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De novo Assembly and Annotation of the Blood Transcriptome of the Southern Elephant Seal *Mirounga leonina* from the South Shetland Islands, Antarctica

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Abstract – The southern elephant seal *Mirounga leonina* is the largest member of the phocid seals and is a highly sexually dimorphic predator at circumpolar regions. In this study, we generated the first high-quality *de novo* assembly of the blood transcriptome of *M. leonina* using the Illumina MiSeq platform. A total of 40.5 million raw reads were obtained and assembled using the Oases assembly platform, resulting in 46,445 contigs with an N50 value of 634 bp. We performed functional gene annotations through pathway analyses of the Gene Ontology (GO), Eukaryotic Orthologous Groups (KOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Ortholog comparison revealed that a high proportion of the *M. leonina* blood transcriptome had significant sequence homology within pinnipeds. *M. leonine*, a deep and long-diving seal, is routinely exposed to progressive hypoxia during dives. KEGG analyses detected an intact hypoxia-inducible factor-1 (HIF-1) signaling pathway, which is a key metabolism pathway in the adaptive homeostatic responses to hypoxia. In the blood transcriptome, many essential genes involved in innate immunity were detected, which suggests that the blood serves as a host defense modulator against exogenous infections in *M. leonina*. This genomic resource will be useful for understanding adaptive metabolism upon repeated breath-hold dives and determining the health status of southern elephant seals without the need to sacrifice them in experiments.

Keywords – southern elephant seal, *Mirounga leonina*, blood, transcriptome, hypoxia

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1. Introduction

The southern elephant seal *Mirounga leonina* is the largest of all pinnipeds; adult males can reach masses of two to four tons and lengths of up to 5.8 m. *M. leonine* has served as an important model for studying physiology, behavior, reproduction, and population dynamics. *M. leonine* has a nearly circumpolar distribution in the Southern Ocean with four distinct populations and is found mostly in sub-Antarctic islands (Laws 1994; Campagna 2008). Previously, *Mirounga leonina* was listed as Vulnerable under the predecessor to the Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act). Although the stability of the *M. leonina* population is a somewhat controversial subject due to the absence of current measurements, its population size was estimated at approximately 650,000 in the 1990s (Laws 1994), and this species was registered as 'least concern' by the International Union for Conservations of Nature (IUCN) Red List of Threatened Species (http://www.iucnredlist.org) in 2008 (Hofmeyr 2015). Because a recent reexamination suggested that fluctuations in the marine environment were the most obvious factor for previous population declines of *M. leonina* that occurred at key colonies during the 1950s–1970s (McMahon et al. 2005), monitoring and conservation for *M. leonina* are required.

Most air-breathing vertebrates (e.g., penguins, seals, and whales) routinely suffer from acute hypoxia (a reduction in

convective oxygen delivery in blood and tissues), hypercapnia (abnormally elevated CO_2 levels in the blood), or acidosis (an abnormally high level of acidity or a low level of alkalinity in the blood) during deep and long dives (Elsner and Gooden 1983). The diving capacity of seals varies, as northern elephant seals (*Mirounga anguistirostris*), hooded seals (*Cystophora cristata*), and Weddell seals (*Leptonychotes weddellii*) have deep- and long-diving characteristics, while ringed seals (*Phoca hispida*) and California sea lions (*Zalophus californianus*) perform short and shallow dives (Zenteno-Savín et al. 2012). Southern elephant seals are also deep-diving and long-ranging predators for squid, octopus, and fish, as they can undertake lengthy dives from 30~45 min to over 2 h and dive more than 2,000 m in depth (Biuw et al. 2007). Although adaptation mechanisms for hypoxia tolerance have continuously been studied in cetaceans, the basis for molecular physiological adaptations has not been well addressed, and several studies recently suggested potential molecular adaptive mechanisms towards low-oxygen conditions in seals (Khudyakov et al. 2015; Crocker et al. 2016; Fabrizius et al. 2016; Hoff et al. 2017).

