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## Change in plasma cortisol and metabolites during the attendance period ashore in fasting lactating subantarctic fur seals

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### Abstract

Lactating fur seals (*Arctocephalus tropicalis*) alternate foraging trips at sea and pup attendance periods ashore. During the onshore nursing periods, lactating females do not have access to food and meet both their own metabolic requirements and milk production from their body reserve. Blood and milk samples were collected from females captured soon after their arrival ashore from a foraging trip and before their departure. Milk lipid but not milk protein content was positively related to the body condition index (BCI) of the female. During the 4-day attendance period ashore, females lost body mass, and plasma cortisol levels increased, whereas plasma urea concentration decreased and  $\beta$ -hydroxybutyrate ( $\beta$ -OHB) remained unchanged. The increase in cortisol level took place while blood urea concentration decreased and  $\beta$ -OHB remained at a low level suggesting that it was independent from the transition from phase II to phase III that is indicative of the depletion of lipid body store as described in penguins. Thus, our results suggest that the increase in cortisol level in relation to decreasing BCI may either contribute to the mobilization of protein stores to ensure milk production when easily mobilized stores are used and/or could act as a re-feeding signal which is triggered well before females have depleted their body store.

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### 1. Introduction

In otariids (fur seals and sea lions), maternal care consists of lactation during a 4-month to 3-year pup rearing period (Gentry and Kooyman, 1986). During lactation, females alternate foraging trips at sea and attendance periods ashore. At sea, mothers acquire the nutrients to cover their own

energy expenditure and to store body reserve. During the onshore nursing periods, adult females do not have access to food and meet both their metabolic requirements and milk production from stored body reserve.

On Amsterdam Island, subantarctic fur seals *Arctocephalus tropicalis* nurse their pups during a 10-month rearing period (Tollu, 1974; Guinet and Georges, 2000) and perform long foraging trips (on average from 11 to 23 days at sea in summer and winter, respectively) while remaining approximately 4 days ashore suckling their pups (Georges and Guinet, 2000a). The duration of the foraging

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trips of *A. tropicalis* females on Amsterdam Island is with the Juan Fernandez fur seals *Arctocephalus philippii* (Francis et al., 1998) among the longest observed for any fur seal species studied to date. During their foraging trips, lactating subantarctic females gain mass that is lost over their visit ashore (Georges and Guinet, 2000a).

The duration of the shore visits and the decision of these females to leave the shore to feed at sea appear to be related to the body store reaching some critical nutritional point (Gentry and Kooyman, 1986; Georges and Guinet, 2000a). This hypothesis is supported by several observations. For a given mass gain over a foraging trip, females with smaller pups (i.e. less efficient to extract maternal resources) were found to stay longer ashore to transfer their reserves to their pup (Georges and Guinet, 2000a). Furthermore, females whose pups had died while foraging at sea stayed ashore for extended periods of time (up to 9 days) following that foraging trip and departed to sea at a similar mass to females, which had suckled a pup (Guinet, unpublished data).

However, the lactating-fasting fur seal situation is very different from the long-term fast of seabirds and other mammals, as fur seal females have to mobilize their body store while fasting to ensure lactation. Metabolic requirements for fasting and lactating seem to be highly conflicting; lactation requires an increase in oxygen consumption (Costa et al., 1989) and the mobilization of large quantities of fatty and amino acids, while fasting requires sparing energy. For instance, lactating subantarctic fur seals on Amsterdam Island produce one of the richest milks ever reported in otariids (Georges et al., 2001) of similar lipid content ( $42 \pm 6\%$ , mean  $\pm$  S.D.) to that reported in phocid seals (Costa, 1991). The milk is also rich in proteins ( $12 \pm 1\%$ ) and that may represent an important part of the total body protein loss.

