DNA synthesis in mammary epithelial cells of Swiss mice during lactation

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Danielle E. Croke-Auldist
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Abstract

Proliferation of extra secretory epithelial cells in mammary glands during lactation could potentially increase milk production, with flow-on benefits such as improved weaning weights of young or increased exports from the dairy cow industry. The primary objective of the research reported in this thesis was to increase proliferation of mammary epithelial cells during lactation so that the mechanisms associated with this phenomenon could be studied. Induction of increased proliferation in mammary glands was attempted by applying challenges to mice which were used as a laboratory model for agriculturally important species such as cows and pigs.

The experiments reported in this thesis also included refinement of methodologies developed to study proliferation of secretory cells in mammary glands during lactation. The first was to improve techniques for describing the chemical composition of mammary glands collected during lactation. This was achieved by collecting and analysing the composition of mouse milk at 3 stages of lactation (Chapter 3). While milk protein and milk fat remained constant throughout, the concentration of lactose increased with time. These data were critically important for correcting the weights of mammary glands for milk content. A second investigation was carried out to compare different methods of calculating the milk production of mice (Chapter 3). Three methods were evaluated with the best based on calculating the maintenance energy requirements of the metabolic weight of the litter which was added to the energy required for measured litter growth. The total energy required was then converted to a quantity of milk. The third methodology developed during the course of the work in this thesis was sample preparation for analysis of lactating mammary cells using flow cytometry.

One approach to increasing proliferation of mammary epithelial cells during lactation was to increase the suckling intensity of the mice. This challenge was accomplished by either increasing litter size (Chapter 6) or by increasing the ratio of pups per gland by taping over 5 of the 10 glands (Chapter 5). Suckling intensity was increased to 2 pups per gland but the effect was to accelerate mammary gland development in terms of cell number and milk synthesis status. Once a suckling intensity of >1 pup per gland was reached, there was no additive effect on the size of mammary glands or

milk production at mid lactation. Mammary glands appeared to have a limit on their size and output which is reached at a suckling intensity of 1 pup per gland. Manipulation of suckling intensity did not produce a suitable model of elevated proliferation of mammary epithelial cells during lactation.

Another approach tested was to use exogenous steroids as these had previously caused increased proliferation in mammary glands (Nagasawa and Yanai, 1978; Knight and Peaker, 1982d). The work reported herein showed that the response of mammary glands of mice to administration of steroids was dependent on stage of lactation and the dose (Chapter 4). In mid lactation, mammary glands were unresponsive for the parameters measured but in late lactation, incorporation of [^{3}H] thymidine into DNA increased and milk production decreased in response to higher doses of estrogen. The high estrogen dose did not however yield a suitable model for the study because the elevated incorporation of [^{3}H] thymidine was associated with early involution of mammary glands rather than proliferation leading to a net increase of epithelial cells.

The most promising method of analysis came from histological studies of lactating glands of mice labelled for DNA synthesis. Labelling indices of epithelial cells were >1.5 times greater on the edges of glands on D1 of lactation compared to the inner zones of glands. This within mouse variation was much greater than any between mouse variation arising from the suckling intensity and steroid experiments. An attractive feature is that tissues are derived from the same gland and have therefore been exposed to the same factors such as systemic mitogens and nutrition. In addition, the differences in labelling indices were measured in glands of mice suckling litters of 10 pups which is an easily repeatable treatment compared to some of the more complicated treatments tested during the course of this thesis. Dissection of mammary glands into outer and inner zones could provide useful tissue for the study of local factors involved with increased DNA synthesis of epithelial cells during lactation. Histological studies also revealed that following labelling of mammary epithelial cells for DNA synthesis on the day after parturition, the proportion of cells labelled decrease at a constant rate over the next 23 days (Chapter 8).

This project has increased the knowledge of manipulations of mouse mammary glands during lactation. It was found that growth of mouse mammary glands during lactation is difficult to increase experimentally and may have limited application as a model system to study regulation of growth of mammary glands during lactation. However, the work completed in this thesis will allow similar work to continue, with a high chance of success of investigating factors involved in mitosis of epithelial cells in lactating mammary glands.

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UNITS

S.I. (Syteme International d'Unites) abbreviations for units are listed below.

cpm	counts per minute
g	rcf = gravitational force
J, kJ, MJ	joule, kilo joule, mega joule
kDa	kilo Dalton
n	number of observations
nm	nano metres
°C	degrees Celcius
rpm	revolutions per minute
sec, min, h, D	second, minute, hour, day
v/v	volume per volume
w/v	weight per volume
µCi	micro Currie
µg, mg, g, kg	micro gram, milli gram, gram, kilo gram
µl, ml, l	micro litre, milli litre, litre
µM, mM, M	micro molar, milli molar, molar

ABBREVIATIONS

[^{3}H] thymidine	[methyl-^{3}H] thymidine
BCA	bicinchoninic acid
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CDK	cyclin dependent kinase
CMF	calcium magnesium free media
CO_2	carbon dioxide
DAB	3,3'-diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
DPX	DPX mountant
E	estrogen
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
FCS	fetal calf serum
FdU	5-fluoro-2'-deoxyuridine
FITC	fluorescein isothiocynate
GH	growth hormone
IGF-1	insulin like growth factor, type 1
JAK	Janus kinase
Milli Q	Milli Q water
MOPS	3-N-morpholino propanesulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
P	progesterone

ABBREVIATIONS continued

PBS	phosphate buffered saline
PBSE	phosphate buffered containing EDTA
PBSF	phosphate buffered saline containing formalin
PCNA	proliferating cell nuclear antigen
PE	phyco erythrin
PI	propidium iodide
PL	placental lactogen
PNA	peanut agglutinin
Prl	prolactin
RNA	ribonucleic acid
RSD	residual standard deviation
RT	room temperature
SDS	sodium dodecyl sulphate
SED	standard error of difference
SEM	standard error of the mean
STAT	signal transducer and activator of transcription
TUNEL	terminal deoxynucleotide end labelling
TCA	trichloroacetic acid
TEB	terminal end bud
tris	tris(hydroxymethyl) aminomethane
UV	ultra violet
wt	weight

Chapter 1 Literature review

The function of mammary glands is to synthesize and secrete milk that is essential for the nourishment of mammalian young. This literature review describes normal mammary development and the mechanisms controlling this development from the embryonic stage up until lactation in sexually mature animals. The focus of the first section of the review is on control and development of mammary development in rodents, as mice were used for the experimental work in the thesis. The next section specifically examines aspects of proliferation of epithelial cells in mammary glands, including detection of cells synthesizing deoxyribonucleic acid (DNA). The subsequent section presents a description of successful manipulations that result in increased growth of mammary glands during lactation. Finally the purpose and scope of the project described in this thesis is defined.

1.1 Mammary gland growth and development

This section of the review describes normal mammary gland development in rodents and the mechanisms controlling each stage from embryonic until lactation in mature animals. These events have been described in reviews by Cowie *et al.* (1980), Topper and Freeman (1980), Knight and Peaker (1982b), Tucker (1987) and Imagawa (1994). The morphology at each stage of mammary development is well described by Richert *et al.* (2000).

1.1.1 Embryonic and fetal

Development of the mammary gland rudiment in the fetal mouse occurs in three distinct phases beginning with 1) initiation of gland formation (Balinsky, 1950), followed by 2) a resting phase during which sexual dimorphism occurs (Kratochwil, 1986) and finally 3) a phase of rapid proliferation and development (Knight and Peaker, 1982b). Development is controlled by local factors of mesenchymal origin (Propper, 1968), epidermal-mesenchymal cell interactions (Kratochwil, 1977) and the maternal endocrine system (Ceriani, 1970). In addition to the previously

mentioned articles, this stage of development and its control is fully reviewed by Raynaud (1971) and Kratochwil (1986).

1.1.1.1 Description of development

The first phase of mammary development in the fetal mouse is on D10 when cells migrate along the mammary streak in preparation to form mammary buds (Balinsky, 1950; Cowie *et al.*, 1980). On D11, there is a thickening of the epidermis along the ventro-lateral body wall (Balinsky, 1950). The epidermis continues to thicken and buds form and grow into the underlying mesenchyme (Balinsky, 1950) (Figure 1.1). Each bud and underlying mesenchyme is termed the mammary rudiment. The position and number of mammary buds that occur along the ventro-lateral body wall determine the position and the number of mammary glands to be formed (Knight and Peaker 1982). The mammary bud later gives rise to the mammary parenchyma, which includes secretory epithelial cells, ductal epithelial cells and myoepithelial cells (Imagawa *et al.*, 1994). The mesenchyme is the origin of all the mammary stroma including connective tissue, blood vessels, fibroblasts and adipocytes (Knight and Peaker, 1982b; Imagawa *et al.*, 1994).

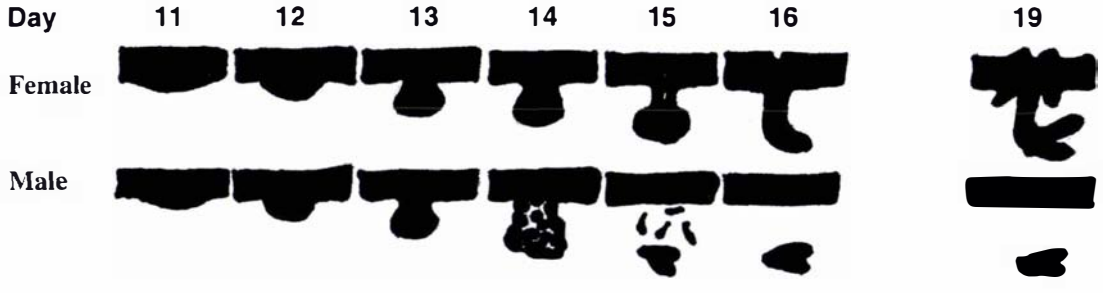


Figure 1.1: Morphological stages of mammary gland development in the female and male fetal mice.

Sexual dimorphism becomes apparent on D14, about 36-48 h after morphological differentiation of the testis. Adapted from Kratochwil (1986).

The second phase of mammary development in the fetal mouse is a resting phase, which occurs between D12 and D16 of gestation. Growth is slow during this time, with the mammary rudiment increasing only 4.5 fold compared with a 10 fold increase in fetal weight during the same period (Balinsky, 1950). This period of relatively slow growth is when sexual dimorphism occurs. Mammary rudiments in

both sexes of the mouse are identical until late on D13, however on D14, mesenchymal cells condense around the mammary buds of the male mouse fetus and either a) rupture the stalk connecting it to the epidermis so that no nipple development occurs but the mammary rudiment is still present in the mesenchyme (Sakakura, 1987), or b) causes necrosis of the mammary bud leaving no mammary rudiment or nipple (Cowie *et al.*, 1980; Kratochwil, 1986)(Figure 1.1).

The third phase of mammary development in the female fetal mouse is a period of rapid growth of the mammary buds to form ducts. This period lasts from D16 of gestation until parturition at D20. Mitotic activity is approximately five times greater than during the previous resting stage and there is positive allometric growth of mammary rudiments (Balinsky, 1950). Initially over this period, the mammary bud elongates and forms a primary sprout that extends from the epidermis and penetrates the underlying mesenchyme (Imagawa *et al.*, 1994). Once a lumen is formed, the primary sprout is called a duct. The primary duct then branches to form secondary and tertiary sprouts, which also become ducts. Each duct ends in a bulbous structure called an end bud. Mitotic activity is greatest at the end bud but is not exclusive to this area (Balinsky, 1950). The mammary glands of newborn female mice consist of a rudimentary ductal tree embedded in mesenchyme. There is no lobule alveolar development and the ducts are lined with two layers of undifferentiated epithelial cells. The primary duct will later form an opening to the nipple through which milk drains (called a galactophore).

1.1.1.2 Control of development

The control of mammary development in the mouse fetus is not well understood owing to difficulties in accessibility while *in utero.* It is known however that the three phases of development and growth are controlled by endocrine mechanisms and/or epidermal-mesenchymal cell interactions.

Formation of mammary buds is the first phase of fetal mammary development and this appears to be initiated by the mammary mesenchyme tissue (Propper, 1968). The most extensive studies of this phase have been done by Propper (1968) who co-cultured mammary and non-mammary epidermal and mesenchyme tissues from 12 and 13 day old fetal rabbits, and observed whether the *in vitro* experiments resulted

in formation of mammary buds. Propper (1968) concluded that mammary development is induced in the epidermis by the underlying mammary mesenchyme between D12 and 13 of gestation in the fetal rabbit and once it has been initiated, the epithelium is capable of proceeding with mammary development even in association with non-mammary mesenchyme. The signal for the initiation of mammary bud formation appears to be a paracrine factor released from the mesenchyme that stimulates epidermal cells (Cowie *et al.*, 1980). While no studies have been conducted on fetal mice on D11 when buds first appear, it is known that by D16 the mammary epidermal cells are sufficiently cyto-differentiated that they can synthesise the milk protein α-lactalbumin in response to mammogenic and lactogenic hormones (Cowie *et al.*, 1980). It may be that the mammary mesenchyme in the fetal mouse is similar to that in the rabbit which determines development of the epidermal tissue at a very early stage.

Sexual dimorphism is the second stage of fetal mammary gland development and this is controlled by both endocrine and mesenchyme-parenchyme cell interactions. On D12, the testis of the male fetus differentiates and by D14 there are changes in the mammary buds (Kratochwil and Schwartz, 1976). Androgen receptors with high binding affinity also appear in the mesenchymal cells next to the mammary bud in increasing numbers from D12 to 14 in fetuses of both sexes (Wasner *et al.*, 1983). The mammary bud is responsive to androgens only on D14 whereas buds collected from D12, 13, 15 and 16 are not affected (Kratochwil, 1977). Lack of responsiveness by the mammary bud before D14 is attributed to low androgen receptor number and binding affinity while no response after D14 is important for normal mammary development in female mice, which later secrete testosterone from the ovaries (Kratochwil, 1977). The sole target for androgens is the mesenchymal cell population next to the epidermal mammary bud (Drews and Drews, 1977). After the receptors of the mesenchymal cells bind to androgens, they condense tightly around the mammary bud and stimulate apoptosis of the epidermal cells. Both the female and male mouse fetuses are equally susceptible to the effects of androgens at D14 as injection of testosterone into the female fetus causes mesenchymal condensation and necrotic epidermal cells while conversely, the removal of testis before D14 will save male mammary buds from destruction (Imagawa *et al.*, 1994). Explant studies show that the cytotoxic, androgen-responsive mesenchymal cells can

only react to androgens if they have been adjacent to epidermal mammary buds since D12. This shows that the mammary epithelium governs development of the androgen responsiveness of the mesenchyme by inducing formation of androgen receptors (Kratochwil and Schwartz, 1976).

The last of the three stages of mammary gland development in the fetal mouse is the period of rapid extension of the mammary bud to form ducts. The rapid growth during D16 to 19 of gestation is due to exposure to maternal hormones as proliferation ceases after birth when the stimulus is removed (Knight and Peaker, 1982b). Fetal mammary development appears not to require fetal peptide hormones as normal development still occurs in fetal mice which have had their pituitary glands destroyed at D12 of gestation (Raynaud, 1971). Hormones which are mammogenic to adult mammary tissue are also mammogenic to fetal mammary tissue as demonstrated by studies where mammary explants are cultured with insulin, adrenal steroid and prolactin (Ceriani, 1970; Cowie *et al.*, 1980). The transient secretory activity of mammary epithelial cells of newborn humans (Oxender *et al.*, 1972) and ruminants (Oxender *et al.*, 1972) is also evidence of the fetal mammary gland being responsive to elevated mammogenic and lactogenic hormones in the mother's blood. Although it is the maternal hormones that cause the rapid proliferation in this phase, the sprouting of ducts of epithelial cells will only occur from mammary buds that are at least 16 days old (Kratochwil, 1986). The culture of explants *in vitro* and transplantation experiments *in vivo* combining epithelium and mesenchyme at different stages of development found that the intrinsic 'clock' for sprouting resides in the epithelial cells of the mammary bud (Kratochwil, 1986).

