Comparative Genomics of Hypoxia and Barotrauma Tolerance in Diving Mammals

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Hypoxia and barotrauma (pressure) tolerance are two main challenges for marine mammal evolution. Marine mammals have evolved many changes in their anatomy and physiology to combat hypoxia and barotrauma risks they are exposed to with their diving lifestyle. Changes in structures across vascular, pulmonary, musculoskeletal, and several other systems work together to prevent and delay injuries that are typically fatal to land mammals. Genomics studies on hypoxia tolerance in marine mammals have focused so far on a limited number of species or consider a limited number of genes. At the same time, genomic studies of barotrauma tolerance are almost absent due to limited physiological understanding of the relevant marine mammal phenotypes. This comparative genomic analysis explored the possibility of convergent evolution of members of pinnipeds, cetaceans and sirenians based on their diving patterns. Shared genes were extracted from complete genomes of 25 marine mammal species and used to infer a phylogenetic tree. I then assessed the evidence for positive selection between the deeper-diving members and their shallow-diving counterparts. Evidence of selection was detected in 315 genes in deeper-diving species, and these genes were significantly enriched for functions relating to hematopoietic (blood cell production) and immunological pathways. This result agrees with existing physiological studies of hypoxia and barotrauma tolerance. It supports existing working theories, such as preventing embolism caused by free-floating fibrin fragments in blood vessels and the intense, prolonged onset of the inflammatory response during dives and surfacing.

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Statement of Copyright

Acknowledgements

I would like to thank Dr Andreanna Welch and Dr Rus Hoelzel for their mentorship throughout the process of this project, Mark Dixon and Karen Bower for assistance with Hamilton High-Performance Computer, Wenbin Wei for assistance with bioinformatics modules, Rodger Wang, Christoph Patterson, and Laura Campbell for their assistance with the scripts. Lastly, I would like to thank Arknights for reminding me of what I am working towards.

To Luby, who was a good boy.

Chapter 1. Introduction

Diving

With the evolution of amniotic eggs and keratinised skin, amniotes represent an important evolutionary step to life away from the aquatic environment. Yet, at many points in the vertebrate tree of life, many species return to the ocean to take advantage of its resources, changing their bodies drastically to achieve this goal. Even humans, such as the Ama people from Japan and Korea (Lindholm and Lundgren, 2009) and the Bajau people of Southeast Asia (Ilardo *et al.*, 2018), routinely engage in breath-hold as part of their livelihood, resulting in physiological adaptations favouring this lifestyle. Unassisted competitive breath-hold divers have been known to dive to depths of 80 m for up to 4 mins (Lindholm and Lundgren, 2009). However, that pales in comparison to other mammalian divers, as most marine mammals can easily surpass this limit without experiencing any adverse health effects associated with prolonged diving. Despite being one of the most popular research topics in vertebrate evolution, diving itself is a very loosely defined behaviour, with a working definition largely dictated by observational experience and specific study methods (Womble *et al.*, 2013). Boyd (1997) provided a general definition of diving: a unit of behaviour in which an animal makes an excursion from a central point (e.g. the surface) to achieve a specific goal over a specific time period. The main reason diving behaviour is so hard to define originates from the diversity in diving styles, which tend to be loosely associated with body sizes and can differ even between closely related species (Ponganis, 2015).

The underwater environment represents numerous physiological challenges for diving animals. Achieving such an impressive dive involves subjecting the body to oxygen-scarce conditions.

Hypoxia – the presence of lower than normal oxygen content and pressure in the cell (MacIntyre, 2014) is a condition often experienced by mammals during breath-hold dives. Animals will also experience drastic changes in water pressure when diving and resurfacing. This can cause nitrogen gas bubbles to form in the diver's blood vessels, leading to a condition known as gas emboli pathology or bends (Fahlman et al., 2021). In addition, amphibious marine mammals that stay on both the land and in water have to cope with the different refractory index of light between water and air. Many species, such as pinnipeds, have modified eye anatomy that provides flexibility so that their lens can focus light while diving in water, especially in low-light conditions typical during deep dives (Hanke et al., 2008). Finally, temperatures are usually much colder in water than at the surface. Marine mammals largely cope with the risk of hypothermia through the blubber layer and countercurrent heat exchange vasculature at their distal body parts, among other thermoregulatory adaptations (Favilla et al., 2021).

Of course, such a drastic shift in lifestyle and even ecological niche for some diving species suggests a sizeable benefit - and implies sizable selection pressure. Marine mammals engage in deep dives mainly to exploit food sources in deep water and to avoid open sea or pelagic predators. Deeper parts of the ocean are typically colder but carry more nutrients. Therefore, the energetic benefit of prey in deep water can outweigh the cost of diving. Fin whales (*Balaenoptera physalus*), for example, are found to quadruple their energy intake when targeting deep krill patches (Friedlaender et al., 2020). At the same time, pinnipeds engage in deep dives to avoid sharks, one of their predators, that are more common in the pelagic sea (Weller, 2009).

Diving Animals

Anatomical and physiological adaptations for diving are a case of convergent evolution (Yuan et al., 2021). Three main lineages of mammals display a clear transition from the ancestral terrestrial to aquatic lifestyle — members of the order *Carnivora* (including members of *Pinnipedia*, family *Ursidae*, and family *Mustelidae*), members of order *Cetartiodactyla* (precisely, members of infraorder *Cetacea*, which was previously a separate order) and members of order *Sirenia* (Ponganis, 2015). In addition, though not as extreme, members of other lineages also display adaptations to aquatic lifestyles, which often consist of short dives. Examples include platypus (*Ornithorhynchus anatinus*), star-nosed mole (*Condylura cristata*), and some members of the order *Rodentia*: muskrat (*Ondatra zibethicus*), beaver (*Castor canadensis*), and capybara (*Hydrochaeris hydrochaeris*) (Ponganis, 2015).

There are two major lineages of pinnipeds: phocid seals (also known as true seals) and eared seals, which include sea lions, fur seals and walruses. Most of the phocid seals dive less than 100m for 10-15mins. Species such as the Weddel seal (*Leptonychotes weddellii*), hooded seal (*Cystophora cristata*) and northern and southern elephant seals (*Mirounga angustirostris, M. leonina*) can dive routinely for 30 minutes to 400-600m deep (Hindell et al., 1992; Costa et al., 2010). On the other hand, Otariid seals are shallow divers compared to phocids, with dives rarely exceeding 4 minutes in duration and 100m in depth. (Ponganis, 2015). Likewise, the walrus (*Odobenus rosmarus*) typically dives to 30-70m deep for 4-6 minutes (Born and Knutsen, 1997; Gjertz et al., 2001; Jay et al., 2001; Nowicki et al., 1997; Wiig et al., 1993). Other than pinnipeds, whose diving patterns were outlined in the previous section, the most notable members of the order Carnivora are polar bears (*Ursus maritimus*) and otters, particularly sea otters (*Enhydra lutris*). However, none of these species engages in diving deeper than 30m

below sea level for over 3 minutes (Dyck & Romberg, 2007; Stirling & Meurs, 2015; Ralls et al., 1995; Tinker et al., 2007; Yeates et al., 2007).

Among cetaceans, sperm whales and beaked whales are the most notable groups of divers, with typical dives to 400m and 800m for 40-60 minutes, respectively (Baird et al., 2008; Hooker and Baird, 1999; Schorr et al., 2014; Tyack et al., 2006; Watkins et al., 1993). Other large-toothed whales, such as beluga whales (*Delphinapterus leucas*) and narwhals (*Monodon monoceros*), are also notable deep divers, though their dives usually last less than 20 minutes. Smaller toothed whales, more commonly known as dolphins, and most baleen whales, mostly dive less than 200 meters for less than 10 minutes (Ponganis, 2015).

Sirenians, in accordance with their grazing lifestyles, are shallow divers. They dive to a maximum of 12m for 2-3 minutes per dive (Chilvers et al., 2004; Gallivan and Best, 1980; Gallivan et al., 1986; Marsh et al., 1978, Reynolds III, 1981, Reynolds III and Odell, 1991). However, sirenians possess some of the densest bones of all mammals, vital in countering buoyancy when they suspend themselves in water for a prolonged time (de Buffrénil et al., 2010).

Animals from other major clades also display adaptations for aquatic or marine lifestyles involving diving. Several species of birds have been observed to take deep, long dives as part of routine foraging behaviour, such as penguins (Ponganis, 2009), while other non-avian reptiles, such as sea snakes, marine iguanas and sea turtles, have transitioned nearly completely to aquatic habitats, rarely, if ever, coming out onto land (Rasmussen *et al.*, 2011). However, having undergone distinct evolutionary pathways, mammals, birds, and reptiles have adopted physiological pathways and structures so different that it becomes difficult to link homologous structures to similar functions (e.g., different mechanisms of ventilation systems).

Therefore, to allow informative comparisons, the scope of this investigation is limited to diving animals within the class *Mammalia*.

Generally, the phylogenetic relationships between the major lineages in question are well-established based on morphological and molecular phylogenetic analyses (Foley et al., 2016; Yuan et al., 2021). Placental mammals are divided into four major superorders: Afrotheria, Xenarthra, Euarchontoglires and Laurasiatheria. Both cetaceans and pinnipeds belong to the superorder Laurasiatheria, with cetaceans part of the order Cetartiodactyla and pinnipeds part of Carnivora. Sirenians belong to the superorder Afrotheria, the superorder's only fully aquatic lineage. This study mainly focuses on marine mammals that heavily depend on aquatic life to the point that they have altered their anatomy and sacrificed their mobility on land. This excludes polar bears and sea otters because they are less adapted to an exclusively marine lifestyle.

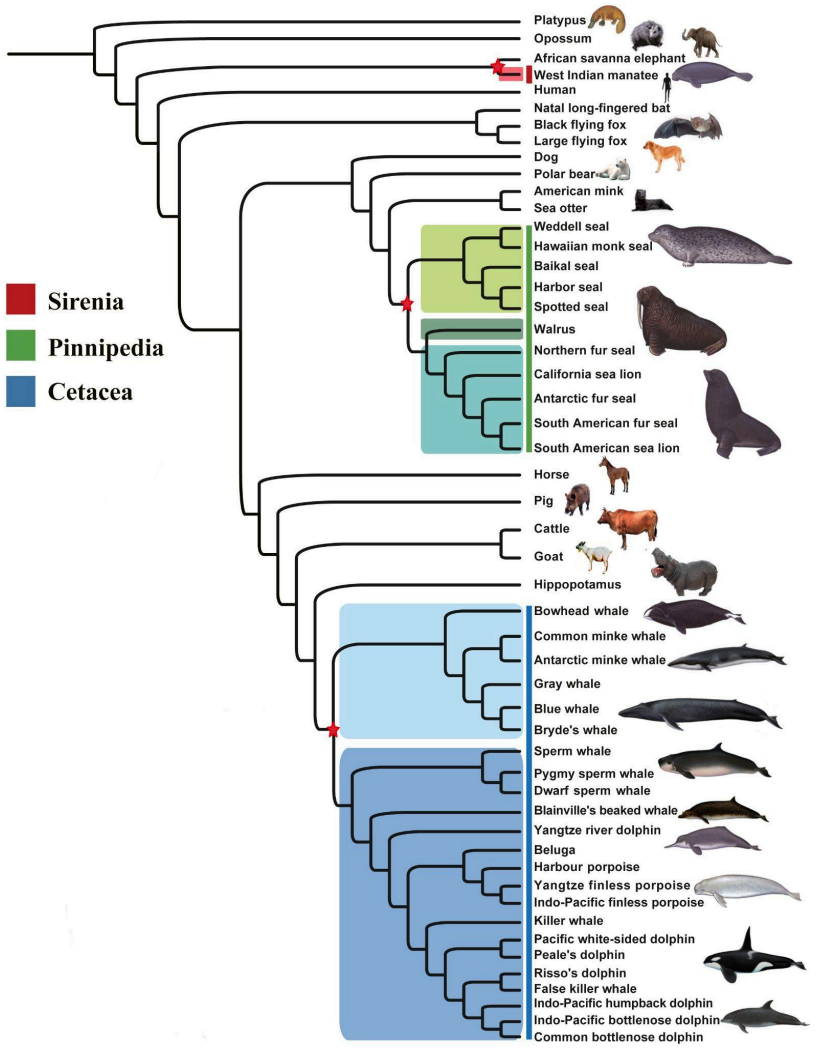


Fig. 1. A maximum likelihood phylogenetic tree of 35 marine mammal species and 16 outgroup mammal species reconstructed from genomic data. Three lineages of marine mammals are distinguished by columns of different colours: Cetacea (blue), Pinnipedia (green), and Sirenia (red). Red stars represent the points of differentiation of the focal lineages from their sister groups. (Yuan et al., 2021; only Figure 1A is shown)

Challenges of Breath-hold Diving

Every diving mammal faces two broad types of challenges during breath-hold dives: limited gas exchange and change of pressure. The urge to breathe during a breath-hold is mainly regulated

by the partial pressure of carbon dioxide (Brubakk *et al.*, 2003). It is mainly dependent on four factors: (1) tolerance to hypercapnia (elevated level of blood carbon dioxide) and hypoxia, (2) oxygen and carbon dioxide storage capacity, (3) metabolic rate and (4) the animal's voluntary decision to hold breath during a dive (Brubakk *et al.*, 2003). Human divers may prolong breath-hold duration by either training to improve carbon dioxide tolerance or hyperventilating before diving to lower the partial pressure of carbon dioxide in their lungs. The latter approach, however, exposes them to shallow-water blackouts as they resurface (Ponganis, 2015). As divers resurface from deep dives, change in ambient pressure drops partial pressure of oxygen in the lungs below the threshold of maintaining consciousness, causing the diver to black out (Ponganis, 2015). Shallow-water blackout has a high fatality rate if not rescued and resuscitated within a short while. It can occur irrespective of the diver's age, fitness, and level of experience (Lane, 2017) — drastic pressure change during diving can lead to excess nitrogen absorption into the bloodstream. Excess blood nitrogen can form bubbles when resurfacing, causing decompression sickness and nitrogen necrosis (Brubakk *et al.*, 2003). After repeated, long dives, the effects of decompression sickness can lead to vertigo, syncope (temporary loss of consciousness caused by a fall in blood pressure), partial paralysis, and even death (Cross, 1965).

Lung Structures

Marine mammals routinely engage in breath-hold diving since they spend much of their life in aquatic environments. However, it is surprising to many that most marine mammals have a similar range of mass-specific lung volumes to terrestrial mammals. Exceptions to this trend are sea otters (with larger lung volumes than expected) and deep-diving whales (with smaller lung volumes than expected; Kooyman, 1973; Fahlman *et al.*, 2011; Piscitelli *et al.*, 2013). The large lung volume of the sea otter is presumed to be associated with buoyancy rather than diving, as

sea otters spend a lot of time feeding, grooming, and performing other activities while floating on the surface. High buoyancy also elevates their body higher, thus reducing heat loss to seawater through conduction (Ponganis, 2015). Despite similar lung volumes, marine mammals can achieve diving depths and durations far more significantly than humans due to a series of adaptations.

Perhaps the most significant adaptation is the modified lungs of marine mammals. Pinnipeds and cetaceans can collapse their lungs under pressure during deep dives, squeezing the air inside into their airways and stopping gaseous exchange when ventilation is impossible (Ponganis, 2015). In phocid seals, distal ends of bronchioles are not supported by cartilage but have smooth muscles to aid this process. Sea otters and walruses have some distal bronchioles supported by cartilages, while other bronchioles are supported all the way until the final 1 mm before alveolar sacs. Sea lions and fur seals lack bronchioles but have cartilaginous support from the trachea to the alveoli. Lung surfactant proteins are typically secreted on the inner layer of alveoli to moisturise the surface, facilitate gas exchange, and ensure the alveoli stay inflated. In marine mammals, surfactant reduces surface tension and adhesion of the inner surface and allows easy reinflation during ascent (Blix, 2018). Most dolphins and some odontocete whales have bronchiolar myoelastic sphincters. Their functions are not fully understood but are also suspected of aiding alveolar collapse and reinflation (Ponganis, 2015). Likewise, histological studies have revealed that deep-diving cetaceans have vascular plexus, an elaborated network of fine blood vessels along the airway. These vasculatures are believed to be engorged with blood to fill the space of thoracic cavities to equalise pressure (Piscitelli et al., 2013). Other hypotheses about their functions include engorging to reinforce the trachea and bronchi, warming air, reducing airway pressures, oxygen storage, and preventing barotrauma (Ponganis, 2015).

