REVIEW ARTICLE

The immune response and diving: conservation considerations for belugas *(Delphinapterus leucas)* in a changing Arctic environment

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Abstract

Diving is a critical behaviour of marine mammals, including belugas, which dive to forage and travel under Arctic sea ice. While the limitations of dive behaviour and physiological dive adaptations have been the focus of several studies, cellular adaptations, particularly those of the immune system, have been little considered. However, diving itself presents several challenges that can impact immune responses, leading to disease or injury. As beluga dive their behaviour changes in response to human activity or environmental shifts. It is necessary to better understand how the beluga's immune system functions during diving. This review provides a brief overview of what is known about beluga's diving behaviour and physiology and discusses the first efforts to understand the link between diving and health via immune function in belugas. This new area of research is an important consideration regarding potential sub-lethal impacts of a rapidly changing Arctic environment on beluga's diving behaviour, health and disease susceptibility.

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Keywords

Dive physiology; decompression sickness; marine mammal; cetacean; immunity; health

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Abbreviations

atm: atmospheres
CD11b/18: cluster of differentiation 11b/18
DCS: decompression sickness
Hb: haemoglobin
IL2R: interleukin 2 receptor
m/s: metres per second
N_2: nitrogen
O_2: oxygen
OWE: out of water examination
SD: standard deviation
SE: standard error

Introduction

With the Arctic region demonstrating greater sensitivity and faster warming compared to other global regions (Post et al. 2019), Arctic species are increasingly exposed to the impacts of anthropogenic activities, whether directly through increased shipping, noise and pollution and oil and gas exploration and mining, or indirectly through the effects of environmental/ecosystem shifts (Huntington 2009; Kovacs et al. 2011; Hauser et al. 2018). Such potential stressors can alter dive behaviour and, ultimately, health. Diving is essential in marine mammals, enabling foraging and travel (Martin et al. 1998), as well as helping in avoidance of potential threats such as boats (Constantine et al. 2004; Williams et al. 2017) and predators such as killer whales (Jefferson et al. 1991). While the importance of diving for foraging may be apparent, the relationship between diving and health via disease or injury in marine mammals is less clear. This paper is not intended to be an exhaustive review of dive behaviour and physiology; rather, it provides an overview and discussion of the first research to consider adaptation of the immune system for diving and the implications of a changing Arctic environment on health in the beluga (*Delphinapterus leucas)*.

Interest in marine mammal dive physiology appears to have had several boosts since the early observations of whalers. The latest resurgence in interest of dive physiology could perhaps be traced to reports of gas-bubble injury and DSC-like injury in several species of cetaceans in the early 2000s (Jepson et al. 2003; Fernandez et al. 2005; Jepson et al. 2005) and has been aided by the development and improvement of tagging technologies that improved our understanding of dive behaviour and physiological responses (e.g., Johnson & Tyack 2003; Cooke et al. 2004; Evans et al. 2013; McIntyre 2014; Williams et al. 2015).

Amongst the challenges associated with diving, issues of aerobic capacity and gas management have been the focus of a great deal of research that aims at helping us understand how marine mammals are able to make prolonged deep and repetitive dives without a direct source of O_2 (e.g., Scholander 1940; Kooyman 1973; Kooyman

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et al. 1981; Kooyman & Ponganis 1998; Noren & Williams 2000; Kanatous et al. 2002; Bostrom et al. 2008; MacDonald & Ponganis 2013). Such studies also seek to elucidate the circumstances under which DCS-like injury is possible by investigating gas dynamics and cardiovascular and pulmonary function to determine the potential for gas-bubble formation (Ridgway & Howard 1979; Hooker et al. 2012; Fahlman et al. 2014; Hodanbosi et al. 2016; Garcia Parraga et al. 2018).

Other health challenges that may be associated with diving include the effects of pressure changes, temperature changes and hypoxia/re-oxygenation on cellular processes and immune responses. For human divers, dive-related changes in immune function include cell aggregation and activation (Nyquist et al. 2004), increased occurrence of certain infections (Brenner et al. 1999) and inflammation, which also plays an important role in the development of injury from DCS (Ward et al. 1987; Nyquist et al. 2004; Barack & Katz 2005; Montcalm-Smith et al. 2007).

Beluga dive behaviour

The beluga is a mid-sized odontocete demonstrating variable dive behaviour both between and within individual populations. Belugas in the Beaufort and Chukchi seas dive several 100 m to over 900 m in off-shelf regions (Citta et al. 2013; Hauser et al. 2015); an adult male made the deepest recorded dive: 1160 m (Citta et al. 2013). The majority of dives are often much shallower, and near-shore and coastal populations may be limited in terms of dive depth by constraints of local bathymetry. For example, Hauser et al. (2015) reported that belugas on the shelf of the Beaufort Sea often dove to the bottom, which was not greater than 100 m depth, and dives of less than 10 m are common among belugas in the shallow Bristol Bay and Cook Inlet, Alaska (Goetz et al. 2012; Laidre et al. 2017). Male belugas off Svalbard dive to less than 5 m approximately 60% of the time, even in deep water (Vacquie-Garcia et al. 2019). However, six belugas tagged near Devon Island, Canada, spent only 20–39% of their time at a depth less than 5 m and had a maximum dive depth of 872 m (Heide-Jørgensen et al. 1998).

