



## Differential changes of fat-soluble vitamins and pollutants during lactation in northern elephant seal mother–pup pairs

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### ABSTRACT

We investigated the changes of vitamins A and E as well as PCBs and DDTs during lactation in northern elephant seal (*Mirounga angustirostris*) mother–pup pairs. On average, milk vitamin A concentrations were 6 times higher during late lactation than during early lactation, a pattern that differs dramatically from terrestrial mammals. Vitamin A concentrations also significantly increased in the inner blubber throughout lactation, whereas they remained constant in the outer blubber. Similar dynamics were observed for PCBs and DDTs in maternal blubber and milk. Blubber appears to be an important storage site for vitamin A and organochlorines in seals and a direct transfer of those molecules to the mammary gland may occur. The dynamics of vitamin A, PCBs and DDTs differed from those of vitamin E. There was a significant drop in milk vitamin E concentrations between early and late lactation, which is the usual pattern observed in terrestrial mammals. The dynamics of vitamin E in the blubber layers also differed from those of vitamin A, suggesting different mechanisms of mobilization and transfer into the milk.

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### 1. Introduction

Phocid seal milk contains high amounts of vitamins A and E as compared to the milk of terrestrial mammals (Debier et al., 1999, 2002a,b). These essential nutrients are involved in several important biological processes such as immunity, reproduction, protection against tissue damage, growth and development (Debier and Larondelle, 2005). They are of utmost importance during the early stages of life and must be provided in adequate amounts to ensure proper pre- and postnatal development of the offspring (Debier and Larondelle, 2005; Debier, 2007). A longitudinal study on grey seals (*Halichoerus grypus*) revealed discrepancies between the changes of vitamin A and vitamin E concentrations in milk (Debier et al., 2004). A dramatic drop followed by constant levels was observed between

colostrum and mature milk for vitamin E. This phenomenon is commonly seen in the milk of terrestrial mammals. Quite surprisingly, the dynamics of milk vitamin A were completely different from those of vitamin E and from the usual pattern of terrestrial, non-fasting mammals. Levels remained stable during the first half of lactation and then increased at the end of the nursing period. These data suggest differences in the mechanisms involved in the mobilization of vitamin A and vitamin E from storage sites and their transfer into the milk.

Vitamins A and E are not the only fat-soluble molecules to be transferred to the offspring through the milk. The mobilization of lipids from blubber releases fat-soluble pollutants that are then transferred into the milk (Pomeroy et al., 1996; Debier et al., 2003; Vanden Berghe et al., 2010). Persistent, fat-soluble pollutants, like organochlorines (e.g. PCBs and DDTs), have been related to toxic effects such as endocrine disruption, immunotoxicity, reproductive impairment and cancer development (Reijnders, 1986; Ross, 2000; Nyman et al., 2003; Debier et al., 2005a; Ylitalo et al., 2005; Letcher et al., 2010; Routti et al., 2010a). Previous work on lactating grey seals revealed that, contrary to general belief, the dynamics of transfer of organochlorines in the milk differed from those of total lipids (Debier et al., 2003). In addition, a curious parallelism was noticed

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between the changes of PCBs and those of vitamin A in milk (Debier et al., 2004; Vanden Berghe et al., 2010). However, as for vitamins, the exact biochemical mechanisms involved in the mobilization of organochlorines from adipose tissue and incorporation into the milk remain unknown.

Numerous studies in marine and terrestrial animals show that vitamin A metabolism can be disrupted by organochlorines (Zile, 1992; Brouwer et al., 1998; Rolland, 2000; Simms et al., 2000; Debier et al., 2005a; Mos et al., 2007; Novák et al., 2008; Letcher et al., 2010). The mechanisms have only been partly elucidated and involve among others a disruption of blood transport, liver storage, catabolism, as well as synthesis of retinoic acid nuclear receptors. To a lesser extent, vitamin E metabolism may also be disrupted by environmental pollutants. Indeed, several xenobiotics have been shown to induce oxidative stress and decrease vitamin E in animal tissues (Saito, 1990; Toborek et al., 1995; Palace et al., 1996; Slim et al., 1999). This phenomenon is amplified in an environment rich in polyunsaturated fatty acids (Hennig et al., 1998), which is the case for marine organisms such as seals (Strandberg et al., 2008; Wheatley et al., 2008). Vitamin A and, to a lesser extent, vitamin E may thus be useful biomarkers of effects due to organochlorine contamination. However, as described above, the physiological state of an animal (fasting and/or lactating) may exert a significant effect on the concentrations of both pollutants and vitamins encountered in the tissues examined (Debier et al., 2003; Routti et al., 2010b; Vanden Berghe et al., 2010). It is thus very important to take into account nutritional and reproductive status when assessing the possible effects of pollution on marine mammals (Routti et al., 2010b).

In the present study, we investigated the impact of lactation on the levels of fat-soluble vitamins (vitamins A and E) and fat-soluble pollutants (PCBs and DDTs) in northern elephant seal (NES – *Mirounga angustirostris*) mother–pup pairs. To our knowledge, it is the first time that the lactational transfer of vitamin A, vitamin E, PCBs and DDTs is presented and compared within the same longitudinal study using recaptured animals. It is also the first time that the dynamics of fat-soluble vitamins as well as fat-soluble pollutants are reported in lactating NES. The northern elephant seal provides an excellent model to study the mobilization of fat-soluble molecules from maternal body lipid stores and their transfer to offspring. Mothers fast during lactation (24–28 days) and secrete a fat-rich milk synthesized from endogenous nutrients (Le Boeuf and Ortiz, 1977). They lose almost 60% of their body fat (Crocker et al., 2001) during lactation and the mass transfer to pups is very efficient (>65%; Ortiz et al., 1984). During the nursing period, the pup gains approximately 90 kg, more than half of it being composed of lipids (Crocker et al., 2001). Lactation in NES is thus characterized by an important mobilization and transfer of maternal lipid stores. Twenty NES mother–pup pairs were captured and sampled at day 4 and day 21 of lactation in order to get longitudinal samples of blubber, serum and milk from the mothers and serum from their pups. Samples were analyzed for vitamins A and E, 19 PCB congeners, DDT and its metabolites.