Diving with collapsed lungs can also help animals cope with changes in pressure as the animal becomes incompressible (Blix, 2018). Furthermore, cessation of alveolar gas exchange can limit nitrogen intake during dives, thus slowing down nitrogen build-up in the bloodstream. This adaptation can delay decompression sickness and the onset of nitrogen necrosis (Blix, 2018). Certain cetacean species also have more compressible tracheas that support further air compression into the larynx and nasal passages, which is used for echolocation (Davenport *et al.*, 2013).

Oxygen Storage in Diving Mammals

It is logical to expect that the circulatory system, which distributes oxygen and other gas to various body tissues, would demonstrate adaptations in diving mammals. Perhaps unexpectedly, the structure and oxygen affinity of haemoglobins in marine mammals do not differ much from terrestrial mammal haemoglobins, with a few exceptions (Lenfat, 1969; Lenfant *et al.*, 1970; Meir *et al.*, 2009; Qvist *et al.*, 1981). Manatee haemoglobins have exceptionally low oxygen affinity compared to other mammals (Farmer et al., 1979; White et al., 1976). Investigation of Weddell seals also found that foetal blood may have slightly lower oxygen affinity than adult blood. However, this difference is attributed to a lower concentration of 2,3-diphosphoglycerate, a protein that increases the maternal-foetal oxygenation efficiency (Webb et al., 2022), in foetal plasma instead of the structure of haemoglobins (Qvist *et al.*, 1981). Despite that, blood volumes and haemoglobin concentrations of marine mammals can be 50-70% higher than those of humans, with some species of phocid seal reaching 2-3 times those of humans (Ponganis, 2011; Ridgway and Johnson, 1966). Additionally, marine mammals tend to have lower blood viscosity than terrestrial mammals when blood flow is slow and blood cell concentrations are relatively high, which may lower the workload of cardiac tissues when they experience bradycardia during dives (Ponganis, 2015).

Early understandings of the cardiovascular system activities of animals during dives were primarily derived from forced submersion studies (Ponganis, 2015). Both marine and terrestrial mammals, except manatees, experience some extent of lowering heart rate or bradycardia when forced to submerge. In free dives, however, though the heart rate of most still drops below resting level, the extent is smaller. During deep dives, vasoconstriction of peripheral tissues (i.e. shunting) also occurs to some extent, directing blood flow to brains while skeletal muscles rely more on anaerobic respiration. Shunting can be regulated by the sympathetic nervous system (Ponganis, 2015). Heart rate appears to correlate with stroke rates for shallow dives but does not exceed resting level (Williams *et al.*,1999).

The role of myoglobin in diving has also been extensively studied. Myoglobin is vertebrate striated muscles' principal oxygen storage protein (Ordway & Garry, 2004). Marine mammals share similarly structured myoglobins with terrestrial mammals. Wright and Davis (2015) characterised 25 aquatic and terrestrial birds and mammals. They found them remarkably conserved except for the melon-headed whale (*Peponocephala electra*), which has myoglobins of exceptionally high oxygen affinity. They also found that the myoglobin oxygen affinity of pinnipeds does show a higher trend with longer diving durations (Wright & Davis, 2015). However, many distantly related diving species have evolved an elevated net positive surface charge of myoglobin. Thanks to this property, myoglobin concentrations in marine mammal muscles can be significantly higher than in other mammals (Berenbrink, 2021). Deep, long-duration divers tend to have higher myoglobin concentrations, but a species's diving capacity is not proportional to its muscle myoglobin concentrations (Ponganis, 2015).

Other Adaptations

Other than lung collapse and myoglobin, the association of physiological adaptations to hypoxia and pressure tolerance of diving animals is not well-explored. Cetaceans' thoraco-spinal retia (an elaborate network of blood vessels) have also been hypothesised to protect them from decompression sickness by directing excess nitrogen to fat tissues at the end of small arteries and sinusoids (Blix *et al.*, 2013). This can also lower the partial pressure of nitrogen delivered to the brain, decreasing risks of decompression sickness and nitrogen necrosis. Another hypothesis is that fat tissues take up nitrogen from blood, thus minimising the risk of decompression sickness and nitrogen necrosis (Koopman and Westgate, 2012).

Other diving adaptations known so far include neuron tolerance for low oxygen and a high lactate internal environment, although how exactly they achieve this is unclear (Czech-Damal et al., 2014). The immune system has also been suspected of being involved in pressure tolerance since inflammation is often observed in stranded marine mammals displaying symptoms of decompression sickness (Fahlman et al., 2021).

Genomic Studies on Evolution of Diving

Adaptations relating to the shift from terrestrial to aquatic lifestyles have been a popular topic of comparative genomics in marine mammals. For the most part, pinnipeds and cetaceans have been model species for studies of significant aspects of diving response. Yuan et al. produced high-quality genome assemblies of 17 marine mammal species across pinnipeds, cetaceans, and sirenians and found three genes that share convergent amino acid substitutions across all three lineages as well as six other genes (HERC1, MITF, EPG5, FAT1, SYNE1, and ATM) that show convergent mutations across some of those lineages. The three genes are associated with blubber development (NFIA), vascular development (SEMA3E), and heat production by

brown adipose tissue (UCP1), respectively. Additionally, Yuan et al. also found that ALAS2, one of the genes interacting with hypoxia HIF-α, is also under positive selection in deep-diving species (Yuan et al., 2021).

Comparative genome analyses by Foote *et al.* on killer whale, walrus, and manatee genomes suggest that although convergent amino acid substitutions are relatively common among marine mammals, linking adaptive convergence to phenotypical convergence is rare (Foote *et al.*, 2015). Chikina et al. (2016) compared the relative rates of evolution of 18,049 protein-coding genes between 5 marine mammal species and 54 other placental mammal species of all major branches. They discovered hundreds of genes experiencing either relaxed or positive selection in response to aquatic lifestyles. These genes tended to be highly enriched in pathways that govern sensory systems, integument, lipid metabolism, pulmonary systems, and muscle physiology, while specific sensory systems, such as the olfactory system, experience a loss of functions. Though many of those genes have also experienced accelerated evolutionary rates in terrestrial mammals, suggesting strong selection pressures, marine mammals had a higher relative evolutionary rate than terrestrial mammals.

Several comparative genomics studies have also been conducted on a smaller scale, mainly among pinnipeds and cetaceans, to identify genetic changes related to aquatic adaptations. Such investigations of cetacean species reveal genes associated with nutrient sensing pathways (Derous *et al.*, 2021), lipid transport and localisation, ATPase activity, sound perception, and muscle contraction are under positive selection compared to terrestrial relatives (Sun et al., 2013). A similar investigation of the Weddell seal genome also reveals evidence of positive selection of genes associated with lipid metabolism and cardiovascular phenotype (Noh et al., 2022). A notable finding is that some of the genes appear to be under different selection pressures, even among members of the same order with different diving styles. For example,

the haemoglobin β gene appears to have higher dN/dS ratios for long-diving than for short-diving cetaceans (Tian et al., 2016).

The genetic changes associated with myoglobin in marine mammals have been well-investigated. Though structurally similar to their terrestrial relatives, the MB gene, particularly the portion responsible for coding amino acids contributing to the net surface charges of myoglobin, was found to be under greater selection pressure in long-diving cetaceans (Nery et al., 2013; McGowen *et al.*, 2014), pinnipeds (McGowen *et al.*, 2014) and other diving mammals (Mirceta *et al.*, 2013). In addition to the adaptive evolution of specific genes, analyses also reveal gene loss following the transition to aquatic life, such as loss of genes regulating detoxification and antioxidant defences (Tian *et al.*, 2018), blood clot formation, DNA repair, and oxidative stress-induced lung inflammation, among others (Huelsmann *et al.*, 2019).

On the other hand, genomic convergence associated with pressure tolerance remains relatively under-explored in marine mammals. This could be attributed to limited information on specific anatomical and physiological adaptations to cope with pressure changes, making specific phenotypes hard to define. Foot et. al. (2007) have demonstrated, through maximum-likelihood models of codon and amino acid substitution, that lung-specific surfactant protein C (SP-C) is under positive selection at different sites in all diving mammals and some semiaquatic mammals. Though each lineage shows substitutions on different sites of SP-C, all of the resulting proteins feature stronger binding of the N-terminal domain to the surfactant phospholipid film and increased absorption of the protein to the air-liquid interface, properties crucial to allowing repeated collapse and reinflation of lungs discussed previously.

Objectives

In this study, I conduct a genome-wide analysis of the major lineages of diving marine mammals to identify positive selection and functional divergence associated with hypoxia tolerance and pressure tolerance in deep diving species. Semiaquatic species outside of group pinnipeds, cetaceans and sirenians are excluded in this study. As discussed above, some aspects of diving adaptations, such as thermoregulative blubber, are well explored in comparative genomics. In contrast, others like hypoxia and pressure tolerance remain less well understood for various reasons, such as the availability of marine mammal genomes, particularly pinniped genomes (Fahlman et al., 2021). Further, previous investigations have been limited regarding species coverage, rarely comparing marine mammalian species amongst themselves between different shallow and deep diving styles.

Given what is known about deep-diving mammalian physiology, I expect the ventilation system to have undergone significant changes in deep-diving marine mammals. Since the target phenotype is too complex to be associated with any single gene, this investigation will attempt to locate genes associated with ontogenic pathways of related organ systems. As previously discussed, the functions of many structures in marine mammals are a matter of speculation. Most of the understanding of adaptations for pressure tolerance also came from early forced submersion studies, which do not represent natural behaviours well (Ponganis, 2015). This investigation thus aims to provide a robust alternative way to study functions of anatomical features starting from the genetic level. A total of 28 species are selected to represent all major branches within the three lineages.

The following hypotheses were tested:

- Of all species tested, those with longer dive duration have more genes associated with hypoxia tolerance under positive selection
- Of all species tested, deep divers have more genes associated with barotrauma tolerance under positive selection

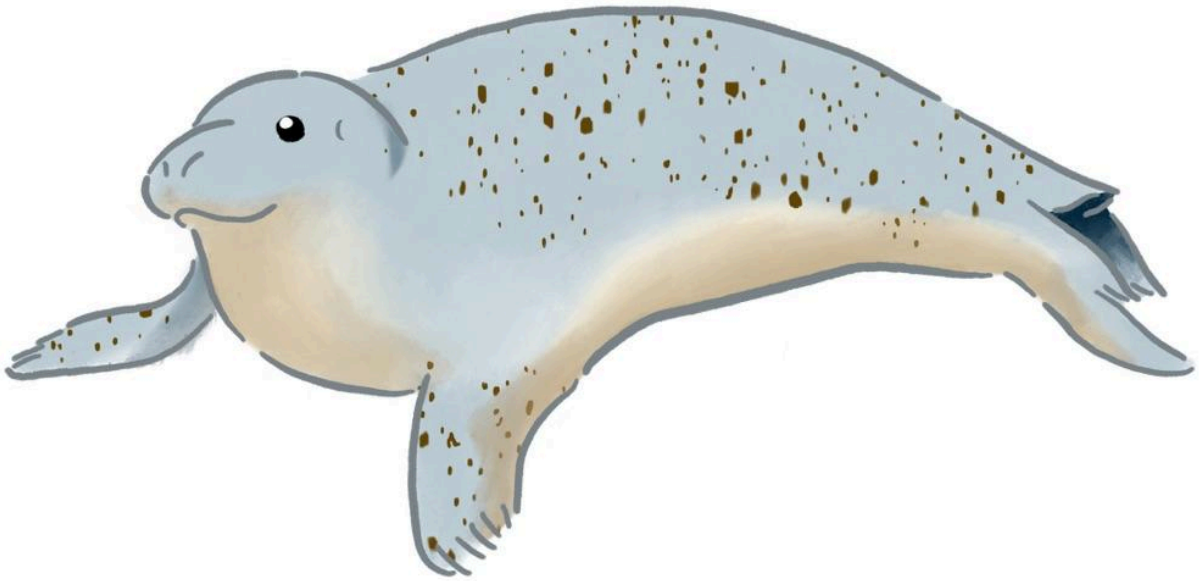
I anticipated finding evidence for positive selection in genes coding for musculoskeletal systems (particularly cartilage development), circulatory systems, lung tissues, lung surfactant proteins, and metabolic and respiration pathways.

In Chapter 2, I describe how I tested these hypotheses: I obtained previously published genome sequences from deep and more shallow diving marine mammals, identified homologous genes, created alignments, and then tested for positive selection. I conducted a GO enrichment test for genes with significant evidence of positive selection ($\omega > 1$) to elucidate their functions. I used knowledge from the literature to discuss whether or not these might be related to hypoxia and barotrauma tolerance. For some candidate genes previously identified in the literature as potentially crucial for deep diving, I further investigated if there was evidence for positive selection on them or not.

In Chapter 3, I describe the results of these analyses, which revealed that over 300 genes showed evidence of positive selection and GO analyses highlighting adaptations in blood chemistry and immune systems.

In Chapter 4, I discuss what is known about the function of these genes and their relationship to hypoxia and barotrauma tolerance in the physiological context.

In Chapter 5, I conclude the thesis with an overview of the limitations of the protocol employed in this study, reflections on the broader field and what can be done to address them.



Harbour Seal (*Phoca vitulina*)

Chapter 2. Methods

In all parts of this chapter involving any programs or scripts, default parameters were used unless otherwise specified.

Species Selection

There are 34 extant species of pinnipeds, 89 extant species of cetaceans and four species of sirenians (Wilson & Reeder, 2005). A total of 28 species with genomes available at scaffold or higher completeness levels were selected in this investigation so that they may represent a concise version of the whole phylogenetic tree. The phylogenetic tree of Peterson et al. (2020) produced on pinnipeds and Geisler et al. (2011) on cetaceans was used as a taxonomic reference. One sirenian species was selected for each family. Each species was selected to represent a significant branching event between groups, so the phylogenetic tree was equally represented (i.e. if multiple species are sister species of each other, only one of them was selected). Yangtze River dolphin (*Lipotes vexillifer)* was also included despite the species being extinct because it is the sole representative of its family with an available genome. The list of species included in this study is shown in Table 1 below, with 7 pinnipeds, 19 cetaceans and 2 sirenians. The candidate species are separated into “shallow divers” and “deep divers”. The exact criteria for this designation are discussed in the section on Positive Selection Tests below.