Several studies have shown that during long-term fasting in birds and non-lactating mammals, protein utilization initially decreases (phase I), is thereafter maintained at a low value (phase II) and then further increases (phase III) when lipid stores are depleted (Cherel et al., 1988a; Cherel and Groscolas, 1999). Hormonal and metabolic changes occurring during the fast are thought to contribute to a re-feeding signal supported by behavioural observation showing an increase in locomotor activity below a threshold body mass corresponding to an increase in body protein catabolism

(Sclafani and Rendel, 1978; Koubi et al., 1991). Protein sparing (phase II) was found to be associated with low levels of corticosterone, while the increase of protein utilization (phase III) was associated with an increase in plasma corticosterone in king penguins (*Aptenodytes patagonicus*; Cherel et al., 1988a), emperor penguins (*Arctocephalus fosteri*; Robin et al., 1998) and rats (*Rattus norvegicus*; Cherel et al., 1992). Leptin was suggested to have an important role in the regulation of homeostasis and as a mediator of adaptation to fasting (Ahima and Flier, 2000). However, recent results obtained on lactating Antarctic fur seal (*Arctocephalus gazella*) and elephant seal (*Mirounga angustirostris*) show no relationship between leptin level and body mass or body lipid levels suggesting that leptin has a minimal, if any, role in the regulation of body fat in these animals (Arnould et al., 2002; Ortiz et al., 2003). In a recent review, Groscolas and Robin (2001) hypothesised that the re-feeding signal in long-fasting emperor penguins, which occurred at critical levels of lipid reserves and motivated their at-sea departure for re-feeding to preserve their survival, was associated with an increase in plasma corticosterone and uric acid concentrations and a decrease in plasma  $\beta$ -hydroxybutyrate ( $\beta$ -OHB) concentration when entering phase III.

To our knowledge, no studies have investigated the concurrent change in glucocorticoids cortisol, urea and  $\beta$ -OHB in fasting pinnipeds during lactation. The purpose of our study was first to monitor changes in plasma concentrations of glucocorticoids in lactating subantarctic fur seals fasting through their onshore visit, in relation to the change in female mass, body condition and milk composition, and second to discuss their possible implication in the termination of the fasting bout ashore and in triggering the at-sea departure for re-feeding (Challet et al., 1995; Robin et al., 1998; Groscolas and Robin, 2001).

## 2. Materials and methods

### 2.1. Study site, animals, milk and blood sampling

The study colony was located at La Mare aux Elephants, northeast of Amsterdam Island ( $37^{\circ}55'$  S,  $77^{\circ}30'$  E) in the Southern Indian Ocean. Pups are born from late November to early January and weaning takes place between the end of September and late October, the mid-departure date of the

pups for sea is about mid-October, i.e. a 300-day nursing period (Tollu, 1974).

The study was carried out during the entire 1995–1996 reproductive season. Study mothers were known to be lactating because they have been seen giving birth to pups that were identified and marked just after birth (Georges and Guinet, 2000b) and suckling their pups normally: both mothers and pups were double tagged (Rototag, Dalton, Nettbed, UK) as described by Georges and Guinet (2000b). Thus, pup age was known for all individuals used in this study. Maternal attendance pattern was determined to the nearest day directly by observations of tagged mothers and indirectly by monitoring daily changes in body mass of tagged pups. When not observed directly, mothers were considered to be at sea when their pups were losing mass, and considered to be ashore when the pups were gaining mass. No differences were found in maternal attendance pattern in mother–pup pairs determined from either VHF radio transmitters or from the daily mass change of their pup (see details in Georges and Guinet, 2000a). In relation to this daily monitoring, tagged lactating females were captured during their first day ashore and then recaptured opportunistically on their second, third and if possible fourth day ashore as described by Gentry and Holt (1982) as it is impossible to determine a priori the departure to sea of the lactating females. When females were handled more than twice during a given attendance period, we only used the mass and blood samples obtained closest to arrival and departure. We considered that the mother had left shore when not observed ashore and when their pup started to lose mass. Pups were weighed on spring scales ( $20 \pm 0.1$  kg). The time lag (days) between mother's arrival ashore and blood sample was also estimated according to the mass change of the pup and the timing of the capture. Females were only captured early morning (6.30–9.40 h) or late afternoon (15.30–17.30 h) to reduce the risk of heat shock as the temperature can reach up to 18–24 °C on the study colony at mid-day on a sunny day.

