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Development of myoglobin stores in skeletal muscles of hooded seal (*Cystophora cristata*) pups

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Abbreviations

ADL	aerobic dive limit
CS	citrate synthase
CO	carbon monoxide
CV	coefficient of variance
EC	enzyme commission number
Hb	haemoglobin
Hct	haematocrit
HIF-1	hypoxia-inducible factor-1
HOAD	β-hydroxyacyl coenzyme A dehydrogenase
IU	international units for enzyme activity (μM/ml/min/g tissue)
LD	*Musculus longissimus dorsi*
LDH	lactate dehydrogenase
Mb	myoglobin
O_2	oxygen
RPM	revolutions per minute
SSP	*Musculus supraspinatus*
TBO	total body oxygen
TP	total protein

Abstract

The hooded seal is a deep diving phocid seal in the North Atlantic Ocean, possessing the highest oxygen storing capacity and the shortest lactation period of any mammal hitherto reported. Pups are not born expert divers and have to develop relevant physiological adaptations quickly in order to forage independently. To investigate the early development of myoglobin (Mb), a key molecule for diving adaptation, muscles from weaned hooded seal pups were sampled for a period of three months. This revealed a rapid initial rise of Mb levels within the first month accounting for 50 % of the Mb development of the entire first year of life. This developmental pattern coincides with the increase of dive duration of free living hooded seal pups, suggesting that the Mb level influences their diving behavior.

To investigate if activity regulates Mb production, the swimming muscle *M. longissimus dorsi* and flipper muscle *M. supraspinatus* were examined as well as key enzymes for muscular metabolism. This showed that active muscles develop faster and have higher Mb concentrations than idle muscles whereas there is no difference in muscles of similar activity. This suggests that activity rather than hypoxia is influencing the post natal increase of Mb in seals.

I. Introduction

The marine world, especially in the Arctic, represents a challenging environment for mammals. To exploit the food resources of the seas, marine mammals have to spend considerable parts of their life underwater and hence are challenged with insufficient oxygen supply, or hypoxia (Ramirez et al., 2007), on a regular basis. Research over the last century has revealed many fascinating adaptations of marine mammals that allows them to cope with the challenges of underwater activity (for review see: Butler and Jones, 1997; Ramirez et al., 2007; Folkow and Blix, 2010).
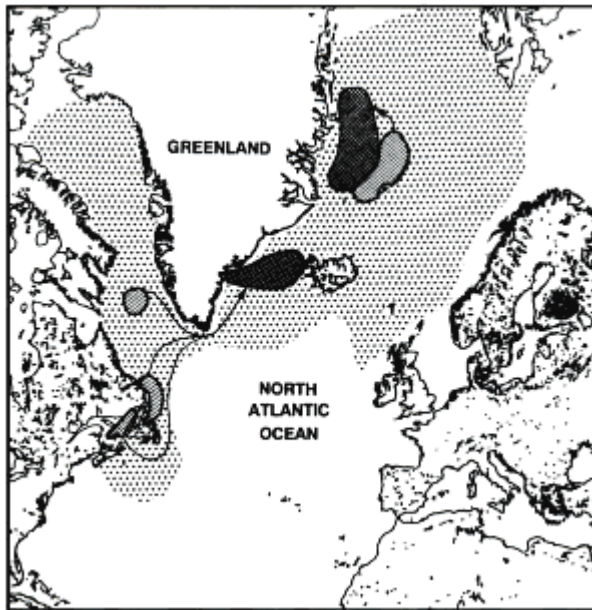
The hooded seal (*Cystophora cristata,* Erxleben, 1777) is a deep diving phocid seal in arctic waters. It reaches depths of more than 1000 m, for durations up to one hour (Folkow and Blix, 1999). This requires extreme physiological adaptations and one of the most important one for diving mammals is the capacity to store large amounts of oxygen. The hooded seal in fact, possesses the largest oxygen stores per body mass hitherto reported in mammals (Burns et al., 2007).

Not being born expert divers, newborn animals have to develop those adaptations in order to be able to forage independently and efficiently. This has to happen as fast as possible, especially for seals in the arctic, breeding on unstable ice floes, to minimize the costs for the mother and ensure survival of the pups. Here again the hooded seal is extreme; having the shortest lactation period of any mammal lasting only three to four days (Bowen et al., 1985) they cannot rely on maternal support while growing up; hence they have to become independent foragers within the shortest time. This requirement of fast maturation, in connection with their extreme diving behaviour, makes the hooded seal an excellent species to study the development of diving adaptations.

Life history and development

The hooded seal is an arctic pinniped species belonging to the family of true seals or earless seals (Phocidae). The average length and weight of adults is about 2 m and <200 kg for females and 2.5 m and <400 kg for males (Rasmussen, 1960; Folkow et al., 1996). Their distribution in the north Atlantic (fig 1.1) stretches from waters off Newfoundland in the south-west, to the waters around Svalbard in the north-east, including waters around the west and east coasts of Greenland (Davis Strait, Greenland Sea) (Folkow et al., 1996).

Two main breeding stocks have been reported, one east of Newfoundland and one in the West-ice north-west of Jan Mayen (Reeves and Ling, 1980; Kovacs and Lavigne, 1986; Folkow et al., 1996). The following description accounts for the West-ice population, which has been the subject of study in the present project.

Breeding takes place in late March / early April when females give birth on heavy pack-ice to a single 20 ± 10 kg pup. Pups are extremely precocial (Bowen et al., 1985; Bowen et al., 1987). They are born with an insulating blubber layer and a short haired fur as they shed their lanugo fur already in utero (Blix and Steen, 1979; Kovacs and Lavigne, 1986). This enables them to tolerate water contact right after birth. They are capable of coordinated motor control and can swim from the first day on (Kovacs and Lavigne, 1986).

Fig. 1.1 Distribution of hooded seals in the north Atlantic (lightly dotted) with breeding grounds (medium dotted) and molting areas (densely dotted). Arrows indicate main migration routes from breeding grounds to molting areas (Folkow et al., 1996). Figure from Blix (2005).

During the short lactation period the pup gains on average 7.2 kg per day (Bowen et al., 1985) and hence more than doubles its weight. Most of the energy transferred from mother to pup is being stored in the pups blubber layer. This provides it with an energy reservoir sufficient for an up to one month lasting post weaning fast (Bowen et al., 1985; Oftedal et al., 1993). Right after weaning the mother abandons the pup and mating occurs followed by excursions into large areas for foraging before they arrive at the moulting areas in the Denmark strait and north of Jan Mayen between late June and early August (Øritsland, 1959; Rasmussen, 1960; Folkow et al., 1996).

Meanwhile, the newborn pups enter the water within a few days after weaning and start to dive (Folkow et al., 2010). The diving duration and depth increases rapidly, exceeding durations of 15 min and depths of 100 m within the first 3 weeks of life (Folkow et al., 2010). After approximately one month the pups leave the ice edge, following a similar migration pattern to the adult animals of the West-ice stock (Folkow et al., 2010). Hooded seals have their first moult at the age of ~14 months and are sexually mature between 2 and 9 years of age (Kovacs and Lavigne, 1986).

The rapid development of diving behaviour in hooded seal pups suggests a similar rapid maturation of their corresponding physiological adaptations.

Diving adaptations

Kooyman and coworkers (1980) showed that the majority (~90 %) of dives in Weddell seals (*Leptonychotes weddellii*) lasted less than 20 min, while some dives lasted more than an hour. Additionally they found no elevated blood lactate concentration after those short dives, while blood lactate accumulated dramatically with increasing diving duration beyond ~20 min (Kooyman et al., 1980). Since lactate is a product of anaerobic metabolism, he reasoned that metabolism in short dives is primarily aerobic, while long dives require anaerobic ATP production. The time, up to which there is no increase in post dive blood lactate concentration, he described by the term 'aerobic dive limit' (ADL).

It can take up to six times the duration of an anaerobic dive to metabolize accumulated lactate (Kooyman et al., 1980) but only a few breaths to renew depleted blood oxygen stores if no lactate build-up has occurred. Thus, the usual behaviour of many short dives within the ADL allows marine mammals to stay submerged for the longest possible time and hence forage more efficiently (Kooyman et al., 1980; Kooyman and Ponganis, 1998; Davis and Kanatous, 1999; Hindell et al., 2000).