1.1.2 Postnatal

Mammary growth in the postnatal mouse is characterized by regression of terminal end buds soon after birth (Cowie *et al.*, 1980). Growth of mammary glands is then isometric for approximately three weeks after which it becomes allometric (Imagawa *et al.*, 1994). The allometric growth begins approximately a week before the first estrous cycle (Sinha and Tucker, 1966).

1.1.2.1 Description of development

The neonatal mouse has rudimentary mammary glands consisting of primary, secondary and tertiary ducts embedded in stromal tissue that originates from the mesenchyme (Richert *et al.*, 2000). Each duct ends in a solid epithelial bud called a terminal end bud (TEB) that regresses soon after birth (Cowie *et al.*, 1980). The other end of the duct opens at the apex of the incipient nipple (Cowie *et al.*, 1980).

The four major components of mammary glands of newborn mice are luminal epithelial cells, myoepithelial cells, the basement membrane and the stroma (Richert *et al.*, 2000). A cross section of a duct shows the lumen to be surrounded by luminal epithelial cells that are encased in a sheath of myoepithelial cells. The myoepithelial cells rest on the basement membrane, which separates parenchymal and stromal compartments. The basement membrane provides structural support for cells as well as influencing epithelial cell shape, polarity, growth and responsiveness to hormones (Streuli and Bissell, 1990; Lee and Streuli, 1999). The stroma consists of large adipocytes interspersed with fibroblasts as well as blood vessels, lymphatics and the lymph node (Richert *et al.*, 2000).

Growth of mammary glands of young rats is isometric between birth and D22 (Cowie *et al.*, 1980) which means they grow at the same rate as body surface area (Sinha and Tucker, 1966). During this time, the ductal system of 5–15 ducts extends into approximately 15% of the mammary fat pad (Medina and Smith, 1990) and there is a concomitant increase in the stroma (Reece, 1958). On D23, which is in advance of the first estrus, growth of mammary glands becomes allometric, meaning it increases at a rate significantly greater than that for body surface area (Sinha and Tucker, 1966). There is development of a large number of TEBs that grow into the fat pad to extend the ductal system.

1.1.2.2 Control of development

The TEBs in mammary glands of neonatal mice probably result from the residual effects of maternal hormones and those of parturition, since the buds regress once exposure to the maternal environment ceases (Cowie *et al.*, 1980).

The isometric growth of mammary glands that occurs in prepubertal rodents is independent of ovarian hormones (Topper and Freeman, 1980). In contrast, the presence of ovaries is required for the allometric growth of mammary glands that occurs prior to the start of estrous cycles in mice (Sinha and Tucker, 1966; Imagawa *et al.*, 1994), but not rats (Reece and Leonard, 1941). The use of ovariectomised, hypophysectomised and adrenalectomised (called triply operated) female mice has established that estrogen (E), growth hormone (GH) and adrenal corticoid (which can be replaced by progesterone, P) are required for normal prepubertal mammary growth (Lyons *et al.*, 1958; Imagawa *et al.*, 1994).

1.1.3 Puberty and adult

The first few estrous cycles are characterized by a cumulative increase in mammary DNA content owing to the extension of the duct system (Sinha and Tucker, 1969). After that, there is a plateau in mammary DNA content although the epithelium undergoes cyclic proliferation and regression, as well as morphogenic changes in response to the changing profile of hormones during each estrous cycle (Cowie *et al.*, 1980).

1.1.3.1 Description of development

The period of most rapid growth of mammary ducts in the female mouse occurs at puberty, at about four weeks of age, and continues until approximately two months of age (Cowie *et al.*, 1980). This is when ducts lengthen and grow to ultimately fill the mammary fat pad (Richert *et al.*, 2000). The duct system develops a characteristic pattern so that adjacent ducts never approach closer than 0.25 mm to each other, thus allowing space for subsequent development of alveoli (Faulkin and DeOme, 1960; Imagawa *et al.*, 1994). The TEBs are the major sites of proliferation and growth within mammary glands and are responsible for duct elongation and branching in pubertal mice, as well as alveolar cells in sexually mature mice (Topper and Freeman, 1980).

The morphology of TEBs and their growth during puberty and post puberty in mammary glands of mice has been well described by Richert *et al.* (2000) and is

summarized here (Figure 1.2). TEBs consist of multiple layers of epithelium with an outer layer of undifferentiated cap cells that are thought to be pluripotent stem cells (Williams and Daniel, 1983). One of the roles of the cap cells is to break down connective tissue in the stroma surrounding the TEBs. During the pubertal growth phase, the cap cells proliferate and invade the fat pad resulting in elongation of the duct. Cap cells give rise to myoepithelial cells of the ducts and can also migrate into underlying epithelial layers and become luminal epithelial cells (Masso-Welch *et al.*, 2000). The layer of epithelial cells beneath the cap cells proliferate and eventually become the ductal epithelial cells (Richert *et al.*, 2000). The inner most layers of the TEB cells undergo apoptosis resulting in formation of the lumen of the duct. Cell division is highest at the leading edge of the TEB (Daniel and Silberstein, 1987) while at the trailing end, the division of cells slows as they differentiate into myoepithelial and ductal cells (Humphries *et al.*, 1996). The myoepithelial cells lay down the basement membrane and the fibroblasts surrounding the ducts lay down stromal tissue (Richert *et al.*, 2000). The TEBs grow through the fat pad until they reach connective tissue surrounding another epithelial structure or the edge of the fat pad, after which they regress (Faulkin and DeOme, 1960) to form terminal ducts and alveolar buds (Richert *et al.*, 2000). The regressed TEB is encased in basement membrane and stroma and is hollowed to form a single layer of epithelial cells surrounded by myoepithelial cells and basement membrane (Richert *et al.*, 2000).

At approximately three months of age, the mouse is considered to be sexually mature. By this age, the duct tree extends throughout the fat pad (Richert *et al.*, 2000) and the TEBs respond to the estrous cycle by giving rise to only alveolar buds, not duct cells (Bresciani, 1968). Alveolar buds are comprised of a single layer of epithelial cells enveloping a circular hollow center (Richert *et al.*, 2000). They are formed by TEBs as well as by lateral branching of ducts where cell types are similar to those found in the proliferating end of TEBs (Richert *et al.*, 2000). Alveolar buds can progress into fully differentiated units capable of milk secretion when pregnancy induced growth occurs (Robinson *et al.*, 1995). Alveolar buds proliferate, differentiate and then regress in response to the cyclic secretion of ovarian hormones with each estrous cycle. At estrus, the greatest number of alveolar buds are present, DNA content of the gland peaks and mammary glands are most morphologically developed (Sinha and Tucker, 1966; Lotz and Krause, 1978). At metestrus, the

alveolar buds regress and the ducts return to being terminated by TEBs rather than alveolar buds (Knight and Peaker, 1982b). During late diestrus and proestrus, DNA synthesis in the TEBs peaks and existing lobules increase in size as epithelial cells proliferate prior to differentiating during the next stage, which is estrus (Knight and Peaker, 1982b). The mitotic index and rate of DNA synthesis is highest during diestrus and low during estrus and metestrus (Cowie *et al.*, 1980).

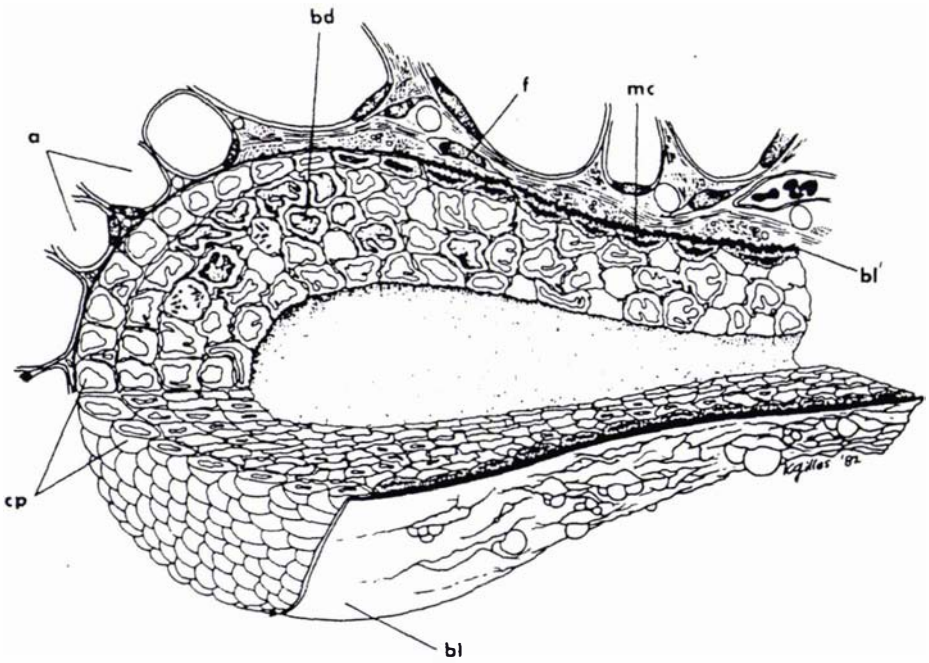


Figure 1.2: Diagram of a terminal end bud in a mouse mammary gland.

The TEB is comprised of cap cells (cp), luminal (bd) and myoepithelial cells (mc). Surrounding the end bud is basal lamina (bl) while the stroma is comprised of adipocytes (a) and fibroblasts (f). Adapted from Williams and Daniel (1983).

1.1.3.2 Control of development

The minimum requirements for duct growth in pubertal rodents are E and either GH or prolactin, Prl (Topper and Freeman, 1980). Some authors state that ovarian and pituitary hormones (GH and Prl) are required (Reece and Leonard, 1941) although others have reported that large quantities of either of the pituitary hormones will promote normal mammary development in young animals even in the absence of steroids (Topper and Freeman, 1980). Maximal duct growth occurs when glucocorticoids are also present (Lyons and Johnson, 1958). Maintenance of the ducts seems to require different hormones to those causing proliferation in that

pituitary hormones are not necessary, provided that either adrenal or ovarian secretion is present (Topper and Freeman, 1980; Imagawa, 1994).

Development of mammary ducts in the mouse is regulated by interplay between local and systemic factors (Silberstein and Daniel, 1987). The inability of E, GH and Prl to cause ductal growth *in vitro* indicates that other factors are involved in order to achieve normal growth (DuBois and Elias, 1984). Silberstein and Daniel (1987) used mammary implants to test the effects of hormones and growth factors. Their studies highlighted the importance of mammary stroma and led to the conclusions that positive or negative growth of ducts is the result of several pathways (Silberstein and Daniel, 1987). Earlier work by Faulkin and DeOme (1960) also illustrated the importance of mammary stroma. The regular spacing of the ducts is probably due to a chemotactic mechanism. It is consistent with the suggestion that mammary duct cells produce a chemical that diffuses into the adjacent fat pad and prevents other ducts from penetrating the area (Faulkin and DeOme, 1960). It only inhibits growth in the immediate vicinity with the result that some TEBs simply turn away and grow in a different direction (Faulkin and DeOme, 1960).

In the sexually mature mouse, the changing profile of hormones associated with the estrous cycle is responsible for alveolar bud formation, differentiation and regression. During proestrus, the release of E and Prl stimulates DNA synthesis (Butcher *et al.*, 1974) while during estrus, Prl in conjunction with the rising P concentrations are responsible for increased DNA content and differentiation (Knight and Peaker, 1982b). The differentiation that occurs during the estrous cycle is not terminal and does not result in formation of rough endoplasmic reticulum as seen during lactation (Topper and Freeman, 1980). After this, Prl declines so that P alone is ineffective as a stimulus for growth. Alveolar buds therefore regress during metestrus (Knight and Peaker, 1982b).

In adult female rodents, the ovarian steroids influence the type of growth that occurs in mammary glands (Imagawa *et al.*, 1994). Ovariectomy causes regression of both ducts and alveoli of the mammary gland. In ovariectomised mice, treatment with E stimulates only ductal growth whereas either E+P or large doses of P alone, cause

both ductal branching and lobuloalveolar development (Bresciani, 1968; Imagawa *et al.*, 1994).

The mode of action of E is largely unknown but it acts directly or indirectly depending on developmental status of the gland (Haslam, 1988). When administered to five week old mice it had a direct effect and stimulated end bud formation while in 10 week old mice it induced P receptors, illustrating a difference in hormonal responsiveness of cells (Haslam, 1988; Haslam, 1989). In the mature female, E may actually induce P receptors on TEB cells (Haslam and Shyamala, 1981). It can also facilitate the effects of P and Prl on ductal and alveolar development by stimulating pituitary Prl secretion, and Prl in turn can raise E and P receptor levels (Fendrick *et al.*, 1998). It is proposed that E stimulates epithelial growth locally but by an indirect mechanism. It is suggested that it acts on the E receptors on stromal cells, which in turn release a paracrine factor that affects the cap cells (Haslam and Shyamala, 1981).

1.1.4 Gestation

Mammary glands undergo enormous growth and differentiation during gestation, which culminates in their ability to lactate after parturition. Approximately 30% of all mammary growth occurs during D6-12 of gestation, and approximately 50% occurs between D12 and parturition in the mouse (Brookreson and Turner, 1959), with the remainder accumulating in early lactation. Exposure to hormones at, or just prior to parturition, is crucial to triggering milk synthesis (lactogenesis) in epithelial cells.

1.1.4.1 Description of development

The first phase of gestational mammogenesis in rodents is the increase in complexity of the structure within the gland (Topper and Freeman, 1980). The duct system grows further into the mammary fat pad and there are increases in the number of duct side branches and alveolar buds (Knight and Peaker, 1982b). The new ducts and alveolar buds gradually replace the shrinking adipose tissue to fill in the interductal spaces present in the mammary glands of virgin mice (Imagawa *et al.*, 1994).

The second half of gestation is characterised by lobuloalveolar differentiation as well as continued growth (Topper and Freeman, 1980). The alveolar buds progressively cleave and differentiate into individual alveoli which will ultimately become milk secreting during lactation (Richert *et al.*, 2000). Vascularisation increases around individual alveoli and terminal ducts and they become surrounded by a basket shaped arrangement of myoepithelial cells (Richert *et al.*, 2000). Some alveolar epithelial cells have direct contact with adipocytes and the basement membrane owing to a decrease in stromal connective tissue and an incomplete sheath of myoepithelial cells around the alveoli (Richert *et al.*, 2000). This direct contact may be critical for full differentiation into alveolar epithelial cells that are capable of milk secretion (Lee *et al.*, 1985; Richert *et al.*, 2000). On the day prior to parturition, the epithelial cells are observed to accumulate rough endoplasmic reticulum, Golgi and secretory vesicles and begin secreting milk proteins and lipids (Richert *et al.*, 2000). The epithelial cells increase in size (hypertrophy) due to accumulation of milk lipid and milk proteins (Knight and Peaker, 1982b).

