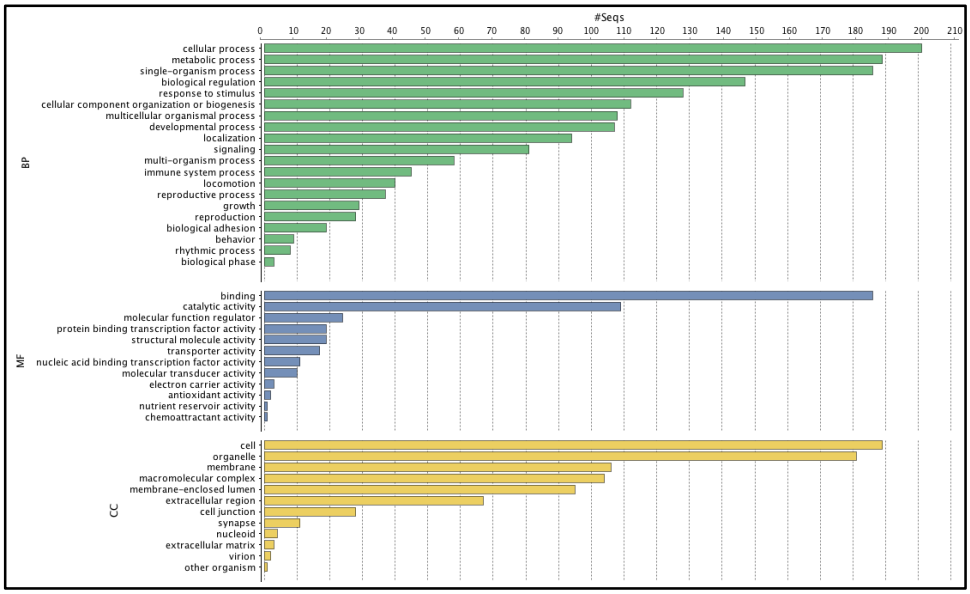

Figure 2.2. Gene Ontology of gene distribution of genes differentially expressed by BPA.
BP: Biological Process. MF: Molecular Function and CC: Cellular Component.

2.3.2. Gene expression profiles of PFOA-treated skin biopsies

PFOA treatment differentially regulated transcription of 428 genes in total (fold > 1.5 and p-value < 0.05), of which 191 were upregulated and 237 were downregulated in both conditions (Figure 2.3)". GO analysis of the annotated genes collocated most of them in the following categories: cellular process (32.5 %), metabolic process (27.4 %), single organism process (25.7 %), biological regulation (20.5 %), immune system process (2.6 %), cellular component organization or biogenesis (15.4 %), localization (12.8 %), multicellular organismal process (9.4 %), developmental process (8.5 %) (Figure 2.4). PFOA treatment caused transcription changes of genes different from that effected by BPA. The different chemical structures and mechanisms of action of the two compounds can likely account for these findings. The genes in the GO involve in response to stress include the uv excision repair protein rad23 homolog and the heat shock protein 90. PFOA has been associated with significant changes in clinical markers of immune and inflammatory process, and exposure to PFOA can adversely affect immune function (DeWitt et al., 2012). We found variations in the genes engaged in the immune system activation. Those genes, like the acidic mammalian chitinase, the interferon induced protein and the interleukin 8 belong to the GO category of inflammatory response (Zhu et al., 2004; Wu et al., 2016).

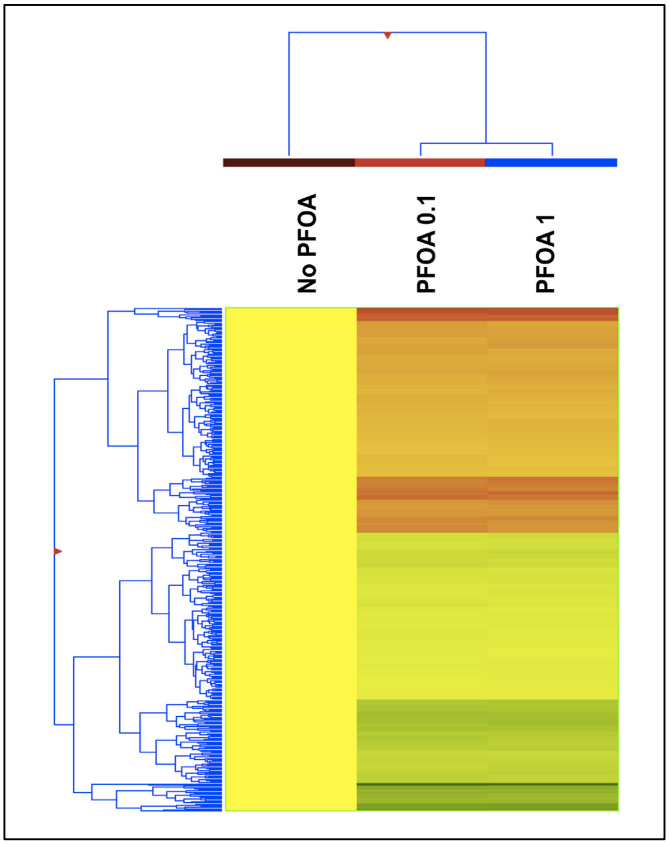

Figure 2.3. PFOA-treated clustering of microarray.
Hierarchical clustering of microarray data showing the differentially expressed genes in dolphin skin organotypic cultures treated with different concentrations of PFOA (0.1 and 1 µg/ml). The dendrogram on the left depicts the 428 genes differentially expressed (fold change > 1.5 and p-value 0.05). Red: up-regulated genes, green: down-regulated genes.

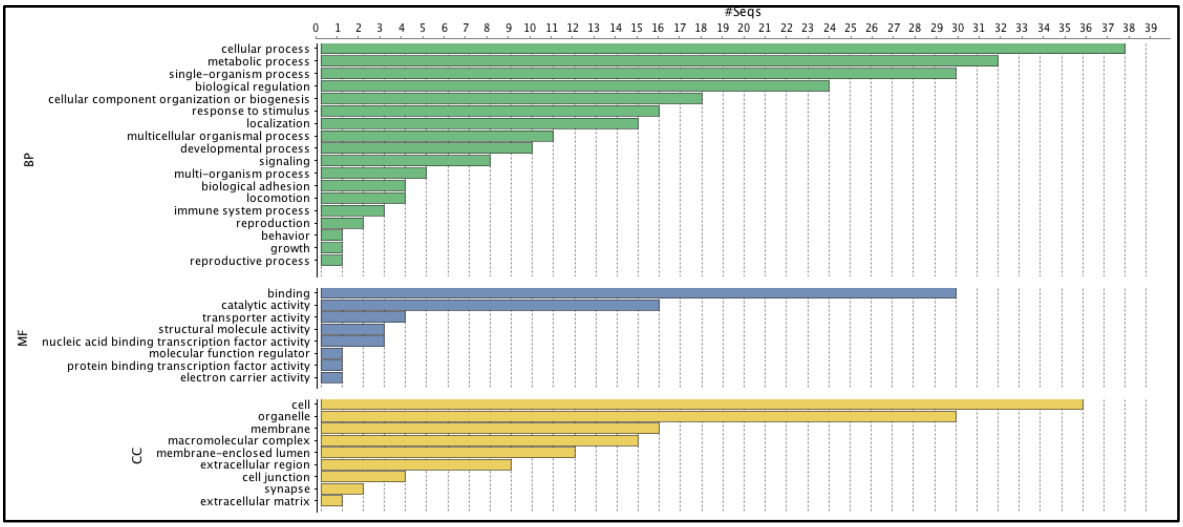

Figure 2.4. Gene Ontology of gene distribution of genes differentially expressed by PFOA.
BP: Biological Process. MF: Molecular Function and CC: Cellular Component.

2.3.3. Validation of microarray results on treated organotypic cultures

Semi-quantitative gene expression analysis resulting from the microarray study was validated through qPCR. Four genes among those differentially expressed were chosen for the analysis testing the same RNA used in microarray analysis. qPCR confirmed microarray data for the selected genes at a contaminant concentration (1 µg/ml) closer to that measured in the environment. At this dose, the contaminant is probably more toxic and poisoning, hence causing a stronger cellular response. From microarray analysis, the adipogenesis regulatory factor (*ADIRF)* is downregulated (1.7 fold) and B-cell receptor-associated protein 31 (*BCAP31)* is upregulated (2.13 fold) in BPA treatments; regulator of G-protein signaling 2 (*RGS2)* and cell division cycle 42 (*CDC42)* were found to be upregulated (3.2 and 4.5 fold, respectively) in PFOA treatments. In Figure 2.5 qPCR and microarray results are compared: there are differences in the quantification of the expression of the genes between the two methods of analysis, with the qPCR being the more quantitatively reliable method then the microarrays.

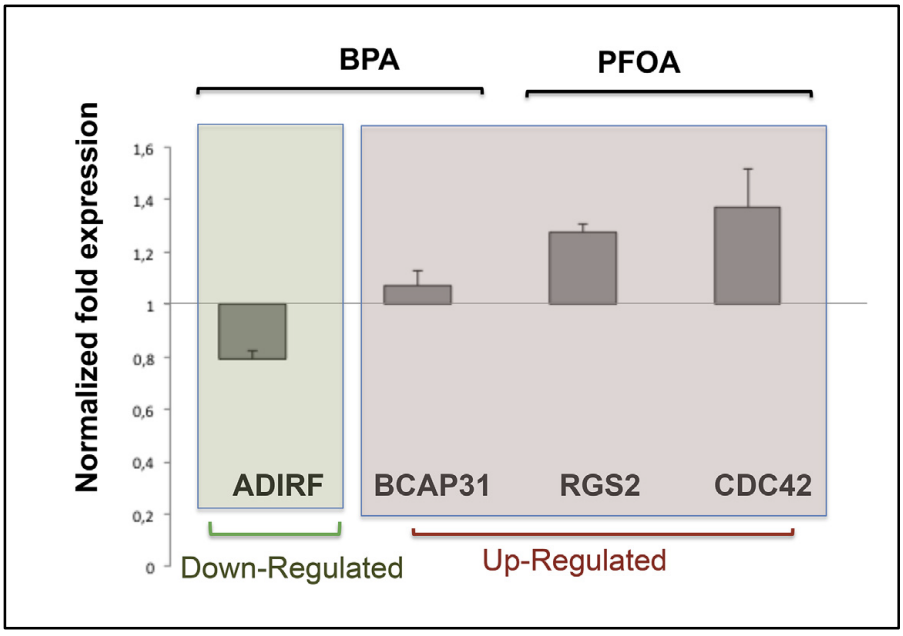

Figure 2.5. Validation of microarray data.
From left to right: results from qPCR for genes: *ADIRF*, downregulated by BPA, *BCAP31*, upregulated by BPA, *RGS2* and *CDC42* upregulated by PFOA. Bottom: green and red brackets, genes up- and down-regulated in microarray analysis. Data were normalized to *GAPDH* and *YWHAZ* genes.

2.3.4. Wild marine mammals sample quality and quantity: problems and solutions.

One major objective of this investigation was to develop “gene markers” using a non-invasive method through both skin biopsies of beached free-ranging dolphins and dart collection from live marine mammals during survey campaign to assess the effects of a specific contaminant exposure on the health status of the animals inhabiting the Mediterranean Sea. Samples were in fact collected from three principal basins in the Mediterranean Sea: Ionian, Adriatic and Tyrrhenian Seas.

A total number of 31 skin biopsies were collected from the side of the animal, close to the dorsal fin, during stranding events from 3 different Italian basins and 3 more samples were collected from free-ranging cetaceans in the *Pelagos Sanctuary,* in the northern part of the Tyrrhenian sea. Of the 33 total samples, 2 were from the *T. truncatus* species, 29 from the S. *coereouloalba* species, one from *B. physalus,* and one from *G. griseus.* The most critical part of the experiment was the RNA extraction for several reasons: first of all, natural degeneration of RNA occurs very quickly and RNA quality is indeed linked to the promptitude of the sampling after the stranding event and to the conditions of the animal stranded; also, samples conservation and mailing to the processing laboratory was, in many occasions, challenging. RNAlater, the preservation buffer, needs to perfuse completely the tissue in order to preserve it for long term and there are specific instructions by manufacturer on the size of the sample and the volumes of buffer to be used in order to obtain the optimum conditions of preservations. Finally, the marine mammal skin tissue has a very hard texture, and can be very resistant to rupture and homogenization. We were able to extract total RNA from only 14 skin biopsies, 7 from the Ionian Sea (6 *S. coeruleoalba* and 1 *T. truncatus*), 5 from the Tyrrhenian Sea (*B. physalus*, *G. griseus,* 2 *S. coeruleoalba and* 1 *T. truncatus)* and 2 from the Adriatic Sea (both *S. coeruleoalba)* (Table 2.1). In order to retrieve information of the gender of the free-ranging animal sampled through dart biopsy, the amplification test previously published by Bèrubè et al. (2006) was employed. The test relies on the amplification of the genes *ZFY/ZFX*. Females express only *ZFX*, while males express both *ZFX* and *ZFY*. The forward primer is complementary to both *ZFX* and *ZFY* sequence while the reverse primer is specific for each gene. The assay was

successful as we were able to identifying the gender of each animal sampled during the scientific cruise. The amplification products of 328 bp and 257 bp were present for *B. physalus and G. griseus*, identifying the gender as male. The amplification product for *S. coeruleoalba* was only one, the fragment of 328 bp, identifying the female gender (Figure 2.6). Gender of animals stranded was easily identified by physical examination.

Sampe ID	Species	Sex	Location of sampling	RNA [ng/µl]	RNA OD (260/280)	RNA OD (260/230)
Sc17IZSS	*S. coeruleoalba*	female	Ionian	173.06	2.1	1.96
Sc19IZSS	*S. coeruleoalba*	male	Ionian	105.75	1.99	1.44
Sc20IZSS	*S. coeruleoalba*	female	Ionian	74.76	1.77	1.14
Sc07IZSS	*S. coeruleoalba*	female	Ionian	70.54	2.43	1.87
Sc26IZSS	*S. coeruleoalba*	male	Ionian	301.62	2.11	1.75
Sc32IZSS	*S. coeruleoalba*	female	Ionian	263.67	2.12	1.9
Tt02IZSS	*T. truncatus*	female	Ionian	121.75	3.16	1.6
Sc01LER	*S. coeruleoalba*	female	Adriatic	696.76	2.15	2.22
Sc34IZSLER	*S. coeruleoalba*	female	Adriatic	411.44	2.14	2.47
Sc01PB	*S. coeruleoalba*	female	Tyrrhennian	117	2.15	1.91
Sc06IZSPLVA	*S. coeruleoalba*	female	Tyrrhennian	100.35	2,13	1.8
Bp01PB	*B. physalus*	male	Tyrrhennian	263.08	2.17	1.25
Tt01LT	*T. truncatus*	female	Tyrrhennian	1072.2	2.5	NA
Gg01PB	*G. griseus*	male	Tyrrhennian	612	2.16	2.18

Table 2.1. List of the samples used in biomarker testing.
The table shows animal ID, gender, geographic location of sampling, and concentration and absorbance of RNA successfully extracted from skin biopsy of 13 marine mammals.

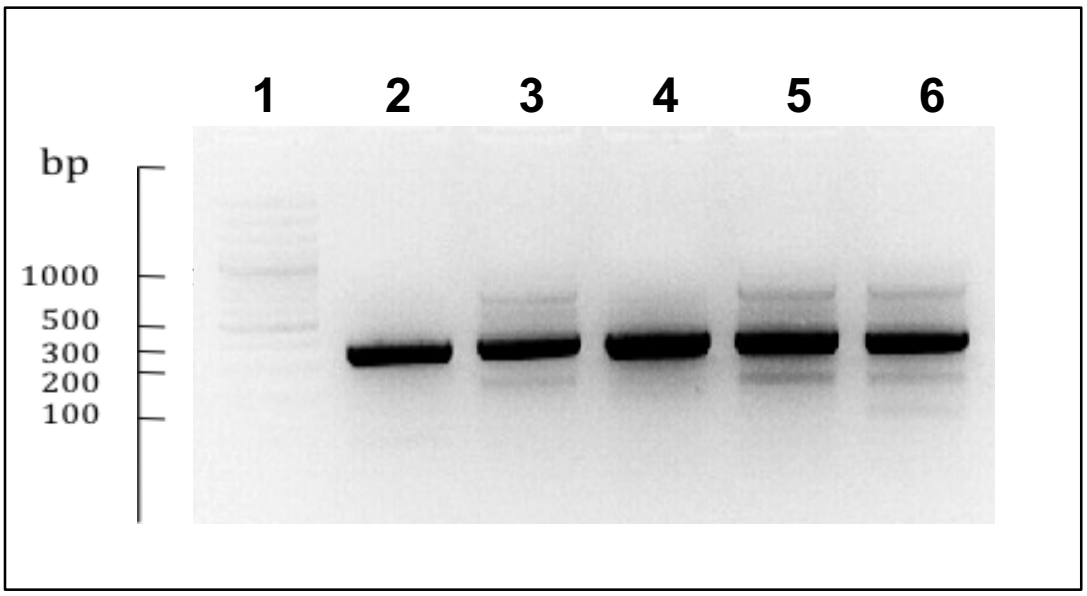

Figure 2.6. Expression of *ZFX/ZFY* in the skin of free-ranging marine mammals. Electrophoresis on 2 % agarose gel of the amplification products of 2, a female control; 3, male control; 4, *S. coeruleoalba* (Sc01PB); 5, G. *griseus* (Gg01PB); 6, *B. physalus* (Bp01PB). 1, DNA ladder, 100bp.

2.3.5. Expression trends of potential biomarkers in wild animals

In order to tests the validity of the selected genes as markers of exposure for BPA and PFOA, 12 of 14 total samples were selected and put to test. The RNA sample from Tt02IZSS was contaminated, even after re-purification and was not included in future analyses. After a first comparison across species, also the sample collected from *B. physalus*, Bp01PB, was not included in future analyses because of the great difference in the expression of the genes tested when compared to all the others species (Figure 2.7).

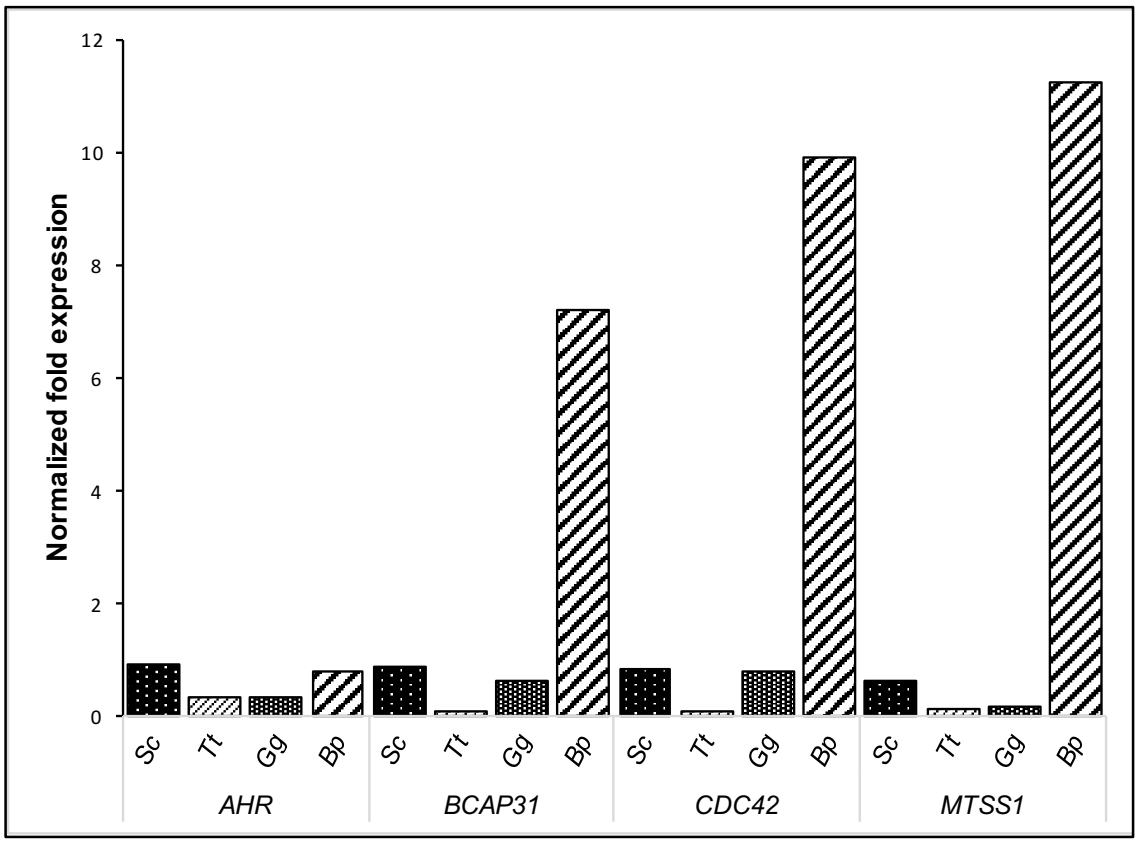

Figure 2.7. Comparison of the expression level of 4 selected genes in the 4 different species tested.
Mean values, where possible, across species is shown. Sc, *S. coereuleoalba*; Tt, *T. truncatus*; Gg, *G.griseus*; Bp, *B.physalus.*

In fact, differently from *T. truncatus, S. coereuleoalba* and *G. griseus*, Odontoceti of the family Delphinidae, *B. physalus* belongs to the family Balaenopteridae, sub-order Mysticeti, a group of cetaceans with very different dietary and migration behaviors, indicating potential differences in the mechanisms of response to contaminants.

The 4 genes selected were in the list of the genes that showed differential expression in the microarray study. The first gene tested was the cell division control protein 42 homolog *(CDC42),* upregulated in PFOA treatment. *CDC42* is a member of the Rho GTPase family, and regulates several cellular functions, such as differentiation, proliferation, migration and polarity; *CDC42* is a key mediator of actin-based cytoplasmatic extension and is implicated in propagation of apoptotic signals (Melendez et al., 2013; Qadir et al., 2015). *CDC42* seem to have a role in helping cancer progression: in non-small cell lung cancer and in cervical cancer in fact, *CDC42* is overexpressed (Stengel et al., 2011; Ye et al., 2015). In mouse

models, the overexpression of *CDC42* in the developing mammary induces gland hyperbranching (Bray et al., 2013) and cardiac hypertrophy (Huang et al., 2016).

The second gene selected was the metastasis suppressor 1 (*MTSS1*), downregulated by BPA exposure. This gene may play a role in the aggressiveness of cancer cells (Du et al., 2011). Initially *MTSS1* was proposed as potential suppressor of metastasis, but some studies highlighted the correlation of its expression to cytoskeleton and actin filament organization during organ development (Glassman et al., 2007; Kayser et al., 2015). *MTSS1* upregulation correlate with malignant transformation such as melanoma, and head and neck squamous cell carcinoma (Dawson et al., 2012; Mertz et al., 2014). *MTSS1* expression is also increased during the progression of hepatocellular carcinogenesis (Ma et al., 2007; Kaysera et al., 2015).

The third selected gene was B-cell receptor 31 (*BCAP31*), upregulated after exposure to BPA. *BCAP31* is a multi-pass transmembrane protein of the endoplasmatic reticulum involved in the anterograde transport and also in caspase-8 mediated apoptosis (Wang et al., 2011). Mutation in *BCAP31* was recently described in optic atrophy, deafness, congenital dystonia and central hypomyelination (Vittal et al., 2015). *BCAP31* might contribute to the regulation of neuronal apoptosis (Limviphuvadh et al., 2007).

AHR is a ligand-activated transcription factor with a key role in the regulation of xenobiotic compound detoxification (Hahn, 1998; Panti et al., 2011). *AHR* interacts with polycyclic and planar halogenated aromatic hydrocarbons (PAH or PHAH) and dioxins, activating the cytochrome P4501A, a member of the superfamily of enzymes involved in Phase I of the oxidative metabolism of exogenous compounds, with a key role in the biotransformation of contaminants. Given its critical role and its absence from the features on the microarray, it was included in the gene marker panel tested.

The expression levels of the four selected genes were compared for geographic area of sampling and gender.

Gender differences were not significant, with only 2 specimens in the experimental male group.

Levels of mRNA expression for the selected genes have different trends in the three different Mediterranean Sea basins, suggesting differences in the marine environment of the three geographic locations where animals live, feed and breed. Gene expression levels of *AHR* and *BCAP31* were significantly higher in the samples from the Adriatic Sea when compared to those of the Tyrrhenian Sea. Moreover, *BCAP31* has significantly higher mRNA levels also between Adriatic Sea and Ionian Sea (*AHR*, $p = 0.0183$; *BCAP31*, $p = 0.01069$) (Figure 2.8). *CDC42* had the higher level of expression in the animals sampled in the Adriatic Sea and the lower in those sampled in the Tyrrhenian Sea, but this difference was not statistically significant. Conversely, *MTSS1* had higher levels of expression in the animals from the Ionian Sea, but the difference was not statistically significant (Figure 2.8).