Since our objective was to collect the gene pool from the blood specimen of the southern elephant seal *M. leonine* for further gene expression application (e.g. health status monitoring), we analyzed the whole blood transcriptome of *M. leonina* using basic bioinformatics tools. To avoid subjecting *M. leonine* to too much stress and to prevent their unnecessary death, a blood sample was collected from an individual. Although the sample size was very small, we assumed that the analysis of a single sample would be enough to annotate and establish a blood gene set in this species, as the quantity and quality of mammalian genes registered in GenBank are relatively high compared to other animals. Blood is highly sensitive to numerous endogenous and/or exogenous stimulations. Because the integrity of blood can be challenged by circulating parasites or pathogens, analyzing blood status, including transcriptome profiling or the measurement of target gene/pathway-specific expression, can be useful for estimating individual homeostasis, disease resistance, and herd immunity. Among mammals, blood transcriptome profiling has successfully been employed to identify pathological markers in cows, horses, pigs, and sheep (Mohr and Liew 2007; Chaussabel 2015). Although genomic information on blood in marine mammals is still limited, the application of high-throughput transcriptomics has been conducted in the blood tissues of beluga whales, bottlenose dolphins, and Steller sea lions (Spitz et al. 2015; Morey et al. 2016, 2017). Thus, genomic information on *M. leonina* will allow future investigations of blood-specific pathways in pinnipeds, with a particular focus on understating the genetic basis for the adaptation of seals to hypoxia and determining the immune status of southern elephant seals without the need to sacrifice them in experiments.

2. Materials and Methods

Sample collection and Illumina sequencing

A blood specimen (ca. 1 mL) was collected from an individual on King George Island, South Shetland Islands, Antarctica (Table 1). The species was identified by reference to morphological characteristics and the mitochondrial cytochrome oxidase subunit 1 (*CO1*) sequence. The blood sample was immediately transferred to an RNAlater vial (QIAGEN, Valencia, CA, USA) and stored at -20°C until RNA extraction. Total RNA was extracted using an RNeasy® Micro Kit (QIAGEN) according to the manufacturer's protocol and stored in RNAstable (Biometrica, San Diego, CA, USA). The quality and quantity of the total RNA were determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). High-quality mRNA (2 μg) was used to generate a double-stranded cDNA library using poly A selection. A NuGEN Encore Complete RNA-Seq Library System (NuGEN, San Carlos, CA, USA) was used to construct paired-end libraries (PE500) of sheared cDNA (500 bp fragments) that were sequenced on an Illumina MiSeq® System platform (300 × 2 paired-end reads) (Kim et al. 2018). Index and adaptor sequences were trimmed using Trimmomatic (Bolger et al. 2014), and low-quality reads were removed using the FASTX toolkit (Gordon and Hannon 2010) with parameters set at -t, 20; -l, 70; and -Q, 33.

De novo assembly

Sequencing produced 40.5 million reads. Low-quality reads (average quality score < 10), adapters, linkers, and polymerase chain reaction (PCR) primers were removed via quality filtering. High-quality reads were assembled *de novo* using Oases (ver. 0.2.08; Schulz et al. 2012) with default parameters. TransDecoder was used to identify coding regions. Unclustered transcripts and the longest sequences were considered unigenes. We identified 46,445 contigs of an average length of 672 bp and an N50 length of 634 bp. All unigenes were searched against the National Center for Biotechnology Information (NCBI) nonredundant database using BLASTx with an E-value threshold of 1.00E-04 to identify functional transcripts.

Table 1. Characteristics of the *Mirounga leonina* blood transcriptome sequencing project in compliance with the MIxS standard

Item	Description
Investigation type	Eukaryote transcriptome
Classification	Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Deuterostomia; Chordata; Craniata; Vertebrata; Gnathostomata; Teleostomi; Euteleostomi; Sarcopterygii; Dipnotetrapodomorpha; Tetrapoda; Amniota; Mammalia; Theria; Eutheria; Boreoeutheria; Laurasiatheria; Carnivora; Caniformia; Phocidae; Mirounga
Project name	*Mirounga leonina* blood transcriptome sequencing
Geographic location name	King George Island (South Shetland Islands), Antarctica
Geographic location	62°14'30.8"S, 58°44'47.7"W
Collection data	13 Jan 2017
Environment (biome)	OMIT_0002267 (Antarctic region)
Environment (feature)	ENVO_00000098 (island)
Environment (material)	ENVO_01000428 (rocky shore)
Tissue type	Blood
Developmental stage	Juvenile female
Sequencing method	Pyrosequencing
Sequencing platform	Illumina Miseq
Assembly program	Oases (ver. 0.2.08)
Assembly method	*De novo* assembly
Finishing strategy	Contig
Bioproject number	PRJNA473905
Data accessibility	SRR7250895