1.1.4.2 Control of development

Studies with triply operated mice (hypophysectomised, ovariectomised and adrenalectomised) show that a minimum of E, P and Prl are required for growth similar to that observed during gestation (Knight and Peaker, 1982b; Imagawa *et al.*, 1994). The addition of only E and Prl results in growth of ducts, while the inclusion of P will advance development to the lobuloalveolar stage (Imagawa *et al.*, 1994).

The signal secreted by secretory epithelial cells in the mammary glands of sexually mature, virgin, mice that maintains spacing of 0.25 mm between ducts (Faulkin and DeOme, 1960) is overcome during gestation. During early gestation P shows a marked and sustained elevation (Virgo and Bellward, 1974) and when it is administered to mature, virgin mice, there is alveolar growth similar to that seen in mid pregnancy (Topper and Freeman, 1980) suggesting P is the hormone that abrogates the signal inhibiting growth.

After coitus in rats and mice, there are twice-daily surges of Prl which continue for the first third of pregnancy (Knight and Peaker, 1982b). These Prl surges together

with elevated P concentrations cause mammogenesis (Knight and Peaker, 1982b). The peak in DNA synthesis on D4 correlates with periods of high P and Prl concentrations (Desjardins *et al.*, 1968).

In the last two thirds of gestation, the concentration of Prl decreases (Cowie *et al.*, 1980). Although the concentrations of E and P remain high, they are ineffective in the absence of a peptide mammotroph (Knight and Peaker, 1982b). It is at this stage that the role of Prl is supplanted by placental lactogen, a hormone secreted by the placenta (Desjardins *et al.*, 1968; Forsyth and Jones, 1976). Placental lactogen (PL) binds to the same receptors as Prl (Kelly *et al.*, 1977) and has similar mammogenic properties (Knight and Peaker, 1982b). Its concentration rises over the last two thirds of gestation and is maintained at a high level until just before parturition (Kelly *et al.*, 1976). The second peak of DNA synthesis in mammary glands coincides with high concentrations of PL (Desjardins *et al.*, 1968). The concentration of PL increased with fetal litter size (Knight and Peaker, 1982b). Increased mammary growth and fetal number are correlated up to a maximum of eight feto-placental units; thereafter the secretion of PL is either not enhanced or the glands cannot respond further (Nagasawa and Yanai, 1971).

During gestation, the development of alveolar buds into lobules of alveoli capable of milk synthesis may be ascribed to two hormonal changes. These changes are firstly, the marked and sustained elevation of P and secondly, the early but short lived elevation of Prl (Cowie *et al.*, 1980). These events are thought to confer differentiative properties and insulin responsiveness to epithelial cells which up until gestation are insulin insensitive (Oka and Topper, 1972; Topper and Freeman, 1980). The early studies of Voytovich *et al.* (1969) suggested insulin, together with Prl and a glucocorticoid can affect terminal differentiation of mouse mammary epithelial cells and this has been confirmed by others (Oka *et al.*, 1974; Vonderhaar, 1977). Insulin and a glucocorticoid cause insulin responsive mammary epithelial cells to accumulate rough endoplasmic reticulum while Prl causes an increase in ribonucleic acid (RNA) for caseins. Glucocorticoids are thought to prolong the effect of Prl on cells to cause an increase in RNA accumulation (Topper and Freeman, 1980).

The ratio of thyroid hormones to Prl is also thought to be of importance to mammary development during gestation (Topper and Freeman, 1980). Relaxin is known to stimulate growth of mammary glands in young rats that have no ovaries or pituitary but it inhibits such growth in goats indicating its role may be species specific (Knight and Peaker, 1982b).

1.1.5 Lactation

During early lactation, epithelial cells in mice and rats continue to proliferate and develop into milk secretory cells. As much as 20-40% of the total DNA content of mammary glands is synthesised during the first four to five days of lactation (Munford, 1964; Knight and Peaker, 1982b). DNA synthesis peaks on D2–3 of lactation (Traurig, 1967a) and milk production and the DNA content of mammary glands peak in mid lactation (Munford, 1964). The suckling of the litter affects both the maternal endocrine and autocrine systems (Knight and Peaker, 1982a) which influence the continued secretion of milk from differentiated epithelial cells.

1.1.5.1 Description of development

During lactation, mammary glands consist of lobules of alveoli that are composed of alveolar epithelial cells that are responsible for the production and secretion of milk (Topper and Freeman, 1980). The epithelial cells contain rough endoplasmic reticulum, Golgi, secretory vesicles containing lactose and milk proteins and lipids (Chepko and Smith, 1997; Richert et al., 2000). At the onset of lactation, alveoli are engorged with milk and the lumens of the ducts become filled with milk (lactogenesis). Each alveolus is surrounded by myoepithelial cells in a basket-like configuration and it is the contraction of the myoepithelial cells in response to oxytocin that forces milk from the alveoli into the ducts. A thin layer of connective tissue and capillaries separate the alveoli. At the start of lactation, approximately 30% of the gland is filled with adipocytes but as lactation progresses the adipocytes shrink as the lipid in the cells is metabolized and alveoli fill the gland (Richert *et al.*, 2000).

Milk yield is a function of epithelial cell number and milk output per cell (Knight *et al.*, 1984). The dynamics of cell number and milk output per cell on milk yield curves has been described by Knight and Peaker (1984) for goats but the general relationship is applicable to most species (Figure 1.3). In a typical milk yield curve, the ascending portion is characterized by an increase in cell number and then an increase in milk output per cell. After peak milk yield, the number of cells declines and in late lactation, the milk output of any remaining cells also decreases (Knight and Peaker, 1984).

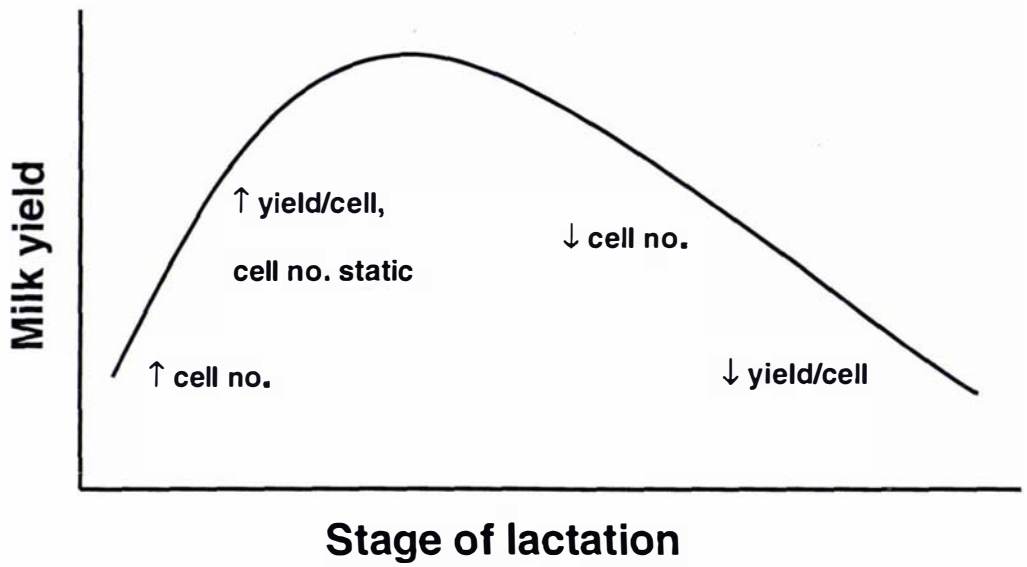


Figure 1.3: Dynamics of mammary epithelial cell number and output per cell in a stylised milk yield curve.

In a typical milk yield curve, the ascending portion is characterized by an increase in cell number and then an increase in milk output per cell. After peak milk yield, the number of cells declines and in late lactation, the milk output of any remaining cells also decreases. Adapted from Knight and Peaker (1984).

1.1.5.2 Control of development

After parturition there is an increase in milk secretion. At this stage, the concentrations of the progesterone and estrogen in plasma are low while Prl and glucorticoid are high (Topper and Freeman, 1980). Mammary epithelial cells retain their insulin sensitivity that began during gestation (Voytovich *et al.*, 1969).

Milk removal is crucial in maintaining lactation. Suckling by the young triggers the milk ejection reflex, which expels milk from alveoli into ducts so that it may be removed. The tactile stimulation of the nipple or teat causes oxytocin to be released from the posterior pituitary gland which stimulates the myoepithelial cells around the alveoli in the mammary glands to contract and force milk out into ducts (Cowie *et al.*, 1980). In species such as mice and rats that have no storage cistern, sucking will not remove milk unless this reflex has been triggered. In species with large gland cisterns, a considerable portion of milk may be removed in the absence of milk ejection (Cowie *et al.*, 1980).

The mammary glands of mice and rats continue to grow in early lactation and in an effort to explain this mechanism, many early experiments studied the administration of hormones to rodents (Cowie *et al.*, 1980) and the subsequent effect on mammary development. Griffith and Turner (1963) injected rats with either E, P, GH, Prl, or cortisol alone, or in various combinations and concluded that GH and adrenal corticoids stimulated mammary growth during lactation. GH was also suggested to be involved in mammary growth and lactation by Moon (1965a). Contrary to that, Ferreri and Griffith (1977) concluded that cortisol was the only effective trigger of mammary growth and lactation and its effect was inhibited by E and P. Prl is another hormone that plays a major role in mammary growth during lactation, as outlined below.

Mammogenesis during lactation in mice and rats requires Prl. Prolactin concentration increases *post-partum* and in proportion to the suckling stimulus of the young (Tucker *et al.*, 1967; Grosvenor and Mena, 1974) and is highly correlated with the DNA content of lactating mammary glands (Grosvenor and Mena, 1974). Recently autocrine factors have also been implicated in mammary growth during lactation (Wilde *et al.*, 1990; Wilde *et al.*, 1995). The feedback inhibitor of lactation (FIL) is a locally produced protein that is secreted into milk (Wilde *et al.*, 1990). It is suggested that frequent and complete removal of milk by young pups reduces the concentrations of local factors such as FIL, thereby making cells more sensitive to the elevated levels of Prl that circulate in response to a high suckling intensity (Wilde *et al.*, 1990). Incomplete milk removal results in accumulation of FIL which acts on

epithelial cells to decrease milk synthesis as well as the number of Prl receptors on the epithelial cells (Wilde *et al.*, 1990).

1.2 Cell proliferation

The main objective of the research reported in this thesis is to study the growth of the mammary gland during lactation. Growth can be caused by either cells undergoing mitosis (Lehninger, 1982) resulting in an increase in cell population or by cells increasing in size with no increase in cell number. Cell types that are able to proliferate (because they are not terminally differentiated) follow the cell cycle and its regulatory steps (Chepko and Smith, 1997).

1.2.1 The cell cycle

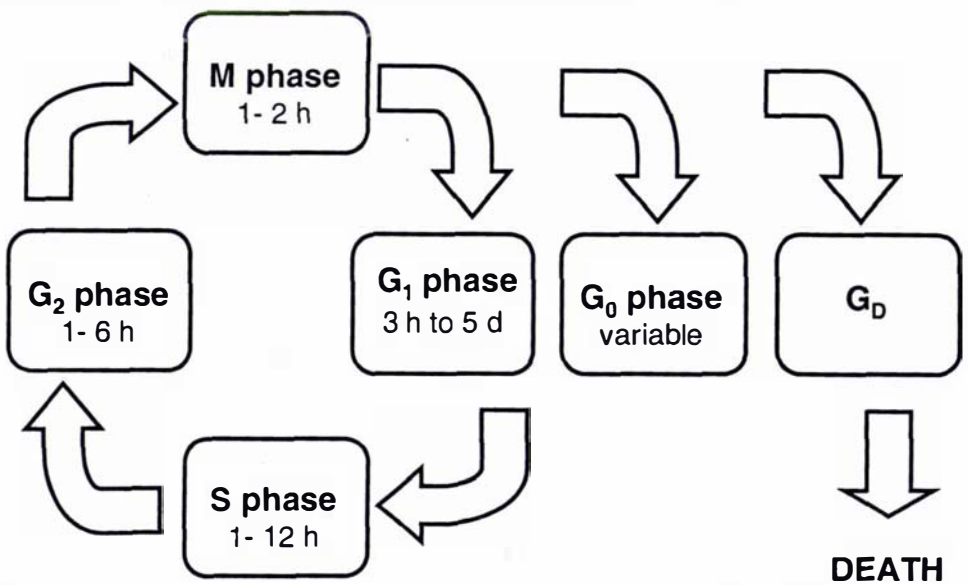
The eukaryotic cell cycle allows the exact duplication of DNA followed by the precise segregation of the DNA and the cell contents into two daughter cells. It consists of four biochemical phases; mitosis (M), gap phase (G_1), synthetic phase (S) and G_2 (Figure 1.4) (Baserga, 1985).

The M phase is the culmination of the cell cycle where a single cell gives rise to two daughter cells and can therefore be viewed as either the start or end of the cycle. At the end of M phase, cells can enter a non-proliferating state (G_0), a terminally differentiated state (G_D) or a new cell cycle (G_1). The two factors that usually determine which path the cell enters are external factors in the cell's environment, such as growth factors or limiting nutrients, and the state of differentiation, such as if the cell is a stem cell or a terminally differentiated alveolar cell (Oshima and Campisi, 1991).

The G_1 phase is the gap phase where the cell prepares for DNA synthesis. It will only proceed with DNA synthesis once it passes several regulatory steps. It is the most variable interval of the cell cycle and can range from a few hours to several days, depending on the cell type (Oshima and Campisi, 1991). Exogenous E can

accelerate secretory epithelial cells of the mammary gland through G_1 while anti-Es have the opposite effect (Sutherland *et al.*, 1998). This variation therefore influences the rate at which a population can proliferate (Pardee *et al.*, 1978).

Figure 1.4: The cell cycle for mitosis in eukaryotic cells.



Following M phase, cells may become terminally differentiated (G_D), quiescent (G_0) or enter another round of mitosis (G_1). Cells prepare to synthesis DNA in G_1 phase. After synthesis (S phase) is the G_2 phase during which cells prepare to divide (M phase). Diagram adapted from Hall and Levison (1990) and Baserga (1985).

The S phase is the interval in which the genome is replicated and usually takes several hours to complete. Once DNA replication is started, a cell is committed to complete the process. Regulatory proteins that may act late in G1, control the activity of the many enzymes involved in synthesising, unwinding and repackaging the DNA (Oshima and Campisi, 1991).

The G_2 phase follows S phase and allows cell to prepare for the M phase. This is a time when regulatory factors can accumulate in preparation for the processes involved during M (Oshima and Campisi, 1991).

The cell cycle is regulated at each phase with the best characterised check points occurring at the G_1-S and G_2-M transitions. For instance, cells cannot proceed to the S stage until conditions are appropriate such as the cell having enlarged sufficiently

since the last cytokinesis. Another example is that cells do not enter M phase from G_2 unless DNA synthesis is complete and any damaged DNA has been repaired. Cyclin dependent protein kinases (CDKs), which have little or no activity until they bind to cyclins, guard the checkpoints (Harter, 1996). There have been seven CDKs and five types of cyclins (A, B, C, D and E) identified to date. Only the A and B type cyclins are involved in mitosis while C, D and E types occur during G_1 (Jacobs, 1995). Over expression of the D types of cyclins accelerates G_1 phase (Harter, 1996). It is a requirement that all cyclins are destroyed before the cell can progress out of mitosis (Harter, 1996). Inhibitors of CDKs have been discovered recently. These bind to CDKs and inhibit their kinase activity, which may explain why some cells arrest in G1.

