



Mobilisation of blubber fatty acids of northern elephant seal pups (*Mirounga angustirostris*) during the post-weaning fast

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ABSTRACT

Northern elephant seal pups were longitudinally sampled at Año Nuevo State Reserve during the post-weaning fast, in order to evaluate the changes of fatty acid (FA) profiles in serum as well as in the inner and outer layers of blubber. The major FAs of inner and outer blubber layers were broadly similar to those found in NES maternal milk previously measured, suggesting a direct deposit of dietary FAs in the blubber during the suckling period. The outer blubber layer contained more medium-chain monounsaturated FAs that contribute in keeping the fluidity of this tissue at cold temperatures. It was compensated by higher proportions of saturated FAs in the inner blubber layer. The FA signature of inner blubber, the layer that is mainly mobilised during energy deprivation, slightly differed from the signature of serum. There were greater proportions of medium-chain saturated FAs and ω -6 polyunsaturated FAs, and lower proportions of long-chain saturated FAs, medium-chain monounsaturated FAs and long-chain monounsaturated FAs in serum as compared to inner blubber. We also demonstrated that lipophilicity is the main factor governing the mobilisation of FAs from blubber. The least lipophilic FAs were preferentially hydrolysed from blubber, leading to an enrichment of the more lipophilic FAs in this tissue with the progression of the fast. The expression levels of HSL and ATGL, which are two enzymes involved in the lipolytic process, remained stable during the post-weaning fast. This suggests that the pups have developed the enzymatic mechanisms for an efficient lipolysis as soon as the first week of fast.

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1. Introduction

Northern elephant seal (NES) females give birth to a single pup. For around 26 days after the birth, NES pup ingests fat-rich milk (20–55% lipid) allowing them to form a large subcutaneous blubber layer (Crocker et al., 2001). During this nursing period, pups gain about 90 kg, and approximately 55% of this mass gain is composed of fat (Crocker et al., 2001). The life of NES is then punctuated by long periods of fasting. The first one occurs after the 27-day suckling period when NES pups are abruptly weaned (Le Boeuf et al., 1994). At this time, they are not able to forage yet and thus begin a period of fast ashore for around 10 weeks, in which they grow and acquire the capabilities to swim and dive (Thorson et al., 1994). NES pups highlight a remarkable dependency on lipids during the fast since more than 30% of lipid mass is lost during this period (Noren et al., 2003). Lipids not only provide buoyancy and thermal insulation (Strandberg et al., 2008) to NES pups, they are also the main source of energy and provide important metabolic by products for both diving physiological and neural development.

The blubber of phocid seals exhibits a typical composition of fatty acids (FAs), with a stratification of the FA profile between the outer (close to the skin) and inner (close to the muscle) layers (Best et al., 2003; Strandberg et al., 2008; Fowler et al., 2014). The outer blubber layer is composed of a higher proportion of medium-chain monounsaturated FAs (MC-MUFAs) (i.e. ≤ 18 carbon atoms). The inner blubber layer contains a higher proportion of saturated FAs (SFAs) and long-chain monounsaturated FAs (LC-MUFAs). The trend for polyunsaturated FAs (PUFAs) is less clear; some studies show higher proportions in inner compared to outer blubber, while others report uniform distribution across both layers (Best et al., 2003; Strandberg et al., 2008; Fowler et al., 2014).

During the fast, the triglycerides stored in the lipid droplets of blubber are hydrolysed by the lipolytic pathway to provide FAs, and thus energy to other tissues by β -oxidation of FAs (Ryg et al., 1988). Adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) are the lipases involved in the lipolytic steps (Lass et al., 2011; Viscarra et al., 2013a, 2013b). ATGL hydrolyses triglycerides (TGs) into diglycerides (DGs) and FAs, HSL mainly hydrolyses DGs into monoglycerides (MGs) and FAs, and MGL hydrolyses MGs into free glycerol and FAs (Brasaemle et al., 2009; Viscarra et al., 2013a, 2013b). The central nervous system, endocrine and autocrine/paracrine factors are able to affect the rate of TG hydrolysis in adipocytes

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(Thompson et al., 2010). Contrary to ATGL and HSL, the activity of MGL is regulated neither by hormones nor by the energy state of the fat cells (Lass et al., 2011).

In vitro and in vivo studies on several species (e.g. human, rat, rabbit, American mink and wild racoon dog) report a selective mobilisation of FAs from adipose tissue during lipolytic periods (Conner et al., 1996; Mustonen et al., 2007; Nieminen et al., 2006a; Raclot, 2003; Raclot et al., 1993, 1995, 1997). The relative mobilisation of FAs from adipose tissue increases with the degree of unsaturation for a given chain length and decreases with the chain length for a given degree of unsaturation, whatever the FA composition of adipose tissue. These rules seem to be confirmed, at least in part, for phocid seals such as lactating NES (Fowler et al., 2014), NES pups during the post-weaning fast (Noren et al., 2013), lactating grey seals (Arriola et al., 2013) and lactating Weddell seals (Wheatley et al., 2008).

In this paper, we describe the dynamics of FA mobilisation from blubber to blood in NES pups during the post-weaning fast. The blubber core was divided into the inner and outer layers and only FAs from TGs were analysed. This allowed determination of the degree to which the blubber is stratified and considered only the lipid fraction (i.e. TG fraction) involved in the lipolytic process. In addition, we evaluated the pattern of free FAs in serum. Coming from the hydrolysis of TGs, the free FA fraction of serum directly reflects the FAs released from blubber. In order to study the FA changes relative to blubber reserves, all results were expressed per unit of wet weight instead of percentages of total FAs. We also investigated the relative mobilisation of FAs from both blubber layers between early and late fast. An analysis of mRNA corresponding to enzymes involved in the lipolytic process (ATGL and HSL) was carried out in parallel in order to better understand the regulation of lipolysis and its influence on FA mobilisation.

2. Experimental procedures

2.1. Animal sampling

The sampling took place at Año Nuevo State Reserve, CA, USA (37°06'30"N, 122°20'10"W) from January to April 2012, under the National Marine Fisheries Service Marine Mammal Permit #14636-02. The technique of sampling was described elsewhere (Louis et al., 2014). Twenty free-ranging pups were specifically marked with dye and were followed by observing the colony each day. At weeks 1, 4, 7 and 10 during the post-weaning fast, blood was collected from the extradural vein into Vacutainer serum tubes (Becton-Dickinson, Erembodegem, Belgium). Samples were centrifuged at 3000 ×g for 15 min at 4 °C and serum was aliquoted into microtubes and stored at −70 °C until analysis. At weeks 1, 4 and 7 of post-weaning fast, two blubber biopsies extending the full depth of the blubber layer were taken in the lateral pelvis area using 6-mm biopsy punches (Miltex, Help Medical, Paris, France). Each biopsy was cut into three equal parts determined with a ruler, and the middle section was discarded. The outer (close to the skin) and inner (close to the muscle) sections from the first biopsy were stored separately in dried microtubes at −70 °C until lipid analysis. The inner and outer blubber layers from the second biopsy were stored separately into microtube with QIAzol Lysis Reagent (Qiagen, Antwerp, Belgium). Blubber tissue was minced, kept at 4 °C overnight and then stored at −70 °C until RNA analysis.

Animals were captured with a canvas bag fitted with an adjustable rope in order to determine their body mass at each capture using a metal tripod and 250 kg capacity digital scale at weeks 1, 4 and 7 (accuracy to 0.1 kg; Measurement Systems International, Seattle, Washington, USA). Standard lengths were measured at weeks 1, 4 and 7. The averages are presented with ±SEM.

2.2. Lipid assessment

2.2.1. Lipid extraction and free fatty acid isolation from serum

After thawing, 800 µL of serum was transferred into Pyrex tubes to which 5 mL of a chloroform/methanol/water (2:2:1, v:v:v) mixture (Biosolve, Valkenswaard, The Netherlands) was added. Tridecanoic acid (C13:0) (Sigma-Aldrich, Bornem, Belgium) was used as an internal standard to quantify the free FA fraction of the serum. After shaking, 1 mL of a solution of 0.88% KCl in deionized water was added. Samples were then centrifuged at 1450 ×g for 10 min. The supernatant was removed and transferred to a new Pyrex tube in order to carry out a second extraction with 2 mL chloroform. Samples were shaken and centrifuged. The supernatant of the second extraction was then removed and placed in a new Pyrex tube. A third extraction was performed, similarly to the second one. The lower phases from the three extractions were pooled in a new Pyrex tube and the solvent was evaporated under a moderate nitrogen flux. Samples were suspended into 200 µL chloroform and loaded on solid-phase extraction (SPE) columns (Bond Elut NH₂, 200 mg, Varian, Middelburg, The Netherlands) previously conditioned with hexane (Biosolve). After the samples were pulled through, neutral lipids (NLs) (corresponding to TGs, DGs, MGs and cholesterol esters) were eluted with 1.8 mL chloroform/2-propanol (2:1, v:v) (Biosolve) and discarded. The column was then loaded with 2.4 mL diethyl ether/acetic acid (98:2, v:v) (Biosolve) to elute free FAs. This fraction was collected into new Pyrex tubes.