Data deposition

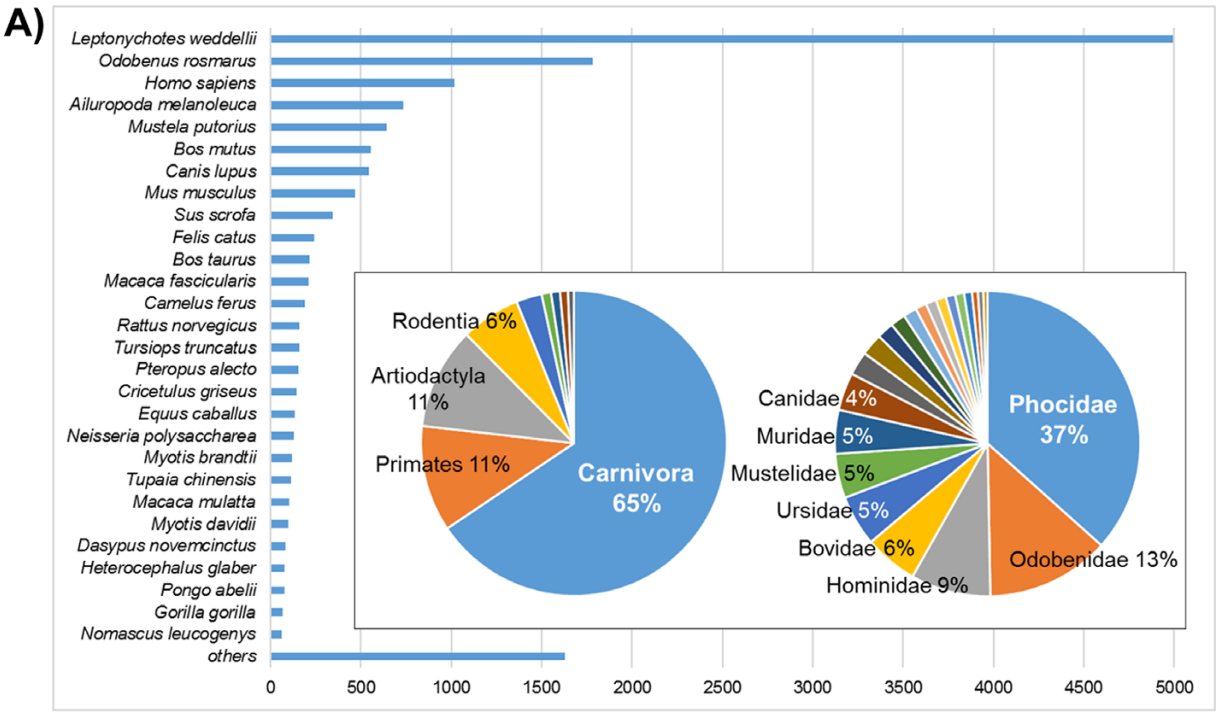
The obtained raw RNA-seq data were deposited in the NCBI Sequence Read Archive (SRA; accession number SRR7250895) under bioproject number PRJNA473905. This Transcriptome Shotgun Assembly (TSA) project was deposited at DDBJ/ENA/GenBank under accession number GGPR00000000 (GGPR01000001-GGPR01046444) (https://www.ncbi.nlm.nih.gov/nuccore/GGPR00000000).

3. Results and Discussion

The principal BLAST hits indicated that approximately 4,991 *M. leonina* contigs exhibited sequence similarities to transcripts of the Weddell seal *Leptonychotes weddellii* and 1,719 exhibited similarities to transcripts of the walrus *Odobenus rosmarus* (Fig. 1A). Of the top hits, 65% and 11% were homologous to transcripts from the classes Carnivora and Primates, respectively (Fig. 1A). At the family level, *M. leonina* contigs showed high similarity to Phocidae (37%), followed by Odobenidae (13%) (Fig. 1A). Thus, we assumed that blood sample preparation and sequencing were successful, as the raw read assembly was undoubtedly seal.