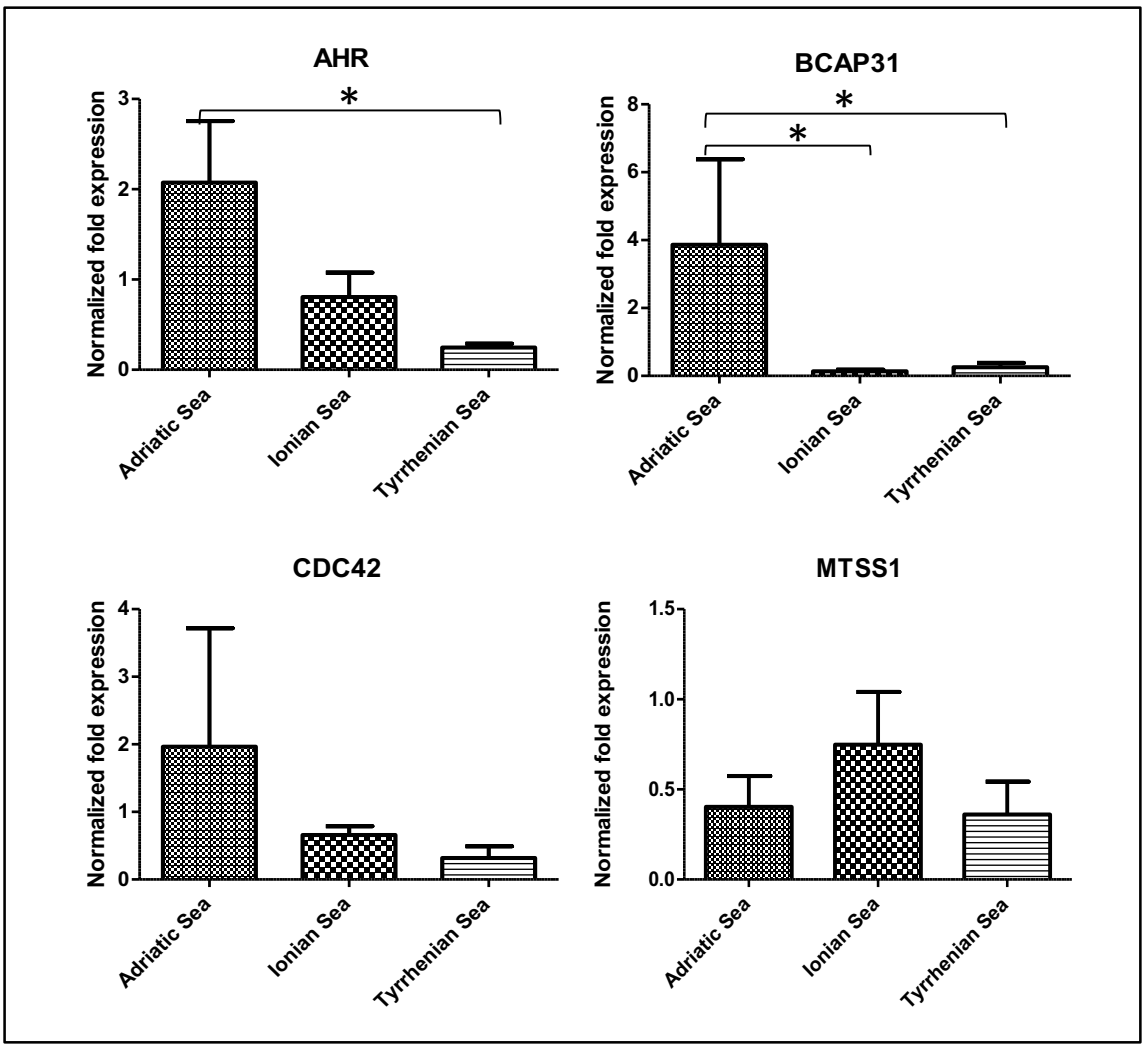

Figure 2.8. Gene expression levels of *AHR, BCAP31*, *CDC42* and *MTSS1* in 12 skin biopsies.

Each bar corresponds to the mean expression of all samples from the same geographical location ± SD. Brachets show statistically significant comparison after Tukey test (*p-value < 0.05). (One-way ANOVA p-value: AHR: 0.0183; BCAP31: 0.0106).

The results suggest a higher expression of *AHR* in the animals resident of the Adriatic Sea which may also be indicative of the higher exposure to PAHs, dioxins, and anthropogenic contaminants that have been previously described in the Adriatic basin (Garritano et al., 2005; Perra et al., 2011). *BCAP31* was upregulated in BPA treated samples analyzed by microarray, and its higher expression in the samples from the Adriatic Sea when compared to the samples of both the Tyrrhenian and Ionic Sea, may be indicative of the presence of this chemical in the Adriatic basin as well. The higher concentration of the contaminants in the Adriatic Sea is probably related to its geographical characteristics: it is, in fact, the northernmost basin of the Mediterranean Sea; it is a semi-enclosed sea, surrounded by the Italian Peninsula in the West and North-West part, and by Slovenia and Greece in its North-East part, with only one connection to the Ionian Sea through the Strait of Otranto. In the Adriatic Sea, currents are counter-clock from the Strait of Otranto, making water exchange difficult thus allowing accumulation of contaminants, especially in the northern part. Conversely, the samples from the Tyrrhenian Sea were collected from the *Pelagos Sanctuary,* a more pristine marine area, protected since 1992, characterized by a management plan focused on the reduction of human activities and pollution (http://www.sanctuaire-pelagos.org/en/).

BCAP31 expression can be altered also by other anthropogenic contaminates, like methylmercury (MeHg), which has well-known toxic effects in humans (Cuello et al., 2012), and by natural toxins, like okadaic acid (Valdiglesias et al., 2012). Okadaic acid is a toxin produced by several species of dinoflagellates, and is known to accumulate in both marine sponges and shellfish, in particular during algal bloom events (Regueraet al., 2014). In the Mediterranean Sea, okadaic acid is produced by *Prorocentrum lima* as described in the Italian Health Ministry reports (Ministero Della Salute, ITALIA, 2006: http://www.salute.gov.it/imgs/c_17_pubblicazioni_641_allegato.pdf)

The preliminary results shown reflect geographical difference in gene expression that may be correlated to different environmental conditions. Indeed, samples from a more known contaminated area such as the Adriatic Sea, are those that have showed the most significant differences, but in order to draw any final conclusions more samples need to be collected and tested.

2.4. Conclusions

In the first part of this investigation we successfully employed a novel approach: the use of the dolphin skin biopsy, an *ex vivo* assay, coupled with a species-specific microarray analysis, to assess the effects of two environmentally threatening contaminants on wild dolphin health. Although this is a preliminary study, the impact of BPA and PFOA exposure on dolphin gene expression at the skin level can lead to hypotheses on how it could impact the health of the entire organism. With both contaminants we observed changes in expression of genes involved in the activation of both an immune and stress response. We have also observed changes in molecular pathways involved in lipid homeostasis and development, most likely linked to the ability of the chemicals to interfere with dolphin's natural hormones, that travel in the bloodstream and regulate many important physiological activities. Of course the interference with the body endocrine system can increase risks of developing metabolic diseases or cancer susceptibility, and can have adverse effects on fertility and reproduction, and on the proper functioning of the nervous system. In the second part, we selected and tested genes that can act like markers of exposure on 13 skin biopsies from stranded and free-ranging dolphins. Our preliminary results showed consistency between the levels of expression of two of the genes tested with the most contaminated geographic location. Although the sample set needs to be increased, the approach taken is promising. The skin is the first barrier at the environment interface and it is also the easiest tissue that can be obtained from marine mammals through a non-invasive sample collection method. We demonstrated that exposure to endocrine disruptors, such as BPA and PFOA, even if used individually, can alone alter skin gene expression. Testing a mixture of contaminants (and toxins, where present) more closely related to the environmental conditions, could lead to a more specific selection of potential marker of exposure location-specific. The use of gene expression microarray with *ex vivo* assay and the selection of markers of exposure could be very informative and useful method to better understand the underlying mechanisms that alter health status of dolphin, helping us in the health status management of the Mediterranean Seas and its inhabitants.

*A part of the results of this study are now in the form of a published manuscript (see **PAPER I** in Appendix).*

3. RNA-sequencing technology: establishment of baseline data for the study of the bottlenose dolphin blood transcriptome.

3.1. Introduction

The blood transcriptome of an organism reflects the physiological status of all the organs of the body. The immune cells in blood have an active role in 1) communication with hormones, cytokines and metabolites, and in the 2) migration between lymphoid and any injured tissues, thus giving important information about any pathological condition of the organism. In mammals, blood cellular components are made up to 96 % red blood cells and 4 % leukocytes. Because leukocytes composition varies in response to the need of the organism, the blood is one of the most dynamic tissues. Due to this variation, the blood transcriptome can change quickly in response of stimuli thus being informative of an organism disease, of an organism exposure to a specific environmental condition and of the organism general health status. It is established that exposure to toxicants alters gene expression in peripheral blood (Mhor et al., 2007) and in human's blood transcriptomic signatures are widely used to identify medical conditions (Mhor et al., 2007). Longitudinal study in healthy human blood transcriptomes shown minimal intra-individual variations over one month, but significant variability over three or six months (Karlovich et al., 2009; De Boever et al., 2014), highlighting how the baseline data can change and that the variations need to be considered. Recent study shown seasonal change in human blood transcriptome, correlated to hematological fluctuations during the year (De Jong et al., 2014). Genes involved in the circadian clock are shown be responsive to day length while the immune function genes, involved in the maintaining of a pro-inflammatory state are highly expressed during the winter in populations sharing high latitudes. In contrast, the same genes in tropical populations are highly expressed in rainy season (Dopico et al., 2015). Many of these transcriptomic changes are correlated with changes in blood composition (De Jong et al., 2014; Dopico et al., 2015). In veterinary medicine, blood transcriptome analysis is used to identify infections in companion animals and economically relevant species (Chaussabel et al., 2015). However, to date, application of high throughput transcriptomics in non-model organism is limited and the lack of sequenced genomes is surely one major constraint. In non-

model organisms, such as wild free-ranging animals, often even obtaining samples can be challenging. In the marine mammal field, transcriptomic analysis of peripheral blood identified potential markers for nutritional stress in California and Stellar sea lions (Spitz et al., 2015; Mancia et al., 2012). In bottlenose dolphin, gene expression microarray analysis described the activation of a stress response in wild animals during capture-released study (Mancia et al., 2008; Mancia et al., 2010), highlighting the importance of the considerations needed in the generation of baseline data for health assessment. Blood gene expression microarrays have also been used to describe the impacts of long-term chemical contaminants exposure linked to the geographic location of resident populations (Mancia et al., 2014; Mancia et al., 2015). Skin transcriptome analysis has also been used to characterize the potential effects of the exposure to endocrine disruptors compounds (such as bisphenol A, BPA, and perfluorooctanoic acid, PFOA) (Lunardi et al., 2016).

The aim of this investigation was to contribute to the establishment of baseline data for blood transcriptome analysis, in order to accurately describe the health status of the bottlenose dolphin, *T. truncatus.* Four healthy and managed dolphins (two males and two females) in Hawaii were sampled monthly during the year 2013. Dolphin's hematological parameters were known, and were correlated to the respective blood transcriptomic profiles. Preliminary tests revealed high quantity of hemoglobin transcripts, so it was necessary to perform a specific protocol for globin depletion, in order to have access to all the transcripts of interest, even those less represented in the transcriptome. A parallel analysis was conducted: one using the publicly available dolphin genome as guide and the other using *de novo* assembling.

3.2. Materials and methods

3.2.1. Sampling

Blood samples were collected in PAXgene tubes (Qiagen, Valencia, California, USA) from the ventral side of the flukes of four managed *T. truncatus* residing at Dolphin Quest, Waikoloa, Hawaii, USA, at approximately monthly intervals during the year 2013. The dolphins sampled for this study included two males, ages 5 (Hua, n = 8) and 17 (Kainalu, n = 7), and two females, ages 12 (Keo, n = 9) and 28 (Pele, n = 7). All animals were trained to participate in monthly veterinary checkups including routine blood draws, conducted in the mornings after overnight fasting. Hematological parameters were measured quarterly on samples collected in parallel with the transcriptome samples.

3.2.2. RNA extraction

Whole blood RNA was extracted using a PAXgene Blood RNA Kit (Qiagen, Valencia, California, USA), according to the manufacturer's protocol. RNA concentrations were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, California, USA). Only samples with a RIN (RNA Integrity Number) 7 were sequenced.

3.2.3. Hemoglobin depletion

RNA-seq analysis of a test sample revealed a high percentage of globin transcripts in the dolphin peripheral blood transcriptome (65 – 75 % of reads). We applied a modified Affymetrix globin depletion protocol utilizing an RNase H (Invitrogen, Carlsbard, California, USA) treatment in order to improve the diversity of transcripts detected by RNA-seq (Wu et al., 2007; Choi et al., 2014; Kommadath et al., 2014). RNA quality of globin-depleted samples was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, California, USA).

3.2.4. Reverse transcription and qPCR

To determine the extent of globin depletion and any non-specific impact on transcript levels, mRNA levels of HBA and four additional genes (delta-aminolevulinate synthase 2, ALAS2; FK506 binding protein 8, FKBP8; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; ribosomal protein L13, RPL13) were assessed by quantitative real-time PCR in samples pre- and post-globin depletion. The specificity of qPCR primers and the size of the amplicon were verified by analysis with an Agilent Bioanalyzer 2100 and further confirmed by melting curve analysis. The reaction efficiency was determined using a standard curve of cDNA from total RNA. Ct values of the pre- and post-globin depletion samples were used to determine the relative expression of the gene in each sample.

3.2.5. Sequencing

Globin depleted (n = 31) and total RNA (n = 6) samples were sent to North Carolina State University Genomics Service Laboratory for library preparation using a NEBNext Ultra Directional RNA Library Prep Kit for Illumina and indexed with the NEBNext Mulitplex Oligos for Illumina (New England Biolabs, Ipswich, Massachusetts, USA). Sequencing was performed on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, California, USA), at a targeted depth of 28 million, 100 nucleotide (nt) single end reads for globin depleted samples. Total RNA from samples that lacked sufficient RNA concentration to perform the globin depletion step were sequenced at a depth of 45 million reads, with the exception of Kainalu May which was sequenced at a depth of 28 million reads for a direct comparison with a globin depleted aliquot of the same sample (Table 3.1).

Animal	Sex	Age (years)	Sample date	Season	Globlin depleted	Millions Reads seq.	Monthly Mean Water Temp	Day length (hours)
Hua	m	5	02/06/13	Winter	Si	28	24.6	11.36
			03/21/13	Spring	Si	28	24.4	11.78
			04/27/13	Spring	Si	28	24.8	12.78
			05/23/13	Spring	Si	28	25.2	13.15
			06/12/13	Spring	No	45	25.4	13.24
			07/08/13	Summer	Si	28	25.9	13.25
			08/23/13	Summer	Si	28	26.4	12.65
			10/11/13	Autumn	Si	28	26.4	11.75
			12/02/13	Winter	Si	28	25.1	11
Kainalu	m	17	02/05/13	Winter	Si	28	24.6	11.36
			04/14/13	Spring	Si	28	24.8	12.58
			05/25/13	Spring	Si	28	25.2	13.18
			06/15/13	Spring	Si	28	25.6	13.3
			07/08/13	Summer	Si	28	25.9	13.25
			09/13/13	Summer	Si	28	26.6	12.27
			12/02/13	Winter	Si	28	25.1	11
Keo	f	12	02/13/13	Winter	Si	28	24.6	11.48
			03/08/13	Spring	Si	28	24.4	11.88
			04/01/13	Spring	Si	28	24.8	12.3
			05/17/13	Spring	Si	28	25.2	12.95
			06/12/13	Spring	Si	28	25.9	13.3
			07/14/13	Summer	No	45	26.2	13.25
			08/28/13	Summer	Si	28	26.4	12.57
			09/29/13	Summer	No	45	26.3	12
			10/29/13	Autumn	Si	28	26.4	11.45
			11/27/13	Autumn	Si	28	25.9	11.05
			12/09/13	Winter	Si	28	25.1	11
Pele	f	28	02/13/13	Winter	Si	28	25.1	11
			03/21/13	Spring	Si	28	24.4	12.13
			04/18/13	Spring	No	45	24.8	12.40
			05/24/13	Spring	Si	28	25.2	13.16
			06/24/13	Spring	No	45	25.5	13.26
			07/13/13	Summer	Si	28	25.9	13
			08/22/13	Summer	Si	28	26.4	12.67
			09/07/13	Summer	Si	28	26.6	12.37
			12/13/13	Winter	Si	28	25.1	10.95

Table 3.1. List of all samples used and parameters measured.
Male dolphins, m; female dolphins, f.

3.2.6. Genome-guided transcriptome assembly and *de novo* assembly

Sequence processing and analysis was carried out in iPlant Collaborative's Discovery Environment (http://www.cyverse.org/) using the High-Performance Computing applications (Goff et al., 2011). The Illumina bcl (base calls per cycle) output files were converted to text-based FASTQ file format and sequence quality trimming was performed using Trimmomatic (Bolger et al., 2014) with a minimum phred quality score > 20 over the length of the reads. The trimmed reads were then quality checked using the FASTQC tool. To assess the effectiveness of globin depletion, reads were mapped to the Ensembl *T. truncatus* genome, turTru1 v76.1, using Tophat2 v 2.3.13 (Kim et al., 2013) with Bowtie2 v 2.2.4 (Langmead et al., 2012) as the alignment engine. Mapped read counts, as FPKM (fragments per kilobase of transcript per million mapped reads), were generated using Cufflinks v 2.2.0 (Trapnell et al., 2012) with the genome as a reference. Differential expression analysis was performed using Cuffdiff v 2.1.1 (Trapnell et al., 2012) and visualization generated by CummeRbund (Trapnell et al., 2012).
Differential expression analyses were performed in EBSeq (Leng et al., 2013) using a false discovery rate (FDR) of 0.05. The raw reads and summarized FPKMs for all samples are available on GEO (accession # GSE78770).

The processed and trimmed reads were also used to construct a *de novo* transcriptome using the Trinity assembler (Grabherr et al., 2011) on iPlant Collaborative Discovery Environment. The read files from one summer and one winter globin depleted samples from each animal (n = 8; Hua: February and September, Kainalu: February and August, Keo: February and August, Pele: February and September) were concatenated into a single fastq.

3.2.7. Statistical analysis

Principal component analysis (PCA) was performed on log2 transformed FPKM values for all genes that had an FPKM > 0 in at least half of the samples and an average FPKM value across all samples of 1 FPKM.

Seasonal expression patterns of genes found to be differentially expressed between summer (July, August, and September) and winter (December and February) months were explored by transforming longitudinal data from all samples (n = 31) to estimate a cosinor linear model (Tong, 1976).

A gene co-expression network was generated using WGCNA (v 1.51) (Langfelder et al., 2008) in R (v 3.3.0) on log2 transformed FPKM values for all genes that had an FPKM > 0 in at least half of the samples and an average FPKM value across all samples of 1 FPKM.

3.3. Results and discussion

3.3.1. Preliminary test and globin depletion

The first dolphin blood sample was sequenced at the target depth of 15 million reads and revealed approximately 75 % of the reads to be hemoglobin sequences. In order to increase sequence detection, a protocol for hemoglobin depletion was performed using a modified Affymetrix globin depletion protocol. The protocol reduces the globin effect by blocking reverse transcription of globin mRNA during the target preparation procedure thus enabling sensitive gene expression analysis. Recovery of RNA was high, with a median of 100.4% and RIN number decreasing from 8.4 ± 0.06 to 7.8 ± 0.009. Decrease in RIN values are common, while recovery is reported to be very variable and often low (Choi et al., 2014). Depletion was confirmed using qPCR: the hemoglobin subunit alpha (*HBA*) was reduced by 286.4 ± 1.3 fold (t-test, $p < 0.05$, $n = 5$), while control genes did not significantly change (t-test, $p > 0.05$, $n = 5$) (Figure 3.1A). To further investigate goblin depletion only one sample was sequenced at the depth of 28 million reads both pre- and post-depletion (Kainalu May) showing a reduction of 99.5 % for *HBA*, 92 % for hemoglobin subunit beta (HBB) and 35.8 % for hemoglobin subunit mu (*HBM*). Five additional pairs of samples were analyzed, depleted and non-depleted, obtaining 98.8 % of reduction for *HBA*, 80 % for *HBB* (Figure 3.1B), while *HBM* shown a non-significant decrease of 1.3 fold. A gene ENSTTRG00000012084 annotated as a novel protein coding gene in dolphin genome was highly expressed in non-depleted samples and is mostly undetected in depleted one (99.9 % of reduction). This gene is on a gene scaffold containing only hemoglobin genes, and blastx searches on NCBI database revealed this gene to be HBA. Also ENSTTRG00000009506 was annotated as novel gene, was reduced by 71.6 %, and is located on scaffold with *HBB* and hemoglobin E (*HBE*), and a blastx search identified it as HBB. Globin depletion showed little impact on others genes, with only 3.7 % ($n = 790$) of the genes showing significantly different expression between pre- and post- samples (Cuffdiff, FDR < 0.05, $n = 5$). Only 357 of these genes are highly expressed in depleted samples. With globin depletion, 2244 additional genes (10.6 %) were detected at a FPKM 1 (Figure 3.1C); similar results were obtained with Affymetrix protocol in porcine or human (Wu et al., 2007). Five samples were sequenced at depth of 45 million reads due to RNA quantity limitations. These samples (Pele June; Hua July;

Keo September; Keo July; Pele April) were compared with depleted samples sequenced at 28 million reads, showing only 268 additional gene detected (1.3 % of the entire genome): when RNA-seq is performed at a sufficient depth, it can overcome high signals of hemoglobin reads. For dolphin blood transcriptome studies using RNA-seq, 45 million reads could be a sufficient sequencing depth.

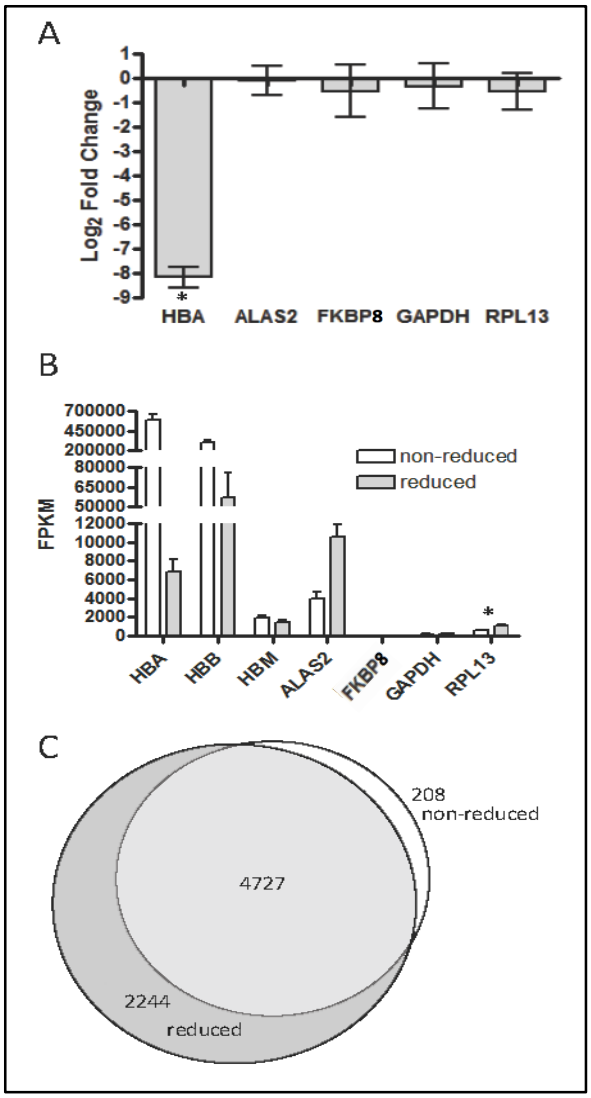

Figure 3.1. Globin depletion of dolphin blood RNA.
A Quantification of *HBA, ALAS2, FKBP8, GAPDH, RPL13* by real-time PCR (n = 5). Statistical significance is denoted by an asterisk. **B** Comparison of quantified level of expression of *HBA*, *HBB*, *HBM*, *ALAS2*, *FKBP8*, *GAPDH*, *RPL13* in globin reduced and non-reduced samples (n = 5). Statistical significance is denoted by an asterisk. **C** Comparison of blood RNA genes identified in globin-reduced and globin-non-reduced samples**.** White, globin-non-reduced samples; grey, globin reduced samples.

3.3.2. Genome guided and *de novo* assembly of dolphin blood transcripts

Approximately 85 % of reads were mapped back to the genome, and only 28.5% of reads were mapped back to annotated genes with Bowtie2. Consequently, many reads were mapped outside annotated regions and were excluded from analyses. In order to more accurately compare the dolphin blood transcriptome to the Ensembl genome, we selected a suite of 17,475 sequences, comprised of coding sequences and pseudogenes, from the genome. Of these, 9610 reads were mapped (45.2 %), with a FPKM > 0 in at least half samples and an average FPKM 1. The 100 highly expressed genes were all annotated, comprise transcripts associated to translation, ribosome, RNA and DNA binding. Similar results have been described in the human blood (Dopico et al., 2015). Transcripts mapping to GO terms included genes involved in immune response, transcription, cell cycle and proliferation, signaling and structural components and were highly express both in human and in dolphin blood. Immune functions, basic cellular functions and processes were well represented in blood transcriptome according to pathway mapping. 137 pathways, identified in WEB-based GEne SeT AnaLysis Toolkit (http://www.webgestalt.org/), were significantly enriched in the blood transcriptome relative to the genome (Benjamini- Hochberg p-value < 0.05). In the blood transcriptome GO terms analysis found to be enriched in process and functions associated to the ribosome, translation, transcription, cell cycle, protein processing, cellular homeostasis and abiotic immune response (Fisher's exact test, FDR < 0.05). Many GO terms related to tissue-specific process and functions are not expressed in blood, including sensory process, skeletal and cardiovascular (Fisher's exact test, FDR < 0.05). Notably, only 3 of 47 genes are mapping to hormone activity, hormone mediated signaling pathway and hormone metabolic process were present in dolphin blood transcriptome. In human blood transcriptome hormone related processes are well documented, while in the dolphin were significantly under-represented (Mhor et al., 2007; Nicholson et al., 2004). It is possible that hormone related transcripts are poorly annotated in the current genome, because data sets were made to ensure that sex-specific expression not excluding these hormone related transcripts.

De novo assembly was performed with Trinity combining eight samples (male, female and winter and summer samples). We obtained 49925 contigs with a minimum length of 400 nucleotides, a N50 of 1331 nt and the longest contig of

12295 nt. *De novo* transcripts were aligned to the coding subset of genome via blastn, resulting in 31.5 % of transcript hits with an E-value < $1e^{-4}$. This percentage is similar to the one obtained when mapping back to the annotated-transcripts in the genome based analysis previously described. Blast alignment of the *de novo* transcriptome to the full genome highlighted minimal novel sequence discovery in the *de novo* assembly. Many transcripts in the *de novo* assembly mapped outside the annotated regions of the Ensembl dolphin genome: the reduced mapping to the genome reflects the limited annotation available rather than an absence of sequence data. Eighty-eight % of reads mapped back to the *de novo* transcriptome using Bowtie2, a significantly higher percentage than the 28.5 % observed mapping the annotated genes in the genome, yielding a substantial increase in usable read data for downstream analyses (Wilcoxon signed rank test, $p < 0.0001$). Thus, all further analyses presented herein utilize the *de novo* assembly.

