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RESEARCH ARTICLE

Primiparous and multiparous females differ in mammary gland alveolar development: implications for milk production

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SUMMARY

Mammary gland capacity is influenced by the number of secretory cells in the gland, the activity of those cells and the size and arrangement of the alveoli that they form. Although reproductive experience has been shown to affect the total number of secretory cells in the gland, its potential effect on the structural development of lobulo-alveolar tissues has not been directly investigated. To examine whether reproductive experience affects lobulo-alveolar development, we took mammary gland biopsies at early and peak lactation from primiparous and multiparous grey seal (*Halichoerus grypus*) females and used histological techniques to compare cell density, alveolar density and alveolar size within secretory lobules. Primiparous females had a significantly higher cell density compared with multiparous females throughout lactation, suggesting that primiparous females have smaller, less-developed secretory cells. Primiparous females had a significantly smaller average alveolar size compared with multiparous females throughout lactation. Although alveolar density was higher in primiparous females compared with multiparous females at early lactation, there was no significant difference between the groups at peak lactation. These results suggest that the mammary gland of primiparous females may have both a lower secretory capacity and a lower storage capacity on a relative basis than those of multiparous females and demonstrate, for the first time, that reproductive experience has a significant effect on both the rate and pattern of mammary gland alveolar development and, potentially, on a female's capacity for milk production.

Key words: grey seal, *Halichoerus grypus*, lactation, mammary gland development, alveolar size, alveolar density, cell density, reproductive experience, primiparous, multiparous.

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INTRODUCTION

In mammals, both offspring growth rate during lactation and the mass of offspring at weaning have a significant impact on their subsequent survival, growth and reproductive success (e.g. Wauters et al., 1993; Festa-Bianchet et al., 2000; Hall et al., 2001). The most significant determinant of offspring growth during the lactation period is the ability of females to transfer milk energy to their neonates. Lactation is the most energetically expensive period in the life of a female mammal (Gittleman and Thompson, 1988) and females must offset these costs by increasing energy intake and/or mobilizing body energy stores. As a result, it is frequently assumed that variation among females in milk production and, thus, in offspring growth is a function of variation in energy acquisition prior to and/or during the lactation period. However, females may vary in their ability to deliver milk energy to their offspring independent of variation in factors such as resource availability, levels of body energy stores or body size (e.g. Yang et al., 1989; Lang et al., 2009) as a consequence of differences in the capacity of their mammary glands for milk production (Knight, 2000). Thus, identifying sources of variation in mammary gland capacity is important to our understanding of variation in reproductive success both among and within individuals.

The functional development of the mammary gland occurs in distinct stages. Prior to first conception, the mammary gland consists of a system of ducts branching through the mammary fat pad with no true secretory tissue present (Cowie et al., 1980; Knight and Peaker, 1982). Following first conception, there is an increase in ductal growth and a rapid proliferation of secretory cells to form the first lobulo-alveolar tissues (Knight and Peaker, 1982). This massive proliferation of cells continues throughout gestation and, in some species, into the initial stages of lactation (Knight and Peaker, 1982). Immediately prior to parturition, mammary epithelial cells are converted from a non-secretory to a secretory state, with a gradual increase in the secretory activity of these cells occurring through the early stages of lactation up until peak lactation (Knight and Peaker, 1984; Shipman et al., 1987). Following weaning, the glands undergo a period of gradual involution during which the alveolar secretory cell population regresses through apoptosis.

Although it was initially believed that all alveolar cells underwent apoptosis following weaning, a significant number of differentiated secretory cells bypass apoptosis and remain in the gland following involution (Vonderhaar et al., 1978; Wagner et al., 2002). These permanently differentiated cells are carried over into the next reproductive cycle, where they function as precursors, giving rise to clonal populations of alveolar cells during subsequent pregnancies (Wagner and Smith, 2005). Because this carryover of differentiated cells corresponds to a significantly larger amount of secretory tissue in the gland at the onset of the subsequent lactations (Wada and Turner, 1959; Anderson and Sheffield, 1983; Fowler et al., 1990), greater daily milk outputs over the course of lactation in multiparous

females compared with primiparous females (Fowler et al., 1990; Nielsen et al., 2001; Miller et al., 2006) have frequently been attributed to differences between the groups in the number of secretory cells in the gland alone. However, mammary gland capacity is also substantially influenced by the activity of the secretory cells and the size and arrangement of the alveoli that they form (Knight, 2000; Akers, 2002). Studies in laboratory species indicate that the differentiated alveolar cells that remain in the gland following the end of a female's first lactation are more responsive to hormonal stimuli than the undifferentiated cells found in the mammary glands of females prior to first conception (Wagner and Smith, 2005). Since hormones play a critical role in the growth and development of the alveoli and in the functional development of the alveolar secretory cells (Heald, 1974; Tucker, 1981; Hennighausen and Robinson, 1998; Neville et al., 2002), there is a potential for both the rate and pattern of lobulo-alveolar development to differ between primiparous and multiparous females. Although results from lactating dairy cattle suggest that reproductive experience affects both the DNA concentration (cell density) and metabolic activity of the secretory tissues (Miller et al., 2006), whether reproductive experience affects the functional development of lobulo-alveolar tissues has not been directly investigated. Thus, whether the observed increases in the rate of milk production with reproductive experience are solely a consequence of increases in the total number of cells in the gland or whether changes in secretory cell development or alveolar density and size may also contribute is unknown. In the present study, we used mammary gland biopsies obtained from primiparous and multiparous grey seal (*Halichoerus grypus* Fabricius 1791) females to examine whether reproductive experience influences the development of lobulo-alveolar tissues.