The longest reported dive duration for a beluga is 31.4 min (Vacquie-Garcia et al. 2019); average dive durations range from 1.1 to 18 min (Ridgway et al. 1984; Martin et al. 1998; Martin & Smith 1999; Kingsle et al. 2001; Martin et al. 2001; Goetz et al. 2012; Citta et al. 2013; Vacquie-Garcia et al. 2019). Deeper dives are usually longer in duration (Martin & Smith 1999; Citta et al. 2013) but may have shorter bottom times or time spent foraging (Martin & Smith 1999). Increased bottom time and, thus, maximal foraging opportunity may be achieved through 'square' dives, which have been reported to be the most common dive type (Martin et al. 1998). Other dive shapes may be indicative of the function of each dive, such as drifting, travelling or exploring (Lefebvre et al. 2018). The occurrence of what is described as 'interrupted square dives' supports the idea that planned dives can be altered and highlights the need to understand the resilience of physiological and immune responses to such events.

Overall, the speed of descent and ascent appears to average around 2 m/s or less (Table 1), with variability based on dive type (Lefebvre et al. 2018). Deeper, longer-lasting dives require a greater vertical distance travelled and result in increased swim speed during descent/ascent (Martin & Smith 1999). A progressive slowing was reported on decompression following long dives, as animals approached the surface (Martin &

Table 1 Summary of reported vertical swim speeds during diving in belugas.

Reference	Descent rate (m/s)	Ascent rate (m/s)
Ridgway et al. 1984[a]	2.27 ± 0.19 (M) 1.99 ±1 .47 (F)	2.25 ± 0.21 (M) 2.29 ± 0.26 (F)
Martin & Smith 1992[b]	1.43 to 2.2	1.23 to 1.84
Shaffer et al. 1997[b]	<2	2.2 to 3
Martin et al. 1998[c]	0.5 ± 0.24 to 1.7 ± 0.02	0.45 ± 0.12 to 1.76 ± 0.06
Martin & Smith 1999[b]	1.5 to1.85	1.37 to 1.97
Martin et al. 2001[d]	1.42 (1.1 to 1.7)	1.73 (1.7 to 1.8)
Lefebvre et al. 2018 [b]	0.15 to 0.87	0.22 to 0.63
	Vertical speed (m/s)	
Heide Jørgensen et al. 1998[b]	0.5 to >2	
Hauser et al. 2015[b]	<2	
Kingsle et al. 2001[e]	1.99 (SE 0.025) –2.19 (SE 0.028)	

[a]Reported as mean ± SD. [b]Reported as range. [c]Reported as mean ± 2 SD. [d]Mean (range). [e]Means and SE.

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Smith 1992), and a series of shallow 'recovery' dives have also been reported following prolonged deep dives (Richard et al. 1997). These behaviours may help recovery from dives that reach or exceed the ADL or help regulate muscle washout of N_2, though there is little evidence to support this (Tyack et al. 2006). Where deep dives do occur, they have been linked to foraging (Martin & Smith 1992; Citta et al. 2013; Hauser 2016; Stafford et al. 2016) and movement under ice cover (e.g., Suydam et al. 2005), so they vary by season and in connection with shifting distribution of prey species (Watt et al. 2016), with more deep and prolonged dives occurring in the winter (Heide-Jørgensen et al. 1998; Watt et al. 2016).

Recent studies suggest that deep dives occur more often in some populations in response to shifts in the vertical distribution of prey, driven by ocean warming and shifting currents (Stafford et al. 2016; Watt et al. 2016; Hauser et al. 2018); there is also a suggestion that dives exceeding 20 min in duration are becoming more frequent (Hauser 2016). Surface behaviour of belugas can be interrupted by human presence, as illustrated by animals in Bristol Bay observed to end milling or feeding behaviours at the water surface and submerge for longer periods of time in response to approaching boats and in some cases swimming at increased speeds as a potential avoidance behaviour (L. Thompson, pers. obs. 2014, 2016). Belugas may also show extreme avoidance of large ships, such as ice breakers, though a lot of variability is noted in the response of these animals to human activities (Richardson & Wursig 1997).

While belugas have been shown to vary their dive behaviour, these forced changes in dive activity in response to anthropogenic stressors or environmental changes may have unforeseen health implications. It is possible that occasional dives to 1000 m are not a problem for belugas but increasing the necessity for such deep dives may push an animal past its physiological limits. Ridgway et al. (1984) noted that the deepest dives of 600 m appeared challenging for the adult female in their study. This was a captive managed individual maintained in sea-pens in San Diego Bay. San Diego Bay is significantly shallower than 600 m, with a maximum depth of about 18–20 m, which may have limited this individual's previous experience with deep dives. Physiological responses may then be related to the dive history or experience of an individual animal and will therefore vary in wild populations: if generally shallow-diving populations need to make increasingly deeper foraging dives, they may struggle more than those with a history of diving deeper. However, before we can evaluate the impact of altered dive responses, it is first necessary to determine how the immune system functions during normal dive behaviours.