2.2.2. Lipid extraction and triglyceride isolation from blubber

After thawing, blubber sections were weighted before being inserted in a tube with ceramic beads (MagNa Lyser Green Beads, Roche Applied Biosystem, Penzberg, Germany) and 800 µL buffer composed of 60 mM Tris(hydroxymethyl)aminomethane (Sigma-Aldrich) and 10 mM ethylenediaminetetraacetic acid (Sigma-Aldrich). Samples were crushed with MagNa Lyser Instrument at 5800 ×g for 30 s and then, transferred into Pyrex tubes. A mixture of chloroform/methanol/water (2:2:1, v:v:v) (Biosolve) was added. Triheptadecanoin (a TG formed by three C17:0) (Larodan, Malmö, Sweden) was used as an internal standard in order to quantify FAs from TGs. Two extractions were performed as described above (see section 2.2.1. Lipid extraction and free fatty acid isolation from serum). Two hundred microlitres of lower phases from the two extractions were pooled in a new Pyrex tube and evaporated under a moderate nitrogen flux. Samples were then suspended in 200 µL of chloroform and loaded on SPE columns previously conditioned with hexane. After the samples were pulled through, NLs were eluted with chloroform/2-propanol (2:1, v:v) and collected into Pyrex tubes. The solvent was evaporated under a moderate nitrogen flux and samples were suspended in 200 µL of chloroform. A second SPE column was conditioned with hexane and the NL fraction was loaded. After the samples in chloroform were pulled through the column, the cholesterol ester fraction was eluted with 1.2 mL hexane and discarded. The column was then loaded with 3.6 mL hexane/diethyl ether/dihychloromethane (Biosolve) in order to collect TG fraction in new Pyrex tubes.

2.2.3. Fatty acid analysis

The solvents of serum and blubber samples were evaporated under a moderate nitrogen flux. A methylation step was performed firstly by adding 1 mL 0.1 M KOH in methanol at 70 °C for 1 h and secondly, by adding 0.4 mL 1.2 M HCl in methanol at 70 °C for 15 min. FA methyl esters (FAMES) were then extracted by addition of 2 mL of hexane followed by 1 mL of deionized water and centrifugation (1000 ×g for 5 min). FAMES were separated by gas chromatography as described in Dang Van et al. (2011). Thirty-three FAMES were analysed in the NES samples (Table 3). Three FAs (C6:0, C8:0 and C24:0) are however not listed since their detection frequency was less than 1%. For technical reasons, C20:1n-11 was not analysed in our samples. Each FA peak

was identified by comparison of retention times with pure methyl ester standards purchased from Larodan and from Nu-Check Prep (Elysian, MN, USA). Data processing was operated with the ChromQuest 4.2 software (ThermoFinnigan, Milan, Italy). The concentrations of FAs are expressed per unit of wet weight in serum ($\mu\text{g FAs/mL serum}$) and blubber ($\text{mg FAs/g blubber section}$). The averages are presented with $\pm \text{SEM}$.

FAs were classified according to the length of carbon chain and the degree of unsaturation: medium-chain ($\leq 18\text{C}$) saturated FAs ($\Sigma \text{MC-SFAs}$), long-chain ($>18\text{C}$) saturated FAs ($\Sigma \text{LC-SFAs}$), medium-chain monounsaturated FAs ($\Sigma \text{MC-MUFAs}$), long-chain monounsaturated FAs ($\Sigma \text{LC-MUFAs}$) and polyunsaturated FAs ($\Sigma \omega\text{-3 PUFAs}$ and $\Sigma \omega\text{-6 PUFAs}$) (adapted from Fowler et al., 2014).

2.2.4. Rate of mobilisation of FAs

In order to illustrate the rate of mobilisation of FAs from blubber as a function of their initial concentrations, we calculated the fractional mobilisation (FM) values for both inner and outer blubber layers separately as following (adapted from Mustonen et al. (2007) and Noren et al. (2013)):

$$\text{Concentrations of FAs at week 1} - \text{Concentrations of FAs at week 7} \\ \text{Concentrations of FAs at week 1}$$

FAs with a positive FM value tend to be mobilised from the blubber layer over the fast, whereas those with a negative FM tend to be conserved in the blubber layer.

In order to investigate if the mobilisation of FAs from blubber over the fast was affected by their lipophilicity, the logarithm of partition coefficient P ($\log P$), also called $\log K_{ow}$, which corresponds to the value of the octanol–water partitioning coefficient, was estimated for each detected FA using Advanced Chemistry Development, Inc. software (ACD/Labs, Toronto, Ontario, Canada) (ACD/PhysChem Suite version 12.01). The $\log P$ value is known as a measure of lipophilicity (Mannhold et al., 2009).

2.3. Gene analysis

Total RNA was extracted from the outer and inner blubber layers with RNeasy Lipid Tissue Mini kit (Qiagen) following the manufacturer's instructions. RNA integrity was tested based on microcapillary electrophoresis with Agilent Bioanalyzer 2111 (Agilent Technologies, Diegem, Belgium). No RNA degradation was observed (data not shown). cDNAs ($0.5 \mu\text{g}$) were then synthesised with oligo(dT) (Qiagen) using Ready-To-Go You-Prime First-Strand Beads kit (GE Healthcare, Diegem, Belgium) according to the manufacturer's guidelines. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays were performed at a final volume of $20 \mu\text{L}$ per well with the GoTaq® qPCR Master Mix (Promega, Leiden, The Netherlands) using StepOne Plus thermocycler (Applied Biosystem, Foster City, CA, USA).

Target genes were ATGL and HSL. Succinate dehydrogenase complex, subunit A (SDHA) and ribosomal protein L8 (RPL8) genes were chosen as reference genes. Because of the lack of knowledge about the sequences for the targeted transcripts in NES, consensus regions between species were identified with Clustal W2 (EMBL-EBI; <http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Primer pairs were then designed on these regions with Primer Express v2.0 (Applied Biosystem) (Table 1). Amplicons were then sequenced by MacroGen

(Seoul, Korea) and compared with known sequences using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) in order to confirm that the desired targets were amplified (results not shown).

2.4. Statistical analysis

Data were log transformed to achieve normality. Statistical analyses were conducted using SAS 9.3 software (SAS Institute Inc., Cary, USA). Linear mixed models were used to test the differences in FA levels throughout the post-weaning fast period within the same tissue as well as to compare the FA profiles between tissues for each capture. A Bonferroni correction was applied for comparisons between fatty acids. Animal ID was modelled as a random effect. The level of statistical significance was set at $p\text{-values} < 0.05$ for all analyses. Results are presented as means $\pm \text{SEM}$.

The stability of reference gene expression was tested using qBase PLUS (Biogazelle, Zwijnaarde, Belgium). The log transformation of the ratios between the expression levels of two reference genes has to be similar between two samples, whatever the experimental conditions (Vandesompele et al., 2002). Gene-stability values (M) and coefficients of variation were calculated (Hellemans et al., 2007; Vandesompele et al., 2002) and the stability of reference genes was confirmed ($M < 1$ and coefficients of variation < 0.5 ; results not shown). The evolution of target gene expressions was evaluated using qBase PLUS in order to transform cycle threshold values into normalised relative quantities (NRQs). This method takes into account the use of several reference genes. The stability of NRQs was then evaluated after a log transformation with a linear mixed model. The level of statistical significance was set at $p\text{-values} < 0.05$ for all analyses.

