Oxygen and the diving seal.

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INTRODUCTION

Diving seals are extraordinary animals. They are able to avoid hypoxia and the effects of oxygen deprivation far more efficiently than the vast majority of mammals. One of the kings of the diving world is the elephant seal (*Mirounga angustirostris*). These animals are capable of performing dives of up to two hours in duration (1) and have been recorded diving to depths of 1.5 kilometers (2). Perhaps more impressive are their routine diving behaviors exhibited during the 5 to 8 month migrations to the sea. During the biannual migrations between foraging grounds and the beaches where they moult and breed, these animals spend 80-95% of their time submerged (3). They follow a pattern of long, deep, continuous dives interspersed with brief surface intervals of 1-3 minutes (4).

It was probably in the early '30s and '40s that we really began to understand the physiology behind the impressive breath hold ability of these animals. Per Scholander, Lawrence Irving and their colleagues investigated the physiology of diving in a wide variety of organisms, subjecting them to forced diving protocols and facial immersion (5, 6). Their findings revealed that across species, there are three main physiological responses to facial immersion: 1) apnea; 2) bradycardia; and 3) peripheral vasoconstriction and hyperperfusion of the peripheral tissues. Over time, this triad of physiological events became known collectively as the mammalian diving response. The events that occur during diving are under the control of multiple reflexes, rather than the result of one single reflexive action. Experimentally, these physiological responses can be elicited through facial immersion. In marine mammals, the use of a diving helmet has been as effective as total body immersion in producing diving bradycardia (Figure 1).

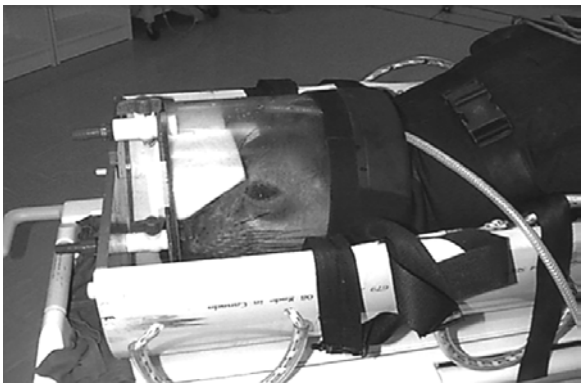

Fig. 1. Northern Elephant Seal in acclimation phase prior to imaging**.** The foam block situated in the upper margin of the helmet prevents the seal from raising its nostrils into the air pocket formed during exhalation. During acclimation, both valves are in the "open" position and a vacuum hose under the neck seal ensures adequate airflow through the helmet.

Bradycardia

Bradycardia and peripheral vasoconstriction act in concert to allow hypoxia-sensitive tissues such as the heart and brain to receive a constant delivery of oxygen. The dramatic onset

of bradycardia, illustrated in the ECG in Figure 2, was obtained from a captive harbor seal and indicates a 90% reduction in heart rate in the first 30 seconds of the dive.

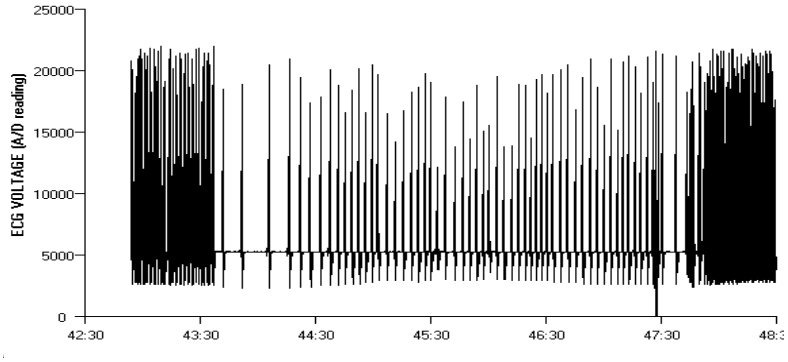

Fig. 2. Electrocardiogram from a experimentally dived harbour seal (***Phoca vitulina)***. EKG from a experimentally dived harbour seal Diving heart rate averaged 12 beats per minute. Note the long interbeat intervals in the first minute of the dive (Thornton, unpublished data).

The benefits of bradycardia include reduced cardiac muscle workload (and thus reduced metabolic demand); a reduction in oxygen delivery to hypoxia-tolerant tissues, resulting in reduced oxygen consumption; and a reduction in cardiac output, which assists in maintaining blood pressure when the peripheral arteries are constricted.

Peripheral Vasoconstriction

In the periphery, tissues exhibit a reduced hypoxia sensitivity and are able to function partly or exclusively using localized oxygen reserves. Per Scholander first unraveled the concept of peripheral vasoconstriction by measuring circulating blood lactate levels. Scholander and Irving hypothesized that hypoperfused tissues will eventually have to rely on anaerobic metabolism. Initially, blood lactate levels appeared to tell a different story. Blood samples obtained during diving did not demonstrate an elevation in lactate. Instead, a striking increase in lactic acid production appeared during the *post dive* period. However, by obtaining muscle samples from animals in the predive, dive and post dive state, Scholander demonstrated that a marked increase in lactic acid formation occurs in the muscles during diving, but is not released into general circulation until the animal surfaced. He then hypothesized that a reduction in muscle perfusion during diving is behind the observed pattern of blood lactate (Figure 3).

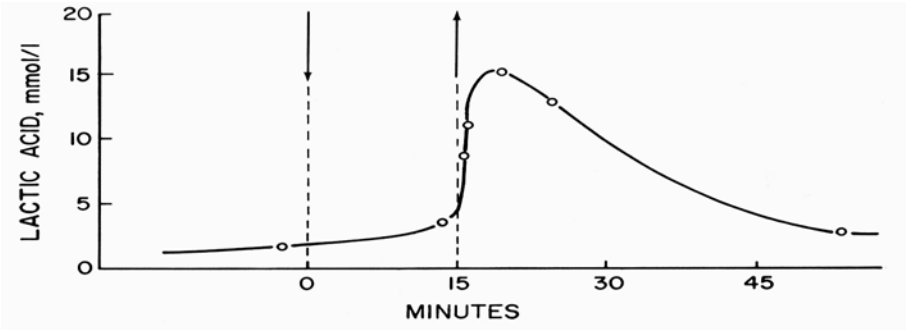

Fig. 3. Lactic acid concentration in the arterial blood of an experimentally dived grey seal *Halichoerus gryphus*. Dive indicated by arrows. (Redrawn from Scholander, 1940).