To be able to stay submerged for extended periods, marine mammals have various physiological adaptations. Those include altering of circulatory patterns (e.g. peripheral vasoconstriction), drop of body temperature and bradycardia, all leading to low metabolic rates which may be similar to resting metabolic rates or lower and resulting in a reduced oxygen consumption during dives (Scholander, 1940; Blix and Folkow, 1983; Castellini et al., 1992; Ponganis et al., 1997; Hurley and Costa, 2001; Ramirez et al., 2007). Low oxygen consumption, however, is not sufficient to maintain prolonged diving; hence it is not surprising to find large oxygen stores in diving mammals. Those are not only generally higher than in terrestrial mammals, but also distinctively distributed.

Generally, oxygen stores are found in the lung, blood, and muscle compartments. While the lungs are quite important oxygen stores for most terrestrial mammals, for marine mammals they represent just a fraction of the total body oxygen stores (TBO) (Scholander, 1940). In hooded seals they represent only 7 % of TBO (Burns et al., 2007). In addition the lungs collapse 25-50m below sea level in deep diving seals (Falke et al., 1985), decreasing the function as oxygen stores even further, but also reducing the risk of nitrogen narcosis and gas bubble formation due to decompression sickness or so called 'diver's sickness' (Scholander, 1940; Kooyman and Ponganis, 1998).

The blood with the oxygen binding molecule haemoglobin (Hb) serves as a far more important O_2 storage. This is represented by increased Hb concentration and blood volume,

resulting in three to four times higher blood O_2 stores (on ml/kg basis) in diving than in terrestrial mammals (Snyder, 1983; Ramirez et al., 2007). It composes 51 % of TBO in hooded seals (Burns et al., 2007). In hooded seal pups those stores are similar to adult values, indicating a development already in utero (Burns et al., 2007).

Another hallmark of hypoxia defence in diving mammals is an elevated myoglobin (Mb) concentration in skeletal muscles, being 10-30 times higher concentrated in aquatic animals than in their terrestrial counterparts (Kendrew et al., 1954; Kooyman, 1989; Kooyman and Ponganis, 1998; Noren and Williams, 2000).

Myoglobin is a cytoplasmatic haemoprotein in cardiac myocytes and skeletal muscles. Like haemoglobin it reversibly binds oxygen. Both molecules contain an iron-porphyrin complex known as the haem group, which actually combines with O_2 (Kendrew et al., 1958; Wittenberg and Wittenberg, 2003). Unlike Hb, Mb contains only one haem group, which is responsible for the more hyperbolic O_2 binding curve of the monomeric Mb vs. the sigmoid shaped binding curve of the tetrameric Hb (Collman et al., 2004; Ordway and Garry, 2004). The resulting higher affinity of Mb in muscular tissue supports the extraction of blood O_2 (Wittenberg, 1970). An increasing lactate level, as it occurs in anaerobic muscular tissue, facilitates O_2 release from Mb (Giardina et al., 1996). It is classically seen as an oxygen storing molecule (Irving, 1939; Scholander, 1940; Scholander et al., 1942; Kooyman and Ponganis, 1998; Noren et al., 2005). Recent research, however, expands the function of Mb and it is suggested to facilitate oxygen diffusion from the blood to the mitochondria of muscle cells, and to serve as a buffer for O_2 supply if blood O_2 delivery decreases (Wittenberg, 1970; Brunori, 2001; Wittenberg and Wittenberg, 2003; Ordway and Garry, 2004; Ponganis et al., 2008).

Mb bound oxygen represents 42 % of TBO in hooded seals who have the highest yet reported Mb concentration of all marine mammals (Burns et al., 2007). They have the highest Mb levels in the primary locomotory muscles, e.g. the *M. longissimus dorsi* (Lestyk et al., 2009), probably due to the high energy demand of those most active muscles.

Mb concentrations in newborn animals, however, are less than 25 % of adult values, hence reducing their capacity of O_2 storage in the muscles (Burns et al., 2007). The mechanisms and timing of development of those stores towards adult levels is the central theme of the present thesis, as further outlined below.

Hypotheses

Despite the fact that Mb values in hooded seal pups are relatively low at birth, they display a rapid increase in diving duration and depth. A detailed inquiry of early Mb development in seals has, however, not been done so far.

Since Mb levels are an important indicator of diving adaptation, the present thesis investigates the initial development of Mb levels in the skeletal muscles of hooded seal pups during the first three months after weaning.

Furthermore this thesis addresses the question why those muscle oxygen stores are not as well developed as the blood O_2 stores in newborn hooded seals.

The development of the blood oxygen stores is mainly triggered by the hypoxia-inducible factor-1 (HIF-1), which regulates among others the expression of erythropoietin and hence the increase in [Hb] (Gassmann and Wenger, 1997). Hooded seal pups may be already exposed to hypoxia in utero, e.g. when the mother dives. This might trigger the expression of HIF-1 sufficiently for the development of the blood O_2 stores before birth.

Mb expression, however, is regulated by calcium signaling which in turn is partly triggered by hypoxia, but only in combination with muscular exercise (Kanatous et al., 2009; Wittenberg, 2009). Since movement is quite restricted for the hooded seal foetus, muscular activity might be the missing trigger for Mb development in utero.

Accordingly, this thesis examines if activity, rather than hypoxia, is the main trigger for postnatal Mb synthesis in hooded seal pups.

As muscle enzymes can be important indicators of the metabolic activity of the tissue they may tell us something about the activity levels of the muscles in hooded seal pups. Therefore this thesis examines the activity levels of key enzymes to underline the activity pattern in different muscles of hooded seal pups.

Lactate dehydrogenase (LDH) is an enzyme which reduces pyruvate to lactate to support anaerobic ATP production via glycolysis (Kaplan, 1964). Additionally lactate is used for energy production in the muscle itself (Fuse, 1999). LDH is thus often used as an indicator for anaerobic metabolism in seals (Blix and From, 1971; Kooyman et al., 1980; Hochachka and Somero, 2002; Polasek et al., 2006; Kanatous et al., 2008; Prewitt et al., 2010).

Citrate synthase (CS) is the first enzyme of the citric acid cycle and catalyzes the conversion from oxalacetate and Acetyl CoA to citrate. This step determines the flux through the citric acid cycle (Wiegand and Remington, 1986) and CS is frequently used as an indicator for aerobic metabolism (Hochachka and Somero, 2002; Polasek et al., 2006; Burns et al., 2010).

β-hydroxyacyl coenzyme A dehydrogenase (HOAD) is involved in the β-oxydation of fatty acids and often used as an indicator for the relative use of lipids for aerobic metabolism (Hochachka and Somero, 2002; Polasek et al., 2006; Burns et al., 2010; Prewitt et al., 2010).

The ratio of LDH to CS gives information about the tendency to use a more anaerobic (higher LDH) or a more aerobic (higher CS) metabolism for ATP production (Polasek et al., 2006; Burns et al., 2010; Prewitt et al., 2010).

In general there is a higher oxidative enzyme activity in muscles used for sustained locomotion than in muscles with less sustained activity (Close, 1972; Pette and Staron, 1990). A shift towards a more oxidative enzyme activity could therefore be an indication for higher muscular activity. Additionally, since Mb is important for oxygen delivery in skeletal muscles, a shift towards more oxidative metabolism could confirm Mb increase.

The ratio of CS to HOAD gives information about the energy source for ATP production, being carbohydrates in the case of low HOAD levels in comparison to citrate synthase and vice versa (Polasek et al., 2006; Burns et al., 2010; Prewitt et al., 2010).

Since working seal muscles rely mainly on aerobic lipid based metabolism for sustained activity (Reed et al., 1994; Polasek et al., 2006; Kanatous et al., 2008), a shift towards a more lipid based metabolism could be an additional hint towards higher activity.