Since a large number of genes were annotated in *M. leonina* blood transcriptome, our next task was to learn how many genes are specifically associated with those of pinnipeds as *M. leonine*-specific genes. Ortholog comparison with the *M. leonina* blood transcriptome showed extensive similarity within pinnipeds (Fig. 1B). Genomic information of four pinniped species was retrieved from published transcriptome data or annotated genes from the following genomes: Antarctic fur seal (*Arctocephalus gazelle*) (Humble et al. 2016), northern elephant seal (*Mirounga angustirostris*) (muscle transcriptome; Khudyakov et al. 2015), walrus (*Odobenus rosmarus*) (Foote et al. 2015), and Weddell seal (*Leptonychotes weddellii*) (Humble et al. 2016). Most transcripts of *M. leonina* matched at least one of the other pinniped species, as 93% contigs (#10,647) showed similarity, while only 758 contigs (6.6%) remained as unique for *M. leonina* from a total of 11,405 contigs. In the case of *M. leonina*, approximately 40% of contigs (#4,511) were commonly matched to those of all pinniped species. This result was quite surprising given the greater evolutionary relatedness and similarity in the genomic sequences among pinniped species. Although the *M. leonina* blood transcriptome contains many genes encoding proteins that carry out blood-specific functions, the high similarity is likely attributed to the high sequencing coverage of pinniped species.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of all contigs were



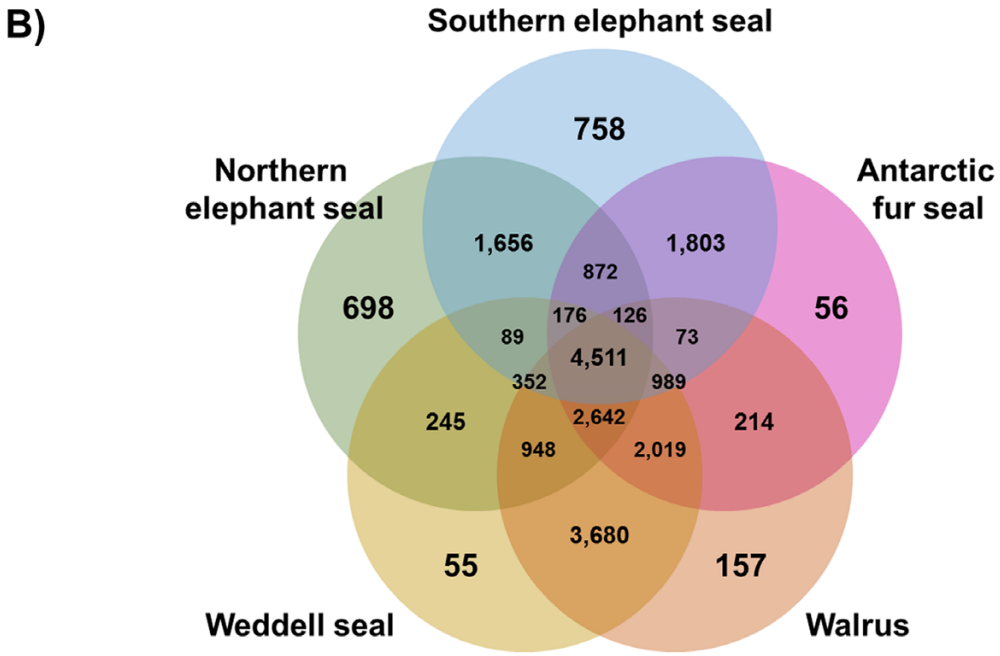


Fig. 1. A) Numbers of major BLAST hits matched to *Mirounga leonina* blood transcripts at the phylum and species levels. Each number indicates the number of orthologous gene families shared by the indicated genomic database. B) Five-way Venn diagram of orthologue gene conservation in five species of pinniped: Antarctic fur seal (*Arctocephalus gazelle*), northern elephant seal (*Mirounga angustirostris*), southern elephant seal (*Mirounga leonine*), walrus (*Odobenus rosmarus*), and Weddell seal (*Leptonychotes weddellii*). The diagram was constructed with orthologous genes identified by the best reciprocal hit method with tBLASTx (E-value < 10^{-10}) pair-wise ortholog matches. Numbers represent unique or shared genes between pinniped species

performed using the Blast2GO sequence annotation tool (ver. 4.0; Conesa et al. 2005). The specific GO composition of each category is presented as a Level 2 percentage. After aligning the contigs, we analyzed three principal categories (i.e., biological processes, cellular components, and molecular function) using default parameters. The BLAST search and functional domain

Table 2. Information on representative genes and their basal mRNA expression levels involved in hypoxia-inducible factor-1 (HIF-1) signaling pathway