Lactation energetics and various factors influencing the transfer of milk energy to offspring have been well studied in the grey seal. Like most other large-bodied phocid seals (Family Phocidae), grey seal females are capital breeders, supporting all of the energetic costs of lactation from the body energy reserves acquired prior to parturition. Females give birth to a single pup, there is no alloparental or paternal support, and pups consume only milk during the lactation period. Females remain ashore with their pup throughout the relatively brief lactation period (16–18 days) (Bowen et al., 1992), during which females secrete large quantities (average 3.2 kg day^{-1}) of high-energy milk (40–60% fat) (Iverson et al., 1993; Mellish et al., 1999a; Lang et al., 2011a). At the end of lactation, females abruptly wean their pups and depart the breeding colony. Pups must then rely on the energy stores acquired during the lactation period to survive a 3–4-week post-weaning fast and the transition to nutritional independence (Noren et al., 2008). In this species, both the body mass and condition of pups at weaning have a significant influence on post-weaning survival, with larger, fatter pups having a greater probability of survival to 1 year of age (Hall et al., 2001). Therefore, the ability of females to rapidly transfer milk energy to their offspring is a critical determinant of maternal reproductive success in grey seals.

Previous work in grey seals demonstrated that, consistent with results in domestic species (e.g. Fowler et al., 1990; Nielsen et al., 2001; Miller et al., 2006), multiparous grey seal females have a higher physiological capacity for milk production, and thus a higher daily milk output over the course of lactation, than primiparous females (Lang et al., 2011a). In the present study, we obtained mammary biopsies at early and peak lactation from free-ranging primiparous and multiparous grey seal females and used histological techniques to compare cell density, alveolar density and alveolar size. We tested the hypothesis that reproductive experience has a significant effect on the patterns of development within lobulo-alveolar tissues, which may, in part, explain the higher rate of milk production in multiparous females compared with primiparous females. Because the majority of mammary gland development occurs during pregnancy, reproductive experience is defined here as including any previous observation of pregnancy regardless of whether a female subsequently nursed a pup. Studies in other, closely related phocid seals (Weddell seal, *Leptonychotes weddellii*; harp seal, *Phoca groenlandica*; hooded seal, *Cystophora cristata*) demonstrate that the gross morphology and microscopic structure of the mammary glands of mature virgin, non-lactating and lactating females are consistent with similar stages in other mammalian species (Belov, 1971; Tedman and Bryden, 1981; Tedman, 1983; Tedman, 1985). Thus, what we learn from grey seals should contribute to our understanding of how reproductive experience influences the development of mammary gland lobulo-alveolar tissues in other mammals.

MATERIALS AND METHODS

The study was conducted on Sable Island (45°55′ N, 60°00′ W), located approximately 300 km east–southeast of Halifax, Nova Scotia, Canada during the 2005 and 2006 breeding seasons. Females in this population begin reproducing at 4–6 years of age and can continue to reproduce to age 30 or beyond (Bowen et al., 2006; Bowen et al., 2007). Study females were a subset of those that were permanently marked between 1985 and 1989 and between 1998 and 2002 with unique, hot-iron brands shortly after weaning, and thus were of known age. As with other grey seal colonies (Allen et al., 1995; Pomeroy et al., 2000), the Sable Island grey seals exhibit a strong philopatry with an estimated fidelity rate of 98.4% (W.D.B., unpublished). Weekly whole-island censuses of all branded individuals combined with daily surveys throughout the colony during the breeding season have been conducted in this population since 1983 (for details, see Bowen et al., 2006), and thus the reproductive histories of all females in the study were known.

The primiparous females in the study (N=12) were 4–7 years of age. Females were considered primiparous if they had not been observed pregnant or rearing a pup in a previous breeding season. Given the frequency of whole-island censuses and colony surveys, it is highly unlikely that a female returning to the breeding colony would not have been detected. Of the 311 females from the 1998–2002 cohorts that recruited to Sable Island, none were sighted on Sable Island or at any other colony in breeding seasons prior to the first year they were observed with a pup. Therefore, we are confident that the first year a female was observed, she was primiparous. The multiparous females in the study (N=10) were 18–21 years of age and had been observed pregnant and/or rearing a pup in a minimum of nine previous breeding seasons (see Table 3 for details of individual reproductive histories). All females had known parturition dates.