The daily mass gain of the pup was determined for pups for which the attendance of the mother was known and for which the mass change of the pup was measured for at least two consecutive days. The mass gain of the pup for a given day of the maternal attendance was then calculated as the difference in pup mass measured on that day and

the previous one. Adults were physically restrained on a wooden board to be weighed ( $\pm 1$  kg), and measured (standard body length, from nose to tail,  $\pm 1$  cm) in order to assess body condition index (BCI). BCI was expressed as a percentage by calculating the individual residual value of the linear regression between body mass and body length (Georges and Guinet, 2000a) and divided by the theoretical body mass according to the length of the mother and multiplied by 100. BCIs calculated this way are independent from body length and mass (Georges and Guinet, 2000a).

When adult females were restrained, a 5-ml blood sample was collected into a heparinized syringe by venipuncture of an interdigital vein in a hind flipper. The blood was then transferred to polypropylene microtubes and kept in a cool box before being centrifuged. The plasma was then collected and kept frozen ( $-20$  °C).

## 2.2. Cortisol and metabolite assays

Cortisol is the major glucocorticoid in the plasma of seals (Liggins et al., 1979). Plasma was analysed for cortisol (ng/ml) by radioimmunoassay in ether-extracted samples (100  $\mu$ l) using a specific antiserum raised in rabbit injected with cortisol 21 HS thyroglobulin (supplied by Dr S. Mamas, Inst. Pasteur, France). Duplicate aliquots of the extracts were incubated overnight at 4 °C with 6000 cpm of [ $^3$ H]cortisol (Amersham Pharmacia Biotech, France) and antiserum. The bound and free cortisols were separated by the dextran-coated charcoal method and after centrifugation the bound fraction was counted in a liquid scintillation counter (Packard). Minimal detectable cortisol level was 0.1 ng/ml. The intra-assay and inter-assay coefficients of variation were 5.5 and 12%, respectively.

Urea is the major nitrogen excretory product in mammals and is used as an index of protein breakdown in mammals (Cherel et al., 1995). An increase in plasma concentration of  $\beta$ -OHB, the main ketone body in mammals, used as an index of fatty acid oxidation, characterizes that part of the fast during which most of the energy is derived from lipids (Cherel et al., 1988a,b; Le Ninan et al., 1988; Robin et al., 1998; Cherel and Groscolas, 1999). Plasma  $\beta$ -OHB and urea concentrations (mmol/l) were measured enzymatically on whole plasma at 340 nm. Commercial kits used were from Sigma Diagnostic France (procedure No.

310-UV) for  $\beta$ -OHB and Biomerieux France (urée cinétique UV 250) for urea.

Glucocorticoid hormones are known to respond to stress and crowding, including fasting stress. However, lactating females have been shown to be less sensitive to stress than control ones for a number of species (Silbolboro Mezzacapa et al., 2003). To reduce the potential restraint effect, all females were handled identically and blood samples were collected within 10 min of the initiation of the capture process. For monitoring change in cortisol concentration over the attendance period ashore, we sampled blood for a given female as close as possible to her arrival ashore and her departure for sea (see above). The females were handled under the same conditions over the two capture events and no difference in the timing of blood sampling after the capture of the female was found between the initial and final blood samples. We thus assumed that changes in cortisol level observed between the two sampling sessions reflected an actual change independent of capture. However, studies have shown that cortisol levels may vary in animals depending on the number of times they have been captured even when the conditions of capture remain identical. So, in a way to control a posteriori for that effect, we performed the analysis for independently sampled females (i.e. the first sampling for females that were sampled several times) and we looked at variation of cortisol concentration in relation to the number of days elapsed since the arrival of the female ashore to suckle its pup, the female mass, length and BCI.

Change in urea,  $\beta$ -OHB and cortisol concentration over the attendance period was investigated for the females that were paired-sampled. Also, these hormones and metabolites were further assayed for females sampled only once through the attendance period. For most of these females, milk composition was also previously measured. Detailed analyses were presented by Georges et al. (2001).