Genus	**Species**	**Common name**	**WGS accession No.**	**Completeness Level**
Phocidae	*Phoca vitulina**	harbour seal	RXNX01	Scaffold
Phocidae	*Mirounga leonina**	southern elephant seal	JAAMPH01	Scaffold

Phocidae	*Halichoerus grypus**	grey seal	JAAXOB01	Scaffold
Otariidae	*Zalophus californianus*	Californian sea lion	WPOA02	Chromosome
Otariidae	*Arctocephalus townsendi*	Guadalupe fur seal	JAQQSM01	Chromosome
Otariidae	*Callorhinus ursinus*	northern fur seal	QLOG01	Scaffold
Odobenidae	*Odobenus rosmarus*	walrus	ANOP01	Scaffold
Balaenidae	*Eubalaena glacialis*	North Atlantic right whale	JAQRBK01	Chromosome
Balaenopteridae	*Balaenoptera musculus*	blue whale	VNFC03	Chromosome
Balaenopteridae	*Balaenoptera ricei*	Rice's whale	JAQMHA01	Scaffold
Physeteridae	*Physeter catodon**	sperm whale	PGGR02	Chromosome
Physeteridae	*Kogia breviceps*	pygmy sperm whale	JAPMIF01	Chromosome
Platanistidae	*Platanista minor*	Indus River dolphin	RJWK01	Scaffold
Ziphiidae	*Mesophlodon densirostris**	Blainville's beaked whale	JANXIL01	Chromosome
Ziphiidae	*Ziphius cavirostris*	Cuvier's beaked whale	RJWS01	Scaffold
Ziphiidae	*Hyperoodon ampullatus**	northern bottlenose whale	JALGBC01	Scaffold
Lipotidae	*Lipotes vexillifer*	Yangtze River dolphin	AUPI01	Scaffold
Pontoporiidae	*Pontoporia blainvillei*	franciscana	PGGH01	Scaffold

Iniidae	*Inia geoffrensis*	boutu	RJWO01	Scaffold
Monodontidae	*Monodon monoceros**	narwhal	SIHG01	Scaffold
Monodontidae	*Delphinapterus leucas**	beluga whale	NQVZ02	Scaffold
Phocoenidae	*Phocoena phocoena*	harbour porpoise	RJWQ01	Scaffold
Phocoenidae	*Neophocaena asiaeorientalis*	narrow-ridged finless porpoise	MKKW01	Scaffold
Delphinidae	*Tursiops truncatus*	common bottlenose dolphin	JAAOMD01	Chromosome
Delphinidae	*Globicephala melas**	long-finned pilot whale	SWEB01	Scaffold
Delphinidae	*Orcinus orca*	killer whale	CAKZJT01	Chromosome
Sirenia	*Dugong dugon*	dugong	CAJQER01	Scaffold
Sirenia	*Trichechus manatus*	West Indian manatee	AHIN01	Scaffold

Table 1. Candidate species selected for this study, their common name, and their WGS accession numbers in the NCBI database. Species designated “deep divers” are marked with an asterisk beside their Latin names.

Gene Extraction and Prefiltering

The whole genomes of all candidate species were downloaded from the NCBI database. BUSCO v 5.3.2 (Manni et al., 2021) with AUGUSTUS gene predictor (Stanke et al., 2008) was used to extract protein-coding regions of each genome and convert them into individual gene files using the lineage database eutheria_odb10, which covers all genes shared by placental mammals. For analyses running longer than three days on the high-performance computing

cluster, checkpoint and restore were applied when necessary using the “--restart” argument in BUSCO.

Then, a list of single-copy protein-coding gene sequences was created for each genome, and the output was formatted using a custom script (see Appendix 1), with hmms folder from the lineage file eutheria_odb10 specified as input to produce a table detailing the presence and absence of all single-copy genes in all genomes.

Next, the single-copy genes were pre-filtered using another custom script (Appendix 2). This step selects all the species genes that possess a single copy, extracts the gene sequences from all the genome outputs and rearranges them into files containing all homologs of each gene to be used in subsequent analyses. A separate genome statistics list was also generated detailing the completeness of each candidate genome. I implemented a threshold of 50% for missing data. Three genomes with >50% missing data were discarded (see Results). The genes found in all the remaining genomes had all their homologs concatenated into one file named after the respective BUSCO group. An improved version of the protocol described in this section is made available on GitHub, courtesy of Rodger Wang: https://github.com/Onion-Skins/phylociraptor/tree/master.

Phylogenomic Analysis

For each BUSCO group that passed this prefiltering process, multiple sequence alignment was performed using PRANK v 170427 (Löytynoja, 2014). The sequences that PRANK could not process were BLASTed using the megablast algorithm against the NCBI nucleotide database (Camacho et al., 2009) to identify the coding region. Manually curated sequences were then concatenated with other sequences of the BUSCO group and successfully aligned again with

PRANK. Alignments were trimmed through MSA trimmer (Kremer, 2019). All trimmed alignments were concatenated into a supermatrix, and IQtree (v 2.2.2.3 COVID-edition, Minh et al., 2020; ModelFinder Kalyaanamoorthy et al., 2017) utilised a stochastic algorithm to construct a maximum-likelihood species tree using the supermatrix as input with 1000 ultra-fast bootstrap repeats (Hoang et al., 2018).

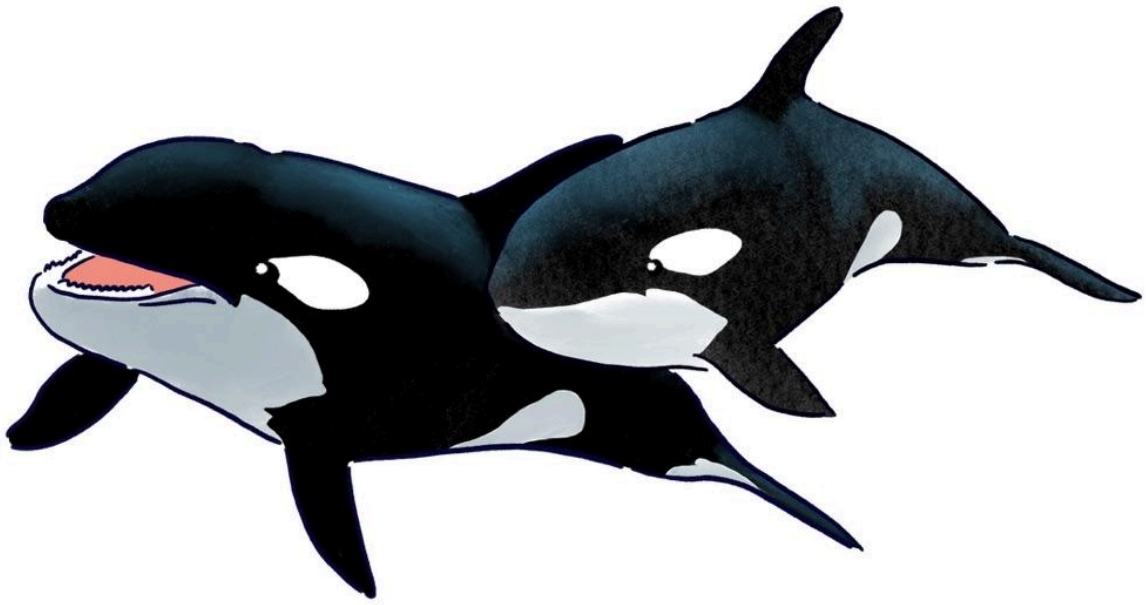
Positive Selection Tests

All the trimmed alignments were then analysed using PAML v 4.10.7 (Yang, 2007) to detect evidence for positive selection. PAML infers positive Darwinian selection through phylogenetic comparison of protein-coding genes. Comparisons of model M7 (beta distribution only) with M8 (rates at sites following a beta distribution with a category for positive selection) were conducted for each gene file (Welch et al., 2014).

Species considered deeper and longer divers relative to their sister species were selected as foreground branches, while the others were designated as background branches. For example, phocid seals dive on average for 10-15 mins, which is significantly longer than their eared seals, who rarely dive for longer than 4 mins (Ponganis, 2015). Therefore, phocid seals are selected as foreground branches and eared seals as background branches. Other study species were designated either foreground or background species based on similar principles, and the selected foreground species are marked with an asterisk in Fig. 2 in the Results chapter. For each test, a Likelihood Ratio Test was conducted using log-likelihood values (lnL) with two degrees of freedom (Álvarez-Carretero et al., 2023). The direction and magnitude of selection in PAML models are reported as nonsynonymous/synonymous codon substitution ratio (d_N/d_S ratio), also known as the omega (ω) value. Genes with significant evidence for positive selection ($\omega > 1$) were retained for further analyses (Álvarez-Carretero et al., 2023).

Gene Ontology Analysis

A Gene Ontology (GO) analysis was conducted to determine if genes under positive selection were enriched for particular functions. Tests were conducted using the selected list of genes from PAML (see above) and PANTHER v 18.0 (Thomas et al., 2022). The analysis was conducted for biological processes, molecular function, and cellular components using *Homo sapiens*, *Bos taurus,* and *Canis lupus familiaris* as model species. *Homo sapiens* was chosen because of the abundance of gene annotations. *Bos taurus* and *Canis lupus familiaris* were chosen for their close evolutionary relationship with cetaceans and pinnipeds, respectively. Significance was assessed using the Fisher's Exact Test. Correction for the false discovery rate using the Benjamini-Hochberg procedure.



Killer whales (*Orcinus orca*)

Chapter 3. Results

A total of 28 genomes (mean coverage 74.5x) were analysed for 11,366 BUSCO groups within the lineage database eutheria_odb10. Of all the genomes, *Platanista minor* (46.7% completeness), *Pontoporia blainvillei* (25.2% completeness), and *Ziphius cavirostris* (34.9% completeness) have completeness levels below the cutoff point of 50%. Therefore, these three genomes were excluded from the following analyses. On average, the remaining genomes have 9483 (83.4%) of all BUSCO groups as single-copy sequences, 159 (1.40%) as multi-copy sequences, 389 (3.43%) fragmented sequences and 1335 (11.7%) missing sequences. Of all single-copy sequences, 2553 sequences were shared by all 25 remaining genomes and were therefore selected for tree-making. The small number of genes after filtering can be attributed to the relatively distant phylogenetic relationships between the three lineages. Afrotheria, in particular, is very distantly related to the other two lineages. Therefore, it is reasonable to expect all 25 species to share very few genes. Alternatively, the shared genes might have been rendered indetectable due to the distant relationships. This is discussed in detail in Discussion.

Phylogenetic Tree

ModelFinder Plus (Kalyaanamoorthy et al., 2017) selected GTR+F+I+R8 as the appropriate model for inferring the maximum likelihood tree. The topology of the resulting tree (Figure 2) agrees with the reference (Peterson et al., 2020; Geisler et al., 2011). Cetaceans and pinnipeds were shown to be more closely related to each other than either of them to the sirenians. Among pinnipeds, phocid seals and otariid sea lions were clearly separated. Likewise, cetaceans were correctly separated into baleen whales and toothed whales. All the branches had 100% bootstrap support. The resulting phylogenetic tree is displayed in Fig. 2.

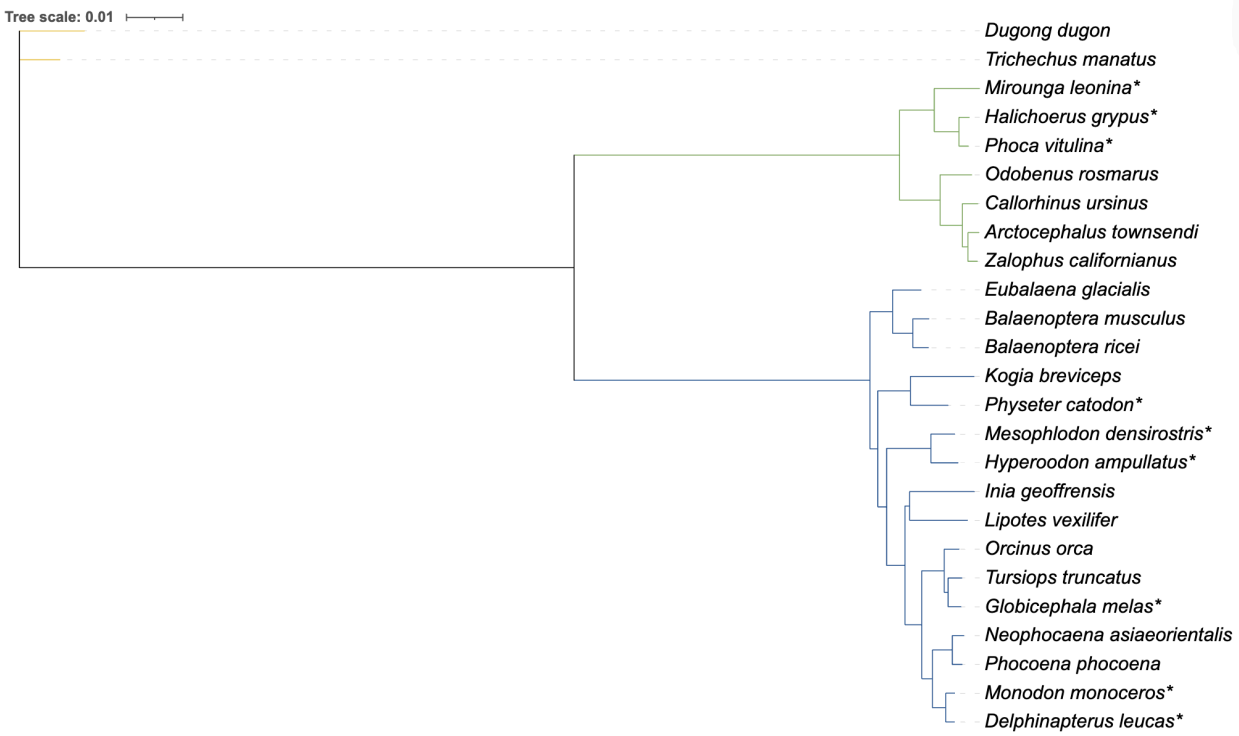


Fig. 2. Maximum-likelihood gene tree based on candidate genomes, with the three major lineages coloured in red (Sirenians), green (pinnipeds) and blue (cetaceans). The foreground branches for tests of positive selection are designated with an asterisk.

Positive Selection Tests

Of the 2553 genes analysed with PAML, 567 were significant for tests with M7 against M8, and 1986 were not. Among all the significant genes, 339 display omega values greater than 1 for Model 8 (Fig. 3), 154 of which had omega values between 1 and 2, 136 between 2 and 5, 23 between 5 and 10, and 26 above 10.

The gene CFB and CTU2, which code for complement factor B and cytosolic thiouridylase subunit 2, had the highest omega value of 999 and the second highest of 174.1171, respectively. However, CTU2 does not appear to be related to hypoxia or pressure tolerance, as

it is mainly involved in the post-transcriptional modification of tRNAs (Xue et al., 2024). CTU2 was not found to be associated with any highlighted pathways in subsequent GO analyses either. CFB, on the other hand, is part of the pathway proliferation of preactivated B lymphocytes (Shimazaki et al., 2021). Despite potentially related to immune adaptations, it is not found to be associated with any GO terms highlighted either. Both genes have omega values beyond two standard deviations (22.0) from the mean (3.5). Therefore, they are considered outliers that are irrelevant to this study. Other than CFB and CTU2, notable genes with high omega values are BRCA1 (Breast cancer type 1 susceptibility protein homolog, ω=42.0855), HIP1 (huntingtin-interacting protein 1 related protein, ω=33.8376), GMEB1 (Glucocorticoid modulatory element binding protein 1, ω=29.9334), CSF1R (colony stimulating factor 1, ω=14.3700), IL12B (Interleukin-12 subunit beta, ω=16.0057), and ERCC2 (ERCC excision repair 2, ω=13.3984).