General dive adaptation of marine mammals

To dive, belugas and other marine mammals have developed physiological, anatomical and behavioural adaptations to combat the issues of O_2 availability and pressure, as has been reviewed elsewhere (e.g., Kooyman et al. 1981; Kooyman & Ponganis 1998; Ponganis 2015). A markedly pronounced dive response, including vagally mediated bradycardia and sympathetically controlled peripheral vasoconstriction, reduces cardiac output and blood flow to peripheral tissues, conserving blood O_2 for critical tissues (Kooyman et al. 1981). Initially termed the dive reflex, it is now known that this response can occur on a gradient, based on dive characteristics such as a forced submersion versus free diving (Kooyman & Campbell 1972), depth or duration (Hindle et al. 2010; McDonald et al. 2018) and activity levels (Davis & Williams 2012; Noren et al. 2012; McDonald et al. 2018), and can even be cognitively controlled (Ridgway et al. 1975; Elmegaard et al. 2016). Increased O_2 stores, including circulating red blood cells and haemoglobin per cell, and myoglobin in muscle (Kooyman et al. 1981; Ponganis &Williams 2015), reduce metabolic activity, possibly indicated by decreased body temperature (e.g., Weddell seals [Kooyman et al. 1980]), and balancing stroke and gliding behaviours (Williams et al. 2000) also aid in prolonging the duration of aerobic dives. Increased capacity for anaerobic metabolism also has been reported for some species (Elsner et al. 1998).

Marine mammals possess collapsible lungs, which, along with the dive response, also protect against DCS, due to reduction of gas exchange in the lungs as well as tissue perfusion (Kooyman & Ponganis 1998). Recent studies suggest that N_2 levels may reach supersaturation under certain conditions (Houser et al. 2001; Zimmer & Tyack 2007) and more questions have arisen about how marine mammals balance the physiological adjustments of the dive response, exercise response and escape response. Furthermore, species differences in dive behaviour and physiology have become a focus of discussion, as shallow- and deep-diving species and even ecotypes may display variable responses (e.g., Hindle et al. 2010; Cantu-Medellin et al. 2011). Our understanding of dive responses, and how marine mammals avoid dive-related injury and disease, remains incomplete.

Beluga dive physiology

Studies specifically focused on beluga dive physiology are relatively few but make use of access to belugas both in professional human care or tissues collected post mortem from subsistence hunts through collaboration with indigenous communities. As early as 1974, greater blood O_2 capacity was reported in belugas compared to other cetaceans, such as the killer whale (Dhindsa et al. 1974). Open-water studies conducted by the US Navy Marine Mammal Program appear to confirm high haemoglobin concentration and haematocrit, which would increase O_2 storage capacity, allowing longer aerobic dives (Ridgway et al. 1984). Table 2 summarizes several haematological parameters that aid in O_2 storage and therefore in aerobic dive ability in belugas as compared with humans and the bottlenose dolphin (*Tursiops truncatus*), generally considered a shallow-diving species; the table also shows the similarity between values in belugas and the deep-diving northern elephant seal (*Mirounga angustriostris*). However, despite belugas' increased blood O_2 stores, Hedrick & Duffield (1991) report limited O_2 transport and conclude that belugas may be limited in their activity level (speed) during a dive. Belugas also possess increased capacity for muscle O_2 storage (myoglobin), muscle buffering capacity, red blood cell counts, haemoglobin and haematocrit, which have been reported to reach mature levels prior to weaning in belugas, which is faster than observed in other marine mammals (Noren & Suydam 2016; Noren et al. 2018; Choy et al. 2019).

The aerobic dive limit for belugas was estimated to be 9–10 min based on post-dive lactate measurements during an open-water study and estimates of body O_2 stores and metabolic rate (Shaffer et al. 1997). However, Martin & Smith (1999) purport that this is an underestimation based on study design and individual animal behaviour. They suggest the ADL is at least twice that estimated, which matches with the range of average reported dive durations (1–18 min; see above) and would agree with most dives remaining aerobic (Martin & Smith 1998). If belugas do dive anaerobically, adaptation in nitric oxide metabolism may aid in protection during such dives (Fago et al. 2017). As with other species, body size appears to influence dive ability based on O_2 stores, as smaller animals have been reported to perform fewer deep dives, spend less time at depth and require longer surface intervals (Heide-Jørgensen et al. 1998; Martin & Smith 1999).

Further adaptations to diving include the anatomy of the beluga heart (Bisaillon et al. 1988) and aspects of cardiac and pulmonary function (Fahlman et al. 2019; Fahlman et al. 2020). To the best of our knowledge, however, cellular adaptations to diving have not previously been of focus in beluga studies, and, with the exception of the connection to foraging, the direct relationship between diving and health has been little considered.

Beluga immune function during diving

Multiple aspects of dive behaviour and physiology have the potential to impact or alter immune activity during, or

Table 2 Reported haematology values for increased blood O_2 storage in belugas as compared with elephant seals, bottlenose dolphins and humans.

Reference	Species	Hb g/dl	Mb mg/g	HCT[a]%	MCV[b]	MCH[c]	MCHC[d]	Total O_2 stores ml/kg
Dhindsa et al. 1974	Beluga[e]	19.3		46	134.13	57.36	42.77	
Hedrick & Duffield 1991	Beluga[e]	21.6		59	177	64.07	36	
Ridgway et al. 1984	Beluga[f]	20.9 ± 0.9 (M) 20.3 ± 0.2 (F)		52.6 ± 4.3 (M) 52.2 ± 2.6 (F)				
St. Aubin et al. 2001	Beluga[g]	21.3–22		58–60	159–164	58–61	36–37	
Choy et al. 2019	Beluga[h]	21.2±1 (M) 23.4 ± 0.4 (F)	83.2 ± 2.1 (M) 84.1 ± 0.9 (F)	55 ± 2.1 (M) 59.6 ± 1.3 (F)				58.7
Noren & Williams 2000	Beluga[h]		34.4 ±3.9					51.9
Hedrick & Duffield 1991	N. elephant seal[e]	25.3		64	182.5	69.75	38.2	
Hassrick et al. 2010	N. elephant seal		78 ± 13 (F)	57 ± 4 (F)				92.8 (F)
Goldstein et al. 2006	Bottlenose dolphin[f]	11.3–18.2		37.13–47.27	107–129	37–44	32–36	
Ponganis & Williams 2015	Bottlenose dolphin							34
	Human							24
Wakeman et al. 2007	Human[g]	12–17.2		37–50	83–98	28–33	32–36	

[a]Haematocrit. [b]Mean corpuscular value. [c]Mean corpuscular haemoglobin. [d]Mean corpuscular haemoglobin concentration. [e]Reported as mean. [f]Reported as mean ± SD. [g]Range. [h]Mean + SE.