We conducted paired-sampled *t*-test for females sampled twice over an attendance period. The Kolmogorov–Smirnov test was used to determine whether the data were normally distributed and an *F*-test to confirm the homogeneity of variances. We used the General Linear Model (GLM) to investigate the relationship between cortisol levels with the explanatory variables for females sampled once; for those which were handled several times,

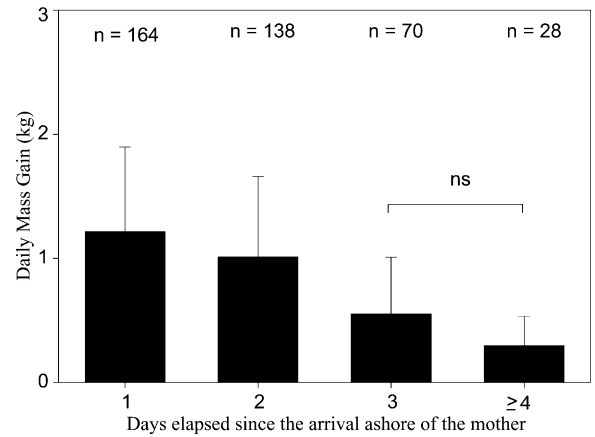


Fig. 1. Pup daily mass gain (kg) during the maternal attendance period in summer and fall. The mass gain during the first day was measured for 164 different pups, and among them we were able to measure the mass gain for the second, third and fourth day (and more) for 138, 70 and 28 pups, respectively. The difference in daily mass gain between days were all significant (*t*-test) except for day 3 and day 4 and beyond.

we only took into account the sample that was obtained when the female was first captured. SYSTAT statistical packages (SYSTAT, 9.0 statistics, SPSS Inc., USA) was used for the analyses and statistical significance was considered to be  $P < 0.05$ . Results are given as mean  $\pm$  S.D.

### 3. Results

#### 3.1. Change in pup mass gain and female mass loss during the attendance period

The mean absolute pup mass gain of 164 independently sampled pups was  $3.1 \pm 0.9$  kg over a  $4 \pm 1$  day attendance period (Georges and Guinet, 2000a). When we summarized the daily mass gain of the pup measured on the first, second, third and fourth day of the attendance period, 72% of the absolute pup mass gain took place over the first 2 days (Fig. 1).

Twenty-four paired-sampled females were heavier when first weighed after their arrival ashore compared to their last weighing before their departure for sea (initial mass:  $49.7 \pm 6.8$  kg, final mass:  $43.0 \pm 6.0$  kg; paired *t*-test:  $t = 12.08$ ,  $P < 0.001$ ,  $n = 24$ ; Fig. 2).

#### 3.2. Cortisol, urea and $\beta$ -OHB concentration

Cortisol was assayed for a total of 71 different females, from which 24 females were sampled

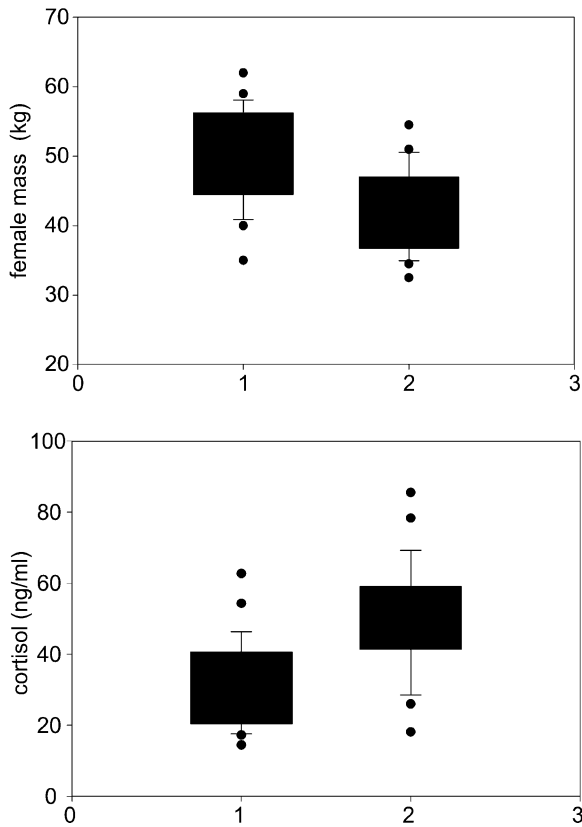


Fig. 2. Mean mass (kg) and blood cortisol concentration (ng/ml) for the 24 lactating subantarctic females when first captured (initial) after their arrival ashore and last captured (final) before their departure to sea during the attendance period (see text for details).

twice (i.e. an initial and a final blood sample) over a given attendance period ashore.