3. Results

3.1. Biometry of NES

At week 1, the mean mass of weaned NES pups was $122 \pm 4 \text{ kg}$. NES pups lost weight throughout the fast and reached a mean mass of $94 \pm 4 \text{ kg}$ at week 7 ($p < 0.001$). During the first 7 weeks of fast, standard length of weaned NES pups significantly increased ($p < 0.001$) and their body mass index significantly decreased ($p < 0.001$) (Table 2). The sex ratio of targeted NES was 1:1.

3.2. Blubber fatty acids

The main classes of FAs were MC-MUFAs, followed by MC-SFAs, $\omega\text{-3 PUFAs}$, LC-MUFAs, $\omega\text{-6 PUFAs}$ and LC-SFAs in both blubber layers across the fasting period (Fig. 1). The proportion of LC-SFAs being much lower than those of the other FA classes, it was not visible within the bars in Fig. 1. C18:1n-9 composed almost one half of the FA content in both blubber layers. It was followed in decreasing order by C16:0, C16:1n-7, C20:1n-9 and C22:6n-3 in the outer blubber layer and by C16:0, C20:1n-9, C16:1n-7 and C22:6n-3 in the inner blubber layer (Table 3).

In both blubber layers, the concentration of ΣFAs expressed per unit of wet weight remained similar over the fast (Table 3). However, even if the ΣFA levels did not change, several temporal trends were observed within the different FA classes.

Table 2

Biometry of northern elephant seals throughout the post-weaning fast period; mean $\pm \text{SEM}$ (range).

	<i>n</i>	Mass [kg]	Standard length [cm]	BMI [kg/m^2]
Week 1	20	122 ± 4^a (88–154)	145 ± 2^a (127–156)	61 ± 2^a (50–75)
Week 4	20	106 ± 4^b (73–133)	148 ± 2^b (133–165)	48 ± 1^b (37–58)
Week 7	20	94 ± 4^c (60–122)	155 ± 2^c (143–164)	42 ± 1^c (37–48)

Values within a column followed by different letters are significantly different ($p < 0.05$). Sex ratio (female:male) = 1:1; BMI = body mass index.

Table 1
Sequences of primer for reference genes (RPL8 and SDHA) and target genes (ATGL and HSL).

Genes	Forward primers	Reverse primers
RPL8	5'-TCGTGTAAGCGGCAAGAAA-3'	5'-TCCAGACAGCACACGATGGT-3'
SDHA	5'-AGGAATCAATGCTGCTCTGGG-3'	5'-AGCCAGTCGGAGCCCTTCA-3'
ATGL	5'-ATGGTGCCTACACTCTGCC-3'	5'-TCCTTCATCCACCGGATGTC-3'
HSL	5'-GCTGAGTTTGACGGGATCAT-3'	5'-TGCCATGTTTGCTAGAGACG-3'

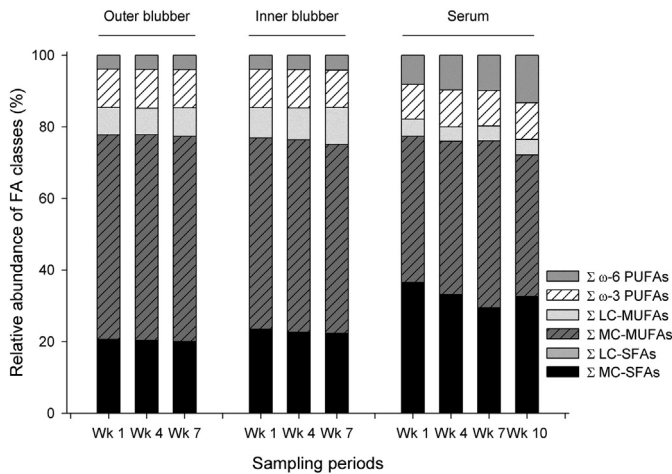


Fig. 1. Proportions of different fatty acid (FA) classes in the outer and inner blubber layers as well as in serum of northern elephant seals throughout the post-weaning fast period (i.e. weeks 1, 4, 7, and 10). MC-SFAs = medium-chain (≤ 18 C) saturated FAs; LC-SFAs = long-chain (> 18 C) saturated FAs; MC-MUFAs = medium-chain monounsaturated FAs; LC-MUFAs = long-chain monounsaturated FAs; PUFAs = polyunsaturated FAs; Wk = week of fast. The LC-SFA class represents a negligible component and is thus not visible within the bars.

In outer blubber, the levels of Σ MC-SFAs remained constant throughout the fast, whereas the Σ LC-SFA content significantly increased between weeks 1 and 7 ($p = 0.029$). The concentrations of Σ MC-MUFAs and Σ LC-MUFAs also rose between weeks 1 and 7 ($p = 0.019$ and $p = 0.005$, respectively). The classes of Σ ω -3 PUFAs and Σ ω -6 PUFAs remained constant throughout the fast. Overall concentrations of the different FA classes remained similar or increased with time in the outer blubber layer.

In inner blubber, the levels of Σ MC-SFAs decreased between weeks 1 and 7 ($p = 0.012$), while the Σ LC-SFA content increased ($p < 0.001$). The concentrations of Σ MC-MUFAs remained constant between early and late fast, while those of Σ LC-MUFAs significantly increased during the study period ($p < 0.001$). Overall, the class of Σ ω -3 PUFAs decreased between weeks 1 and 7 ($p = 0.018$) and the class of Σ ω -6 PUFAs remained constant throughout the fast. Changes in concentrations of the different FA classes were more variable in inner blubber compared to outer blubber.

3.3. Serum fatty acids

We found mainly Σ MC-MUFAs in serum, followed by Σ MC-SFAs, Σ PUFAs, Σ LC-MUFAs and Σ LC-SFAs (Fig. 1). The proportion of LC-SFAs being much lower than those of the other FA classes, it was

Table 3

Concentrations of fatty acids (FAs) in the outer and inner blubber layers of weaned northern elephant seal pups throughout the post-weaning fast (i.e. weeks 1, 4 and 7). Values were expressed in mg of FAs per g of wet weight $\pm$ SEM.