When filtered to ensure FRKM > 1 we found 29702 transcripts, corresponding to the 59.5 % of *de novo* transcriptome; 13889 genes had homology to human genes using blastx, higher than the homology obtained mapping to dolphin genome (45.2 % of genome). 38% of these transcripts were fully annotated in blast2GO and 21% mapped unambiguously to Entrez Gene IDs for pathways mapping in WebGestalt. This set of 29702 transcripts was defined as the “blood transcriptome” and used as the background set of transcripts expressed in blood for all further analyses.

Among the 100 most highly expressed genes in this blood transcriptome, 92 % were annotated, most of them were transcript associated with ribosome, protein and nucleic acid binding, and translation, with a little expression of genes involved in hormone biosynthesis, degradation and signaling, and only 12 genes mapped to hormone activity (GO:0005179), hormone-mediate signaling pathway (GO:0009755) or hormone metabolic process (GO:0042445). Significant mapping to ribosome pathways were identified using KEGG or WikiPathways (p-value < $5e^{-70}$). The lack of hormone related transcripts in dolphin blood may reflect differences in hormone related transcript expression between humans and dolphins, or may be due to the lack of homology, at the sequence level, between human and dolphin transcripts (more information on the KEGG pathways can be found in Appendix, PAPERII, Table 3).

3.3.3. Changes in gene expression by gender and seasonality

Principal component analysis (PCA) was employed to reveal any strong clustering for sex, season or any other measured parameter. PC1 accounted for 21.8 % of the variance and was slightly correlated with sex, with females clustering together while the expression profiles from the two males were more variable (Figure 3.2). PC2 only weighted for 7.8 % of variance and was not associated with animal, sex, or season. Samples from individual animals did not cluster on either the PC1 or PC2 axes. The samples from males that clustered with the females on PC1 were not consistent with regard to season of collection. Neither PC1 nor PC2 were correlated with day length, water temperature, month of collection, nor with any of the hematological parameters measured.

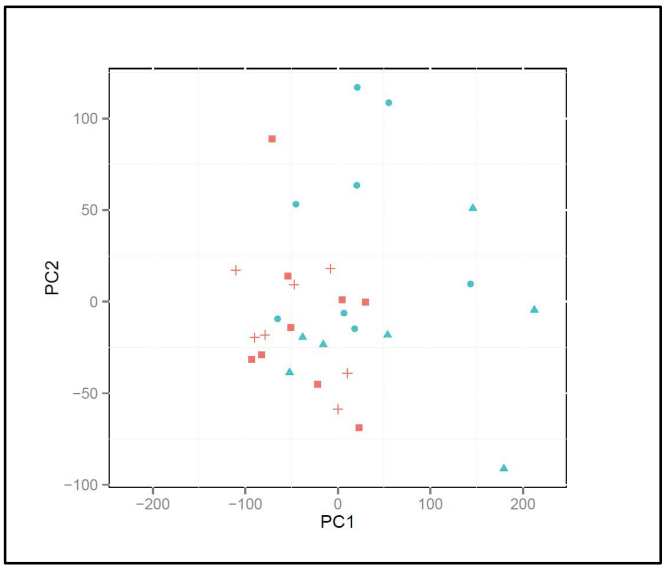

Figure 3.2. Principal components analysis of 31 transcriptomic profiles.
Animals: circle, Hua; triangle, Kainalu; square, Keo; cross, Pele. Sex: pink, females; blue, males.

In the blood transcriptome, 499 transcripts were differentially expressed between males and females (1.7 %; EBseq; FDR < 0.05; n = 15 for males, and n = 16 for females). Of these, 204 (41 %) had annotation. 93 genes were mostly expressed in males (female FPKM < 1), and only 11 were annotated: *BATF1, CENPF, CREG1, GALM, HSF1, NDRG3, NOXO1, RAB22A, SLC18A2, SPAST*, and *TSLNG* all are autosomal in humans, while a total 261 genes were highly expressed in males (log2 fold change 0.28 to 11.9, average = 1.65). 238 genes were expressed highly in

females (male average FPKM < 1). Ten annotated female expressed genes, *C20orf112, CHMP1A, CNN2, PPP4R2, SAMHD1, SNX19, TXLNG, ULK1, VPS13C, ZDHHC21*, are all autosomal in humans. Two genes were expressed slightly higher in females (log2 fold change 0.45): *SMC1A* and *CD40LG*, both are homologs to X chromosome linked genes in humans. *SMC1A* is involved in sister chromatid cohesion during cell division and is known to be located in an area of the X chromosome that is not subject to X inactivation (Brown et al., 1995). No homologs to human Y chromosome genes were found among the differentially expressed transcripts. In fact, most dolphin chromosomes display substantial homology to human chromosomes by chromosome painting, while the Y chromosome in dolphins is minute, and does not display any cross-hybridization with human chromosome probes (Bielec et al., 1998). The absence of Y chromosome genes anticipated in sex biased gene expression may reflect a lack of homology to human genes, resulting in unannotated dolphin transcripts.

All other genes were present in both sexes and were expressed at different levels and only 10 are in common with those differentially expressed in human peripheral blood (Jansen et al., 2014). There was no enrichment of any GO term among the annotated genes highly expressed in male or female dolphins, nor in the combined set of genes differentially expressed by sex in this study. Significant seasonal changes in transcript expression were previously observed in human blood and in dolphin skin (Dopico et al., 2015; Van Dolah et al., 2015), so we analyzed genes differentially expressed between winter and summer. Based on local temperatures, we grouped samples from July, August and September as summer samples, and samples from December to February as winter samples, with n = 8 in both cases. EBSeq reported a very small percentage of the dolphin blood transcriptome exhibiting significant changes in expression between summer and winter months, with only 0.7 % of the transcriptome has significantly changes between summer and winter and only 53 genes were annotated. 53 genes were expressed only in winter, while 102 were expressed only in summer. There is no significant enrichment of any GO category or pathway in these sets of seasonally changing transcripts. Then gene expression was queried for males and females, separately. 0.8 % of the transcriptome changed in males, while 2.4 % in females, only 7 genes are in common, and only one are annotated, and there isn't significant enrichment of any GO terms or pathways.

The results have been compared to previous seasonal expression studies. In human blood the percentage of genes that exhibit a seasonal change is higher than in dolphin blood: 23 % in human and 0.7- 2.4 % in dolphins. This may be due to: 1) minimal change in day length (1.5 hr) or in temperature (1.5 °C) in Waikoloa, Hawaii; and 2) small dataset in this investigation. Nonetheless, 16 % of genes assigned a gene symbol via blast searches in dolphin blood are listed in seasonal genes in human blood, and 42 % of annotated genes in dolphin are represented by different member of the same protein family in human blood. In male-only gene dataset this trend is more marked, with 40 % of annotated genes also found in human, and another 40 % represented within the protein family in human blood. In female-only dataset 47 % are same genes, and 32 % within the same family. This overlap indicates that gene expression in dolphin, despite the tropical climate or the small sample size, may undergo seasonal variation that must be taken into account when assessing gene expression changes associated with clinical parameters, disease or toxic exposure. To this end, in order to visualize any seasonal expression cycles genes that significantly differed in expression between summer and winter months were subjected to a cosinor analysis. Gene differentially expressed in cosinor shown peak of expression in either in the cooler (November-February) or in warmer months (June-September) (Figure 3.3), while patterns and extent of cycles of cyclic changes in expression varied between dataset. On full dataset cosinor analysis, only two transcripts shown highest expression in cooler months, both annotated (Figure 3.3A). Using male-only dataset shown more stable annual expression patterns (Figure 3.3B), and the two highly expressed genes, with a peak in the cooler months, are *coactosin* and *coronin*, both associated with cytoskeletal processes and actin binding. Females-only dataset exhibit the greatest annual rhythmicity (Figure 3.3C). The highest expression and the greatest amplitude of change is a 60S ribosomal protein L31 (RPL31), while in the warmer months an erythroid associated factor (ERAF) exhibited high expression levels, and CD79B exhibited seasonal rhythmicity peaking in cooler months. These seasonal expression changes in ERAF and CD79B may reflect changes in the cellular composition of blood (De Jong et al., 2014; Dopico et al., 2015). A larger sample set, as well as samples from dolphins in regions with greater environmental fluctuations over the year may reveal cyclic patterns of gene expression not observed in the current study.

Comparison with gene expression changes in dolphin skin from Gulf of Mexico revealed much less overlap: less than 5 % are the same genes and approximately 33 % within the same family. The difference between dolphin blood and dolphin skin may reflect tissue specific differences in gene expression and the difference in environmental exposure to temperature fluctuations. Another difference is the origin of the samples; skin was in fact collected from wild dolphin in the Gulf of Mexico while blood analysis was carried out in managed dolphins from Waikoloa, Hawaii.

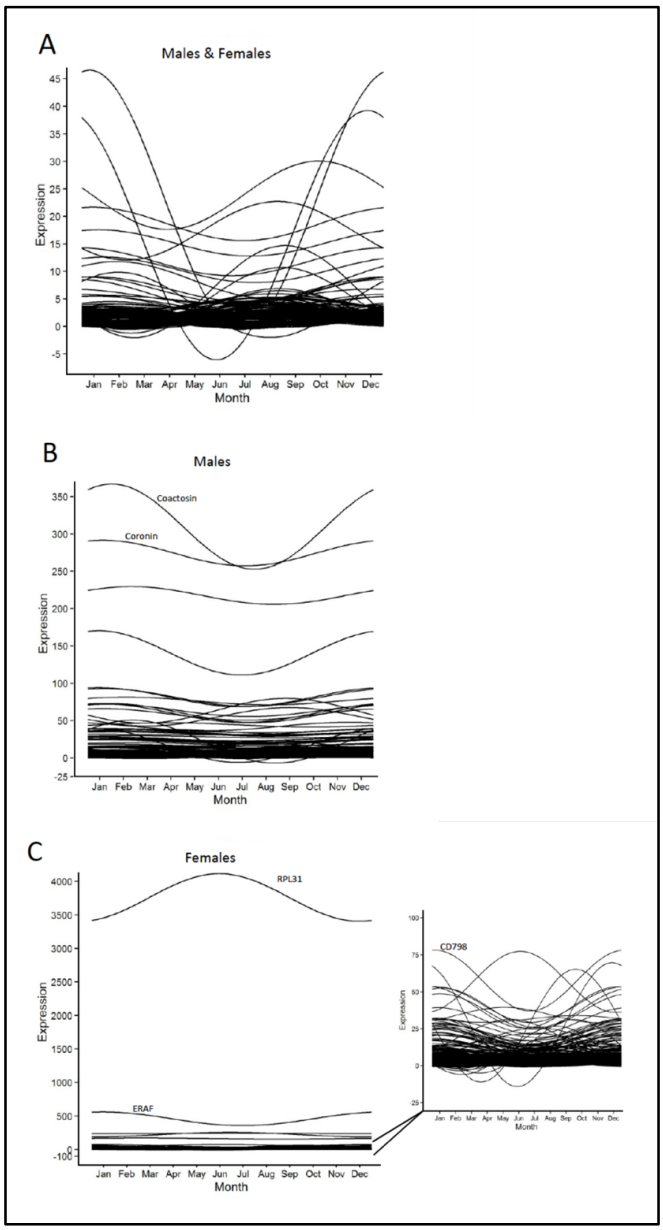

Figure 3.3. Cosinor analysis of seasonally expressed genes in the dolphin blood transcriptome.
Genes differentially expressed between summer (July, August, September) and winter months (December and February) (EBseq FDR < 0.05) are plotted for males and female (**A**, n = 31, 210 transcripts), in males only (**B**, n = 15, 252 transcripts), or in females only (**C**, n = 16, 699 transcripts).

3.3.4. Gene co-expression network analysis

In order to use blood transcriptome to identify any physiological perturbations, such as those resulting from disease or toxic exposure, a better understanding of healthy transcriptome over time and differences between males and females, or age and hematological parameters, is necessary. For this reason, we constructed a gene co-expression network in WGCNA using all samples (n = 31) as independent measures. Fifteen co-expressed gene modules were identified (Figure 3.4A and B), while the majority of genes, represented by the grey module, were not significantly co-regulated. Pairwise correlations between each module eigengene and each of the physical or hematological parameters measured revealed several modules with significant associations (Figure 3.4C).

The ability of the network analysis to identify modules of co-expressed genes that correlate with clinical measurements in this small sample set of healthy and managed dolphin may be informative for the identification of metabolic perturbations indicative of infections, disease, or toxic exposures in bottlenose dolphins. The independence of many co-expressed modules from the individual animal and month of collection suggests that these may not be confounding factors for identifying transcriptomic responses to adverse health impacts. Validation of the results using a larger sample set will be the next immediate step but so far the results are promising. This study has direct practical implications: any medical doctor or veterinarian needs to have reference values and baseline data species-specific in order to establish the significance of changes in any patient, and how the degree and quality of these changes can be correlated to any abnormal condition such as an infection or a disease status.

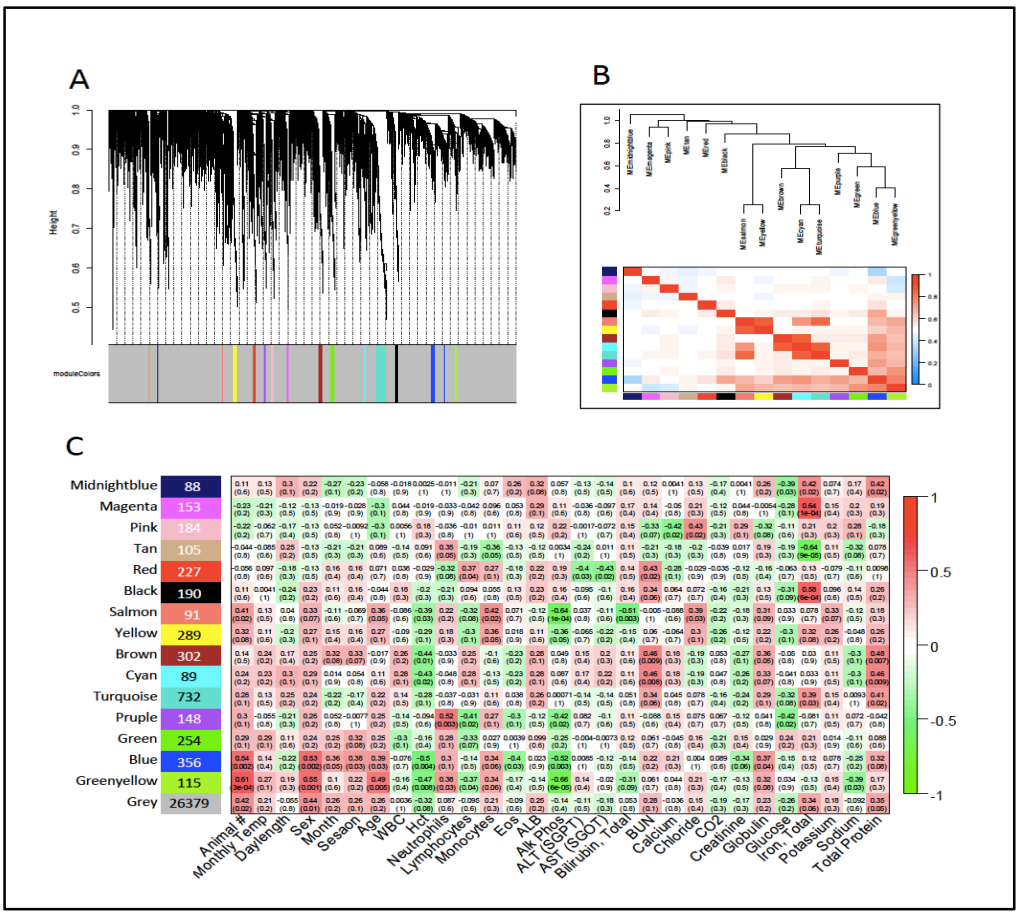

Figure 3.4. Weighted gene expression co-variance network analysis.
WGCNA identified 15 co-expressed gene modules. **A** Average link hierarchical clustering dendrogram of the network with color bands identifying module membership. **B** Hierarchical cluster of module eigengenes identifies closely related modules. **C** Correlation matrix of modules with sample traits and hematological parameters: red, positively correlated, green, is negatively correlated. The correlation coefficient between the module eigengene and the measured trait is listed for each pairwise correlation, with significance in parentheses (p-value). Number of genes in each module is listed at left.

3.4. Conclusions

This investigation provides information on the blood transcriptome content and on the transcript expression changes associated with sex and season in four healthy and managed bottlenose dolphins. Both a seasonal component to changes in blood gene expression, consistent with studies in humans, and an association of gene co-expression modules with age, sex or hematological parameters measured were found. The proportion of genes exhibiting changes in expression along with the degree of change observed was limited, demonstrating the relative stability of the dolphin blood transcriptome within and between animals throughout the course of one year. Although in this investigation was used a small dataset of healthy and managed dolphins, the observed correlations to hematological parameters with an otherwise stable transcriptome and precedence from human medicine suggests that blood transcriptome analysis may be useful for identifying exposures, infections and pathological changes that cannot be readily monitored in protected marine mammal species. In order to use the blood transcriptomics for diagnostic purposes in bottlenose dolphins is necessary to establish normative values in healthy animals using a robust database of gene expression in dolphins from different environments, both managed and wild. This database would facilitate the use of blood transcriptomics for biomonitoring wild and managed populations of bottlenose dolphins.

*This study is now in the form of a published manuscript (see **PAPER II** in Appendix).*

4. Stem cells cultures: bottlenose dolphin's models for *in vitro* testing

4.1. Introduction

Stem cells are undifferentiated cells with remarkable characteristics: they are able to proliferate while maintaining their pluripotency state (self-renewal) and they can differentiate into multiple cell lineages.

Stem cells are found in all multicellular organisms, from the early stages of development to the end of life. They can be classified according to 1) potency, the extent to which they can differentiate into different cell types and 2) source, derived from embryos, adult or fetal tissues.

1) Potency is taken from the Latin term "potens" which means, "having power"

i) totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism;
ii) pluripotency refers to a stem cell that has the potential to differentiate into any of the three germ layers: endoderm, mesoderm or ectoderm;
iii) multipotency describes progenitor cells which have the gene activation potential to differentiate into discrete cell types (e.g. blood cell types);
iv) unipotency refers to that one stem cell that has the capacity to differentiate into only one cell type (e.g. hepatocytes).

2) Source:

i) Cells comprising the inner mass cell of the blastocyst during development are named embryonic stem cell (ESCs), but there are embryonic germ stem cell and fetal tissues stem cells, specifically named from the specific stage of the embryo development. Embryonic stem cell research and use poses a moral dilemma, because in order to obtain them, the early embryo has to be taken.

ii) Cells that are found in differentiated tissues at any stage of development and can divide to repair and/or replace damaged tissues, such as the gut and the bone marrow are called adult stem cells (ASCs). In other organs, pancreas and the heart, adult stem cells only divide under special conditions.

Stem cell research offers unique models for studying a variety of processes that occur in the early development of mammals whilst holding tremendous promise for the development of novel therapies for many serious diseases and injuries. Stem cell-based treatments have been established as a clinical standard of care for some conditions, such as hematopoietic stem cell transplants for leukemia and epithelial stem cell-based treatments for burns and corneal disorders (Szilvassy et al., 2003, Gratwohl et al., 2005). The scope of potential stem cell-based therapies has expanded in recent years due to advances in stem cell research.

Until recently, ESCs were the only well–studied source of pluripotent stem cells. Nonetheless, on the ESCs use there are constraints, such as the immune incompatibility and, most importantly, ethics. The immune incompatibility problem can be solved by therapeutic cloning, consisting in the somatic cell nuclear transfer (SCNT) into the egg cell and then obtaining the embryo and the ESCs from the inner mass of the blastocyst. However, with therapeutic cloning reprogramming yield is very low and there is still the ethic problem of the human ovarian cell donation (Medvedev et al., 2010).

In 2006 Takahashi and colleagues first reported the “reprogramming” of adult specialized cells (skin fibroblasts) to a stem cell-like state: this new type of stem cells was called induced pluripotent stem cells (iPSCs) (Takahashi et al., 2006). New techniques to generate iPSCs were rapidly generated in humans and many other species (Koh et al., 2014), from fibroblasts and from several other differentiated cell types (Prasad et al., 2016), creating a powerful new protocol to create "de-differentiate" cells similar to ESCs.

Another alternative to the use of ESCs are stem cells extracted from bone marrow, umbilical cord and the adipose tissues: these are called mesenchymal stem cell (MSCs), or multipotent stromal cells, and represent a rare population of multipotent progenitors capable of supporting hematopoiesis and differentiating into three (osteogenic, adipogenic, and chondrogenic) or more (myogenic, cardiomyogenic, etc.) lineages, including lineages derived from neuroectoderm (Friedenstein et al., 1974; Bianco et al., 2001; Phinney et al., 2007). MSCs have several advantages: they are not associated with ethical issues and they can be easily isolated and expanded *in vitro*. For these reasons to this date there are 672 clinical trial testing the potential of MSCs therapy worldwide (http://www.clinicaltrial.gov/).

The International Society for Cellular Therapy (ISCT) established a list of minimal criteria to define multipotent mesenchymal stem cells: (I) the capacity to adhere to plastic (II) the ability to differentiate into fibroblast colony-forming-units (CFU-F) and into three cell lineages of mesoderm origin: osteocytes, chondrocytes and adipocytes and (III) specific surface markers: CD73, CD90, CD105 and CD44 and negative for CD11b, CD14, CD34, CD45 and human leukocyte antigen (HLA)-DR (Dominici et al., 2006). Due to their trilineage differentiation, MSCs are tested for their capability to regenerate damaged tissues both in humans and in animals. Tissue healing and regeneration can be supported locally and over distance through the secretion of trophic factors, which act on decreasing cells and cytokines involved in inflammation and modulating the immune system response (Carrade et al., 2013).

In case of injury, MSCs are involved in the attenuation of the tissue damage, through the inhibition of fibrotic remodeling and of apoptosis, stimulation of endogenous stem cell recruitment, proliferation, activation of angiogenesis and reduction of the immune responses (Maumus et al., 2013). MSCs are considered primarily as a source in therapeutic uses but they also have a key role in ecotoxicology and developmental toxicology (Kopras et al., 2014; Il'yasova et al., 2015).

Unfortunately, MSCs are only found in limited amounts: at present, the most used MSCs are those derived from bone marrow (BM-MSCs). Due to the clinical limitations of bone marrow biopsies alternative sources have been sought. This led to the discovery of related MSCs in adipose tissue, namely adipose-derived stem cells (AD-SCs), which have shown the same biological capabilities as the BM-MSCs (Zuk et al., 2002). AD-SCs are obtained in large amount from adipose tissue through liposuction: they are easily expanded in culture, they have a low immunogenicity and they have the capability to maintain their phenotype after long-term culture *in vitro* (Zuk et al., 2002). AD-SCs are capable of differentiating in various mature cell lineages, reproducing processes of osteogenesis, chondrogenesis or adipogenesis (He et al., 2013).

In order to generate a sufficient number of stem cell for clinical application new approaches for AD-SCs amplification and isolation are being developed, including autologous transplant approaches from minimal tissue isolation to avoid undesired

immunoreactions. In fact, during the last decade, great effort is being made to increase the yield, the efficiency and their therapeutically capability. AD-SCs have been harvested and characterized not only in human, but in many species as rats, mice, rabbits, pigs, dogs horse, bears and dolphin (Gimble et al., 2010; Fink et al., 2011; Johnson et al., 2012). AD-SCs isolation from liposuction, after local anesthesia, is considered an invasive methodology, and, especially in animals, it can be cause of stress. Moreover, in marine mammals, this protocol can be difficult to perform (Johnson et al., 2012).

The aim of this investigation is to develop an efficient protocol for isolation, characterization and cryoconservation of mesenchymal and hematopoietic cells in bottlenose dolphin from a source different from BM-MSCs or AD-MSCs, the placental and umbilical cord tissues (mesenchymal stem cells) and umbilical cord blood (hematopoietic stem cells). In fact, in dolphins, cells can be directly isolated from tissues easily collected right after the expulsion of the placenta, which makes the process less invasive than any other protocol of MSCs collection. Mesenchymal and hematopoietic stem cells can be easily accessible from these tissues, expanded *in vitro*, and cryopreserved for future therapeutic uses, without any stress for the animals.

The final goal of this project is the creation of a cell bank for bottlenose dolphin for future therapeutic use with an unlimited source of mesenchymal and hematopoietic cells for research and biomedical applications.

4.2. Materials and methods

4.2.1. Tissue origin

Bottlenose dolphin umbilical cord (UC) and placental (PL) tissues were collected between the year 2014 and 2016 from dolphins housed in 4 different marine parks thanks to the collaboration with Edutainment park, that manage many marine parks in Italy and Europe and the collaboration with the Oceanografic and the Centro de Investigation Principe Felipe, in Valencia, Spain. During this time, 5 dolphins gave births: 2 in Oltremare, Riccione, Italy (ID: Tt01OR; Tt03OR), 2 in the Genoa Aquarium, Genoa, Italy (ID: Tt02AG; Tt04AG), and 1 in the marine park Oceanographic, Valencia, Spain (ID: Tt05OV). Blood from UC, and tissue samples of PL and UC were collected during each birth event following the placenta expulsion. The *postpartum* time elapsed ranged between 5 hrs 40 minutes and 9 hrs 30 minutes; the weight of the placenta collected was between 1.6 Kg and 2.25 Kg and the umbilical cord length was between 30 cm and 50 cm. The cord blood collected was from a minimum of 5 ml to a maximum of 30 ml.