All sampled females showed detectable cortisol levels. For the 24 females that were sampled twice, cortisol concentrations were significantly lower in the initial sampling compared to the final sampling (initial cortisol concentration:  $37.9 \pm 12.9$  ng/ml, final cortisol concentration:  $60.0 \pm 12.1$  ng/ml; paired  $t$ -test:  $t = -6.1$ ,  $P < 0.001$ ,  $n = 24$ ; Fig. 2).

When looking at the effect of maternal characteristics (body mass, length, BCI), the duration of the previous foraging trip, the number of days elapsed since the arrival ashore of the female, the timing of the blood sample (morning vs. afternoon) and the age of the pup on cortisol (ng/ml), we found that cortisol concentration was only negatively related to BCI (cortisol =  $44.6 - 0.53[\text{BCI}]$ ;  $r^2 = 0.12$ ,  $P = 0.008$ ,  $n = 71$ ).

Urea and  $\beta$ -OHB concentrations were measured

on 44 different females (the 24 paired-sampled plus 20 that were sampled only once). The mean urea concentration in paired-sampled females was significantly lower in the final sample compared to the initial sample (initial urea concentration:  $14.6 \pm 4.1$  mmol/l, final urea concentration:  $11.3 \pm 2.2$  mmol/l; paired  $t$ -test:  $t = -3.4$ ,  $P = 0.002$ ,  $n = 24$ ; Fig. 3), while no variation in  $\beta$ -OHB was detected between the initial and the final blood sample (initial  $\beta$ -OHB concentration:  $0.22 \pm 0.09$  mmol/l, final  $\beta$ -OHB concentration:  $0.22 \pm 0.09$  mmol/l; paired  $t$ -test:  $t = -0.042$ ,  $P = 0.96$ ,  $n = 24$ ; Fig. 3). When taking only independent samples into account to investigate which variable may correlate with change in urea or  $\beta$ -OHB concentrations (mmol/l), we found that urea decreased over the attendance period (AP, days) (urea =  $-1.3[\text{AP}] + 14.8$ ;  $r^2 = 0.20$ ,  $P = 0.002$ ,  $n = 44$ ), but no relationships were found in relation to maternal size, mass or BCI or cortisol.  $\beta$ -OHB

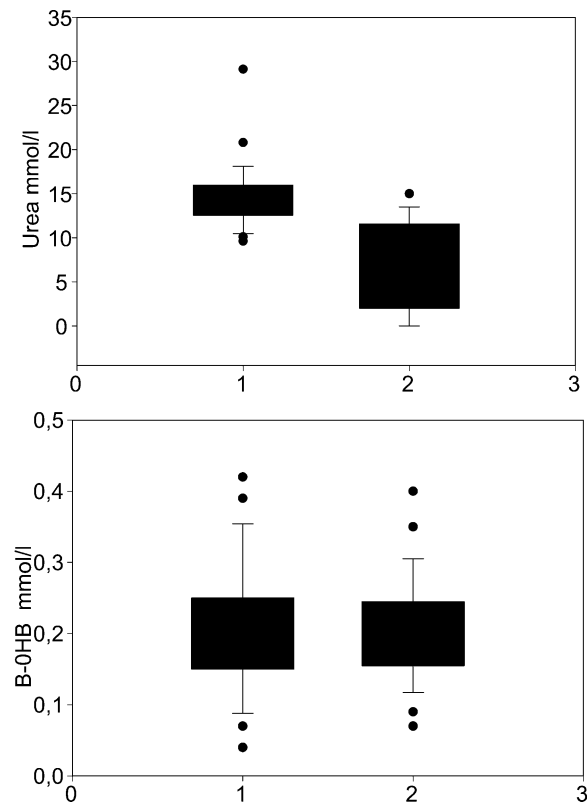


Fig. 3. Change in urea and  $\beta$ -OHB concentration (mmol/l) for the 24 lactating subantarctic females when first captured (initial) after their arrival ashore and last captured (final) before their departure to sea during the attendance period.