	Outer blubber (mg FAs/g ww)				Inner blubber (mg FAs/g ww)			
	Week 1	Week 4	Week 7	Trends	Week 1	Week 4	Week 7	Trends
C10:0	0.228 $\pm$ 0.006 ^a	0.223 $\pm$ 0.006 ^a	0.210 $\pm$ 0.008 ^a	=	0.234 $\pm$ 0.004 ^a	0.220 $\pm$ 0.008 ^a	0.184 $\pm$ 0.004 ^b	↓
C12:0	0.74 $\pm$ 0.01 ^a	0.72 $\pm$ 0.01 ^a	0.72 $\pm$ 0.01 ^a	=	0.70 $\pm$ 0.01 ^a	0.70 $\pm$ 0.2 ^a	0.67 $\pm$ 0.2 ^a	=
C14:0	18.6 $\pm$ 0.4 ^a	18.9 $\pm$ 0.4 ^a	18.8 $\pm$ 0.5 ^a	=	19.6 $\pm$ 0.5 ^a	19.0 $\pm$ 0.5 ^b	18.9 $\pm$ 0.6 ^{a,b}	=
C16:0	78.8 $\pm$ 1.9 ^a	80.5 $\pm$ 1.7 ^a	80.0 $\pm$ 1.4 ^a	=	93 $\pm$ 2 ^a	87 $\pm$ 2 ^b	83 $\pm$ 3 ^b	↓
C18:0	13.8 $\pm$ 0.5 ^a	13.9 $\pm$ 0.4 ^a	14.2 $\pm$ 0.3 ^a	=	20.7 $\pm$ 0.6 ^a	20.2 $\pm$ 0.5 ^a	21.5 $\pm$ 0.4 ^a	=
Σ MC-SFAs	107 $\pm$ 3 ^a	114 $\pm$ 2 ^a	114 $\pm$ 2 ^a	=	135 $\pm$ 3 ^a	128 $\pm$ 3 ^b	124 $\pm$ 4 ^b	↓
C20:0	0.59 $\pm$ 0.02 ^a	0.61 $\pm$ 0.02 ^{a,b}	0.65 $\pm$ 0.02 ^b	↑	0.70 $\pm$ 0.02 ^a	0.74 $\pm$ 0.03 ^a	0.87 $\pm$ 0.3 ^b	↑
C22:0	0.155 $\pm$ 0.008 ^a	0.158 $\pm$ 0.009 ^a	0.162 $\pm$ 0.009 ^a	=	0.17 $\pm$ 0.01 ^a	0.20 $\pm$ 0.01 ^{a,b}	0.22 $\pm$ 0.01 ^b	↑
Σ LC-SFAs	0.75 $\pm$ 0.02 ^a	0.77 $\pm$ 0.3 ^{a,b}	0.82 $\pm$ 0.02 ^b	↑	0.87 $\pm$ 0.03 ^a	0.93 $\pm$ 0.04 ^a	1.09 $\pm$ 0.04 ^b	↑
C14:1n-5	1.60 $\pm$ 0.05 ^a	1.66 $\pm$ 0.05 ^a	1.65 $\pm$ 0.05 ^a	=	0.95 $\pm$ 0.04 ^a	0.94 $\pm$ 0.04 ^a	0.85 $\pm$ 0.04 ^a	=
C16:1n-7	54.5 $\pm$ 0.7 ^a	56.0 $\pm$ 0.9 ^a	54.3 $\pm$ 1.1 ^a	=	43 $\pm$ 1 ^a	39 $\pm$ 2 ^b	33 $\pm$ 2 ^c	↓
C18:1n-7	23.6 $\pm$ 0.5 ^a	25.7 $\pm$ 0.5 ^b	26.2 $\pm$ 0.4 ^b	↑	25.1 $\pm$ 0.5 ^a	25.6 $\pm$ 0.7 ^a	25.1 $\pm$ 0.8 ^a	=
C18:1n-9	240 $\pm$ 5 ^a	253 $\pm$ 7 ^{a,b}	256 $\pm$ 5 ^b	↑	249 $\pm$ 5 ^a	248 $\pm$ 7 ^a	244 $\pm$ 5 ^a	=
Σ MC-MUFAs	320 $\pm$ 5 ^a	336 $\pm$ 8 ^{a,b}	338 $\pm$ 5 ^b	↑	318 $\pm$ 5 ^a	313 $\pm$ 9 ^a	303 $\pm$ 7 ^a	=
C20:1n-9	40 $\pm$ 1 ^a	41 $\pm$ 1 ^{a,b}	43 $\pm$ 1 ^b	↑	46 $\pm$ 1 ^a	48 $\pm$ 1 ^b	54 $\pm$ 2 ^c	↑
C22:1n-9	3.1 $\pm$ 0.1 ^{a,b}	2.9 $\pm$ 0.1 ^a	3.2 $\pm$ 0.1 ^b	=	3.7 $\pm$ 0.1 ^a	3.8 $\pm$ 0.2 ^a	4.5 $\pm$ 0.2 ^b	↑
C24:1n-5	0.82 $\pm$ 0.02 ^a	0.80 $\pm$ 0.02 ^a	0.83 $\pm$ 0.02 ^a	=	0.92 $\pm$ 0.02 ^a	0.94 $\pm$ 0.04 ^a	1.08 $\pm$ 0.03 ^b	↑
Σ LC-MUFAs	44 $\pm$ 1 ^a	44 $\pm$ 1 ^{a,b}	47 $\pm$ 1 ^b	↑	51 $\pm$ 1 ^a	53 $\pm$ 2 ^a	60 $\pm$ 2 ^b	↑
C18:3n-3	3.5 $\pm$ 0.1 ^a	3.6 $\pm$ 0.1 ^{a,b}	3.9 $\pm$ 0.1 ^b	↑	3.58 $\pm$ 0.09 ^a	3.56 $\pm$ 0.13 ^a	3.42 $\pm$ 0.13 ^a	=
C18:4n-3	1.32 $\pm$ 0.07 ^a	1.34 $\pm$ 0.06 ^a	1.29 $\pm$ 0.06 ^a	=	1.45 $\pm$ 0.07 ^a	1.32 $\pm$ 0.08 ^b	1.15 $\pm$ 0.07 ^c	↓
C20:3n-3	1.05 $\pm$ 0.05 ^a	1.02 $\pm$ 0.03 ^a	1.07 $\pm$ 0.03 ^a	=	1.16 $\pm$ 0.03 ^a	1.13 $\pm$ 0.04 ^a	1.16 $\pm$ 0.04 ^a	=
C20:4n-3	2.5 $\pm$ 0.1 ^a	2.6 $\pm$ 0.9 ^a	2.5 $\pm$ 0.9 ^a	=	2.8 $\pm$ 0.1 ^a	2.6 $\pm$ 0.1 ^b	2.4 $\pm$ 0.1 ^c	↓
C20:5n-3	9.9 $\pm$ 0.6 ^a	10.1 $\pm$ 0.5 ^a	9.3 $\pm$ 0.5 ^b	↑	9.4 $\pm$ 0.5 ^a	7.7 $\pm$ 0.6 ^b	5.8 $\pm$ 0.6 ^c	↑
C22:5n-3	8.7 $\pm$ 0.4 ^a	9.4 $\pm$ 0.4 ^{a,b}	9.7 $\pm$ 0.4 ^b	↑	9.0 $\pm$ 0.5 ^a	9.7 $\pm$ 0.5 ^{a,b}	10.1 $\pm$ 0.5 ^b	↑
C22:6n-3	34 $\pm$ 1 ^a	35 $\pm$ 1 ^a	35 $\pm$ 1 ^a	=	36 $\pm$ 1 ^a	36 $\pm$ 1 ^a	36 $\pm$ 1 ^a	=
C24:5n-3	0.17 $\pm$ 0.01 ^a	0.17 $\pm$ 0.07 ^{a,b}	0.18 $\pm$ 0.06 ^b	↑	0.186 $\pm$ 0.007 ^a	0.195 $\pm$ 0.005 ^a	0.215 $\pm$ 0.006 ^b	↑
C24:6n-3	0.39 $\pm$ 0.01 ^a	0.41 $\pm$ 0.01 ^a	0.41 $\pm$ 0.01 ^a	=	0.36 $\pm$ 0.01 ^a	0.39 $\pm$ 0.01 ^a	0.385 $\pm$ 0.01 ^a	=
Σ ω -3 PUFAs	62 $\pm$ 2 ^a	63 $\pm$ 2 ^a	63 $\pm$ 2 ^a	=	64 $\pm$ 2 ^a	62 $\pm$ 2 ^{a,b}	60 $\pm$ 2 ^b	↓
C18:2n-6	11.5 $\pm$ 0.3 ^a	12.0 $\pm$ 0.2 ^{a,b}	12.1 $\pm$ 0.2 ^b	↑	12.0 $\pm$ 0.2 ^a	12.1 $\pm$ 0.2 ^a	12.2 $\pm$ 0.3 ^a	=
C18:3n-6	0.19 $\pm$ 0.01 ^a	0.19 $\pm$ 0.01 ^a	0.19 $\pm$ 0.01 ^a	=	0.19 $\pm$ 0.01 ^a	0.19 $\pm$ 0.01 ^a	0.18 $\pm$ 0.01 ^a	=
C20:2n-6	2.65 $\pm$ 0.09 ^a	2.70 $\pm$ 0.06 ^a	2.70 $\pm$ 0.06 ^a	=	3.0 $\pm$ 0.1 ^a	3.0 $\pm$ 0.1 ^a	3.2 $\pm$ 0.1 ^b	↑
C20:3n-6	0.50 $\pm$ 0.01 ^a	0.51 $\pm$ 0.01 ^a	0.52 $\pm$ 0.01 ^a	=	0.56 $\pm$ 0.01 ^a	0.54 $\pm$ 0.01 ^a	0.57 $\pm$ 0.01 ^a	=
C20:4n-6	4.17 $\pm$ 0.09 ^a	4.29 $\pm$ 0.08 ^a	4.28 $\pm$ 0.07 ^a	=	4.2 $\pm$ 0.1 ^a	3.8 $\pm$ 0.1 ^b	3.4 $\pm$ 0.1 ^c	↓
C22:4n-6	0.71 $\pm$ 0.02 ^a	0.74 $\pm$ 0.03 ^{a,b}	0.78 $\pm$ 0.02 ^b	↑	0.73 $\pm$ 0.03 ^a	0.76 $\pm$ 0.03 ^a	0.82 $\pm$ 0.03 ^b	↑
C22:5n-6	1.32 $\pm$ 0.03 ^a	1.31 $\pm$ 0.03 ^a	1.35 $\pm$ 0.03 ^a	=	1.45 $\pm$ 0.04 ^a	1.48 $\pm$ 0.05 ^a	1.64 $\pm$ 0.04 ^b	↑
Σ ω -6 PUFAs	21.0 $\pm$ 0.4 ^a	21.7 $\pm$ 0.3 ^a	21.9 $\pm$ 0.3 ^a	=	22.4 $\pm$ 0.4 ^a	21.9 $\pm$ 0.4 ^a	22.0 $\pm$ 0.4 ^a	=
Σ FAs	559 $\pm$ 9 ^a	581 $\pm$ 11 ^a	585 $\pm$ 8 ^a	=	591 $\pm$ 9 ^a	579 $\pm$ 13 ^a	571 $\pm$ 12 ^a	=

For a defined tissue, values within a row (which correspond to a fatty acid or a fatty acid class) followed by different letters are significantly different ($p \leq 0.05$). MC-SFAs = medium-chain (≤ 18 C) saturated FAs; LC-SFAs = long-chain (> 18 C) saturated FAs; MC-MUFAs = medium-chain monounsaturated FAs; LC-MUFAs = long-chain monounsaturated FAs; PUFAs = polyunsaturated FAs.

not visible within the bars in Fig. 1. Similarly to the blubber, C18:1n-9 was the major FA in the serum. It was followed in the decreasing order by C16:0 > C18:0 > C20:4n-6 > C22:6n-3 ≈ C16:1n-7 over the post-weaning fast.