Biopsy instrument

Although mammary gland biopsies had previously been obtained from both grey seals and hooded seals through a relatively simple modification of a 6 mm tissue biopsy tool (crimping of the cutting blade on both sides) (Mellish, 1999; Mellish et al., 1999b), a pilot study we conducted in 2004 (four grey seal females) demonstrated that, because this modification did not reliably sever connective tissues within the gland, core samples of adequate size for histological examination could not be consistently obtained. Therefore, we developed a single-use biopsy instrument that could sever the tissue core (Fig. 1). Using this biopsy design, we were

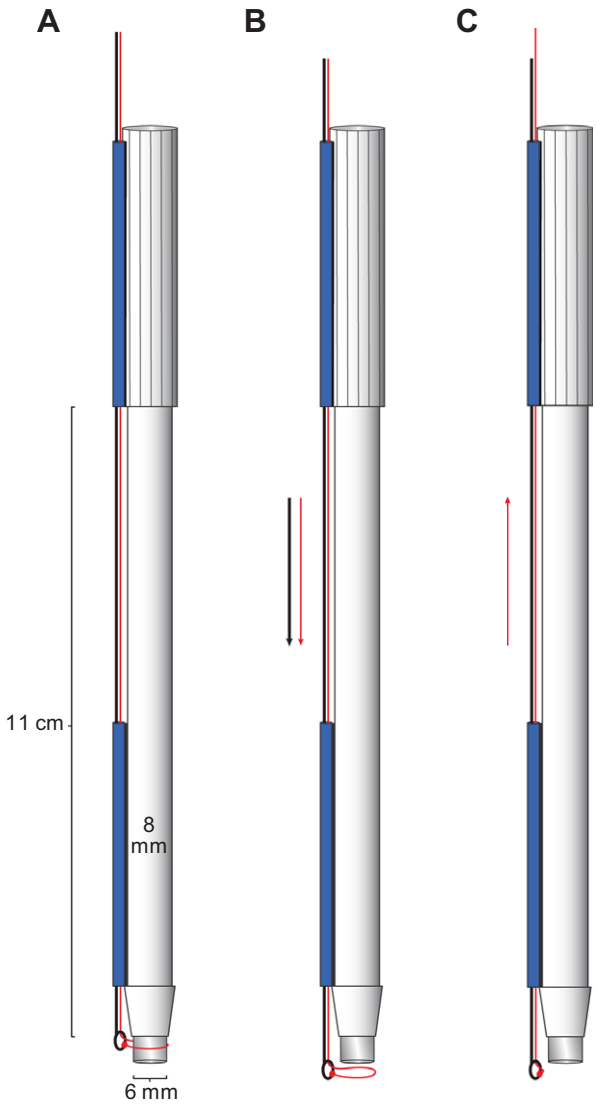

Fig. 1. Schematic of the biopsy instrument used to obtain cores of grey seal mammary gland tissue. (A) The biopsy instrument consists of a hollow cannula with tapered end (11 cm×8 mm i.d.) attached to a seamless, stainless steel tip (6 mm i.d.) with a razor-sharp cutting edge. An 18 gauge stainless steel guide wire (black) with a closed loop at the cutting end runs the full length of the biopsy tool through two hollow guides (blue). A 30 gauge stainless steel suture wire (red) is secured to the loop in the guide wire, wrapped around the cutting tip and threaded through the loop in the guide wire and up through the two guides. (B) With the biopsy tool fully inserted in the tissue, the guide wire and its associated suture wire are slid downwards until the loop of suture wire is below the cutting edge of the biopsy tool. (C) With the guide wire held steady, the suture wire is pulled upwards, closing the loop at the cutting edge and severing the tissue core.

able to reliably obtain mammary tissue samples of 30–50 mm in length and 6 mm in diameter.

Field procedures

Samples were obtained from each female at day 3–4 postpartum (early lactation) and again at day 10–12 postpartum (peak lactation). Day 3–4 postpartum was selected as the sampling point for early lactation in order to allow the pair bond to develop between females and their pups and thereby minimize the risk of abandonment as a result of handling. Day 12 postpartum was selected as being

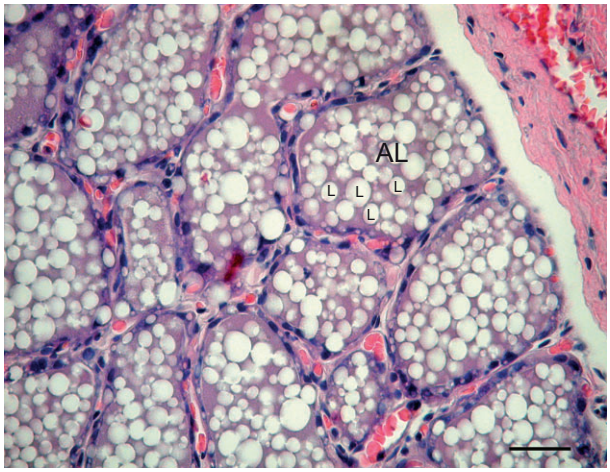

Fig. 2. Histological section of grey seal mammary gland tissue showing alveoli that are full of secreted milk. AL, alveolar lumen; L, lipid droplets. Tissues stained with hematoxylin and eosin. Scale bar, 100 μm.

representative of peak milk energy composition and output in grey seals (Iverson et al., 1993).