Contig ID	Description	Abbreviation	RPKM
Seal_contig_15058	interleukin-6 receptor subunit alpha	IL6Ra	32.7789
Seal_contig_16785	interleukin-6 receptor subunit beta	IL6Rb	6.7228
Seal_contig_23097	toll receptor 4	TLR4	8.9148
Seal_contig_34407	interferon gamma receptor 2	IFN-gR	25.7374
Seal_contig_14502	solute carrier family facilitated glucose transporter member partial	GLUT	5.5714
Seal_contig_6770	signal transducer and activator of transcription 3 isoform 2	STAT3	24.6022
Seal_contig_21241	nuclear factor nf-kappa-b p105 subunit isoform 1	NFkB	19.0787
Seal_contig_73430	mitogen-activated protein kinase kinase 2	MEK2	2.4987
Seal_contig_7343	dual specificity mitogen-activated protein kinase kinase	MEK	37.0250
Seal_contig_1355	phosphatidylinositol -bisphosphate 3-kinase catalytic subunit delta	PI3Kd	72.6178
Seal_contig_2051	phosphatidylinositol -bisphosphate 3-kinase catalytic subunit beta	PI3Kb	9.6813
Seal_contig_36184	phosphatidylinositol -bisphosphate 3-kinase catalytic subunit alpha	PI3Ka	5.6887
Seal_contig_13553	rac-alpha serine threonine-protein kinase	AKT	58.8076
Seal_contig_3246	mitogen-activated protein kinase 3	ERK	30.9468
Seal_contig_63892	serine threonine-protein kinase mtor	mTOR	7.1154
Seal_contig_6181	map kinase-interacting serine threonine-protein kinase 1	MNK	24.8695
Seal_contig_10408	eukaryotic translation initiation factor 4e-binding protein 1	4E-BP1	99.0189
Seal_contig_13562	eukaryotic translation initiation factor 4e-binding protein 2	4E-BP2	39.6256
Seal_contig_4066	ribosomal protein s6	rpS6	202.5135
Seal_contig_66609	protein kinase c alpha type	PKCa	1.6616
Seal_contig_22196	protein kinase c beta type isoform x2	PKCb	28.8386
Seal_contig_11950	protein kinase c delta type	PKCd	24.1520
Seal_contig_59227	protein kinase c epsilon type	PKCe	4.4243
Seal_contig_23915	protein kinase c eta type	PKCh	11.6518
Seal_contig_42826	protein kinase c theta type isoform x2	PKCt	4.9117
Seal_contig_12745	hypothetical protein PANDA_016272	PKCb	30.2247
Seal_contig_6540	1-phosphatidylinositol -bisphosphate phosphodiesterase gamma-1	PLCG1	17.7588
Seal_contig_929	1-phosphatidylinositol -bisphosphate phosphodiesterase gamma-2	PLCG2	14.0032
Seal_contig_8744	low quality protein: pyruvate dehydrogenase isozyme 1	PDK1	5.6176
Seal_contig_2593	cytochrome b-245 heavy chain	NOX2	44.1271
Seal_contig_2343	cytochrome b-245 light chain	NOX3	190.8212
Seal_contig_17700	pyruvate dehydrogenase e1 component subunit mitochondrial	PDH	10.5498
Seal_contig_25487	eukaryotic translation initiation factor 4e	elf4E	17.8241
Seal_contig_4066	ribosomal protein s6	RPS6	202.5135
Seal_contig_14148	hypoxia inducible factor 2alpha	HIF-1a	3.1288
Seal_contig_33558	hypoxia-inducible factor 1-alpha	HIF-1a	10.1974
Seal_contig_11583	ring-box protein 1	Rbx1	20.0824
Seal_contig_3235	transcription elongation factor b polypeptide 2	elonginB	45.6079
Seal_contig_36551	Cullin-2	CUL2	6.2027
Seal_contig_6403	egl nine homolog 2	PHD2	17.9113
Seal_contig_26036	egl nine homolog 1	PHD1	12.9020
Seal_contig_26801	egl nine homolog 3	PHD3	11.3375
Seal_contig_48164	calcium calmodulin-dependent protein kinase type 1 isoform x2	CamK	16.7698
Seal_contig_26206	histone acetyltransferase p300	EP300	10.2617
Seal_contig_34161	metalloproteinase inhibitor 1	TIMP1	12.8920
Seal_contig_2933	heme oxygenase 1	HMOX1	59.9636
Seal_contig_8744	low quality protein: pyruvate dehydrogenase isozyme 1	PDK1	5.6176
Seal_contig_6137	hexokinase-1	HK	19.4262
Seal_contig_26625	hexokinase-3	HK	17.9796
Seal_contig_79	glyceraldehyde-3-phosphate dehydrogenase isoform x2	GAPDH	452.7474
Seal_contig_983	alpha-enolase isoform x1	ENO	134.0467
Seal_contig_224	cyclin-dependent kinase inhibitor 1 isoform x1	p21	21.2032
Seal_contig_13933	cyclin-dependent kinase inhibitor 1b	p27	33.8961