Hypometabolism

In addition to documenting the mobilization of lactic acid from the muscles of diving animals, Scholander calculated the contribution of anaerobic metabolism to the overall metabolic "debt" incurred during diving. The metabolic cost of diving is difficult to measure, but may be

estimated by evaluating the excess oxygen consumption in the post dive period. Scholander found that the aerobic contribution to diving is often less than the resting metabolic rate; therefore it was thought the balance of energy utilized during the dive would be supplied through anaerobic metabolism. However, the calculated total energy consumption (anaerobic and aerobic ATP production) for the duration of the dive was often below the level of a resting animal.

Studies on terrestrial animals have shown that there exists a linear relationship between blood flow and oxygen consumption at both the cellular and organism level (7, 8). This relationship holds true over a wide range of activity, suggesting that reduced perfusion results in an overall suppression of metabolism. It is likely, albeit difficult to demonstrate, that seals experience a significant reduction in overall metabolic rate related to peripheral vasoconstriction.

Morphology

The seal's physiological arsenal for the fight against hypoxia is supplemented by a number of morphological characteristics. Seal muscle is rich in myoglobin, containing 5-12 times the amount found in human muscle. Seals have a higher circulating blood volume and a higher resting hematocrit than terrestrial organisms. With a total blood volume in the range of 15% of body mass (human blood volume is ~5-7% of body mass), a considerable increase in oxygen storage is realized. During diving or periods of apnea, a significant and rapid rise in circulating red blood cells is observed (9, 10, 11; Figure 4). This variation in red cell mass indicates that seals have some method of sequestering red cells during non-apneic events. It was widely suspected that the source of these cells was the spleen.

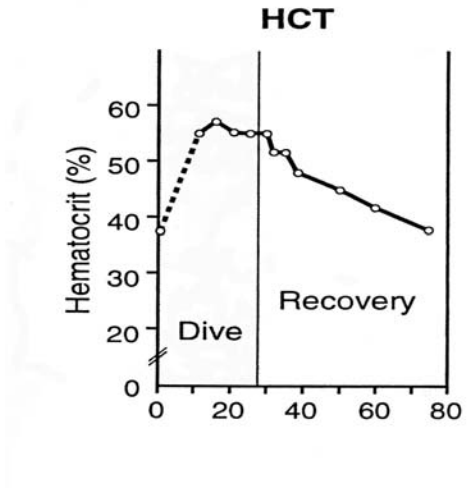

Fig. 4. Changes in hematocrit**.** Figure 4 illustrates changes in hematocrit during diving and recovery in a representative dive in the Weddell seal. After Hurford et al, 1995.

The Pinniped Spleen

Anatomical observations of seals dating back to the 1800s consistently remark on the size of the spleen. Autopsy data indicate that the spleen is approximately 1% body mass in the large seal species (Weddell, northern and southern elephant seal). As the spleen is composed of a smooth muscle capsule, which may contract at the time of death, these data most likely underestimate the working volume of this organ. Histological studies reveal that seal spleens are capable of sequestering significant quantities of red blood cells and possess contractile properties in both the smooth muscle capsule and the internal structural cells (12). In Weddell seals, epinephrine injection was followed by an increase in hematocrit and a decrease in splenic volume, as measured by ultrasound (13). As increased catecholamine levels are observed during diving in seals, a correlation between diving, splenic contraction and increased hematocrit seems likely. Although much evidence points toward the splenic role in diving, no measurements have been obtained during a dive.

At this point the field of diving physiology was dependent on indirect evidence. We began searching for methods that would allow for direct interrogation, and turned to magnetic resonance imaging (MRI). Now, if you've ever seen an elephant seal you will realize that this was a bit of a leap of faith, but through the collaborative efforts of this diverse group of individuals, we were, for the first time, able to view the physiological changes that occur during diving. In conjunction with the University of California Santa Cruz's elephant seal group, physicists from Stanford University's Center for MR Imaging, and physiologists from University of British Columbia, this project came to fruition (14). Five juvenile elephant seals were collected from Año Nuevo State Reserve (National Marine Fisheries Service Marine Mammal Permit # 786-1463) and were held at Long Marine Laboratory, UCSC for up to 8 days. The seals were released at the site of capture at the conclusion of the study. Images were obtained from 5 seals over 24 simulated dives. Facial immersion was achieved by slowly filling the helmet through the top valve and simultaneously closing the drain valve on the bottom of the helmet (Figure 5)**.** Images were obtained before the dive (baseline splenic volume), sequentially during the dive (initiated as soon as the animal's nostrils were submerged) and continued until the helmet was drained and the animal took its first breath. Post dive times were recorded from the first breath and post dive imaging began 1 minute post dive. Images obtained between 15 and 20 minutes post dive were considered baseline.

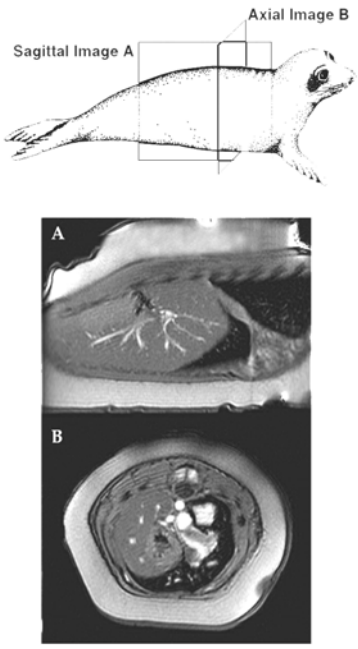

Fig. 5. Sagittal and axial images of the spleen of a northern elephant seal pup. Sagittal localizers were used to define the upper and lower image slice location and calculation of the number of axial slices required to image the total spleen. A series of 29-34 1.5 cm "slices" were used to image the organ completely, requiring less than a minute of scan time. Image A is from 5 cm left of the midline (spine) and image B is 8 cm below the diaphragm.