II. Material and methods

Design of Study

To investigate Mb concentration and enzyme activities, muscle samples were collected at regular intervals from weaned hooded seal pups until they were three months old. To acquire more detailed information about the initial Mb increase, the sampling was more frequently performed at the beginning of the sampling period.
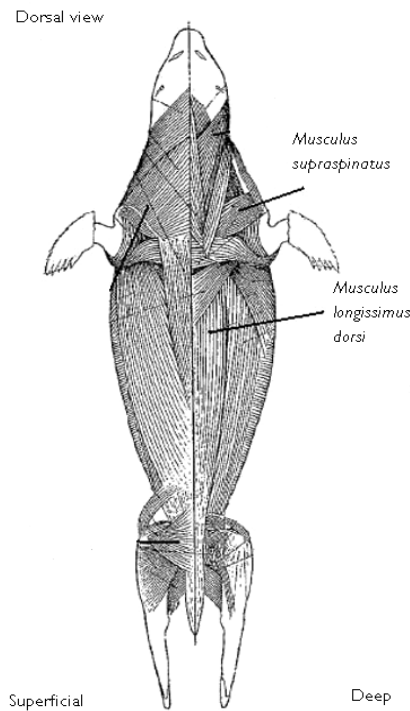
Fig 2.1 Skeletal muscle anatomy of seals, dorsal view, showing the two muscles sampled for this thesis. From Howell (1929)

To investigate the influence of activity and hypoxia on the development of Mb levels, two groups of animals were placed in different conditions: one group was kept in a pool and thus had swimming/diving activity while the other group was kept on land, to prevent swimming/diving activity.

In addition, muscle samples were taken from two different locations; the main swimming muscle *M. longissimus dorsi* (LD) and the flipper muscle *M. supraspinatus* (SSP) (fig 2.1).

Animal handling

Eight hooded seal pups (tab 2.1) were collected in the pack ice north-west of Jan Mayen (West-ice) during a research cruise with the R/V 'Jan Mayen' end of March 2010 under the permits from Norwegian and Greenland authorities. The animals collected were weaned and weighed ≥ 38.5 kg. The pups were brought to Tromsø/Norway where they were kept at the approved research animal facilities at the Arctic Biology building of the Department of Arctic and Marine Biology. They were held in two groups: four had access to a 40'000 L sea-water pool (pool group) and four on a snow covered outdoor area (land group). The land group got access to a second 40'000 L sea-water pool when, at the end of May (day 61 since the first sampling), the snow cover on which the animals were maintained had almost melted completely. The pools were equipped with a water cleaning system (650 l/min) and the
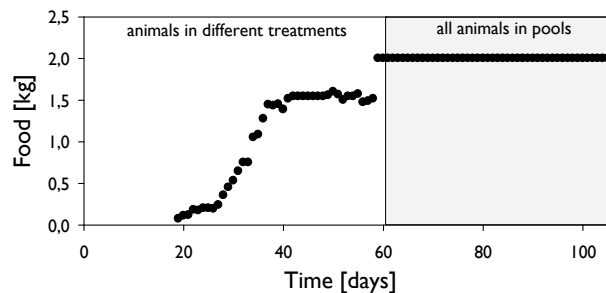
water temperature was kept at ~5°C. A wooden platform in the pools allowed the seals to haul out of the water.

Tab 2.1 Origin of the eight seal pups. All pups were weaned and hence already abandoned by the mother

Seal ID	Date of capture	Position of capture	Weight at capture [kg]	Sex	Group
K1/10	26.03.10	71°53'N, 14°20'W	46.5	♀	pool
K2/10	26.03.10	71°51'N, 14°23'W	51.5	♂	pool
K3/10	27.03.10	71°40'N, 14°26'W	45.0	♂	pool
K4/10	27.03.10	71°34'N, 14°43'W	40.5	♂	land
K5/10 [†]	27.03.10	71°34'N, 14°38'W	44.5	♂	land
K6/10	27.03.10	71°34'N, 14°38'W	44.5	♂	pool
K7/10	23.03.10	71°39'N, 13°32'W	42.5	♂	land
K8/10	22.03.10	71°47'N, 13°58'W	38.5	♂	land

† animal deceased the 10th of Mai 2010

Feeding started the 23rd day after the first sampling. The amount of food was kept low until all animals started to feed voluntarily and then gradually increased. From day 40 onwards the amount of food was kept on a steady level (fig 2.2). The first food was small capelin (*Mallotus villosus*), later herring (*Clupea harengus*) (as soon as they could eat the bigger fish i.e. ~ from day 28 onwards) with vitamin and mineral supplement (Sea Tabs® II for marine mammals, Pacific Research Laboratories, CA, USA). All experiments were approved by the National Animal Research Authority of Norway (permit # 2402).

Fig 2.2 Average amount of food consumed by the hooded seal pups (per animal). Day zero marks first sampling date and aprox. age of 5-10 days.

Sample collection

The sampling began the 27th of March 2010, which was set to day zero in this study. The hooded seal pups had an age of 5-10 days at that time. The sampling period for this study lasted from day 0 until day 87 when the animals were approximately three months old. Further sampling was performed until the animals were one year old to get an overview of the entire first year (not in the scope of this study). The initial sampling was done in 10 day

intervals to get a more detailed overview over that time period, i.e. until day 30. The following sampling was done on day 30, 44, 61 and 87 after the first sampling.

The first sampling was done on board R/V Jan Mayen in the field; the remaining sampling was performed in Tromsø/Norway at the research animal facilities at the Arctic Biology building of the Department of Arctic and Marine Biology, University of Tromsø (UiT).

Prior to sampling the animals were sedated with an intra muscular injection of Zoletil Forte Vet (1-1.5 mg/kg; tiletamin-zolazepam, Virbac, Carros Cedex, FR). The sedated animals were then transported to the laboratory/operation room, weighed (model 235 suspended weight; Salter, UK), and the locations for the biopsies cleaned and disinfected with Klorhexidin (5%, Fresenius Fabi, NO).

Blood samples were collected with a central venous catheter (Secalon T, 16G/1.7x160mm, Becton Dickinson, SG) from the extradural intravertebral vein, 30-40 cm above the tail of the animals. The catheter was also used for additional intra venous injections of the sedative (0.5-0.7 mg/kg) when the previous dose wore off. The blood was centrifuged in haematocrit (Hct) glass capillaries (Brand, GER) using a EBA 12 Hct centrifuge (Hettich, GER) for Hct measurement (10k RPM for 15 min) and in heparinised vials (BD vacutainer, UK) using a vial centrifuge (KS-8000, Kubota, JP) for plasma collection (3k RPM for 10 min). The plasma (supernatant) was frozen subsequently at -80°C for future analyzing (not in the scope of this thesis).

Muscle biopsies were collected from the LD and SSP under local anaesthesia (subcutaneous) with ~3 ml Xylocaine (10mg/ml; Astra Zeneca Södertälje, SE). A small incision was made with a scalpel (blade No. 11) to be able to penetrate the subcutaneous blubber layer. The sample was then collected using a sterile one way 6 mm biopsy punch (Miltex, PA, USA). A slight vacuum was applied with a 10 ml syringe to the punch to ensure effective sampling. The samples had a volume of 1-2 µl and were immediately frozen at -80°C. Thereafter the incision wounds were closed, using resorbable endo stitch type sutures (polysorb lactomer 9-1, Tyco, USA).

For the sake of sterility the sterile biopsy punches, catheters and needles were only used once. All other tools were disinfected with 95% ethanol prior to use.

During all handling (incl. everyday feeding) the animals behaviour was routinely observed. Until day 40 the general behaviour and activity level was observed every day for at least 30 min and a summary was noted.

Biochemistry and analyses

Analysis

All muscle sample analyzes were performed during a scientific visit in J.M. Burns laboratory in Anchorage, AK at the facilities of the department of biology at the University of Anchorage, Alaska (UAA), USA. Besides enzyme and Mb analyzes, total protein (TP) content was measured to normalize the results in the case of age related increase of tissue TP (Burns et al., 2010).

Frozen muscle samples were thawed, cleaned from connective tissue and blood and then sonicated (Fisher Scientific, Sonic Dismembrator model 500, NJ, US) in ice-cold buffer (description below) until no chunks were left in the solution.

For Mb and TP analyzes a 0.04 M phosphate buffer (19.25 ml/g tissue, pH 6.6) was used as described previously (Lestyk et al., 2009).

For the more sensitive enzyme assays muscle samples were initially homogenized in an imidazole homogenization buffer (50 mM imidazole hydrochloride, 1 mM EDTA, 2 mM $MgCl_2$, pH 7.0 at 37°C) as described previously (Polasek et al., 2006; Prewitt et al., 2010).

This buffer was also used for additional Mb and TP analyses from the same muscle samples. The different buffers give the same results for Mb (Richmond, 2004) as well as for TP (Lestyk K., personal communication). Paired t-tests on the results of this study confirmed (r = 0.88, p < 0.001) that both buffers give the same results for Mb and TP.