annotation by InterProScan of the Blast2GO software package assigned 3,528 contigs to at least one GO term. Finally, the assembled data were arranged in terms of read length, gene annotation, GenBank number, E-value, species, and species accession number. The mRNA expression level was calculated using the reads per kilobase of the transcriptome per million mapped reads (RPKM) method (Mortazavi et al. 2008).

The diverse GO assignments analyzed by Blast2GO revealed that blood performs many complex biological functions. Overall, the *M. leonina* blood transcriptome encompasses a wide range of cellular metabolisms. The vast majority of transcripts in the biological processes category were assigned to cellular processes (16%), metabolic processes (14%), and single-organism processes (13%; Fig. 2A). Of the GO terms related to cellular components, many genes were categorized as cell (28%) and organelle (24%) components (Fig. 2B). In terms of the molecular function category, many genes were classified as binding (49%), followed by catalytic activity (27%; Fig. 2C). Blood is one of the most dynamic tissues in the mammalian body, as cellular components of blood can be

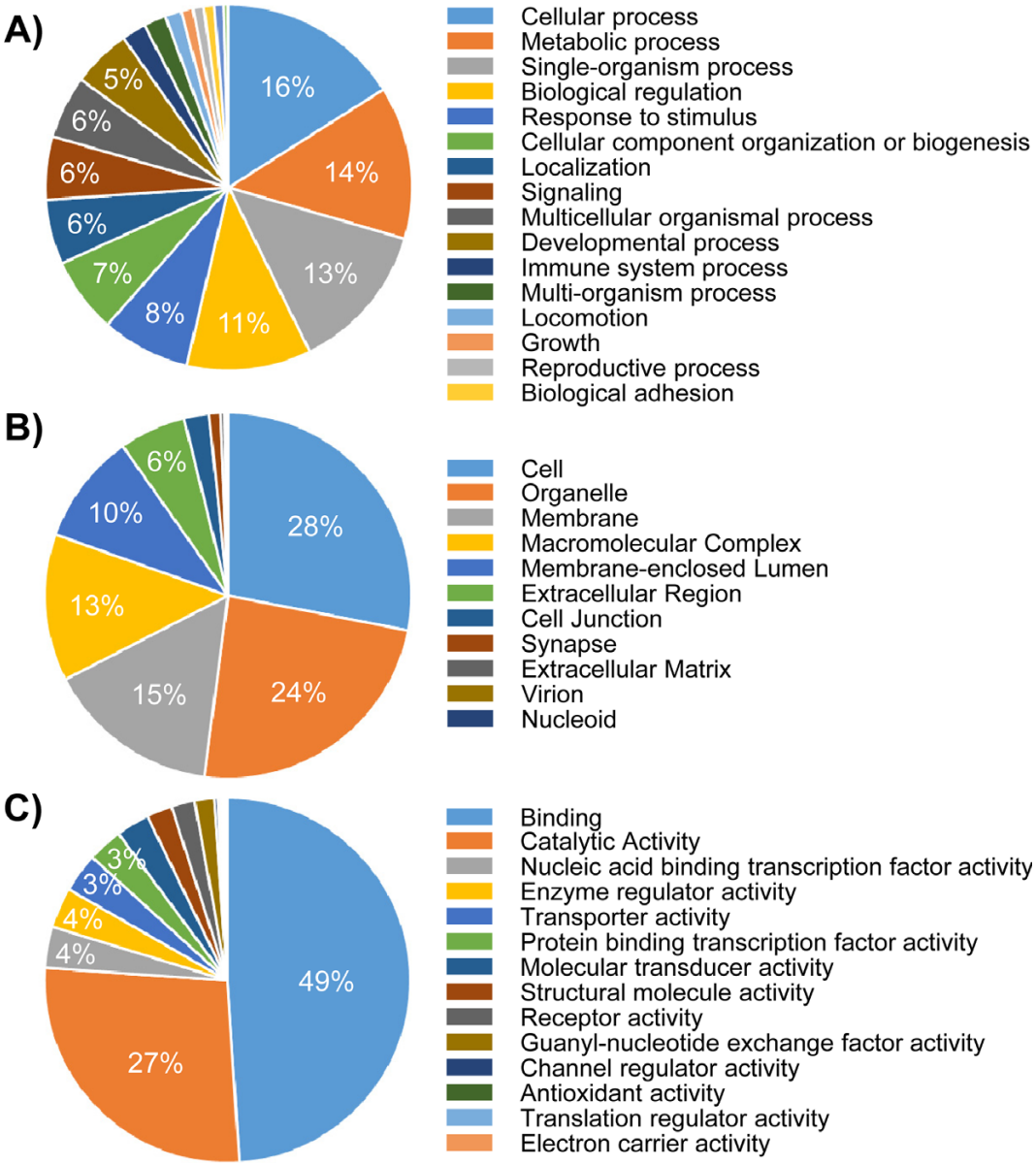


Fig. 2. Gene Ontology (GO) analyses: A) biological processes, B) cellular components, and C) molecular functions enriched in the blood transcriptome of *Mirounga leonina*. D) Comparison of the compositions (percentages) of two subcategories: response to stimuli and immune system processes of avian blood transcriptomes

significantly altered in composition by numerous exogenous or endogenous factors, resulting in changes in the global blood transcriptome.