The most striking observation from these images is the rate at which the spleen contracted. By dive minute 3, the spleen had reduced to approximately one-fifth of resting volume and remained contracted for the duration of the dive (Figure 6).

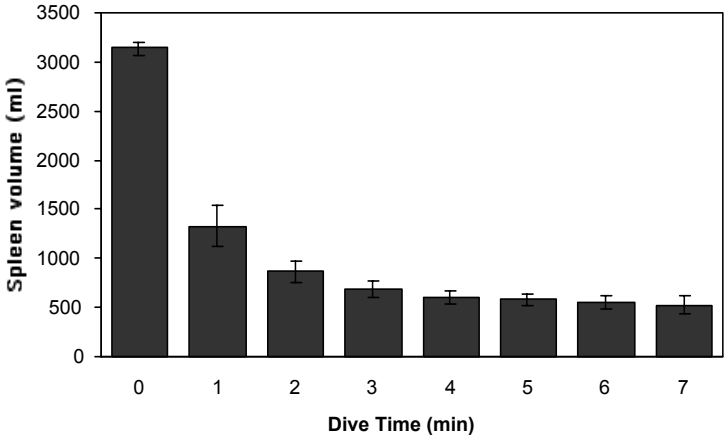

Fig. 6. Northern elephant seal spleen volume during rest. Northern elephant seal spleen volume during rest (Min 0) and diving (Min 1-7) was obtained using MR imaging techniques (n = 5, each individual's value is the average of four dives). Splenic volume does not decrease significantly after minute 2 (ANOVA, $F_{(6,28)}$= 33.94, P < 0.0001; Tukey Kramer HSD, P = 0.05). Error bars indicate SD. Thornton et al, 2001.

These data clearly support the existence of a diving-induced sympathetic contraction of the spleen and subsequent release of the stored erythrocytes; however, a discrepancy exists in the timing of splenic contraction and the rise in

circulating hematocrit. Complete splenic contraction occurs within 3 minutes of catecholamine stimulation, yet peak Hct is not observed until 15-25 minutes after the spleen has contracted (11, 13).

The second defining observation of this study was the appearance of a fluid-filled structure within the abdominal cavity: the hepatic sinus (Figure 7). Formed by the dilation of the hepatic veins, the thin walled sinus lies caudal to the diaphragm, draining from its midpoint through the diaphragm and into the thoracic portion of the posterior vena cava. The inferior vena cava and the hepatic sinus may contain up to one fifth of the animal's total blood volume and is a significant storage depot of oxygenated blood during dives.

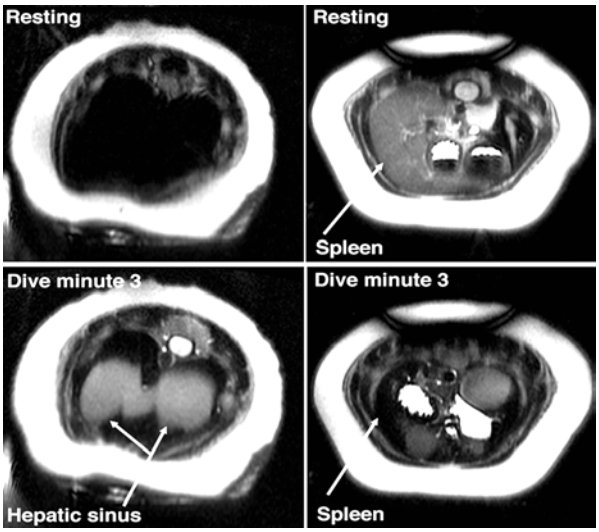

Fig. 7. Thoracic images of a northern elephant seal during rest and diving. Images on the left are from the region immediately caudal to the diaphragm; images on the right are 12 cm caudal to the diaphragm. Rapid contraction of the spleen and simultaneous filling of the hepatic sinus are observed. After Thornton et al, 2001.

Filling of the sinus is dependent on the closure of a muscular vena caval sphincter located on the cranial aspect of the diaphragm. In experimental dives using harp seals (*Pagophilus groenlandicus*), Hol et al (15) reported a marked constriction of the sphincter occurred 20 seconds after commencement of the dive, with dilation of the posterior caval vein and hepatic sinuses occurring before as well as during the 40 seconds following constriction. They also demonstrated a temporary relaxation of the caval sphincter during the dive and subsequent mixing of the blood in the sinus with that returning from the anterior part of the body.

The interplay between the spleen and hepatic sinus serves to explain a number of observed physiological events. Although the spleen has long been suspected as the source of the RBCs released during diving, the rate of splenic contraction has presented an apparent contradiction to the gradual diving-induced rise in Hct. In this study, maximal Hct occurred after the 7 minute dive had concluded, whereas the spleen had released the majority of its RBCs by Dive Min 2. The involvement of the sphincter-controlled sinus serves to delay the release of RBCs into general circulation and may abrogate the potentially deleterious effects of an acute rise in red cell mass. In northern elephant seal pups, contraction of the spleen in the first minute of the dive would result in an increase in vena caval blood volume at a rate of 23.6 ml/second (Min 0 to Min 1 decrease in splenic volume = 1417 ml/60 sec). Relocating the RBCs from the spleen into the sphincter-controlled venous sinus results in a gradual metering of oxygenated RBCs into the heart, protecting it from a drastic increase in right ventricular pressure at a time when diving bradycardia is most profound.

From the evidence presented herein, it appears that the system works as follows: facial immersion causes stimulation of the trigeminal nerve, leading to vagal stimulation, bradycardia, peripheral vasoconstriction and caval sphincter contraction. Circulating catecholamine levels rapidly increase, resulting in splenic contraction and the maintenance of peripheral vasoconstriction. The oxygenated RBCs of the spleen are then released into venous circulation. Venous blood returning to the heart is prevented from passing cranially through the diaphragm

by the occlusion of the sphincter, causing the hepatic sinus to fill. As the dive progresses, red blood cells are gradually metered out into general circulation via relaxation of the caval sphincter (Figure 8).