Females were captured using a hinged pole net. Following capture, females and pups were weighed to the nearest 0.5 kg. Females were then mildly sedated with an intravenous injection of diazepam (25 mg, Sandoz Canada Inc., Boucherville, QC, Canada). Because it was not possible to control at what point following a suckling event sampling occurred, and thus the degree to which alveoli within the gland may be full (Fig. 2), sampling was standardized by administering an intramuscular injection of oxytocin (1.5 ml at 20 IU ml^{-1}; Vétoquinol Canada, Lavaltrie, QC, Canada) to facilitate milk letdown followed by the complete evacuation of one of the two mammary glands by suction (using a 60 cm^3 syringe with the tip removed) prior to the biopsy procedure. Two 30 ml vials of milk were subsampled and stored at –20°C until analysis for proximate composition. The remaining milk collected was subsequently delivered to the pup *via* stomach tube prior to release of the pair to ensure that the evacuation of the female's mammary gland did not negatively impact the pup's energy intake. Following evacuation of the gland, a 4 cm^2 area over the centre of the gland was shaved, cleaned with alcohol and Prepodyne Solution (West Penetone Inc., Montréal, QC, Canada), and a subcutaneous injection of lidocaine hydrochloride (Xylocaine, 1.5 ml at 20 mg ml^{-1}; Astra Zeneca Canada, Mississauga, ON, Canada) was administered as a local anaesthetic. Subsequently, a small incision (~1 cm) was made in the skin using a sterile scalpel. A biopsy was then taken using a sterile biopsy core tool (Fig. 1) through the full depth of the blubber layer (3–4 cm) and into the mammary gland. The mammary gland tissue was immediately separated from the blubber tissue and placed in a fixative (10% acetate buffered formalin). The incision was closed with two interrupted sutures using absorbable suture and sprayed with a topical antiseptic. At peak lactation, the biopsy procedure was repeated on the other gland. Mammary biopsies were removed from the fixative after 36 h, washed twice with 70% ethanol and then stored in 70% ethanol until processing.

Prior to release on day 3–4 postpartum, pups were given an individually numbered hind-flipper tag (Rototag, Dalton ID Systems Limited, Henley-on-Thames, Oxfordshire, UK) to permit post-weaning identification. Pairs were sighted daily throughout the lactation period to ensure that the female and pup were still together

and to obtain an accurate date of weaning. On the day of weaning, which was marked by the departure of the female, pups were weighed to the nearest 0.5 kg.

All sampling protocols were conducted in accordance with the requirements of the Canadian Council on Animal Care and were approved by Dalhousie University's Animal Care Committee (protocol numbers 03-095 and 05-115).

Sample analyses

Mammary gland tissues were prepared for histological analysis following standard techniques. The fixed tissues were dehydrated in a series of graded ethanol solutions, cleared in xylene and embedded in paraffin wax. The tissues were sectioned at 5 µm on a Reichert Jung rotary microtome (Leica Microsystems Inc., Wetzlar, Germany). For cell counts per unit area (cell density), sections were stained with DAPI (4′-6-Diamidino-2-phenylindole; Vectashield Mounting Medium with DAPI; Vector Laboratories, Inc., Burlingame, CA, USA), a fluorescent stain that binds to the DNA of the cell nuclei (Fig. 3A). For alveolar counts and size measurements, sections were stained with Mayer's hematoxylin and eosin (H&E; Fig. 3B). Photographs of histological slides were taken on a Nikon Eclipse E600 compound microscope fitted with a Nikon DXM1200F digital camera (Nikon Instruments, Inc., Melville, NY, USA). DAPI-stained sections were photographed at 40× magnification (UV light) and all photographs were taken within the boundaries of the secretory lobules, which are composed of clusters of alveoli. H&E-stained sections were photographed at 20× magnification (Fig. 3).

Cell counts, alveolar counts and alveolar areas were analysed manually using NIS Elements (AR 3.0) imaging software (Nikon Instruments, Inc.). Cell counts were conducted on five randomly selected images from each sample. For each image, the area of all alveolar lumens (AL in Fig. 3A) was subtracted from the total image area, and the cell density was calculated as the number of cells per unit of net area (#mm^{-2}). The area of the alveolar lumens (AL in Fig. 3B) was measured on 250–310 alveoli per sample from four to seven randomly selected images (alveolar size, µm^2). The same images were used to estimate alveolar density (#mm^{-2}) by dividing the number of alveoli in each image by the total area of the image. Areas of connective tissue (C in Fig. 3B) or portions of the image that did not contain tissues (e.g. images taken near the edge of histological samples) were subtracted from the total image area prior to the calculation of alveolar density.

All milk samples were analysed for their percentage protein, fat and dry matter content. Single subsamples were analysed for protein content by macro-Kjeldahl (AOAC, 2000a). Milk fat content was determined gravimetrically following sequential petroleum ether and diethyl ether extractions using the standard Roese-Gottlieb procedure for milks (AOAC, 2000b). Dry matter (water content) was determined gravimetrically following forced convection drying for 5 h at 100°C. All analyses for milk fat and dry matter content were done in duplicate and the values averaged. Milk samples were not analysed for carbohydrate content as it has been previously demonstrated that this is a very minor component of phocid milks (Oftedal and Iverson, 1995), as confirmed by the low residuals from the sum of fat and protein compared with dry matter.