Diving mammals have developed several adaptive metabolisms for breath-holding periods, such as elevated hemoglobin levels and tolerance to hypoxia (Elsner 1999). We confirmed that contigs coding for two hemoglobin genes (hemoglobin subunit alpha [HBA] and beta [HBB]) show high RPKM values (HBA: contig_31798 with 5,286, contig_32923 with 698; HBB: contig_45291 with 14,367, contig_8284 with 7,718) in the *M. leonina* blood transcriptome. High expression values of HBA and HBB may enhance the capacity for oxygen storage and transfer during routine diving. Acute hypoxia is recognized as one of the biggest challenges for diving seals. KEGG pathway analyses revealed that the most vital members involved in the HIF-1 signaling pathway (KEGG pathway number: 04066) are annotated in the transcriptome (Fig. 3; Table 2). HIF-1 proteins are key regulators that coordinate the adaptive homeostatic responses to hypoxia. HIF-1α protein is constitutively expressed in tissues of ringed seals during nonhypoxic conditions (Johnson et al. 2005). The nuclear content of HIF-1α significantly increases in the blood of northern elephant seal pups after breath-hold trials (Vázquez-Medina et al. 2011). Thus, the intact HIF-1 signaling pathway of *M. leonina* blood may contribute to its physiological adaptation to regular exposure to hypoxia in its natural environment.

The blood transcriptome can reflect pathological changes as immune cells circulate through the animal body. In the *M. leonina* blood transcriptome, most of the crucial members of innate immune systems were retrieved by KEGG pathway analyses as annotated sequences, although a full complement of the transcripts was not annotated. Since blood is known to be highly sensitive to endogenous and/or exogenous stimulations, transcriptional absences of several components in each pathway would be explained if it were the case that the *M. leonine* used in this study is not highly influenced by environmental factors or infection. However, further studies are very much needed in order to know whether these components can be