## 2. Material and methods

### 2.1. Sample collection

The study was conducted on the colony of Año Nuevo, CA, USA (37°06'30"N, 122°20'10"W) in January and February 2005. Twenty mother–pup pairs were captured at day 4 and day 21 of lactation. Dates of birth were recorded by marking the mothers with hair dye and observing the colony each day. Mothers were immobilized by intramuscular injection of Telazol (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA) at a dose of 1 mL per 100 kg of estimated body mass. At both captures, blood, milk and blubber were taken from the mothers. Blood samples were collected by placement of an 18 g

spinal needle in the intravertebral space and into the extradural vein. Two blubber biopsies extending the full depth of the blubber layer were taken in the lateral pelvis area using a 6 mm biopsy punch (Uni-Punch, Premier Medical, Plymouth, PA, USA). After a subcutaneous injection of 40 IU of oxytocin (American Pharmaceuticals Partners, Los Angeles, CA, USA) injected near the mammary gland, milk was collected from the teat by palpitation using a syringe with the tapered portion cut off. For pups, blood samples were collected from the extradural vein at both captures. Mothers and pups were reunited after the procedure and observed to ensure they remained together. Whole blood samples were centrifuged for 30 min and serum was aliquoted into 1.5 mL Nunc tubes (Nalge Nunc International, Rochester, NY, USA). Blubber samples for organochlorine analyses were stored at –20 °C in aluminum foil. Blubber samples for vitamin analyses were stored at –80 °C in Nunc tubes.

### 2.2. Milk fat content

Milk lipids were extracted using an accelerated solvent extractor with a mixture of hexane, dichloromethane and methanol at 80 °C under a pressure of 10.34 GPa. The fat content of milk samples was then determined gravimetrically (see Debier et al., 2003 for more details).

### 2.3. Organochlorine analyses

Blubber biopsies (approximately 6 cm long) were cut into three equal parts. Inner (closest to the muscle) and outer (closest to the skin) layers were analyzed separately. Blubber, serum and milk were analyzed for 19 PCB congeners (IUPAC 52, 101, 105, 110, 118, 128, 138, 143, 149, 153, 156, 170, 180, 183, 187, 194, 195, 206, 209) as well as *p,p'*DDT, *p,p'*DDE and *p,p'*DDD (DDTs) by gas chromatography using a Thermo Quest Trace 2000 gas chromatograph equipped with a 63Ni ECD (electron capture detector; Thermo Quest, Trace 2000, Milan, Italy). The details of sample preparation, clean-up and analysis, including quality assurance are provided in Debier et al. (2003). Results are expressed per unit of wet weight, except for blubber, where they are expressed per unit of lipid weight.

### 2.4. Vitamin A and E analyses

As for organochlorine analyses, blubber biopsies were cut into 3 equal parts and inner and outer layers were analyzed separately. Sample preparation and extraction were performed as described in Debier et al. (2002a,b) and Vanden Berghe et al. (2010). Milk and blubber samples underwent saponification with ethanolic KOH under nitrogen flow before vitamin A and E extraction and analysis. Vitamin A as well as vitamin E were analyzed by HPLC using a RF-551 spectrofluorometric detector (Shimadzu, Kyoto, Japan) (vitamin A: excitation 325 nm, emission 475 nm; vitamin E: excitation 290 nm, emission 325 nm). Vitamin A quantification corresponded to retinol in serum and to retinol and retinyl esters in blubber and milk. Vitamin E quantification corresponded to  $\alpha$ -tocopherol in serum and to  $\alpha$ -tocopherol and  $\alpha$ -tocopheryl esters in milk and blubber. Results are expressed per unit of wet weight.

### 2.5. Data analyses

Results were analyzed using the General Linear Model (GLM) procedure (Statistica 7.1). In order to lower the variance heterogeneity and normalize the data, all the tests were conducted on the logarithms ( $\log_e$ ) of PCB, DDT, vitamin A and vitamin E concentrations. The variations of concentrations in the blubber of mothers were analyzed using a three-way mixed ANOVA, crossed design, with the following factors: individual, stage of lactation (early or late) and blubber layer (inner or outer). Stage of lactation and blubber layer

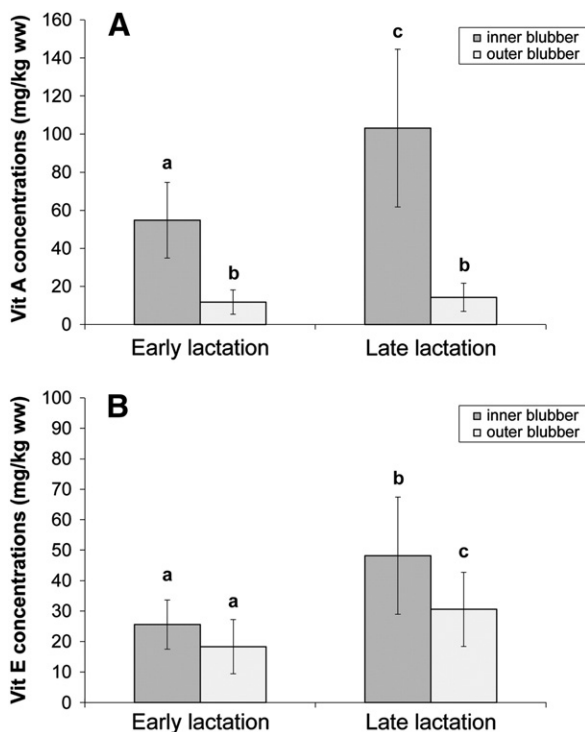
were then combined into a single 4 level-factor (stage-layer), whose levels were compared pairwise, using Bonferroni's t-test for paired samples. The variations of PCB or DDT concentrations in the serum (mother or pup) and in the milk were tested using a two-way mixed ANOVA, crossed design, with the following factors: individual and stage of lactation (early or late). The level of statistical significance was set at  $p \leq 0.05$  for all analyses.