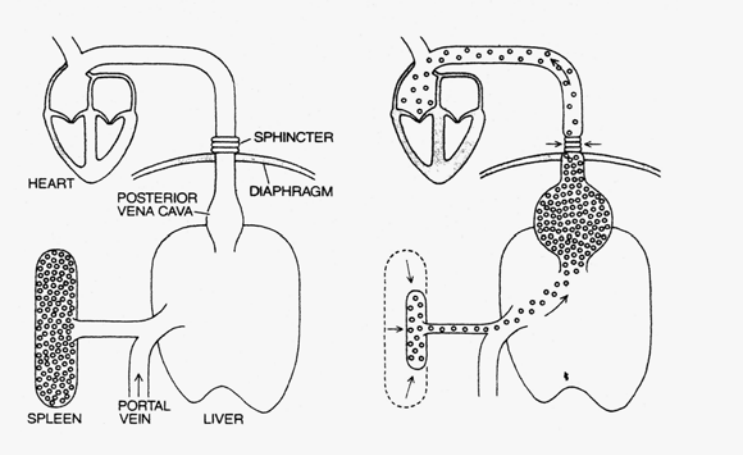

Fig. 8. Oxygen-rich blood cells released from the spleen. Oxygen-rich red blood cells (RBCs) are released from the spleen during contraction when the animal dives. The caval sphincter constricts the venous return to the heart, causing an expansion of the hepatic sinus. As the dive progresses, the oxygenated RBCs are slowly metered out into circulation via relaxation of the caval sphincter. After Zapol, 1987.

In 1987, Warren Zapol speculated on the interplay between the spleen and hepatic sinus, and these data essentially support his supposition (16). Zapol equated the seal spleen to a "SCUBA tank", providing the animal with continuous supply of oxygenated RBCs as the dive progressed. Based on this elegant system of storage, transfer, and metering of RBCs, it appears that the spleen does indeed function as a SCUBA tank, and increases the fitness of the species through elevated oxygen stores, increased dive time, and thus increased foraging success, predator avoidance and efficiency of locomotion.

Diving Adaptations

The field of comparative physiology was eager to label the spleen as a morphological SCUBA tank; a trait specifically adapted to an aquatic environment. Logically, we argue that you and I do not have a large spleen, nor do we possess a caval sphincter or hepatic sinus. And also obvious is our inability to perform substantial breath hold dives.

However, there are a number of caveats when labeling the spleen as an "adaptation to diving." The field of evolutionary physiology has become more rigorous in its definition of adaptation, requiring that the feature be a product of natural selection in the true Darwinian sense, provide an increase in the fitness of the bearer, and exhibit complexity and purpose. In order to evaluate whether the spleen is an adaptation to diving, we had to establish the purpose for which it was selected. An accepted means to establish evolution of a trait is to evaluate the structure and function of the trait in closely related species. In phocid seals, comparisons between species of varying diving ability should reveal traits that correlate with increased maximum dive time.

A fundamental problem exists when comparing related species: the closer the phylogenetic relationship, the more likely they are to exhibit similar traits. To remove the factor or relatedness and allow for the examination of each species as an independent data point, we use a phylogenetically independent contrast analysis. A study conducted by Mottishaw et al (17) examined a number of traits that have been traditionally referred to as "diving adaptations". This process allows us to take away the factor of "relatedness" and look at each species as an independent data point. The transformed data (standardized independent contrasts) may then be used in ordinary statistical procedures.

In order to stay submerged for a longer period of time, an air-breathing mammal must either increase the amount of oxygen carried within the body, or decrease the amount of oxygen

used. This study examined up to a maximum of 17 phocid and 15 otariid species, evaluating factors that would potentially extend diving time: blood volume, body mass, hematocrit, maximum bradycardia and splenic volume (Figure 9).

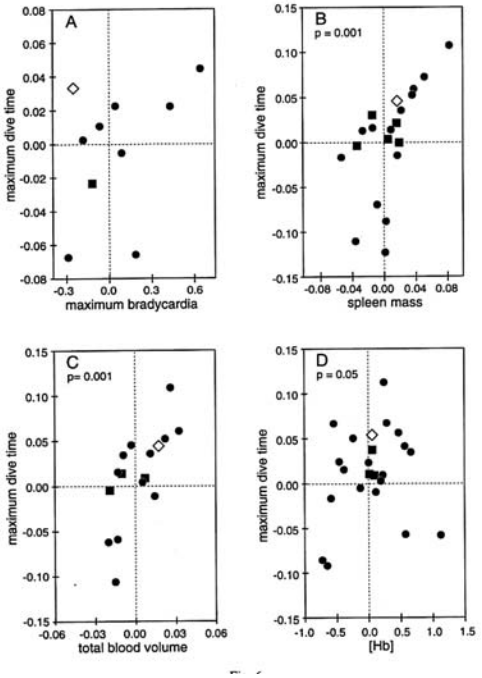

Fig. 9. Correlation of residuals**.** A. The correlation of residuals generated by regression of log maximum dive time contrasts and maximum bradycardia (not statistically significant; P=0.15). B. Significant positive correlation between residuals generated by regressions of log maximum dive contrasts and log spleen mass contrasts on body mass contrasts (r=0.69; P<0.001). C. Significant positive correlation between residuals generated by regressions of log maximum dive time contrasts and log total blood volume contrasts on log body mass contrasts (r=0.74, P<0.001). D. Significant positive correlation between residuals generated by regression of log maximum dive time contrasts and [Hb] contrasts on log body mass contrasts (r=0.46, P=0.05). In all graphs, circles (●) represent contrasts within the phocid species; squares (■) represent contrasts within the otariid species; and the diamond (◊) represents the root node, or contrasts between phocids and otariids. After Mottishaw et al, 1999.

As expected, body mass was positively correlated with diving ability, as the more oxygen you carry, the longer your expected dive duration. Also, the larger the organism, the lower the mass specific oxygen consumption; therefore, dive duration should be longer. Analysis of spleen size, blood volume and blood hemoglobin revealed similar positive correlations. In order to remove the influence of body size on each variable, residuals were generated and were then regressed on the residuals of each physiological or morphological variable; therefore, these correlations are independent of body mass. Again, the data seem to support the categorization of these characters as adaptations to diving.