Calculations and data analyses

For each female, daily milk output between early and peak lactation was estimated from her average milk energy concentration and her pup's rate of mass gain as described below. In grey seals, milk fat increases from parturition until reaching peak values at approximately day 8 postpartum and then remains relatively stable over the remainder of lactation (Iverson et al., 1993). To account for the non-linear change in milk fat content over the measurement period, we calculated a weighted harmonic mean for a female's percent milk fat (see Lang et al., 2009). Following an initial decline shortly after parturition, milk protein content remains relatively constant throughout the remainder of lactation in grey seals (Iverson et al., 1993) and, therefore, early and peak lactation values were averaged. The average milk energy concentration (MJ kg^{-1}) was then calculated using the values of 39.3 MJ kg^{-1} and 23.6 MJ kg^{-1} for fat and protein, respectively (Blaxter, 1989). Daily milk energy output (MEO, MJ day^{-1}) was estimated for each female from her pup's mass gain (PMG, kg day^{-1}) between early (day 3 or 4 postpartum) and peak (day 10–12 postpartum) lactation, based on the regression equation from Lang et al. (Lang et al., 2011a) (r^2=0.952, N=29), where PMG=(MEO×0.027)+0.011. Daily milk output (kg day^{-1}) was then estimated by dividing the daily milk energy output by the average milk energy concentration for each female.

Maternal postpartum mass for each female was estimated from day 3–4 postpartum mass using the rate of mass loss per day for that female. For pups weighed on days 4, 10 or 11 postpartum, mass was adjusted to day 3 or day 12 postpartum prior to analysis using the rate of mass gain per day for that pup.

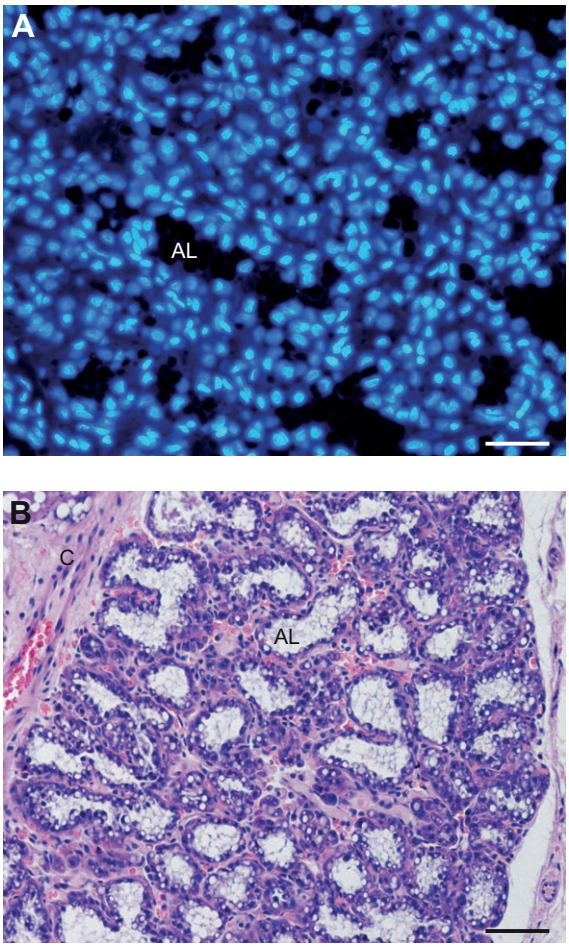

Fig. 3. Histological sections of grey seal mammary gland tissue stained with (A) DAPI and (B) hematoxylin and eosin. AL, alveolar lumen; C, connective tissue. Scale bars: (A) 100 µm; (B) 200 µm.

All statistical analyses were conducted in R (version 2.13.0; R Foundation for Statistical Computing, Vienna, Austria). Four primiparous females weaned their pups prior to day 12 postpartum; thus, only the early lactation data for milk composition and histological characters from these females are included in the analyses. Two early lactation biopsy samples (one primiparous female, one multiparous female) consisted largely of connective tissue and did not contain sufficient lobulo-alveolar tissue for analysis; thus, there are only late lactation histological data for these females. A milk sample could not be obtained from one primiparous female at early lactation.

To account for the serial correlation present in the histological data collected within females and the unbalanced sample numbers across lactation (early *vs* peak), comparisons of cell densities, alveolar densities and alveolar sizes across reproductive status (primiparous *vs* multiparous) and lactation stage (early *vs* peak) were analysed using linear mixed-effects models [nlme package, version 3.1-100 (Pinheiro et al., 2011)]. Female identity was entered into the models as a random effect, with reproductive status and lactation stage entered as fixed effects. The models were fit using the restricted maximum likelihood (REML) method with a compound symmetric covariance structure. To allow the variation in the histological characters within samples to be incorporated into the model outcomes, all observations for each sample were used in the models. Thus, for each female at each sampling point there were five observations for cell density, 4–7 observations for alveolar density and 250–300 observations for alveolar area used in the analyses. Predicted group means for cell density, alveolar density and alveolar size were obtained from the linear mixed-effects models. Standard errors for the predicted group means from the linear mixed-effects models were estimated using the AICcmodavg package [version 1.17 (Mazerolle, 2011)]. To ensure that the values for the females selected for this study were consistent with our previous observations for multiparous and primiparous females (Lang et al., 2009; Lang et al., 2011a) and to verify that our biopsy procedure did not adversely affect mammary gland function, we compared milk composition, daily milk output and pup growth patterns between the groups. Because changes in these characters with lactation stage have already been well characterized for this species (Iverson et al., 1993; Mellish et al., 1999a, Lang et al., 2009), comparisons were made within lactation stages across reproductive status using the *t*-test for independent samples. Percentage values (milk composition) were arcsine transformed prior to analysis. Standard errors are reported throughout. For all analyses, differences across reproductive status and/or lactation stage were considered significant at $P \leq 0.05$.