### 3. Results

Milk lipid content increased from  $28.2 \pm 5.0\%$  at day 4 to  $45.8 \pm 6.3\%$  at day 21 ( $P < 0.01$ ,  $df = 1,19$ ) but did not vary among lactating females ( $p = 0.34$ ,  $df = 19,19$ ).

#### 3.1. Vitamin A

Vitamin A concentrations in maternal blubber significantly varied among individuals ( $p < 0.01$ ,  $df = 19,57$ ). There was also a difference of concentrations between inner and outer blubber, during both early and late lactation ( $p < 0.01$ ,  $df = 3,57$ ) (Fig. 1A). The inner blubber contained from 4.5 to 7.5 times higher vitamin A levels than the outer blubber throughout lactation. In inner blubber, vitamin A concentrations increased significantly ( $p < 0.01$ ,  $df = 3,57$ ) whereas they remained constant in outer blubber ( $p = 1.00$ ,  $df = 3,57$ ) between early and late lactation (Table 1 and Fig. 1A). In maternal serum, concentrations of retinol significantly varied among lactating females ( $p < 0.01$ ;  $df = 19,19$ ) but did not change with lactation stage ( $p = 0.57$ ,  $df = 1,19$ ) (Table 1). There was a dramatic increase of vitamin A concentrations in milk between early and late lactation ( $p < 0.01$ ,  $df = 1,19$ ) (Table 1 and Fig. 2A). On average, levels were 6 times higher at late lactation. Milk vitamin A concentrations varied significantly among individuals ( $p = 0.03$ ,  $df = 19,19$ ). In pups, circulating levels of vitamin A varied significantly among individuals ( $p < 0.01$ ,  $df = 19,19$ ) and increased between day 4 and day 21 ( $p < 0.01$ ,  $df = 1,19$ ) (Table 1).



**Fig. 1.** Changes in vitamin A (A) and vitamin E (B) concentrations (mean  $\pm$  standard deviation) in inner and outer blubber of 20 NES females at early (day 4) and late (day 21) lactation. Bars that do not share the same letter show significant differences ( $p < 0.05$ ). Values are expressed per unit of wet weight (ww).

**Table 1**

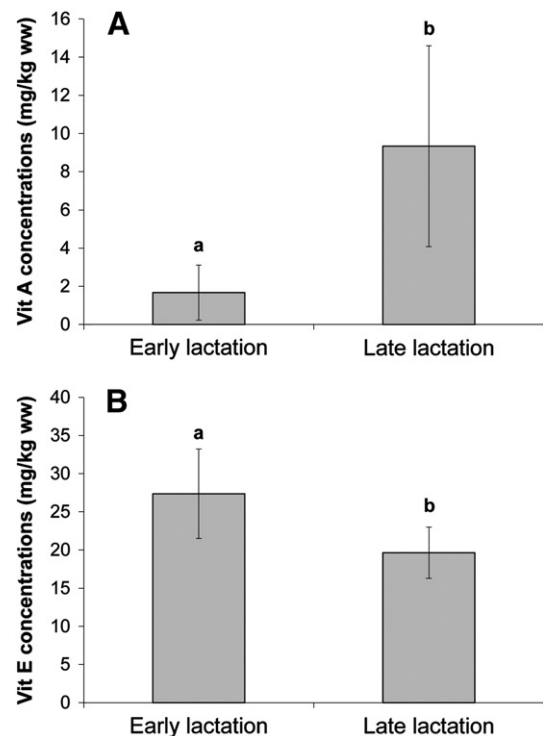
Mean ( $\pm$  standard deviation) vitamin A and vitamin E levels at early and late lactation in the different compartments of transfer<sup>a</sup>.

| Compartment                    | Vitamin A                    |                               | Vitamin E                   |                              |
|--------------------------------|------------------------------|-------------------------------|-----------------------------|------------------------------|
|                                | Early                        | Late                          | Early                       | Late                         |
| Maternal outer blubber (mg/kg) | 11.8 $\pm$ 6.4 <sup>a</sup>  | 14.2 $\pm$ 7.4 <sup>a</sup>   | 18.3 $\pm$ 8.9 <sup>1</sup> | 30.6 $\pm$ 12.2 <sup>2</sup> |
| Maternal inner blubber (mg/kg) | 54.8 $\pm$ 19.9 <sup>a</sup> | 103.2 $\pm$ 41.4 <sup>b</sup> | 25.6 $\pm$ 8.1 <sup>1</sup> | 48.2 $\pm$ 19.2 <sup>2</sup> |
| Maternal serum (mg/L)          | 0.42 $\pm$ 0.09 <sup>a</sup> | 0.43 $\pm$ 0.12 <sup>a</sup>  | 30.7 $\pm$ 7.9 <sup>1</sup> | 30.2 $\pm$ 9.6 <sup>1</sup>  |
| Milk (mg/kg)                   | 1.7 $\pm$ 1.4 <sup>a</sup>   | 9.3 $\pm$ 5.3 <sup>b</sup>    | 27.4 $\pm$ 5.9 <sup>1</sup> | 19.6 $\pm$ 3.3 <sup>2</sup>  |
| Pup serum (mg/L)               | 0.25 $\pm$ 0.07 <sup>a</sup> | 0.53 $\pm$ 0.14 <sup>b</sup>  | 21.2 $\pm$ 6.2 <sup>1</sup> | 21.2 $\pm$ 4.6 <sup>1</sup>  |

<sup>a</sup> Values followed by different letters/numbers are significantly different ( $p < 0.05$ ) between early and late lactation. All results are expressed per unit of wet mass.