The concentrations of total FAs expressed per unit of wet weight significantly rose until week 7 ($p < 0.001$) and then remained constant until week 10 (Table 4).

Within the different FA classes, the concentrations of all FA classes (Σ MC-SFAs, Σ LC-SFAs, Σ MC-MUFAs, Σ LC-MUFAs, Σ ω -3 PUFAs and Σ ω -6 PUFAs) as well as all constituent FAs increased significantly between week 1 and either week 7 or week 10 ($p < 0.001$) (Table 4). On average, levels of individual FAs rose from 2 to 3 fold in serum over the fast.

3.4. Comparison of fatty acid classes between tissues

The FA profiles were compared between adjacent tissues (outer blubber vs. inner blubber and inner blubber vs. serum) (Fig. 1).

Regarding outer and inner blubber, at each sampling period within the post-weaning fast (weeks 1, 4 or 7), Σ MC-SFA and Σ LC-SFA and Σ LC-MUFA percentages were significantly lower in the outer blubber layer compared to the inner blubber layer ($p < 0.001$), whereas Σ MC-MUFA proportions were significantly higher in the outer blubber layer compared to the inner blubber layer ($p < 0.001$). The proportions of Σ ω -3 PUFAs were comparable between both blubber layers at week 1. They were lower in the outer blubber layer than in the inner blubber

layer at week 4 ($p = 0.017$) and were higher in the outer blubber layer than in the inner blubber layer at week 7 ($p < 0.001$). Finally, the percentages of Σ ω -6 PUFAs were comparable between both blubber layers at weeks 1 and 4 but were lower in the outer layer compared to the inner layer at week 7 ($p = 0.007$).

The inner blubber being more vascularized and considered as the layer preferentially mobilised in case of negative energy balance, its FA profile was compared to the one of serum (Fig. 1). For all sampling periods over the post-weaning fast (weeks 1, 4 or 7), Σ MC-SFA proportions in the inner blubber layer were lower than in serum ($p < 0.001$), while Σ LC-SFA, Σ MC-MUFA and Σ LC-MUFA proportions were higher in the inner blubber layer compared to serum ($p < 0.001$). The percentages of Σ ω -3 PUFAs were higher in the inner blubber layer than serum at week 1 ($p = 0.013$), became similar at week 4, and then returned to being higher in the inner blubber layer at week 7 ($p = 0.022$). The proportions of Σ ω -6 PUFAs were lower in the inner blubber layer relative to serum throughout the fast ($p < 0.001$) (Fig. 1). The differences of FA profiles were thus more pronounced between inner blubber layer and serum.

3.5. Fractional mobilisation

The results of fractional mobilisation (FM) for all targeted FAs are illustrated in Fig. 2. The range of FM values was lower in the outer blubber (−0.14 – 0.08) than in the inner blubber (−0.41 – 0.42), reflecting a different amplitude of FA mobilisation between the two

Table 4
Concentrations of fatty acids (FAs) in serum of weaned northern elephant seal pups throughout the post-weaning fast (i.e. weeks 1, 4, 7 and 10). Values were expressed in μg of FAs per ml of wet weight $\pm$ SEM.

	Serum (μg FAs/mL ww)			
	Week 1	Week 4	Week 7	Week 10
C10:0	0.65 $\pm$ 0.04 ^a	0.82 $\pm$ 0.03 ^b	1.03 $\pm$ 0.06 ^b	1.23 $\pm$ 0.04 ^c
C12:0	0.73 $\pm$ 0.04 ^a	1.10 $\pm$ 0.06 ^b	1.25 $\pm$ 0.12 ^b	1.92 $\pm$ 0.16 ^c
C14:0	5.49 $\pm$ 0.46 ^a	8.71 $\pm$ 0.52 ^b	16.46 $\pm$ 1.06 ^c	16.04 $\pm$ 1.75 ^c
C16:0	53.57 $\pm$ 3.02 ^a	85.50 $\pm$ 1.19 ^b	143.35 $\pm$ 7.24 ^c	138.66 $\pm$ 11.48 ^c
C18:0	26.97 $\pm$ 1.80 ^a	49.50 $\pm$ 4.45 ^b	80.61 $\pm$ 6.73 ^c	90.03 $\pm$ 7.64 ^c
Σ MC-SFAs	87.41 $\pm$ 4.11 ^a	145.64 $\pm$ 8.27 ^b	242.71 $\pm$ 16.69 ^c	247.87 $\pm$ 22.18 ^c
C20:0	0.09 $\pm$ 0.03 ^a	0.20 $\pm$ 0.04 ^a	0.33 $\pm$ 0.05 ^b	0.41 $\pm$ 0.06 ^b
C22:0	N.D.	0.20 $\pm$ 0.03	0.43 $\pm$ 0.04	0.53 $\pm$ 0.06
Σ LC-SFAs	0.11 $\pm$ 0.07 ^a	0.40 $\pm$ 0.05 ^a	0.76 $\pm$ 0.07 ^b	0.94 $\pm$ 0.09 ^b
C14:1n-5	0.13 $\pm$ 0.02 ^a	0.31 $\pm$ 0.03 ^b	0.78 $\pm$ 0.07 ^c	0.63 $\pm$ 0.11 ^{b,c}
C16:1n-7	13.44 $\pm$ 1.09 ^a	25.21 $\pm$ 2.04 ^b	53.51 $\pm$ 3.81 ^c	41.83 $\pm$ 5.71 ^{b,c}
C18:1n-7	10.04 $\pm$ 1.00 ^a	19.62 $\pm$ 1.25 ^b	36.46 $\pm$ 2.15 ^c	28.73 $\pm$ 2.79 ^{b,c}
C18:1n-9	77.01 $\pm$ 6.81 ^a	143.98 $\pm$ 9.34 ^b	294.07 $\pm$ 17.75 ^c	243.29 $\pm$ 24.00 ^c
Σ MC-MUFAs	100.63 $\pm$ 8.61 ^a	189.13 $\pm$ 12.20 ^b	384.82 $\pm$ 22.90 ^c	314.48 $\pm$ 32.02 ^c
C20:1n-9	8.79 $\pm$ 0.72 ^a	14.54 $\pm$ 0.93 ^b	28.88 $\pm$ 1.83 ^c	28.48 $\pm$ 3.02 ^c
C22:1n-9	1.52 $\pm$ 0.19 ^a	1.69 $\pm$ 0.08 ^a	2.83 $\pm$ 0.19 ^b	2.87 $\pm$ 0.23 ^b
C24:1n-5	1.04 $\pm$ 0.04 ^a	1.08 $\pm$ 0.04 ^a	1.46 $\pm$ 0.08 ^b	1.59 $\pm$ 0.07 ^b
Σ LC-MUFAs	11.35 $\pm$ 0.90 ^a	17.31 $\pm$ 1.02 ^b	33.18 $\pm$ 2.03 ^c	32.93 $\pm$ 3.14 ^c
C18:3n-3	1.11 $\pm$ 0.11 ^a	2.47 $\pm$ 0.21 ^b	5.09 $\pm$ 0.33 ^c	3.87 $\pm$ 0.43 ^{b,c}
C18:4n-3	0.35 $\pm$ 0.04 ^a	0.71 $\pm$ 0.07 ^b	1.44 $\pm$ 0.13 ^c	1.14 $\pm$ 0.16 ^{b,c}
C20:3n-3	0.28 $\pm$ 0.05 ^a	0.44 $\pm$ 0.03 ^b	0.91 $\pm$ 0.06 ^c	0.70 $\pm$ 0.09 ^c
C20:4n-3	0.76 $\pm$ 0.08 ^a	1.41 $\pm$ 0.13 ^b	2.54 $\pm$ 0.17 ^c	2.10 $\pm$ 0.25 ^{b,c}
C20:5n-3	4.39 $\pm$ 0.37 ^a	8.06 $\pm$ 0.66 ^b	14.91 $\pm$ 1.04 ^c	12.61 $\pm$ 1.75 ^{b,c}
C22:5n-3	1.63 $\pm$ 0.13 ^a	3.72 $\pm$ 0.26 ^b	7.42 $\pm$ 0.44 ^c	6.96 $\pm$ 0.62 ^c
C22:6n-3	14.13 $\pm$ 1.26 ^a	29.66 $\pm$ 2.74 ^b	50.83 $\pm$ 3.65 ^c	53.89 $\pm$ 5.71 ^c
C24:5n-3	N.D.	N.D.	N.D.	N.D.
C24:6n-3	0.51 $\pm$ 0.32	N.D.	N.D.	N.D.
Σ ω -3 PUFAs	23.15 $\pm$ 1.67 ^a	46.48 $\pm$ 3.65 ^b	83.17 $\pm$ 5.21 ^c	81.98 $\pm$ 8.51 ^c
C18:2n-6	5.09 $\pm$ 0.31 ^a	10.29 $\pm$ 0.60 ^b	19.94 $\pm$ 1.04 ^c	21.04 $\pm$ 1.87 ^c
C18:3n-6	N.D.	N.D.	N.D.	N.D.
C20:2n-6	0.61 $\pm$ 0.05 ^a	1.16 $\pm$ 0.09 ^b	2.32 $\pm$ 0.16 ^c	2.35 $\pm$ 0.29 ^c
C20:3n-6	0.38 $\pm$ 0.06 ^a	0.75 $\pm$ 0.09 ^b	1.36 $\pm$ 0.11 ^c	1.76 $\pm$ 0.19 ^d
C20:4n-6	12.36 $\pm$ 1.47 ^a	30.83 $\pm$ 3.40 ^b	58.39 $\pm$ 5.73 ^c	77.88 $\pm$ 7.52 ^c
C22:4n-6	0.31 $\pm$ 0.02 ^a	0.54 $\pm$ 0.04 ^b	0.98 $\pm$ 0.07 ^c	1.05 $\pm$ 0.08 ^c
C22:5n-6	0.42 $\pm$ 0.06 ^a	0.53 $\pm$ 0.04 ^a	1.20 $\pm$ 0.08 ^b	1.41 $\pm$ 0.16 ^b
Σ ω -6 PUFAs	19.18 $\pm$ 1.74 ^a	44.10 $\pm$ 3.93 ^b	84.18 $\pm$ 6.82 ^c	105.49 $\pm$ 9.90 ^c
Σ FAs	241.48 $\pm$ 14.37 ^a	443.06 $\pm$ 25.19 ^b	828.82 $\pm$ 47.09 ^c	783.70 $\pm$ 72.32 ^c