About a year after we published this paper (17), I began to look at these correlations in a more rigorous light. The correlated evolution of these traits does not prove that they have been selected specifically for the task of increasing dive time. To evaluate the functional significance of a trait, the physiology must be examined in context with the life history of the animal. We have clearly demonstrated that splenic contraction accompanies facial immersion, and the larger the spleen, the greater the breath hold ability. In the wild, elephant seal pups leave the beaches of Año Nuevo in the spring and immediately enter into a continuous diving bout, averaging 20 minute dives and 1-3 minute surface intervals. The brevity of the surface interval illustrates a substantial paradox: although the spleen contracts and releases its contents into circulation within three minutes of submergence, in the post-dive situation the spleen takes approximately 20 minutes to passively refill. If the same physiology occurred in a free diving elephant seal pup off the coast of California (and there is no reason to believe it doesn't), a 1 to 3 minute surface interval is not sufficient to resequester the red blood cells and reduce hematocrit to resting levels. Therefore, if the spleen contracts at the beginning of a dive bout (which may last up to 8 months!), but is unable or unlikely to refill due to short surface intervals, is the spleen actually serving a useful purpose during diving? In fact, if we splenectomized a seal, would it affect the animal's diving ability?

The answer is *probably not*. The benefit of the spleen is its ability to release RBCs, which increase the oxygen carrying capacity within the vascular system during submergence. As it was previously thought that the spleen supplemented circulating blood with oxygenated RBCs during the dive, the inability to resequester RBCs during the surface interval brings this role into question. Elevated hematocrit throughout a diving bout has been observed in phocids both in the field (Weddell seals; (9, 11)) and the laboratory (northern elephant seal pups; (18)), lending further evidence to the lack of participation of the spleen during an extended diving bout. In fact, decreasing the circulating blood volume during the surface interval would delay replenishment of tissue oxygen stores and removing metabolic byproducts from the periphery, therefore any selective pressure for rapid resequestration of RBCs would be minimal. The question then becomes what is the selective pressure acting upon organogenesis and influencing spleen size in a diving pinniped? It has been suggested that these animals may utilize the spleen to sequester RBCs when the benefit of increased oxygen is offset by the cost of transporting blood of higher viscosity (19). In light of the time it takes to remove the supplemental RBCs from circulation and return them to the spleen, this alternate explanation has significant merit.

During diving, periods of high hematocrit are accompanied by vasoconstriction and bradycardia. The combined effect of these events would serve to decrease shear rate and result in elevated viscosity. Upon returning to land, these animals would experience a dramatic and prolonged increase in heart rate, leading to deleterious viscosity effects on the vascular system. Based on the data presented here, it appears that the spleen allows the seal to maintain a higher circulating hematocrit during periods of hypoxia, yet effectively reduce hematocrit and circulating blood volume when oxygen is not limiting, thus avoiding any deleterious effects of increased blood viscosity. In essence, the spleen is more likely to be an adaptation to the terrestrial portion of a pinniped's live history than a character that increases diving time.

Bradycardia Revisited

Although many of the morphological features related to oxygen storage correlated with diving ability, the PIC analysis revealed that bradycardia, often referred to as the key to the mammalian diving response, did *not* correlate with maximum dive time (Figure 10). In fact, the lowest heart rates found in the field show little variation within the pinniped species and are similar to those observed during forced dive studies.

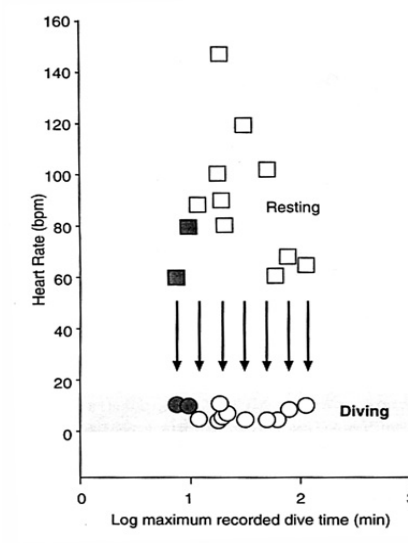

Fig. 10. Relationship between maximum diving duration and degree of bradycardia expressed in several species of sea lions and seals. It is evident that all of these species, both short and long duration divers, are able to activate bradycardia to the same extent (minimum heart rate 4-10 bpm). There is no statistically significant relationship between maximal bradycardia and dive duration. (After Mottishaw et al, 1999).

This surprising result contrasts with the prevalent view of bradycardia as an obvious adaptation for diving. To clarify, we agree that bradycardia is likely an integral component of a seal's diving ability; however, these results suggest that it is an ancestral and conserved trait, not specifically acted upon by the process of natural selection with the purpose of increasing breath hold ability. In other words, it is not an adaptation to diving, but is instead a hard-wired response found throughout

air-breathing vertebrates, possibly as an asphyxial defense mechanism. Indeed, within my own experience during 2nd year physiology labs, I have recorded students' heart rates in the 10 -12 bpm range during facial immersion, yet the maximum breath hold ability for humans (at least, for 2nd year physiology students!) averages less than one minute. Clearly, diving ability is more complicated than mere heart rate reduction.

A recent study by Elliot et al (20) from the University of British Columbia serves to further downplay the role of bradycardia in phocid diving ability. During voluntary diving in an 11 m deep tank, the cardiovascular responses to submergence of five harbour seals were manipulated using specific pharmacological antagonists, and the effects on diving behaviour were observed. Using a muscarinic blocker methoctramine, (diving bradycardia); the α-adrenergic blocker prazosin (diving vasoconstriction); and the β-adrenergic blocker metoprolol (post-dive tachycardia), they assessed the necessity of diving bradycardia, vasoconstriction and surface tachycardia in the performance of short dives and short surface intervals in harbour seals.

None of the pharmacological blockers had any effect on average dive or surface interval duration, and seals maintained a high percent dive time in all treatments (Figure 11). Thus, it appears that during short dives, harbor seals do not need to invoke bradycardia or peripheral vasoconstriction in order to maintain an efficient dive strategy.