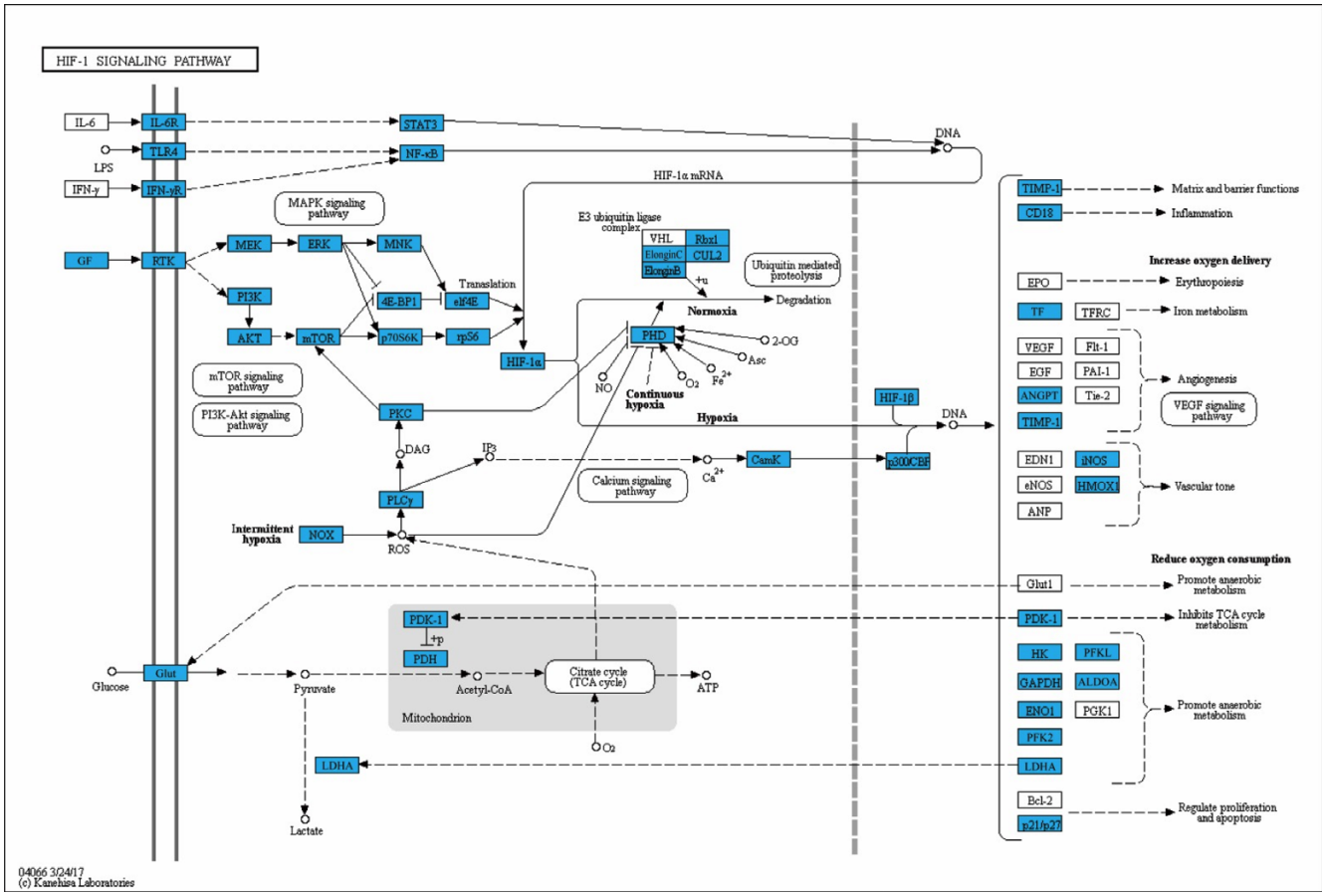


Fig. 3. Transcriptional coverage of *Mirounga leonina* transcripts on the vertebrate hypoxia-inducible factor-1 (HIF-1) signaling pathway (#04066). Matched homologues are highlighted with a lighter shade of cyan

transcriptionally induced or reduced by stimulations. Of the innate immune system sequences, four major signaling pathways, nuclear factor kappa B (NF-κB) (#04064), Toll-like receptor (TLR; #04620), Janus kinase/signal transducers and activators of transcription (JAK-STAT; #04630), and tumor necrosis factor (TNF; #04668), corresponded to vertebrate canonical signaling. The transcriptional involvement of immune-related genes on each pathway is highly matched on each pathway. In mammals, the four major innate immunity signaling pathways are important for responding to exogenous pathogens and maintaining homeostasis (Akira et al. 2006). The core members of each pathway revealed that the four innate immune systems are likely expressed to serve as a host defense modulator against exogenous infections in blood. *M. leonina* is a sentinel species for the circumpolar region of the Southern Ocean; thus, assessing its health status is crucial to estimate the disease resistance ability of seal populations and facilitate or promote their protection. Taken together, blood transcriptome profiling and the analyses of hematological parameters may be a useful indicator for determining health status, susceptibility to disease, and monitoring responses to exposure in pinnipeds.

In this study, we present the first whole-blood transcriptome of the southern elephant seal and analyze the pathways putatively involved in hypoxia regulation and the immune system. Although the small sample size is a weakness in this study, library construction and assembly were successfully conducted with *M. leonine* blood and the transcriptome covers the essential gene repertoire. We believe that this transcriptome information can be applied for understanding the molecular adaptation and immune homeostasis of southern elephant seals and facilitate their protection.

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