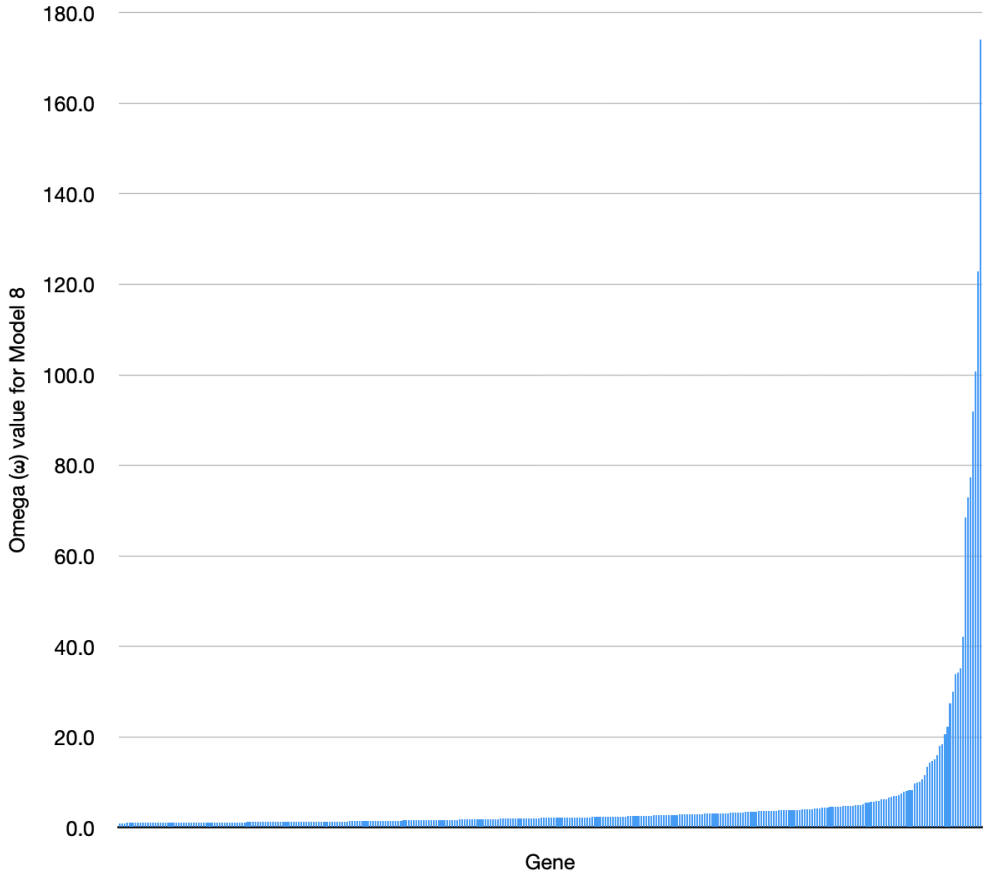


Fig. 3 Omega (ω) values from 338 positive selection tests in PAML where model 8 (rates at sites following a beta distribution with a category for positive selection) was a significantly better fit than model 7 (beta distribution only). CFB has been excluded in this graph for scaling. Each bar corresponds to a gene. Bars have been ordered by omega value.

Since the candidate species cover a wide range of the mammalian tree, it was rare to find positions in the alignment where deep diving species shared the same amino acid that differed from the shallow diving species (convergent SNPS).. However, there are some instances, particularly for genes with high omega values, where disparate deep-diving lineages do show convergent SNPs, such as IL12B shown in Fig. 3. On position 578, the majority of the deep-diving species have guanine in this position, while shallow-diving species tend to have adenine. More data from outgroup species would be required to confirm that this apparent case

of convergence among the deep diving species is an adaptation. However, according to the NCBI database, IL12B orthologs of several terrestrial lowland ruminant species (*Bos taurus*, *Ovis aries*, *Bos javanicus*, etc.) have adenine at the same position, as do several terrestrial mustelids (*Mustela erminea*, *Mustela lutreola*, *Mustela putorius furo*, etc.). Ruminants and mustelids are closely related outgroups to cetaceans and pinnipeds, respectively (Wilson & Reeder, 2005). Therefore, this suggests that adenine is the ancestral condition while guanine substitution is the derived condition for aquatic adaptations—the substitution of adenine with guanine results in a change from glutamic acid to glycine upon translation. Since glutamic acid has a longer tail and carries a slight negative charge than glycine, this may alter the resulting protein's properties.

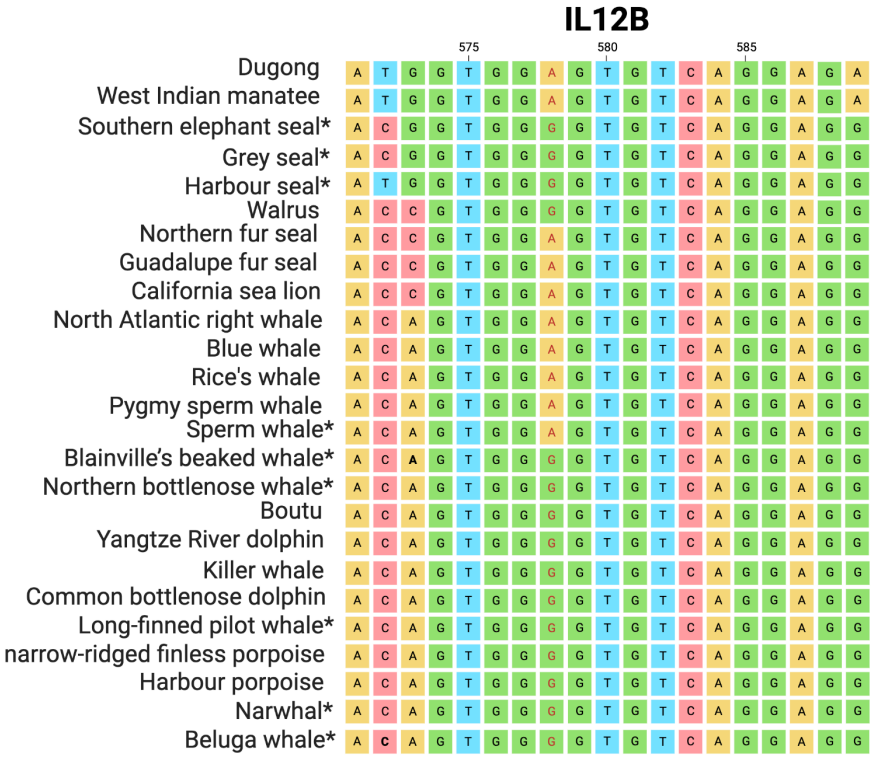


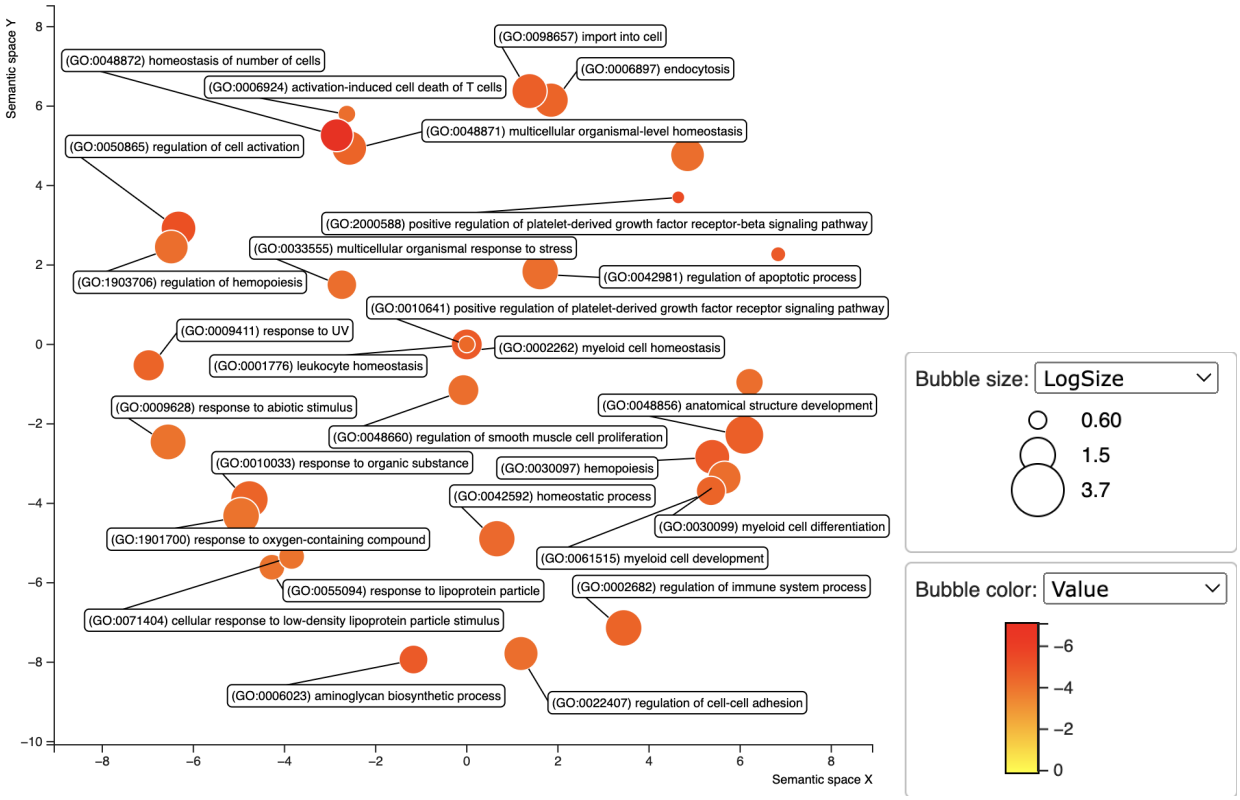
Fig. 4 Alignment of the IL12B gene among candidate species, with deep divers shown with an asterisk. Convergent base change between deep divers is highlighted in red at position 578.

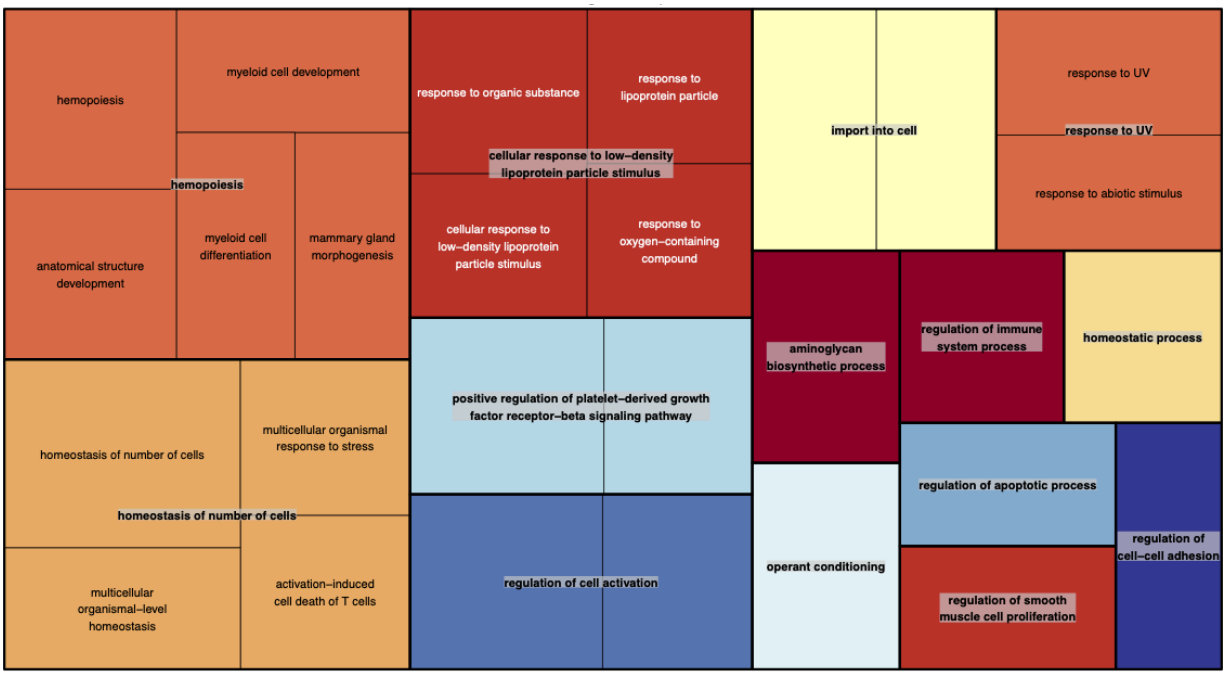
GO Analysis

Considering the false discovery rate, the gene list was significantly associated with 31 GO terms relative to humans, 21 GO terms relative to cows and 110 GO terms relative to dogs for Biological Processes (Appendix 3). For molecular functions, 1 GO term relative to cows was highlighted. For cellular components, 2 GO terms relative to humans were highlighted. All other GO analyses returned no statistically significant results.

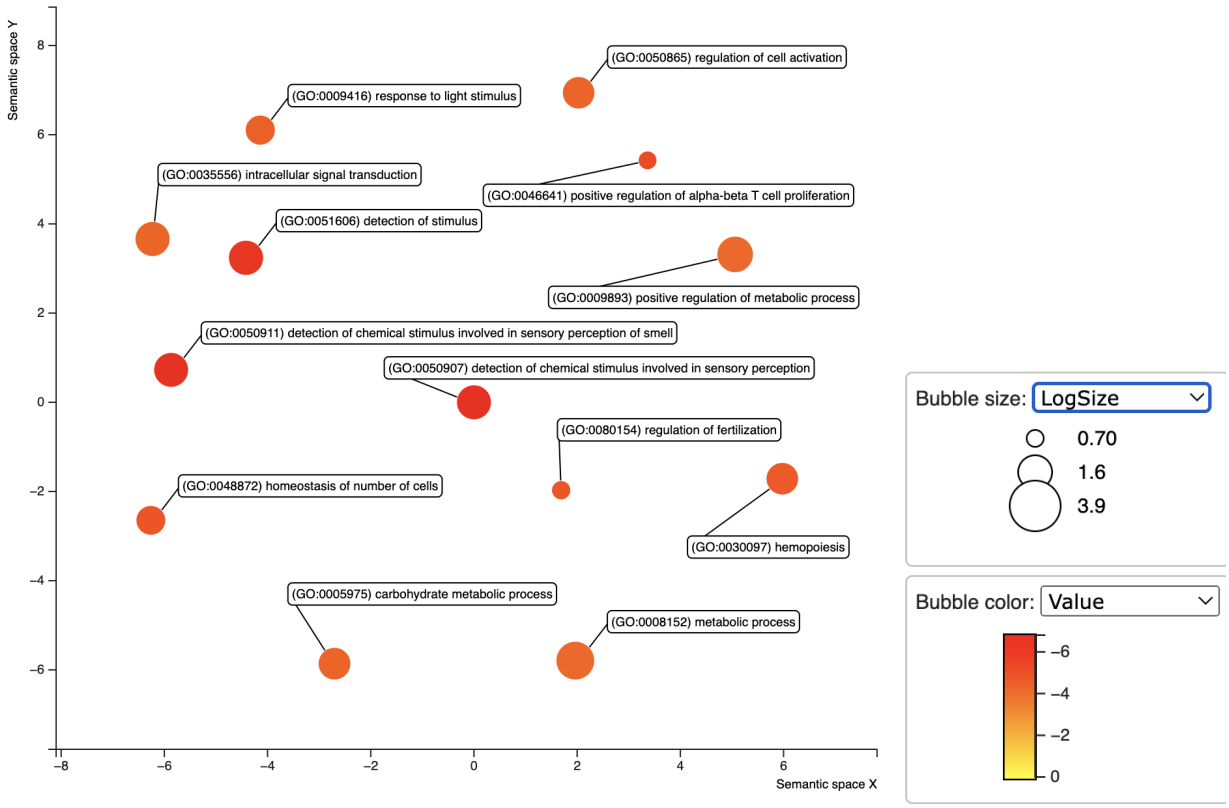
With the exception of the detection of chemical stimulus involved in sensory perception of smell, which was underrepresented relative to dogs and cows, all other significant GO terms were overrepresented. Fig. 5 summarises notable enriched GO terms plotted by ReviGO (Supek et al., 2011). The majority of enriched terms are involved in immune systems, particularly cellular response to inflammatory agents. In addition, genes under positive selection were enriched for functions related to apoptosis and hemopoiesis and their corresponding regulatory processes (see discussion below).