Values within a row followed by different letters are significantly different ($p \leq 0.05$). N.D. = not detected; MC-SFAs = medium-chain ($\leq 18\text{C}$) saturated FAs; LC-SFAs = long-chain ($> 18\text{C}$) saturated FAs; MC-MUFAs = medium-chain monounsaturated FAs; LC-MUFAs = long-chain monounsaturated FAs; PUFAs = polyunsaturated FAs.

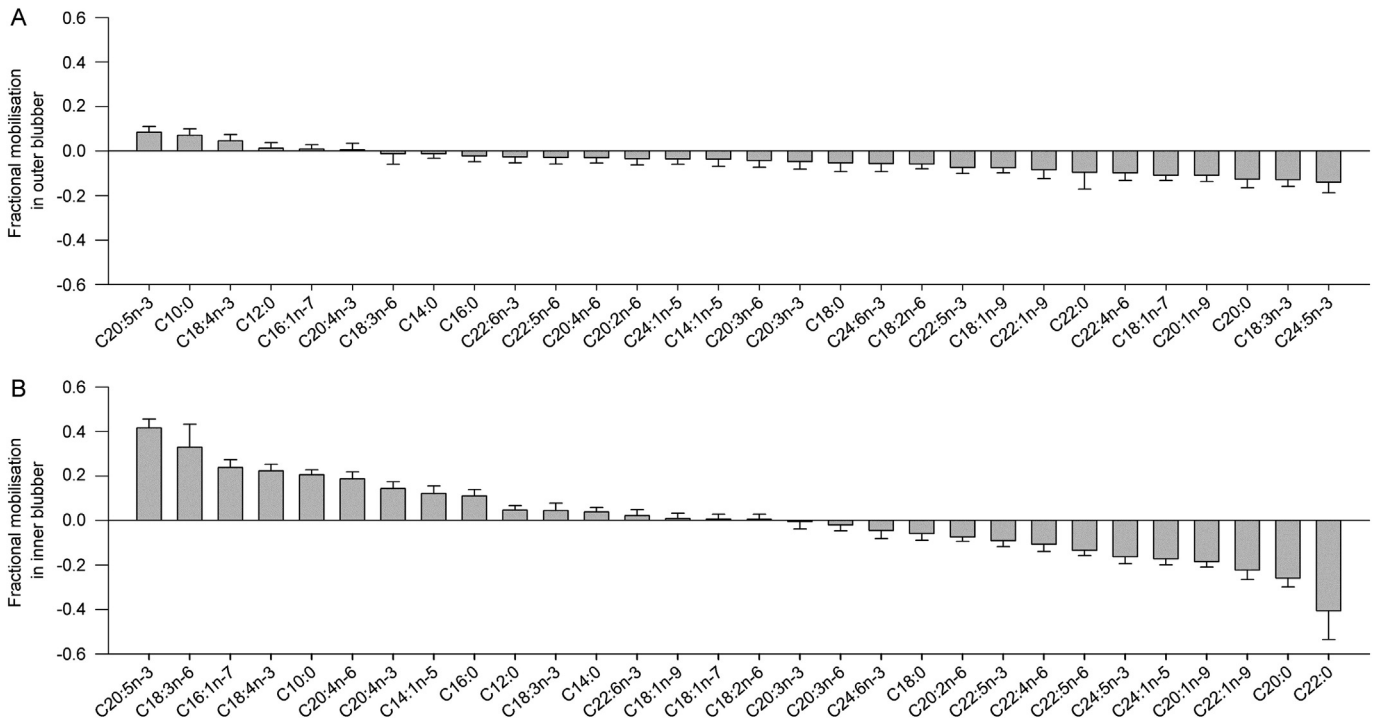


Fig. 2. Fractional mobilisation of fatty acids of northern elephant seal pups during the post-weaning fast in the outer (A) and inner (B) blubber layers.

layers. Among the studied FAs, only 6 FAs (1 MC-MUFA, 2 SFAs and 3 ω -3 PUFAs) had positive FM values in the outer blubber layer compared to 16 FAs (5 ω -3 PUFAs, 4 MC-MUFAs, 4 SFAs and 3 ω -6 PUFAs) in the inner blubber layer (Fig. 2 A–B). Most MC-MUFAs were mobilised from the inner blubber to some extent. The FA with the greatest positive FM value was C20:5n-3 in both blubber layers. This FM value was

however markedly higher in inner blubber than in outer blubber (Fig. 2 A–B). All targeted ω -6 PUFAs were conserved in the outer blubber layer as well as all LC-MUFAs, in both blubber layers. C24:5n-3 and C22:0 had the lowest FM values in the outer and inner blubber layers, respectively.