4.2.2. Blood and tissue processing protocols

The UC blood was processed within 12 hours, using Histopaque 1077 (Sigma-Aldrich, St. Louis, Missouri, USA), a density gradient cell separation medium of polysucrose and sodium diatrizoate used for the separation of lymphocytes from whole blood. Briefly, mononuclear cells extraction from blood is performed using centrifugation, because erythrocytes can aggregate to the polysucrose and rapidly sediment meanwhile granulocytes become hypertonic and pellet at the bottom of centrifuge tube. Lymphocytes and mononuclear cells remain in the plasma/Histopaque interface. 1 ml of Histopaque are added to 1 ml whole blood in conical centrifuge tube, centrifuged for 30 minutes at room temperature at 400 xg. After centrifugation, the upper layer of the opaque interface was aspirated and transferred in a new centrifuge tube, and washed with 10 ml of Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen, Carlsbad, California, USA) solution, mixed by inversion and centrifuged for 20 minutes at 250 x g at room temperature. The supernatant was aspirated and the cell pellet wash was repeated twice. In the

last step, the cell pellet was resuspended in 0.5 ml in buffered solution containing Dulbecco's modified eagle's medium (DMEM) (Invitrogen, Carlsbad, California, USA) and stored at - 80°C for future use.

The tissue samples collected were cubes of the size of 2 x 2 cm^3. Four replicate samples were collected for both PL and UC. Of the 4 replicate samples, 3 were processed in order to obtain cultures of cells (1 was immediately used for direct culture and 2 were frozen and stored for future MSC cultures, to test the cryopreservation protocol) and 1 sample was stored in RNAlater (Qiagen, Hilden, Germany) for total RNA extraction.

UC and PL samples of 2 x 2 cm^3 were excised, placed in a sterile Petri dish and cut into smaller cubes using sterile scalpel and forceps. Cubes were washed twice in 1X DPBS (Invitrogen, Carlsbad, California, USA) supplemented with 100 units/ml penicillin/ 100 µg/ml streptomycin (Invitrogen) (DMEM/Ab). DMEM/Ab was used to clean tissue samples and to remove any residual blood before transferring into a sterile syringe (BD Biosciences, San Jose, California, USA) containing freshly prepared cryopreserving medium, CM (CM: Dulbecco's Modified Eagle Medium—DMEM [Invitrogen], supplemented with 10 % high quality dimethyl sulfoxide [DMSO; Sigma Aldrich, St. Louis, Missouri, USA] and 10 % fetal bovine serum [FBS; Sigma Aldrich]). The syringe was equipped with a stopcock to create a partial vacuum in the syringe chamber to perfuse the cord tissue with CM. Briefly, after all the air was removed from the CM/tissue mix, the stopcock was closed and the cubes were perfused with CM by moving the syringe plunger slowly backwards to increase chamber volume (partial vacuum/tissue expansion) and forwards to reduce chamber volume. This process was repeated a total of 7 times to insure complete perfusion. After perfusion with CM, UC and PL cubes were differentially used for 1) direct digestion and culture and 2) transfer into sterile cryovials (Thermo Scientific, Rochester, New York, USA) containing CM, and subjected to slow freezing (~ 1 °C/min) controlled by an isopropanol bath container (Nalgene/Thermo Scientific) in a − 80 °C freezer.

4.2.3. PCR on total RNA from tissue sampled

Thirty nanograms of UC and PL samples isolated and stored in RNAlater were lysed using IKA homogenizator T25 (Staufen Im Breisgau, Baden-Wurttemberg, Germany) at approximately 10000 - 15000 rpm for 30 seconds. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

RNA quality and quantity were assessed using BioSpec NANO Spectrophotometer (Shimadzu Biotech, Kyoto, Japan). Samples with a concentration 100 ng/ul, OD260/280 ratio of ≈ 2 were considerate acceptable. The same procedure was applied to extract RNA from bottlenose dolphin skin used as control, collected from a bottlenose dolphin stranded in the coast of the Adriatic Sea in 2014 (TtSskin), thanks to the collaborations with the Italian Institutes for Animal Protection, IZS (*Istituto Zoo Profilattico della Lombardia e dell'Emilia Romagna*). One microgram of total RNA was reverse transcribed using iScript Select cDNA Synthesis Kit (BioRad, Hercules, California, USA) according to manufacturer's instructions, obtaining cDNA in an adjusted final concentration of 10ng/µl. PCR was performed using DreamTAQ Master Mix (Thermo Fisher Scientific, Massachusetts, USA) in a total volume of 50 µl containing 10 ng cDNA, 19 µl of nuclease-free water (Qiagen), 0.5 µM forward primer (REX1F and GAPDH-F), 0.5 µM reverse primer (REX1R and GAPDH-R) and 25 µl of 2X DreamTaq Green PCR Master Mix (Thermo Fisher Scientific). PCR reactions were run as follow: 1 cycle of 95 °C for 3 minutes, 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, 1 final cycle of 72 °C for 5 minutes. The sequence of the primers for the gene *REX1* and *GAPDH* were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) on the gene-specific *T. truncatus* sequences obtained searching the dolphin genome database at www.ensembl.org (Acc.nos.: XM_004328739, REX1 and XM_004322381.1, *GAPDH*, at www.ncbi.nlm.nih.gov). The sequences of the primers were: REX1F, ACGGCAAGCTCACTTCTTGA; REX1R, TCGGGGACTATGAACTCGGA; GAPDH: GAPDHF, CGACCACTTTGTCAAGCTCA; GAPDHR: CGGAGGACCTCTCTCTTCCT.

4.2.4. Isolation and culture of MSCs

UC tissue samples used in this work were collected from a bottlenose dolphin kept under human care in the aquaria at Oceanografic, in Valencia, Spain. Once perfused, the PL and UC tissue were transferred into a sterile 60 mm dish containing DMEM/Ab. The tissue was divided into smaller pieces in a laminar flow hood using sterile scalpel and forceps and washed 3 times in DMEM/Ab (Invitrogen). Tissue was then dissociated enzymatically at 37 °C in DPBS/Ab containing collagenase type IA (0.07 %, Sigma, California, USA) overnight. The following day the digested tissue was collected and washed 3 times in DPBS/Ab. Then the tissue was centrifuged (500 g, 3 minutes) and the pellet was resuspended in growth medium GM (DMEM/Ab supplemented with 10% heat inactivated FBS [GIBCO], 2mM L-glutamine [Thermofisher, 30% L-glucose [Sigma-Aldrich]) and plated in a petri 60 mm diameter petri dish, incubated overnight at 37 °C in 5 % CO_2, 21 % O_2. The following day the medium was removed and replaced with fresh GM. The cells that remained attached to the dish were the mesenchymal stem cells, MSCs; MSCs were allowed to grow until nearly confluent for two subsequently passages, and were cryopreserved at a concentration of $1x10^6$ cells/ml/vial in CM at – 80 C.

The same protocol has been performed on UC and PL tissue stored in a −80 °C freezer after sampling. For thawing, cryovials were transferred from −80 °C freezer storage into an ice container, brought to a 37 °C in water bath, and rapidly thawed with agitation at 37 °C.

Cell count assessment of disaggregated cells was performed using a hemocytometer on an inverted light microscope with Trypan blue exclusion as the viability assay after trypsinization, an incubation of 10 minutes at 37 °C in 5 % CO_2, 21 % O_2 with 0.5 % trypsin-EDTA. All thawed cells at passage 3 were cultured for 4 - 5 more passages prior reaching senescence. For growth curves, cells were thawed and plated at 2 × 100 cells/35 mm dish and expanded to 80 – 90 % confluence prior to counting and passage at the same plating density (2 × 100 cells/35 mm dish). Cell morphology analysis were performed at passage 2 using ImageJ software (http://imagej.nih.gov/ij/) on images collected by bright-field

microscopy on a Leica DM 6000 microscope supported by the Leica Application Suite (LAS) software v4.5 (Leica, Buffalo Grove, Illinois, USA).

4.2.5. PCR on total RNA from MSCs cultured

A total of $1x10^6$ cells from passage 4 was used for RNA extraction using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. PCR were performed in 50 µl total reaction mix containing 10 ng cDNA, 19 µl of nuclease-free water (Qiagen), 0.5 µM forward primer (REX1F, NANOGF, SOX2F, OCT4F and GAPDH-F), 0.5 µM reverse primer (REX1R, NANOGR, SOX2R, OCT4R and GAPDH-R) and 25 µl of 2X DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA) and run as follow: 1 cycle of 95 °C for 3 minutes, 30 cycles of 95 °C for 30 seconds, 53 °C for 30 seconds, 72 °C for 30 seconds, 1 final cycle of 72 °C for 5 minutes. The sequence of the primers for the gene *REX1, GAPDH* were the same as described above. *NANOG, SOX2* and *OCT4* were designed on the gene-specific *T. truncatus* sequences obtained searching the dolphin genome database at www.ensembl.org (Acc.nos.: XM_004325830.1, *NANOG*; XM_004311784.1, *SOX2*; XM_004311235.1, *OCT4*; at www.ncbi.nlm.nih.gov). The sequences of the primers were: NANOGF, CCTGAGATTTATGGGCCTGA; NANOGR, CGGTTCTGGTCTTCTGCTTC; SOX2F, GTTTGCAAAAGGGGGAAAGT; SOX2R, GCCAGGAAAATCAGACGAAG; OCT4F, CTCGAACAATTTGCCAAGGT; OCT4R: CTCCAGGTTGCCTCTCACTC. PCR products were visualized after an electrophoretic run on agarose gel 2 %, at 120V for 25 min. PCR products were quantified and Sanger sequenced (BMR Genomics, Padova, Italy). The sequences obtained were analyzed by MEGA6 software (http://www.megasoftware.net/) and BlastX (www.ncbi.nlm.nih.gov) to confirm gene identity.

4.2.6. Karyotyping

A total of $1.5x10^6$ cells from passage 4 were subcultured from T25 flasks into three T75 flasks (Corning, Sigma-Aldrich Missouri, USA). Metaphases were harvested 48 hours after dividing confluent flasks and used for the karyotyping procedure. The cells were harvested according to the following procedure: after 3 hrs of treatment with 0.01 μg/ml Colcemid, the cells were centrifuged at 1200 rpm for 10 min to remove supernatant. Followed a hypotonic treatment of 20 minutes exposure to a 1:1 mixture of 0.075 M KC1 and 0.4 % sodium citrate at 37 °C; the cells were then pelleted by centrifugation and resuspended in 500 μl normal methanol: acetic acid fixing fluid (at a ratio of 3:1). Then cells were centrifuged at 1200 rpm for 10 minutes, supernatant was removed and finally cells were resuspended in 5 ml of fixative fluid and incubated at room temperature for 30 minutes. Centrifugation and resuspension were repeated twice. Slides were prepared according to standard procedures. After final centrifugation, cells were resuspended in small amount of fixative and dropped in slides cleaned in ethanol with 1 % HCl, and let resting horizontally at 65 °C overnight. Slides were treated with a Giemsa solution (10% Sorensen's buffer [0.1 M KH2PO4, 0.1 M Na2HPO4] and 2% Giemsa) (adjusted from Perry and Wolff, 1974). A test slide was placed in trypsin for 2 minutes, rinsed in HBSS - Hank's balanced salt solution (HBSS) for 15 seconds and stained with the Geimsa solution for 30 minutes. The test slide was examined for correct trypsin digestion and the time adjusted accordingly for the final sample slides staining.

4.2.7. Fluorescence-activated cell sorting (FACS)

A total of $3x10^5$ cells form passage 4 was used for flow cytometry. Cells were pelleted and resuspended in warm medium minus non-essential amino acid, NEAA (Thermo Fisher Scientific, Massachusetts, USA) in order to obtain three vials of 100 μl each. To vial 1 was added 5 μl of FITC mouse anti-human CD45 (catalog # 560976, BD Pharmigen Biosciences, San Josè, California, USA). To vial 2, were added: 5 μl of MsmAb anti CD105 (catalog # ab53321, abcam, Cambridge, UK), 5 μl of PE-Cy7 mouse anti-human CD44 (catalog # 560533, BD Pharmigen Biosciences), and 5 μl of APC mouse anti-human CD29 (catalog # 561794, BD

Pharmigen Biosciences). To vial 3, was added: 5 µl of CD90-PC5 (catalog # PN IM3703, Beckman Coulter). All vials were incubated for 1 hr at room temperature, in the dark. After 1 hr, 1 ml DPBS was added to each vial and the vials were centrifuged at 300 x g for 5 min. The pellet was resuspended in 500 µl of fresh DPBS and covered with aluminum foil prior analysis in Cytomics FC 500 (Beckman Coulter, Brea, California, USA). The antibodies used in this experiment were previously tested and validated (Johnson et al., 2012; Griffeth et al., 2014).

4.3. Results and discussion

4.3.1. Identification of MSC populations in tissues sampled

RNA was extracted from tissues sampled in 4 animals, Tt01OR, Tt02AG, Tt03OR and Tt04AG. The process of RNA extraction from the 2 tissues, PL and UC, resulted in very different outcomes. RNA extraction from all the PL tissues processed was successful. OD ratios described good quality RNA (2 < OD260/280 < 2.1) and the RNA concentrations were respectively 508 ng/µl for Tt01OR, 478.58 ng/µl for Tt02AG, 112.54 ng/µl for Tt03OR and for 257.43 ng/µl Tt04AG. RNA extraction from UC was not successful in any of the 4 animal's samples tested. Probably the nature of the tissue mostly characterized from a hardened texture related to the high concentration of mucopolysaccharides of the Wharton's jelly made the isolation of the cells without a proper enzymatic digestion very difficult.

The RNA isolated from the PL was tested for the presence of MSCs in an amplification reaction, looking for the expression of *REX1 (Zpf-42)*, a well-known marker of pluripotency (Shi et al., 2006). A band corresponding to the molecular weight expected of 318 bp was observed in all samples but in the differentiated tissue RNA used as negative control, the skin RNA. Expression of housekeeping *GAPDH*, with an amplified product of 380 bp, was observed in all samples tested (Figure 4.1).

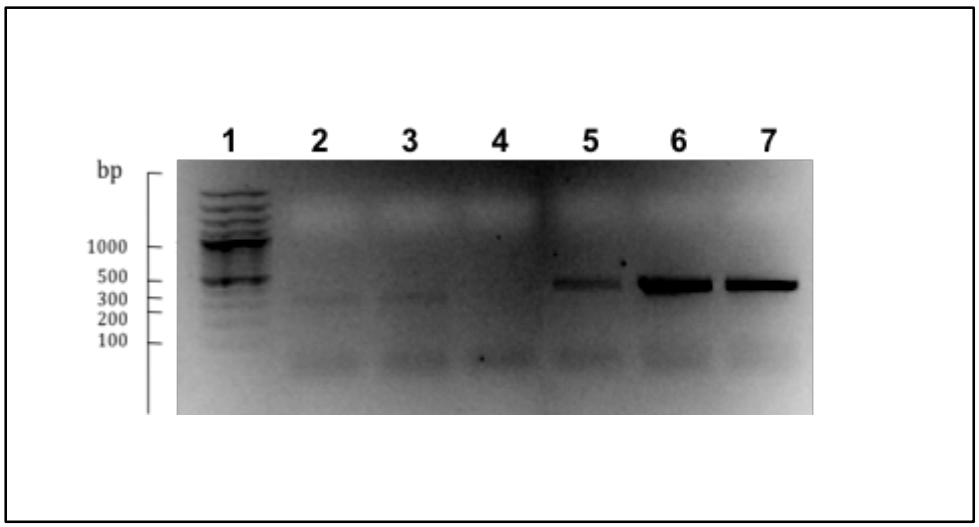

Figure 4.1. PCR products of REX1 on 2 % agarose gel in bottlenose dolphin tissues. 2-4 Amplification product for *REX1*. 5-7, amplification product for *GAPDH*. 2, 5 Tt01OR1; 3, 6 Tt02AG; 4, 7 TtSskin. 1, DNA ladder, 100bp.

REX1 is a zinc finger protein expressed primarily in undifferentiated stem cells, both in the embryo and the adult: *REX1* influences differentiation, cell cycle regulation, and cancer progression. It is a transcription factor and its regulation is critical in maintaining a pluripotent state. As the cell begins to differentiate, *REX1* is abruptly downregulated. We have observed expression of *REX1* in the RNA from the PL tissue. Besides the MSCs, the placental tissue is composed by different cell types (e.g. fibroblasts, epithelial cells, vascular smooth muscle cells, perivascular cells, endothelial cells) but only the MSCs express *REX1*. That is probably why the level of transcript present is much lower than the housekeeping gene *GAPDH*, which is ubiquitously expressed by any cell populating the placental tissue. Moreover, transcription factors expression is quantitatively very different from that of a housekeeping gene, having a much finer mechanism of regulation.

4.3.2. Isolation and characterization of the MSCs

PL and UC tissues were processed for MSCs isolation from both fresh tissues (e.g. right after sample collection) and cryopreserved tissues (e.g. frozen after collection and perfusion). Direct cultures were attempted on the samples collected from Tt05OV and were successful from the UC tissue but not from the PL tissue. Cryopreserved PL and UC tissues were tested for Tt01OR and Tt02AG and were

not successful. Cryopreserved tissues from Tt03OR and Tt05OV have to be tested. Tissues from Tt04AG are no longer viable due to technical problems.

Bottlenose dolphin umbilical cord MSCs (DUC-MSCs) isolated from direct culture have been characterized by quantitative analysis and morphology. DUC-MSCs were able to grow up to 6 - 7 passages before reaching senescence. DUC-MSCs average spread area was 1812 μm^2 ± 448 μm^2 and the cells were plastic adherent with a spindle morphology defined by their circularity and axial ratio (major/minor axis) measurement, of 0.310 ± 0.096 and 2,93 ± 1,28 respectively (Figure 4.2). Throughout the culture process, the cells retained their characteristic spindle shape and reached 80 % – 90 % confluence with a doubling time of seven days. The shape and size of DUC-MSCs were similar to the AD-SCs from dolphin and from other marine and terrestrial mammal (Johnson et al., 2012; Hoogduijn et al., 2013; Griffeth et al., 2014). Differently from other MSCs described in the same or other species, DUC-MSCs show a very slow growth, suggesting a unique species-specific characteristic, or, most likely, indicating adjustments on the tissue culture media composition and/or culture parameters, which are currently standardized on terrestrial mammal culture conditions.

DUC-MSCs were also characterized for their chromosome number and appearance in order to confirm absence of aberration thus making sure that culture conditions were not affecting cell properties. The G-banded karyotype showed a normal (2 n = 44) G-banding pattern for the *T. truncatus* species as previously described (Yu et al., 2005; O'Brien et al., 2006) (Figure 4.3). The karyotype also showed the sex chromosome to be XY, confirming the male gender of the baby dolphin born in Valencia.

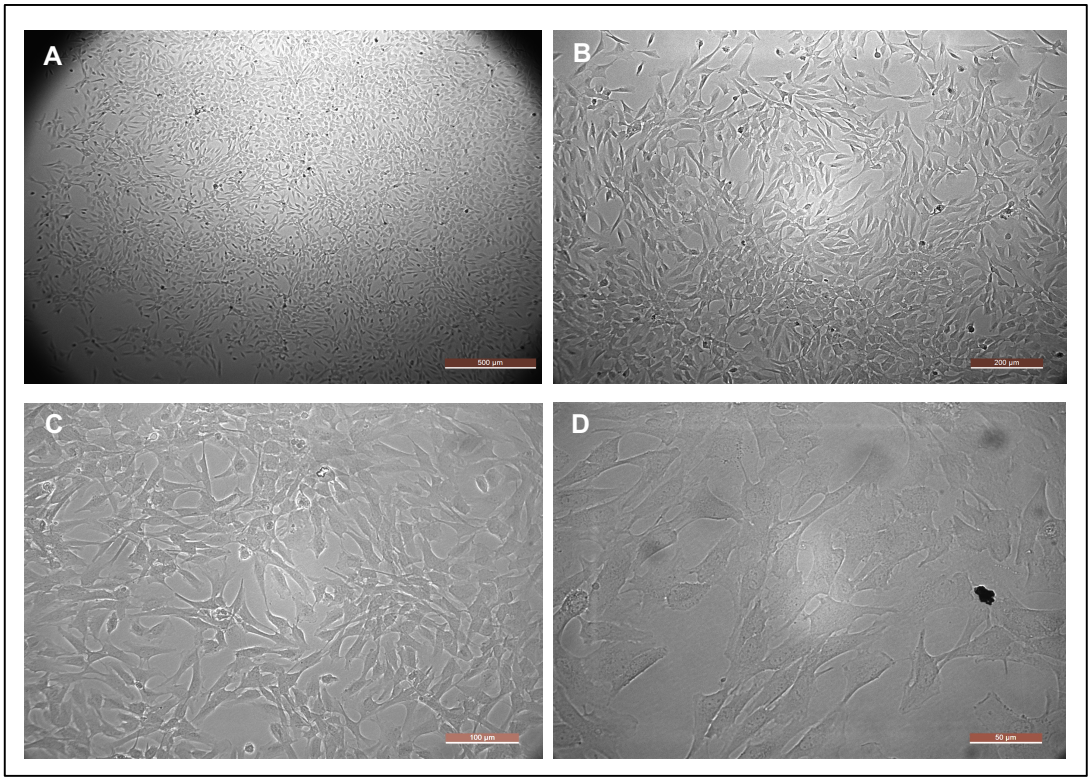

Figure 4.2. Dolphin umbilical cord mesenchymal stem cells, DUC-MSCs.
DUC-MSCs on tissue culture polystyrene imaged at passage 5 on bright-field microscopy (**A**, 5X; **B**, 10X; **C**, 20X; **D**, 40X objective).

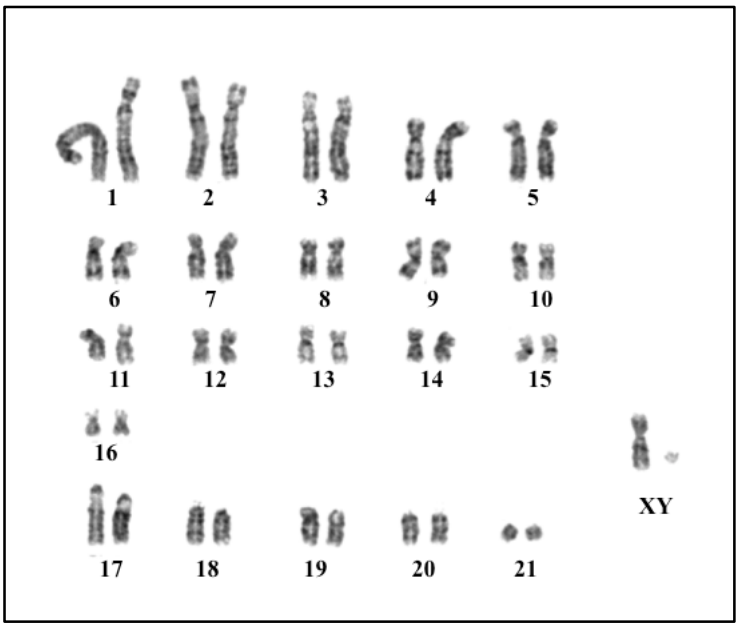

Figure 4.3. G-banded karyotype of DUC-MSCs.

4.3.3. Minimal Criteria for *bona fide* MSCs

The International Society for Cellular Therapy (ISCT) has established a set of minimal criteria for defining multipotent MSCs (Dominici et al., 2006): i) cells must adhere to plastic when placed in culture conditions; ii) cells must express the Cluster of Differentiation (CD) markers such as CD73, CD90 and CD105 and lack expression of CD34, CD45, CD14, CD11b, CD79, CD19 and HLA-DR (major histocompatibility class II); iii) cells must differentiate into adipocytes, chondroblasts and osteoblasts *in vitro*.

We established that these cells are plastic-adherent and are able to be expanded up to 6 - 7 passages in culture (Figure 4.2). Moreover, we were able to show the expression of several CD markers of stemness. In Figure 4.4 are shown the results of FACS analysis on DUC-MSCs for CD90, CD44 and CD105. DUC-MSCs express all the stem cell surface protein markers and specifically 85% showed expression of CD90, 99 % of CD44 and 35 % of CD105. CD29, used as negative control, was expressed only by a small fraction (3 %) of the cell tested. The low expression of CD105 is probably due to the antibody used, which is designed for human and may lack of specificity for the dolphin protein (Carrade et al., 2013).

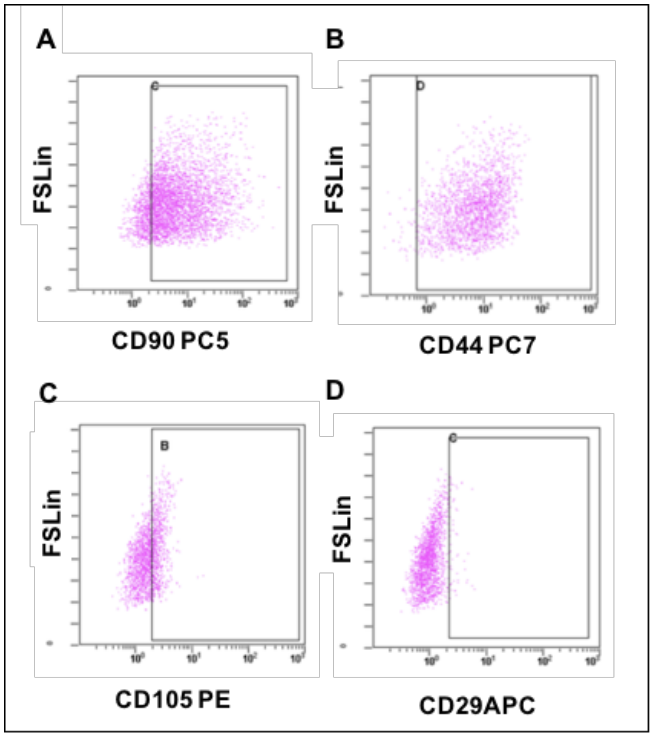

Figure 4.4. Flow cytometry analysis of CDs expression on DUC-MSCs.
Positive cells for the specific CD marker are boxed inside each plot. X axis, number of cells; Y axis, fluorescence measured by forward scatter FS light; CD marker (A) CD90, (B) CD44, (C) CD105, (D) CD29.