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following, a dive including: pressure; changes in O_2 availability; metabolic rate and activity level and bi-products in muscle; presence of a stressor; circulating hormone levels (such as catecholamines, which help regulate vasoconstriction) and potential formation of gas bubbles upon decompression. There is a complicated relationship between each of these factors (e.g., stressors may directly impact the dive response or may alter activity which then alters the dive response (Fig. 1). During a dive, effects may occur on cells found in tissue (e.g., macrophages) or in circulation. Even though drastic reductions in peripheral perfusion occur during the dive response, the variability in this response (as described above) suggests that some circulation and perfusion may persist during natural dives. While the direct effect of pressure on cells may be restricted to deeper depths (>500 m), belugas have been shown to reach such depths within their natural repertoires (Citta et al. 2013; Hauser et al. 2015). The challenges related to O_2 availability, activity level, metabolic rate and hormones may be of consideration for comparatively shallower dives. Work conducted in our laboratory has been the first to consider beluga immune function in the context of diving, aimed at a better understanding of the adaptation of the immune system to the aquatic environment, and to evaluate the potential for stressors to impact marine mammal health. To date, there have been only four studies directly evaluating immune function in response to the challenges associated with diving in belugas, and these have only begun to consider the effects of pressure, gas bubbles, apnea and activity level.

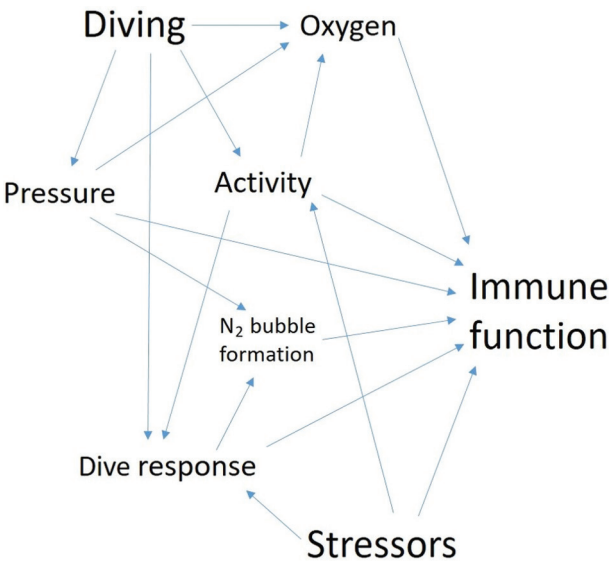

Fig. 1 A simplified diagram illustrating the relationship between physiological challenges associated with diving that may alter immune function. Environmental and anthropogenic stressors may also have indirect impacts (e.g., stressors alter activity that may modulate the dive response in such a way as to allow the development of N_2 bubbles, which then trigger immune responses).

Effects of pressure on immune function

Over the course of a dive, pressure increases by 1 atm for every 10 m of depth. A main consideration of pressure is the effects on air-filled spaces; pressure can also alter cell function by reducing process volumes (Bartlett 2002), ordering of membrane structures and reducing membrane fluidity, which in turn alters ion channel function, permeability, receptor binding and therefore the transmission of external to internal signalling (reviewed in Macdonald 1982; Heinemann et al. 1987; Somero 1992; Haskin & Cameron 1993; Kato & Hayashi 1999; Daniels & Grossman 2003; Pradillon & Gaill 2007). In addition, gene expression and protein synthesis may be altered (Bartlett et al. 1995; Pradillon & Gaill 2007) and the progression of the cell cycle and cell division may be slowed (Macdonald 1982).

Innate immunity

Present from birth, innate immunity includes non-specific defense mechanisms that recognize general signals of 'non-self' and include inflammatory processes, as well as certain cell functions. Phagocytosis is a process by which immune cells engulf infectious agents, foreign particles and cell debris, after which such particles can be destroyed intracellularly. Phagocytosis involves membrane receptor binding and membrane fluidity, which allows for membrane extension to surround and engulf the foreign particle (Murphy et al. 2008). The phagocytic response can be 'primed' or 'activated' as compared with a resting state, resulting in increased expression of cell surface proteins that facilitate binding, leading to stronger responses when an actual challenge is present (Downey et al. 1995). One such protein is the adhesion complex macrophage 1 antigen, which is composed of two heterodimers; CD11b and CD18. This protein enables phagocytosis (Graham et al. 1989) and has also been implicated in tissue damage (Orr et al. 2007).