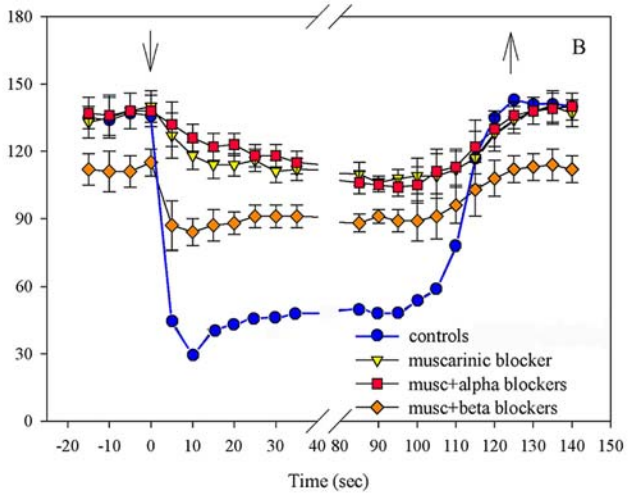

Fig. 11. Heart rate profiles.Heart rate profiles before, during and after voluntary dives in muscarinic-, muscarinic plus α-adrenergic-, and muscarinic plus β-adrenergic-blocked seals. Arrows denote the beginning and end of the dive. Each data point represents the mean heart rate for the preceding 5 s interval. For each treatment, mean heart rates during two dives (approximately 120 s) were averaged for each animal. Data from all seals (N=5) were then combined to give the means (±S.E.M) illustrated. The data were normalized so that dives of different lengths ended at the same time. (After Elliott et al, 2002).

Most of the original data on bradycardia was derived from forced diving experiments, complicating the physiological response to facial immersion with stress and fear response. Over the last 20 years we have witnessed significant advances in microprocessors and the scientific literature is rife with data from deployments on a vast array of diving animals. Measurements of velocity, swim speed, depth, and heart rate indicate that the classic mammalian diving response occurs in freely diving animals, but is more plastic and variable than what we have witnessed in the laboratory. Although the profound and dramatic response to facial immersion was somewhat abrogated in the field diving situation, the data from these animals were dramatic in their own right, documenting a life of underwater existence for months at a time, punctuated by surprisingly brief moments on the surface. For an air-breathing mammal to spend 4 to 8 months at sea, and 90% of this time submerged, one cannot fail to be impressed.

The plasticity of the bradycardic response brings into question how these animals modify their heart rate in response to various situations. This particular trace (Figure 12; (21)) shows a depth pattern of an animal diving at about 100 meters when it begins to ascend.

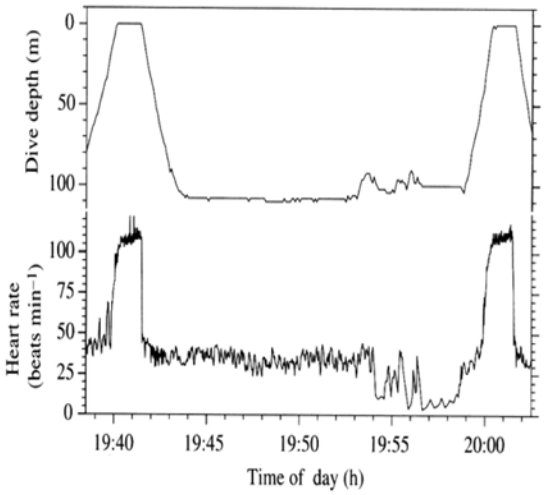

Fig. 12. Dive depth and instantaneous heart rate record from a northern elephant seal. This trace details a single dive. Note that, after ascending for one minute at 19:53:39h, the seal reversed direction. An abrupt decrease in heart rate accompanied the descent. This pattern was repeated 2.5 min later. After Andrews et al, 1997.

After a brief period of ascent, the depth meter indicated a reversal of direction and the animal began to descend, possibly in response to sighting a predator or school of fish. Correlated with that descent was a dramatic bradycardia. This animal's heart rate dropped from approximately 35 bpm down to 4 bpm. The pattern of ascent and descent continued, with concomitant changes in heart rate. Data such as these caused us to speculate on the animal's cognitive control over the diving response.

Cognitive Influence

To examine the cognitive influence on diving bradycardia, we raised 10 harbour seal pups from three weeks of age and using operant techniques and trained them to dive on command for various durations. Heart rates during one-minute apneic periods (slow wave sleep, awake, free dive, trained dive, and forced dive) were recorded and preliminary data are documented below. As expected, animals exhibited the highest heart rate during slow wave sleep, assumed to be the state of lowest cognitive input. Seals normally breathe in an apneustic pattern, pausing on the inhalation for minutes at a time, then exhibiting a rapid breathing pattern before initiating another period of apnea. Heart rates during 1 minute "awake" apneas (55 sec to 1 min 5 sec) demonstrated a greater degree of suppression over sleep apnea. The addition of facial immersion stimuli (i.e. apnea during diving) caused a further decrease in heart rate. Although quantification of "motivation" in a diving seal is impossible, the assumption is that a freely diving seal is less motivated to exhibit a profound bradycardia than a trained or forcibly dived seal. The data reflect this assumption, demonstrating an increase in bradycardia with decreasing control over the dive. In the forced dive situation, the animals were not exposed to any signals or visual cues as to the duration of the dive, therefore we hypothesized that a maximum bradycardic response would be exhibited to aid in survival of what was an unknown period of submergence.

In an attempt to quantify diving "motivation", the seals were trained to recognize two targets: a white circle indicating a short (1 min) dive, and a black square, signifying a long (>5 min) dive. The animals were also trained to surface into a respiratory dome at the completion of the dive. Catheters placed in the extradural intravertebral vein allowed for assessment of lactate. This system enabled the assessment of both the anaerobic and aerobic contribution to diving. In addition to physiological measurements, dives were recorded on video to assess differences in activity in response to the dive duration (Figure 13)**.**

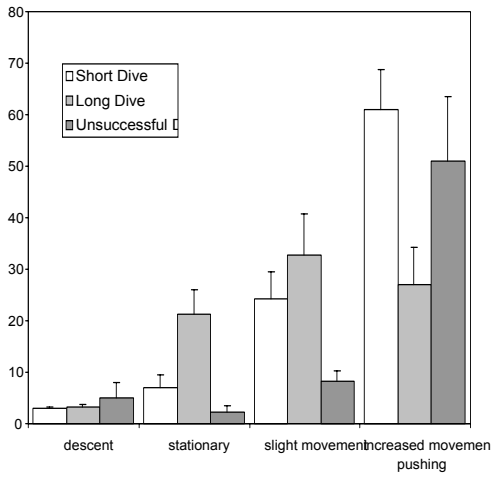

Fig. 13. Dive Assessments**.** Activity during short (1 min), long (>5 min) and unsuccessful (aborted long) trained dives (mean ± SEM; n=6). Short dives are not significantly different from unsuccessful dives in any category (ANOVA, $p<0.05$; Tukey Kramer HSD, a=0.05).