Cell populations are continuously being produced by mitosis and lost by cell death (Hall and Levison, 1995). Any factor which alters either mitosis or cell death will alter the net size of the population in the tissue (Knight *et al.*, 1984). The DNA content of murine mammary glands increases in early lactation due to mitosis occurring at a greater rate than apoptosis (Knight and Peaker, 1984; Knight *et al.*, 1984). After mid lactation, the rate of mitosis decreases to very low values while apoptosis increases resulting in a diminishing population of secretory cells in the mammary glands (Knight and Peaker, 1984; Knight *et al.*, 1984).

1.2.2 Detection of proliferating cells

The proportion of cells in the mitosis cycle can be analysed by methods that detect mitotic figures during the S phase. These methods include labelling precursors of the DNA (Hall and Levison, 1990), or by immunohistochemical detection of proteins that occur naturally during mitosis (McCormick and Hall, 1992) or by flow cytometry (Shapiro, 1988).

1.2.2.1 Thymidine labelling

Thymidine is catalysed to thymidine monophosphate by the enzyme thymidine kinase and then incorporated into replicating DNA. Thymidine kinase assays using labelled thymidine, such as [^{3}H] thymidine, or analogues, such as

bromodeoxyuridine (BrdU) are routinely used as indices of DNA synthesis (Hall and Levison, 1990; Meyer *et al.*, 1986).

[^{3}H] thymidine has been used in animal experiments to indicate proliferation of mammary epithelial cells since the 1950s (Hall and Levison, 1990). Only 5-methyl labelled thymidine should be used since any other position can be demethylated and degraded to form uracil which is incorporated into RNA and hence does not label DNA specifically (Lehninger, 1982). [^{3}H] thymidine can be either administered *in vivo* or used in cell culture models with either mammary explants or isolated cells and its incorporation into DNA detected in homogenized tissue using a β counter. Alternatively [^{3}H] thymidine incorporation into DNA can be assessed histologically in tissue sections. Studies of breast carcinoma indicate that [^{3}H] thymidine labelling indices closely correlate with the rate of proliferation of tumours (Meyer *et al.*, 1986).

BrdU is a non-radioactive analogue of thymidine that is incorporated into replicating DNA during the S phase and is administered as for [^{3}H] thymidine. Its presence is detected histochemically using specific antibodies on tissue sections or by flow cytometry but unlike [^{3}H] thymidine, a method has not yet been developed for its detection in homogenized tissues. BrdU has the advantage over [^{3}H] thymidine in that detection can be within hours compared to the weeks of exposure required for autoradiography. In addition, it does not cause damage to cellular DNA and is therefore suitable for long term labeling studies.

1.2.2.2 Immunohistochemical methods

Antibodies to proteins that are synthesized at specific stages of mitosis are another important means of assessing cell proliferation in tissues sections (Hall *et al.*, 1990; Hall, 1993).

An antigen that occurs during the cell cycle is called the proliferating cell nuclear antigen (PCNA). PCNA is a 36 kDa protein (Mathews *et al.*, 1984) that has been highly conserved during evolution with only four amino acids differing between the rat and the human forms (McCormick and Hall, 1992) and belongs to the large family of cell cyclins. It is essential for DNA synthesis as it is a co-factor for DNA

polymerase γ in the S phase and also for DNA synthesis associated with DNA repair (McCormick and Hall, 1992). When interpreting results obtained from using antibodies to PCNA, it is important to be aware that PCNA has a very long life and is still detectable approximately 20 h after the M phase. In addition, there are small amounts of PCNA present even in non cycling cells with only long term quiescent cells totally down regulating their PCNA production. Results when using PCNA have been comparable to those when flow cytometry was employed (Garcia *et al.*, 1989). Antibodies to PCNA have been developed for use on fixed and wax embedded tissues (Hall *et al.*, 1990; Hall, 1993).

Another antigen that occurs in normal and neoplastic cells during the cell cycle is recognized by an antibody called Ki-67. Ki-67 recognises a large protein doublet of molecular weight 345–395 kDa that is thought to be a structural protein that maintains the order of DNA during mitosis (Sawhey and Hall, 1992). It is expressed in mid G_1 and increases through S and G_2 to peak in M. The protein has a very short life and none is detectable 1 h after M phase. There is no expression of Ki-67 during DNA repair processes so it is purely associated with DNA synthesis for mitosis (Hall, 1993), unlike other proteins such as PCNA. Several studies have shown a close correlation between Ki-67 immunoreactivity and cellular proliferation as measured by [^{3}H] thymidine or BrdU incorporation, or flow cytometry (Isola *et al.*, 1990). Production of different Ki-67 antibodies enable detection of the antigen associated with mitosis in cryostat sections as well as formalin fixed sections (Hall, 1993).

1.2.2.3 Flow cytometry

The principles of flow cytometry are reviewed elsewhere (Shapiro, 1988; Gabriel and Kindermann, 1995) but are briefly described here. Cells in a single cell suspension are passed in front of a laser beam and any light that is reflected or transmitted is detected and converted to electronic signals. The electronic signals are translated to yield information about cell number, cell size and granularity. Further information about the cells can be obtained by the use of fluorescent dye markers linked to antibodies or that bind to nucleic acids. Propidium iodide (PI) is a red fluorescent dye that binds to the DNA in cells. Antibodies or lectin markers can be used to identify differentiation status of mammary epithelial cells. For instance, the

lectin peanut agglutinin (PNA) binds to an oligosaccharide on the apical membrane of active secretory cells from alveoli of mammary glands (Newman *et al.*, 1979; Rudland, 1992). When the lectin is linked to a fluorescent marker it will give information regarding the proportion of the cells that are differentiated. A sample of mammary epithelial cells that has been prepared with PI and PNA and analysed by flow cytometry could yield information such as the proportion of cells in S phase and the proportion of cells that are differentiated.

Flow cytometry is a method of analysis that can measure five parameters of approximately 5000 cells per sec (Shapiro, 1988). The rapidity of this method is an obvious advantage over the traditional method of preparing and manually assessing labelling indices in tissue sections (Hall and Levison, 1990). A disadvantage of flow cytometry for the current project is that samples must be single cell suspensions. This is the reason why most flow cytometry work is conducted on naturally occurring single cell suspensions such as blood and sperm (Gabriel and Kindermann, 1995). This problem has been overcome by the partial enzyme digestion and physical disruption of mouse and rat mammary glands which results in single cell suspensions that can be analysed using flow cytometry (Dundas *et al.*, 1991; Kim *et al.*, 1993; Pulland and Steul, 1996).

1.2.3 Cell types responsible for proliferation of mammary parenchyma

The cell types responsible for proliferation of mammary parenchyma in mature rodents have been well discussed by Smith and Medina (1988), Medina and Smith (1990), Zeps *et al.* (1996), Chepko and Smith (1997) and Smith and Chepko (2001).

The sometimes dramatic changes observed in the size of the population of mammary epithelial cells at different stages of the reproductive cycle led to the suggestion of a regenerative cell population that is capable of repeatedly populating mammary glands. During each estrous cycle, there is extensive cell division of the mammary epithelium followed by its regression (Zeps *et al.*, 1996). Similarly, repeated lactations are characterized by proliferation and differentiation of mammary epithelium followed by near complete involution and disappearance of secretory

tissue which then reappears in the subsequent lactation (Topper and Freeman, 1980). The cells that can repopulate mammary glands with epithelial cells are termed mammary stem cells.

A mammary stem cell is defined as a cell that has the capacity to repopulate the mammary fat pad with parenchyma that undergoes the entire range of morphological and differentiation changes exhibited in the normal development cycle of the mammary gland (Medina and Smith, 1990). Mammary gland stem cells retain their undifferentiated characteristics and give rise to non-mitotic cells that can perform specialized functions such as milk synthesis (Smith and Chepko, 2001). Evidence for the existence of stem cells in all parts of the mammary gland came from transplantation studies. Segments of mammary glands taken from primary duct, tertiary duct, end bud, alveoli from pregnant and lactating mice, regressed gland or aged gland all give rise to normal epithelial outgrowths with complete developmental capacity when transplanted into a permissive site of a host (DeOme *et al.*, 1959; Ormerod and Rudland, 1986; Smith and Medina, 1988; Smith and Chepko, 2001). These studies show that mammary epithelial stem cells must exist in the mouse mammary gland throughout life.

A model of proliferation and regeneration of the mammary gland by stem cells has been proposed by Chepko and Smith (1997) and is similar to the model of the more widely studied stem cell proliferation in the epidermis (Loeffler *et al.*, 1987). Stem cells, which are devoid of differentiation characteristics, generate daughter cells that are morphologically similar to the mother cell. One remains a stem cell while the other becomes a primary progenitor cell that can undergo multiple divisions. The daughters of primary progenitor cells differentiate into secondary progenitor cells which are then committed to either secretory or myoepithelial differentiation. Whichever differentiation path is chosen, the secondary progenitor cells can divide multiple times to produce many more secretory or myoepithelial cells (but great numbers of the latter are not required) which become terminally differentiated and are incapable of mitosis.

The cell types above have been characterized using electron microscopy (Chepko and Smith, 1997). Stem cells and primary progenitor cells are described as small,

amoeboid shaped cells that are pale and contain few organelles while the terminally differentiated cells are large, dark cells that contain many organelles and can synthesis milk. The differentiated, secondary progenitor cells that occur between these two extremes can contain differentiation characteristics, such as being large, dark and able to synthesis milk, as well as progenitor characteristics, such as being highly mitotic. It is probably differentiated, secondary progenitor cells that have been identified several times in earlier literature. Mammary epithelial cells that appeared to be both secretory and mitotic in rodents and ruminants have been reported by several groups (Franke and Keenan, 1979; Capuco and Akers, 1990). This was perplexing as secretory cells were considered to be terminally differentiated and therefore unable to be mitotic. In view of the observations made by Chepko and Smith (1997) it may be, however, that these cells were actually differentiated, secondary progenitor cells and therefore capable of milk synthesis which can be paused when the cell becomes mitotic.

Chepko and Smith (1997) estimated that stem cells and primary progenitor cells make up approximately 3% of epithelial cells in mammary glands while secondary progenitor cells and differentiated cells comprise 5% and 75% respectively. It has also been noted that the time taken for completion of the cell cycle varies between these groups and ranges from less than three days to longer than 14 days (Christov *et al.*, 1993). This is consistent with intestinal stem cells, which were found to divide less frequently than the progenitor and differentiated cells so that they were never more than 60 generations from the original cell of the zygote (Morris, 1994; Zeps *et al.*, 1996).

As evident in the transplantation studies described earlier, stem cells are distributed throughout mammary glands (Smith and Chepko, 2001). The primary candidate for stem cells in the rodent mammary gland appears to be the cap cells of TEBs. Although TEBs regress when ductal growth ceases they are the sites of future alveolar buds. Alveolar buds also arise from specific side branching of ducts (Topper and Freeman, 1980). Both these sites from which alveolar buds arise have been examined. Cells have been found to be resting in G_2 phase and conform to the description of stem cells being small and pale (Smith and Medina, 1988). Thus, it appears that as the ductal tree extends into the mammary fat pad, stem cells or

progenitor cells are left at specific locations along ducts and at the terminal end of ducts so that they can populate glands with epithelial cells when required (Smith and Chepko, 2001).

1.3 Manipulation of mammary growth during lactation

The first section of this literature review described the normal mammary growth of mice and rats, which included growth during early lactation. The typical growth and milk yield of a normal lactation can be increased, however, by use of strategic manipulations of the dam or her litter (Knight and Peaker, 1982e; Moon, 1969; Tucker, 1966). In the following section some of these manipulations are discussed.

1.3.1 Litter size

The mammary glands of rodents can respond to litter size by increasing size and/or milk yield (Bateman, 1957). In rats with litters adjusted on the day of parturition, the mammary glands of those with 12 pups contained 39% more per DNA by D13 of lactation than those with only six pups (Moon, 1969). These results are similar to those of Tucker (1964) who reported that the DNA content of rat mammary glands on D21 of lactation increased as litter size increased from four to 10 pups. Another study also showed a close correlation between DNA content of rat mammary glands at peak lactation and number of young suckled (Tucker, 1966). Knight and Peaker (1982e) reported that when lactating mice were hemihysterectomised to reduce the number of feto-placental units from 14 to five, the DNA content of the mammary glands was reduced by 45% in late gestation. After parturition, mice were given extra pups or suckled their own reduced-size litters and compared to control mice. Mice that were hemihysterectomised and suckled their own reduced-size litters had small mammary glands that contained only 65% of the DNA of glands of control mice. The mice that were hemihysterectomised and suckled large litters experienced such extensive mammary growth that the DNA content of their glands did not differ to control mice by D5 of lactation (Knight and Peaker, 1982e).

The above studies, and many others in the literature, clearly show a strong relationship between litter size and rodent mammary gland size. This relationship is based on ratios of ≤1 pup per gland. The few studies in which the pup/gland ratio does exceed 1 do not clarify whether the increased suckling stimuli can cause further increases in mammary growth and output. Litters of 18 pups caused increased milk production in mice in early lactation but in mid and late lactation, production was less than for mice suckling only 10 pups (Knight *et al.*, 1986b). Litters of 16 pups also caused increased milk production in rats in early lactation. However, in mid and late lactation there was no difference in milk production compared to rats suckling 10 only pups (Fiorotto *et al.*, 1991). These results suggest that rodent mammary glands can respond to stimuli of >1 pup per gland in early lactation but not in mid and late lactation. However, further research is required to verify this conclusion.

The cellular mechanisms by which mammary growth occurs during lactation in response to increased suckling intensity are controlled by both the autocrine and endocrine factors. Frequent and complete removal of milk from glands removes local factors such as FIL so that milk synthesis increases (Wilde and Peaker, 1990) and cells become more sensitive to systemic hormones such as Prl (Wilde *et al.*, 1990). Prolactin, released in proportion to the suckling stimulus (Grosvenor and Mena, 1974), interacts with receptors on the surface of cells to activate the JAK/STAT signal transduction pathway that can result in cell proliferation, cell differentiation and milk synthesis (Bole-Feyston *et al.*, 1998).

1.3.2 Exogenous hormones

The importance of hormones in mammary development and output has long been recognised (Topper and Freeman, 1980). This has led to numerous studies in which hormone concentrations are manipulated in an effort to alter mammary development and output (Bauman *et al.*, 1985; Fulkerson and McDowell, 1974).

Growth hormone administered to lactating cows increases milk yield by up to 40% (Bauman *et al.*, 1985) and improved lactation persistency (Burton *et al.*, 1994). Prolactin, which is in the same super family as growth hormone, fails to increase

milk yield when given to ruminants during lactation (Plaut *et al.*, 1987). In contrast to this, Prl is more important than growth hormone for the maintenance of lactation in rodents. Suppression of Prl release by twice daily injections of bromocriptine will decrease milk production to 45% (Knight *et al.*, 1986a) whereas reduction of effective GH concentrations with a specific antibody to GH reduces milk yield at peak lactation by only 20% in rats (Flint *et al.*, 1992). Prolactin can completely restore milk production in bromocriptine and antibody treated rats whereas GH is only partially effective (Flint *et al.*, 1992). The mode of action of these two hormones is probably different, although they may trigger some of the same parts of the JAK/STAT signal transduction pathway. While Prl receptors are found on mammary epithelial cells, none have been found for GH. It is thought that GH acts on stromal cells to release insulin-like growth factor type 1 (IGF-1), which then stimulates increased activity by the epithelial cells (Ruan *et al.*, 1992).