The samples were then centrifuged (Marathon 3200R, Fisher Scientific, NJ, US) at 10'000 RPM for 5min at 4°C and the supernatant used for Mb, TP and enzyme assays. The assays (described in detail below) were read at various wavelengths using a plate reader (Spectra Max 340PC, Molecular Devices, CA, USA).

Myoglobin

Myoglobin (Mb) concentration was determined as described by Reynafarje (1963) and Lestyk et al. (2009): the supernatant (from both initial buffers, see above) was transferred to a 96 well flat bottom immuno plate (Nalge Nunc int., Rochester, NY, USA) and diluted further to a total volume of 110 μl in 0.04 M phosphate buffer (pH 6.6) to adjust for appropriate optical density (i.e. an OD within the reading range of the plate reader). The plate was then placed in a vacuum chamber which was first gassed with CO (99.5 %) for 30 sec, then filled with CO for 15 sec and closed. After 20 min of CO incubation 10 μl of 10 % sodium dithionite solution was added and the plate vortexed to ensure full reduction of Mb for correct absorption measurement. After an additional 5 min of CO incubation the OD was read at λ 538 nm and 568 nm (see below). Assays were run in triplicates and each run included lyophilized horse standards and tissue controls from an adult harbour seal (*Phoca vitulina*) with known Mb levels (Burns et al., 2007) to validate the results. To estimate the variance within the muscles, three samples from the sampling locations of both the LD and SSP were collected from the deceased animal K5/10 (tab 2.1) and analyzed. The precision of the assay was estimated from the tissue control.

Mb concentration was then calculated following the established Reynafarje method (Reynafarje, 1963; Polasek et al., 2006; Burns et al., 2007; Lestyk et al., 2009).
It is assumed that the supernatant from blood perfused muscular tissue contains both HbCO and MbCO as pigments, if treated with pure CO as described above. Such a supernatant has two absorption peaks, one at 538 nm and one at 568 nm (Reynafarje, 1963). The following equation describes the signal intensity difference at 538 nm and 568 nm between Hb and Mb

$$OD_{538} - OD_{568} = \left(\varepsilon_{538,HbCO} - \varepsilon_{568,HbCO}\right) * C_{HbCO} + \left(\varepsilon_{538,MbCO} - \varepsilon_{568,MbCO}\right) * C_{MbCO} \qquad \text{(Eq. 1)}$$

Where OD = optical density, ε = extinction coefficient and C_{HbCO} and C_{MbCO} are the concentration of HbCO and MbCO respectively (mol/L).
The Reynafarje method further assumes an identical Hb extinction coefficient at 538 nm and 568 nm, so the first term on the right cancels. Also it assumes for Mb an extinction coefficient of $14.7*10^3$ cm^{-1}M^{-1} at 538 nm and $11.8*10^3$ cm^{-1}M^{-1} at 568 nm. This leads to the following calculation which was used to calculate Mb concentration (mol/L):

$$C_{MbCO} = \frac{OD_{538} - OD_{568}}{14.7*10^3 - 11.8*10^3} = (OD_{538} - OD_{568}) * 3.45 * 10^{-4} \qquad \text{(Eq. 2)}$$

Enzymes

Enzyme in vitro activities were measured at 37°C for citrate synthase (CS), β-hydroxyacyl coenzyme A dehydrogenase (HOAD) and lactate dehydrogenase (LDH) under substrate saturating conditions, following previously described methods (Polasek et al., 2006; Burns et al., 2010; Prewitt et al., 2010). The assay mix formulae were as following:

For CS (EC 4.1.3.1): 0.25 mM 5,5'-dithio-bis(2-nitrobencoic acid), 0.4 mM acetyl CoA, 0.5 mM oxalacetate, 50 mM imidazole buffer pH 7.5 at 37°C, ΔA_{412}, millimolar extinction coefficient ε_{412}=13.6.

For HOAD (EC 1.1.1.35): 0.3 mM NADH, 1 mM ethylenediaminetetra-acetic acid (EDTA), 0.2 mM acetoacetyl CoA (trisodium salt), 50 mM imidazole buffer pH 7.0 at 37°C, ΔA_{340}, millimolar extinction coefficient ε_{340}=6.22.

For LDH (EC 1.1.1.27): 0.3 mM NADH, 1 mM pyruvate, 50 mM imidazole buffer pH 7.0 at 37°C, ΔA_{340}, millimolar extinction coefficient ε_{340}=6.22.

Each assay mix was prepared in aluminium foil wrapped flasks just prior to analyzing; the supernatants were diluted with imidazole buffer (pH 7 for CS, pH 7.5 for LDH and HOAD) to the appropriate concentrations to produce an optimal reaction (see below) and 10 μl of each sample dilution were put in 4 wells of a pre-heated (37°C) 96 well plate. The assay mix was then quickly heated to 37°C and 150 μl of it were added to the wells with the diluted supernatant. The plate was then instantly read for 180 sec in 5 sec intervals in the pre-heated (37°C) plate reader (Spectra Max 340PC, Molecular Devices, CA, USA). Absolute activities were calculated from the change in absorbance at the maximal linear slope of the assay reaction (Lineweaver and Burk, 1934; Burns et al., 2010). Harbour seal tissue of known enzyme activities (Burns and Lestyk, unpublished data; Prewitt et al., 2010) was used as tissue control. The assay run was accepted if the enzyme activity produced a maximal linear slope, the tissue control was within the expected activity range and the results had a CV of less than 10 % (based on a triplicate run). Precision of the assays was calculated from the tissue control values.

Total Protein

Total protein (TP) content was determined using the Pierce Coomassie Blue 'The Better Bradford' Total Protein Assay (Pierce Chemicals, Rockford, IL, USA). 10 μl of the initial supernatant was diluted to the concentration range of the assay in 0.04 M pH 6.6 phosphate buffer. 10 μl of the resulting ~300x dilution was pipetted onto a 96 well plate and mixed with 300 μl dye. After 10 min incubation the OD was read at λ 595 nm. TP levels were

calculated from plate specific standard curves derived from bovine serum albumin standards. A tissue control of an adult harbour seal with known TP levels (as determined by the same Bradford kit) was included in every run, and runs only accepted if the standards had a precision >98 %.

Data handling and statistics

The data for the different age classes of this project is coming from repeated measurements of the same animals and is hence not independent while the data for each age class in itself is independent. To take care of those properties a linear mixed model approach was used and a p-value of 0.05 (95% confidence level) was set as threshold for significance. For all analyses the data was graphically screened (histogram) for equal distribution. For the analyzes on the effect of group location, only the data until sample day 60 was used since after that day the land group got access to a pool.

To determine the effect of muscle type, group location and time of the development of Mb, Enzymes, Hct and TP, each was tested in a full model including all parameters as fixed factors. If the muscle type had a significant effect, i.e. there was a significant difference between the muscle types, the data was split and analyzed for both types separately. If the group location had a significant effect, the file was split further and analyzed for each group to determine the effect of time in the model, as well as performing a pair-wise comparison of the different age classes. To determine the effect of group location for each age class the data was split to analyze each separately. If a parameter had no significance, it was removed from the model. Pairwise comparisons were adjusted for multiple comparisons with the Bonferroni method. Values are given in mean ± standard deviation unless otherwise noted.

All statistical analyses were made using SPSS v.19.0 (SPSS inc, Chicago, IL).

III. Results

General data

Body mass

Body mass of the hooded seal pups decreased during the fasting period from 44.2 ±3.8 kg at day 0, by approximately 30 % to 32.3 ±2.2 kg at day 30 and rose after feeding start again to 39.4 ±1.7 kg at day 87 (fig 3.1). The animals from the pool group tended to be slightly heavier at the beginning (5.3 ±1.3 kg at day 0) and their body mass tended to increase faster after the feeding started than the body mass of the land group. From sampling day 61 (pool – land = 1.4 ±1.0 kg) on both groups tended to have the same body mass.

Observed behaviour

The pool group had an overall higher activity than the land group and the animals were diving frequently.

There was no sleep apnea (Castellini, 1996) observed for neither group, which, however, cannot be excluded entirely, since no data for hooded seal pups is available.