The effects of pressure on phagocytic activity in belugas were investigated by exposing fresh blood samples to simulated dives utilizing a steel benchtop pressure chamber and hydraulic pump (Thompson & Romano 2015). Two target pressures (depths) were used to represent an extreme dive to 1360 m (2000 psi) and a deep dive to 640 m (1000 psi), which may be more common for wild belugas. In addition, the speed of compression and decompression (descent/ascent) and the duration spent at each target pressure (30 min, 5 min or two repeated 5 min) were

controlled and varied. Results varied with the characteristics of each simulated dive (depth, duration, rate of compression and decompression); in general, larger changes were observed for exposures to greater pressure (deeper) and with faster rates of compression/decompression (Fig. 2). An overall decrease in phagocytic activity was observed in these baseline samples, suggesting a pressure-induced down-regulation of function, possibly mediated by the direct effect of pressure on membrane characteristics. Increased pressure can decrease membrane fluidity and reduce the fusion of membranes, limiting both cellular ingestion and degranulation (Heinemann et al. 1987). This is further supported as expression of CD11b displayed minimal changes, suggesting that the overall activation state was not influenced. While a dive to 1360 m (2000 psi) would represent an extreme dive for belugas, the decreased innate function seen in this study immediately following pressure exposures either returned to normal or increased following a 20-min post-exposure recovery period (at ambient pressure), suggesting that the effect of pressure on the cells is reversible and may therefore not result in serious health implications. However, this was a single exposure, and the cumulative effects of repeated deep and prolonged dives were not explored.

Adaptive immunity

Adaptive, or acquired, immunity develops through exposure to potential health threats and is responsible for immunological memory leading to more efficient responses with repeated exposure. Recognition of nearly any possible pathogen is accomplished through high diversity of receptors on T lymphocytes, yet few cells express any given combination of receptors (Murphy et al. 2008). In order to mount an effective response, cells that recognize a pathogen must then be able to rapidly produce clones with the same recognition capability, a process called lymphocyte proliferation (King & Stott 2002; Murphy et al. 2008).

Similar to phagocytes, lymphocytes can be activated through binding of cell membrane receptors, and the clustering of cell membrane components. Such activation results in production of the cell signalling molecule interleukin 2 (IL2), which acts as both an autocrine and paracrine signal, as well as an up-regulator of the IL2 receptor in the cell membrane (Tizard 2000).

Thompson & Romano (2016) used the same in vitro approach as described for the innate immune function study to evaluate the effect of simulated dives on lymphocyte proliferation and activation. Similar to the phagocytosis results, a general decrease in function was detected following pressure exposure in baseline beluga samples (Fig. 3). However, in contrast to phagocytes, which did not display pressure-induced activation (increased CD11b), expression of IL2R increased in response to pressure exposures. This presents an interesting disconnect between activation state and cell activity, suggesting that even though cells are activated, they may be unable to proliferate because of the mechanical effects of pressure on membrane function or intracellular processes.

Combined effect of pressure and additional stressors

Diving itself is a challenging behaviour that requires several physiological adjustments. Increased hormone

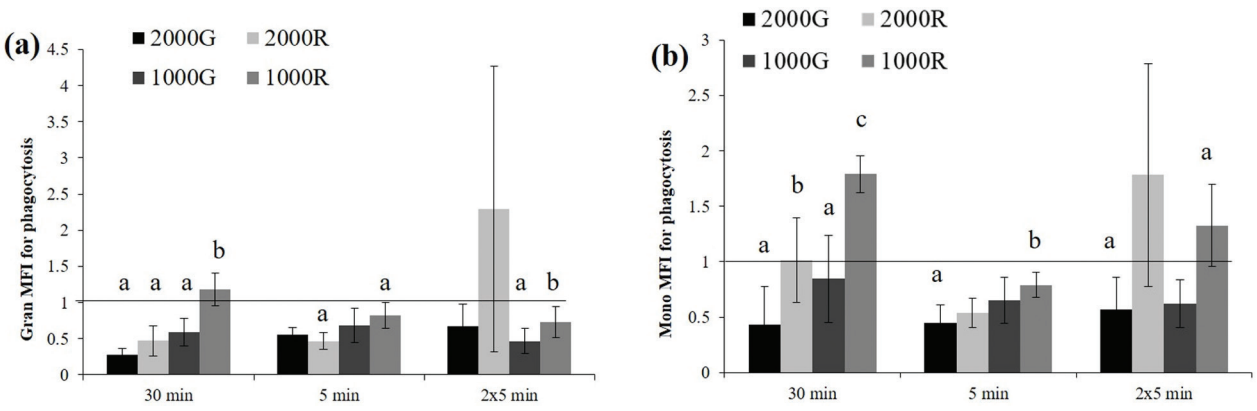

Fig. 2 Changes in (a) granulocyte and (b) monocyte phagocytosis measured in beluga blood samples (n = 4) following simulated dive exposures (i.e., pressure). Samples were exposed to either 2000 psi (1360 m) or 1000 psi (640 m), with compression and decompression occurring either gradually over two min (G) or rapidly in 15 s (R). Data have been normalized to controls (represented by the solid line at 1) and presented as the mean change in function ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposure. Significant differences between exposures ($p < 0.05$) are indicated by letters (a, b, c). (Reprinted from Thompson & Romano 2015; figures 1a, 2a.)