RESULTS

Milk composition, and thus milk energy concentration, at early and peak lactation did not differ significantly between primiparous and multiparous females (Table 1). The estimated daily milk output of primiparous females between early and peak lactation (2.4±0.16 kg day^{-1}, range 2.0–3.0 kg day^{-1}, N=7) was lower than that of multiparous females (3.7±0.13 kg day^{-1}, range 3.0–4.2 kg day^{-1}, N=10; P<0.001). Primiparous females weighed significantly less (157.0±6.83 kg, range 128.0–181.0 kg, N=8) than multiparous females at parturition (227.0±7.01 kg, range 186.0–256.0 kg, N=10; P<0.001). Pup growth followed the same pattern in both groups throughout lactation. Pups of primiparous females weighed significantly less than those of multiparous females throughout lactation (Table 2). However, as a proportion of maternal postpartum mass, pup mass did not differ between primiparous and multiparous females at day 3 or 12 postpartum or at weaning (Table 2).

Cell density decreased between early and peak lactation in both primiparous and multiparous females, but cell density was significantly higher in primiparous compared with multiparous females throughout lactation (Table 3). Alveolar density did not differ by lactation stage (Table 3). Primiparous females had a higher alveolar density at early lactation than multiparous females, although there was no difference between the groups at peak lactation. Thus, there was a significant interaction between reproductive status and lactation stage (Table 3). Multiparous females had significantly greater alveolar size compared with primiparous females throughout lactation (Table 3). Although alveolar size increased significantly between early and peak lactation in multiparous females, this was not the case in primiparous females, resulting in a significant interaction between reproductive status and lactation stage (Table 3).

DISCUSSION

To our knowledge, the present study is the first to investigate the structural development of mammary gland lobulo-alveolar tissue in primiparous and multiparous females. Our results indicate that multiple cycles of pregnancy and lactation affect both the rate and pattern of lobulo-alveolar development in grey seals.

Table 1. Proximate composition of the milk of primiparous and multiparous grey seal females at early (day 3–4 postpartum) and peak lactation (day 10–12 postpartum)

	Reproductive status		
	Primiparous	Multiparous	*P*
Early lactation	*N*=11	*N*=10	
Water (%)	38.2±1.06	38.9±1.27	0.668
Dry matter (%)	61.8±1.06	61.1±1.27	0.668
Protein (%)	8.6±0.21	8.7±0.16	0.754
Fat (%)	49.9±1.05	49.3±1.13	0.719
Energy (MJ kg^{-1})	21.6±0.43	21.4±0.46	0.754
Peak lactation	*N*=8	*N*=10	
Water (%)	29.3±1.12	27.4±0.65	0.146
Dry matter (%)	70.7±1.12	72.6±0.65	0.146
Protein (%)	8.9±0.17	8.9±0.21	0.979
Fat (%)	60.3±1.39	60.7±0.72	0.772
Energy (MJ kg^{-1})	25.8±0.55	25.9±0.24	0.768

Values are means ± s.e.m.

Table 2. Body mass of pups of primiparous and multiparous grey seal females at days 3 and 12 postpartum and at weaning

	Reproductive status		
	Primiparous	Multiparous	*P*
Day 3			
Mass (kg)	17.3±0.64 (12)	24.6±0.92 (10)	<0.001
Mass (% of MPM)	10.7±0.59 (8)	11.0±0.64 (10)	0.722
Day 12			
Mass (kg)	30.4±1.24 (8)	47.0±1.06 (10)	<0.001
Mass (% of MPM)	19.5±0.73 (8)	20.9±0.92 (10)	0.253
Weaning			
Mass (kg)	32.6±1.71 (12)	52.0±1.44 (10)	<0.001
Mass (% of MPM)	22.3±0.65 (8)	23.1±0.88 (10)	0.495

Values are means ± s.e.m. Sample sizes are given in parentheses. MPM, maternal postpartum mass.

Although some mammary biopsy procedures can result in a reduction in the subsequent rate of milk production (see Farr et al., 1996), there was no evidence that the biopsy procedure used in this study negatively affected the mammary gland function of the females studied. The values for milk composition (Table 1), estimated daily milk output and the relative growth of pups over lactation (Table 2) were consistent with previous observations for primiparous and multiparous grey seal females (Lang et al., 2009; Lang et al., 2011a). These results indicate that the transfer of milk energy to pups was not significantly affected following the biopsy procedures at either early or peak lactation and that the females selected for this study were representative of the population.