The animals on land were propelling themselves mostly by undulating body movements. Moreover the flippers were not used as much as expected for movement on land, the undulating movement seemed to be employed often exclusively.
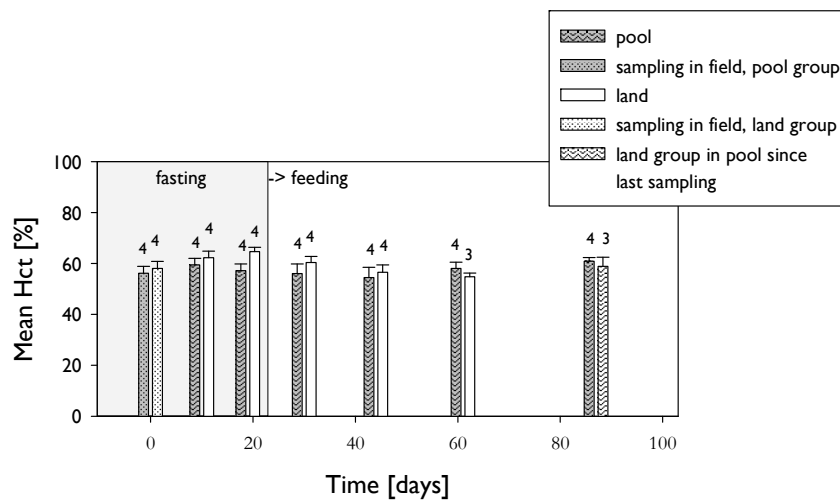
Haematocrit

Generally the levels remained stable over the whole sampling period, ranging around 59 % ($mean_{pool}$= 58.561 ±0.333 %, $mean_{land}$= 59.815 ± 0.358 %) (fig 3.2). The haematocrit (Hct) level was statistically significant higher in the land group than in the pool group ($F_{1, 70}$= 16.713, p < 0.001), that difference is, however, rather small (average Hct_{land}-Hct_{pool}= 1.253 ± 0.489 %). Time had a significant influence on the mean Hct of both groups (pool: $F_{5, 42}$= 2.534, p = 0.043; land: $F_{5, 40}$= 17.176, p < 0.001). Even though the values tended to stay on the same levels; eyeballing reveals a slight rise in Hct for the land group at the beginning, later the values returned to initial levels. The pool group showed almost no variation.
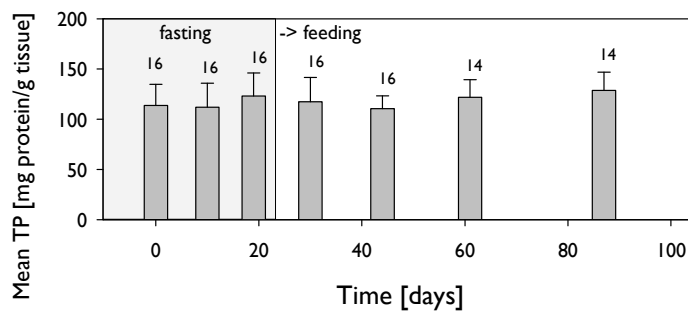
Total protein

The values (mean = 117.97 ± 20.88 mg protein/g tissue) show no significant change over time ($F_{6,78}$= 1.585, p = 0.163) and neither a significant difference between the groups (pool, land) ($F_{1,78}$= 0.700, p = 0.405) or the muscles (LD, SSP) ($F_{1,78}$= 2.494, p = 0.118) (fig 3.3).
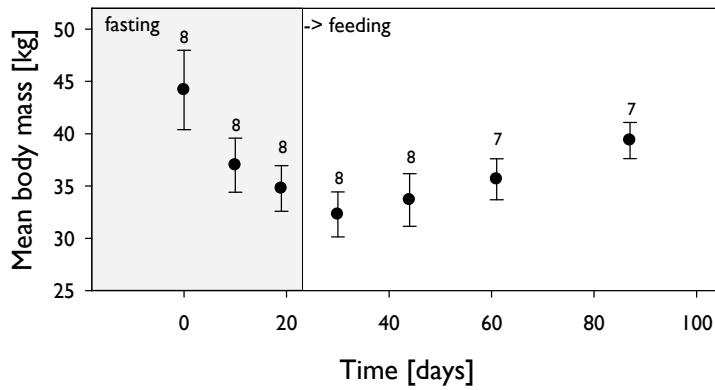
Fig 3.1 Mean Body mass vs time ± 1 SD. For all animals the Body mass decreases during post weaning fast (PWF). After feeding-start the Body mass increases again. Numbers give n.

Fig 3.2 Comparison of haematocrit (Hct) of the different groups. Mean values ± 1 SD. The land group had a higher mean Hct than the pool group ($F_{1, 70}$ = 16.713, $p < 0.001$). The values tend to stay at the same level. Numbers give n.

Fig 3.3 Mean total protein (TP) contend of the muscles, including all animals and muscles. Mean values ± 1 SD. No significant change over time. Numbers give n.

Myoglobin

The results showed a significant difference in mean myoglobin levels between *M. longissimus dorsi* and *M. supraspinatus* in both groups (pool: $F_{1,35}$= 23.883, p < 0.001, land: $F_{1,34}$= 4.434, p = 0.043). The average difference was higher in the pool group than in the land group (pool: LD-SSP = 7.952 $\pm$ 1.627 mg Mb/g tissue, land: LD-SSP = 3.585 $\pm$1.702 mg Mb/g tissue).

M. longissimus dorsi

There was a significant difference between the groups at LD ($F_{1,35}$= 10.9, p = 0.002, mean difference pool-land = 5.912 $\pm$ 1.791) i.e. the mean Mb levels were higher in the animals which were staying in the pool. Time was a significant factor for both groups at the LD (pool: $F_{5,18}$=6.878, p = 0.001; land: $F_{5,17}$=4.912, p = 0.006). Especially the beginning was marked by a strong rise of Mb levels, supported by pair-wise comparison (tabs 3.1&3.2). In the pool group the rise continued until the 30th sample day, whereas the development tended to generally level out at the later sampling dates (> day 30), even though a small peak was visible at sampling day 61. In the land group, the rise was only visible until sampling day 19, followed by a similar general levelling out of Mb levels and a peak value at sampling day 44 (fig 3.4).

M. supraspinatus

There was no statistically significant difference between the two groups at the SSP. Fig 3.5 confirms this for the first three sampling dates. Later on, however, the pool group had a tendency towards higher Mb levels than the land group. Looking at the groups separately reveals, that time had a significant influence only on the Mb values on the SSP of the pool ($F_{6,17}$= 4.077, p = 0.013, see tab 3.3 for pair-wise comparison). Similar to the LD, Mb levels tended to steadily rise at the beginning of the sampling period, followed by a levelling out.

Estimated variation within the muscles: CV_{LD}= 5.7 %, CV_{SSP}= 6.1 %. Estimated precision of the assays: 95 %.

Tab 3.1 All statistically significant pair-wise comparisons between the sampling days for *M. longissimus dorsi* of the pool group

day -	day	mean difference [mg Mb/ g tissue]	SD	df	Sig.[a]
0	30	-16.831	4.629	21	0.032
	61	-25.022	4.629	21	0.000
	87	-23.291	4.629	21	0.001
10	61	-18.305	4.629	21	0.015
	87	-16.574	4.629	21	0.037

a: Adjustment for multiple comparisons: Bonferroni, SD: Standart deviation, df: degrees of freedom, mean difference=day from left column – day from right column.

Tab 3.2 All statistically significant pair-wise comparisons between the sampling days for *M. longissimus dorsi* of the land group

| day -	day	mean difference [mg Mb/ g tissue]	SD	df	Sig.[a]
0	44	-18.322	4.009	19	0.004
10	44	-18.305	4.009	19	0.045

a: Adjustment for multiple comparisons: Bonferroni, SD: Standart deviation, df: degrees of freedom, mean difference=day from left column – day from right column.