#### 3.2. Vitamin E

Vitamin E concentrations in maternal blubber were different among females ( $p < 0.01$ ,  $df = 19,57$ ). At early lactation, there was no significant difference in concentrations between the inner and outer blubber ( $p = 0.11$ ;  $df = 3,57$ ) (Fig. 1B). By contrast, during late lactation, inner blubber concentrations were higher than outer blubber concentrations ( $p < 0.01$ ,  $df = 3,57$ ) (Fig. 1B). In both blubber layers, vitamin E concentration increased between early and late lactation ( $p < 0.01$ ,  $df = 3,57$ ) (Table 1 and Fig. 1B). As observed for vitamin A, vitamin E concentrations in maternal serum differed among females ( $p < 0.01$ ,  $df = 19,19$ ), but remained stable throughout lactation ( $p = 0.57$ ,  $df = 1,19$ ) (Table 1). There was a drop of vitamin E concentration in milk between day 4 and day 21 ( $p < 0.01$ ,  $df = 1,19$ ) (Table 1 and Fig. 2B). Concentrations varied among individuals ( $p < 0.01$ ,  $df = 19,19$ ). Vitamin E concentration in pup



**Fig. 2.** Changes in vitamin A (A) and vitamin E (B) concentrations (mean  $\pm$  standard deviation) in the milk of 20 NES females at early (day 4) and late (day 21) lactation. Bars that do not share the same letter show significant differences ( $p < 0.05$ ). Values are expressed per unit of wet weight (ww).

serum differed among individuals ( $p < 0.01$ ,  $df = 19,19$ ), but did not vary between early and late lactation ( $p = 0.81$ ,  $df = 1,19$ ).

### 3.3. PCBs and DDTs

In all compartments, the major congeners were hexa-chlorobiphenyls (hexa-CBs) followed by hepta- and penta-CBs. Tetra-CBs were present in very low concentrations. Octa- and nona-CBs were always situated under the detection limit. Penta-CBs were mostly represented by PCB-101 and 118, hexa-CBs by PCB-128, -138, -149, -153, and -156, and hepta-CBs by PCB-170, -180, -183, and -187. These congeners accounted for more than 95% of total PCB concentrations.

PCB concentrations in maternal blubber differed among lactating females ( $p < 0.01$ ,  $df = 19,57$ ). PCB levels in inner blubber increased significantly between early and late lactation ( $p < 0.01$ ,  $df = 3,57$ ) (Table 2 and Fig. 3A). On the other hand, no significant difference in PCB concentrations was noted between early and late lactation in outer blubber ( $p = 1.00$ ,  $df = 3,57$ ). During early lactation, inner blubber was significantly less contaminated by PCBs than the outer blubber ( $p < 0.01$ ,  $df = 3,57$ ). The inverse tendency was observed during late lactation, where inner blubber PCB levels were higher than outer blubber concentrations ( $p < 0.01$ ,  $df = 3,57$ ) (Fig. 3A). PCB concentrations in the serum of lactating females varied among individuals ( $p < 0.01$ ,  $df = 19,19$ ) and increased with time ( $p < 0.01$ ,  $df = 1,19$ ) (Table 2). PCB concentrations in milk increased between early and late lactation ( $p < 0.01$ ,  $df = 1,19$ ) (Table 2 and Fig. 4A). Concentrations did not vary among individuals ( $p = 0.25$ ,  $df = 19,19$ ). PCB concentrations in pup serum increased between early and late lactation ( $p < 0.01$ ,  $df = 1,19$ ) (Table 2). Concentrations did not vary among individuals ( $p = 0.13$ ,  $df = 19,19$ ).

The proportions of the main groups of congeners (penta, hexa and hepta-CBs) remained fairly constant between early and late lactation in all body compartments (Fig. 5). Some slight differences of PCB profiles among compartments can be observed in Fig. 5. In general, female serum, milk and pup serum contained a lower proportion of hepta-CBs than maternal blubber.

*p,p'*DDE accounted for more than 95% of all forms (*p,p'*DDT, *p,p'*DDD and *p,p'*DDE). The ratios of DDTs to PCBs in the different compartments of transfer ranged from 1.5 to 2.5. DDT concentrations in maternal blubber showed differences among lactating females ( $p < 0.01$ ,  $df = 19,57$ ). As with PCBs, DDT concentrations in inner blubber increased between early and late lactation ( $p < 0.01$ ,  $df = 3,57$ ), whereas they remained constant in outer blubber ( $p = 1.00$ ,  $df = 3,57$ ) (Table 2 and Fig. 3B). During early lactation, inner blubber was less contaminated by DDTs than outer blubber ( $p < 0.01$ ,  $df = 3,57$ ). The difference disappeared during late lactation ( $p = 0.68$ ,  $df = 3,57$ ) (Table 2 and Fig. 3B). As with the PCBs, DDT concentrations in maternal serum varied among individuals ( $p < 0.01$ ;