Immediately before parturition, mammary cells are converted from a non-secretory to a secretory state and there is a gradual increase in the secretory activity of the cells through the early stages of lactation up until the time of peak milk yield (Knight and Peaker, 1984; Shipman et al., 1987). This increase in secretory activity is accompanied by proliferation of the rough endoplasmic reticulum, hypertrophy of the Golgi apparatus, increases in the number of mitochondria and a growth in the cytoplasmic volume of the cells (Heald, 1974; Tucker, 1981). As a result of the high density of secretory cells in grey seal mammary glands, we were unable to directly assess cell sizes; however, the observed decrease in cell density with stage of lactation in both primiparous and multiparous females (Table 3) is consistent with an increase in cell development (and thus cell size) and may partly account for the increase in daily milk output over the course of lactation observed in grey seals (Mellish et al., 1999a). The greater cell density in the mammary gland tissue of primiparous females compared with multiparous females throughout lactation (Table 3) suggests that primiparous grey seal females may have smaller, less-developed secretory cells and thus may have a lower level of secretory activity per cell throughout lactation. This is consistent with the lower levels of metabolic activity observed in the mammary tissues of primiparous females compared with multiparous females at both the onset and the peak of lactation in dairy cattle (Miller et al., 2006). Although smaller alveolar cells may also be associated with a lower frequency of milk removal from the gland (Hillerton et al., 1990), primiparous grey seal females nurse their offspring more frequently than multiparous females throughout lactation (Lang et al., 2011b) and, thus, the higher cell density observed in primiparous females cannot be attributed to a lower frequency of milk removal. The differences in cell density between primiparous and multiparous grey seal females suggest that, consistent with the observations from tissue cultures in mice (Wagner and Smith, 2005), the secretory cells of multiparous females may be more responsive to the hormonal stimuli associated with the onset of lactogenesis (Heald, 1974; Tucker, 1981) and thus may show a greater and more rapid level of development compared

Table 3. Individual and predicted group means (±s.e.m.) for cell density, alveolar density and average alveolar size in the mammary gland tissues of multiparous and primiparous grey seal females at early (day 3–4 postpartum) and peak (day 10–12 postpartum) lactation

Female brand	Cohort	Age at recruitment[a]	Age at time of study	Prior reproductive experience[b]	Cell density (# mm^{-2})		Alveolar density (# mm^{-2})		Alveolar size (μm^2)	
					Early	Peak	Early	Peak	Early	Peak
Mulitparous										
F201	1985	4	20	13	1103±16.4 (5)	1126±43.8 (5)	25±1.0 (5)	23±1.0 (5)	8278±458.6 (281)	11,774±639.0 (261)
F380	1985	5	20	12	1180±24.3 (5)	929±15.0 (5)	26±1.5 (6)	22±0.3 (6)	6297±340.1 (294)	9172±400.8 (283)
F456	1985	5	21	14	1300±32.6 (5)	1329±38.0 (5)	21±1.2 (6)	24±1.4 (6)	7004±440.9 (275)	9309±582.4 (283)
F463	1985	5	20	13	1205±40.7 (5)	998±27.5 (5)	25±2.8 (5)	20±0.9 (7)	5901±278.4 (261)	9382±415.8 (302)
F576	1986	4	19	13	1204±67.2 (5)	1034±39.9 (5)	25±0.9 (7)	22±0.8 (6)	8682±375.4 (284)	8747±412.1 (281)
F710	1986	5	19	9	1125±13.3 (5)	1102±25.5 (5)	22±2.1 (6)	22±1.1 (5)	7857±381.5 (279)	7920±409.5 (276)
F913	1986	4	20	14	1519±52.1 (5)	1210±16.3 (5)	31±1.8 (4)	32±1.3 (4)	5955±344.2 (250)	9537±495.9 (310)
E227	1987	4	19	14	1318±30.0 (5)	1375±57.3 (5)	27±1.5 (5)	30±2.7 (4)	7138±582.8 (289)	8559±695.9 (272)
E353	1987	4	18	10	1200±56.1 (5)	1122±35.9 (5)	25±2.1 (5)	29±0.9 (4)	7422±389.4 (279)	9340±490.3 (282)
E449	1987	5	18	12		940±12.8 (5)		21±1.2 (5)		8219±497.3 (254)
		Predicted group mean[c]			1223±41.2	1116±40.5	25±1.1	24±1.1	7134±310.9	9206±305.9
Primiparous										
02X	1998	7	7	0		1150±41.5 (5)		22±1.2 (5)		5978±350.2 (259)
39V	1999	7	7	0	1466±60.4 (5)	1482±24.6 (5)	31±1.5 (4)	23±1.1 (6)	4663±288.9 (266)	5675±314.6 (278)
4V0	1999	6	6	0	1341±31.5 (5)		26±0.9 (4)		4509±249.2 (253)	
6V2	1999	6	6	0	1303±15.9 (5)	1660±29.2 (5)	28±1.5 (5)	32±1.1 (4)	4100±210.9 (252)	4231±194.4 (252)
9T4	1999	6	6	0	1282±31.4 (5)	1079±45.1 (5)	32±2.7 (5)	30±3.5 (4)	5051±275.3 (262)	4681±237.5 (262)
44D	2000	6	6	0	1357±29.3 (5)		28±2.0 (5)		5110±264.5 (263)	
D98	2000	5	5	0	1287±21.7 (5)	1149±21.1 (5)	27±1.5 (4)	23±1.4 (5)	6122±313.8 (253)	4648±198.1 (253)
2L0	2001	5	5	0	1371±33.5 (5)		29±1.1 (5)		7788±444.9 (265)	
4L3	2001	5	5	0	1461±22.8 (5)		34±1.9 (4)		5370±327.6 (282)	
8J5	2001	4	4	0	1168±60.2 (5)	1084±39.3 (5)	20±2.0 (6)	22±2.6 (4)	6620±340.2 (258)	8233±471.8 (292)
2N2	2002	4	4	0	1607±38.3 (5)	1219±44.9 (5)	30±1.3 (5)	30±1.8 (4)	4534±300.8 (251)	6836±425.2 (286)
8N8	2002	4	4	0	1481±58.9 (5)	1293±21.7 (5)	36±2.5 (4)	21±0.6 (6)	7270±498.5 (306)	5983±328.5 (262)
		Predicted group mean[c]			1366±37.5	1272±39.8	29±1.0	25±1.1	5590±285.1	5741±308.5
					Status: P=0.019 Stage: P<0.001 Status × Stage: P=0.734		Status: P=0.028 Stage: P=0.415 Status × Stage: P=0.030		Status: P=0.002 Stage: P<0.001 Status × Stage: P<0.001	