We verified the expression of stem cell markers not only at protein level, but also at transcriptomic level to investigate the gene expression mechanisms regulating the pluripotency property of the cells. The RNA tested was the RNA extracted from cultures of DUC-MSCs. Specifically, *REX1, OCT-4, NANOG* and *SOX2* were chosen from literature as marker of stemness. *OCT4* (octamer-binding transcription factor 4 or *POU5F1* - POU domain, class 5, transcription factor 1) and *NANOG* are transcription factors required to maintain the pluripotency and self-renewal of embryonic stem cells through the control of a cascade of pathways that are intricately connected to govern pluripotency, self-renewal, genome surveillance and cell fate determination (Loh et al., 2006). *SOX2* (SRY sex determining region Y - box 2) a member of the SoxB1 transcription factor family, is an important transcriptional regulator in pluripotent stem cells. Together with *OCT4* and *NANOG*, *SOX2* co-operatively control gene expression in pluripotent stem cells and maintain their pluripotency. Furthermore, *SOX2* plays an essential role in somatic cell reprogramming, reversing the epigenetic configuration of differentiated cells back to a pluripotent embryonic state (Zang et al., 2014). Amplification products of the

molecular weight corresponding to the expected size of 318 bp for *REX1*, 232 bp for *NANOG*, 267 bp for *OCT4* and 244 bp for *SOX2* were observed confirming their expression in the DUC-MSCs isolated (Figure 4.5). The amplified products of the 4 genes have been sequenced thus confirming the identity of all the stem cell markers tested in DUC-MSCs.

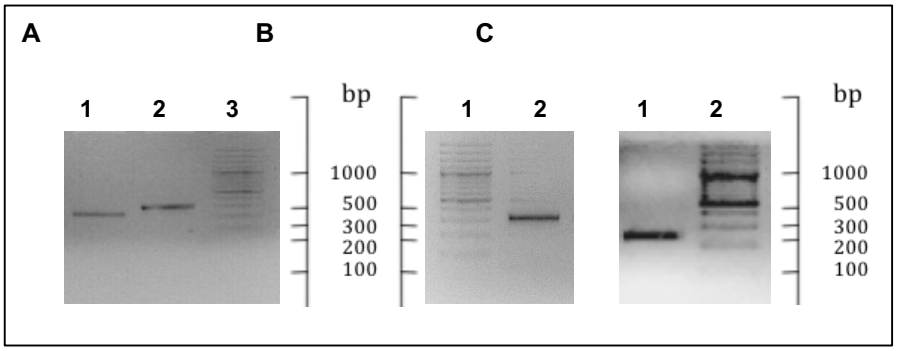

Figure 4.5. PCR products of markers of pluripotency in DUC-MSCs on 2 % agarose gel.
A Amplification product for 1, NANOG and 2, OCT4. **B** Amplification product for 2, REX1. **C** Amplification product for 1, SOX2. **A** 3, **B** 1, and **C** 2, DNA ladder, 100bp.

4.4. Conclusion

The results presented here, although preliminary, show a promising protocol for the isolation of mesenchymal stem cell from the umbilical cord of bottlenose dolphin. While we were able to confirm the presence of stem cell populations in the placental tissue, the direct culture of mesenchymal stem cell was successful only from the umbilical cord tissue. The mesenchymal stem cells isolated were plastic adherent, with a spindle morphology and with 2 n = 44 karyotype. The identity of the mesenchymal stem cell isolated was confirmed not only by the expression of gene markers of stemness (*REX1, OCT4, NANOG* and *SOX2*) but also by the expression of cellular surface markers (*CD44, CD90* and *CD105*), despite the lack of available species-specific antibodies and reagents. While the differentiation potential into adipocytes, chondroblasts and osteoblasts still needs to be completed and details need to be refined for optimal conditions for culture growth, the protocol developed is already showing its potential and applicability. Indeed, it is a much easier to perform and less invasive protocol from those currently used to isolate MSCs from marine mammal adipose tissue. Most importantly, the work presented has the potential of generating an unlimited source of stem cells for scientific research and clinical applications. Accelerated wound healing in injured wild or captive dolphins may help minimize infection and other side effects associated with open soft tissue wounds. Treatments using stem cells has an incredible potential to treat injuries of mesenchymal origin, such as soft tissue, bone, cartilage, or tendon in dolphins, following what is already being done in companion (dogs) or economically important (horses) animals. Moreover, the dolphin may serve as a human model for several disease processes, as was recently proposed for type 2 diabetes (Venn-Watson et al., 2011a) and aging studies (Venn-Watson et al., 2011b), where new treatment modalities using stem cells could be realized. Additionally, these treatments have the potential of being applicable to other Cetaceans and marine mammals.

5. Final conclusions

Model organisms have been crucial for in-depth analysis of cellular and molecular processes of life. The study of non-human species to research human biology and disease was made possible by the conservation of metabolic and developmental pathways and genetic material over the course of evolution. An organism must have specific characteristics in order to be considered a model to study a specific biological phenomenon, such as the size, the generation time, the accessibility, the manipulation, as well as the genetics and the conservation of molecular mechanisms. Unfortunately, these characteristics cannot be fund in the majority of organisms, many of which are of great ecological and biotechnological significance. Conventionally, genetic and transcriptional studies of these organisms, namely non-model organisms, have been restricted, due to the lack of reference genomes that impede their analyses. Until recently, marine mammal cellular and molecular biology technologies, specifically in relation to dolphins, were not greatly developed if compared to what was currently used in humans or in model organisms research. Luckily, high-throughput sequencing technologies are revolutionizing the life sciences technologies; genome sequences of non-model organisms are accumulating at an unprecedented rate, in each case representing a fundamental source of data of significant importance to biological research. Current tools work in diverse organisms, characterized by their unique metabolic pathways. Genome sequencing and the exploration of its coding and non-coding regions, is rapidly yielding meaningful answers to diverse questions. Cetaceans (including dolphins) evolved from land mammals millions of years ago. Dolphins have adapted to a completely dissimilar environment than that of terrestrial mammals. To aid in swimming they have developed a fusiform body shape; to prevent heat loss, they have developed a thick layer of blubber which provides insulation and also a food reserve and buoyancy. To dive deep, they have developed the capability to collapse their lungs, to reduce their heart rate and the blood flow to the non-essential organs together with increased ability to store oxygen (in both blood and muscle). To find prey, they have developed echolocation by which they emit rapid sound pulses and listen to their echo using the melon on their forehead to focus and direct waves. However, very little is known about the physiology of their unique adaptations to this environment. The understanding of the link between genes in molecular pathways

and between genes and proteins in the cellular context are necessary to understand the biology of an organism. In the study of non-model organisms, which also are protected species, like dolphins, cell and molecular approaches capable of sensitive and real-time pictures of environmental impacts (and interactions) of stressors are going to be even more useful.

The work presented in this dissertation represents advances in the development and applications of tools for the study of the biology, health and physiology of the dolphin, *Tursiops truncatus*, and their relevance for future studies of the impact of the environment changing conditions on the marine coastal ecosystem and its inhabitants, humans included.

In particular, the focus was 1) on the analysis of the bottlenose dolphin transcriptomes using gene expression microarray and RNA-seq technologies and 2) on the isolation and characterization of stem cells cultures, as an inestimable resource for basic and applied scientific research.

5.1. Methods for the investigations of wild dolphin's transcriptome

In order to better understand the connection of the dolphin health status to the marine environment and its correlation to human's health, we applied techniques for transcriptome analysis, typically used for model organism, to a non-model organism, which is also a protected species, making the investigation even more challenging. Transcriptomics focuses on the gene expression at the RNA level and offers the genome-wide information of gene structure and gene function in order to reveal the molecular mechanisms involved in specific biological processes.

In this work, we analyzed the blood and the skin transcriptomes of the bottlenose dolphin, using two different technologies: gene expression microarrays and RNA-seq.

The skin is the first barrier at the environment interface and it is also the easiest tissue that can be obtained from a wild dolphin through a non-invasive sample collection method. Variation in the dolphin skin transcriptome was investigated in correlation to environmental threats, such as the presence of contaminants of emerging concerns, most of which act as endocrine disruptors. We demonstrated

that exposure to endocrine disruptors, such as BPA and PFOA, even if used individually, can alone alter dolphin skin gene expression. Testing a specific contaminant aid the understanding (and discovery) of the effects of exposure at molecular level, thus leading to the discovery of proper biomarkers. Although we tested potential gene markers on a limited sample data set, our preliminary results are promising. We have observed trends in gene expression that correlate with different geographic locations, each one characterized by different levels of anthropic contamination. A bigger number of samples is needed, because there could be difference in gene expression also introduced by variables such as the animals age, sex, potential infection or diseased status, of which we do not have any information. These difference could be confounding factors in the analysis with a small sample set; certainly to increase the number of samples and data will make the conclusion more confident. Another important future step in the validation of the biomarkers will require sampling not only the skin, but also the blubber of few selected individuals. The blubber is the repository for many chemical contaminants and would be highly informative to directly quantify the presence of the BPA and/or PFOA in blubber and to correlate these values with the variation in expression of the contaminant-specific biomarkers selected in skin. Here we presented a preliminary study, where the effects of the exposure to two contaminants are described. A mixture of contaminants (and toxins, where present) more closely related to the natural environmental conditions, can lead to a better picture of the impacts on the health status of the organisms and on the long-term effects of environmental contamination exposure.

The conservation biology of marine mammals faces many practical problems, prominent amongst which is the difficulty in establishing criteria diagnostic of a healthy normal animal. An important message from the challenge faced in the transcriptomic study presented as well as from published transcriptomic studies of non-model, wild organisms, is the necessity of baseline data. A robust database of gene expression in dolphins from different environments, both managed and wild, would facilitate the use of transcriptomics for biomonitoring wild and managed populations of bottlenose dolphins describing their health status. The method used to evaluate the correlation between variations in the transcriptome associated with measured parameters was RNA-seq, a more comprehensive and less biased approach than the microarrays. The hematological parameters were evaluated for

correlation with season, gender and age. The transcriptome tested was the blood transcriptome in healthy managed bottlenose dolphins. Thanks to the active role of the immune cells, the blood is informative of an organism disease, exposure to a specific environmental condition and of the health status in general. Both a seasonal component to changes in blood gene expression, consistent with studies in humans, and an association of gene co-expression modules with age, sex or hematological parameters measured were found. The proportion of genes exhibiting seasonal changes in expression along with the degree of change observed was limited, demonstrating the relative stability of the dolphin blood transcriptome within and between animals throughout the course of one year. The major limitation of the study was the small difference in temperature observed throughout the four seasons in the specific location of sampling. Another limit of the study was the small dataset of healthy dolphins, restricted to managed animals. The dolphins at the Dolphin Quest facility are under human care and fed by humans, habits that could introduce differences with free-ranging dolphins. In order to explore the full possibility of seasonal changes a bigger number of samples, including those from wild, free-ranging dolphins that inhabit regions with higher changes in temperature and daylight are needed. In this study, we observed correlations between hematological parameters and variation in global gene expression, suggesting that blood transcriptome analysis may be also informative of infections and pathologies, thus showing its potential as a diagnostic tool.

5.2. Method for the in vitro study of dolphin's unique adaptations

Because opportunities for studies on wild and protected organisms such as dolphins are limited, it is critical to have cell lines from these organisms to study as surrogates for the intact animal. Isolated cells are common models for the study of protein functions, cellular mechanisms, organ-specific functions and responses to environmental parameters. Conditions must be determined for culturing the cells, such as optimal culture medium and other factors in order to maintain physiological characteristics and viability. The monitoring of cell growth and the characteristics that define their changes is now being accomplished thanks to the development of technologies that enable scientists to look at a living cell in the population but also

inside the single cell, gathering information of its behavior in different culture conditions. Specifically, advances in microscopy, image analysis and statistical software over the last few years provide for more accurate assessment of baseline quantitative information needed to understand processes both at a cellular and molecular level. Primary cells isolated from tissues mimic the physiological state of cells *in vivo* and generate more relevant data representing living systems. Unfortunately, primary cells typically can be kept in culture for a limited period of time, before they reach senescence. Primary cells can undergo mutations causing de-regulation of the normal cell cycle controls leading to uncontrolled proliferation *in vitro*. These cells are called immortalized cells and share characteristics with cancerous cells. The main advantage of using an immortal cell line for research is that it can be grown indefinitely in culture. But the immortalization can alter the biology of the cells leading to phenotypic differences and potentially different experimental results. A different group of cells that are cultured *in vitro* and can be propagated indefinitely is represented by the stem cells. Stem-cell lines are derived from any organism and come from one of three sources: embryonic stem cells, induced stem cells, or adult stem cells. Given the difficulty of culturing embryonic stem cells for ethical issues and the troubles of generating an accurate induced stem cell line from reprogramming events, the attention was focused on adult stem cells, in particular mesenchymal stem cells. Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types including: osteoblasts, chondrocytes, myocytes and adipocytes. In animal medicine, for therapeutic purpose, they are derived mainly from bone marrow and adipose tissue.

Here we present a protocol for the isolation of mesenchymal stem cell from a different source, the umbilical cord tissues of the bottlenose dolphin. The mesenchymal stem cells isolated plastic adherent, with a spindle morphology and with 2 n = 44 chromosomes. The identity of the mesenchymal stem cell isolated was confirmed not only by the expression of gene markers of stemness but also by the expression of cellular surface markers, despite the lack of available species-specific antibodies and reagents. While the differentiation potential into adipocytes, chondroblasts and osteoblasts still needs to be tested for the study to be complete and the statements fully supported, the protocol developed is already showing its potential and applicability. For instance, it is a much easier to perform and less invasive protocol from those currently used to isolate mesenchymal stem cells from

marine mammal adipose tissue. Each animal, right after birth, could have its own cell bank for any therapeutic purposes. Mesenchymal stem cells are an attractive cell population for regeneration of musculoskeletal tissues and wound healing in dolphins. Multiple sources of mesenchymal stem cells have been now described including not only the adipose tissue or umbilical cord, but also the bone marrow, ligaments or lung. While for most cases the procedure to isolate stem cells from tissues of source is laborious and invasive, this is not the case of the umbilical cord derived stem cells, since the placenta is expelled upon birth. Stem cells hold great potential for the development of new and exciting therapeutic strategies against diseases and injuries of animals. Vet-Stem, a company based in California, has provided stem-cell treatments to more than 5000 horses, 4300 dogs and 120 cats since treating its first patient in 2004. Stem cells have also been used to repair a lumbar fracture in a Bengal tiger and to treat arthritis in pigs. In managed dolphins, or wild dolphins under treatment in rehabilitation centers, stem cells could be used for medical therapies to repair, replace, restore and regenerate damaged or diseased tissues. The unlimited source of cells that can be obtained from stem cells in culture is also enabling the study of stem-cell therapies in large animals, which are more physiologically similar to humans than the mice often used in preclinical research. They could be a powerful model for medicine, if we only think to the recent finding that stem cells in the fat of bottlenose dolphins raise hopes for treating the marine-mammal versions of liver disease and type 2 diabetes are some examples.

5.3. Final considerations

When the first complete draft of the human genome was released in 2003 we entered the post-genomic era. While it took less than 15 years from the announcement of the quest to sequence the human genome to its completion, the post-genomic era can be expected to last much longer. The genome projects are generating an unprecedented amount of information regarding the identification and structure of genes. The comprehensive catalog of the known genes together with their nucleotide sequence is intensifying research efforts on exploration of gene

function, both individually and collectively, at the molecular, cellular, organism and population levels.

Eight years after the first whole-genome assembly of the human genome, the sequencing of a first, light version of the bottlenose dolphin genome was completed, thus initiating a new era of discoveries in the marine mammal field. Since then, the bottlenose dolphin genome has been greatly implemented thanks to the assembly of new high-throughput sequences data and a new high-coverage genome is about to be released. To date, genomes from 16 species of marine mammals have been sequenced and are currently being analyzed. Genome-guided transcriptome assembly as well as *de novo* transcriptome assembly are providing novel insights into the mechanisms underlying the variety and diversity of marine mammals, revealing novel genes, functions, connections of molecular and physiological mechanisms related to unique biological phenomena.

Genomics and transcriptomics are to be considered only an entry point for looking at the other -omics sciences. The study of the genome and of the information that is encoded within the full DNA sequence of an organism seem not be able alone to unlock the code of life. The information in the genes of an organism, its genotype, is largely responsible for the final physical makeup of the organism, referred to as the phenotype. However, the environment also has some influence on the phenotype. About a decade ago, scientists started looking into the “epigenome,” chemical modifications of the DNA, associated with a heritable phenotype, affecting its organization and regulation in the cell, without alterations in the DNA sequence. The study of the epigenome involves genome-wide mapping of DNA methylation, histone modifications, nucleosome positioning and three-dimensional architecture. The integration of genome and epigenome, of the RNA information from coding (mRNA) and non-coding RNA (epigenetic related RNAs, such as micro RNA, short interfering RNA, piwi-interacting RNA and long non-coding RNA) is necessary to understand how environment and genetic inclinations can intertwine in the complexity of cell biology.

The science of marine mammals goes back at least to Aristotle, who described in 400 BC dolphins giving birth to live young nursed with the mother’s milk. Accurate natural history observation on the biology of marine mammals expanded throughout the time mixed by misconceptions, imagination and superstition. Marine mammals

have been hunted for a long time for their oil, meat, baleen, but also with the intention of reducing their predation on valued resources such as fish, thus reducing many populations to a fraction of their former size. Only after the Second World War the marine mammal science started to grow and studies started combining aspects of mammology, ethology, ecology, animal conservation, oceanography, evolutionary biology, geology and, finally, molecular biology. Emerging diseases and contamination levels described in free-ranging animal's studies or observed during stranding events are now being addressed more thoroughly, thanks to the possibility of linking molecular and cellular mechanisms to a physiological function and to its perturbations (e.g. for an infection or a disease), caused by the continuous changes in the marine environment induced by human activities. The patterns and the correlation that we are observing combining the data can now be used in predictive models to identify potential risks and propose solutions, making the new technologies relevant as prognostic and diagnostic tools.

The exploitation of new techniques applied to the study of the genome and epigenome, along with focused bottlenose dolphin cell-based experiments, will allow difficult research questions to be addressed. We will be able to better understand the interaction between dolphin, human and the ocean, to discover a unique and challenging environment, and to solve problems associated with the quality and quantity of living marine resources.

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Appendix

PAPER I

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PAPER II

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Short communication

Transcriptomic analysis of bottlenose dolphin (*Tursiops truncatus*) skin biopsies to assess the effects of emerging contaminants

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ABSTRACT

Chemicals discovered in water at levels that may be significantly different than expected are referred to as *contaminants of emerging concern* (CECs) because the risk to environmental health posed by their occurrence/frequency is still unknown. The worldwide distributed compounds perfluorooctanoic acid (PFOA) and bisphenol A (BPA) may fall into this category due to effects on endocrine receptors.

We applied an *ex vivo* assay using small slices of bioptic skin from the bottlenose dolphin, *Tursiops truncatus*, cultured and treated for 24 h with different PFOA or BPA concentrations to analyze global gene expression. RNA was labeled and hybridized to a species-specific oligomicroarray. The skin transcriptome held information on the contaminant exposure, potentially predictive about long-term effects on health, being the genes affected involved in immunity modulation, response to stress, lipid homeostasis, and development. The transcriptomic signature of dolphin skin could be therefore relevant as classifier for a specific contaminant.

1. Introduction

The health decline of the marine ecosystem can be mostly related to anthropogenic impacts such as overfishing, coastal habitat destruction, deep sea mining, oil and gas exploration, and ocean acidification, but it is also strictly correlated to industrial application and the release of chemical contaminants and pollutants. The exposure to those named as *contaminants of emerging concern* (CECs, http://water.epa.gov/scitech/cec/) is unavoidable: after their release into the environment, they can be transported through air, water and soil, and be a threat to both ecosystem and human health. Several of these chemical products are classified as endocrine disruptors (EDs), since they can interfere modifying the synthesis, circulating levels, and peripheral action of hormones (Casals-Casas and Desvergne, 2011).

Among the CECs is bisphenol A [2,2 bis(4-hydroxyphenyl) propane; BPA], an ubiquitous, high-volume-production (>2.5 × 10^6 kg $year^{-1}$) monomer used in the manufacture of polycarbonate plastics (Casals-Casas and Desvergne, 2011). BPA has been shown to leach out of products such as plastic containers, utensils, toys, water bottles and fax paper, and high levels of monomer have been identified in human and animal samples (McLachlan, 2001). In humans, high urinary concentrations of BPA are associated with an increased occurrence of cardiovascular disease, diabetes, and liver enzyme abnormalities (Lang et al., 2008) and a large body of evidence links BPA to adverse health effects in perinatal, childhood and adult (Rochester, 2013). It can bind estrogen receptors and promote both agonist and antagonist activities. BPA binds to aryl hydrocarbon receptors and has diverse endocrine effects on mammalian and non-mammalian health (Kharrazian, 2014). The contaminant concentration for BPA already measured in the environment is 1 μg/ml (http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/bpa.html).

Another CEC is the perfluorooctanoic acid (PFOA), one of the

Abbrevations: ADIRF, adipogenesis regulatory factor; BCAP31, B-cell receptor-associated protein 31; BPA, bisphenol A; CDC42, cell division cycle 42; CECs, contaminants of emerging concern; EDs, endocrine disruptors; f, fold; GADPH, glyceraldehyde 3-phosphate dehydrogenase; GAP, GTPase-activating protein; GO, Gene Ontology; GPCR, G protein-coupled receptor; MTSS1, metastasis suppressor 1; PFCs, perfluorinated compounds; PFOA, perfluorooctanoic acid; qPCR, quantitative real-time polymerase chain reaction; RGS2, regulator of G-protein signaling 2; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

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most important synthetic perfluorinated compounds (PFCs). The chemical structure of PFCs gives them unique properties, such as thermal stability and the ability to repel both water and oil, that make them useful components in a wide variety of consumer and industrial products including paper, leather and fire-fighting foam. Due to its persistence and bioaccumulation PFOA has been listed as emerging persistent organic pollutant in 2009 Stockholm Convention (Wang et al., 2009). High production volumes led to widespread distribution in the environment, and once absorbed PFOA does not undergo biotransformation, distributing primarily in the liver and plasma, and to a lesser extent in the kidneys and lungs (Kudo and Kawashima, 2003). PFOA has been found to exert acute and sub-chronic toxic effects with the liver as a primary target organ in mice and rats, rabbits and birds (Kannan et al., 2002; Betts, 2007). Several recent studies detected PFOA in a variety of wildlife animals including fresh water species, marine mammals and shellfish, and suggested that can be biomagnified at the top levels of the food chain (Lau et al., 2004). PFOA concentrations up to 10 ng/ml have been detected worldwide in human serum (Haug et al., 2009). PFOA can be considered an ED, since the exposure of rodents led to serious impact on phospholipid metabolism, reductions in serum cholesterol (Peng et al., 2013), hormonal perturbations with decreased testosterone and increased estradiol levels (Shi et al., 2007).

Some CECs can bioaccumulate through the food chain, leading to highest levels of exposure in predator species. The bottlenose dolphin (*Tursiops truncatus*) spends its life in marine coastal environment, feeds at a high trophic level and is exposed to contaminants of human concern. Due to their long lifespan, dolphins could be chronically exposed to CECs and as top-level predators concentrate the contaminants in their body, being also endowed with large blubber stores that can serve as depots for anthropogenic chemicals and toxins. In addition, its populations display high site fidelity for coastal locations thus have the potential to be sentinel species for emerging contamination in that areas (Aguirre and Tabor, 2004; Wells et al., 2004; Moore, 2008; Bossart, 2011; Kucklick et al., 2011).

Here we report about the effects of BPA and PFOA on bottlenose dolphin skin biopsies analyzed by transcriptomics. This tool allows the response to treatment of thousands of genes to be examined simultaneously, providing a comprehensive information on the pathways modulated. The analysis of genes in the cellular context under controlled conditions would allow identifying those differentially expressed and classifying as exposure biomarkers. This approach could be informative not only of the impact of CECs on dolphins (and marine mammals in general), but also on the threats posed to the marine ecosystem and have a practical outcome due to the development of real-time tools for rapid detection of contaminant exposure.

2. Methods

2.1. Bottlenose dolphin samples

Skin samples were obtained 4 h after death of a juvenile female (length 144 cm, weight 31.6 Kg) stranded on Tyrrhenian shores (Italy) in October 2011 (CITES permit: Nat. IT025IS, Int. CITES IT 007 issued to Accademia dei Fisiocritici and University of Siena). No relevant pathology, parasites and lesions were detected at *post mortem* examination and the cause of death is unknown.

2.2. Ex vivo assay

Slices (about 2 mm-thick) spanning the epidermis and dermis were cut from skin samples of the stranded specimen immediately after collection, to set up the organotypic cultures exposure experiments in 5 ml culture tubes, as previously described (Godard et al., 2004). Distinct slices were separately incubated for 24 h at room temperature (24–28 °C) in cell culture media (Fossi et al., 2006) containing BPA (0.1 or 1 μg/ml), or PFOA (0.1 or 1 μg/ml), or the vehicle (final concentrations: 0.01% ethanol for BPA and 0.1% methanol for PFOA) in a final volume of 3 ml. Thereafter, they were homogenized using a Tissue Lyser (Qiagen) and RNA was extracted using the Aurum™ Total Fatty and Fibrous Tissue kit (Bio-Rad) following the manufacturer's instructions.

2.3. Dolphin microarray hybridization and gene expression analysis

The microarray used was a species-specific, custom 4 × 44K Agilent oligo array representing 24,418 unigene sequences (Mancia et al., 2015).