The different molecular structures of FAs give them different degrees of lipophilicity that can be estimated by log *P* (Table 5). The predicted log *P* values increased with the number of carbon atoms, for a similar degree of unsaturation, and decreased with the degree of unsaturation, for a similar number of carbon atoms. The comparison of FA lipophilicity with the FM from inner blubber highlighted that the less lipophilic FAs (with a predicted log *P* ranging from 4.0 to 7.2) tended to be selectively mobilised from the inner blubber layer (FM > 0), whereas the more lipophilic FAs (with a predicted log *P* ranging from 7.6 to 10.9) tended to be conserved (FM < 0). An intermediate state of lipophilicity could be considered for the FAs with a predicted log *P* $\approx$ 7 and FM values close to 0 (e.g. C18:1n-7, C18:1n-9, C18:2n-6, C20:3n-3 and C20:3n-6). The rule of lipophilicity was however not applicable to the outer blubber layer, probably due to its lower metabolic activity and consequently, fewer mobilised FAs.

3.6. Gene expression

The levels of expression of ATGL and HSL were evaluated throughout the post-weaning fast. No gene profile underwent significant changes within a blubber layer between two consecutive periods of fast. In addition, there was no significant difference in expression levels between both blubber layers at each sampling period (Table 6).

4. Discussion

4.1. Patterns of fatty acids in blubber and serum

During the post-weaning fast, NES pups mobilised their lipid stores in order to meet physiological needs. Consequently, they lost more than 30 kg over 6 weeks. Nevertheless, there were stable concentrations of total FAs in TGs within the inner and outer blubber layers

Table 5

Predicted log *P* values for each studied fatty acids, which are sorted by ascending order. Symbols “+” and “–” refer to positive and negative values of fractional mobilisation (FM) shown on Fig. 2.

Fatty acids	Predicted log <i>P</i>	FM outer blubber	FM inner blubber
C10:0	4.0 $\pm$ 0.2	+	+
C12:0	5.0 $\pm$ 0.2	+	+
C14:1n-5	5.6 $\pm$ 0.2	–	+
C18:4n-3	5.9 $\pm$ 0.4	+	+
C14:0	6.1 $\pm$ 0.2	–	+
C20:5n-3	6.2 $\pm$ 0.5	+	+
C18:3n-3	6.5 $\pm$ 0.3	–	+
C16:1n-7	6.6 $\pm$ 0.2	+	+
C18:3n-6	6.6 $\pm$ 0.3	–	+
C22:6n-3	6.8 $\pm$ 0.5	–	+
C20:4n-3	6.9 $\pm$ 0.3	+	+
C20:4n-6	6.9 $\pm$ 0.4	–	+
C16:0	7.2 $\pm$ 0.2	–	+
C18:2n-6	7.2 $\pm$ 0.3	–	+
C20:3n-3	7.6 $\pm$ 0.3	–	–
C20:3n-6	7.6 $\pm$ 0.3	–	–
C22:5n-3	7.6 $\pm$ 0.5	–	–
C18:1n-7	7.7 $\pm$ 0.2	–	+
C18:1n-9	7.7 $\pm$ 0.2	–	+
C24:6n-3	7.7 $\pm$ 0.5	–	–
C22:5n-6	7.8 $\pm$ 0.4	–	–
C22:4n-6	8.0 $\pm$ 0.4	–	–
C18:0	8.2 $\pm$ 0.2	–	–
C20:2n-6	8.2 $\pm$ 0.3	–	–
C20:1n-9	8.8 $\pm$ 0.2	–	–
C24:5n-3	9.2 $\pm$ 0.5	–	–
C20:0	9.3 $\pm$ 0.2	–	–
C22:1n-9	9.8 $\pm$ 0.2	–	–
C22:0	10.3 $\pm$ 0.2	–	–
C24:1n-5	10.9 $\pm$ 0.2	–	–

Table 6
The mean levels of expression of ATGL and HSL in the outer and inner blubber layers of northern elephant seal pups throughout the post-weaning fast (i.e. weeks 1, 4 and 7). Data are expressed in normalised relative quantities $\pm$ SEM.

	Outer blubber			Inner blubber		
	Week 1	Week 4	Week 7	Week 1	Week 4	Week 7
ATGL	1.08 $\pm$ 0.21	1.49 $\pm$ 0.43	1.26 $\pm$ 0.24	2.24 $\pm$ 0.84	1.56 $\pm$ 0.27	1.59 $\pm$ 0.29
HSL	0.94 $\pm$ 0.13	0.99 $\pm$ 0.12	1.06 $\pm$ 0.12	1.69 $\pm$ 0.33	1.33 $\pm$ 0.19	1.15 $\pm$ 0.11

(concentrations being expressed per unit of wet weight). Since TGs are composed of by FAs, and TGs are the main lipid fraction of adipose tissue, the present results may be compared to the total lipid percentages of blubber. Similar trends in the inner blubber layer of weaned NES pups were observed for the total lipid percentages, which were determined by gravimetry (Louis et al., 2014). This means that TG content remains stable in blubber throughout the fast, which may be surprising because TGs are heavily mobilised. Indeed, the lipid mass of weaned NES pups is reduced by 24–49% (Noren et al., 2003) and a net increase of free FA concentration between early and late fast is noted in their serum (our study and Louis et al. (2014)). Moreover, a morphometric study of the weaned NES pup blubber was conducted earlier and showed that the proportions of small adipocytes increased in both blubber layers, whereas the proportions of large adipocytes decreased over the fast, reflecting a reduction of lipid content in the adipocytes (Louis et al., 2014). The 6-mm diameter biopsy punch that was used allowed sampling blubber cores of same size. As a result, the sample contains a higher number of smaller adipocytes at late fast as compared to early fast. As the proportions of connective tissue are very low in the phocid blubber (<5%), blubber mass is mainly composed of adipocytes and thus, lipids. This could explain the stable level of the Σ FA concentrations within the inner and outer blubber layers when concentrations are based on weight. However, there were variations of temporal trends within the different FA classes, especially in the inner blubber, as illustrated by the wide range of fractional mobilisation.

With the exception of C20:1n-11 that was not analysed in the present study, the patterns of FA classes and major individual FAs in NES pup blubber broadly followed those observed in marine mammals (Σ MC-MUFAs > Σ SFAs > Σ PUFAs $\approx$ Σ LC-MUFAs) (Best et al., 2003; Fowler et al., 2014; Lambert et al., 2013; Noren et al., 2013). Those studies determined the FA profiles in the blubber without distinction of FA origin (TGs vs. phospholipids), whereas it seems more relevant to know the exact composition of FAs from TGs, with the aim of studying the lipid class involved in the lipolytic process. The contribution of FAs from phospholipids could indeed become more significant with the progression of the fast. Although more than 90% of blubber is composed of TGs (Best et al., 2003; Wheatley et al., 2008), the separation of different lipid fraction should help to better understand and compare FA changes during the lipolytic period (Price et al., 2012). However, despite the fact that the other studies did not separate lipid fractions for FA analyses, our findings remained similar to previous work.

The FAs stored in blubber may originate either by direct deposition from the circulation, or by de novo biosynthesis within the layers (Best et al., 2003). Iverson et al. (1995) suggested that ingested FAs are directly incorporated without modification in the blubber of marine mammals during periods of extensive fattening. In the case of NES pups, the unique dietary source of FAs was the milk transferred from the mother during the suckling period. The FA profile of NES milk is mostly formed by C18:1n-9, C16:0, C20:1n-11, C16:1n-7 and C20:1n-9 (Fowler et al., 2014), which is very similar to the FA composition of pup blubber. The FA composition of milk clearly determines the FA pattern of blubber in NES pups.

Data regarding FA profiles in phocid seal blood are scarce. To our knowledge, only one study reports the FA profiles in the plasma of Weddell seal pups during the post-weaning fast (Rea et al., 1997). Plasma FA pattern from the phospholipid fraction of weaned Weddell

seal pups is mainly composed of C18:0 > C20:4n-6 > C16:0 > C18:1n-9 and the TG fraction mainly contains C16:0 > C20:5n-3 > C22:6n-3. In the present study, the free FA fraction in the serum of weaned NES pups was composed of C18:1n-9 > C16:0 > C18:0 > C20:4n-6 > C22:6n-3 > C16:1n-7 at late fast. The different FA patterns between both species may be explained by the origin of FAs. The serum free FA fraction comes mainly from the hydrolysis of TGs in adipose tissue, whereas circulating phospholipids and TGs are found in lipoproteins and do not directly represent the release of FAs from adipocytes. In order to draw strong conclusions regarding the FA pattern in the serum of weaned animals, a comparison of FAs from similar lipid fractions should be carried out.