Values in parentheses indicate the number of observations per individual. Status: multiparous *vs* primiparous, Stage: early *vs* peak.
[a]First year a female was observed pregnant and/or rearing a pup; [b]number of breeding seasons a female was observed pregnant and/or rearing a pup prior to the present study; [c]obtained from linear mixed-effects models (see Materials and methods for details).

with the secretory cells of primiparous females following parturition. Although direct measures of cell activity (e.g. RNA concentration or enzyme activity) are needed, these results suggest that primiparous grey seal females may have a lower secretory capacity on a relative basis (per mass of mammary gland tissue) compared with multiparous females.

Although the majority of mammary gland growth and structural development occurs during pregnancy, the degree to which this process is complete at the time of parturition varies substantially among species (Cowie et al., 1980; Knight and Peaker, 1982). The observed increase in alveolar size between early and peak lactation in multiparous grey seal females (Table 3) indicates that alveolar development is not complete at parturition and that it continues at least over the early part of lactation in multiparous grey seals. The lack of change in alveolar density with increasing average alveolar size over lactation in multiparous females (Table 3) suggests that the regions between alveoli become compressed as alveoli increase in size within the lobule, which is consistent with observations of alveolar development in rats (Masso-Welch et al., 2000) and mice (Richert et al., 2000). The specific mechanisms that regulate the size of alveolar lumens remain poorly understood (Reginato and Muthuswamy, 2006); however, the smaller average alveolar size in primiparous grey seal females compared with multiparous females at early lactation and the lack of a significant increase in the average size of alveoli in primiparous females over lactation (Table 3) suggest that reproductive experience does have a significant influence on the development of alveoli. Neighbouring alveoli may fuse during development (Pitelka, 1998), and the decrease in alveolar density between early and peak lactation in primiparous females (Table 3) suggests that this process may not be complete at parturition and that it continues to occur over the early part of lactation in primiparous females. However, alveolar density did not differ between primiparous and multiparous females at peak lactation, suggesting that reproductive experience does not affect the number of alveoli that form within a fully developed secretory lobule in grey seals. Given that secreted milk is stored within the lumen of the alveoli and that alveolar density did not differ between the groups at peak lactation, the smaller average alveolar size in primiparous females indicates that the relative storage capacity of the mammary gland per unit mass will be significantly less for primiparous grey seal females compared with multiparous females during the period of highest milk output.

Differences in the rate of milk production between primiparous and multiparous females have frequently been attributed to differences between the groups in the total number of cells in the gland (Wada and Turner, 1959; Anderson and Sheffield, 1983; Fowler et al., 1990; Nielsen et al., 2001). However, the results of the present study suggest that, in addition to the increases that have been observed in the total number of secretory cells in the glands (Wada and Turner, 1959; Anderson and Sheffield, 1983; Fowler et al., 1990), reproductive experience has a significant influence on the development of both secretory cells and the size of alveoli. As a result, the mammary glands of primiparous females may have both a lower secretory capacity and a lower storage capacity on a relative basis (i.e. per mass of mammary secretory tissue) than those of multiparous females. To date, nothing is known about the relationship between mammary gland size or mass and the rate of milk production in grey seals or phocid seals in general. Therefore, to what extent the differences in cell density and average alveolar size between primiparous and multiparous females (Table 3) may contribute to the observed differences in daily milk output (Lang et al., 2011a; present study) remains to be determined. Nevertheless, our results provide insight into possible mechanisms contributing to variation in milk production capacity in phocid seals.

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