The proportion of time spent in each behavior category varied significantly with the type of dive. When the animal was presented with a short dive target, it would comply by placing its nose to the target and submerging with it to the bottom of the pool. Presentation of the long dive target often resulted in reduced compliance, departure from the pool or training area, or departure from the target once submerged. The behavior during a successful long dive was noticeably different, with a "settling in" period, followed by a reduction in activity for the duration of the dive. Only the first minute of each dive was used for analysis (i.e., short dive activity is compared with the first minute of a long dive).

One interpretation of these data is that in the first part of the dive, the animal makes a "decision" as to whether it will commit to the long dive. In an unsuccessful dive (a long dive aborted within two minutes of submergence), activity remains high and the animal never achieves the "settling in" that is so apparent from the videos of successful long dives. However, one may also hypothesize that the animal has expended too much energy in the first minute and is physically unable to perform a dive of long duration (unlikely, as the oxygen stores in these animals are sufficient to support aerobic dives of ~8-10 minutes).

Metabolically, these dives were aerobic in nature. In both long and short dives, diving and postdive blood lactate levels were not elevated over predive levels (as these dives are within the aerobic diving limit of this species, these data are consistent with our expectations). However, the post-dive oxygen consumption data were very interesting, suggesting that short dives resulted in diving metabolic rates that were in excess of resting metabolic rate. When the excess post-dive oxygen consumption is averaged over the duration of the dive (Figure 14), the data indicate that somewhere between a one minute and a five minute period of submergence, a dramatic suppression of metabolism was occurring. Although intriguing, these experiments were unable to shed further light on the mechanism of metabolic downregulation that occurs in a diving animal.

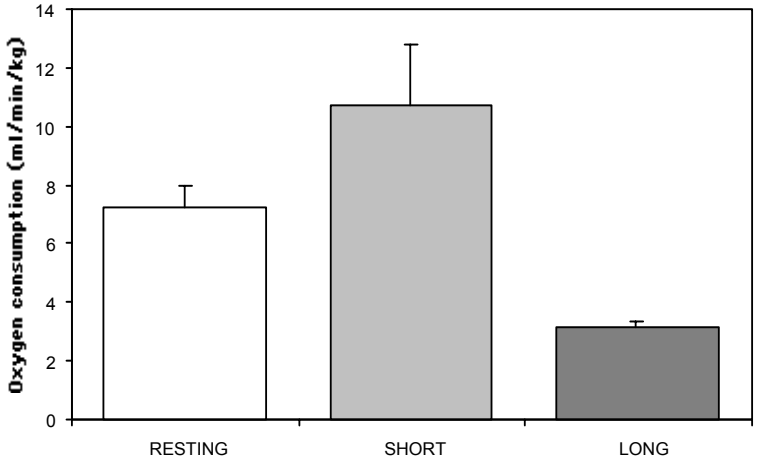

Fig. 14. Diving metabolic rate of harbor seals**.** Diving metabolic rate (ml O_2/kg/min) of harbor seals during resting (in water) and after long (>5 min) and short (1 min) trained dives (mean ± SEM; n=6). The quantity of post dive oxygen in excess of resting (predive) rate was assumed to be the aerobic cost of diving. Mass-specific oxygen consumption is significantly lower during long dives than short dives (t-test; P=0.05).

Phosphocreatine

For a more precise, real time analysis of diving metabolism, we turned again to the high-tech world of magnetic resonance and employed a technique called 31-phosphorous magnetic resonance spectroscopy (^{31}P MRS). This allowed us to interrogate high-energy phosphate flux within the muscle of northern elephant seal pups during a dive. Using the diving helmet described earlier, each seal was subjected to 3 to 5 dives of 8 minutes in duration. Data were acquired using a transmit/receive surface coil (diameters 20 cm and 10 cm respectively) placed on the dorsal surface of the animal. The *longissimus dorsi* muscle was evaluated, as it is the largest muscle involved in phocid locomotion.

The hydrolysis of phosphocreatine (PCr) is coupled to glycolysis through ADP and ATP, and through H^+ (formed during *in vivo* glycolysis) according to the equation,

$$H^+ + PCr + ADP \leftrightarrow ATP + Cr$$

PCr acts as an ATP buffer, maintaining cellular ATP levels when the rate of oxidative phosphorylation is insufficient to meet the short-term requirements. PCr hydrolysis may also be caused by an increased proton load, so if the tissue is affected by respiratory or metabolic acidosis, a decline in PCr may occur.

In diving seals, it has been assumed that muscle hypoperfusion causes a reliance on myoglobin-associated oxygen stores, with anaerobic compensation occurring when tissue oxygen stores are near exhaustion. We evaluated MRS-visible metabolite concentration changes that occur *prior* to tissue oxygen depletion (i.e. within the aerobic diving limit) to assess the hierarchy of metabolic pathways within ischaemic muscle tissue, therefore we did not expect to see significant hydrolysis of PCr in the quiescent muscle of a diving seal.

The results, however, were surprising. End dive PCr values from individual dives ranged from 36.27 – 116.63% of predive values. In 4 animals, PCr declined continuously from Dive Min 1 through 7. These animals should not be exhibiting elevated metabolic rates, as have sufficient myoglobin in their muscles for at least 12 minutes of aerobic metabolism in a completely ischaemic muscle. Yet if we assume that this PCr decline is driven by adenylates and calculate the muscle metabolic rate based on PCr hydrolysis, these seals are actually sprinting! I can assure you they were not sprinting when lying in the magnet; therefore we had to examine alternate explanations.