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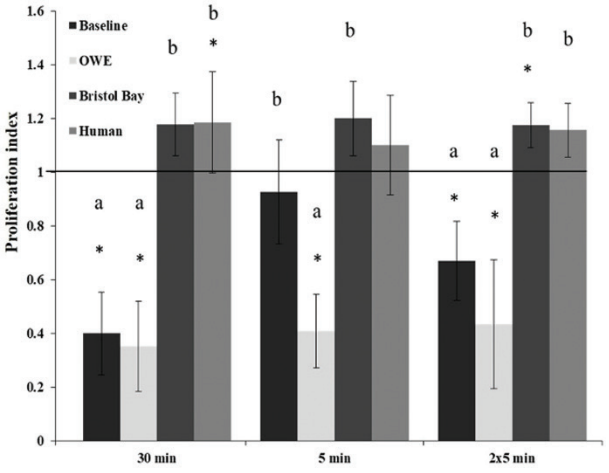

Fig. 3 Lymphocyte proliferation in aquarium belugas during baseline (n = 4) and OWE conditions (n = 3) in belugas from Bristol Bay (n = 9) and humans (n = 4) following exposures to 2000 psi (1360 m) with two min of compression and decompression. Data are normalized to controls (represented by the solid line at 1 and are presented as the mean ± SE). Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Within each duration, significant differences between conditions in belugas or between belugas and humans are indicated with letters (a, b) (p < 0.05). Significant differences from controls are indicated with an asterisk (p < 0.05). (Reprinted from Thompson & Romano 2016; figure 1d.)

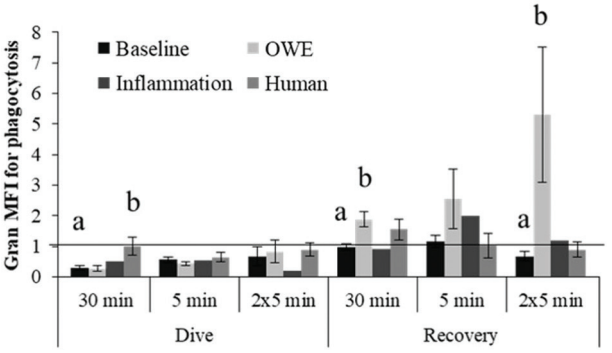

Fig. 4 Changes in granulocyte phagocytosis following exposures to 2000 psi (1360 m) with two min of compression and decompression, measured in belugas baseline (n = 4), OWE (n = 3) and inflammation (n = 2) conditions, and humans (n = 4). Data have been normalized to controls (represented by the solid line at 1) and presented as the mean change in function ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between belugas and humans or between conditions in belugas are indicated with letters (a, b) (p < 0.05). (Reprinted from Thompson & Romano 2015; figure 4a.)

expression, for example, serves to regulate certain aspects of the dive response, such as vasoconstriction (Hance et al. 1982; Hochachka et al. 1995; Butler & Jones 1997). Catecholamines, such as epinephrine and norepinephrine, as well as neuroendocrine activation are also important regulators of the mammalian stress response and can directly and indirectly modulate immune responses (Romano et al. 2002; Padgett & Glaser 2003). There is interest in the interplay of these two responses, and concern that a sudden stress response during a dive could result in abnormal or extreme physiological responses (Talpalar & Grossman 2005).

In studies on pressure effects on immune function, several paradigms were used to sample belugas under opportunistic 'stressor' conditions. For aquarium belugas, an OWE, where animals were stretchered and removed from the water for a full 30-min veterinary examination, resulted in increased measures of catecholamines and significantly higher serum cortisol (Thompson & Romano 2015) and had previously been reported to increase adrenocorticotropic hormone and cortisol (Schmitt et al. 2010). Samples collected following this 30-min OWE resulted in greater changes in granulocyte phagocytosis following in vitro pressure exposures, including significant increases in function during the recovery periods (Fig. 4). Lymphocytes also displayed larger pressure-induced changes in function following the OWE (Thompson & Romano 2016).

Wild belugas in Bristol Bay sampled under restraint in shallow water during live-capture/release health assessment efforts, provided an additional stressor paradigm for testing lymphocyte function (Thompson & Romano 2016). These samples displayed higher proliferative responses as compared to both aquarium baseline and OWE samples, which resembled those measured in human samples more closely (see Fig. 3). Under such a situation, this similarity may indicate greater risk of dive-related disease in belugas that dive while facing additional significant stressors, such as shipping, pollution, increased sound levels, oil spills and other effects of human activities.

Response to N_2 bubbles

Thompson & Romano (2015, 2016) present the first data supporting the hypothesis that marine mammals, such as the beluga, may display a down-regulation of certain immune functions (e.g., phagocytosis and proliferation) during diving and that this may protect against unwanted immune activity, such as inflammation leading to tissue damage. Because the development of DCS injury has been linked to changes in immune activity, this down-regulation may be one mechanism through which marine mammals avoid DCS-like injury during diving. Increased cellular function measured in opportunistic stressor samples, particularly

during recovery periods (i.e., after decompression) and responses that resemble those observed in human samples may indicate conditions during which belugas are more susceptible to dive-related injury.

While the possibility of gas-bubble formation during decompression in diving cetaceans is still under question, DCS is not limited only to SCUBA or saturation dives and can occur during breath-hold diving (Wong 2006; Schipke et al. 2006) and there is some evidence that N_2 supersaturation could occur under certain circumstances (Houser et al. 2001; Jepson et al. 2003). Yet for human divers, the occurrence of gas bubbles alone does not equal DCS and so-called 'silent bubbles' can occur without the development of symptoms (Barack & Katz 2005), possibly due to reduction in immune responsiveness (e.g., Kayar et al. 1996).