Tab 3.3 All statistically significant pair-wise comparisons between the sampling days for *M. supraspinatus* of the pool group

day -	day	mean difference [mg Mb/ g tissue]	SD	df	Sig.[a]
0	44	-10.893	3.115	20	0.048
	87	-15.598	3.115	20	0.001
10	87	-13.410	3.115	20	0.003
19	87	-10.761	3.115	20	0.028

a: Adjustment for multiple comparisons: Bonferroni, SD: Standart deviation, df: degrees of freedom, mean difference=day from left column – day from right column.
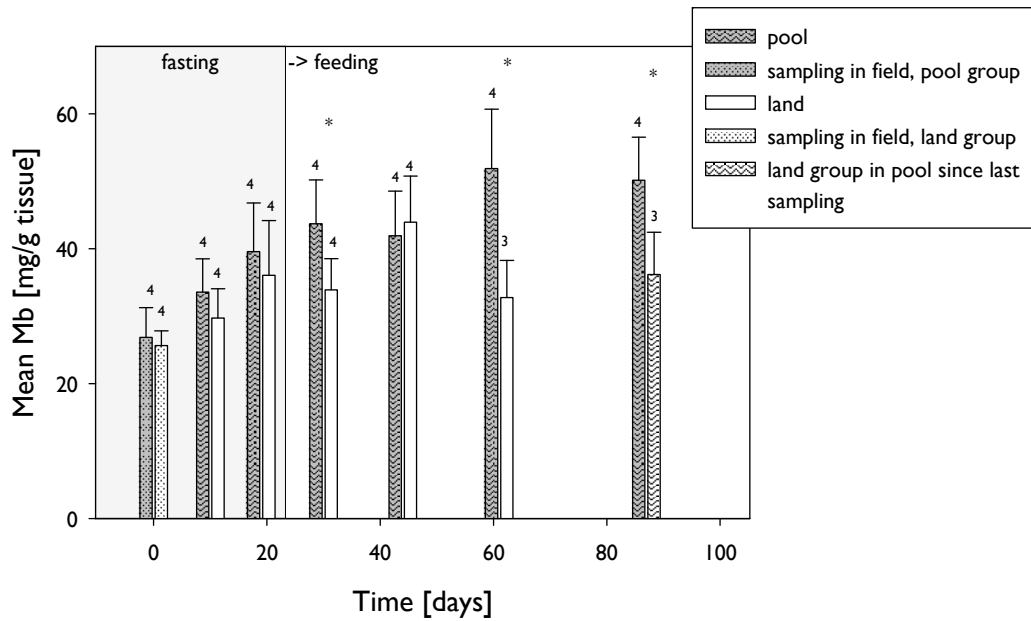
Fig 3.4 Myoglobin development over time at *M. longissimus dorsi.* Mean values ± 1 SD. The animals from the pool goup showed significantly higher mean values than the animals from land group ($F_{1,35}= 10.9$, p = 0.002). Steady increase until day 30 followed by more or less steady levels. The land group had a steady increase of Mb levels the first 20 days, followed by changing levels. Asterisk marks pair-wise significant difference (post-hoc comparison). For pair-wise comparison between days see tabs 3.1&3.2. Numbers give n.
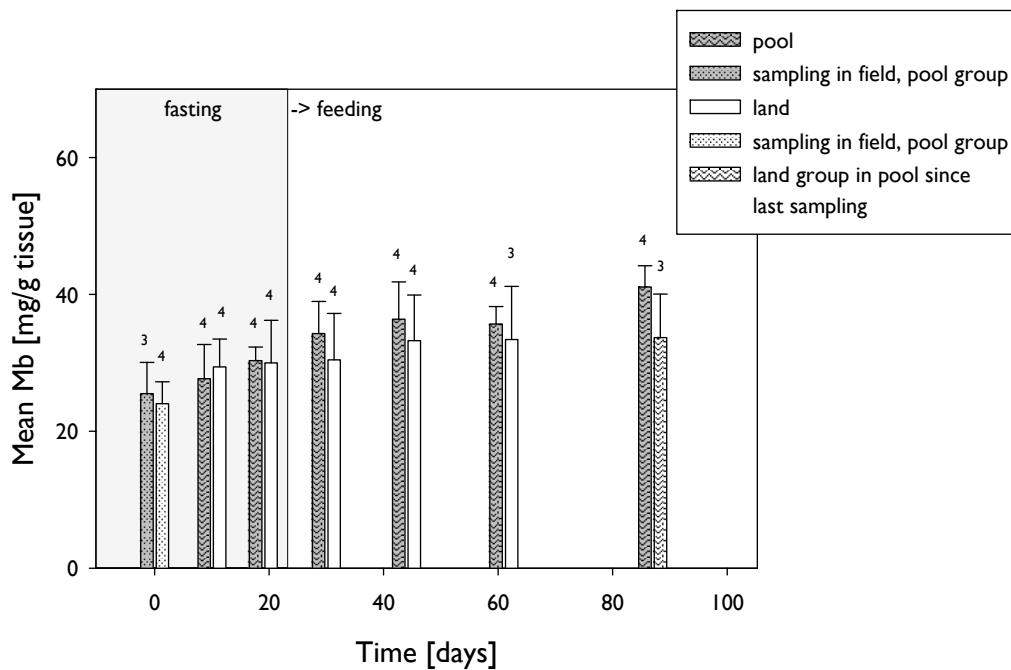
Fig 3.5 Myoglobin development over time at *M. supraspinatus.* Mean values ± 1 SD. No statistically significant difference between the groups, even though the pool group tended to have slightly higher values than the land group. For pair-wise comparison between days see tab 3.3. Numbers give n.

Enzyme activities

The activity levels citrate synthase (CS), lactate dehydrogenase (LDH) and β-hydroxyacyl coenzyme A dehydrogenase (HOAD) had varying levels with no relevant significant influence of muscle (LD, SSP), group (pool, land) or time on the activity (see attachment 2). The overall means for day 0 (i.e. weaned pups) was as follows:

CS: 34.02 ±5.5 IU/g tissue, LDH: 537.11 ±121.7 IU/g tissue, HOAD: 51.86 ±14.0 IU/g tissue.

There was no significant difference between the pool group and the land group in regard to the LDH:CS ratio (Fig 3.6). However, between the muscle types, there was a significant difference ($F_{1,68}$= 88.253, p < 0.001), i.e. the LD had a significantly lower mean CS:LDH ratio than the SSP.

In the LD time had no statistically significant influence on the ratio, even though it tended to increase at day 10 and return afterwards to previous levels before it tended to increase from day 60 on again. Time had a statistically significant influence on the ratio in the SSP ($F_{5,33}$= 6.731, p < 0.001). At first it tended to increase, later on the ratio tended to remain more or less stable. In a pair-wise comparison day 0 had a significantly lower CS:LDH ratio than day 61, underlining the increase of the ratio.

The CS:HOAD ratio (fig 3.7) was below one throughout the sampling period. There was no statistical significance between the groups, but a significantly lower mean CS:HOAD ratio in the LD compared to the SSP ($F_{1,63}$= 4.245, p = 0.044). In neither muscle was a statistically significant change over time.