4.2. Selective mobilisation of FA from blubber

Because circulating free FAs originating from hepatic synthesis are reduced during conditions of food deprivation (Aarsland et al., 1998), most of the circulating free FAs measured in fasting NES pups result from the lipolytic process in the adipose tissue. Since the inner blubber layer exhibited the highest range of FM, we can expect that this layer is the most metabolically active (compared to the outer one), as already suggested elsewhere (e.g. Louis et al., 2014) and thus, participates largely to the increase of FAs in serum. Most of the major FAs encountered in serum (C18:1n-9 > C16:0 > C16:1n-7 $\approx$ C22:6n-3) were indeed similar to those of inner blubber. However, there were greater proportions of Σ MC-SFAs and Σ ω -6 PUFAs, and lower proportions of Σ LC-SFAs, Σ MC-MUFAs and Σ LC-MUFAs in serum as compared to inner blubber. Those differences of FA profiles between inner blubber and serum suggest that the hydrolysis of TGs from adipose tissue is not a homogeneous process and that some FAs may be more efficiently mobilised than others. Studies on fasting terrestrial carnivorous mammals found differences of FA composition between plasma and adipose tissue (Nieminen et al., 2006a, 2006b). However, in those studies, the FA profiles were assessed in the total lipid fraction of each tissue. In weaned NES pups, the total lipid fraction of blood is dominated by phospholipids (52%), followed by cholesterol (31%) and TGs (10%) (Louis et al., 2014), for which the liver is the main source. The free FA fraction, which comes from the lipolytic process in blubber, represents only 5% of the total lipids of blood (Louis et al., 2014). Such data underline the importance to specifically study the free FA fraction of blood to follow the FAs mobilised from the adipose tissue.

The selective mobilisation of FAs from blubber (Noren et al., 2013), reflected by a wide range of FM values, may explain the differences of FA composition between inner blubber and serum. Previous in vitro and in vivo studies on terrestrial mammals have pointed out a relative mobilisation of FAs from adipose tissue according to their molecular structure (i.e. chain length and degree of unsaturation). The molecular structures of FAs give them different physico-chemical characteristics, such as different degrees of lipophilicity. It thus appears from our data that the mobilisation of FAs from adipose tissue is selective and this selection depends on the lipophilicity of FAs (estimated by log *P* values). TGs containing more hydrophilic FAs may thus be situated closer to the lipid–water interface, where the lipases act, and therefore be hydrolysed first (Raclot, 2003). This phenomenon leads to a modification of FA pattern in blubber layers throughout the fast through an accumulation of more lipophilic FAs. These results match the lipolytic process. Indeed, we know that the lipolytic enzymes (ATGL, HSL and MGL) are

water-soluble and that their action occurs at the water–lipid interface formed by the cytosol on one hand and by the lipids on the other (Derewenda et al., 1993). It is reasonable to think that the TGs containing the less lipophilic FAs are situated closer to the interface than those containing the more lipophilic FAs. Raclot (2003) put forward a similar hypothesis suggesting that the selective FA mobilisation from fat cells occurs according to their polarity, with a preferential hydrolysis of TGs containing more polar FAs. However, even if log *P* values are a good coarse indicator of FA mobilisation (FAs with log *P* values above 7 tended to be characterised by a less pronounced mobilisation, whereas FAs with log *P* values below 7 tended to show a higher degree of mobilisation), there was some discrepancy between the actual order of the mobilised FA (from highest to lowest) and the log *P* predictions, especially in outer blubber.

As already suggested in Noren et al. (2013), some FAs mobilised from NES pups may be particularly important in various physiological processes. C20:5n-3, which was highly mobilised from inner and outer blubber, is involved, among others, in neurobiological function (Innis, 2005; Kim et al., 2004). C20:4n-6, which was mobilised from inner blubber, may be the precursor of bioactive molecules, such as eicosanoids (Smith et al., 2002). Furthermore, ω -3 PUFAs and ω -6 PUFAs are required for structural growth and brain function (Innis, 2005). They are strong ligands of peroxisome proliferator-activated receptor (PPAR), which monitor genes involved in the lipid metabolism (Desvergne et al., 1999). The metabolism of PUFAs utilizes less oxygen than MUFAs and SFAs (Trumble et al., 2012), that may play an important role for the young animal learning to dive.

4.3. Stratification of fatty acids in blubber

The FA profiles varied within blubber layers. This stratification could be explained by the different roles played by the tissue (Lambert et al., 2013). The outer blubber layer was characterised by higher relative proportions of MC-MUFAs compared to the inner blubber layer. They were conserved in this layer throughout the fast. It is advantageous for NES pups to maintain this class of FAs with low melting points (Knothe et al., 2009) in order to preserve the fluidity of this tissue at cold temperatures as well as insulation and streamlining (Enser, 1984; Knothe et al., 2009). In contrast, the inner layer exhibited higher relative abundance of MC-SFAs than the outer layer. This proportion however decreased during the first half of the fast, with C10:0, C12:0, C14:0 and C16:0 showing a positive fractional mobilisation.

4.4. Genes involved in lipid mobilisation

Intracellular enzymes have a key role in the regulation of the breakdown of TG storage. ATGL and HSL are indeed responsible for 95% of the lipolytic activity in mice (Schweiger et al., 2006), but the expression of these two genes has never been quantified in weaned NES pups. Our results pointed out that their expression levels remained stable during the period of post-weaning fast in both blubber layers of NES. The first sampling was done during the first week of fasting. We can assume that the weaned NES pups have already developed all the enzymatic mechanisms for an efficient lipolysis and thus, the maximum expression levels of ATGL and HSL were already reached at the first week of fasting and maintained over the fast period. Investigating the expression levels of ATGL and HSL in suckling NES pups may confirm this hypothesis.

To date, no work has studied the gene expression of intracellular lipases in NES. In contrast to our RNA data, previous investigations reported an increase of ATGL protein levels across the fast in weaned pup adipose tissue (Viscarrà et al., 2012, 2013a, 2013b). This difference may reflect post-transcriptional regulation. However, the lack of consistent changes in gene expression in the current study was influenced by wide individual variation, suggesting that caution should be taken when drawing conclusions based on the small sample size used in the protein expression studies. Consistent with our findings, ATGL activity was

stable across the fast in lactating NES females (Fowler, 2012). HSL protein levels in weaned NES pups were stable during the post-weaning fast (Viscarrà et al., 2012), which corroborates the results from our study. Stable levels of lipolytic enzyme gene expression across the fast suggest that the free FA increases in serum across the fast are strongly influenced by changes in rates of re-esterification (Crocker et al., 2014; Viscarrà et al., 2013a, 2013b).

Levels of expression of lipolytic enzymes have been studied extensively in fasting rats. Levels of ATGL and HSL mRNA in epididymal adipose tissue increased over 4 days of fasting. They then remained constant during the next 2–3 fasting days for ATGL and decreased for HSL (Bertile et al., 2011). In contrary, Palou et al. (2010) have shown a stability of ATGL mRNA in the mesenteric and inguinal adipose tissue of rats over 24 h of fasting and an increase in the retroperitoneal adipose tissue. The levels of HSL mRNA in the three depots of white adipose tissue were stable over 24 h of fasting (Palou et al., 2010). These last studies show that a fast of only a few days does not necessarily cause increased expression of lipolytic enzymes in rats, which are non-fasting-adapted animals. NES naturally undergo extended fasting periods throughout their life history. It seems reasonable that they have developed some physiological adaptations to sustain long periods of fasting, leading to constant expression levels of lipolytic enzymes throughout the fast. Further longitudinal experiments coupling the study of mRNA expression and protein activity are needed to confirm this hypothesis.

To conclude, weaned NES pups exhibit a stratification of FAs within the blubber column in terms of profiles and rates of mobilisation. For this kind of study, it is thus critical to distinguish inner from outer blubber. Firstly, more MC-MUFAs were found in outer blubber compared to inner blubber, while proportions of Σ MC-SFAs were higher within inner blubber compared to outer blubber. This specific pattern promotes the fluidity of the outer layer in cold temperatures. Secondly, it appears from our data that FAs were preferentially released from inner blubber, confirming the higher metabolic activity of this layer. This trend was however not observed in the levels of expression of lipolytic enzymes (ATGL and HSL). The mobilisation of individual FAs was not homogeneous over the fast. The more lipophilic FAs were preferentially retained within the blubber, whereas the less lipophilic FAs were readily released. This observation could be compared to the dynamics of mobilisation of lipophilic pollutants stored in blubber, which are also influenced by the lipophilic character of the molecules.

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