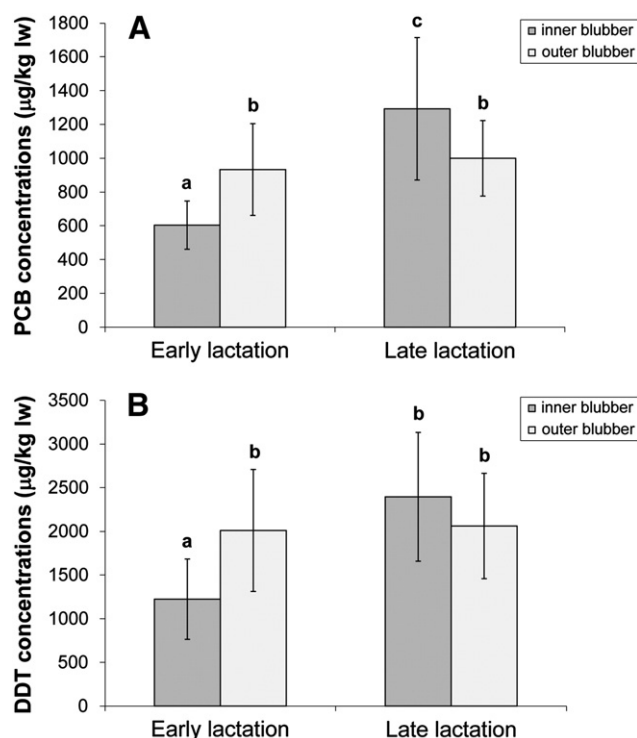


Fig. 3. Changes in PCB (A) and DDT (B) concentrations (mean  $\pm$  standard deviation) in inner and outer blubber of 20 NES females at early (day 4) and late (day 21) lactation. Bars that do not share the same letter show significant differences ( $p < 0.05$ ). Values are expressed per unit of lipid weight (lw).

$df = 19,19$ ) and increased between early and late lactation ( $p < 0.01$ ;  $df = 1,19$ ) (Table 2). Concentrations in milk increased between early and late lactation ( $p < 0.01$ ,  $df = 1,19$ ) (Table 2 and Fig. 4B). Milk DDT concentrations did not vary among individuals ( $p = 0.15$ ;  $df = 19,19$ ). DDT concentrations in pup serum did not change between early and late lactation ( $p = 0.12$ ;  $df = 1,19$ ) and among individuals ( $p = 0.14$ ;  $df = 19,19$ ) (Table 2).

## 4. Discussion

### 4.1. Vitamin A

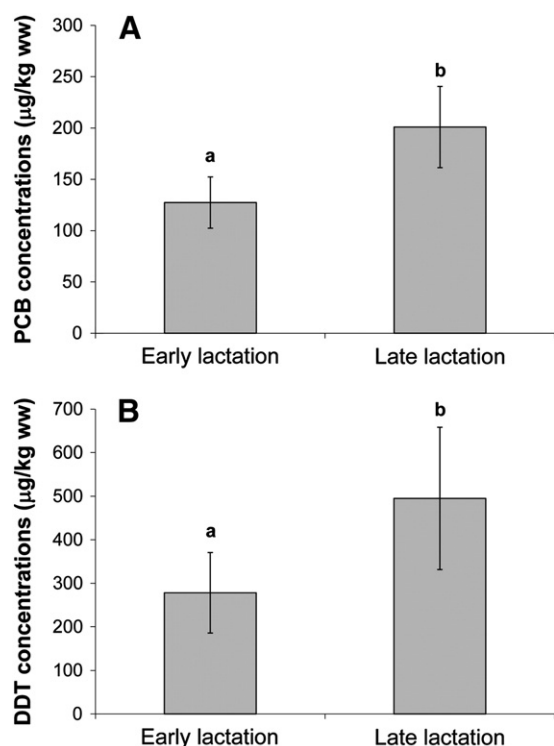
Contrary to terrestrial mammals, a large proportion of vitamin A (40–66%) appears to be stored in adipose tissue in seals (Schweigert et al., 1987; Käkälä et al., 1997; Mos and Ross, 2002).

Table 2

Mean ( $\pm$  standard deviation) PCB and DDT levels at early and late lactation in the different compartments of transfer<sup>a</sup>.

| Compartment                    | PCBs                           |                                 | DDTs                            |                                 |
|--------------------------------|--------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                | Early                          | Late                            | Early                           | Late                            |
| Maternal outer blubber (µg/kg) | 933.2 $\pm$ 271.8 <sup>a</sup> | 999.7 $\pm$ 223.5 <sup>a</sup>  | 2010.7 $\pm$ 698.1 <sup>1</sup> | 2061.9 $\pm$ 603.5 <sup>1</sup> |
| Maternal inner blubber (µg/kg) | 604.2 $\pm$ 142.8 <sup>a</sup> | 1292.5 $\pm$ 421.6 <sup>b</sup> | 1223.9 $\pm$ 460.2 <sup>1</sup> | 2395.9 $\pm$ 737.3 <sup>2</sup> |
| Maternal serum (µg/L)          | 5.4 $\pm$ 0.8 <sup>a</sup>     | 7.7 $\pm$ 1.4 <sup>b</sup>      | 7.8 $\pm$ 2.4 <sup>1</sup>      | 11.9 $\pm$ 4.1 <sup>2</sup>     |
| Milk (µg/kg)                   | 127.3 $\pm$ 25.0 <sup>a</sup>  | 200.9 $\pm$ 39.6 <sup>b</sup>   | 278.2 $\pm$ 92.4 <sup>1</sup>   | 494.9 $\pm$ 163.4 <sup>2</sup>  |
| Pup serum (µg/kg)              | 5.8 $\pm$ 1.4 <sup>a</sup>     | 6.1 $\pm$ 2.5 <sup>b</sup>      | 8.3 $\pm$ 2.5 <sup>1</sup>      | 9.9 $\pm$ 4.3 <sup>1</sup>      |

<sup>a</sup> Values followed by different letters/numbers are significantly different ( $p < 0.05$ ) between early and late lactation. All results are expressed per unit of wet weight, except for concentrations in blubber, which are expressed per unit of lipids.