With the aim of directly testing the potential response of beluga inflammation to potential N_2 gas bubbles, Thompson et al. (2020) exposed serum to various flows of N_2 gas in vitro. While human samples were not included in this study, a significant activation of the complement response was reported, measured as a significant increase in the protein C5a, in human and rabbit sera exposed to air flow at a rate of 0.5 ml/min for 30 min (Bergh et al. 1993). This flow rate failed to trigger a similar significant increase in C5a (inflammatory activation) in either aquarium beluga baseline samples or wild live-capture/release belugas (stressor) samples (Fig. 5). However, a higher flow rate of 1 ml/min did result in a significant increase in C5a in wild beluga samples, suggesting that the additional stressor of chase and restraint may alter the sensitivity of the complement system. This work also suggests that there may be a threshold of bubble load before belugas (or other marine mammals) are at risk, and this may be greater than that of humans and other terrestrial animals.

Interestingly, in this study, serum from the closely related narwhal (*Monodon monoceros*) also sampled under restraint conditions, were subject to the same in vitro N_2 exposures but did not display any significant activation of complement, even at the highest flow rate, which significantly affected wild belugas (Fig. 5). Narwhals are comparatively deeper divers than belugas, reaching maximum depths of over 1500 m (Heide-Jørgensen et al. 2015) and these results suggest species-specific responses to stress (coping styles), varying susceptibility to dive-related injuries and sensitivity to forced changes in dive behaviour.

The need for in vivo studies

These early studies were carried out in vitro, utilizing both fresh and frozen archived samples. In vivo investigations are a critical next step to place results within the context of various physiological adjustments that occur during diving. Preliminary in vivo studies utilizing a single beluga (Thompson & Romano 2019) collected blood samples following two different trained dive behaviours: (1) stationary dive in which the whale remains submerged under water and stations on a target, and (2) active dive in which the whale swims between targets while submerged. Only one dive behaviour occurred in a single day. Each dive behaviour lasted for a duration of three min and each behaviour was sampled three times. Blood samples were collected by veterinary staff from the ventral aspect of the fluke under behavioural participation by the individual whale. Plasma catecholamines were measured using high-performance liquid chromatography and C5a was measured with and without in vitro exposure to N_2 bubbles.

While no statistical analysis can yet be run on this data, Thompson & Romano (2019) showed an observed increase in norepinephrine during each dive behaviour, suggesting that 3 min was sufficient to initiate a mammalian dive response (Fig. 6). Additionally, there appeared to be an increase in circulating C5a following both dive types, which was greater and less variable following the stationary dives as compared with the active dives (Fig. 7).

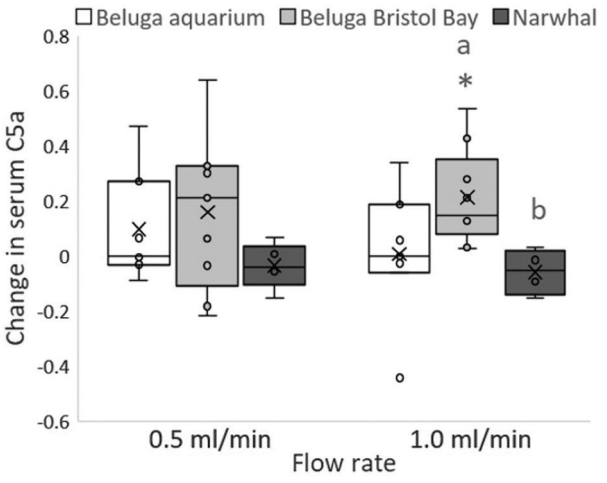

Fig. 5 Change in serum C5a with N_2 bubble exposures compared to controls in narwhal ($n = 5$) and beluga (aquarium $n = 7$, Bristol Bay $n = 9$). Bubble exposures were of 30 min at either 0.5 or 1 ml/min N_2. Data were normalized to control values for each sample and are represented as the mean ΔC5a ± SE. Box and whisker plots indicate the median and quartile ranges for each sample group. Means are indicated by an "x". The significant exposure-induced increase in mean C5a for Bristol Bay belugas is denoted with an asterisk. Significant differences in the response of C5a between species are indicated with letters (a, b). (Reprinted from Thompson et al. [2020] with permission from Springer Nature Customer Service Centre GmbH: Springer Nature [*Journal of Comparative Physiology B*. Variation in hemostatic complement (C5a) responses to in vitro nitrogen bubbles in monodontids and phocids].)

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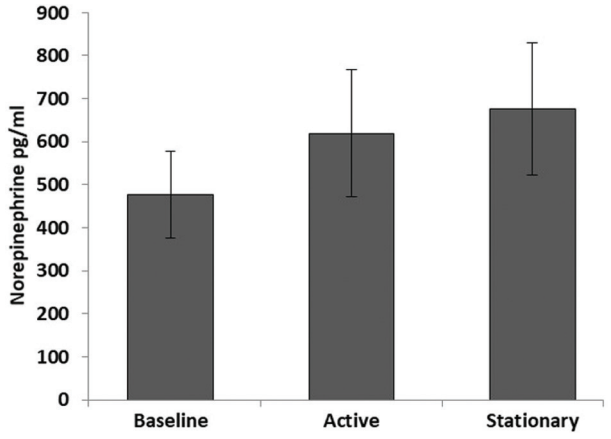

Fig. 6 Plasma norepinephrine measured in a single beluga during monthly blood draws (n = 7; baseline) and following specific active and stationary dive behaviours. Each dive behaviour was replicated three times.