Estimated precision of the assays: CS = 95.9 %, LDH = 92.2 %, HOAD = 90.9 %.
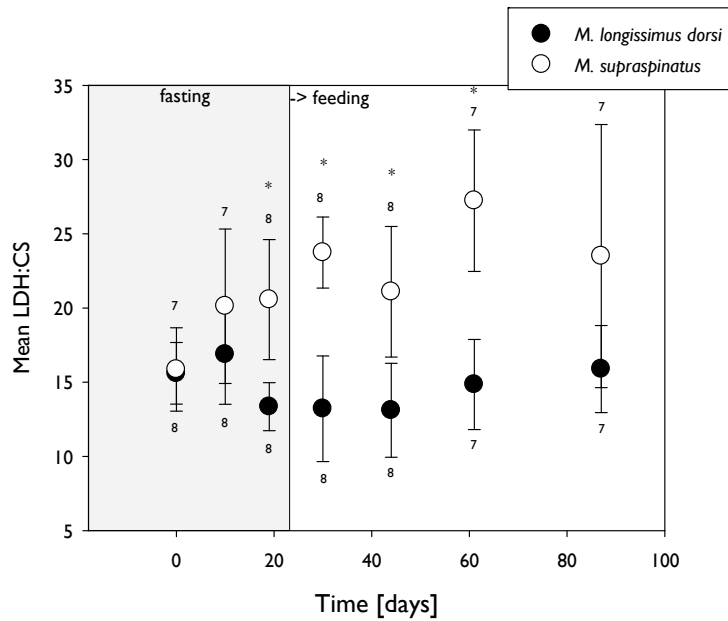
Fig 3.6 Development of the ratio between lactate dehydrogenase (LDH) and citrate synthase (CS). Mean values ± 1 SD, The mean ratio is more on the side of CS in the *M. longissimus dorsi* (LD) in comparison to *M. supraspinatus* (SSP) ($F_{1,68} = 88.253$, $p < 0.001$). No statistically significant influence of time in LD. Statistically significant influence of time in SSP ($F_{5,33} = 6.731$, $p < 0.001$), the ratio tends to change towards higher LDH activity until day 30. Asterisks mark significant difference between LD and SSP (pair-wise comparison). Numbers give n.
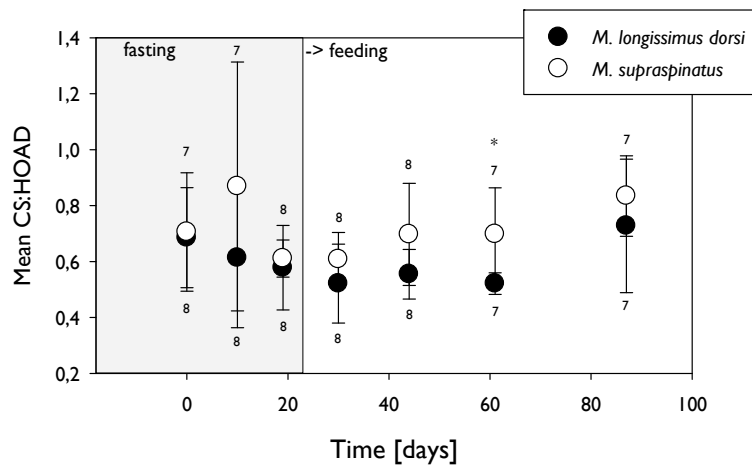
Fig 3.7 Development of the ratio between citrate synthase (CS) and β-hydroxyacyl coenzyme A dehydrogenase (HOAD). Mean values ± 1 SD. No significant influence of time but a significantly higher mean ratio at *M. supraspinatus* (SSP) compared to *M. longissimus dorsi* (LD) ($F_{1,63} = 4.245$, $p = 0.044$). Asterisk marks significant difference between LD and SSP (pair-wise comparison). Numbers give n.

IV. Discussion

General physiology

The body mass development of the animals in this study (fig 3.1) follows the expected pattern: a constant decline during the post weaning fast, indicating the use of endogenous energy deposits, followed by a constant rise after feeding start. The extend and timing of change in body mass concurred with previously published values (Bowen et al., 1987), indicating that the pups in this study had a similar food availability as pups in the wild. The fact that there were only minor individual differences in body mass development, underlines that the present study was conducted with a fairly homogenous and healthy group of animals.

The haematocrit (Hct) values (fig 3.2) indicate that the hooded seal pups have already quite developed blood oxygen stores as already shown for this species (Burns et al., 2007). This is also supported by the stable values over the whole sampling period. The measured differences are probably artefacts since Hct values are influenced by the level of arousal and accompanying contraction/relaxation of the spleen. In addition this influence is unusually large in seals where the spleen serves as a temporary store of erythrocytes (Cabanac et al., 1997), and the sampled animals had a quite different personality influencing the level of arousal.

The total protein levels (fig 3.3) concur with previously published values as well (Burns et al., 2010). The fact that the total protein content of the samples remained at the same levels during the entire sampling period, suggests that protein catabolism played no significant role during the initial fasting period. Therefore stable TP levels supports the Mb results since muscle degradation due to protein catabolism could influence the Mb levels relative to muscle weight.

Myoglobin development

This study showed for the first time that there is a rapid initial increase of Mb levels in the muscles of hooded seal pups within their first month of life. In that short period of time, the Mb levels in the swimming muscle *M. longissimus dorsi* (fig 3.4) rose from less than 25 % to over 50 % of adult values (Lestyk et al., 2009; Burns et al., 2010). In addition the data suggest that further Mb production occurred at a much slower pace, also considering that they need

the following 11 months after the initial increase to reach ~75 % of adult values (Lestyk et al., 2009). The presented pattern of a rapid initial increase of Mb within the first month of life seems to be present in all sampled muscles (figs 3.4&3.5) and coincides with the rapid early increase in diving duration of free living hooded seal pups of the West-ice population (figs 4.1&4.2).
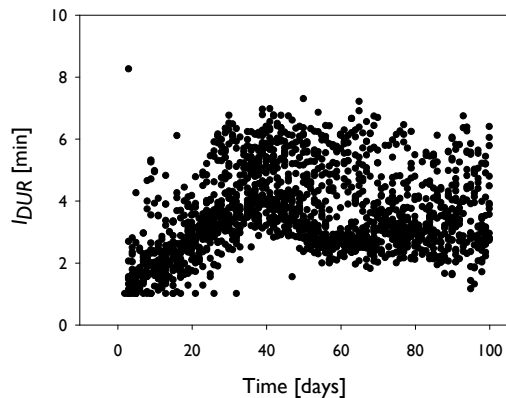
Fig 4.1 Development of diving duration from hooded seal (*Cystophora christata*) pups. Modified after Folkow et al. (2010). I_{DUR}= diving duration index. Day 0 corresponds to 27th of March (various years) being also the first sampling date in this study. The diving duration increases rapidly the first 40 days before the duration levels off.
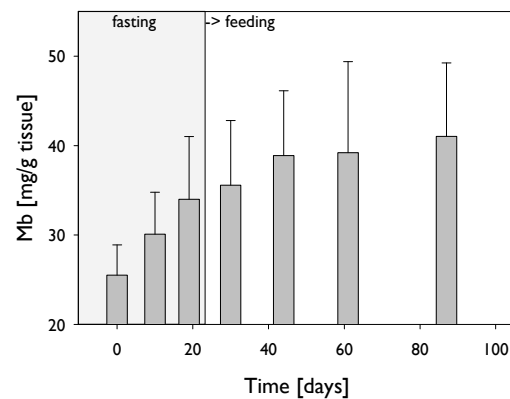
Fig. 4.2 Myoglobin development over time. Overall mean ± 1 sd, from all animals, regardless of group or muscle. The general Mb level tends to increase rapidly within the first 40 days before the values level off.

This similarity in development may suggest that the development of Mb levels, and hence the development of muscular oxygen storage, is influencing the early diving behaviour of hooded seal pups.

There might be one factor responsible for this halt in Mb development after the rapid initial rise. A very important ingredient for the production of Mb is iron. Since the most obvious rise in Mb levels occurs within the fasting period, all the iron for the production of Mb must originate from endogenous iron reserves. The presented data can be interpreted to suggest that those endogenous reserves are depleted after approximately one month. It probably takes some time until the iron supply due to feeding is sufficient for further Mb production due to the learning process to hunt fish and the development/maturing of iron absorption mechanisms. Therefore depleted iron stores could be responsible for the halt in Mb level increase.

A follow up study to investigate iron stores in hooded seal pups is already in progress.

Muscular activity

Muscular metabolism

The activity level of all the investigated enzymes were below previously published values of the same species (Burns et al., 2010). One possible explanation could be differences in the breeding stocks, since the previously sampled animals came from the Newfoundland stock and not from the West-ice like the animals of this study. The ratio, however, of LDH:CS as well as CS:HOAD concurs with the literature (Burns et al., 2010). Furthermore the enzyme activity showed no relevant significant change, while the ratios seemed to be connected to muscular activity as well as Mb levels.

This study showed that there was a significantly lower LDH:CS ratio in the LD than in the SSP (fig 3.6), presumably reflecting a higher reliance on aerobic in relation to anaerobic metabolism in LD than SPP. This could be due to the higher Mb levels in LD, providing more oxygen for aerobic metabolism. Furthermore this is supported by the early shift towards higher LDH:CS ratios (day 10) and could be explained by the faster growing oxygen demand and the insufficient oxygen supply due to still low Mb levels. That pattern is especially expressed in the SSP. In the LD the ratio tends to follow that patter as well, to a lesser extent though, which is probably due to faster growing Mb levels (see second sampling point i.e. day 10 in figs 3.4, 3.5 and 3.6). At day 19, the metabolism switched towards a more aerobic metabolism in the LD while at the same time the Mb levels increased, a tendency which reversed towards day 87, when the Mb production slowed down. In the SSP the metabolism tended more towards an anaerobic metabolism (high LDH:CS ratio), which might be explained by the rather low Mb levels in the SSP (compare figs 3.4, 3.5 and 3.6).