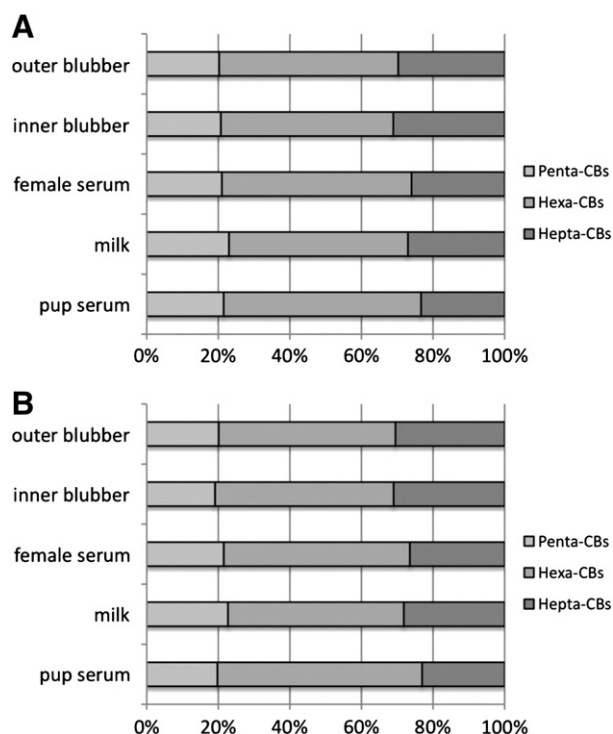


**Fig. 4.** Changes in PCB (A) and DDT (B) concentrations (mean  $\pm$  standard deviation) in the milk of 20 NES females at early (day 4) and late (day 21) lactation. Bars that do not share the same letter show significant differences ( $p < 0.05$ ). Values are expressed per unit of wet mass (ww).

In the NES, the inner blubber was systematically much more concentrated in vitamin A than the outer blubber, both during early and late lactation. These variations may be explained by vertical

metabolic divergence of the blubber layers. Indeed, differences in the fatty acid profile between the inner and outer blubber have been shown in southern elephant seals (*Mirounga leonina*) (Best et al., 2003) and are confirmed in NES from the present study (results not shown). Vitamin A being stored as retinyl esters in adipose tissue, its esterification and storage may occur preferentially with some fatty acids as compared to others. In a previous study on ringed seals (*Pusa hispida*), it was suggested that the vertical distribution of vitamin A could exhibit stratification patterns similar to those of fatty acids (Strandberg et al., 2008). An analysis of the different forms of retinyl esters should be conducted and compared to the fatty acid profile encountered in each layer to confirm this hypothesis. A higher expression of intracellular transporters (cellular retinol binding protein CRBP I and III – Bellovino et al., 2003; Piantedosi et al., 2005) as well as enzymes involved in the esterification of retinol into retinylesters might also contribute to the sharp difference between the layers. Vanden Berghe et al. (2010) also noticed a difference of vitamin A concentrations between inner and outer blubber in grey seals. However, during early lactation, the tendency was different than in our study, with lower concentrations in the inner blubber compared to the outer blubber. In general, outer blubber was also more concentrated in vitamin A than in the present study. These discrepancies might result from dietary and metabolic differences between the 2 species.

In marine mammals, lipids are mainly mobilized from inner blubber in cases of negative energy balance such as occurs during fasting and/or lactation. On the other hand, outer blubber, which is involved in thermoregulation and buoyancy, is usually considered as more stable (Koopman et al., 1996; Best et al., 2003; Strandberg et al., 2008). The increase of vitamin A concentrations with time in NES inner blubber reflects the fact that this nutrient is less efficiently mobilized from blubber than are triacylglycerols. However, during late lactation, the mobilization rate of vitamin A from blubber appears to increase, as is apparent in the dramatic rise in vitamin A within the milk. Milk vitamin A concentrations were indeed, on average, more than 5 times higher at day 21 than at day 4. This tendency was also observed in UK grey seals (Debier et al., 2002b; 2004; Vanden Berghe et al., 2010). The increase in milk vitamin A did not directly result from the increase of milk lipids. Indeed, even when expressed per unit of milk lipids, vitamin A levels were significantly higher during late lactation as compared to early lactation (results not shown). In Debier et al. (2002b), grey seal females were sampled up to 5 times between birth and late lactation. Results revealed different dynamics between milk fat and vitamin A. Milk triacylglycerols indeed increased during the first half of the nursing period and then stayed constant thereafter, whereas milk vitamin A levels expressed per unit of wet weight remained constant during the first half and then increased with the progression to late lactation (Debier et al., 2002b, 2004). It must however be noted that when vitamin A was expressed per unit of milk lipids, concentrations in colostrum were higher than in milk collected 3 days later (Debier et al., 2002b). Schweigert and Stobo (1994) observed comparable results for Canadian grey seals. Such changes could not be tested in the present study because of the absence of multiple sampling at early lactation. Contrary to the present study, as well as to the longitudinal studies on UK grey seals (Debier et al., 2002b; Debier et al., 2004; Vanden Berghe et al., 2010), Schweigert and Stobo (1994) did not report an increase in milk vitamin A concentrations at late lactation. The fact that this study did not use longitudinal samples might be at the origin of these differences. Indeed, the authors based their study on shot animals sampled once, at various lactation stages, instead of animals serially captured and sampled between early and late lactation. Temporal trends might be more difficult to visualize in the former case, due to inter-individual differences. The increase in milk vitamin A concentrations at late lactation is not reported in



**Fig. 5.** Mean PCB patterns in the different compartments of transfer (outer blubber, inner blubber, female serum, milk and pup serum) at early (day 4 – A) and late (day 21 – B) lactation (20 mother–pup pairs).

terrestrial mammals which exhibit higher vitamin A levels in colostrum (Macias and Schweigert, 2001; Schweigert et al., 2004; Calderon et al., 2007). However, those animals do not fast during lactation. As a consequence, postprandial vitamin A transported as retinylesters in chylomicrons might also be taken up by the mammary gland through the contribution of lipoprotein lipase (Van Bennekum et al., 1999; Ross et al., 2004). In addition, milk vitamin A originates mainly from the liver rather than from the blubber in terrestrial mammals.