All RNA labeling and microarray hybridizations were performed according to the manufacturer's instructions in the One-Color Microarray-Based Gene Expression Analysis manual (Agilent Technologies, Santa Clara, CA). Five hundred nanograms of RNAs from each treated slice were hybridized. One-color gene expression was performed according to the manufacturer's procedure. Briefly, total RNA fraction was obtained from samples by using the RNeasy kit (Qiagen). RNA quality was assessed by the use of Agilent 2100 Bioanalyzer. Low quality RNAs (RNA integrity number <6) were excluded from microarray analyses. Fluorescent complementary RNA (cRNA) was synthesized from 200 ng of total RNA using the Low Input Quick-Amp Labeling Kit, one color (Agilent) in the presence of cyanine 3-CTP. Hybridizations were performed at 65 °C for 17 hs in a rotating oven. Images at 5 μm resolution were generated by Agilent scanner and the Feature Extraction 10.7.3.1 software (Agilent) was used to obtain the microarray raw-data. The microarray build and hybridization data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO accession numbers GSM1712791- GSM1712796 (www.ncbi.nlm.nih.gov/geo/).

Microarray results were analyzed using GeneSpring GX v.12 software (Agilent). Data transformation was applied to set all the negative raw values at 1. Data were normalized using quantile normalization. A filter on low gene expression was used to keep only the probes expressed in at least one sample (flagged as detected). Then, samples were grouped in accordance to their treatment status. BPA- and PFOA-treated samples were analyzed compared with samples incubated in cell media containing the specific vehicles. Differentially expressed genes were selected as having a 1.5-fold expression difference (geometrical mean) between the groups of interest and a statistically significant p-value (<0.05) at moderated t-test statistic, followed by the application of the Benjamini and Hoechberg correction for false positives reduction. Differentially expressed genes were employed for cluster analysis of samples using the Manhattan correlation as a measure of similarity. Gene Ontology (GO) analyses were carried out using Blast2go pro software (www.blast2go.com).

2.4. Quantitative real time PCR: validation of microarray data

Results from the microarray analysis were validated by qPCR by measuring mRNA expression of four selected genes in all the treated samples. *GADPH* (glyceraldehyde 3-phosphate dehydrogenase) and *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) genes were used as internal controls.

Relative mRNA levels were determined on a CFX Connect (Bio-Rad) with specific primers designed using *Tursiops truncatus* sequences on the arrays and in the NCBI public database. Each primer

set was optimized for efficiency and specificity by running standard curves on cDNA resulting highly correlated ($R^2 > 0.969$) with efficiencies between 90 and 110%. Quantitative qPCR efficiencies were calculated using the equation m = (1/logE), where m is the slope of the line and E is the efficiency (Dhar et al., 2009) (Table S1).

One microgram of total RNA for each treated samples was reverse transcribed using iScript Select cDNA Synthesis Kit (BioRad) according to manufacturer's instructions.

Optimized qPCR parameters for each genes were determined using diluted (1:10) cDNA reverse transcribed from 1 μl of total RNA using SsoFast™ EvaGreen® Supermix (BioRad) in a total volume of 10 μl of a reaction mix containing 1 μl cDNA, 0.6 μl of each primer (300 μM), 5 μl Evagreen enzyme and 3.4 μl DNase-free sterile water.

qPCR reactions were run as follows: 1 cycle of 98 °C for 30 min, 49 cycles of 95 °C for 5 min, 60 °C for 10 min; melting curve 65 °C–95 °C: increment 0.5 °C every 5 min. Each reaction was run in triplicate, together with a triplicate of no-template control. The average Ct values were normalized to the corresponding measured mRNA Ct value of the housekeeping genes *GADPH* and *YWHAZ*. Comparative Ct method of analysis ($2^{-\Delta\Delta ct}$) was used to determined changes of expression between control and treated samples.

3. Results and discussion

3.1. Gene expression profiles of BPA-treated skin biopsies

BPA treatment of organotypic cultures differentially regulated (fold ≥1.5 and p value <0.05) 1146 genes, of which 449 were up regulated and 697 down regulated (Fig. 1, Table S1). Based on Gene Ontology (GO) analyses, the differentially expressed genes annotated are involved in metabolic and cellular process (81.4%), immune response (18.2%), developmental process (43.3%), cellular component organization and biogenesis (45.3%), cellular signaling (32.8%) (Supplementary Fig. 1).

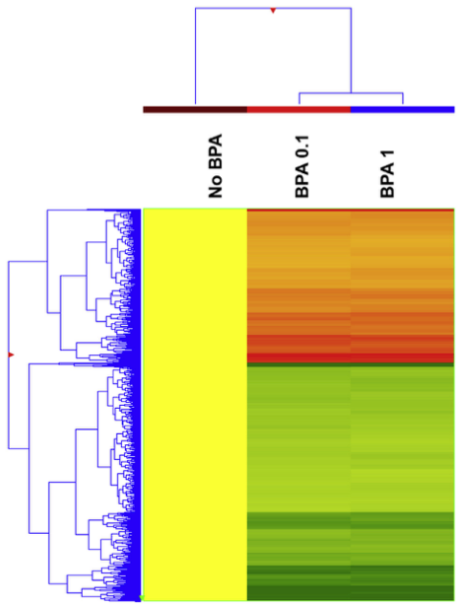

Fig. 1. Hierarchical clustering of microarray data showing the differentially expressed genes of dolphin skin organotypic cultures treated with different concentrations of BPA (0.1 and 1 μg/ml). The dendrogram on the left depicts the 1146 genes differentially expressed (fold change ≥1.5 and p-value ≤0.05). Red: up-regulated genes, green: down-regulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the 'response to stress' GO category we found genes like the *programmed cell death protein 4* (down, 1.53 fold, f), a tumor suppressor that plays a role in apoptosis and has a role inhibiting neoplastic transformation. Alterations in the concentration of the cellular product of this gene can lead to tumor development and malignant progression, as well as to change in regulation of cellular processes such as proliferation, differentiation and DNA repair (Jansen et al., 2005; Yang et al., 2006; Mudduluru et al., 2007). The activation of the genes involved in this GO category after BPA exposure can be linked to a generalized response of the cells to an emergency condition where the cellular pathways involved in the malfunctioning of proteins are interrupted by the change in regulation of key genes, and defected cells keep dividing, avoiding physiological apoptosis.

Because of its estrogenic activity, BPA can impact immune signaling pathways in many ways, activating T- and B-lymphocytes and dendritic cells, stimulating TNF-α production by macrophages, and leading to chronic activation of antigen-presenting cells (Kharrazian, 2014). We found many down regulated genes in the 'immune system process' category: *complement factor H* (down, 1.6 f), involved in negative regulation of the inflammatory response, *class I histocompatibility alpha chain* (down, 2.1 f), involved in the presentation of foreign antigens, *alpha 2 macroglobulin precursor* (down, 2.4 f), able to inactivate an enormous variety of proteinases, functioning as an inhibitor of fibrinolysis and coagulation, *melanoma inhibitory activity protein 3* (down, 1.7 f), a protein with growth factor activity involved in skin melanoma, *pleckstrin* (down, 5.9 f), a modular platelet protein. Some immune genes were also up regulated, such as *B-cell receptor-associated protein 31* (up, 2.1 f), involved in antigen processing, and *lymphocyte antigen 96* (up, 1.5 f), cooperating with TLR4 in the innate immune response to bacterial lipopolysaccharide.

BPA exposure, even in low doses, can result in adverse effects both pre- and post-birth. Estrogens have a key role in early development and prenatal exposure of BPA can affect estrogen-responsive tissues. A study in mice showed that maternal behavior can be altered, in part affecting the CNS, in particular the neuroendocrine-gonadal axis, which regulates neuronal development (Palanza and Howdeshell, 2002). According to GO, we found changes in the transcription of genes involved in embryonic development and growth, like *titin* (down, 5.57 f), a key component in the assembly and functioning of both adult and embryonic striated muscles and muscle tendons, *nuclear distribution protein nude-like 1* (up, 1.64 f), involved in organization of the cellular microtubule array and microtubule anchoring at the centrosome, and in brain development for the migration of newly formed neurons which plays a role in the regulation of neurite outgrowth (Vergnolle and Taylor, 2007).

Moreover, it is well known that estrogenic effects of BPA can impact adipogenesis and body weight, through complex interactions with the estrogen, thyroid hormone and glucocorticoid receptors, causing metabolic disturbances (Casals-Casas and Desvergne, 2011; Wang et al., 2012). We found that BPA may affect pathways involved in fat (blubber) differentiation, down regulating several genes involved in lipid metabolism: *adipogenesis regulatory factor* (down, 1.80 f), which promotes adipogenic differentiation and plays a role in fat cell development, *fatty aldehyde dehydrogenase isoform 2* (down, 1.62 f), responsible for the catalysis of the oxidation of long-chain aliphatic aldehydes to fatty acids,

apolipoprotein B 100 (down, 2.39 f), whose function is to recognize the signal for the cellular binding and internalization of LDL particles. Alterations in apolipoprotein B 100 can lead to disorder in lipoprotein metabolism and hypercholesterolemia, and increased proneness to coronary artery disease (van der Graaf et al., 2011).

3.2. Gene expression profiles of skin biopsies treated with PFOA

PFOA treatment of organotypic cultures differentially regulated (fold ≥1.5 and p value <0.05) transcription of 428 genes in total, of which 191 were up regulated and 237 were down regulated (Fig. 2, Table S2). GO analysis of the annotated genes collocated most of them in the following categories: cellular process (32.5%), metabolic process (27.4), single organism process (25.7%), biological regulation (20.5%), immune system process (2.6%), cellular component organization or biogenesis (15.4%), localization (12.8%), multicellular organismal process (9.4), developmental process (8.5%).

The genes up regulated involved in response to stress were: *uv excision repair protein rad23 homolog a* (up, 1.59 f), which plays a central role both in proteasomal degradation of misfolded proteins and DNA repair, and *heat shock protein 90* (up, 1.8 f), a molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved in cell cycle control and signal transduction. Differently, we found in the same category a down regulated gene: *complement c5* (down, 5.79 f), a mediator of local inflammatory process. In humans, high levels of PFOA have been associated with significant changes in clinical markers of immune and inflammatory process, and exposure to PFOA can adversely affect immune function (DeWitt et al., 2012). Besides *complement c5*, that can be included also in the inflammatory response GO category, we found variations (down/up) in transcripts of genes engaged in immune system activation, like *acidic mammalian chitinase* (down, 5.23 f), involved in the defense against nematodes, fungi and other pathogens, or *interferon-induced protein* (up, 1.86 f) and *interleukin 8* (up, 6.80 f), which is a chemotactic factor for neutrophils, basophils and T-cells and it is also involved in neutrophil activation.

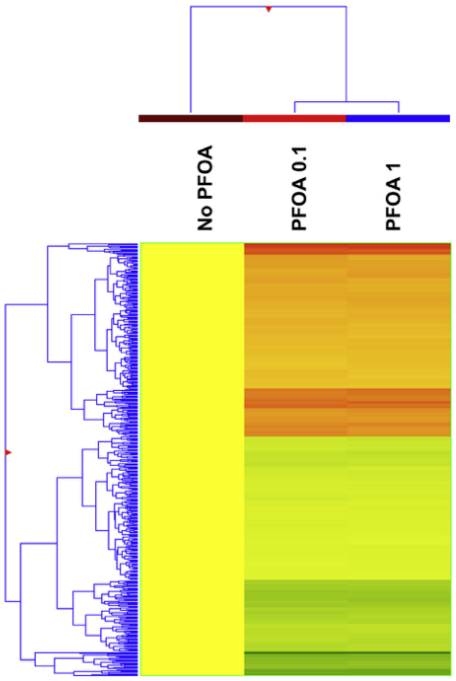

Fig. 2. Hierarchical clustering of microarray data showing the differentially expressed genes in dolphin skin organotypic cultures treated with different concentrations of PFOA (0.1 and 1 μg/ml). The dendrogram on the left depicts the 428 genes differentially expressed (fold change ≥1.5 and p-value ≤0.05). Red: up-regulated genes, green: down-regulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It is worth noting how PFOA treatment evoked transcription changes of a panel of genes different from that effected by BPA. The different chemical structures and mechanisms of action of the two compounds can likely account for this findings.

Some studies in adult rats suggest that PFOA interferes with a normal lipid metabolism, affecting serum concentrations of sex hormones (decreasing testosterone and increasing estradiol) and lipids (reducing cholesterol and/or triglycerides) (Shi et al., 2007; Thibodeaux et al., 2003). We have found variations of transcripts of genes regulating lipid metabolic process, like *choline phosphotransferase 1* (down, 1.50 f), which is involved in phospholipid metabolism, or *trifunctional enzyme subunit beta mitochondrial like* (up, 1.58 f) which is involved in lipid metabolism and fatty acid beta oxidation. Deficiency of this latter gene can lead to hypoglycemia and cardiomyopathy (Spiekerkoetter et al., 2003).

Furthermore, exposure to PFOA during pregnancy leads to developmental toxicity, reduced birth weight and neonatal mortality in mice (Lau et al., 2006; Fuentes et al., 2006; Luebker et al., 2005). This is consistent with our findings, since we report about up regulation of genes involved in embryonic development, such as *vascular endothelial growth factor* (up, 1.54 f), and *Rho GTPase-activating protein 35* (up, 1.62 f), which may participate in the regulation of retinal development, and *non-lysomal glucosylceramidase* (up, 2.19 f) which plays a role in central nervous system development (Martin et al., 2013) (Supplementary Fig. 2).

3.3. Quantitative real time PCR

Semi-quantitative gene expression analysis resulting from the microarray study was validated through qPCR. Four genes among those differentially expressed were chosen for the analysis testing the same RNA used in microarray analysis (Table 1). Two genes were selected from the BPA exposure study: 1) *ADIRF* (adipogenesis regulatory factor), that plays a role in fat cell development and promotes adipogenic differentiation, 2) *BCAP31* (B-cell receptor-associated protein 31), involved in the anterograde transport of membrane proteins from the endoplasmic reticulum to the Golgi and in caspase 8-mediated apoptosis (Fig. 3). The two genes selected from the PFOA exposure study were both up regulated: 1) *RGS2* (regulator of G-protein signaling 2), encoding a protein that binds to heterotrimeric G proteins by way of its RGS domain and acts as GAP (GTPase-activating protein) to turn off G protein-coupled receptor (GPCR) signals, and 2) *CDC42* (cell division cycle 42), involved in regulation of the cell cycle.

qPCR confirmed microarray data for the selected genes at a contaminant concentration (1 μg/ml) already measured in the environment (http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/bpa.html). At this dose, the contaminant is probably toxic and poisoning, hence causing a strong cellular response. A linear correlation ($r = 0.993$; $P < 0.001$) between variations of expression of the four genes selected for analysis, assessed by

Table 1
List of genes used in real time PCR, differentially expressed in microarray analysis.

Gene name	Conting number	Treatment	Regulation	Fold change (microarray)
Adipogenesis regulatory factor	Contig-c2315-3	BPA	down	1.7
B-cell receptor-associated protein 31	Contig-c9258-1	BPA	up	2.13
Regulator of G-protein signaling 2	Contig-c23388-1	PFOA	up	3.2
Cell division cycle 42	SRR027946.159665	PFOA	up	4.5

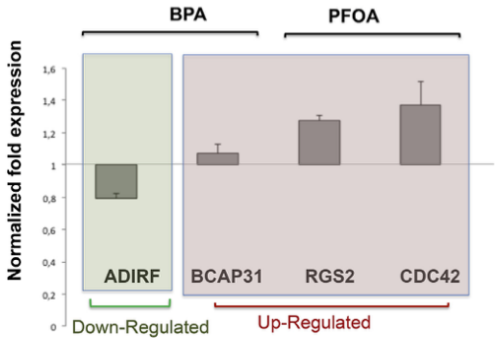

Fig. 3. Real time PCR data for 4 selected genes on treated organotypic cultures. RNA from 2 treated samples (1 μg/ml) was individually reverse transcribed, and each gene was amplified in triplicate from the cDNA. Data were normalized to *GAPDH* and *YWHAZ* genes by ΔΔct method. From left to right: *ADIRF, BCAP31, RGS2, CDC42*. Bottom: green and red brackets, genes up- and down-regulated in microarray analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microarray analysis and qPCR, validated both analytical approaches.

BPA is a selective estrogen receptor modulator that shows a different binding affinity for/and regulation of ERα and ERβ in target cells and can interact differently with transcriptional co-regulators. Furthermore, there is evidence that BPA can elicit rapid cell responses via non-genomic signaling, like estradiol does. Very low concentration of BPA and estradiol can activate cell-signaling pathways via plasma membrane receptors and this very rapid response can produce additive effects to that mediated by nuclear receptors, which takes longer to occur and at higher concentration of BPA (Welshons et al., 2006). In rat hippocampus, instead, BPA has been shown to antagonize the action of estradiol by locking its stimulatory effect (MacLusky et al., 2005). In another study, BPA seems to disrupt the actions of estradiol acting as potent estradiol mimetic, during cerebellar development (Zsarnovszky et al., 2005). Considering the complex spectrum of mechanisms potentially exerted by BPA, our findings are clearly suggestive of an overall effect of the contaminant, but the underlying mechanisms obviously require more detailed analysis.

PFOA is not metabolized in the body and, unlike BPA, is not lipophylic but binds to serum albumin and is excreted primarily from the kidney. PFOA induces various types of tumors, reduces birth weight in mice and causes neonatal death in rats (Lau et al., 2007). In mice it decreases the B- and T-cell immune responses and impairs thyroid hormone homeostasis (Lau et al., 2007; Butenhoff et al., 2002).

qPCR data for the BPA and PFOA differentially expressed genes confirmed microarray data: ADIRF is downregulated and BCAP is upregulated (BPA); RGS2 and CDC42 were found to be upregulated (PFOA). As expected, there are differences in the quantification of the espression of the genes between the two methods of analysis, with the qPCR being more quantitatively reliable method. The observed effects at the concentration tested agreed with previously published data. Unlike most other well-studied water contaminants, PFOA effects can be observed at low exposure levels in humans. Some effects can be recorded in both humans and animals while others such as on lipid metabolism, can not (Post et al., 2012). In fact, besides the activation of the immune and stress responses we have observed the differential regulation of a suite of genes involved in such pathways. Many studies showed positive correlation between serum level of PFOA and other lipids (i.e., LDL cholesterol and triglycerides) but the underlying mechanisms are not yet clarified. Taken together, our findings suggest that PFOA has an impact on the skin tissue treated, which was not described before and deserves further investigation.

3.4. Technology potential for cetacean health studies

The implication of the long-term effects of chemical pollution on the marine fauna, and on cetaceans in particular, is mostly unknown. This work describes the successful employment of two novel approaches: 1) the use of the dolphin skin biopsy, an *ex vivo* assay, coupled with a transcriptomic analysis, using a species-specific custom made microarray, and 2) the assessment of the effects of two non-canonic but environmentally threatening contaminants on wild dolphin health. We know from literature that exposure to chemical contaminants in the long-term does cause immunosuppression and health problems to marine mammal populations (Wells et al., 2004; Bossart, 2011; Schwacke et al., 2014, 2012), so the hypothesis to be tested was the activation of contaminant-specific gene signature. The molecular pathways activated by BPA and PFOA had a profile consistent with the cellular response to EDs exposure. Genes involved in the activation of the immune system and response to stress are differentially expressed and mechanisms linked to lipid homeostasis and development seem to be activated. Nevertheless we found a broad spectrum of differentially expressed genes to be contaminant specific. BPA activates a stress response and many genes affected are also involved in development, which may be inferred to the estrogenic activity of the chemical. PFOA exposure triggers mainly genes related to the immune response, the lipid metabolism and homeostasis, probably depending on its chemical nature and action mechanisms.

We identified some potential biomarkers that will have to be tested on wild animals in order to establish a reliable diagnostic tool. Skin/blubber biopsies from marine mammals inhabiting areas with different level of contamination are being collected to test which, between the genes differentially expressed upon treatment, would substantiate BPA and PFOA exposures.

Other applications would reside in skin biopsy analysis: a diagnostic tool for stress diagnosis in cetaceans, or for the detection of the nature of contamination by the quantification of known and emerging contaminants. Target genes selected as biomarkers could be usefully employed for fast assessment of exposure and health of free-ranging mammals, and possibly even to ascertain causes of stranding. The skin is an effective barrier at the interface with the environment and at the same time offers many advantages: easy to

collect from stranded animals but also the easiest tissue to collect (dart biopsies) during sampling in the wild, in a rather non-invasive way. Moreover, studies on the skin may lead to biomedical applications. The amazingly effective regeneration of dolphin skin is well documented (Zasloff, 2011) and transcriptomic tools would allow specific gene networks and pathways to be identified, with potential for therapeutic aids of wound healing in humans as well. A better understanding of dolphin's wound healing may lead to the identification of new antimicrobial, analgesic and tissue-regenerating mechanisms, that may apply to new drugs and therapeutics for debilitating and life-threatening diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2016.01.002.

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PAPER II

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RESEARCH ARTICLE Open Access

RNA-Seq analysis of seasonal and individual variation in blood transcriptomes of healthy managed bottlenose dolphins

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Abstract

Background: The blood transcriptome can reflect both systemic exposures and pathological changes in other organs of the body because immune cells recirculate through the blood, lymphoid tissues, and affected sites. In human and veterinary medicine, blood transcriptome analysis has been used successfully to identify markers of disease or pathological conditions, but can be confounded by large seasonal changes in expression. In comparison, the use of transcriptomic based analyses in wildlife has been limited. Here we report a longitudinal study of four managed bottlenose dolphins located in Waikoloa, Hawaii, serially sampled (approximately monthly) over the course of 1 year to establish baseline information on the content and variation of the dolphin blood transcriptome.

Results: Illumina based RNA-seq analyses were carried out using both the Ensembl dolphin genome and a *de novo* blood transcriptome as guides. Overall, the blood transcriptome encompassed a wide array of cellular functions and processes and was relatively stable within and between animals over the course of 1 year. Principal components analysis revealed moderate clustering by sex associated with the variation among global gene expression profiles (PC1, 22 % of variance). Limited seasonal change was observed, with < 2.5 % of genes differentially expressed between winter and summer months (FDR < 0.05). Among the differentially expressed genes, cosinor analysis identified seasonal rhythmicity for the observed changes in blood gene expression, consistent with studies in humans. While the proportion of seasonally variant genes in these dolphins is much smaller than that reported in humans, the majority of those identified in dolphins were also shown to vary with season in humans. Gene co-expression network analysis identified several gene modules with significant correlation to age, sex, or hematological parameters.

Conclusions: This longitudinal analysis of healthy managed dolphins establishes a preliminary baseline for blood transcriptome analysis in this species. Correlations with hematological parameters, distinct from muted seasonal effects, suggest that the otherwise relatively stable blood transcriptome may be a useful indicator of health and exposure. A robust database of gene expression in free-ranging and managed dolphins across seasons with known adverse health conditions or contaminant exposures will be needed to establish predictive gene expression profiles suitable for biomonitoring.

Keywords: *Tursiops truncatus*, Bottlenose dolphin, Blood transcriptome, RNA-seq, Globin-reduction

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Abbreviations: AMMPA, Alliance of marine mammal parks and aquariums; BAR, Bright, alert responsive; BLAST, Basic local alignment search tool; CEG, Core eukaryotic gene; CEGMA, Core eukaryotic genes mapping approach; FPKM, Fragments per kilobase of transcript per million mapped reads; GEO, Gene expression omnibus; GO, Gene ontology; PC, Principal component; PCA, Principal component analysis; PCB, Polychlorinated biphenyl; PCR, Polymerase chain reaction; RIN, RNA integrity number; RSEM, RNA-seq by expectation maximization; WGCNA, Weighted gene co-expression network analysis

Background

High-throughput blood gene expression profiling has been broadly applied in human medicine for the identification of health status and disease, monitoring responses to drug therapies, defining disease prognosis, and identifying exposures to environmental toxicants [1, 2]. Blood plays a central role in physiological homeostasis and immunity; transporting nutrients, hormones, metabolites, and cytokines to all tissues in the body. Immune cells recirculate between the blood and lymphoid tissues and migrate to sites of injury or pathological insult, where genes responsive to specific exposures or disease states are induced. The blood transcriptome therefore has the capacity to reflect both systemic exposures and pathological changes in other organs of the body. In humans distinct blood transcriptomic signatures have been demonstrated for over 35 different medical conditions [2] and have been successfully employed to gain insight into processes and/or prognoses of cancer, heart disease, stroke, autoimmunity, neurological disorders, and responses to vaccines [1].

Blood transcriptome analysis has met similar success in veterinary medicine to identify markers of infectious disease and pathological conditions in economically relevant species (cow, pig, horse, sheep) and companion species (dog, cat) (for review see [1]). The application of high throughput transcriptomics to wildlife has to date been more limited, in part because of the lack of sequenced genomes for many species, as well as the difficulty associated with obtaining samples. In marine mammals, a microarray study in California sea lions identified blood gene expression profiles that distinguished between two prevalent disease states, domoic acid poisoning and leptospirosis [3], while qPCR of selected genes expressed in blood from California sea lions identified signatures of polycyclic aromatic hydrocarbons (PAH) exposures associated with wildfires [4]. Transcriptomic analysis of peripheral blood identified potential markers of nutritional stress in Steller sea lions [5]. Microarray based blood gene expression profiles in bottlenose dolphins identified stress responses associated with handling during capture-release studies [6] and were able to classify animals according to polychlorinated biphenyl (PCB) exposure levels [7].

Mammalian blood is composed of approximately 55 % plasma and 45 % cellular material. The cellular components are made up of 96 % red blood cells and 4 % leukocytes, which can vary in composition over time, and is thus one of the most dynamic tissues in the body. Because the gene expression profiles of leukocyte classes differ significantly, changing cellular composition can significantly alter the global blood transcriptome. Further development of blood transcriptomics as a tool for identifying signatures indicative of disease, exposures, or health status in wildlife requires knowledge of both the natural intra- and inter-animal variation in gene expression. For example, longitudinal studies of gene expression in human blood have found minimal intra-individual variation over the course of 1 month, but significant variability was observed by 3 to 6 months and this baseline variation must be taken into account [8, 9]. Recent studies suggest that seasonal patterns in human behavior, physiology, and disease susceptibility may be related to underlying fluctuations in hematological parameters, as blood cell composition shows annual variation [10]. Studies on ethnically and geographically diverse populations identified significant seasonal variation in over 4000 blood gene transcripts that are biologically and clinically relevant [11]. Circadian clock genes responsive to changes in day length are among the seasonally expressed genes, as well immune functions suggestive of a proinflammatory status during the winter months among populations living at high latitudes. In contrast, seasonal trends in gene expression from tropical populations correlate with rainy season during which individuals experience higher exposure to infectious agents [11]. Many of the observed differences reflect seasonal changes in cellular composition of the blood [10, 11].