An additional explanation for those differences could be the generally higher oxidative enzyme activity in muscles used for sustained locomotion than in muscles not used for sustained locomotion as discussed below.

Energy source for metabolism

Since CS and HOAD levels are proportional to the maximal flux through the citric acid and β-oxidation cycle, a CS:HOAD ratio below one indicates a oxidation of fatty acids to produce acetyl-CoA for oxidative metabolism close to the ability of the citric acid cycle to consume it (Winder et al., 1974; Stanley et al., 2005). This study suggest that this is the case throughout the sampling period, since the CS:HOAD is never above 1, which is in accordance with previous findings (Burns et al., 2010). In comparison with the CS:HOAD ratio of ~7 in the locomotory muscles of dogs (*Canis familiaris*) (Polasek et al., 2006) the

extremely low ratio indicates an almost exclusive reliance on fat metabolism, which could be the reason that there is no significant change during the sampling time.

There is, however, a tendency for the CS:HOAD ratio to decrease towards day 30 (fig 3.7). Considering the dependency on endogenous blubber reserves for energy production in hooded seal pups during the fasting period, it is not surprising that the ratio seems to go towards a maximum dependency on fatty acids during the fasting period. Therefore the tendency of an increasing CS:HOAD ratio towards day 87, especially after the feeding start, is an indicator of beginning protein metabolism due to food intake.

Taken together, the presented enzyme ratio can be used to estimate the activity level of the muscles. Since working seal muscles rely mainly on aerobic lipid based metabolism for sustained activity (Reed et al., 1994; Polasek et al., 2006; Kanatous et al., 2008) the higher HOAD activity in the LD compared to the SSP indicates a higher activity in the LD, keeping in mind the above suggested higher aerobic metabolism in the LD.

Influence of muscular activity on Mb development

The results showed significantly higher Mb levels in the more active swimming muscles *M. longissimus dorsi* (LD) than in the less active flipper muscles *M. supraspinatus* (SSP) (figs 3.4&3.5). In the pool group this might be due to the fact that the LD was used for swimming, and hence obviously more active than the SSP which was used for steering under water. Even though the muscles were differently used by the land group, they had a more active LD than SSP as well, since, for their undulating moving behaviour the animals on land must have used the back muscles, including LD. In addition, the function of the SSP is just to pull the flippers towards the front, a considerably less forceful movement than the LD triggers by setting the whole body into undulating motion.

The pool group was generally much more active and had a more sustained, continuous activity in the LD due to swimming than the land group. Therefore the difference in muscular activity could account for the more rapid rise in Mb levels in the LD of the pool group compared to the land group. The flippers were used to approximately the same extend by both groups which could account for the similar Mb values and development.

Alternatively, the hypoxia exposure due to the diving behaviour of the pool group could have contributed to their generally higher Mb levels. However, the fact, that the Mb levels increased at a similar rate in the SSP, but at a different rate in the LD of the two groups, supports that activity had a stronger influence on Mb production than hypoxia. If hypoxia would be the critical trigger, both the LD and SSP should show different development of Mb levels.

V. Conclusion

The data of this study supports the hypothesis that sustained muscular activity is an important trigger for post-natal production of myoglobin (Mb) and hence the development of muscular oxygen stores in young hooded seal pups. The activity pattern, as indicated by the different behaviour of the two groups (pool and land), the muscle types and the different enzyme activities, suggests that those muscles that were most active had significantly higher Mb levels/production than muscles with less activity.

Furthermore this study showed that the Mb levels in the swimming muscle *M. longissimus dorsi* of hooded seal pups increased from less than 25 % to more that 50 % of adult values within only one month. Considering that Mb levels reach ~75 % after one year, it means that more than half of the Mb production of the entire first year happens within the first month in hooded seal pups. This suggests that a postnatal trigger for Mb production, such as the onset of activity, is responsible for the initial increase rather than general maturation. The question remains, where the pups got the ingredients, e.g. iron, for the Mb production from, since most of the initial Mb production happens within the post weaning fast. This suggests that some endogenous store of iron must be present. Therefore the cease in the rapid initial Mb increase after one month could be explained by the depletion of such stores.

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Attachments

Attachment 1: List of chemicals

Imidazole hydrochloride (Sigma-Aldrich CO, St Louis, MO, USA)

EDTA (Fisher Scientific, NJ, USA)

$MgCl_2$ (Fisher Scientific, NJ, USA)

5,5'-dithio-bis(2-nitrobencoic acid) (Sigma-Aldrich CO, St Louis, MO, USA)

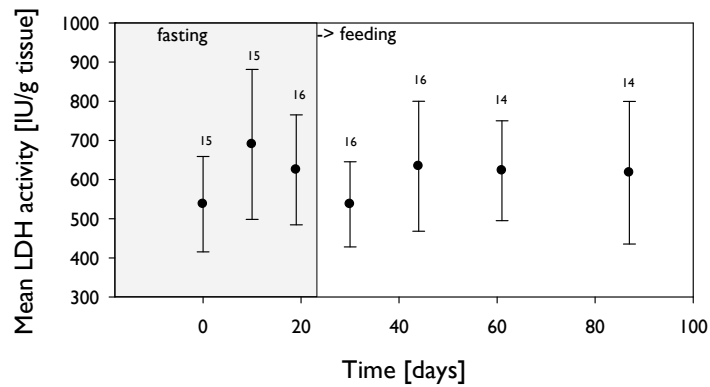Acetyl CoA, 0.5 mM oxalacetate (Sigma-Aldrich CO, St Louis, MO, USA)

NADH (MP Biomedicals, Solon, OH, USA)

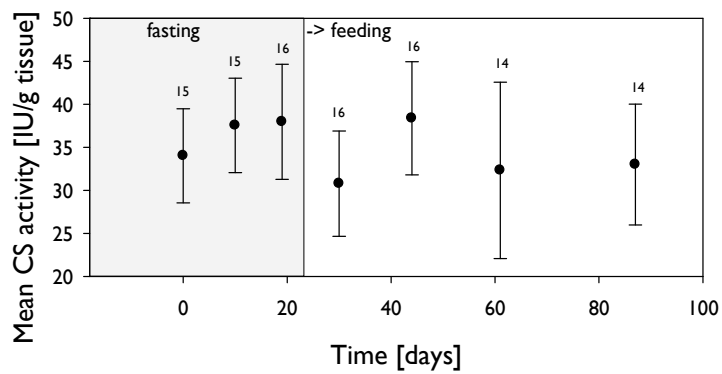Ethylenediaminetetra-acetic acid (EDTA) (Sigma-Aldrich CO, St Louis, MO, USA)

Acetoacetyl CoA (trisodium salt) (Crystal Chem inc, IL, USA)
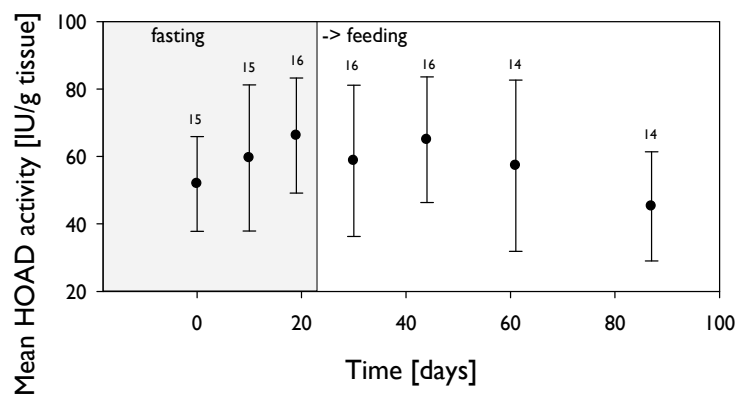
Pyruvate (Acros Organics, NJ, USA)

Attachment 2: Enzyme activities

Fig A.1 Overall mean of lactate dehydrogenase (LDH), including all animals and muscles. Mean values ± 1 SD. No significant change over time. Numbers give n.

Fig A.2 Overall mean of citrate synthase (CS) activity, including all animals and muscles. Mean values ± 1 SD. No significant change over time. Numbers give n.

Fig A.3 Overall mean of lactate dehydrogenase (LDH) activity, including all animals and muscles. Mean ± 1 SD. No significant change over time. Numbers give n.