A higher rate of mobilization of vitamin A from the inner blubber at late lactation was already suggested for grey seals (Debier et al., 2002b; Vanden Berghe et al., 2010) and might be due to several factors. Circulating vitamin A is transported in great part bound to retinol binding protein (RBP), its specific transport protein. RBP is synthesized in adipose tissue (Tsutsumi et al., 1992), the RBP-retinol complex can therefore be mobilized from the blubber and directed to the mammary gland. A higher expression of RBP as well as enzymes involved in the hydrolysis of retinylesters in the adipose tissue might contribute to the increased mobilization of vitamin A at late lactation. The rate of mobilization of vitamin A might also be linked to the one of fatty acids. Indeed, the mobilization of fatty acids from triacylglycerols has been shown to be a selective process in adipose tissue (Raclet, 2003). Short and unsaturated fatty acids are mobilized first, followed by less hydrophilic fatty acids, which are liberated later. It is possible that the rate of mobilization of vitamin A into the blood is linked to this differential mobilization of fatty acids, as detailed in Vanden Berghe et al. (2010). At the mammary gland level, an increase of expression of RBP-retinol receptors as well as cellular retinol binding proteins (CRBP I and III) might also explain the increase of vitamin A in milk at the end of lactation (independently or in combination with the higher release from blubber).

In case of a higher mobilization of vitamin A from blubber at the end of lactation, one could expect to find an increase of circulating levels. However, retinol levels did not change between day 4 and day 21. In the grey seals, vitamin A levels decreased slightly between day 0 and day 3 of lactation and then increased slightly until the end of lactation (Debier et al., 2002b; Vanden Berghe et al., 2010). Perhaps such a profile also occurred in the serum of lactating NES, but could not be tested because there were only two sampling points per animal and no capture on the day of parturition. Alternatively, considering that circulating retinol levels are under homeostatic regulation (Debier and Larondelle, 2005), the higher release of retinol by the blubber during late lactation might have been balanced by a higher uptake at the mammary gland level, as mentioned above. Uptakes by the liver or the kidneys, which both play important roles in vitamin A metabolism, might also have occurred (Käkelä et al., 2003; Debier and Larondelle, 2005).

After intestinal absorption of vitamin A by the suckling pups, the retinylesters are transported into chylomicrons and might in part be taken up by extrahepatic tissues such as the adipose tissue through the contribution of lipoprotein lipase (Van Bennekum et al., 1999; Debier and Larondelle, 2005). After clearance of chylomicron remnants, the liver stores part of the vitamin A as retinylesters in stellate cells and a highly regulated amount will be secreted as retinol bound to its transport protein (RBP) (Debier and Larondelle, 2005). During early lactation, NES suckling pups exhibited low retinol concentrations, probably resulting from a low placental transfer. However, at the end of lactation, retinol levels increased and were even higher than in maternal serum. This increase most probably results from the ingestion of large volumes of milk with high vitamin A concentrations. A rapid and consistent supply of vitamin A is important for neonatal growth and development. In addition, it is essential that the newborn quickly establishes its own reserves before the 2–3 month post-weaning fast. The increase in circulating vitamin A in suckling pups was also noticed in grey seals and harbour seals (*Phoca vitulina*) (Simms and Ross, 2000; Debier et al., 2002b; Vanden Berghe et al., 2010).

## 4.2. Vitamin E

Vitamin E concentrations increased in inner and outer blubber layers between early and late lactation. The increase was however sharper in the inner blubber. This increase might result from the mobilization of lipids from the blubber and the concentration of vitamin E in the remaining layer as lactation progresses. Part of the inner blubber vitamin E might also migrate to the outer layer, resulting in an increase of concentrations in that layer too. Contrary to the observations made on vitamin A, vitamin E did not seem to be mobilized from the blubber during lactation. The main source would rather be situated at the level of liver as suggested by Schweigert et al. (2002). These authors have indeed noticed a drop of vitamin E concentration in the liver of grey seals during the lactation period.

In terrestrial mammals, vitamin E circulates in the serum as  $\alpha$ -tocopherol associated to lipoproteins (very low density lipoproteins or VLDL, low density lipoproteins or LDL and high density lipoproteins or HDL). The uptake of  $\alpha$ -tocopherol by the cells appears to be allowed by several receptors (scavenger receptor class B, type I, (SR-BI), phospholipid transfer protein (PLTP)) (Spector and Johanson, 2007; Tachikawa et al., 2007; Takada and Suzuki, 2010). If present in the mammary gland of NES, those receptors might participate in the uptake of  $\alpha$ -tocopherol by the mammary cells. Lipoprotein lipase may also play a role, as it appears to be important for the delivery of  $\alpha$ -tocopherol carried in VLDL to the mammary gland (Martinez et al., 2002). In maternal NES serum, vitamin E levels remained constant. The study of Debier et al. (2002a) on UK grey seals showed a slight drop of vitamin E levels soon after birth. The authors suggested that this drop was due to an increase of lipoprotein receptors in the mammary gland in order to incorporate large amounts of vitamin E in the milk. Similar observations were also reported with Canadian grey seals in Schweigert et al. (2002). The decrease may also have happened in NES females from the present study but could not be tested, as our females were not sampled near parturition.