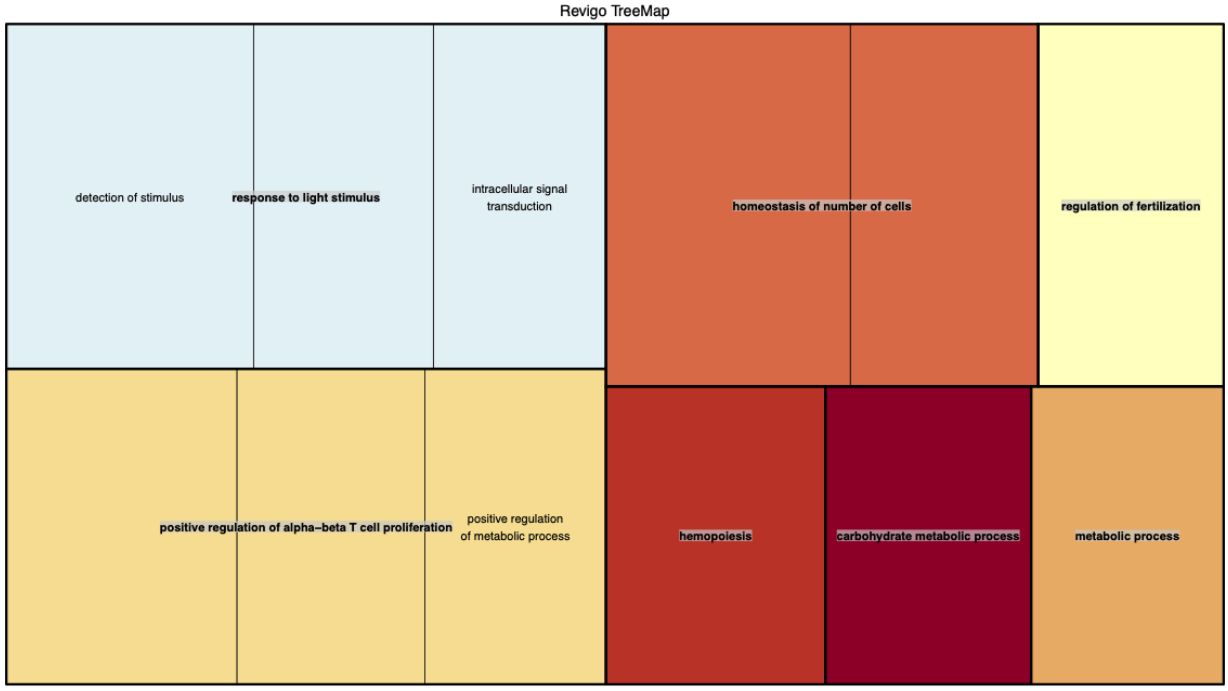
a) Human


Semantic space Y
Semantic space X
(GO:0048872) homeostasis of number of cells
(GO:0098657) import into cell
(GO:0006897) endocytosis
(GO:0006924) activation-induced cell death of T cells
(GO:0048871) multicellular organismal-level homeostasis
(GO:0050865) regulation of cell activation
(GO:2000588) positive regulation of platelet-derived growth factor receptor-beta signaling pathway
(GO:0033555) multicellular organismal response to stress
(GO:0042981) regulation of apoptotic process
(GO:1903706) regulation of hemopoiesis
(GO:0010641) positive regulation of platelet-derived growth factor receptor signaling pathway
(GO:0009411) response to UV
(GO:0002262) myeloid cell homeostasis
(GO:0001776) leukocyte homeostasis
(GO:0009628) response to abiotic stimulus
(GO:0048856) anatomical structure development
(GO:0048660) regulation of smooth muscle cell proliferation
(GO:0010033) response to organic substance
(GO:0030097) hemopoiesis
(GO:0042592) homeostatic process
(GO:0030099) myeloid cell differentiation
(GO:1901700) response to oxygen-containing compound
(GO:0061515) myeloid cell development
(GO:0055094) response to lipoprotein particle
(GO:0002682) regulation of immune system process
(GO:0071404) cellular response to low-density lipoprotein particle stimulus
(GO:0006023) aminoglycan biosynthetic process
(GO:0022407) regulation of cell-cell adhesion
Bubble size: LogSize
0.60
1.5
3.7
Bubble color: Value
−6
−4
−2
0


hemopoiesis
myeloid cell development
hemopoiesis
anatomical structure development
myeloid cell differentiation
mammary gland morphogenesis
response to organic substance
response to lipoprotein particle
cellular response to low−density lipoprotein particle stimulus
cellular response to low−density lipoprotein particle stimulus
response to oxygen−containing compound
import into cell
response to UV
response to UV
response to abiotic stimulus
aminoglycan biosynthetic process
regulation of immune system process
homeostatic process
positive regulation of platelet−derived growth factor receptor−beta signaling pathway
homeostasis of number of cells
multicellular organismal response to stress
homeostasis of number of cells
multicellular organismal−level homeostasis
activation−induced cell death of T cells
regulation of cell activation
operant conditioning
regulation of apoptotic process
regulation of cell−cell adhesion
regulation of smooth muscle cell proliferation

b) Cow


Semantic space Y
Semantic space X
(GO:0050865) regulation of cell activation
(GO:0009416) response to light stimulus
(GO:0035556) intracellular signal transduction
(GO:0046641) positive regulation of alpha-beta T cell proliferation
(GO:0051606) detection of stimulus
(GO:0009893) positive regulation of metabolic process
(GO:0050911) detection of chemical stimulus involved in sensory perception of smell
(GO:0050907) detection of chemical stimulus involved in sensory perception
(GO:0080154) regulation of fertilization
(GO:0048872) homeostasis of number of cells
(GO:0030097) hemopoiesis
(GO:0005975) carbohydrate metabolic process
(GO:0008152) metabolic process
Bubble size: LogSize
0.70
1.6
3.9
Bubble color: Value
−6
−4
−2
0


Revigo TreeMap
detection of stimulus
response to light stimulus
intracellular signal transduction
homeostasis of number of cells
regulation of fertilization
positive regulation of alpha−beta T cell proliferation
positive regulation of metabolic process
hemopoiesis
carbohydrate metabolic process
metabolic process

c) Dog

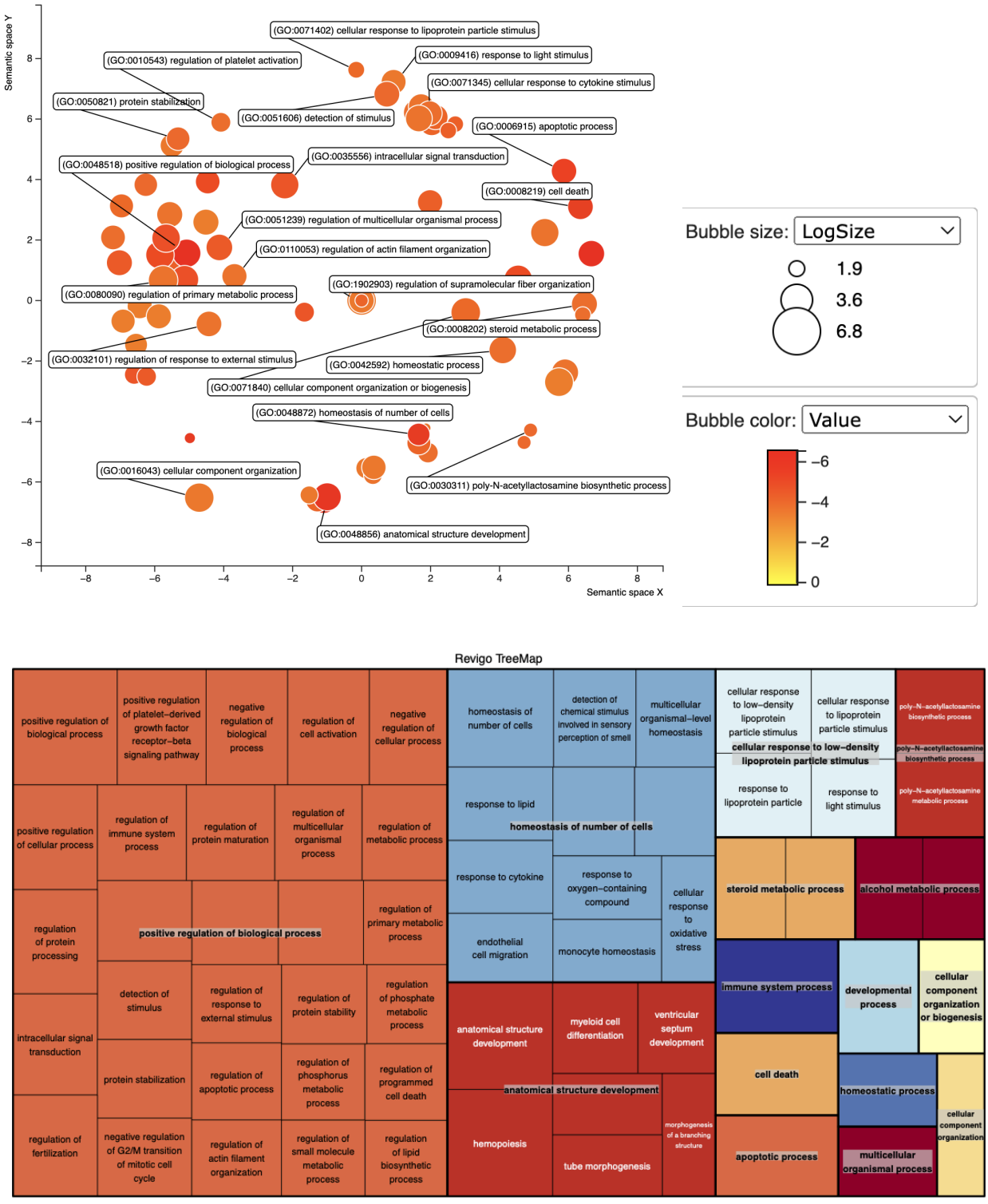
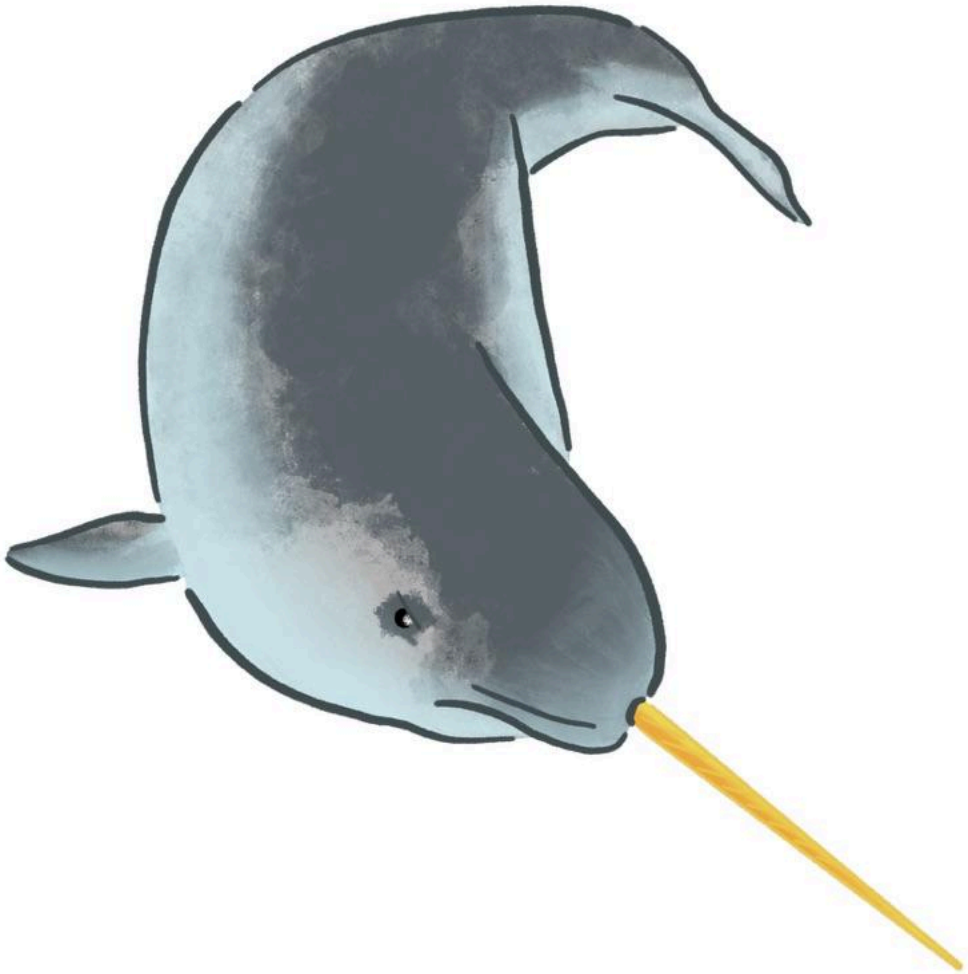


Fig. 5 ReviGO scatterplots and ReviGO tree maps of enriched GO biological process categories with (a) human, (b) cow and (c) dog. The colour gradient of the circles in the scatterplot denotes P values, with the red ones having higher P values. Each box in the

tree map represents a supercluster of enriched GO terms, with superclusters of loosely related biological processes visualised in the same colour. The full table of the results is in Appendix 3.



Narwhal (*Monodon monoceros*)

Chapter 4. Discussion

The results largely coincide with the initial hypotheses. This study was led with the hypothesis that marine mammal species diving longer and deeper are expected to have more genes associated with hypoxia and pressure tolerance under positive selection. Indeed, marine mammal species often engaged in longer and deeper dives (foreground branches in the positive selection tests, Figure 2) share more than 330 genes under positive selection with functions relevant to hypoxia and pressure tolerance compared to their shallow-diving relatives (background branches). Some genes are also relevant to musculoskeletal and circulatory systems, as expected. However, genes relevant to immune systems were also found to be under positive selection. For the following discussion, refer to Fig. 5 for a qualitative representation of the fold enrichment of each GO term or Appendix 3 for their exact values.

Musculoskeletal Development

Since marine mammals have long been known to display high efficiency in obtaining and retaining oxygen in their system, I proposed in my hypotheses that genes directly relevant to the cardiovascular, musculoskeletal, nervous and pulmonary systems responsible for collecting and distributing oxygen should be under selection. Indeed, many genes that are part of the physiological pathways related to these functions were found to be under positive selection. For example, the GO term “regulation of smooth muscle cell proliferation” was identified as having 4.69-fold enrichment relative to humans. This could explain the smooth muscle structures around bronchioles in phocid seals as well as how the smooth muscles lining arteries aid in shunting during dives (Blix, 2018). The GO term “regulation of actin filament organisation” (3.74 fold enrichment relative to dogs) is part of the functional structure of muscle fibre. Similarly, the GO term “ventricular septum development” was found to have 7.46-fold enrichment relative to

dogs. The septum is the muscular structure that separates the heart ventricles into left and right ventricular chambers. In mammals, a fully developed septum should separate the two ventricles completely. The septum also houses Purkinje fibres, specialised conducting fibres that relay electric signals from the pacemaker at the top of the heart to the ventricular wall to initiate heartbeats. Purkinje cell protein 2 homolog isoform X1 (PCP2) is among the genes showing positive selection. Septum development under selection could correlate to marine mammals' frequent need to go through bradycardia as they dive (Ponganis, 2015).

Myoglobin

The MB gene coding for myoglobin was not found to be under positive selection. It was filtered out during the pre-filtering process due to possible duplication or missing homologues in some candidate genomes. This could be seen as a deviation from my hypothesis, but MB is not expected to be under positive selection based on the physiological context outlined in the literature (Berenbrink, 2021). For myoglobin, marine mammal proteins have been documented as being functionally similar to those of other mammalian species except for their higher net surface charges (Berenbrink, 2021). The surface charge difference enables a higher concentration of myoglobin in muscle fibres rather than any changes in myoglobin structure, which is a significant adaptation for diving animals (Berenbrink, 2021). However, some studies have shown that myoglobin concentrations are not proportional to diving abilities (Ponganis, 2015). Therefore, the role of myoglobin in diving remains unclear, and it may differ depending on the species examined.

Lung Surfactant Protein

Although some genes mentioned in previous literature were indeed found to be under significant selection, they do not belong to any significantly enriched pathways. Therefore, these genes

were not highlighted in GO analysis outputs. One example is SFTPB (ω=1.0) coding for lung surfactant protein B. As discussed in the Introduction, lung surfactant protein B performs a vital role in enabling lung collapse during breath-hold dives (Blix, 2018), but that is the extent of its function. Lung surfactant protein does not participate in any biological pathways, so this gene escapes GO analyses, resulting in an apparent deviation from the hypothesis.