Exogenous E has been used successfully to increase milk yield although results are extremely variable and seem associated with stage of lactation and dose. Ewes are unresponsive to a low dose of E in early lactation yet the same dose increases milk yield in late lactation (Fulkerson and McDowell, 1974). In contrast, a 10 fold increase in the dose of E causes a severe decline in milk yield (Fulkerson and McDowell, 1974). The results from studies with mice have also proved to be extremely variable. In late lactation, a low dose of E and P caused litter growth rates and mammary cell proliferation to increase yet the response was reversed in early lactation (Knight and Peaker, 1982d). In contrast to the variable studies of Knight and Peaker (1982d), Shyyamala and Ferenczy (1982) reported that mammary glands of mice did not respond to either a low or high dose of E.

1.3.3 Age and weight

Lactational performance increases with age of mice at parturition. Bateman (1957) studied lactating mice that were 11 to 22 weeks of age. As mice are still growing at 11 weeks, he investigated whether it was actually age or body weight that affected lactation performance. He concluded that body weight increases with age up until maturity is reached and therefore any effect of age on lactation performance is

indirect. Lactation performance increases in older mice as a result of their greater body weight (Bateman, 1957). Gaines *et al.* (1942) reached a similar conclusion for dairy cattle.

The body weight differences across species also accounts for the differences in milk output and mammary gland sizes. Linzell (1972) showed that milk yield per unit body weight was linear across species ranging in size from mice to cows when plotted on a log log graph. A similar relationship exists for milk yield per unit of mammary gland weight. These relationships describe the observation that small animals, such as mice, have larger mammary glands and yield more milk per unit body weight when compared to larger animals such as cows (Linzell, 1972).

1.3.4 Compensatory growth

Compensatory growth is the term used to describe two types of growth of mammary glands. The first is the growth observed in response to the removal of a portion of the organ. This type of compensatory growth also occurs in tissue such as the liver and kidney and is thought to be stimulated by locally produced chalone molecules (Bucher and Malt, 1971). The second type of growth is that observed in mammary glands contralateral to sealed glands from which secretion cannot be removed. This type of growth is linked to suckling intensity (Auldist *et al.*, 2000) and is relevant to the topic of the current thesis.

Mammals raising litters demonstrate the second type of compensatory growth during lactation in response to teat ligation. Sows that suckled 12 piglets per 12 glands were compared to sows suckling 12 piglets (in two litters) from six glands. The latter group suckled more frequently and after 3 weeks of treatment, weights of individual mammary glands were 90% heavier than those suckling 12 piglets from 12 glands (Auldist *et al.*, 2000). In rats, the DNA content of glands contralateral to ligated teats increased with litter size and suckling stimulus (Tucker, 1964; Moon, 1965b). The decrease in DNA content of ligated teats was compensated for by an increase in the DNA content of contralateral non-ligated glands of rats (Moon, 1965b).

The mechanism for this type of growth is likely linked to suckling intensity and its effect on the autocrine and endocrine control of mammary glands (Wilde *et al.*, 1990). Increased milk removal from available glands would remove local factors such as FIL so that milk synthesis can increase (Wilde and Peaker, 1990) and cells are more sensitive to systemic hormones such as Prl (Wilde *et al.*, 1990). Prolactin, released in proportion to the suckling stimulus (Grosvenor and Mena, 1974), interacts with receptors on the surface of cells to activate the JAK/STAT signal transduction pathway that can result in cell proliferation, cell differentiation and milk synthesis (Bole-Feyston *et al.*, 1998).

1.3.5 Reduced apoptosis

Cell number is a function of cell proliferation and cell longevity. The latter is determined by when death occurs and therefore factors that control the timing and how cells die. Altering either the rate of proliferation or time death can result in a net increase in the number of cells within the mammary gland. It follows, therefore, that if cell proliferation remains constant and cell longevity is increased, there will be a net increase in the cell population (Knight and Peaker, 1984). Such an increase in cell population could realise not only a growth of the lactating mammary gland but also an increased milk production, (assuming the cells retained the capacity to synthesise and secrete milk). Decreasing cell death is hence one method of causing growth of lactating mammary glands.

Cell death during lactation occurs mostly in the form of apoptosis. Apoptosis is defined as programmed cell death (Kerr *et al.*, 1972). Apoptotic cells exhibit distinctive nuclear and cytoplasmic changes, where the nucleus condenses and becomes pyknotic, the nuclear envelope blebs and the chromatin breaks into nucleosomes (Kerr *et al.*, 1972). Eventually there is membrane blebbing and the engulfment of the apoptotic cells fragments by macrophages (Kerr *et al.*, 1972). The engulfment of apoptotic cells means no inflammatory response is caused. In contrast, necrotic cell death results in local inflammation owing to the lysis of the cell and release of intracellular contents that are potentially toxic and immunogenic (Savill, 1997). Methods used to detect apoptotic cells are based on the detection of

fragmented chromatin. DNA extracts resolved by electrophoresis result in 'ladders' of oligonucleosomal DNA fragments (Wilde *et al.*, 1997). Nicked or broken DNA can be detected *in situ* in tissue sections by identifying terminal deoxynucleotide end labelling (TUNEL) of fragmented DNA (Quarrie *et al.*, 1995).

Much study of apoptosis has been done on involuting mammary glands. Involution can be initiated by the removal of suckling young or in dairy animals by the cessation of milking. Involution occurs in two phases in rodents after litters are removed. Firstly, there is an accumulation of milk in the alveolar lumen and some limited apoptosis (Walker *et al.*, 1989). Suckling will reverse the process up to 1.5 days after weaning but the increasing levels of apoptosis make involution irreversible by d 4. This marks the second stage of involution. It is morphologically characterised by the degradation of the basement membrane, the collapse of alveoli and infiltration of macrophages (Marti *et al.*, 1999), and peak intensity of DNA laddering (Quarrie *et al.*, 1995). Apoptosis occurs throughout the gland until 50 to 80% of all the epithelial cells have been cleared and the gland is restructured to resemble a virgin like state (Walker *et al.*, 1989) with additional carry-over of undifferentiated epithelial cells. The remodelling of bovine mammary glands between lactations involves only modest loss of mammary epithelial cells (Hurley, 1989). The loss that does occur is accompanied by TUNEL-positive cells and DNA laddering, which indicates that bovine mammary cells die by apoptosis during involution (Stefano *et al.*, 2002).

Gene expression changes during these two stages of involution. During the first stage there is a down regulation of mRNA coding for milk protein genes (Stefano *et al.*, 2002). Other genes that are up regulated during the first stage include caspase-1 and bax, which are putative effector genes for apoptosis, which have maximum expression at d 3 (Boudreau *et al.*, 1995; Heermeier *et al.*, 1996). The second phase of involution is characterised by a change in ratio of extracellular proteinase inhibitors to proteinases (Heermeier *et al.*, 1996). The products of these changes contribute to the destruction of the extracellular matrix components and therefore implement the remodelling process.

More recently, apoptosis has been found to be the cause of cell loss during lactation. Tissue taken from lactating mice has revealed DNA laddering and TUNEL positive cells and therefore the presence of apoptotic cells (Wilde *et al.*, 1999). In lactating cows, approximately 2% of epithelial cells were apoptotic as determined by the TUNEL technique (Wilde *et al.*, 1997). Similarly, nearly 1% of cells were found to be dying in the lactating udders of goats (Li *et al.*, 1999). These percentages may seem negligible but when multiplied by cell population and time, it becomes obvious that a large number of cells die during lactation. Retaining these cells could conceivably result in larger mammary glands and greater milk output.

Apoptosis during lactation is caused by a number of factors. These include nutrition and frequency of milk removal (Stefano *et al.*, 2002). These are well described by Stefano *et al.* (2002) but are briefly described below.

Nutrition influences mammary development during pregnancy and lactation. It is also thought to influence apoptosis during lactation although there are no conclusive reports of varied diets and changes in apoptosis. Studies in rodents suggest a superior dietary regime given at the start of lactation can cause an increase in cell proliferation in early lactation and a decrease in apoptosis in late lactation (Moon and Park, 1999). Studies in ruminants suggest a more complex relationship as high planes of nutrition caused an increase in both proliferation and apoptosis (trend only) in the udders of heifers in early lactation (Sorensen *et al.*, 2000). Although much work needs to be done, it is logical that poor diets incapable of maintaining milk production will ultimately result in a down sized epithelial cell population due to increased apoptosis. Reduced apoptosis in lactating mammary glands should require good nutrition, in addition to other factors.

Frequency of milk removal affects alveolar distension and eventually apoptosis. If milk is not removed frequently and efficiently then alveolar units will accumulate milk. Within this milk is a small whey protein, called feedback inhibitor of lactation (FIL), that acts on secretory cells to decrease milk synthesis (Wilde *et al.*, 1995). FIL also exerts autocrine control of cells to decrease sensitivity to endocrine hormones such as prolactin (unpublished studies by C. J. Wilde and K. O'Reilly cited by Wilde and Peaker, 1990). Prolactin is known to control cell survival in

rodents by repressing IGFBP-5 (Tonner *et al.*, 1997) and influence mammary gene expression in both rodents and ruminants (Stefano *et al.*, 2002). A study of goats milked infrequently in one gland show that after 4 weeks of treatment, there is increased DNA laddering compared to the thrice daily milked gland (Li *et al.*, 1999). Apoptosis is sensitive to milking frequency. An approach to decreased apoptosis is to ensure milking frequency and efficiency is sufficient to keep the concentration of FIL low.

The discussion above highlights that cell population is a function of new cells, cell lifespan and removal of cells. Cell population can be increased by proliferation of new cells. It can also be increased by minimising the number of cells removed by apoptosis.

1.4 Purpose and scope of this project

The strategic manipulation of mammary glands to increase the growth of new epithelial cells during lactation could benefit many agricultural industries that are based on milk production, ranging from dairying to production of weaner piglets. A larger population of secretory epithelial cells could increase milk production while the young age of the cells could increase the persistency of lactation and improve milk quality.

A study of increased mitosis of epithelial cells in lactating glands requires mammary tissue that can be produced experimentally. It is not obvious how to obtain such tissue as there are many contrasting reports in the literature. In order to experimentally produce mammary tissue with high levels of mitosis it was necessary to complete conclusive studies in one species of animal. The mouse was chosen due to its high milk production per unit bodyweight as well as other attractive features such as short gestation and lactation periods and small size.

The objective of the research reported in this thesis was to increase the proliferation of mammary epithelial cells to cause growth of murine mammary glands during lactation.

Chapter 2 Materials and Methods

2.1 Animals

2.1.1 Ethics

All work using experimental animals was conducted with approval from the Ruakura Animal Ethics Committee (Ruakura Research Centre, Hamilton, New Zealand).

2.1.2 Management

The studies described in this thesis utilised Swiss mice housed at the Ruakura Small Animal Colony (AgResearch, Hamilton, New Zealand). All mice had *ad libitum* access to water and a commercial, non-purified, pelleted diet (Alister Osborne, Sharps, Carterton, New Zealand) that contained 17% protein, 5.2% fat, 4% fibre and all essential vitamins and minerals. Mice were kept in an air conditioned room where air temperature ranged from 21 $\pm$ 1.5 °C with a regimen of 12h light followed by 12h dark. All mice were housed in clear Perspex cages containing wood shavings. Pregnant mice were placed in their own cages approximately 3 days prior to parturition.

Nulliparous female mice were mated at 7-10 weeks of age. Mice averaged 19 days of gestation and parturition usually occurred overnight. If new pups were noticed by *circa* 0900h the next day, that day was designated D0 of lactation.

2.1.3 Fostering of pups

On the day of parturition (D0 of lactation) the litter was weighed, the pups were counted and their sex determined. Cross fostering pups of similar ages ($\pm$1 day of age) was performed to establish the required litter sizes. The pups in each litter were manipulated so that both sexes were represented as equally as possible.

2.1.4 Measuring litter growth rate, dam weight gain and fasted weight losses of litters

Litter growth rate was measured by weighing the litters at *circa* 0900h each day and then calculating the average increase in body weight (g) during one day. Dam weight gain was also monitored by weighing the dams at *circa* 0900h each day.

A requirement for the estimation of milk yield during lactation (Chapter 3) was to measure the weight loss of fasted litters. This was done by weighing the litters at *circa* 0900h and recording the time. The dam was then placed in a separate cage for approximately 5h. At *circa* 1400h, the litter and dam were each weighed to the closest 10 mg and reunited. The time of this weighing was recorded, and the elapsed time of separation calculated. This was repeated for each litter at each of D3, 10 and 17. The weight loss of litters over the measured period was used to calculate a weight loss over 24h.

2.1.5 Taping over of teats

Teats were taped over to make them unavailable to litters. A circular piece of tape (diameter = 5 mm) cut from Elastoplast tape (Beiersdorf, Germany), with a small dab of Locktite glue (Locktite Australia, Caringbah, NSW) applied to the adhesive side, was placed over each teat. Taping of teats required one person to hold each mouse with its abdomen upwards, while another person applied tape over each teat and held it in position for 3 sec after which it had adhered firmly to the fur and teat of the mouse. Mice were inspected daily to ensure teats were still covered and any missing tapes were replaced.

2.1.6 Materials

Materials and reagents used are listed in this section under the manufacturer they were sourced from. All chemicals were of analytical grade.

BDH Laboratory Supplies (Poole, England) were the manufacturers and suppliers of acetone, copper sulphate ($CuSO_4$), crystal violet, di-potassium hydrogen orthophosphate anhydrous (K_2HPO_4), disodium hydrogen orthophophate (Na_2HPO_4), DPX mountant, EDTA ($CH_2N(CH_2COOH)_2)_2$, eosin counter stain, ethanol (C_2H_5OH), Folin and Ciocalteu's reagent, formalin (HCHO), glacial acetic acid (CH_3COOH), haemotoxylin counter stain, magnesium sulphate ($MgSO_4$), hydrogen peroxide (H_2O_2), methanol (CH_3OH), methylamine, NaK-tartrate, nickel sulphate ($NiSO_4$), perchloric acid ($HClO_4$), potassium dihydrogen orthophosphate (KH_2PO_4), potassium hydroxide (KOH), sodium carbonate anhydrous (Na_2CO_3), sodium dihydrogen orthophosphate (NaH_2PO_4), trichloroacetic acid (CCl_3COOH), trisodium citrate ($Na_3C_6H_5O_7.2H_2O$), Tween and xylene ($C_6H_4(CH_3)_2$).