Here we sought to establish baseline data on blood transcriptomes in bottlenose dolphins, *Tursiops truncatus*. Four healthy, managed dolphins located in Waikoloa, Hawaii, were sampled approximately monthly for the duration of 1 year. Hematological parameters including blood cell counts and serum chemistry were measured quarterly. Transcriptomic analysis of blood using RNA sequencing can be a challenge because globin transcripts are highly abundant, up to 76 % in human blood, 46 % in porcine blood [12], potentially limiting the detection and coverage of other transcripts of interest or incurring additional cost

of deeper sequencing to obtain adequate transcriptome sampling. In preliminary analyses we found that bottlenose dolphin blood was dominated by globin transcripts and therefore developed a globin depletion protocol specific for bottlenose dolphin.

Because a fully annotated bottlenose dolphin genome is currently lacking, we conducted parallel analyses of the dolphin blood transcriptome, one assembled using the Ensembl dolphin genome (2.59X coverage) as a guide and the other assembled *de novo* using Trinity. Seasonal differences in gene expression were observed and patterns of gene expression over the sampling year were assessed for rhythmicity. In addition, network analysis identified several co-expressed gene modules with correlation to clinical parameters. The observed correlation of gene co-expression modules with clinical measurements suggests that blood transcriptomics may be informative of health status and disease in bottlenose dolphins, once a larger database of blood transcriptomes is established.

Methods

Animals, experimental design and sample collection

Blood samples were collected in PAXgene (Qiagen, Valencia, CA) tubes from the ventral side of the flukes of four managed *T. truncatus* residing at Dolphin Quest, Waikoloa, Hawaii at approximately monthly intervals during 2013. All research was approved by the Dolphin Quest Research Committee and carried out according to standards and guidelines of the AMMPA (Alliance of Marine Mammal Parks and Aquariums). The dolphins sampled for this study included two males, ages 5 (Hua, $n = 8$) and 17 (Kainalu, $n = 7$), and two females, ages 12 (Keo, $n = 9$) and 28 (Pele, $n = 7$). All animals are trained to participate in monthly veterinary checkups including routine blood draws, which were conducted in the mornings after overnight fasting. Hematological parameters were measured quarterly on samples collected in parallel with the transcriptome samples. Only samples from healthy animals were used; defined as bright, alert, responsive (BAR) animals demonstrating baseline behavior and appetite and blood chemistry within normal ranges. All samples and associated physical and hematological parameters collected are listed in Table 1 and Additional file 1: Table S1. Blood tubes were stored at −80 °C until extracted for RNA.

RNA extraction

Whole blood RNA was extracted using a PAXgene Blood RNA Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol with on-column DNase digestion to remove contaminating DNA. RNA concentrations were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). Only samples with a RIN (RNA Integrity Number) ≥ 7 were sequenced.

Hemoglobin depletion

RNA-seq analysis of a test sample revealed a high percentage of globin transcripts in the dolphin peripheral blood transcriptome (65–75 % of reads). Therefore a modified Affymetrix globin depletion protocol utilizing RNase H was performed [12–14] in order to improve the diversity of transcripts detected by RNA-seq. Briefly, 500 or 1000 ng total RNA was hybridized with 2 μM each of 2 HBA oligonucleotides (5'-GGTATTTGG AGGTCAGCACGG-3' and 5'-ATGGACCGAGGGCGT GAAAT-3') and 1 μM each of HBB (5'-CTGAA GCTCCGGGGTGAATTC-3') and HBM (5'-GTCAG GAACTTATCCCACACCAC-3') oligonucleotides in hybridization buffer (100 mM Tris-HCl, pH 7.6, 200 mM KCl) at 70 ° C for 5 m and cooled to 4 ° C. The RNA-DNA hybrids were then digested with 1 U RNase H (Ambion, Thermo Fisher Scientific, Wilmington, DE) in 100 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 0.1 mM DTT, and 40 U SUPERase-In (Ambion, Thermo Fisher Scientific, Wilmington, DE) at 37 ° C for 10 m and cooled to 4 ° C. The reaction was stopped with 2 μl 0.5 M EDTA and the RNA was immediately purified with the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA), according to manufacturer instructions. All samples presented in Table 1 were subjected to globin depletion. Five samples were too dilute (<20 ng RNA/μL) for use in this protocol and were not subjected to globin depletion (Hua: June, Keo: July and September, Pele: April and June). RNA quality of globin depleted samples was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA).

Reverse transcription and qPCR

To determine the extent of globin depletion and any non-specific impact on transcript levels, mRNA levels of HBA and four additional genes (ALAS2, FKBP8, GAPDH, RPL13) were assessed by quantitative real-time PCR in samples pre- and post-globin depletion. Fifty nanograms of RNA was reverse transcribed with EpiScript -RNase H Reverse Transcriptase (Epicentre, Madison, WI) and oligo(dT) priming. Gene specific primers (400 nM, Table 2) were used for qPCR on an ABI 7500 using ABI Power SYBR Green master mix (Applied Biosystems, Foster City, CA). The specificity of qPCR primers and the size of the amplicon were verified by analysis with an Agilent Bioanalyzer 2100 and further confirmed by melting curve analysis. The reaction efficiency was determined using a standard curve of cDNA from total RNA. A cycle threshold (Ct) was assigned at the beginning of the logarithmic phase of PCR amplification and the difference in the

Table 1 All globin depleted samples and associated physical parameters measured

Animal	Sex	Age (years)	Sample Date	Monthly Mean Water Temp (°C)	Daylength (hours)
Hua	male	5	2/6/13	24.6	11.36
			3/21/13	24.4	11.78
			4/27/13	24.8	12.78
			5/23/13	25.2	13.15
			7/8/13	25.9	13.25
			8/23/13	26.4	12.65
			10/11/13	26.4	11.75
			12/2/13	25.1	11
Kainalu	male	17	2/5/13	24.6	11.36
			4/14/13	24.8	12.58
			5/25/13	25.2	13.18
			6/15/13	25.6	13.3
			7/8/13	25.9	13.25
			9/13/13	26.6	12.27
			12/2/13	25.1	11
Keo	female	12	2/13/13	24.6	11.48
			3/8/13	24.4	11.88
			4/1/13	24.8	12.3
			5/7/13	25.2	12.95
			6/12/13	25.9	13.3
			8/28/13	26.4	12.57
			10/29/13	26.4	11.45
			11/27/13	25.9	11.05
			12/9/13	25.1	11
Pele	female	28	2/13/13	25.1	11
			3/21/13	24.4	12.13
			5/24/13	25.2	13.16
			7/13/13	25.9	13
			8/22/13	26.4	12.67
			9/7/13	26.6	12.37
			12/13/13	25.1	10.95

Ct values of the pre- and post-globin depletion samples were used to determine the relative expression of the gene in each sample.

Sequencing

Globin depleted ($n = 31$, Table 1) and total RNA ($n = 6$; 5 samples named above and Kainalu May) samples were sent to North Carolina State University Genomics Service Laboratory for library preparation using a NEBNext Ultra Directional RNA Library Prep Kit for Illumina and indexed with the NEBNext Mulitplex Oligos for Illumina (New England Biolabs, Ipswich, MA). Sequencing was performed on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA), at a targeted depth of 28

Table 2 Primer sequences for qPCR analyses

Primer	Oligonucleotide Sequence (5'-3')
HBA-F	ATGGACCGAGGGCGTGAAAT
HBA-R	GGTATTTGGAGGTCAGCACGG
ALAS2-F	TGATCCAAGGTATCCGCAATAG
ALAS2-R	GTGTCTCAGGGTTAGACTTCTTT
FKBP8-F	CCATCAAGGCCATCACTTCT
FKBP8-R	CCAGGTTGTTCAGACACTTCA
GAPDH-F	TATGACAACCACCTCAAGATCG
GAPDH-R	GCCGAAGTGGTCATGGATAA
RPL13-F	GTACCGCTCCAAGCTCATTCT
RPL13-R	CTCTGTGATGACTCTGGCTTTCT

million, 100 nucleotide (nt) single end reads for globin depleted samples. Total RNA from samples that lacked sufficient RNA concentration to perform the globin depletion step were sequenced at a depth of 45 M reads, with the exception of Kainalu May which was sequenced at a depth of 28 M reads for a direct comparison with a globin depleted aliquot of the same sample.

Genome-guided transcriptome assembly and analysis

Sequence processing and analysis was carried out in iPlant Collaborative's Discovery Environment using the High-Performance Computing applications [15]. The Illumina BCL output files were converted to FASTQ-sanger file format and sequence quality trimming was performed using Trimmomatic [16], with a minimum phred quality score >20 over the length of the reads. The trimmed reads were then quality checked using the FASTQC tool. To assess the effectiveness of globin depletion, reads were mapped to the Ensembl *T. truncatus* genome, turTru1 v76.1, using Tophat2 v 2.3.13 [17] with Bowtie2 v 2.2.4 [18] as the alignment engine and mapped read counts, as FPKM (fragments per kilobase of transcript per million mapped reads), were generated using Cufflinks v 2.2.0 [19] with the genome as a reference. Differential expression analysis was performed using Cuffdiff v 2.1.1 [19] and visualization generated by CummeRbund [19]. For more detailed gene expression analysis of the blood transcriptome, reads from globin depleted samples were mapped to the Ensembl *T. truncatus* genome, turTru1 v76.1, using RSEM v 1.2.18 [20] with Bowtie2 v 2.2.4 [18] as the alignment engine and mapped read counts, as FPKM (fragments per kilobase of transcript per million mapped reads), were generated. Differential expression analyses were performed in EBSeq [21] using an FDR of 0.05. The raw reads and summarized FPKMs for all samples are available on GEO (accession # GSE78770). Gene enrichment analysis and pathway mapping of the differentially expressed gene sets was analyzed using Fishers Exact test in Blast2GO [22–25] (FDR < 0.05) and pathway mapping with the hypergeometric test for enrichment evaluation in WebGestalt [26, 27] (Benjamini & Hochberg adjusted p-value < 0.05) using a background comprised of all genes expressed in blood with an average FPKM ≥ 1 across all samples ($n = 31$) and and FPKM > 0 in at least half of the samples.

de novo transcriptome assembly and analysis

The processed and trimmed reads were also used to construct a *de novo* transcriptome using the Trinity assembler [28] on iPlant Collaborative's Discovery Environment. The read files from one summer and one winter globin depleted sample from each animal ($n = 8$; Hua: Feb and Sept, Kainalu: Feb and Aug, Keo: Feb and Aug, Pele: Feb and Sept) were concatenated into a single fastq file for assembly using a minimum K-mer coverage of 1, a minimum overlap value of 25 and a minimum contig length of 400 nucleotides. The assembly completeness was assessed by mapping a set of highly conserved core eukaryotic genes using CEGMA [29]. The transcriptome was annotated using BLAST+ for blastx searches (E-value ≤ $1e^{-4}$) of the human subset of the UniProt-SwissProt database (downloaded 10Jun2016), as the Ensembl genome is annotated off the human genome, followed by conserved domain mapping and gene ontology assignment using Blast2GO [22–25]. Read mapping, quantification, differential expression, gene enrichment analysis and pathway mapping were carried out using the same methods as in analysis of the genome-guided assembly. Gene enrichment and pathway mapping used a background comprised of all genes expressed in blood with an average FPKM ≥ 1 across all samples ($n = 31$) and FPKM > 0 in at least half of the samples. The Trinity assembly, raw reads, summarized FPKMs, and differential expression results are available on GEO (accession # GSE78770).

Principal components analysis

Principal component analysis (PCA) was performed on log2 transformed FPKM values for all genes that had an FPKM > 0 in at least half of the samples and an average FPKM value across all samples of ≥ 1 FPKM. PCA was performed using the prcomp package from the stats library in RStudio (v 0.99.486). The plots were visualized using ggplot2 (v 1.0.1) [30].

Cosinor analysis

Seasonal expression patterns of genes found to be differentially expressed between summer (July, August, and September) and winter (December and February) months were explored by transforming longitudinal data from all samples ($n = 31$) to estimate a cosinor linear model [31] using the cosinor package (v 1.1) [32] in Rstudio (v 0.99.878) and visualized with ggplot2 (v 2.1.0) [30].

Weighted gene Co-expression network analysis

A gene co-expression network was generated using WGCNA (v 1.51) [33] in R (v 3.3.0) on $\log_2$ transformed FPKM values for all genes that had an FPKM > 0 in at least half of the samples and an average FPKM value across all samples of ≥ 1 FPKM. An unsigned co-expression network was then constructed on all pairwise Spearman correlations of gene expression. To weight highly correlated genes, correlation coefficients were then raised to a soft thresholding power (β) of 10, as determined by scale-free topology [34]. For network construction, a minimum module size of 80 was used with a detect cut height of 0.90 and a merge cut height of 0.25.

The resulting modules were then tested for their association with sample traits by correlating module eigengenes (the first principal component of the module, representative of the gene expression profiles) with clinical measurements as described in [33]. Gene ontology and pathway enrichment analyses were then performed on individual gene co-expression modules compared to a background of all genes expressed in blood using the hypergeometric test in WebGestalt as previously described.

Results and discussion

Effect of globin depletion

As an analysis of a preliminary dolphin blood sample, sequenced at a targeted depth of 15 million reads, revealed that approximately 75 % of reads represented hemoglobin sequences (data not shown), we sought to establish a protocol for globin depletion of dolphin blood to increase the breadth of sequence detection. We modified an Affymetrix globin depletion protocol [12, 14] to yield high quality RNA with greatly depleted levels of hemoglobin transcripts. Recovery of RNA following globin depletion was high (median = 100.4 %) and only a minimal decrease in RIN, from 8.4 ± 0.06 to 7.8 ± 0.09, was observed. It has been reported that RNA recovery is low and variable following globin depletion in human and porcine blood [12, 35] and decreased RINs are also common [12, 36], thus it appears the globin depletion protocol performed exceptionally well in dolphin blood. As measured by quantitative PCR, HBA was significantly reduced by 286.4 ± 1.3 fold (t-test, $p < 0.05$, $n = 5$), whereas expression of other selected genes was not significantly changed (t-test, $p > 0.05$, $n = 5$) (Fig. 1a). Due to limitations in RNA quantity, only a single sample (Kainalu May) was sequenced at a targeted depth of 28 million reads both pre- and post-globin depletion. In this sample HBA was reduced 99.5 %, HBB 92 %, and HBM 35.8 %. To further investigate the effects of globin depletion in our study, five pairs of globin depleted and non-depleted samples, each collected 1 month apart, were analyzed by RNA-seq. HBA and HBB were reduced by 98.8 and 80.9 % respectively (Fig. 1b). This degree of depletion is similar to that observed using this protocol in porcine samples [12]. HBM exhibited a minor, non-significant decrease of 1.3 fold. In addition, ENSTTRG00000012084, annotated as a novel protein coding gene in the dolphin genome, was expressed at exceptionally high levels in non-depleted samples and was virtually undetected in globin depleted samples (99.9 % reduction). While this gene is termed "novel" in the genome annotation, it is located on a gene scaffold containing only hemoglobin genes (HBA, HBM, HBQ1, and HBZ). Blastx searches of the NCBI nr database identify this gene as HBA (*E*-value = $4e^{-37}$). Likewise ENSTTRG00000009506, annotated as a novel protein coding gene in the Ensembl genome, was reduced by 71.6 % following globin depletion. This gene is located on a scaffold with HBB and HBE and blastx searches

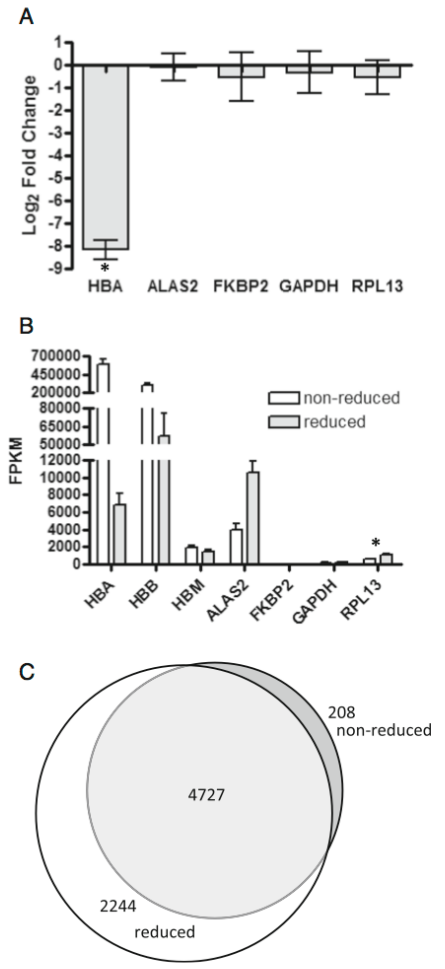

Fig. 1 Globin depletion of dolphin blood RNA. **a** By real-time PCR, HBA was observed to be significantly depleted by nearly 300-fold (t-test, $p < 0.05$, $n = 5$), with little change in other genes (t-test, $p > 0.05$, $n = 5$). **b** Large reductions in HBA and HBB were observed by RNA-seq analysis in the absence of expression decreases in other genes (Cuffdiff, FDR < 0.05, $n = 5$). Due to extremely high expression values, statistical analyses were not performed on HBA and HBB. **c** Globin depletion of blood RNA resulted in a 10 % increase of identified genes. Statistical significance is denoted by an asterisk

identified this gene as HBB (*E*-value = $4e^{-98}$). Despite large reductions in expression, this gene and the annotated HBB were the top two most highly expressed genes in globin depleted samples, indicating that further protocol modifications may improve the reduction of HBB. Overall, globin depletion had little impact on expression of other genes, with only 790 (3.7 %) genes showing significantly different expression between depleted and non-depleted samples (Cuffdiff, FDR < 0.05, $n = 5$). Among these, 357 exhibited 1.6–13.9 fold higher expression in non-depleted samples, while 433 had 1.6–18.4 fold higher expression in globin depleted samples. As these differences may encompass both biological and technical variation, all further analyses were conducted on the globin depleted samples only.

As a result of globin depletion, 2244 additional genes (10.6 % of genome) were detected at a FPKM ≥ 1 (Fig. 1c), similar to the 8.6 % or 9.8 % increase in gene detection following globin depletion of porcine [12] or human [37] blood, respectively. Due to limiting amounts of starting RNA, five samples were not subjected to globin depletion and were instead sequenced at a targeted depth of 45 million reads. These samples were compared to five temporally matched globin depleted samples sequenced at a targeted depth of 28 million reads. Only 268 additional genes (1.3 % of genome) were detected with an average FPKM ≥ 1 in globin depleted samples. Thus, it is likely that globin depletion of dolphin blood is not necessary for RNA-seq studies if sequenced to a sufficient depth to overcome high levels of globin expression, as has been reported for human blood [37]. Our study suggests that approximately 45 M reads is sufficient to overcome globin dominance of the transcript pool.

Genome-guided assembly transcript expression in blood

Overall approximately 85 % of reads mapped back to the dolphin genome. However, only 28.5 % of reads mapped back to annotated genes in the genome with Bowtie2. This indicated that many reads mapped outside of annotated regions of the genome and were consequently excluded from further analyses; therefore improved annotation of the Ensembl dolphin genome may greatly expand data interpretation. In order to more accurately compare the dolphin blood transcriptome to the Ensembl genome, we selected a suite of 17,475 sequences, comprised of coding sequences and pseudogenes, from the genome. Pseudogenes were included after identifying reads from the transcriptome aligning to regions of the genome annotated as such. Reads mapped to 9610, 45.2 %, of these genes identified in the Ensembl dolphin genome, with FPKM > 0 in at least half the samples and an average FPKM ≥ 1, similar to the percentage expressed in human blood [35]. Among the 100 most highly expressed genes in dolphin blood (avg FPKM from globin depleted samples), 100 % were annotated and were dominated by transcripts associated with ribosomes, translation, and DNA and RNA binding (Fig. 2a). Many of these terms are also among the most

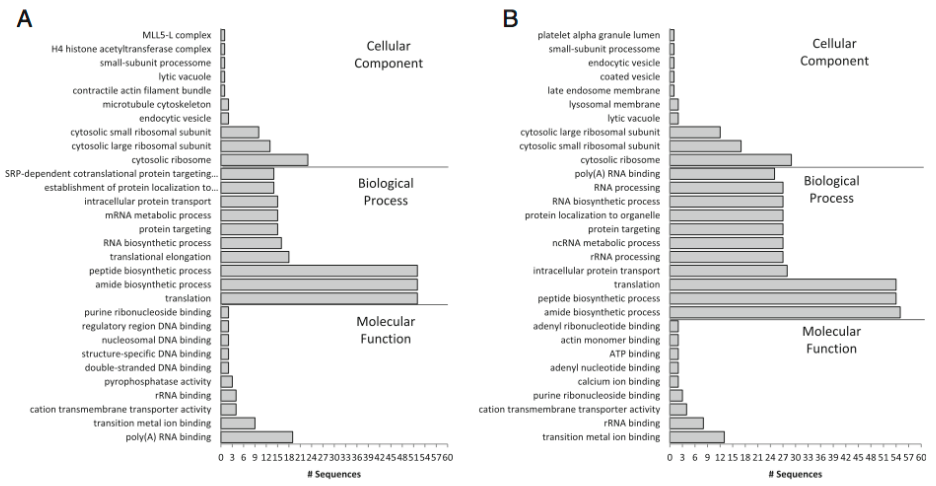

Fig. 2 Top ten Gene Ontology (GO) annotations (level 6) from the 100 most highly expressed transcripts in dolphin blood. **a** All of the top 100 expressed genes mapping to the dolphin genome were annotated in Blast2GO. **b** Ninety-two of the top 100 expressed genes mapping to the *de novo* blood transcriptome were annotated in Blast2GO

highly expressed transcripts in human blood. Likewise, transcripts mapping to GO terms involved with immune response, transcription, cell cycle and proliferation, signaling, and structural components or functions are highly expressed in both human and dolphin blood [38].

Pathway mapping indicates that many basic cellular functions and processes, and in particular immune functions, are well represented within the blood transcriptome (Table 3). In all, WebGestalt identified 137 pathways (KEGG or WikiPathway) significantly enriched in the blood transcriptome relative to the genome (Benjamini-Hochberg p-value < 0.05). In contrast, the 7865 genes not expressed in blood only showed enrichment of 24 different pathways, notably lacking many basic metabolic functions and processes encompassed by blood transcripts (data not shown). Similarly, an analysis of GO terms found the blood transcriptome to be significantly enriched in processes and functions associated with the ribosome, transcription, translation, cell cycle, protein processing, cellular homeostasis, and abiotic and immune responses (Fisher's exact test, FDR < 0.05). Many GO terms related to tissue-specific processes and functions are not expressed in blood including sensory processes (olfactory, visual, taste) and skeletal and cardiovascular system development (Fisher's exact test, FDR < 0.05). Hormone-related GO terms have been documented in the

Table 3 Pathways of interest significantly represented in the blood transcriptome

Pathway	Pathway #	#T/#G	*p*-value
Protein processing in endoplasmic reticulum	K4141	136/149	5.58E-17
Metabolic pathways	K1100	663/916	8.49E-17
mRNA processing	WP411	108/116	1.51E-14
Lysosome	K4142	102/111	2.62E-13
Ubiquitin mediated proteolysis	K4120	114/128	1.59E-12
Ribosome	K3010	68/70	2.93E-12
Electron Transport Chain	WP111	67/69	1.08E-11
Apoptosis	K4210	70/75	4.72E-10
Oxidative phosphorylation	K190	82/92	3.15E-09
B cell receptor signaling pathway	K4662	63/68	7.37E-09
T cell receptor signaling pathway	K4660	85/99	7.68E-08
Neurotrophin signaling pathway	K4722	97/116	1.11E-07
Toll-like receptor signaling pathway	K4620	66/75	3.91E-07
Natural killer cell mediated cytotoxicity	K4650	71/83	1.41E-06
Translation Factors	WP107	41/43	2.56E-06
Androgen receptor signaling pathway	WP138	73/86	6.13E-06
Antigen processing and presentation	K4612	34/36	1.45E-05
DNA replication	K3030	31/33	5.79E-05
MAPK signaling pathway	WP382	117/153	8.20E-05
NOD-like receptor signaling pathway	K4621	42/48	1.00E-04
Proteasome	K3050	38/43	2.00E-04
Phagosome	K4145	84/109	3.00E-04
RIG-I-like receptor signaling pathway	K4622	45/53	3.00E-04
Citrate cycle (TCA cycle)	K20	26/28	4.00E-04
Endocytosis	K4144	127/175	5.00E-04
Protein export	K3060	21/22	5.00E-04
Insulin signaling pathway	K4910	92/123	8.00E-04
Peroxisome	K4146	56/72	2.30E-03
Primary immunodeficiency	K5340	28/33	4.60E-03
IL-7 signaling pathway	WP205	22/24	5.60E-03
Chemokine signaling pathway	K4062	105/152	1.82E-02

WebGestalt analysis using an Benjamini Hochberg adjusted p-value. The genome (G) used for background contained 17,475 coding sequences and psuedogenes. The test (T) set was the 9610 genes expressed in blood. *WP* WikiPathways, *K* KEGG pathway

human blood transcriptome [2, 39], however GO terms for hormone activity and signaling and synaptic functions were significantly under-represented in the dolphin blood transcriptome. Only 3 of 47 genes mapping to hormone activity (GO:0005179), hormone-mediated signaling pathway (GO:0009755), or hormone metabolic process (GO:0042445) were present in the blood transcriptome. As data sets were queried to ensure that sex-specific expression was not causing these transcripts to be excluded from our analysis (i.e., FPKM > 0 was required in at least half of samples) it is unknown why a broad representation of hormone-related transcripts are not present in the dolphin blood transcriptome, but it is possible that hormone-related transcripts are poorly annotated in the current genome.

de novo transcriptome-guided assembly transcript expression in blood

A Trinity assembly of reads combined from eight samples resulted in 49,925 contigs with a minimum length of 400 nucleotides. The *de novo* assembly had an N50 of 1331 nt and the longest contig was 12,295 nt in length. The assembly appears to encompass the breadth of core eukaryotic genes (CEGs), with 87.5 % of full length CEGs identified by CEGMA. This increases to 97.78 % of CEGs when partial-length alignments to CEGs are included. When the *de novo* transcripts were aligned to the coding subset of the genome via blastn, 31.5 % of transcripts returned hits with an *E*-value < $1e^{-4}$, notably similar to the percentage of reads mapping back to annotated transcripts in the genome-based analysis and indicating a number of unannotated coding sequences in the Ensembl genome or novel sequence information identified by the *de novo* transcriptome assembly. However, blastn alignments of the *de novo* transcriptome to the full genome sequence indicate that there are minimal novel sequences in the *de novo* assembly. Rather, many transcripts in the *de novo* assembly map outside of annotated regions of the Ensembl dolphin genome; therefore the reduced mapping to the genome likely reflects the limited annotation available rather than an absence of sequence data. Overall, 88 % of reads mapped back to the transcriptome using Bowtie2. This is significantly higher than the 28.5 % observed mapping to annotated genes in the genome, yielding a substantial increase in usable read data for downstream analyses (Wilcoxon signed rank test, $p < 0.0001$). Thus, all further analyses presented herein utilize the *de novo* assembly.