Reflections on Deviations from Hypotheses

Several factors may have led to the apparent deviations of the results from this study's hypothesised selection of biological pathways. Some genes might be missing from the analysis because they are missing from the genome (deletion) or were absent in the sequencing data (sequencing error). Other genes might be fragmented or duplicated in some of the genomes, and thus, they were excluded during the prefiltering process. When using BUSCO to isolate protein-coding regions of genomes, the lineage file used as benchmarking datasets of single-copy orthologs was chosen as eutheria_odb10. Eutheria, or placental mammals, is the most specific clade that includes all candidate species examined here. However, the lineage file includes only 11366 genes, which may not reflect the average number of genes typical mammalian genomes possess. Since the number of shared genes among all placental mammals is expected to be relatively small, this could lead to genes relevant to diving evading detection due to not being included in the analysis. Since conserved genes are limited in this case, extending the analysis with a de novo orthologue discovery pipeline could be a reasonable way to capture more genes.

Additionally, the prefiltering approach requires all 25 genomes to possess exactly one copy of the gene for said gene to be retained. That means even if one species loses the gene, it will have to be excluded. This could have resulted in some genes being filtered out. Likewise, if the

adaptation is the loss of certain genes, the current method cannot detect this adaptation. It might be of interest to conduct a similar analysis while excluding Sirenians since they are the most phylogenetically distance group and do not contribute much to the foreground branches. Their inclusion might have let more genes be filtered out this way.

Similarly, the research question involved comparing only marine rather than terrestrial and marine species. Some genes previously highlighted in literature may have been found when comparing marine mammals with land mammals; thus, they are shown to be under significant selection among marine mammals in general but not significant when compared among deep and shallow-diving species. Lastly, the reference gene list selected for GO analyses could have contributed to which pathways were highlighted. Of the three reference species selected for this study, humans have the most annotations, yet the analysis using dogs as a reference reported the highest number of significantly enriched pathways. This could be due to the fact that dogs were subject to intense artificial breeding. Dog gene pool, then, displays traits similar to populations that have experienced inbreeding depressions, with many genes altered or lost. Reference gene lists for dogs may have relatively low levels of enrichment overall and, therefore, tend to highlight more pathways.

Circulatory System

Consistent with the hypothesis, the majority of relevant enriched pathways were involved in the circulatory systems. Since the selected marine mammal species tend to take longer and deeper dives, these mechanisms are possibly involved in combating ischemia/reperfusion injury and are under stronger selection than those who take shallower or shorter dives. The “platelet-derived growth factor-beta signalling” pathway, one of the most enriched pathways (72.12 fold enrichment to humans and 65.16 fold enrichment to dogs), is responsible for

postinfarction (after a period when blood supply is blocked) repair of blood vessels and is also known to be responsible for the development of important cardiovascular structures such as the coronary artery (Chen et al., 2014; Mellgren et al.,2008). Notable genes involved in this pathway are huntingtin interacting protein 1 (HIP1), huntingtin interacting protein 1 receptor (HIP1R) and proto-oncogene tyrosine-protein kinase src (SRC). SRC regulates vascular permeability after ischemic reperfusion (Zan et al., 2013), while HIP1 is also involved in endocytosis (Metzler et al., 2001). Deeper-diving marine mammals are exposed to prolonged periods of hypoxia per dive, which could lead to a more extended infarction period across more peripheral tissues. Indeed, an overall reduction in blubber haemoglobin concentration and saturation was observed in harbour seals during voluntary dives within the aerobic diving limit (McKnight et al., 2019), suggesting that they may experience reduced peripheral perfusion during routine dives. Therefore, deep-diving species may experience an infarction-like condition in their peripheral tissues compared to their shallow-diving counterparts. Thus, high enrichment may have led to adaptations in the signalling system responsible for post-infarction repair.

Hematopoiesis

Similar arguments can be made about the apparent selection of hematopoiesis, which is the production of the different cell types associated with blood, including white blood cells, red blood cells, and platelets. Myeloid cells, the precursor to all types of blood cells, are the centrepiece of hematopoiesis. Various stages of myeloid cell development and homeostasis, as well as their regulatory processes, are found to be highly enriched for deep-diving species (see Table 3). Notable genes that participate in myeloid cell homeostasis include family with sequence similarity 210 member B (FAM210B), 5’-aminolevulinate synthase 2 (ALAS2) and SH2B adaptor protein 3 (SH2B3). FAM210B is involved in positive regulation of red blood cell differentiation (Suzuki et al., 2013). ALAS2 encodes a product that catalyses the first step of the biosynthetic

pathway of heme, the functional unit in haemoglobin (Cox et al., 2004). SH2B codes for a protein that is a critical regulator of hematopoiesis (Oh et al., 2010). This result is in agreement with the literature as marine mammals tend to have a relatively high concentration of blood cells (Ponganis, 2015) even when the structure of haemoglobin itself varies widely across all species (Lenfat, 1969; Lenfant *et al.*, 1970; Meir *et al.*, 2009; Qvist *et al.*, 1981).

In addition, the functions of negative regulation of plasminogen activation (43.27 fold enrichment to dogs) and regulation of platelet activation (10.02 fold enrichment to dogs) were enriched. Plasminogen is part of the enzyme cascade that results in the formation of blood clots while platelets activate this cascade, so enrichment of these two pathways suggests the blood clotting process is also under significant selective pressure. This can be related to two points relevant to diving physiology. First, tight regulation of blood clotting may assist in reducing blood viscosity during periods of slow blood flow, which was proposed to lower the workload of cardiac tissues in the event of shunting (Ponganis, 2015). Second, it has been proposed that circulating nitrogen gas bubbles formed as the animal experiences rapid pressure changes might induce activation of the clotting enzyme cascade, which could lead to the formation of free-floating fibrin clots and cause an embolus (i.e. blockage of blood vessels). This is one of the classic symptoms of decompression sickness (Philp, 1974; Philp et al., 1972, 1979; Boussuges et al., 1998). This result from the GO analysis may support this hypothesis since high enrichment on negative regulation of plasminogen activation can inhibit this process and avoid the adverse effects of decompression sickness. Particularly, serpin family member 2 (SERPINF2), which codes for an inhibitor of plasmin (the activated form of plasminogen), is found to be under positive selection (Moroi & Aoki, 1976). This suggests that deep-diving marine mammal species may possess convergent adaptation of pressure tolerance.

Organismal Response to Stress

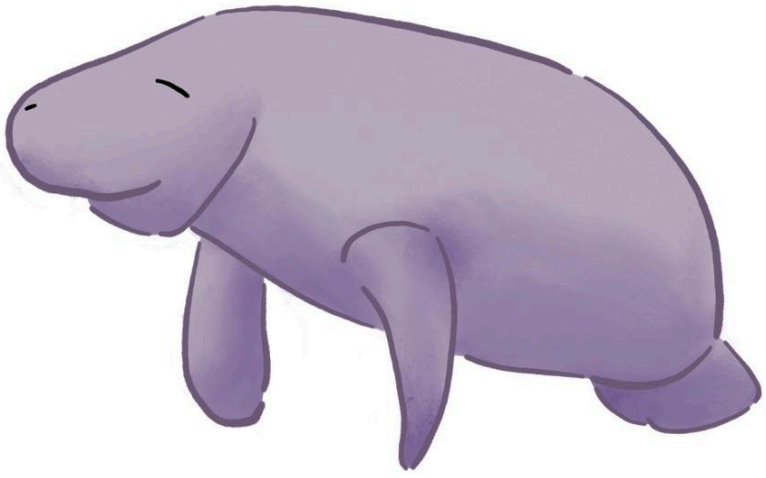
The function of multicellular organismal response to stress also has enrichment (5.92 fold enrichment to humans). This was not expected in the hypotheses but could also be an adaptation for pressure tolerance since the preventative mechanisms of blood clotting regulation and postinfarction repair described above are known to be prone to failing when the animal is stressed or disturbed. Marine mammals were not believed even to be susceptible to decompression sickness until gas bubbles were observed in stranded or otherwise stressed individuals (Jepson et al., 2003; Fernández et al., 2005; Moore et al., 2009; Van Bonn et al., 2011, 2013; García-Párraga et al., 2014, 2018; Fahlman et al., 2017; Fernández et al., 2017; Bernaldo de Quirós, Y. et al., 2019; Parga et al., 2020).

Immune Response

Lastly, the immune system also appears under strong positive selection, which was not expected in the hypotheses. This includes processes such as proliferation and apoptosis of T cells and monocyte homeostasis. It is possible that, collectively, these pathways lead to a faster onset of acquired immunity so that inflammatory response is kept at a minimum. Inflammatory response is an integral part of innate immunity. Normally, it is the body's initial response to any potentially harmful foreign stimuli while paving the way for more efficient and targeted acquired immunity at the same time (Ferrero-Miliani et al., 2007). However, chronic, uncontrolled inflammation can lead to chronic damage, a disease in its own right (Ferrero-Miliani et al., 2007). In the case of marine mammals, inflammation is known to be triggered when tissues undergo periods of hypoxia and upon reperfusion (Chambers et al., 1985; McCord, 1985; Granger, 1988).

For this reason, cellular response to hydrogen peroxide is also under significant selection since hydrogen peroxide is one of the powerful oxidants that tend to be produced from the tissue enzymatic systems upon reperfusion of oxidated blood and subsequently lead to oxidative damage to tissues. Furthermore, gas bubble formation can initiate an acute, almost constant inflammatory response as part of decompression sickness (Bove, 1982). Therefore, it is perhaps advantageous for marine mammals to mitigate the expression of the inflammatory response to assist in preventing barotrauma while compensating the immune system function by selecting for more efficient acquired immunity.

This genomic survey has revealed convergence in adaptations of the immunological and haematological response of deep-diving marine mammal species potentially related to hypoxia and barotrauma tolerance. In particular, the results of this study have provided some promising explanations of the working theories on how marine mammals avoid decompression sickness in addition to speculations based on limited clinical observations.



West Indian manatee (*Trichechus manatus*)

Chapter 5. Conclusion

There are several assumptions or limitations of this research methodology that deserve consideration. The most important limitation of the research question itself is the sheer diversity of diving behaviours and marine mammals. As outlined in the Introduction, many other mammals and reptiles dive routinely, but due to those species being very dispersed on the phylogenetic trees, including those species may complicate the interpretation of the analysis results. Therefore, this study is limited to marine mammals instead. Even so, there is great diversity in diving patterns among marine mammals. Simply defining “shallow divers” and “deep divers” for the sake of this study can be controversial, as there are hardly any universal standards when multiple lineages are considered. Based on behavioural data compiled by Ponganis (2015), deep-diving and shallow-diving species can be separated into two distinct groups for each taxon (e.g. deep-diving seals and shallow-diving seals). If one plots diving depth or time over the number of species with a distribution curve for either pinnipeds or cetaceans, it would present a continuous curve with two distinct peaks. However, the exact cutoff line between shallow and deep diving species differs by lineage. What can be considered a shallow dive for pinnipeds may not be shallow for cetaceans, so the assignment is more of a relative concept than an absolute standard. Since the selection pressures for deep-diving pinnipeds and deep-diving cetaceans may not be identical, this may have caused shared substitutions to be challenging to find in gene alignments, with interspecific differences often overshadowing functional substitutions, an issue with the cross-species comparison protocol that Weitzner et al. (2020) raised.

Fortunately, most species selected for this study had the most complete genome available with at least scaffold-level completeness. Other studies have utilised genomes of similar completeness levels, so this study is on par with field standards. It is unfortunate that three

species had to be excluded since their genome completeness was so low. Cuvier's beaked whale, for example, is known for exceeding its aerobic diving limits by utilising lactate as its energy source in addition to glucose (Tyack et al., 2006). They were selected for potentially interesting implications in extreme physiological diving adaptation, but their genome was excluded due to low completeness.

Another critical problem with this bioinformatic analysis protocol lies in the awkward programmed logic of codon-aware genomic analysis tools used in this study. To ensure accurate results of the phylogenetic tree construction and positive selection tests, both PRANK and PAML are used since they operate with codon information rather than treating genes as simple strings of bases. This system, however, means they are hard-wired to consider any gene sequences not in multiples of 3 as invalid sequences even if they were extracted correctly by BUSCO. Without manual editing of many sequences, this may have led to the loss of genes from the analysis. Both PRANK and PAML are highly robust programs that have withstood the test of time. This flaw is hardly reported in published literature. However, the fact that many groups dismiss it as merely a minor inconvenience is potentially why it is under-reported despite the abovementioned shortcomings. Therefore, it is worth calling attention to updating this programming logic in light of this and many other similar studies.

The limitations of GO analysis also constitute a source of bias. The reference lists for this study were picked based on the closest available relatives of the study lineages (dog for pinnipeds and cow for cetaceans). However, no reference related to sirenians was used because no members of the Afrotheria superorder had their reference gene list available in PANTHER. While neither sirenian species was a deep diver in the context of this study, it was still a biased representation.

Even the selected reference annotations could use more scrutiny. Most of the vertebrate annotations come from either humans or model organisms that are domesticated. The domestication process involves intense artificial selection that results in preservations of alleles that otherwise would not persist in natural populations, as in the case of domestic dogs bred for debilitating conditions for breed traits. Their versions of the genes, then, may not reflect the healthiest versions of the sequence. Therefore, it is a good idea to establish reference annotations with wild species such as grey wolves. The functions listed in these annotations may also inaccurately reflect the actual role of their products when derived from clinical records of those reference species. This discrepancy may lead to misinterpretation of the role of those genes when put into the physiological context of wildlife species. Though not available in this study, methods allowing the duplicated sequences to be assessed may alleviate this problem somewhat because analyses of duplicated versions of the same gene possessed by the same genome may allow divergence of their products to become more readily observable.

As mentioned in the Introduction, using genomics to assess molecular adaptations carries inherent controversies. The strengths of this study lie in part in comparing multiple major marine mammal lineages at once. This also introduces many confounding variables by default because the study species from three distinct, not closely related lineages may have very different life histories and selection forces may act on the same molecular functions for different reasons. Likewise, any evidence of selection detected in this study had to be interpreted based on the current knowledge of marine mammal physiology, which is limited at the current stage. Some biological pathways highlighted by GO analysis that are apparently irrelevant to the research question may very well turn out to be relevant as clinical understanding improves. There is little to be done based on these results alone if they are not followed up by animal clinical trials, but that is beyond the scope of this study.

Another limitation of this methodology is that it only concerns the genome's protein-coding regions. Weitzner et al. (2020) pointed out that genomics investigations using cross-species comparisons have many shortcomings, such as limited high-quality annotations and difficulty parsing phenotype-specific signals from noise. Weitzner *et al.* (2020) suggested that studying single-species regulative pathways of pinniped species as the terrestrial pup transitions to the adult amphibious lifestyle is more appropriate for studying this topic. Indeed, the level of gene expression and changes in genome structures can influence evolution just as much. A survey of small noncoding RNA across four divergent anoxia tolerant (Riggs et al., 2018) and high-altitude species (Hao et al., 2019) yielded few cases of conserved expression. These two studies also highlight the inherent problem of inter-specific variations acting as confounding variables to identify convergence among non-coding homologues in a comparative genomic approach. Chikina et al. (2016) point out that the similarity of pathways, rather than exact gene changes, defines convergence. This kind of similarity, however, is not detectable within the limited scope of this study.

Nevertheless, this study managed a bold attempt at covering a more comprehensive array of marine mammal species compared to genomic surveys of similar scale on this topic and identified more genes under positive selection as well (Yuan et al., 2021; Foote *et al.*, 2015; Chikina et al., 2016). This study is a step forward in guiding future physiology studies, particularly on marine mammals' barotrauma tolerance. Similar topics previously received very little research, and most conclusions were largely speculative based on limited field observations. Having genomic evidence may spur further controlled studies on this topic.