Ajax Chemicals Pty., Ltd. (Auburn, Australia) manufactured the lactose. American National Can (Chicago, Illinois, U.S.A.) manufactured parafilm. Amersham Pharmacia (Sweden) was the supplier of [^{3}H] thymidine, anti-BrdU monoclonal antibody, biotinylated anti-mouse IgG2a from ovine, BrdU, cell proliferation kit (RPN20), NCS and streptavidin biotinylated horse radish peroxidase complex. Biolab Scientific Ltd., (Auckland, NZ) was the supplier of ESCO coverslips and Kimble tubes (borosilicate 13 x 100 mm tubes). Boehringer Mannheim (Germany) was the supplier of epidermal growth factor, galactose, NAD, β-galactose dehydrogenase and β-galactosidase. Daido Sangyo Company Ltd. (Japan) was the manufacturer of Pap pens. Dulbecco (Hampshire, England) manufactured the PBS tablets. Eastman Kodak Company (New York, U.S.A.) manufactured autoradiography developer. Feather Safe Razor Company Ltd. (Japan) manufactured the blades used for cutting histological sections. Gibco BRL Life Technologies Inc. (New York, U.S.A.) was the supplier of fetal calf serum (FCS), M199 media and RPMI media. Hawksley (London) was the manufacturer of glass micro-haematocrit tubes. Ilford Ltd., (Cheshire, England) manufactured the autoradiography emulsion gel and fixer. Intervet (Chemavet Division Pharmaco (NZ) Ltd., Auckland) was the manufacturer of oxytocin. Janssen Animal Health (Buckinghamshire, U.K.) was the manufacturer of Hypnorm. Lockertex (Warringotn, U.K.) was the manufacturer of nylon mesh (50, 100 μm). Nunc (Denmark) was the supplier of 96 well micro titre plates, cell culture chambers, cryovials and petri dishes. Research Organics (Cleveland, Ohio, U.S.A.) manufactured MOPS buffer. Roche Products Ltd.

(Auckland, New Zealand) was the manufacturer of Hypnovel and Dnase. Shanghai Machinery Import and Export Company, (China) manufactured the glass histology slides. Sigma Chemical Company (St Louis, U.S.A.) was the supplier of bovine serum albumin (BSA), β-estradiol 3-benzoate, calf thymus DNA standard, collagenase type V, DAB, glucose, Hoeschst dye (H33258 bisbenzimide), hyaluronidase type I-S from bovine testes, insulin, penicillin, PCNA, poly-L-lysine, sodium bicarbonate, streptomycin and trypsin. United States Biochemical Corporation (Cleveland, Ohio) manufactured the 4-pregnen-3 20 dione. Wallac (Milton Keynes, U.K.) manufactured the Optiphase 'HiSafe' scintillant.

2.1.7 *In vivo* labelling with [^{3}H] thymidine or BrdU

Unless otherwise stated, lactating mice were injected intra-peritoneally with 2 μCi of [^{3}H] thymidine (1mCi/ml) per g of live weight or 20 μl of undiluted aqueous 5-bromo-2'-deoxyuridine (BrdU) containing 5-fluoro-2'-deoxyuridine (FdU) per g of live weight. Mice were then separated from their litters for 1h unless otherwise stated. During the 1h of incubation, up to five mice were housed together in a cage and had *ad libitum* access to food and water. At the end of the incubation time, mice were euthanased by cervical dislocation.

2.1.8 Collection of mammary glands

After death, mice were pinned to a dissecting board and, starting at the abdomen, the skin was cut from the mouth to the vulva. The skin was then peeled from the body to expose the mammary glands. Glands were dissected, blotted free of blood and milk, and weighed to four decimal places. The mammary glands were either snap frozen in cryovials immersed in liquid nitrogen, or put into formalin solution for later histology (Section 2.4.1). Alternatively tissue was digested to single cells for later analysis on the flow cytometer (Section 2.7.2).

Swiss mice have five pairs of mammary glands; three pairs in the thoracic region and two pairs in the abdominal region. For the work reported in this thesis, glands were

identified by their position and side of the body, for instance, the description of a gland being "4th left" meant it was the fourth gland from the head and on the left side of the mouse (Figure 2.1).

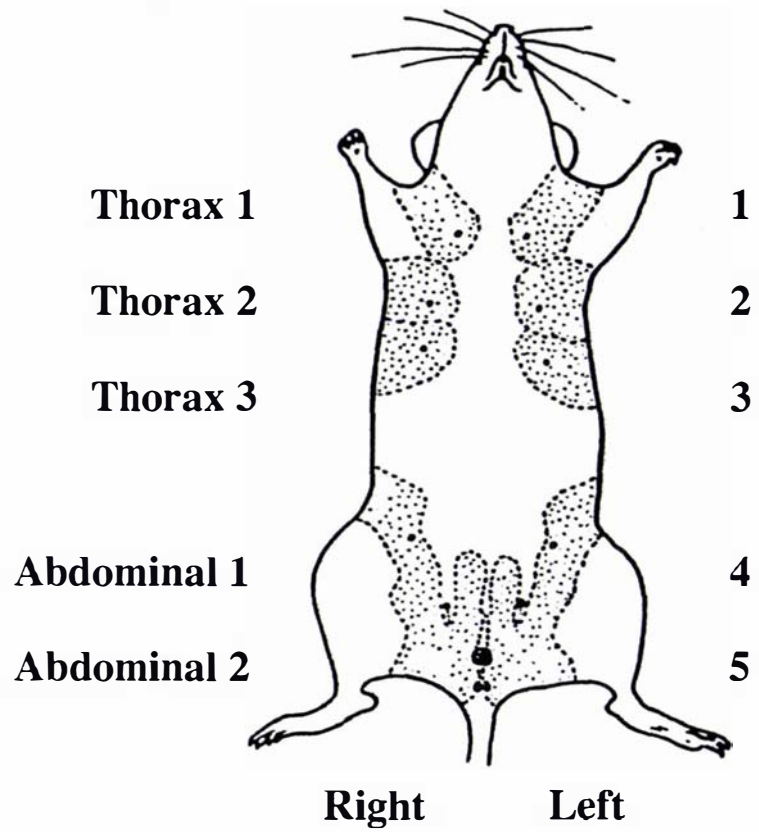


Figure 2.1: Position and labelling of mammary glands of mice.

The large black dots represent the nipples and the stippled areas represent the mammary glands. Diagram from *Biology of the Laboratory Mouse* by Green *et al.* (1966).

2.2 Milk collection and analysis of milk samples

2.2.1 Milk collection

Mice were milked on D3 (early), D10 (mid) and D17 (late) of lactation. Dams were separated from their litters for a timed period ranging from 3-4h. Care was taken to ensure the separation period was not longer than 4h as previous studies reported a

change in milk fat content after longer periods of separation (Keen *et al.*, 1980; Grigor and Gain, 1983). Dams were injected intra-peritoneally with 0.2 ml of anaesthetic. The anaesthetic was made up of 1 ml Hypnovel (5 mg/ml midazolam hydrochloride), 2.5 ml Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone) and 6.5 ml of sterile Milli Q water. After 10 min, the anaesthetised mice were injected intra-peritoneally with 0.15 I.U. of synthetic oxytocin and milked immediately.

Milk was extracted manually from the four inguinal glands by gently massaging the mammary tissue from the base to the nipple using the thumb and forefinger (Ragueneau, 1987). The droplets of expressed milk were collected in a micro-haematocrit tube (75 mm length x 1.5 mm outside diameter). Micro-haematocrit tubes were emptied into eppendorf tubes, which were kept on ice. The milking operation took 10 min per mouse and yielded 50–100 μl of milk from each mouse. Mice recovered on a plate warmed to 37°C and were reunited with their litters approximately 90 min after receiving the anaesthetic injection.

2.2.2 Content of fat in mouse milk

Milk fat content of mouse milk was estimated by first measuring the cream content. Cream content of milk was measured by centrifugation in a standard glass micro-haematocrit tube (75 mm length x 1.5 mm outside diameter). This method has been used to analyse small samples of milk from ruminants, rodents and monogastrics by Fleet and Linzell (1964) and milk from humans by Lucas *et al.* (1978). Approximately 50–75 μl of well-mixed milk was drawn by capillarity into the micro-haematocrit tube. Tubes were sealed at one end by plugging with plasticine and then centrifuged for 10 min at 10,000 *g*. The cream layer was measured to the nearest 0.5 mm and expressed as a percentage of the total length of milk in the tube height. The percentage of cream in the mouse milk was multiplied by 0.868 to give an estimate of the fat concentration in the sample as cream in rodent milk is linearly related to the amount of fat in the milk such that milk fat is 0.868 of milk cream (Knight *et al.*, 1986b).

2.2.3 Content of protein in mouse milk

After milking, an aliquot of the milk from each mouse was diluted 1 in 50 in Milli Q water and stored at –80°C for subsequent analysis of protein content. Total protein concentration of individual mouse milk samples was determined using a method modified from Lowry *et al.* (1951). Stock solutions of 2% Na_2CO_3 and 0.02% NaK-tartrate in 0.1M NaOH (Reagent A) and 0.5% $CuSO_4$ (Reagent B) were mixed 50:1 to make Reagent C. A standard curve was obtained using bovine serum albumin (BSA) solutions ranging in concentration from 0.2 to 2.0 mg/ml. In addition, a quality control sample of bovine skim milk of known protein concentration (30 mg/ml as determined by Kjeldahl digestion) was added to each assay. For the assay, the mouse milk was diluted again 1:2 with Milli Q water to a final dilution of 1:100. The diluted sample (50 µl) was mixed with 50 µl of Folin and Ciocalteu's reagent and 500 µl of Reagent C (made immediately before use) was added to the sample and mixed well. A 200 µl aliquot of this solution was added to a microplate in duplicate wells for each sample. Standards and controls were treated in the same manner. The assay was covered with another microtitre plate, sealed with parafilm and incubated for 60 min at 55°C. Absorbance was measured at 595 nm. Samples with a coefficient of variation of greater than 5% were repeated.

2.2.4 Content of lactose in mouse milk

An enzymatic method was used to measure lactose content in the milk samples (Kulski and Buehring, 1982). The principle of the reaction is the hydrolysis of lactose into glucose and β-galactose in the presence of the enzyme β-galactosidase and water (see a below). The galactose is then oxidised by NAD to galactonic acid in the presence of the enzyme β-galactose dehydrogenase (see b below). The reaction can be summarised as follows:

(a) lactose + H_2O $\xrightarrow{\text{β-galactosidase}}$ glucose + β-galactose

(b) β-galactose + NAD^+ $\xrightarrow{\text{β-galactose dehydrogenase}}$ galactonic acid + NADH + H^+

The formation of NADH is measured by an increase in absorbance at a wavelength of 340 nm, which is proportional to the quantity of lactose. Galactose was assayed as described for lactose except that β-galactosidase was omitted from the reaction mixture. Samples with a coefficient of variation of greater than 5% were repeated.

A 50 μl aliquot of the milk from each mouse was diluted 1 in 50 in 0.5M 3-N-morpholino propanesulfonic acids (MOPS) buffer and stored at –80°C for subsequent analysis of lactose. The diluted milk was further diluted 15 fold with MOPS buffer and a 500 μl aliquot was deproteinised with 1000 μl 1M perchloric acid. After centrifugation at 4°C for 10 min at 2700*g*, 900 μl of the supernatant was neutralised by addition of 300 μl of a KOH/potassium phosphate buffer (0.6M KOH, 0.15M K_2HPO_4, 0.1M KH_2PO_4 at pH 13.6) and centrifuged again at 4°C for 10 min at 2700 *g*. Either 150 μl aliquot of supernatant or standard solutions of lactose covering the range of 0 – 400 μM lactose was added to a 96-well microtitre plate in triplicate. To measure free galactose in the samples, 100 μl of galactose reagent was added to one replicate. Galactose reagent consisted of 1 ml of 0.5M MOPS buffer, 0.5 mg of NAD, 10 μl of 1 M $MgSO_4$ and 1 I.U. β-galactose dehydrogenase. The lactose reagent (100 μl) was added to each of the remaining two replicate wells. Lactose reagent was made by addition of 15 μl β-galactosidase (1500 I.U./ml) to 1 ml galactose reagent. The microtitre plate was covered, sealed with parafilm and left in the dark for 30 min prior to measuring the reduction of NAD to NADH fluorometrically at 340 nm. Samples with a CV of greater than 5% were repeated. The optical density of the galactose well was subtracted from the average of the duplicate lactose wells to give lactose content of the sample in μM. Multiplication by a dilution factor of 750 then yielded the content of lactose in the mouse milk samples.

2.3 Analysis of tissue samples

2.3.1 Homogenisation

Mammary glands were removed from cryovials and chopped on a petri dish into 1 mm^3 pieces. A volume of phosphate buffered saline (PBS) equivalent to four times

the weight of each gland was added to the mammary tissue which was then homogenised in 5 ml plastic vials for 60 sec using an Ultra turrex homogeniser (IKA, Janke and Kunkel, Germany). Aliquots of the homogenate were retained for subsequent analysis of total protein, lactose, DNA and incorporation of [^{3}H] thymidine.

2.3.2 Protein content of mammary tissue

The protein content in tissue samples was estimated using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, Illinois, U.S.A.). The principle of the reaction is the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (see a below) and the highly sensitive colourimetric detection of the cuprous cation using a reagent containing bicinchoninic acid (see b below).

(a) protein (peptide bonds) + Cu^{2+} $\xrightarrow{OH^-}$ tetradentate-Cu^{+} complex

(b) Cu^{+} + 2 BCA ⟶ BCA-Cu^{+} complex (purple colour).

A BSA standard was provided in the kit to make a range of standard concentrations between 0.2 and 2.0 mg/ml. A bovine skim milk sample of known protein content was used as a quality control. For assay, mammary tissue homogenates were thawed and diluted in PBS to give a final dilution of 1 in 90. Either a 10 µl aliquot of this sample, BSA standard or control was pipetted into a microtitre plate and 200 µl of BCA reagent added. Duplicate wells were run for each sample, standard and control. The 96-well plate was covered, incubated at 37°C for 40 min and absorbance measured at 595 nm. Samples with a coefficient of variation of greater than 5% were repeated.

2.3.3 Content of lactose in mammary tissue

The lactose content of homogenised mammary glands was measured using the enzymatic lactose assay to measure the lactose content of milk, except that the homogenised mammary tissue samples were less dilute. Aliquots of the

homogenised glands (which were at a 1 in 5 dilution) were diluted a further 1 in 10 in 0.5M MOPS buffer. An aliquot of 500 µl was then deproteinised and measured for content of lactose as described in Section 2.2.4. Lactose concentration of the aliquot was converted to total lactose in the gland, and then knowing the lactose content of milk at different stages of lactation, mammary gland weight was corrected for milk content.

2.3.4 Content of DNA in mammary tissue

Total DNA was measured by fluorometric assay using Hoechst dye (H33258; Sigma) following the method of Labarca and Paigen (1980) with some modifications (T. B. McFadden, unpublished). Initially, tissues were homogenised for 60 sec in a volume of PBS four times that of the gland weight, to give a 1 in 5 dilution. The homogenate was then centrifuged at 200 *g* for 10 min at 4°C. The supernatant under the fat layer was collected and an aliquot added to an equal volume of 2xPBSE (a high salt phosphate buffer containing EDTA, 0.017M $NaH_2PO_4.2H_2O$, 0.07M $Na_2HPO_4.2H_2O$, 3.86M NaCl, 0.004M EDTA) which was then stored at –80°C until analysed. After thawing, the sample was diluted to a final dilution of 1 in 400, 200 µl was added to glass cuvettes (Kimble tubes) together with 1.5 ml of PBSE containing Hoescht dye and the samples left for a minimum of 2 h (maximum 16 h) at room temperature (RT) in darkness. Calf thymus DNA (100 µg/ml) was used as a standard. Absorbance was measured on a fluorimeter (Perkin Elmer Luminescence Spectrometer, LS50), standard curves were constructed to determine the DNA content of the homogenate and then the DNA content of the mammary tissue was calculated using the appropriate dilution factors.