In the individuals which showed a decline of PCr (and not all animals did), there was a strong positive correlation with intracellular pH. Calculations involving potential sources of proton load indicated that a rise of this magnitude could only be caused by anaerobic metabolism and lactic acid formation. Now we have an animal which was assumed to be quiescent and aerobic, but the PCr data indicated a system that is either highly metabolic or producing copious amounts of lactate. Things were getting even more confusing.

When the data were considered in light of the pH values, a possible explanation emerged. If metabolism were to be inhibited at the mitochondrial level, an accumulation of the products of glycolysis would occur. Pyruvate, in the presence of lactate dehydrogenase, would be converted to lactic acid in the muscle. This mechanism operates under both aerobic and anaerobic glycolytic function (22, 23), would result in lactate accumulation in both tissue and blood, and could explain the elevated proton load in the muscle. However, blood samples obtained from five animals showed no significant increase in blood [La^-] during the dive or in the postdive period.

The presence of elevated [H^+] without a concurrent increase in blood [La^-] is possibly due to lactate recycling to glycogen or lactate oxidation to CO_2 and H_2O within the muscle. It has been assumed that the anaerobic contribution to energy production during diving would be

minimal until tissue oxygen stores are depleted. However, our data suggest that significant lactate production occurs *prior* to muscle oxygen depletion and must occur concurrently with oxidative phosphorylation. In an ischemic muscle, glycolytically generated H^+ would be trapped within the tissue and although buffered, would contribute to the observed pH drop and a shifting of the creatine kinase reaction to the right. In the muscle of a diving seal, the rate of lactate formation may be high enough to account for an elevated [H^+], but low enough to allow for complete further metabolism of lactate to glycogen or to complete oxidation during the course of a dive.

Metabolic Downregulation: Future Directions

This led us to think about possible mechanisms of metabolic downregulation that may occur at the cellular level. To evaluate the mechanisms behind metabolic downregulation in a diving seal, my recent research has moved away from whole animal work and is now focussing on mitochondria.

Recent discoveries in the field of metabolic control indicate that mammalian tissues are not efficient consumers of oxygen. Oxygen is required in order to create the electrochemical gradient of protons across the mitochondrial inner membrane. These protons are then channelled through a chemiosmotic pump termed an ATPase, which is analogous to a hydroelectric dam. Following the same principle, if there are leaks in the dam, energy is lost and the system becomes less efficient. A futile cycle of outward proton pumping and inward proton leak occurs across the membrane, resulting in both heat production and the imperfect coupling of oxygen consumption to ATP synthesis. This proton leak is thought to account for 15-20% of standard metabolic rate (SMR) and represents a biochemical "inefficiency" in the system (24). There are many suppositions as to why this inefficiency exists, but at this time a definitive answer has not been achieved.

Mammals are also are quite inefficient at the cell membrane level. Tony Hulbert and Paul Else (25-28) investigated the cellular metabolic differences between ectotherms and endotherms and discovered that ectotherms are more metabolically efficient. They exhibit a reduction in ionic leak through their cells by maintaining "tight" cell membranes. Mammals, on the other hand, have a greater degree of leakiness in our cell membranes. In order to maintain membrane gradients, our pumps are constantly working overtime and as a consequence, energy (in the form of heat) is released. Hulbert and Else hypothesized that the reason why mammalian cells leak is for the purpose of heat production and the maintenance of endothermy. They found that the factor that correlated most strongly with the leakiness of the membranes is the degree of polyunsaturation in the lipid bilayer.

Up to 60 to 80% of the metabolic rate in a mammalian liver cell is due to maintaining the membrane gradient. The ability to decrease both cellular and mitochondrial leaks during times of reduced oxygen availability would provide a means of reducing oxygen consumption without sacrificing cellular performance. In perfused rat skeletal muscle, 50% of the resting respiration is attributed to proton leak (29). In a Weddell seal, muscle accounts for 35% of total body mass; therefore a reduction in proton leak could result in a significant decrease in whole animal oxygen consumption during diving.

To evaluate the effect of the diving environment on mitochondrial respiration, I am heading down to Antarctica to obtain muscle biopsies from Weddell seals. In a diving animal, vasoconstriction of blood vessels in the periphery, combined with conductive heat loss in water result in >10˚C temperature drop in the muscle bed (30). In Weddell seals, muscle temperature

has not been measured, but the conductive heat loss caused by immersion in -1.8˚C water, combined with significant peripheral vasoconstriction due to the mammalian diving response, are likely to result in dramatic reduction in temperature at the muscle-blubber interface (Figure 15). By subjecting seal muscle cells to reduced temperatures and increased atmospheric pressures, the effects of the diving environment on cellular respiration may be obtained. The second phase of this study involves measuring proton leak under similar conditions, thus enabling the quantification of mitochondrial efficiency at the cellular level.

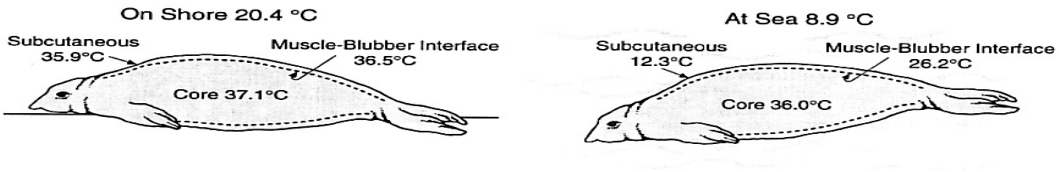

Fig. 15. Diagrammatic summary of temperature profiles of northern elephant seal. Diagrammatic summary of temperature profiles in the body of a northern elephant seal at rest in normoxia, normothermia (on shore) contrasted with regional heterothermy observed during diving at sea, assuming an average water temperature of 8.9˚C (after Andrews, 1999).

A number of other factors may relate to mitochondrial efficiency, one being uncoupling proteins (UCPs), which bypass the ATPases and allow protons to leak through the membrane. Uncoupling protein 3 (UCP3) is found in skeletal muscle and may play a role in metabolic regulation at the cellular level. Quantification of UCP3 mRNA and protein levels in seal muscle will be conducted to reveal a possible role of UCPs in the metabolic plasticity of this species.

In closing I would just like to say that we're very pleased with how far the field has come and especially with our interaction between field physiology and technology and that we've been able to bring these two fairly diverse fields together to aid in our understanding of how oxygen is used in a diving animal.

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