When filtered to ensure FPKM > 0 in at least half of samples and an average FPKM ≥ 1 there were 29,702 transcripts (59.5 % of *de novo* transcriptome) and 13,889 had homology to human genes via blastx searches as described in the methods, a sizable increase from the 9610 (45.2 % of genome) genes mapping to the genome. Thirty-eight percent of these transcripts were fully annotated in Blast2GO and 21 % mapped unambiguously to Entrez Gene IDs for pathway mapping in WebGestalt. This set of 29,702 transcripts was defined as the "blood transcriptome" and used as the background set of transcripts expressed in blood for all further analyses. There was no significant enrichment of any GO terms among this background set relative to the complete *de novo* assembly, likely reflecting the expected breadth of gene expression in the blood transcriptome. Overall, gene expression guided with the *de novo* transcriptome was similar to that guided by the dolphin genome. Among the top 100 most highly expressed genes in the transcriptome (avg FPKM from globin depleted samples), 92 % were annotated, and were likewise dominated by transcripts associated with the ribosome, protein and nucleic acid binding, and translation (Fig. 2b). As in the genome-based analysis, there appeared to be little expression of genes involved in hormone biosynthesis, degradation, and signaling in the blood transcriptome, with only 12 transcripts mapping to hormone activity (GO:0005179), hormone-mediated signaling pathway (GO:0009755), or hormone metabolic process (GO:0042445) in the blood transcriptome. KEGG or WikiPathway analysis in WebGestalt identified significant mapping to ribosome pathways (p-value < $5e^{-70}$). The absence of hormone related transcripts in dolphin blood may reflect differences in hormone related transcript expression between humans and dolphins or may be due to the lack of homology, at the sequence level, between human and dolphin transcripts.

Overall variation between samples using principal components analysis

Principal components analysis did not reveal strong clustering associated with animal, sex, season, or any other measured parameter. PC1 accounted for 21.8 % of the variance and was somewhat correlated with sex, with females clustering together while the expression profiles from the two males were more variable (Fig. 3). PC2 only accounted for 7.8 % of variance and was not associated with animal, sex, or season. Samples from individual animals did not cluster together on either the PC1 or PC2 axes. The samples from males that clustered with the females on PC1 were not consistent with regard to season of collection. Neither PC1 nor PC2 were correlated with day length, water temperature, month or season of collection, nor with any of the hematological parameters measured.

Genes expressed differentially by sex

Of the 29,702 expressed genes the blood transcriptome, 499 (1.7 %) were differentially expressed (EBseq, FDR <0.05) between males ($n = 15$, all samples from Hua and

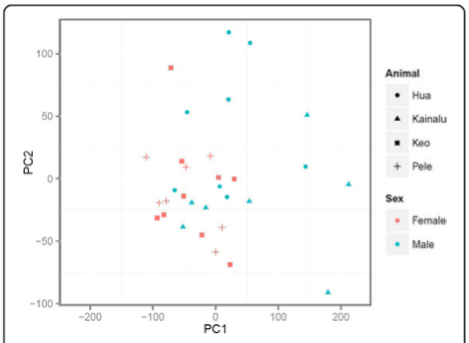

Fig. 3 Principal components analysis of 31 transcript profiles. The transcript profiles from the females (*pink*) largely clustered together, while those from the males (*blue*) were much more variable. Although the profiles from both males were variable, the young male (Hua, 5 years) differed somewhat from those of the older male (Keo, 17 years) along PC2

Kainalu) and females ($n = 16$, all samples from Keo and Pele) (Additional file 2: Table S2). Of these, 204 (41 %) had annotation. Two were homologs to X chromosome-linked genes in humans: SMC1A and CD40LG. Both were expressed slightly higher in females ($\log_2$ fold change 0.45). SMC1A, required for sister chromatid cohesion during cell division is known to be located in an area of the X chromosome that is not subject to X inactivation [40]. No homologs to human Y chromosome genes were found among the differentially expressed transcripts. Interestingly, while most dolphin chromosomes display substantial homology to human chromosomes by chromosome painting, the Y chromosome in dolphins is minute, and does not display any cross-hybridization with human chromosome probes [41]. Thus the absence of Y chromosome genes anticipated in sex biased gene expression may reflect a lack of homology to human genes, resulting in unannotated dolphin transcripts. Ninety-three genes were essentially expressed only in males (female ave FPKM < 1). Of these 11 were annotated: BATF1, CENPF, CREG1, GALM, HSF1, NDRG3, NOXO1, RAB22A, SLC18A2, SPAST, and TSLNG and all are autosomal in humans. Overall, 261 genes were expressed more highly in males ($\log_2$ fold change 0.28 to 11.9, ave = 1.65). Among the 238 genes more highly expressed in females ($\log_2$ fold change 0.28 to 10.4, ave = 1.84), 151 were essentially unexpressed in males (male ave FPKM < 1). The ten annotated female expressed genes, C20orf112, CHMP1A, CNN2, PPP4R2, SAMHD1, SNX19, TXLNG, ULK1, VPS13C, ZDHHC21, are all autosomal in humans. All other genes in the differentially expressed list were present in both sexes, but at different levels. This sex-biased gene set had ten genes in common (among those annotated) with those differentially expressed in human peripheral blood, which included 582 autosomal genes [42], plus 51 X-chromosome linked and 26 Y-chromosome linked genes. In humans the gene ontology processes enriched in the female biased genes included cytokine stimulus, response to interferon, and lymphocyte differentiation, while male specific genes were not enriched for any Biological Process GO category. There was no enrichment of any GO term among the annotated genes expressed more highly in male or female dolphins, nor in the combined set of genes differentially expressed by sex in this study.

Seasonal changes in gene expression

As significant seasonal changes in transcript expression have been observed in human blood [11] and dolphin skin [43], we queried this data set for transcripts differentially expressed between summer and winter. Based on local temperatures, samples from July, August, and September were collectively defined as summer ($n = 8$) while samples from December and February were defined as winter ($n = 8$). EBSeq reported a very small percentage of the dolphin blood transcriptome exhibiting significant changes in expression between summer and winter months (Additional file 3: Table S3). Overall, 0.7 % of the transcriptome exhibited significant changes between summer and winter months (FDR < 0.05, $\log_2$ fold change −4.9 to 4.2) and only 53 were annotated. The majority of genes, 63.8 %, were more highly expressed in summer (ave $\log_2$ fold change 2.18). The 36.2 % of genes more highly expressed in winter exhibited an average $\log_2$ fold change of 2.4. Fifty-three were effectively expressed only in winter (summer ave FPKM < 1), whereas 102 were expressed only in summer (winter ave FPKM < 1). There is no significant enrichment of any GO category or pathway in these sets of seasonally changing transcripts.

Despite the minimal changes in gene expression correlated with sex, seasonal gene expression was queried for males and females separately. Among males only a similar amount of change was observed, 0.8 % of transcriptome, whereas 2.4 % of the transcriptome changed significantly in females with season (FDR < 0.05, Additional file 3: Table S3). These gene sets are distinct, with only seven genes in common between the male and female sets of seasonal changers. Only one of the seven genes was annotated; highly similar to human beta-chimaerin. While there was no significant enrichment of any GO terms or pathways in these data sets, not only did the female only analysis yield the largest number of differentially expressed genes, it also exhibited the greatest degree of change ($\log_2$ fold change −6.86 to 6.55).

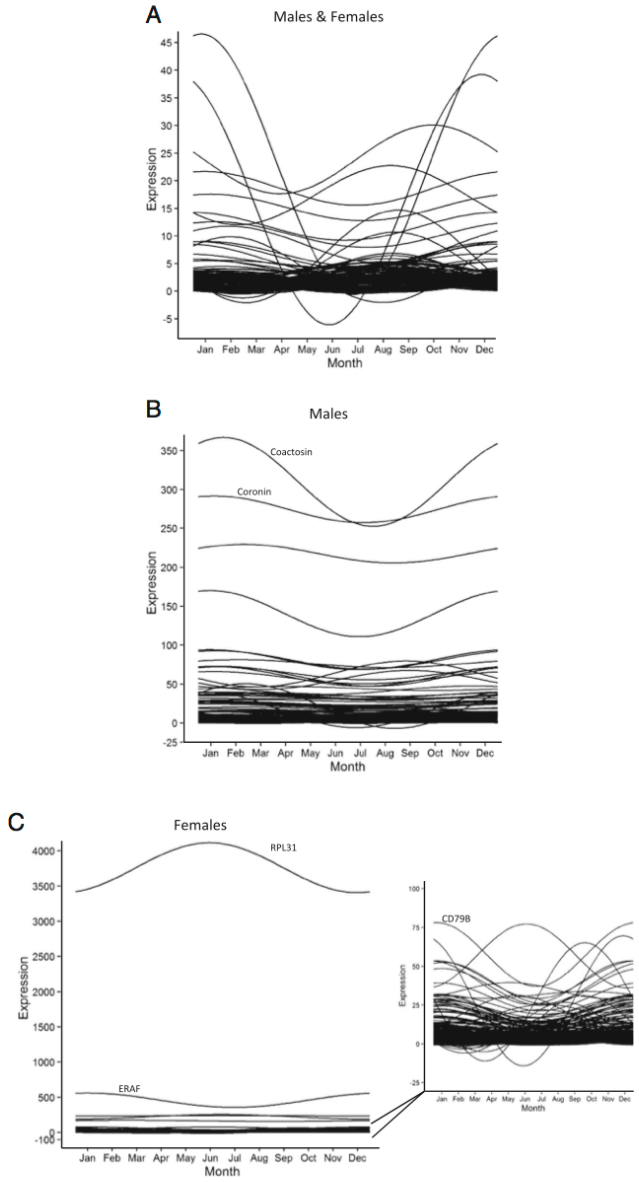

Fig. 4 (See legend on next page.)

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Fig. 4 Cosinor analysis of seasonally-expressed genes in the dolphin blood transcriptome. Genes differentially expressed between summer (July, August, September) and winter months (December and February) (EBseq FDR < 0.05) are plotted for males and female (**a**, $n = 31$, 210 transcripts), in males only (**b**, $n = 15$, 252 transcripts), or in females only (**c**, $n = 16$, 699 transcripts). Expression profiles are varied and there is little overlap between the three gene sets

Similarities with human seasonal gene expression

The percentage of genes exhibiting seasonal change in dolphin blood (approx. 0.7–2.4 %) is markedly less than the 23 % observed in human blood [11] or 25 % in dolphin skin [43]. This may be due to the minimal seasonal fluctuations in temperature (1.5 ° C) or day length (1.5 h) prevailing in the Waikoloa, Hawaii area, and may also reflect the small sample set in the current study (i.e., larger variance). Nonetheless, similarities exist among the gene sets, with 16 % of seasonally expressed genes assigned a gene symbol via blast searches in dolphin blood also present in the list of seasonal genes from human blood. An additional 42 % of annotated seasonal genes in dolphin blood are represented by a different member of the same protein family in human blood. This trend was more apparent in the slightly larger male-only seasonal gene set, with 40 % of annotated genes also found in the human seasonal set and another 40 % represented by another member within the protein family in human blood. The largest seasonal gene set from the female-only analysis had substantial overlap with the human set (47 % same gene, 32 % same family). In contrast, there was much less overlap with a recent study identifying seasonal change in gene expression in dolphin skin from the Gulf of Mexico. Fewer than 5 % of seasonal genes in dolphin blood are the same as seasonal genes identified in dolphin skin via microarray and approximately 33 % are represented by another gene in the same family [43]. The difference between the dolphin blood and skin transcriptomes may reflects tissue specific differences in gene expression as well as differences in environmental exposures of the two tissue compartments to temperature fluctuations. Further, the blood transcriptome utilized samples from managed dolphins in Waikoloa, Hawaii whereas the skin transcriptome utilized samples from wild dolphins in the northern Gulf of Mexico.

While the seasonal impacts on gene expression may be muted by the tropical climate or small sample size in this study, the agreement observed between the dolphin blood and human blood data sets indicates that gene expression in dolphin blood may undergo seasonal variation that must be taken into account when assessing gene expression changes associated with clinical parameters, disease, or toxic exposures. To this end, genes that significantly differed in expression between summer and winter months were subjected to a cosinor analysis to visualize any seasonal expression cycles. Overall, genes differentially expressed by season exhibited peak expression either in cooler months (November – February) or in warmer months, (June - September), however patterns and extent of cyclic changes in expression varied between data sets (Fig. 4). Among the genes differentially expressed when analysis was performed on the full data set (males and females), the two transcripts with the highest expression levels and greatest degree of change exhibited peak expression during cooler months, but both are unannotated (Fig. 4a). Analysis of the differentially expressed genes in males only exhibited more stable annual expression patterns (Fig. 4b). The two most highly expressed genes, which exhibited peak expression in cooler months, are coactosin and coronin, both associated with cytoskeletal processes and actin binding. Differentially expressed genes in females only again exhibited the greatest annual rhythmicity among the analyzed data sets (Fig. 4c). A 60S ribosomal protein L31 (RPL31) has the highest expression and greatest amplitude of change, with peak expression in cooler months. An erythroid associated factor (ERAF) exhibited high expression levels, peaking in warmer months, whereas CD79B exhibited seasonal rhythmicity peaking in cooler months. These seasonal expression changes in ERAF and CD79B may reflect changes in the cellular composition of blood [10, 11]. A larger sample set, as well as samples from dolphins in regions undergoing greater environmental fluctuations over the year may reveal cyclic patterns of gene expression not observed in the current study.

Gene co-expression network analysis

The utility of the blood transcriptome to identify physiological perturbations, such as those resulting from disease or toxic exposure, requires insight into the stability of the healthy transcriptome over time, as well as differences between individuals that may relate to differences in age, sex, or hematological parameters. We therefore constructed a gene co-expression network in WGCNA using all samples ($n = 31$) as independent measures. Fifteen co-expressed gene modules were identified (Fig. 5a and b), while the majority of genes, represented by the grey module, were not significantly co-regulated. Gene membership in these modules is listed in Additional file 4: Table S4. Pairwise correlations between each module eigengene and each of the physical or hematological parameters measured revealed several modules with significant associations (Fig. 5c). There were no

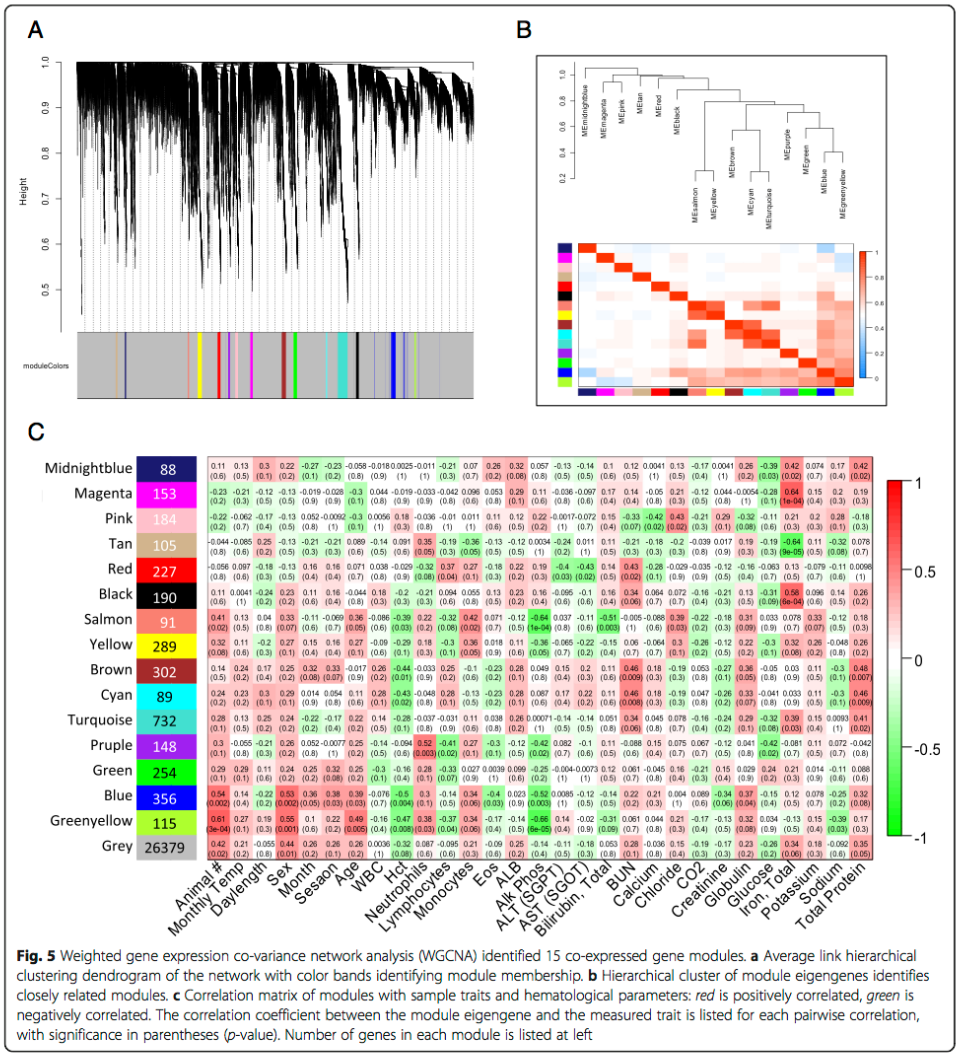

Fig. 5 Weighted gene expression co-variance network analysis (WGCNA) identified 15 co-expressed gene modules. **a** Average link hierarchical clustering dendrogram of the network with color bands identifying module membership. **b** Hierarchical cluster of module eigengenes identifies closely related modules. **c** Correlation matrix of modules with sample traits and hematological parameters: *red* is positively correlated, *green* is negatively correlated. The correlation coefficient between the module eigengene and the measured trait is listed for each pairwise correlation, with significance in parentheses (*p*-value). Number of genes in each module is listed at left

co-expressed gene modules associated with temperature or day length, and only modest correlation to season (blue module, $r = 0.38$, $p = 0.04$). Only two modules showed strong correlation to individual animal, the blue module (352 transcripts, $r = 0.54$, $p = 2e^{-03}$) and the greenyellow module (115 transcripts, $p = 0.61$, $p = 3e^{-04}$). These modules also correlated significantly with the sex and age, and were negatively correlated with hematocrit and alkaline phosphatase. Hematocrit values were significantly different between sexes (mean ± SEM = 38.7 ± 0.49 in females; 43.75 ± 0.53 in males). The correlation with alkaline phosphatase likely reflects the high alkaline phosphatase levels observed in the young male (Hua, 5 years; range 570–848 U/L) and the low alkaline phosphatase levels found in the older female (Pele, 28 years; range 107–190 U/L). Alkaline phosphatase levels have previously been

shown to be high in juvenile dolphins [44]. The KEGG pathway for map kinase signaling ($p = 0.015$) was enriched in the blue module while KEGG pathways for hematopoeitic cell lineage ($p = 7.2^{e-03}$) and regulation of the actin cytoskeleton ($p = 7.2^{e-03}$) were enriched in the yellowgreen module. The salmon module, although more weakly associated with sex and age, was also strongly associated with alkaline phosphatase ($r = -0.68$, $p = 2e^{-05}$), however the small number of annotated transcripts in this module prevented the identification of any enrichment within the module.

The brown module (302 genes) had little correlation with animal, sex or age, but was negatively correlated with hematocrit and positively correlated with blood urea nitrogen (BUN), a proxy for kidney function, and total protein. The brown module was enriched in GO terms for regulation of muscle fiber development ($p = 9.7e^{-03}$) and endothelial cushion morphogenesis ($p = 6e^{-03}$), and KEGG pathways for gluconeogenesis ($p = 6.0e^{-03}$), galactose and starch metabolism ($p = 2.7e^{-02}$), and gluconeogenesis ($p = 3.6e^{-02}$). The cyan module (89 genes) has similar correlations to hematocrit and BUN but had no significant enrichment, probably due to its small size.

The tan module (105 genes) was negatively correlated with total iron ($r = -0.64$, $p = 9e^{-5}$), and enriched in GO terms for circulatory system process ($p = 1.2e^{-02}$) and blood circulatory genes ($p = 1.2e^{-02}$). Two other modules (magenta and black) with positive correlation to total iron had no significant enrichment.

The purple module (145 genes) was positively correlated with neutrophils ($r = 0.52$, $p = 0.003$) and negatively correlated with lymphocyte percentage ($r = -0.41$, $p = 0.02$), alkaline phosphatase ($r = -0.42$, $p = 0.02$), and glucose ($r = -0.42$, $p = 0.02$). There was no significant difference in lymphocyte percentage between the sexes; however, the 17 year old male, Kainalu, was substantially lower in lymphocyte percentage (13.25 ± 3.0) than all other animals (20.9 ± 5.7) and was also below the normal range for dolphins (15–30 %). This module was enriched in GO process categories for negative regulation of sequestration of calcium ($p = 3.2e^{-02}$) and cell cycle ($p = 2.3e^{-02}$).

The ability of the network analysis to identify modules of co-expressed genes that correlate with clinical measurements in this small sample of healthy, managed dolphins suggests that blood transcriptomes may be informative for identifying metabolic perturbations indicative of with infections, disease, or toxic exposures in bottlenose dolphins. The independence of many co-expressed modules from the individual animal and month of collection suggests that these may not be confounding factors for identifying transcriptomic responses to adverse health impacts.

Conclusions

This longitudinal analysis of blood transcriptomes from four managed bottlenose dolphins provides the first information on the blood transcriptome content and sex, seasonal, and individual variation in transcript expression in bottlenose dolphins. The blood transcriptome was found to express a wide array of genes that mapped to diverse pathways, thus demonstrating the potential for broad applications of dolphin blood transcriptomic analysis in marine mammal management. We found both a seasonal component to changes in blood gene expression, consistent with studies in humans, and an association of gene co-expression modules with age, sex or hematological parameters measured. However, the proportion of genes exhibiting changes in expression along with the degree of change observed was limited, demonstrating the relative stability of the dolphin blood transcriptome within and between animals throughout the course of a year. Although this represents a small sample of healthy, managed dolphins, the observed correlations to hematological parameters coinciding with an otherwise stable transcriptome and precedence from human medicine suggests that blood transcriptome analysis may be useful for identifying exposures, infections, and pathological changes that cannot be readily monitored in protected marine mammal species. The utility of blood transcriptomics for diagnostic purposes in bottlenose dolphins will require the establishment of a robust database of gene expression in dolphins from different environments, both managed and wild, with which to establish normative values in healthy animals. The establishment of such an archive would facilitate the use of blood transcriptomics for biomonitoring in wild and managed populations of bottlenose dolphins.

Additional files

Additional file 1: Table S1. All globin depleted samples and associated physical or hematological parameters measured. (XLS 43 kb)

Additional file 2: Table S2. Genes with significantly different expression between males and females (EBSeq, FDR < 0.05). (XLS 85 kb)

Additional file 3: Table S3. Annotated genes with significantly different expression between summer and winter from de novo transcriptome based analyses (EBseq, FDR < 0.05). (XLS 166 kb)

Additional file 4: Table S4. Gene co-expression modules with significant correlation to measured traits. (XLS 427 kb)

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Availability of data and materials
The datasets supporting the conclusions of this article are available in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE78770 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78770).

Authors' contributions
JSM participated in RNA extraction and globin depletion, carried out RNA-seq data analysis, *de novo* transcriptome assembly, differential expression, enrichment, and cosinor analyses and drafted the manuscript. MGN carried out PCA analyses. DL carried out RNA extraction, globin depletion, and qPCR analyses and participated in cosinor analyses. PEA provided computational support and assisted in data analysis. LHS participated in the design of the study. MC participated in the design of the study and provided samples and corresponding hematological and environmental data. FVD conceived of the study, participated in the design of the study, carried out WGCNA analyses and participated in writing the manuscript. All authors read and approved the final manuscript.

Authors' information
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Samples for this study were collected under the auspices of a USDA public display permit issued to Dolphin Quest. Samples were collected as part of routine veterinary care in an effort to advance the animal management practices within Dolphin Quest locations, as afforded by the permit. All sampling and research was carried out according to standards and guidelines of the AMMPA (Alliance of Marine Mammal Parks and Aquariums). All protocols were approved by the Dolphin Quest Research Committee and Animal Wellness Team. The Research Committee oversees all animal care and use in any research activities, in lieu of an IACUC, as authorized under the USDA public display permit.

NOAA disclaimer
The scientific results and conclusions, as well as any opinions expressed herein, are those of the author(s) and do not necessarily reflect the views of NOAA or the Department of Commerce. The mention of any commercial product is not meant as an endorsement by the Agency or Department.

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