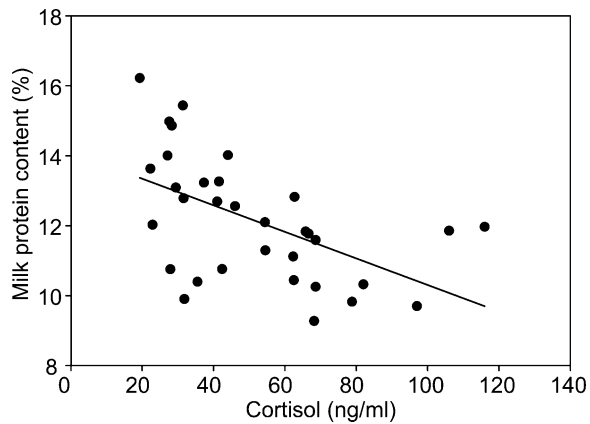


Fig. 4. Relationship between the milk protein (%) content and plasma cortisol level (ng/ml).

concentration did not vary over the attendance duration and was neither related to maternal length, mass or BCI.

### 3.3. Milk composition and cortisol concentration

Detailed results on milk composition were presented by Georges et al. (2001). Milk protein and lipid content were known for, respectively, 33 and 45 independent samples also assayed for cortisol. No relationship was found between cortisol level and milk lipid content ( $r^2=0.02$ ,  $P=0.40$ ,  $n=45$ ) but milk lipid content (%) was positively correlated to BCI (lipid =  $48.9[\text{BCI}] + 44.1$ ;  $r^2=0.57$ ,  $P < 0.001$ ,  $n=44$ ). Milk protein content (%) was negatively related to the cortisol level according to the following equation (Fig. 4):

$$\text{Protein (\%)} = 14.100 - 0.038[\text{cortisol}],$$

$$(r^2=0.28, P < 0.001, n=33).$$

## 4. Discussion

Subantarctic fur seal pups exhibited an important mass gain during the maternal attendance period ashore with 72% of the pup mass gain taking place over the first 2 days of the attendance period. Georges et al. (2001) found that on Amsterdam Island, milk composition of subantarctic fur seal remained roughly constant with high values during the first 2 days ashore (lipids:  $44.9 \pm 4.5\%$ ; proteins:  $13.4 \pm 1.6\%$ ) and then decreased sharply for the subsequent period (lip-

ids:  $32.5 \pm 7.6\%$ ; proteins:  $11.1 \pm 1.9\%$ ). By combining the information regarding the milk composition and the proportion of mass transferred over the first 2 days, we found that 79% of the lipids and 77% of the proteins delivered to the pup are transferred within the first 2 days of the maternal visit ashore. According to these results, and also changes in fatty acid composition (Georges et al., 2001), we suggest that two types of milk of different quality and from different origins are fed to the pup when the mother is fasting ashore.

On return from their foraging trip at sea, lactating subantarctic fur seals are in relatively good condition (Georges and Guinet, 2000a) and they progressively deteriorate through milk transfer and metabolism while fasting ashore. Our study shows that at the same time the cortisol concentration increased. This relationship was also found for independently sampled females and this result supports the idea that the increase in cortisol concentration was not just due to stress. Furthermore, observations do not suggest that females are subject to a higher degree of pup harassment or crowding. At the end of the maternal attendance period, most pups are satiated and harass their mother less compared to when females arrive ashore and at that stage pups are highly motivated to suckle. Crowding only occurs during the breeding season when density is at the highest and all our samples were collected when the breeding-mating season was completed.

Change in BCI was a better predictor of milk lipid content and cortisol levels than the duration of the fast, suggesting that milk lipid content and cortisol concentration are directly related to the level of body reserves at any time of the fast. Similar results were reported in fasting elephant seal pups where a negative relationship was found between body mass and cortisol (Ortiz et al., 2001, 2003).

The absence of any changes in  $\beta$ -OHB blood concentration indicates that the *A. tropicalis* females in our study were in phase I of fasting, and possibly in transition to phase II as suggested by the decrease in urea concentration. However, the decrease in urea that we observed through the shore visit may just result from a reduction of the female metabolic rate when ashore lactating compared to at sea (Costa et al., 1989), or to a decrease in the contribution of protein to metabolism, and thus may not be indicative of protein

sparing for long fast adaptation as described in fasting birds and mammals (Cherel et al., 1988b, 1995; Cherel and Groscolas, 1999). The absence of change in plasma  $\beta$ -OHB concentration in fasting and lactating subantarctic fur seals over the attendance period ashore suggests that adult females exhibited a longer transition from phase I to phase II than in pups (Rea et al., 2000; Arnould et al., 2001), which may reflect difference in body size or adiposity (Cherel et al., 1992; Cherel and Groscolas, 1999). This also suggests that adult females returning to sea for feeding were departing after they had used their easily mobilized reserve, but well before they had completely depleted their body store. Finally, the absence of change of plasma  $\beta$ -OHB concentration may also be explained by the fact that these animals may use ketones as the main source for the central nervous system at a rate fast enough for blood concentration to remain constant but the overall flux to change.