Appendix 1 – Extract and Tabulate Statistics for All BUSCO Groups

```
#!/usr/bin/env python
# written by Philipp Resl
import os
import sys
import argparse
if sys.version_info[0] < 3:
   raise Exception("Must be using Python 3")
pars = argparse.ArgumentParser(prog="extract_busco_table.py", description = """This script will get all busco3 hmms and look in the busco results all specified genomes for the presence of the files""", epilog = """written by Philipp Resl""")
pars.add_argument('--hmm', dest="hmm_dir", required=True, help="Directory of the BUSCO hmms.")
pars.add_argument('--busco_results', dest="busco_results", required=True, help="Results directory containing all BUSCO runs.")
pars.add_argument('-o', dest="out", required=True, help="BUSCO table output file.")
args=pars.parse_args()
hmms = os.listdir(args.hmm_dir)
hmms = [hmm.strip(".hmm") for hmm in hmms]
genomes = os.listdir(args.busco_results)
outfile = open(args.out, "w")
header = "species\t"
header += "\t".join(hmms)
header += "\tpercent_complete"
print(header, file= outfile)
for species in genomes:
	ones = 0
	zeros = 0
	outstring = species
	print("Extracting HMMs for", species, file=sys.stderr)
	try:
		busco_listing_file = open(args.busco_results + species + "/run_busco/single_copy_busco_sequences.txt", "r")
		buscos = []
		for line in busco_listing_file:
			line = line.strip()
			line = line.split(" ")[-1]
			line = line.split("/")[-1]
			if ".faa" in line: # only take aa files, this should be enough
				buscos.append(line)
		buscos = [busco.strip(".faa") for busco in buscos]
		busco_listing_file.close()
		for hmm in hmms:
			if hmm in buscos:
				outstring += "\t"
				outstring += "1"
				ones +=1
			else:
				outstring += "\t"
				outstring += "0"
				zeros +=1
		percent = ones / (ones+zeros)
		outstring += "\t"
```

```
            outstring += str(percent)
            print(outstring, file=outfile)
        except:
            out = species + " not found. Skipped.\n"
            print(out, file=sys.stderr)
            continue
            outfile.close()

#run by the following command:
python3 extract_busco_table.py \
--hmm /pathway/to/hmm/files \
--busco_results pathway/to/busco/results/ \
--o busco_table.tsv#
```

Appendix 2 – Prefiltering and Generating Sequence Files

```
#!/usr/bin/env python
# written by Philipp Resl, modified by Rodger Wang
import os
import sys
import pandas as pd
from Bio import SeqIO
import argparse
import tarfile
from io import StringIO
from io import TextIOWrapper
if sys.version_info[0] < 3:
    raise Exception("Must be using Python 3")
pars = argparse.ArgumentParser(prog="create_sequence_files.py", description = """This script will create fasta files for all the buscos from all species with >% of buscos present""", epilog = """written by Philipp Resl""")
pars.add_argument('--busco_table', dest="busco_table", required=True, help="Path to BUSCO table.")
pars.add_argument('--busco_results', dest="busco_results", required=True, help="Results directory containing all BUSCO runs.")
pars.add_argument('--cutoff', dest="cutoff", default=0, required=True, help="Percent cutoff %% for BUSCO presence. Species below this threshold will be excluded.")
pars.add_argument('--outdir', dest="outdir", required=True, help="Path to output directory.")
pars.add_argument('--minsp', dest="minsp", required=True, help="Minimum number of species which have to be present to keep the sequences.")
pars.add_argument('--type' , dest="type", required=True, help="Type of sequences (aa or nu).")
pars.add_argument('--genome_statistics' , dest="genome_stats", required=True, help="Path to genome statistics output file.")
pars.add_argument('--gene_statistics' , dest="gene_stats", required=True, help="Path to gene statistics output file.")
pars.add_argument('--batchID' , dest="batchid", default=1, type=int, help="Batch ID (start for subsampling)")
pars.add_argument('--nbatches', dest="nbatches", default=1, type=int, help="Total number of batches (step size for subsampling)")
args=pars.parse_args()
extension=""
if args.type == "nu":
        extension = ".fna"
else:
        extension = ".faa"
busco_overview = pd.read_csv(args.busco_table, sep="\t")
genomes = os.listdir(args.busco_results)
#print(busco_overview)
print("Settings:")
print("cutoff: ", args.cutoff)
print("minsp: ", args.minsp)
print("type: ", args.type)
print("outdir: ", args.outdir)
print("batchID: %i / %i" %(args.batchid, args.nbatches))
species_list = busco_overview.species.tolist()
print("Original number of species:", len(species_list))
#print(species_list)
#first remove species with too low busco coverage
busco_overview = busco_overview.set_index("species")
```

```
genome_file = open(args.genome_stats, "w")
for sp in species_list:
	if busco_overview.loc[sp, "percent_complete"] < float(args.cutoff):
		out = sp + "\tFAILED" + "\t" + str(busco_overview.loc[sp, "percent_complete"]) + "\t" + str(float(args.cutoff))
		print(out, file=genome_file)
		busco_overview = busco_overview.drop([sp])
	else:
		out = sp + "\tOK" + "\t" + str(busco_overview.loc[sp, "percent_complete"]) + "\t" + str(float(args.cutoff))
		print(out, file=genome_file)
species_list =  list(busco_overview.index)
print("Species remaining after applying cutoff:", len(species_list))
genome_file.close()
#now loop through each busco and extract sequence for each species
buscos = list(busco_overview.columns.values)
buscos.remove("percent_complete")
target=int(args.batchid)
gene_file = open(args.gene_stats, "w").close()
species_file_lists = {}
for species in species_list:
		tar_file_content = open(args.busco_results + "/" + species + "/run_busco/single_copy_busco_sequences.txt", "r")
		#file_list = pd.read_csv(args.busco_results + "/" + species + "/run_busco/single_copy_busco_sequences.txt",
sep=" ").iloc[:, -1].tolist()
		file_list = []
		for line in tar_file_content:
			line = line.strip()
			a_filename = line.split(" ")[-1]
			file_list.append(a_filename)
		#print(file_list)
		species_file_lists[species] = file_list
#print(species_file_lists)
for i in range(len(buscos)):
	j = i+1
#	 print("i: %i; j: %i; target: %i" %(i,j, target))
	if j != target:
#		 print("Don't do anything (i: %i)" %i)
		continue
	gene_file = open(args.gene_stats, "a")
	target+=args.nbatches
	busco = buscos[i]
	print("Processing: " + busco)
	numseqs = 0
	outstring = ""
	for species in species_list:
		#tar_file_content = open(args.busco_results + "/orthology/busco/busco_runs/" + species +
"/run_busco/single_copy_busco_sequences.txt", "r")
		#file_list = []
		#for line in tar_file_content:
		#	line = line.strip()
		#	a_filename = line.split(" ")[-1]
		#	file_list.append(a_filename)
		#print(file_list)
		"""
		for line in tar_file_content:
			line = line.strip()
			if ' '+busco+extension in line:
				path_to_busco_file = line.split(" ")[-1]
```

```
                        tf = tarfile.open(args.busco_results + "/orthology/busco/busco_runs/" + species +
"/run_busco/single_copy_busco_sequences.tar", "r")
                        #print(path_to_busco_file)
                        #print(tf.extractfile(path_to_busco_file))
                        tar_file_content = TextIOWrapper(tf.extractfile(path_to_busco_file))
                        if tar_file_content:
                              with TextIOWrapper(tf.extractfile(path_to_busco_file)) as handle:
                                    for seq_record in SeqIO.parse(handle, "fasta"):
                                          name = ">" +species+"\n"
                                          #outfile.write(name)
                                          #outfile.write(str(seq_record.seq)+"\n")
                                          outstring = outstring + name
                                          outstring = outstring + str(seq_record.seq) + "\n"
                        else:
                              print(busco, "not found for", species)
                              continue
                  """
            filename = busco+extension
            if filename in species_file_lists[species]:
                  #tf = tarfile.open(args.busco_results + "/orthology/busco/busco_runs/" + species +
"/run_busco/single_copy_busco_sequences.tar", "r")
                  #tar_file_content = TextIOWrapper(tf.extractfile(filename))
                  #if tar_file_content:
                  #       with TextIOWrapper(tf.extractfile(filename)) as handle:
                  #             for seq_record in SeqIO.parse(handle, "fasta"):
                  #                   name = ">" +species+"\n"
                  #                   outstring = outstring + name
                  #                   outstring = outstring + str(seq_record.seq) + "\n"
                  #else:
                  #       print(busco, "not found for", species)
                  file_path = os.path.join(args.busco_results + "/", species,
"run_eutheria_odb10/busco_sequences/single_copy_busco_sequences", filename)
                  if os.path.exists(file_path):
                        for seq_record in SeqIO.parse(file_path, "fasta"):
                              name = ">" +species+"\n"
                              outstring = outstring + name
                              outstring = outstring + str(seq_record.seq) + "\n"
                  else:
                        print(busco, "not found for", species)
      if outstring.count(">") >= int(args.minsp):     # only keep sequences if total number is larger than specified cutoff above.
            print(busco + "\t" + "OK" + "\t" + str(outstring.count(">")) +"\t" + str(int(args.minsp)), file=gene_file)
            outfile = open(args.outdir+"/"+busco+"_all.fas", "w")
            outfile.write(outstring)
            outfile.close()
      else:
            print(busco + "\t" + "FAILED" + "\t" + str(outstring.count(">")) +"\t" + str(int(args.minsp)), file=gene_file)
      gene_file.close()
#old working code when busco sequences are not tared.
"""
      for species in species_list:
            for genome in genomes: # this loop is to get the correct directory name, it is very unelegant
                  #print(args.busco_results+"/"+genome+"/run_busco/single_copy_busco_sequences/"+busco+extension)
                  if species == genome:
                        try:
                              seqfile = open(args.busco_results + "/" + genome + "/run_busco/single_copy_busco_sequences/"
+ busco + extension, "r")
                              for seq_record in SeqIO.parse(seqfile, "fasta"):
```

```
                                name = ">" +species+"\n"
                                #outfile.write(name)
                                #outfile.write(str(seq_record.seq)+"\n")
                                outstring = outstring + name
                                outstring = outstring + str(seq_record.seq) + "\n"
                            seqfile.close()
                        except: # skip missing buscos
                            continue
    if outstring.count(">") >= int(args.minsp):     # only keep sequences if total number is larger than specified cutoff above.
        print(busco + "\t" + "OK" + "\t" + str(outstring.count(">")) +"\t" + str(int(args.minsp)), file=gene_file)
        outfile = open(args.outdir+"/"+busco+"_all.fas", "w")
        outfile.write(outstring)
        outfile.close()
    else:
        print(busco + "\t" + "FAILED" + "\t" + str(outstring.count(">")) +"\t" + str(int(args.minsp)), file=gene_file)
gene_file.close()
"""
#run by the following command:
python3 create_sequence_files.py \
--busco_table busco_table.tsv \
--busco_results /pathway/to/results/files \
--cutoff 0.5 \
--outdir pathway/to/output/directory \
--minsp 25 \
--type aa \
--gene_statistics gene_stats.txt \
--genome_statistics genome_statistics.txt \
>logs/create_sequence_files.log#
```

Appendix 3a – Complete GO Analysis Report: Human

Analysis Type: PANTHER Overrepresentation Test (Released 20240226)
Annotation Version and Release Date: GO Ontology database DOI: 10.5281/zenodo.10536401 Released 2024-01-17
Analyzed List: upload_1 (Homo sapiens)
Reference List: Homo sapiens (all genes in database)
Test Type: FISHER
Correction: FDR

GO biological process complete	Homo sapiens - REFLIST (20592)	upload_1 (316)	upload_1 (expected)	upload_1 (over/under)	upload_1 (fold Enrichment)	upload_1 (raw P-value)	upload_1 (FDR)
positive regulation of platelet-derived growth factor receptor-beta signaling pathway (GO:2000588)	3	3	.05	+	65.16	3.58E-06	2.73E-02
operant conditioning (GO:0035106)	4	3	.06	+	48.87	1.42E-05	3.08E-02
positive regulation of platelet-derived growth factor receptor signaling pathway (GO:0010641)	5	3	.08	+	39.10	3.50E-05	3.14E-02
activation-induced cell death of T cells (GO:0006924)	6	3	.09	+	32.58	6.92E-05	4.22E-02
cellular response to low-density lipoprotein particle stimulus (GO:0071404)	30	5	.46	+	10.86	8.57E-05	4.51E-02
response to lipoprotein particle (GO:0055094)	30	5	.46	+	10.86	8.57E-05	4.36E-02
mammary gland morphogenesis (GO:0060443)	46	6	.71	+	8.50	6.96E-05	4.08E-02
aminoglycan biosynthetic process (GO:0006023)	69	8	1.06	+	7.56	1.04E-05	3.98E-02
myeloid cell development (GO:0061515)	78	8	1.20	+	6.68	2.60E-05	3.05E-02
leukocyte homeostasis (GO:0001776)	97	9	1.49	+	6.05	1.84E-05	3.12E-02
multicellular organismal response to stress (GO:0033555)	88	8	1.35	+	5.92	6.24E-05	4.32E-02
myeloid cell homeostasis (GO:0002262)	140	11	2.15	+	5.12	1.09E-05	3.33E-02
regulation of smooth muscle cell proliferation (GO:0048660)	139	10	2.13	+	4.69	5.84E-05	4.24E-02
response to UV (GO:0009411)	156	11	2.39	+	4.59	3.02E-05	3.07E-02
homeostasis of number of cells (GO:0048872)	282	19	4.33	+	4.39	8.30E-08	1.27E-03
myeloid cell differentiation (GO:0030099)	294	15	4.51	+	3.32	5.34E-05	4.28E-02
negative regulation of response to external stimulus (GO:0032102)	409	18	6.28	+	2.87	6.77E-05	4.49E-02
regulation of hemopoiesis (GO:1903706)	409	18	6.28	+	2.87	6.77E-05	4.30E-02
endocytosis (GO:0006897)	527	22	8.09	+	2.72	2.39E-05	3.03E-02
regulation of cell activation (GO:0050865)	623	26	9.56	+	2.72	4.32E-06	2.19E-02
regulation of cell-cell adhesion (GO:0022407)	487	20	7.47	+	2.68	7.03E-05	3.97E-02
multicellular organismal-level homeostasis (GO:0048871)	611	24	9.38	+	2.56	2.73E-05	2.98E-02
hemopoiesis (GO:0030097)	699	27	10.73	+	2.52	1.15E-05	2.92E-02
import into cell (GO:0098657)	715	27	10.97	+	2.46	1.72E-05	3.27E-02
response to abiotic stimulus (GO:0009628)	1112	35	17.06	+	2.05	7.16E-05	3.90E-02
homeostatic process (GO:0042592)	1430	42	21.94	+	1.91	4.77E-05	4.04E-02
regulation of immune system process (GO:0002682)	1540	45	23.63	+	1.90	3.18E-05	3.03E-02
regulation of apoptotic process (GO:0042981)	1476	43	22.65	+	1.90	5.82E-05	4.44E-02
response to oxygen-containing compound (GO:1901700)	1554	44	23.85	+	1.85	8.88E-05	4.37E-02
response to organic substance (GO:0010033)	2467	64	37.86	+	1.69	2.32E-05	3.21E-02
anatomical structure development (GO:0048856)	5231	114	80.27	+	1.42	2.15E-05	3.28E-02

Appendix 3b – Complete GO Analysis Report: Cow

Analysis Type: PANTHER Overrepresentation Test (Released 20240226)
Annotation Version and Release Date: GO Ontology database DOI: 10.5281/zenodo.10536401 Released 2024-01-17
Analyzed List: upload_1 (Bos taurus)
Reference List: Bos taurus (all genes in database)
Test Type: FISHER
Correction: FDR