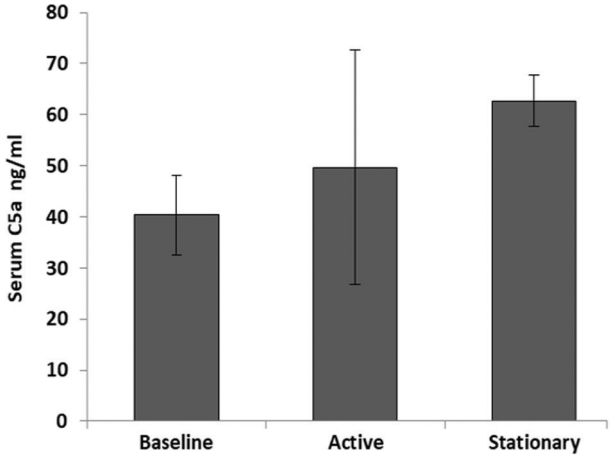

Fig. 7 Serum concentration of C5a measured in a single beluga during monthly blood draws (baseline) and following specific active and stationary dive behaviours. Each category includes three replicates.

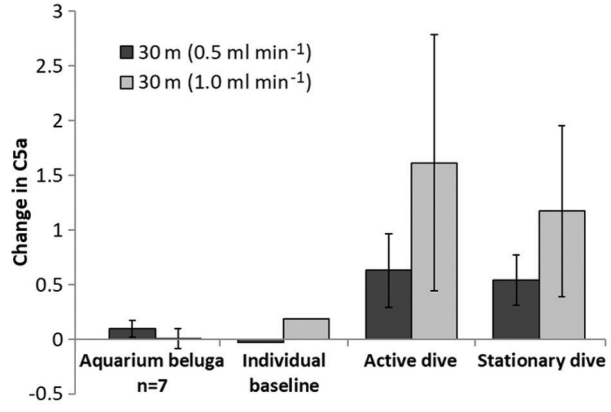

Fig. 8 Calculated changes in C5a following 30 min in vitro exposures to N_2 gas bubbles at flow rates of either 0.5 or 1 ml/min. Aquarium beluga baseline (n = 7) are re-reported from Thompson et al. (2020). The individual baseline presents a single measure, while three replicates were completed for each dive type.

Furthermore, exposure to in vitro N_2 bubbles following these dive behaviours resulted in huge increases in C5a as compared with the individuals' baseline samples and the beluga baseline data reported by Thompson et al. (2020; Fig. 8). Such results indicate that there may be other physiological factors that influence inflammatory activation during diving, including apnea and activity level. As O_2 plays a key role in cell metabolism, certain immune responses (e.g., oxidative burst) and re-oxygenation can induce inflammation; further studies investigating the effects of hypoxia are warranted.

While in vitro approaches allow for the effects of individual parameters (pressure, bubble presence) to be isolated and examined, in vivo studies are needed to understand immune function in the whole animal. Further studies utilizing trained belugas are being developed in order to increase the sample size for this research as well as to target longer duration behaviours. Shaffer et al. (1997) reported behavioural dives of 13 min, with stationary breath-holds of 17 min. These durations would be of particular value as they may begin to reach ADL and would thereby provide information relevant to animals performing at or near their physiological limits.

Summary and conclusion

Diving is a critical behaviour for cetaceans. For belugas, the ability to dive to depths is necessary for foraging as well as prolonged periods when navigating under the Arctic ice and they are well-adapted to diving at various depths and durations. There have been relatively few direct studies of beluga dive physiology, especially at the cellular level. More research is needed to better understand the impacts of changing dive behaviour on beluga health. As with other physiological processes, it is possible that the marine mammal immune system has some dive adaptation as immune activity is not only necessary for maintaining health and homeostasis but also plays an important role in dive-related injury and disease, including DCS. Here we have recounted the first studies to consider immune function in belugas in the context of diving. Results of this effort suggest that beluga immune responses are less sensitive to the challenges associated with diving, as compared with humans, but that these responses can be altered by stressors, including anthropogenic activities. Both in vivo and in vitro aspects of this work measured end-dive changes, and new approaches and technology are needed to investigate mid-dive function.

It is clear that more work is needed to better understand the complicated relationship between immune function, diving and health in belugas. Rapid changes occurring in the Arctic and Subarctic regions may force changes in

beluga dive behaviour. Belugas are diving deeper and longer during foraging (Hauser 2016; Stafford et al. 2016) and it is unknown if this will push animals—such as smaller belugas, which make shallower, shorter dives, with longer inter-dive intervals at the surface (Heide-Jørgensen et al. 1998)—past their physiological limits. Reduced dive ability has also been linked to reduced O_2 stores in animals with poor body condition (Choy et al. 2019). Poor nutrition affects both dive ability and immune function (e.g., Chandra 1999). As the ice cover declines, increased human presence may directly alter beluga dive behaviour, resulting in decreased surface time. If this should interrupt inter-dive or post-dive recovery, lactate levels could increase (Shaffer et al. 1997) with as yet unknown implications for immune responses as lactate has been linked to both perpetuating inflammation (Haas et al. 2015) and regulating inflammation during hypoxia (Ivashkiv 2020). If interruption of natural dive behaviours increases the potential for N_2 bubble formation, this, in combination with the physiological response to environmental stressors or boat noise, may leave belugas more susceptible to dive-related injury.

The consequences of human activity and shifting Arctic environment on beluga health may not be as apparent as mass strandings or physical injury. There is likely a complicated balance between immune activity and diving physiology, behavioural responses and health that can be impacted by abnormalities in any one system. Research investigating the immune system's role in diving is critical in understanding potential sub-lethal impacts of a rapidly changing Arctic on beluga diving behaviour and physiology, health and disease susceptibility.

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