The dynamics of vitamin E differed from those of vitamin A in milk, with higher levels at day 4 compared to day 21. The production of a colostrum with high concentrations of vitamin E as compared to mature milk is the usual pattern of terrestrial and marine mammals (Schweigert and Stobo, 1994; Debier et al., 1999; Macias and Schweigert, 2001; Debier et al., 2002a; Schweigert et al., 2004; Calderon et al., 2007). A rapid vitamin E supply is important to protect the newborn against oxidative stress and to contribute to the development of its immune system. The fact that the drop observed here was less pronounced than in the other phocid seal studies (Schweigert and Stobo, 1994; Debier et al., 1999; Debier et al., 2002a) is most probably due to the timing of the sample collection. Samples collected here were taken 4 days after birth whereas, in the other seal species, samples were collected within the first 48 h following birth. The drop in vitamin E between colostrum and milk is potentially explained by the increase in the diameter of milk fat globules as lactation progresses, which could have a negative impact on the constituents of their membranes, including vitamin E (Boersma et al., 1991; Barbas and Herrera, 1998).

In pups, vitamin E absorbed by the intestine is transported as  $\alpha$ -tocopherol in chylomicrons before being taken up by the liver. Vitamin E is then transported in the circulation in association with VLDL, LDL and HDL, as mentioned earlier (Debier and Larondelle, 2005). A previous study in grey seals (Debier et al., 2002a) showed that serum vitamin E increased dramatically during the very first days after birth, following the ingestion of colostrum containing very high levels of vitamin E. Circulating vitamin E concentrations then decreased and remained stable until the end of lactation. Contrary to vitamin A, the main changes in the dynamics of vitamin E were thus shown to occur at the onset of lactation, during the formation of colostrum in the mammary gland and its ingestion by the

newborn. In the present study, vitamin E concentrations were relatively stable in pup serum, after 4 and 22 days of lactation. Here again, we suspect that changes have occurred during the very first days of lactation but could not be seen due to the absence of samplings during that period.

#### 4.3. PCBs and DDTs

The PCB profile of the NES was mainly composed of penta-, hexa- and hepta-CBs. This profile corresponds to the one observed in NES weaned pups (Debier et al., 2005b, 2006). It differs from the one of lactating grey seals from the North Sea that contained higher proportions of octa and nona-CBs (Debier et al., 2003). The fact that DDT concentrations were twice higher than PCB concentrations is a common pattern of contamination of marine mammals from the west coast of United States (Le Boeuf et al., 2002; Debier et al., 2005a). The very high DDE/ΣDDT ratio is indicative of long-term pollution.

Blubber is the main storage site of organochlorines (98–99%) (Wolkers et al., 2006). Considering a transfer from maternal stores, blubber can thus be reasonably considered as the main site of mobilization and transfer into the subsequent compartments (maternal circulation, mammary gland, milk and pup circulation). The difference in the dynamics of PCBs and DDTs between blubber layers was previously observed in lactating grey seals as well as in NES pups during the post-weaning fast (Debier et al., 2003; Debier et al., 2006; Vanden Berghe et al., 2010). As lactation progresses, the release of fatty acids from the inner blubber drives the increase of the PCBs and DDTs in the remaining layer. As with vitamin A, PCBs and DDTs are less easily mobilized from blubber than fatty acids at early lactation. At late lactation, the increase in PCBs and DDTs in serum most probably results from an increase in their mobilization rate from the blubber. As suggested for vitamin A and detailed in Debier et al. (2006) and Vanden Berghe et al. (2010), the dynamics of organochlorine release from blubber might be linked, at least in part, to the selective mobilization of fatty acids according to their physico-chemical properties. In the present study, the increase in PCB and DDT concentrations in milk reflected the rise observed in maternal serum. By contrast, we suspect that this increase is not directly linked to the increase of milk lipids, as a previous study on grey seals, for which a 3-point sampling was conducted (early, mid and late) showed that longitudinal dynamics of organochlorines in milk were completely unlike those of triacylglycerols (Debier et al., 2003). Levels of organochlorines in milk are thus not simply reflected by the movements of triacylglycerols (Debier et al., 2004). The tendency towards an increase in PCBs and DDTs as lactation progresses is less obvious in pup serum. This phenomenon is quite surprising as milk contamination as well as milk ingestion by the pups increase with the stage of lactation. However, this result does not mean that the body burden of the animals did not rise. As mentioned in Wolkers et al. (2006), blood contaminant concentrations do not reflect the whole body contaminant burden. The contaminants ingested and absorbed by the pups are perhaps rapidly deposited in their blubber or liver, the latter appearing to be a more important storage site for organochlorines than blubber in the particular case of suckling pups (Wolkers et al., 2006).

#### 5. Conclusions

The intriguing similarities between the changes of PCBs, DDTs and vitamin A that were previously noticed in lactating grey seals (Debier et al., 2004; Vanden Berghe et al., 2010) were also observed in NES. Concentrations increased sharply in the inner blubber and milk, whereas they remained constant in outer blubber across lactation. The fact that blubber constitutes the main site of storage and transfer of both organochlorines and vitamin A could explain this parallelism. The dynamics of vitamin E differed from those of the organochlorines

and vitamin A. The discrepancies between the dynamics of vitamin A and vitamin E during lactation suggest that these fat-soluble vitamins are mobilized from storage tissues by different mechanisms. This finding is in accordance with other seal studies during either lactation (Schweigert et al., 2002; Debier et al., 2004) or molting (Routti et al., 2010b).

This study brings additional data about the significant impact that lactation/fasting exerts on the concentrations of both vitamins and organochlorines in tissues. Because food deprivation and negative energy balance are common in seals, it is important to account for the physiological status of the seals when using vitamins as biomarkers of effects. It is also essential to be aware that the fat-soluble vitamins and contaminants are not homogeneously distributed throughout the blubber layer. This fact must be taken into account, among others, in the process of assessing the possible effects of pollution on marine mammals.

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