Later the method was improved by omitting centrifugation and including sonication (Section 2.6). Homogenates were sonicated for 30 sec before being stored at -80°C. In addition, the method was adapted so that a 96 well micro plate could be used. On the day of analysis the samples were thawed, diluted to 1 in 200, 50 µl of either sample or standard added in duplicate to a microtitre plate and 250 µl of the Hoechst dye was added to each well. The plate was covered and left for 2h in darkness and then absorbance measured on the fluorescent plate reader (Biotek SL500) at an

excitation wavelength of 360 nm and an emission wavelength of 460 nm. Standard curves were included and the DNA content of mammary tissue determined after accounting for dilution factors.

2.3.5 Incorporation of [^{3}H] thymidine into DNA

DNA of mice was labelled *in vivo* (Section 2.1.6) and then mammary glands were collected (Section 2.1.7) and homogenised (Section 2.3.1). To precipitate the DNA, 1.5 ml of cold 10% TCA was added to 40 and 80 µl aliquots of mammary homogenates and kept at 4°C for 15 min. The tubes were centrifuged at 1720 *g* for 15 min at 4°C, the supernatant removed and the tubes left inverted for complete draining. Scintillant was added (1.3 ml), the tubes gently mixed and left in the dark overnight. Radioactivity was quantified on a beta counter (Wallac 1409, Wallac, Milton Keynes, UK). In later experiments NCS was added to more rapidly dissolve the pellet prior to the addition of scintillant.

2.4 Histology

2.4.1 Preparation of whole mammary gland tissue sections

Mammary glands from mice were dissected, blotted free of blood and milk, weighed and spread on a glass slide. The whole gland mounts were fixed by immersion in PBSF (4% formalin in PBS) for 24 h after which they were immersed in 70% ethanol. The glands were taken through a series of graded alcohol baths and then embedded in paraffin wax. A microtome (Microtome 2050 Supercut, Richert–Jung, Cambridge Instruments GmbH, Nussloch) and fine stainless blades were used to cut 4 µm sections parallel to the abdominal surface of the mammary gland. Placing the wax embedded tissue on ice helped achieve high quality sections. The sections were floated on water heated to between 40 and 50°C and picked up on poly-L-lysine (0.01% w/v poly-L-lysine solution) coated glass slides. Slides were dried on racks

over a warm element overnight and were then stored until required for histological staining.

2.4.2 Autoradiography

Mammary gland sections were dewaxed in 2x 5–10 min baths of xylene and then hydrated by immersion for 2-5 min each in baths of decreasing alcohol content (two baths of absolute ethanol, 90% ethanol, 70% ethanol, 50% ethanol) and then left in water. In a dark room, 150 µl of melted Ilford Nuclear Research Emulsion gel was applied to the top of each slide and gently spread over the entire surface using the edge of the pipette tip. Coated slides were left standing in a rack to drain as much emulsion as possible so that only a thin layer of approximately 10 µm thickness remained on the slides. Slides were left to drain overnight in a dark box containing silica crystals. The next day they were transferred to a small dark slide box containing silica crystals in the base. The box was sealed, protected from light and stored for exposure at 5°C for 21 days. Exposure time was determined by development of test slides after 7, 14, 21 and 28 days of exposure. Prior to developing of the slides, the boxes were removed from the cold room and allowed three hours to equilibrate to room temperature to prevent condensation forming on the slide surfaces. The developer, stop solution (0.1% v/v glacial acetic acid) and fixer were used fresh each time after being equilibrated to 18-20°C. Under a red safelight filter in the dark room, slides were gently agitated in the developer for 5 min, followed by the stop solution for 30 sec and finally the fixer for 5 min. Slides were left to wash for 2h under gently running water.

2.4.3 Labelling with anti BrdU antibody

All reagents used for the immunohistochemistry described below were provided in the cell proliferation kit purchased from Amersham Pharmacia. Using a PAP pen, a line was drawn around each section to assist blebbing of solutions. Each incubation was conducted in a humidified chamber at RT and slides were carefully dried around the tissue section before each incubation step. The primary antibody (anti-BrdU

monoclonal antibody, subclass IgG_{2a} of murine origin) in tris buffered saline containing BSA, magnesium chloride and nuclease was added to the sections of the mammary glands. The slides were incubated for a minimum of 1h after which they were washed six times in PBS containing 0.05% Tween for a minimum of 3 min each. The secondary antibody (peroxidase anti-mouse IgG_{2a} of caprine origin) in PBS containing BSA was added and the slides incubated for 30 min. Following six washes in PBS containing Tween, slides were immersed in 3,3'-diaminobenzidine tetrahydrochloride (DAB) in a phosphate buffer with heavy metal intensifier containing nickel chloride and H_2O_2 for 2 min. Slides were washed three times in Milli Q water for 3 min each. The PAP pen was removed carefully using xylene and the section was then counter-stained with eosin. Sections were dehydrated through increasing concentrations of alcohol (70% ethanol, 90% ethanol, two washes of absolute ethanol) for 2–5 min in each bath and cleared in xylene for 5 min. DPX mountant was added and a cover slip mounted.

2.4.4 Labelling with anti PCNA antibody

The PCNA primary antibody was diluted to 1 in 50 in PBS and left on the sections of mammary gland overnight in a humidified chamber at 4°C. PBS washes (3x 5min each) were followed by the secondary antibody (biotinylated antimouse Ig from ovine) at a 1 in 500 dilution and left to incubate at RT in the humidified chamber for 30 min. Sections were then washed with PBS (3x 5min each), streptavidin biotin horse radish peroxidase complex was added at 1 in 400 dilution and left at RT in a humidified chamber for 30 min. Sections were then washed with PBS (3x 5min each) and immersed in the DAB reagent (712 µl 0.2 M Pi, 2137 µl MQ, 150 µl DAB 10mg/ml) and H_2O_2 30% 7.5 µl and NiCo 7.5 µl for 5 min. The reaction was stopped by washing with Milli Q. The PAP was removed with xylene, and the sections were counterstained with eosin. Finally, the sections were dehydrated through alcohol baths, cleared in xylene and mounted with DPX and a coverslip as described in Section 2.4.3.

2.4.5 Counter staining

The counter-stains evaluated included alum carmine, methyl green, Loeffler's methyl blue, haemotoxylin and eosin, and nuclear fast red. Haemotoxylin and eosin gave the best results and were used for the majority of counter-stained sections.

2.4.6 Microscopy, image analysis

Generally, stained mammary tissue sections were examined at a magnification of 100 to 400. An image analysis system was developed to assist with data collection to determine labelling indices. The image was focussed under a microscope and then onto a television monitor. Using image analysis software (Snappy, Play Inc.) the image was optimised (for colour and contrast) and captured (Snappy, Play Inc.) and stored on a computer zip disc. Labelled and unlabelled cells were counted using the computer mouse and an automatic counting system in the software used to calculate labelling index.

2.5 Validation of the anti-BrdU antibody labelling method

BrdU is a chemical alternative to radioactive products such as [^{3}H] thymidine that is incorporated into replicating DNA (Hyatt and Beebe, 1992). The BrdU labelling reagent also contained FdU at a ratio of 1:10 of FdU:BrdU. The FdU inhibits thymidilate synthetase and therefore increases BrdU incorporation by lowering competition with endogenous thymidine. The localisation of BrdU in cell nuclei was detected using a specific monoclonal antibody (Section 2.4.3).

2.5.1 Initial problems with anti-BrdU immuno staining of mammary gland sections

In the initial studies, mice were injected on D3, D10, D17 or D24 of lactation with 10 µl/g body weight BrdU labelling reagent. One hour after the injection mice were killed by cervical dislocation and the 4th mammary glands were dissected, fixed,

embedded and 4 μm sections cut. Dewaxed and hydrated sections had primary antibody added (anti-BrdU monoclonal antibody), and were incubated for 60 min before being washed three times with PBS (3 min each wash). The secondary antibody was added (biotinylated anti-mouse IgG_{2a} from ovine) incubated for 30 min and then washed in PBS (3x 3 min washes). Streptavadin biotinylated horse radish peroxidase complex was added to the sections and incubated for 15 min, and then DAB solution (200 μg DAB/ml, 50% hydrogen peroxide in PBS) was added with one drop of heavy metal intensifier for 3 min. Slides were then washed in PBS and tap water, counterstained with eosin for 5 sec, dehydrated, cleared in xylene and mounted with DPX and a coverslip. The positive control sections of BrdU labelled wool follicles were strongly stained while the negative controls and all the mouse mammary sections did not show any positive immuno staining at all.

There were four reasons that may have resulted in no immuno staining of the mouse mammary tissue sections. Incomplete dewaxing of the sections prior to staining was confidently eliminated as correct procedures had been followed. The successful staining of the positive control sections indicated that the DAB solution was effective and that the heavy metal intensifier was still in solution. It was possible, however, that the BrdU labelling reagent used to inject the mice was not active. Alternatively, the mouse mammary sections may have been overfixed in formalin and the cross links formed prevented the primary antibody from reaching its epitope. These two latter possibilities were investigated further.

2.5.2 Testing the reagent for effectiveness of BrdU labelling

To test the batch of BrdU for activity, cells were labelled with the BrdU reagent and then immuno stained for detection of incorporated BrdU. New BrdU labelling reagent was also used to compare results between batches. A mammary cell line of HC11 cells (passages 20–21) were propagated in growth media comprising RPMI with phenol red and supplemented with 44 mM sodium bicarbonate, 10% FCS fetal calf serum, epidermal growth factor, EGF (10 ng/ml), insulin (5 μg/ml), penicillin (100 I.U./ml, maker) and streptomycin (0.1 mg/ml, maker). The cultures were maintained at 37°C in a humidified 5% CO_2 : 95% air atmosphere.

The HC11 cells were harvested after treatment with 5 ml trypsin (0.25% trypsin) and 0.4% EDTA in PBS for 10 min. The cell suspension was centrifuged for 5 min at 80*g* and the supernatant removed. The pellet was resuspended in 8 ml of growth media warmed to 37°C. The concentration of live cells was determined by adding 100 µl of cell suspension to 900 µl of warmed crystal violet solution and counting the number of blue nuclei on a haemocytometer grid. Cells were plated into culture slides at $2x10^4$ cells per well. Each culture slide contained four wells formed by gaskets adhered to chambers. Growth medium containing BrdU labelling reagent (1 µl/ml) was added to each well and the cells were incubated for 4h. The medium was removed and the cells washed in PBS. Chambers were removed from each culture slide and the cells permitted to air dry in the laminar flow unit. The slides were then immersed in acetone for 60 sec and air dried again. The following day, the gaskets were removed and the cell proliferation kit was used to immuno stain the slides (Section 2.4.3) as well as a dewaxed and hydrated positive control wool follicle slide (kindly donated by Dr Alan Nixon).

The results showed the positive control (the wool follicle slide) had black nuclei at the base of the follicle indicating the immuno staining had worked. In contrast, the nuclei of the HC11 cells with the PBS added instead of the primary antibody were all pink indicating that no DAB staining had occurred. The nuclei of the HC11 cells where the primary antibody was added were a mix of both pink and brown in colour indicating that some cells had taken up the BrdU. Highest labelling indexes were for attached cells while the unattached, rounded cells mostly had pink nuclei indicating no mitosis had occurred. The incubation period of 4 h was very short and did not allow sufficient time for all cells to attach which is required before cells can become mitotic. The mix of attached, mitotic cells and unattached, non mitotic cells provided a good internal positive and negative control. Identical results occurred regardless of the batch of BrdU labelling reagent used in the growth media. The conclusion of the trial was that the original BrdU labelling reagent was incorporated into synthesised DNA.

2.5.3 Comparison of methods of fixation of sections

A potential cause of the failure to detect BrdU in the tissue sections was that the tissue was overfixed. In the initial experiment the tissues were fixed for three days rather than the specified 24h and consequently two trials were conducted to determine if overfixing of the tissue was responsible for the poor immuno staining of the sections. The first trial examined another antibody to see if there were also problems associated with reduced binding from extensive cross linking. In the second trial the over fixed tissues were heated in a microwave oven to re-expose the antigen.

2.5.3.1 Immunohistochemistry to detect PCNA

Serial sections of fixed tissue that had stained poorly for BrdU were tested for the presence of proliferating cell nuclear antigen (PCNA). This was based on the assumption that if the antibody to PCNA was able to detect its antigen then the antibody to BrdU should be able to access the BrdU. Dewaxed and hydrated sections were circled with a PAP pen and then blocked with 5% BSA in PBS for 30 min. After washing in PBS, immunohistochemistry was completed as described in Section 2.4.4.

Nuclei in sections incubated with PBS (negative control) were not stained while those of the wool follicle (positive control) were stained. PCNA labelling was dense across the mouse mammary sections from early lactation. It was concluded that the immunohistochemistry successfully detected PCNA and any apparent overfixing of sections did not impede antibody access to PCNA epitopes. The fact that PCNA, but not anti-BrdU, was detected was attributed to the latter molecule having epitopes that were more difficult to access.

2.5.3.2 Microwave treatment

Formalin fixing of tissues is superior to frozen sections for morphological preservation of tissues but it can yield unsatisfactory results in immunohistochemical studies from antigen loss (Tanimoto and Ohtsuki, 1996). Cross-links can form between proteins as a result of formaldehyde fixation and prevent antibodies from binding to their epitopes (Oliver *et al.*, 1997). A novel method used to achieve

antigen retrieval is the heating of dewaxed sections in a microwave oven (Tanimoto and Ohtsuki, 1996; Haas *et al.*, 1998) to disrupt the cross-links and therefore allowing better penetration of the antibodies into the section of mammary tissue.

Dewaxed and hydrated sections of mouse mammary glands were placed in a 10 mM tri–sodium citrate buffer and microwaved on medium-high for 15 min with the buffer being topped up at 5 min intervals. After being allowed to cool for 30 min, the sections were immuno-stained using the cell proliferation kit as detailed previously for the cultured HC11 cells (Section 2.4.3).

Nuclei were stained in sections that were overfixed and microwaved but not in sections that were not microwaved. It was concluded that the BrdU epitope was hidden in overfixed sections but they would be re-exposed by treating the sections with microwaves.

2.5.4 Optimisation of the BrdU immunohistochemistry

Modifications were made to the method of detecting BrdU so that the back-ground staining was reduced and the intensity of tissue staining was increased.

Background staining of sections was reduced by using minimal primary antibody, increasing washes and removing endogenous peroxidase. Minimal primary antibody should be used as excess may bind with lower affinity to other sites. To determine the optimal concentration of primary antibody, the antibody provided in the cell proliferation kit was serially diluted to cover the concentration range of 1/100 to 1/25,000. Sections covered with the range of primary antibody dilutions were incubated at RT for an hour and compared to sections incubated at 4°C for 24h. The best results were obtained using the dilution recommended by the cell proliferation kit (i.e. 1/100) and at this dilution either incubated at RT for 1h or 24h at 4°C showed identical results. The kit recommended 3x 3 min washes in PBS. In an attempt to reduce background three extra washes were included (a total of six) as well as using a mild detergent (0.05 % Tween) in PBS and the results showed a decrease in background staining. Finally sections were pre-treated with H_2O_2 (2 % in methanol)

for 30 min at RT and compared to sections without the pre-treatment to examine whether background staining was due to endogenous peroxidase activity. The results showed no difference between treated and untreated slides and therefore it was concluded endogenous peroxidase activity was not contributing to the background in the wax-embedded sections. As a result of these analyses it was decided to include six washes in PBS plus Tween instead of three washes in PBS alone. No changes were made to the recommended procedure with the primary antibody and no pre-treatment with H_2O_2 was necessary.