GO biological process complete	Bos taurus - REFLIST (23838)	upload_1 (343)	upload_1 (expected)		upload_1 (over/under)	upload_1 (fold Enrichment)	upload_1 (raw P-value)	upload_1 (FDR)
positive regulation of CD4-positive, alpha-beta T cell proliferation (GO:2000563)	4	3	.06	+	52.12	1.17E-05	1.43E-02	
positive regulation of alpha-beta T cell proliferation (GO:0046641)	9	4	.13	+	30.89	5.01E-06	7.48E-03	
regulation of fertilization (GO:0080154)	11	4	.16	+	25.27	1.28E-05	1.44E-02	
myeloid cell homeostasis (GO:0002262)	86	8	1.24	+	6.46	3.39E-05	2.84E-02	
response to UV (GO:0009411)	100	9	1.44	+	6.25	1.43E-05	1.48E-02	
homeostasis of number of cells (GO:0048872)	178	12	2.56	+	4.69	1.11E-05	1.49E-02	
response to light stimulus (GO:0009416)	197	12	2.83	+	4.23	3.06E-05	2.74E-02	
carbohydrate metabolic process (GO:0005975)	413	18	5.94	+	3.03	3.45E-05	2.73E-02	
hemopoiesis (GO:0030097)	469	20	6.75	+	2.96	1.74E-05	1.67E-02	
regulation of cell activation (GO:0050865)	424	18	6.10	+	2.95	4.85E-05	3.43E-02	
intracellular signal transduction (GO:0035556)	1177	35	16.94	+	2.07	4.67E-05	3.49E-02	
positive regulation of metabolic process (GO:0009893)	2707	64	38.95	+	1.64	6.93E-05	4.43E-02	
metabolic process (GO:0008152)	7974	150	114.74	+	1.31	6.67E-05	4.48E-02	
sensory perception (GO:0007600)	1415	3	20.36	-	.15	2.59E-06	4.34E-03	
detection of stimulus involved in sensory perception (GO:0050906)	1120	1	16.12	-	.06	2.02E-06	3.87E-03	
sensory perception of chemical stimulus (GO:0007606)	1143	1	16.45	-	.06	1.36E-06	3.04E-03	
detection of stimulus (GO:0051606)	1193	1	17.17	-	.06	6.19E-07	1.66E-03	
detection of chemical stimulus involved in sensory perception of smell (GO:0050911)	1043	0	15.01	-	< 0.01	3.53E-07	1.58E-03	
detection of chemical stimulus involved in sensory perception (GO:0050907)	1077	0	15.50	-	< 0.01	2.42E-07	1.63E-03	
detection of chemical stimulus (GO:0009593)	1098	0	15.80	-	< 0.01	1.57E-07	2.11E-03	
sensory perception of smell (GO:0007608)	1058	0	15.22	-	< 0.01	3.83E-07	1.29E-03	

Appendix 3c – Complete GO Analysis Report: Dog

Analysis Type: PANTHER Overrepresentation Test (Released 20240226)
Annotation Version and Release Date: GO Ontology database DOI: 10.5281/zenodo.10536401 Released 2024-01-17
Analyzed List: upload_1 (Canis lupus familiaris)
Reference List: Canis lupus familiaris (all genes in database)
Test Type: FISHER
Correction: FDR

GO biological process complete	Canis lupus familiaris - REFLIST (20988)	upload_1 (291)	upload_1 (expected)	upload_1 (over/under)	upload_1 (fold Enrichment)	upload_1 (raw P-value)	upload_1 (FDR)
positive regulation of platelet-derived growth factor receptor-beta signaling pathway (GO:2000588)	3	3	.04	+	72.12	2.64E-06	5.98E-03
monocyte homeostasis (GO:0035702)	2	2	.03	+	72.12	1.92E-04	3.26E-02
negative regulation of plasminogen activation (GO:0010757)	5	3	.07	+	43.27	2.58E-05	1.35E-02
positive regulation of platelet-derived growth factor receptor signaling pathway (GO:0010641)	5	3	.07	+	43.27	2.58E-05	1.30E-02
regulation of platelet-derived growth factor receptor-beta signaling pathway (GO:2000586)	6	3	.08	+	36.06	5.12E-05	1.93E-02
poly-N-acetyllactosamine biosynthetic process (GO:0030311)	7	3	.10	+	30.91	8.86E-05	2.62E-02
poly-N-acetyllactosamine metabolic process (GO:0030309)	7	3	.10	+	30.91	8.86E-05	2.57E-02
regulation of plasminogen activation (GO:0010755)	10	4	.14	+	28.85	7.12E-06	9.69E-03
phosphatidylcholine acyl-chain remodeling (GO:0036151)	8	3	.11	+	27.05	1.40E-04	3.03E-02
regulation of fertilization (GO:0080154)	13	4	.18	+	22.19	2.35E-05	1.33E-02
cellular response to low-density lipoprotein particle stimulus (GO:0071404)	15	4	.21	+	19.23	4.38E-05	1.81E-02
response to lipoprotein particle (GO:0055094)	15	4	.21	+	19.23	4.38E-05	1.75E-02
cellular response to lipoprotein particle stimulus (GO:0071402)	17	4	.24	+	16.97	7.47E-05	2.42E-02
branching involved in mammary gland duct morphogenesis (GO:0060444)	18	4	.25	+	16.03	9.50E-05	2.64E-02
cellular response to hydrogen peroxide (GO:0070301)	33	5	.46	+	10.93	8.55E-05	2.58E-02
regulation of protein processing (GO:0070613)	40	6	.55	+	10.82	1.74E-05	1.19E-02
regulation of protein maturation (GO:1903317)	43	6	.60	+	10.06	2.67E-05	1.30E-02
regulation of platelet activation (GO:0010543)	36	5	.50	+	10.02	1.31E-04	3.08E-02
negative regulation of lipid biosynthetic process (GO:0051055)	39	5	.54	+	9.25	1.94E-04	3.18E-02
mammary gland morphogenesis (GO:0060443)	40	5	.55	+	9.02	2.19E-04	3.31E-02
response to hydrogen peroxide (GO:0042542)	43	5	.60	+	8.39	3.10E-04	4.17E-02
negative regulation of G2/M transition of mitotic cell cycle (GO:0010972)	54	6	.75	+	8.01	9.97E-05	2.66E-02
endothelial cell migration (GO:0043542)	55	6	.76	+	7.87	1.11E-04	2.89E-02
negative regulation of cell cycle G2/M phase transition (GO:1902750)	56	6	.78	+	7.73	1.22E-04	2.97E-02
ventricular septum development (GO:0003281)	58	6	.80	+	7.46	1.49E-04	3.07E-02
myeloid cell development (GO:0061515)	62	6	.86	+	6.98	2.16E-04	3.34E-02
leukocyte homeostasis (GO:0001776)	73	7	1.01	+	6.92	6.83E-05	2.27E-02
cellular response to reactive oxygen species (GO:0034614)	86	7	1.19	+	5.87	1.93E-04	3.21E-02
myeloid cell homeostasis (GO:0002262)	108	8	1.50	+	5.34	1.32E-04	3.04E-02
protein stabilization (GO:0050821)	132	9	1.83	+	4.92	9.58E-05	2.61E-02
response to UV (GO:0009411)	119	8	1.65	+	4.85	2.57E-04	3.73E-02
homeostasis of number of cells (GO:0048872)	218	14	3.02	+	4.63	2.33E-06	6.33E-03
regulation of lipid biosynthetic process (GO:0046890)	127	8	1.76	+	4.54	4.00E-04	4.95E-02
cellular response to oxidative stress (GO:0034599)	145	9	2.01	+	4.48	1.96E-04	3.10E-02
morphogenesis of a branching structure (GO:0001763)	152	9	2.11	+	4.27	2.79E-04	3.95E-02

steroid metabolic process (GO:0008202)	170	10	2.36	+	4.24	1.36E-04	3.03E-02			
myeloid cell differentiation (GO:0030099)	220	12	3.05	+	3.93	6.16E-05	2.20E-02			
alcohol metabolic process (GO:0006066)	211	11	2.93	+	3.76	1.85E-04	3.31E-02			
regulation of actin filament organization (GO:0110053)	212	11	2.94	+	3.74	1.92E-04	3.23E-02			
regulation of protein stability (GO:0031647)	215	11	2.98	+	3.69	2.17E-04	3.32E-02			
response to light stimulus (GO:0009416)	220	11	3.05	+	3.61	2.65E-04	3.80E-02			
mononuclear cell differentiation (GO:1903131)	246	12	3.41	+	3.52	1.77E-04	3.30E-02			
regulation of small molecule metabolic process (GO:0062012)	228	11	3.16	+	3.48	3.59E-04	4.61E-02			
cellular response to organic cyclic compound (GO:0071407)	261	12	3.62	+	3.32	3.04E-04	4.27E-02			
cellular response to lipid (GO:0071396)	284	13	3.94	+	3.30	1.80E-04	3.26E-02			
hemopoiesis (GO:0030097)	510	23	7.07	+	3.25	8.03E-07	2.73E-03			
regulation of cell activation (GO:0050865)	422	19	5.85	+	3.25	7.63E-06	8.66E-03			
regulation of cell-cell adhesion (GO:0022407)	336	15	4.66	+	3.22	7.68E-05	2.43E-02			
regulation of supramolecular fiber organization (GO:1902903)	294	13	4.08	+	3.19	2.52E-04	3.68E-02			
leukocyte differentiation (GO:0002521)	317	14	4.40	+	3.19	1.49E-04	3.11E-02			
response to radiation (GO:0009314)	300	13	4.16	+	3.13	3.06E-04	4.16E-02			
response to lipid (GO:0033993)	410	17	5.68	+	2.99	6.46E-05	2.25E-02			
small molecule biosynthetic process (GO:0044283)	348	14	4.83	+	2.90	3.85E-04	4.80E-02			
multicellular organismal-level homeostasis (GO:0048871)	448	18	6.21	+	2.90	5.92E-05	2.18E-02			
apoptotic process (GO:0006915)	603	24	8.36	+	2.87	4.07E-06	6.15E-03			
programmed cell death (GO:0012501)	638	25	8.85	+	2.83	3.35E-06	6.51E-03			
cell death (GO:0008219)	638	25	8.85	+	2.83	3.35E-06	5.69E-03			
regulation of leukocyte activation (GO:0002694)	389	15	5.39	+	2.78	3.76E-04	4.78E-02			
cellular response to cytokine stimulus (GO:0071345)	518	19	7.18	+	2.65	1.23E-04	2.94E-02			
response to cytokine (GO:0034097)	581	21	8.06	+	2.61	6.69E-05	2.27E-02			
tube morphogenesis (GO:0035239)	499	18	6.92	+	2.60	2.27E-04	3.39E-02			
regulation of cell adhesion (GO:0030155)	558	20	7.74	+	2.59	1.12E-04	2.87E-02			
regulation of response to external stimulus (GO:0032101)	750	24	10.40	+	2.31	1.58E-04	3.07E-02			
regulation of phosphate metabolic process (GO:0019220)	695	22	9.64	+	2.28	3.49E-04	4.57E-02			
regulation of phosphorus metabolic process (GO:0051174)	695	22	9.64	+	2.28	3.49E-04	4.53E-02			
immune system process (GO:0002376)	1329	42	18.43	+	2.28	5.89E-07	4.01E-03			
regulation of immune system process (GO:0002682)	1088	33	15.09	+	2.19	2.42E-05	1.32E-02			
intracellular signal transduction (GO:0035556)	1163	35	16.13	+	2.17	2.32E-05	1.37E-02			
response to oxygen-containing compound (GO:1901700)	900	27	12.48	+	2.16	1.86E-04	3.24E-02			
regulation of apoptotic process (GO:0042981)	954	28	13.23	+	2.12	1.79E-04	3.29E-02			
homeostatic process (GO:0042592)	1070	31	14.84	+	2.09	1.21E-04	2.99E-02			
regulation of programmed cell death (GO:0043067)	990	28	13.73	+	2.04	3.80E-04	4.79E-02			
negative regulation of biosynthetic process (GO:0009890)	1531	43	21.23	+	2.03	1.07E-05	1.04E-02			
cellular response to organic substance (GO:0071310)	1178	33	16.33	+	2.02	1.47E-04	3.12E-02			
negative regulation of cellular biosynthetic process (GO:0031327)	1527	42	21.17	+	1.98	1.94E-05	1.20E-02			
negative regulation of cellular metabolic process (GO:0031324)	1822	48	25.26	+	1.90	1.37E-05	1.16E-02			
negative regulation of nitrogen compound metabolic process (GO:0051172)	1504	39	20.85	+	1.87	1.39E-04	3.05E-02			
response to organic substance (GO:0010033)	1588	41	22.02	+	1.86	1.18E-04	2.97E-02			
negative regulation of metabolic process (GO:0009892)	1984	51	27.51	+	1.85	1.70E-05	1.22E-02			
cell development (GO:0048468)	1650	42	22.88	+	1.84	1.55E-04	3.11E-02			
negative regulation of macromolecule biosynthetic process (GO:0010558)	1493	38	20.70	+	1.84	3.06E-04	4.20E-02			
cellular response to chemical stimulus (GO:0070887)	1658	42	22.99	+	1.83	1.61E-04	3.09E-02			
regulation of multicellular organismal process (GO:0051239)	2094	52	29.03	+	1.79	2.98E-05	1.35E-02			
negative regulation of macromolecule metabolic process (GO:0010605)	1841	44	25.53	+	1.72	3.46E-04	4.57E-02			
positive regulation of nitrogen compound metabolic process (GO:0051173)	2099	50	29.10	+	1.72	1.57E-04	3.09E-02			

positive regulation of metabolic process (GO:0009893)	2657	63	36.84	+	1.71	1.67E-05	1.26E-02				
anatomical structure development (GO:0048856)	3677	85	50.98	+	1.67	7.71E-07	3.50E-03				
positive regulation of macromolecule metabolic process (GO:0010604)	2425	56	33.62	+	1.67	1.32E-04	2.99E-02				
positive regulation of cellular metabolic process (GO:0031325)	2408	55	33.39	+	1.65	1.87E-04	3.23E-02				
system development (GO:0048731)	2452	55	34.00	+	1.62	3.04E-04	4.23E-02				
multicellular organism development (GO:0007275)	2786	62	38.63	+	1.61	1.61E-04	3.05E-02				
negative regulation of cellular process (GO:0048523)	3553	79	49.26	+	1.60	1.25E-05	1.13E-02				
positive regulation of biological process (GO:0048518)	4556	101	63.17	+	1.60	2.90E-07	3.94E-03				
negative regulation of biological process (GO:0048519)	3816	84	52.91	+	1.59	7.57E-06	9.37E-03				
developmental process (GO:0032502)	3970	86	55.04	+	1.56	1.05E-05	1.10E-02				
positive regulation of cellular process (GO:0048522)	4193	89	58.14	+	1.53	1.61E-05	1.29E-02				
regulation of primary metabolic process (GO:0080090)	4180	85	57.96	+	1.47	1.49E-04	3.03E-02				
regulation of nitrogen compound metabolic process (GO:0051171)	4064	82	56.35	+	1.46	2.38E-04	3.52E-02				
regulation of metabolic process (GO:0019222)	4965	100	68.84	+	1.45	3.73E-05	1.64E-02				
cellular component organization or biogenesis (GO:0071840)	4635	93	64.26	+	1.45	8.47E-05	2.62E-02				
cellular component organization (GO:0016043)	4429	88	61.41	+	1.43	2.13E-04	3.34E-02				
regulation of cellular metabolic process (GO:0031323)	4595	90	63.71	+	1.41	3.35E-04	4.47E-02				
multicellular organismal process (GO:0032501)	5090	99	70.57	+	1.40	1.86E-04	3.28E-02				
detection of stimulus involved in sensory perception (GO:0050906)	828	1	11.48	-	.09	1.95E-04	3.15E-02				
sensory perception of chemical stimulus (GO:0007606)	830	1	11.51	-	.09	1.96E-04	3.13E-02				
detection of stimulus (GO:0051606)	894	1	12.40	-	.08	9.43E-05	2.67E-02				
detection of chemical stimulus involved in sensory perception of smell (GO:0050911)	743	0	10.30	-	< 0.01	4.16E-05	1.77E-02				
detection of chemical stimulus involved in sensory perception (GO:0050907)	778	0	10.79	-	< 0.01	2.80E-05	1.32E-02				
detection of chemical stimulus (GO:0009593)	799	0	11.08	-	< 0.01	1.86E-05	1.21E-02				
sensory perception of smell (GO:0007608)	762	0	10.57	-	< 0.01	4.39E-05	1.71E-02				

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