According to our results, there is no evidence that the motivation of the female to leave the colony is motivated by a decrease in blood concentration of  $\beta$ -OHB and an increase of blood urea concentration as observed in penguins entering phase III before leaving their egg or their chick (Groscolas and Robin, 2001).

Glucocorticoids increased in relation to the depletion of body stores during fasting in a number of studies: rats (Challet et al., 1995), emperor penguins (Robin et al., 1998), elephant seal pups (Ortiz et al., 2001, 2003). During prolonged fasting in birds and mammals, increases in plasma glucocorticoid levels were found to coincide with changes in lipid and protein utilization (Cherel et al., 1988c, 1992). In long-term fasting animals, glucocorticoids have an effect on protein catabolism with amino acids being transformed in the liver into glucose to maintain a sufficient circulating glucose level that is essential because the central nervous system must have a continuous supply of glucose if the organism is to survive. This is not the case in our study as urea concentration decreased while cortisol levels increased. In our study we found that the increase in cortisol levels was related with a decrease in milk protein content, suggesting that cortisol concentration, within the range we observed, did not favour protein catabolism to ensure metabolism, but favoured protein mobilization to produce amino acids to ensure milk production. Our first hypoth-

esis would be that cortisol levels increase when the females have used, over the first 2 days of lactation, easily mobilized reserves—such as the ventral fat deposit tightly associated with the mammary gland—and switch to a different pool of reserves more difficult to mobilize to ensure milk production (Allison and Wannemacher, 1965; Goodman et al., 1984). This scenario appears to be consistent with the finding of Georges et al. (2001) of a decrease in milk quantity and quality taking place after the first 2 days ashore.

Several studies have shown a critical role of glucocorticoid hormones in a fasting-induced mobility in pinnipeds, rats (Challet et al., 1995) and emperor penguins (Robin et al., 1998), and it has been suggested that this may be critical for the initiation of foraging during periods of food deprivation (Challet et al., 1995). In mammals, glucocorticoids have been proposed as long-term regulators of both energy intake and storage (Strack et al., 1995) and glucocorticoids have been shown to promote feeding behaviour by stimulating the hypothalamus (Green et al., 1992). The common features between all these studies in fasting seabirds and mammals is the increase of glucocorticoids prior to their at-sea departure or associated with increased mobility. One interesting finding of our study was the fact that the increase in cortisol concentration—in relation to decreasing BCI of the females—is uncoupled from the transition of phase II to phase III as described in king and emperor penguins (Cherel et al., 1988b; Robin et al., 1998) when proteins are used for gluconeogenesis.

A second hypothesis, non-exclusive from the first, regarding the effect of cortisol increase on fur seal female behaviour would be that the increase in cortisol concentration contributes to the at-sea departure of the lactating fur seals well before the females are physiologically adapted to long fast.

Our observations and the previous work conducted on other species of seabirds and mammals make glucocorticoids likely candidates for a re-feeding signal that may drive the at-sea departure of the lactating females after they have depleted their easily mobilized body reserve to ensure milk production, but well before they start using substantial amounts of other body stores that may impact their own survival. On Amsterdam Island, the at-sea departure of the female before the depletion of their body store may allow the females

to travel for 2–3 days before reaching their distant foraging grounds (Georges et al., 2000c).

According to our results and the possible function attributed to cortisol in the short lactation fast of subantarctic fur seals, future studies should try to investigate: (1) the origin of body stores mobilized to ensure lactation in relation to changes in cortisol levels and (2) the possible implication of cortisol in the motivation of the female to leave the colony, by experimental studies such as inhibiting the synthesis of cortisol to observe if females delay their at-sea departure in relation to that treatment.

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