2.6 Validation of the method for detecting DNA

The DNA content of mammary glands collected in Chapter 4 was measured by fluorometric assay using Hoechst dye (H33258) following the method of Labarca and Paigen (1980) with some modifications (T. B. McFadden, unpublished) (Section 2.3.4). However, the DNA content of the mouse mammary tissue was lower than most others reported in the literature (Alston-Mills *et al.*, 1995; Knight and Peaker, 1982e; Shipman *et al*, 1987) and it was decided to ensure the method had been optimised. DNA standard solutions were made in sterile PBSE and gave consistent optical density readings as well as consistent standard curves between runs. Experiments to determine if the method had been optimised examined (a) the volume of PBSE used to homogenise the tissue, (b) centrifugation conditions after homogenisation and (c) the omission of sonication.

2.6.1.1 Volume of PBS used for homogenisation

The tissue was homogenised at a 1 in 5 dilution. A greater dilution may have aided DNA dissociation from cells. Comparison of the DNA content of homogenates when diluted 1 in 5 compared to 1 in 10 was conducted and showed there were no significant differences ($P>0.05$).

2.6.1.2 Centrifugation

Centrifugation at 4°C was included in the original method to eliminate fat in the sample. Tests were conducted to determine if the centrifugation step affected the DNA content of mouse mammary tissue. Mouse mammary tissue was homogenised

as described previously and half was centrifuged. Aliquots of supernatant were taken at three different positions (1/4, 1/2 and 3/4 of the way down the tube) from both the centrifuged and uncentrifuged samples. DNA content was analysed and the results showed that uncentrifuged samples contained 3.5 times more DNA than centrifuged samples. Results also showed that DNA content did not differ when the sample was taken from each site of collection in the tube. It was concluded that the centrifugation step was responsible for a decrease in DNA content and that it should therefore be omitted from any future sample preparation.

2.6.1.3 Sonication

Sonication of samples was also tested to determine its effect on DNA content of mammary tissue. Mammary tissue was homogenised as described previously and half the homogenate was centrifuged. These samples were divided into two, and one set was sonicated for 30 sec. Sonicated samples contained more detectable DNA than unsonicated samples and this was not dependent on centrifugation. It was concluded that samples should be sonicated prior to analysis of DNA content.

2.6.1.4 Correlation

From the above three sets of tests it was evident that homogenates should have been sonicated but not centrifuged prior to analysis of DNA concentration. Prior to this finding, samples collected for the experiment in Chapter 4 had been analysed differently (i.e. they were centrifuged and not sonicated). In order to correct these results, DNA analyses were conducted on a sub set of samples (n = 22) collected for Chapter 4. The samples were treated as for the first method (centrifuged and not sonicated) as well as the improved method (sonication and no centrifugation). It was determined that the improved method was significantly correlated to the first method used, according to Equation 2.1.

Equation 2.1 $Y = 3.76 X + 0.63$ $R^2 = 85\%$ $P = 0.012$

where X = DNA concentration (μg/mg) determined using the first method (Labarca and Paigen, 1980 modified as per T. B. McFadden, unpublished),

and Y = DNA concentration (μg/mg) determined using the improved method (sonication and no centrifugation).

When the correlation was applied to the results of Chapter 4, the conclusions were not different to those when DNA concentration was analysed according to the first method. The improved method was used for analysis of subsequent experiments.

2.7 Development of method for flow cytometry

2.7.1 The principles of flow cytometry

Flow cytometry can measure five physical characteristics of a single cell. Cell size and granularity, and three different fluorescences are measured as each cell passes single file through a laser beam at the rate of 500–5000 cells per sec (Shapiro, 1988). Interaction of a cell with the focussed laser beam will defract light at a low angle (forward scatter) and is proportional to cell size. Some light is reflected at a high angle (side scatter) and is proportional to cell granularity and complexity. Three different fluorescences emitted from the cells can also be detected. The intensity of the fluorescence is proportional to the number of binding sites per cell bound by fluorochromes (Shapiro, 1988). The three colours of fluorescence are green, orange and red which emit at 530, 575 and 675 nm respectively. Fluorochromes that can emit these colours are fluorescein isothiocynate (FITC), phyco erythrin (PE) and propidium iodide (PI) respectively (Gabriel and Kindermann, 1995).

Part of the original aim of the work was to determine how the challenges of nursing demand and exogenous hormones altered the percentage of mitotic cells in the lactating mammary gland. In addition, we were interested in whether the mitotic cells were differentiated. In a report (Franke and Keenan, 1979) it was suggested that differentiated cells can proceed to mitosis; as indicated by a micrograph of a differentiated mammary epithelial cell that was apparently mitotic as it had incorporated [^{3}H] thymidine into its nucleus.

The application of flow cytometry to mammary epithelial cells required tissue to be digested to single cells and then stained with fluorescent labels for detection on the

flow cytometer. The following section details the method used to digest tissue to single cell suspensions.

2.7.2 Digestion of mammary tissue to single cell solutions

Mammary gland tissue was digested to single cells using both enzymatic and physical dissociation techniques. All digestion of cells occurred in sterile medium and in aseptic conditions to minimise bacterial contamination.

Mammary glands were dissected out, blotted and weighed before being placed together on a petri dish with 0.5 ml of warmed calcium and magnesium free media (CMF; 1 mg/ml glucose, 2.2 g/l sodium bicarbonate, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B, in PBS). The glands were diced to approximately $2mm^3$ pieces using scalpels or scissors and the chopped tissue was then washed for 15 min in 15 ml of CMF at 37°C while being shaken at 150 rpm. The tissue was transferred to 5 ml of CMF containing collagenase (0.125% w/v collagenase type V), hyaluronidase (0.1% w/v hyaluronidase type I-S from bovine testes) and BSA (4% w/v) and shaken at 150 rpm for 75 min at 37°C to aid dissociation. The tissue was further digested in 5 ml of M199 media (M199 media, 2.2 g/l sodium bicarbonate, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B, and pH 7.3 to 7.4) containing the enzyme trypsin (25 mg/ml trypsin 1:250 from porcine pancreas) and was shaken at 150 rpm at 37°C for 20 min. At RT, 1 ml of 10% BSA was added and the tissue and media were centrifuged at 70 *g* for 5 min. The fat and supernatant were discarded and the pellet was resuspended in approximately 4 ml of M199 media, and a few drops of DNAse (800 units/ml solution of DNAse I from bovine pancreas in PBS) was also added to digest the DNA in solution that was causing clumping of cells. The media and dissociated tissue were gently filtered through a sterile 100 μm and then a 50 μm nylon mesh filter to omit large clumps of cells. The filtrate was centrifuged again for 5 min at 70 *g*, the supernatant was removed and the cells washed with 1 ml of M199 media before being centrifuged again. The cells were washed and centrifuged a second time, the supernatant removed and cells were resuspended in 1 ml M199 media. A cell count was performed using a haemocytometer. PBS (9 ml) at 37 °C was added to the cell suspension with 3 ml

PBS containing formalin (4 % v/v formalin) and the tube was gently inverted to mix before leaving it on ice for 15 min. Methylamine (200 µl) and 1 ml of 10 % BSA were added and the tube gently inverted to mix again. After centrifuging for 3 min at 650 *g* the supernatant was discarded. The pellet was resuspended in 0.3 ml PBS and placed in an eppendorf tube with 1.2 ml of 75% ethanol. The samples were stored in this at –18°C until subsequent staining for analysis on the flow cytometer.

2.7.3 Verification of cell stains by fluorescent microscopy

Flow cytometry was to be used to obtain information on the epithelial cells DNA content and their differentiation status. Experiments examined potentially suitable stains including FITC labelled peanut agglutinin (PNA) and FITC labelled soybean lectin to determine differentiation status (Hurley, 1996; Rudland and Barraclough, 1988) and propidium iodide (PI) to determine DNA content (Dundas *et al.*, 1991).

PNA-FITC and soybean lectin-FITC were thought to be appropriate for staining differentiated epithelial cells. Both PNA and soybean lectins are galactose specific (Lotan *et al.*, 1975). Peanut agglutinin (Lectin from Arachis Hypogaea, Sigma Chemical Company, St. Louis, U.S.A.) binds to an oligosaccharide containing the terminal sequence β-D-galactose-(1-3)-N- acetyl galactosamine (Kim *et al.*, 1993). PNA binds to rat alveolar epithelial cells (Rudland and Barraclough, 1988) and has been used as a marker for breast epithelial cell differentiation (Newman *et al.*, 1979). Soybean agglutinin (Lectin from Glycine Max, Sigma Chemical Company, St. Louis, U.S.A.) binds to (α and β)-N-acetyl-D-galactosamine and stains the apical surface of epithelial cells in lactating bovine tissue (Hurley, 1996). Both these preferred binding sites are on the apical membrane of differentiated mammary epithelial cells (Nemanic *et al.*, 1983). Both lectins were labelled with FITC which fluoresces a green colour at 530 nm.

To test which of the two lectins would best stain differentiated epithelial cells, staining by the lectin-FITCs were compared on 4 µm formalin fixed, dewaxed and hydrated sections of lactating mammary tissue. The stains were first tested on sections of tissue rather than single cell suspensions as the histological section would

provide location information such as apical side of the cell and whether it contained milk components. The top of each section was left as an unstained control and the bottom of each was stained with either PNA or soybean lectin. Experiments had been done previously to determine the best staining time and concentration of the lectins. Staining was conducted in conditions as dark as possible. When the staining was complete, a drop of glycerol was placed on the section as this had been earlier determined to be the best medium for viewing the section under the microscope. A coverslip was applied and the sections were examined under UV light on a fluorescent microscope. Both lectins demonstrated specificity as they did not stain myoepithelial and adipocytes but did stain the apical membrane of the epithelial cells most intensely and the rest of the cells with lower intensity. This lower intensity staining was attributed to the lower binding affinity of the lectins to other carbohydrates in the cells and milk. The specificity for differentiated epithelial cells was not so clear as some stained cells did not have milk fat droplets in their cytoplasm. This may have been due to them having released fat droplets immediately prior to when the tissue was collected. Both lectins also stained alveolar lumen contents but this was not considered a problem as this component would not be present at the completion of the digestion process. Both lectins varied in intensity of staining across sections showing that binding was stronger in some areas than others. The soybean lectin differed to the PNA in that it had a grainy fluorescent appearance. In conclusion, the PNA and soybean lectin both seemed appropriate stains but the PNA was chosen for future use because it was cheaper, there was a large quantity readily available in the laboratory and because it was well documented in the literature as being specific to differentiated epithelial cells.

PI auto fluoresces a red colour at 650 nm and stains in proportion to content of DNA in cells. PI was tested by using mammary tissue sections and showed specific binding to nuclei of all cells in the section. The optimal results were obtained using a low concentration of PI for only 15 min, immediately prior to examining the section under the microscope.

2.7.4 Staining of single cell suspensions with PNA and PI

The fluorescent PNA and PI stains were successful on tissue sections and the staining method was then adapted to stain single cell suspensions of mammary epithelial cells. A number of different methods were tested but the optimal approach was to stain with PNA first and leave the PI stain until just prior to using the flow cytometer. The single cells suspensions were vortexed to resuspend cells that had formed a pellet during time in storage at –18°C. Then 500 µl of the suspension was added to 500 µl of 1% BSA and left at RT for 15 min. Samples were centrifuged at 110 *g* for 3 min and the supernatant removed. PNA was reconstituted in PBS containing 0.5% BSA to give a final concentration of 200 µg/ml. PNA (1000 µl) was added to the sample and incubated in darkness at RT for 15 min after which it was centrifuged at 110 *g* for 3 min and the supernatant removed. Then 1 ml of 0.5% BSA was added and the cell suspension filtered through 100 µm and then 50 µm nylon mesh. It was left in darkness at 4°C overnight. Prior to analysis on the flow cytometer, 1 ml of PI solution (5µg/ml PI, 0.1 % sodium azide, 200 µg/ml RNAse, in PBS) was added to the sample and incubated in darkness at RT for 15 min. The sample was then analysed on the flow cytometer.

2.7.5 Use of a Coulter flow cytometer

A Coulter flow cytometer (Model Profile II, Coulter), housed at Health Waikato Haematology Department, was pressurised and calibrated with Standard Brite beads (Coulter) and Immunocheck (Coulter). The laser and pressure settings were unaltered but the volume of sample added to the isotonic carrier fluid (Coulter) was manipulated so that approximately 10,000 events per min were run per sample. Options in the software such as amplifier gain, photomultiplier voltage, discriminators for the forward scatter, side scatter and log fluorescence scales were altered until cell events were within range and on the most appropriate scale. At the end of a session, the machine was cleaned using a series of bleach and Milli Q water washes, depressurised and turned off.

An initial problem encountered was a blocked nozzle. On the first attempt to run samples problems compounded until no data were being collected. The problem was found to be a blocked nozzle caused by sticky clumps of cells. Although the cells were in a single cell suspension after digestion, small clumps formed during storage. For this reason two filtering steps were added to the staining method as detailed in Section 2.7.4. The filtering steps reduced the clumps of cells but to the detriment of the number of cells in the sample.

Successful flow cytometry requires that the diameter of each single cell to be 10 times less than the diameter of the nozzle (Shapiro, 1988). The diameters of cells in some of the single cell suspensions were measured. Cell diameter increased with stage of lactation such that early lactation cells were approximately 12.5 μm in diameter, mid lactation were approximately 20 μm and late lactation were approximately 40 μm in diameter (D. Auldist, unpublished observations). The small cells were less than 10 times the diameter of the nozzle (150 μm) but the larger cells caused blockages when doublets, triplets or small clusters were formed.

Data collected were in the form of multiple pages of histograms (forward scatter versus PI and versus FITC, side scatter versus PI and versus FITC, number of events versus each fluorescence) per sample as well as information stored on computer regarding the 10,000 events per sample. The data on disc were unprocessed and required a computer program to eliminate debris and discriminate against doublets. This would allow gating and then back gating of the FITC and PI labelled cells to yield information on the percentage of the cells that were in S phase of mitosis (identified by high PI content), the percentage of cells that were differentiated (identified by PNA-FITC label) as well as the percentage of cells that were dual labelled and therefore both mitotic and differentiated. Familiarity and competency with the data manipulation software were being achieved when the Department's computer package expert left unexpectedly with the software. The Department did not replace the software program. The steroid experiment (Chapter 4) had been run on the Coulter flow cytometer but none of the data were able to be analysed past the raw data histogram stage.

2.7.6 Use of a Becton Dickinson flow cytometer

A Becton Dickinson (BD, San Jose, California, U.S.A.) flow cytometer was purchased by AgResearch and while the principles were the same, the machine and software were different to the Coulter flow cytometer. Expert technical assistance was not available although frequent contact was made with the supplier.

In the experiments using the Becton Dickinson flow cytometer, problems with cells clumping together persisted. Although filtering was effective at obtaining single cell suspension it did greatly reduce the number of cells remaining for analysis and therefore other methods of obtaining a single cell suspension were tested. Tween was added to PBS and extra vortexing steps were introduced to the staining procedure in an attempt to increase the percentage of single cells present. Microscopy and counting of many samples showed these extra steps did not alter the general composition of prepared samples from 50% single cells, 20% doublets or triplets, 15% free nuclei, 7.5% large groups of cells and 7.5% cell fragments.

To improve the procedure of running samples on the flow cytometer, internal standards of beads of known sizes (Calibrite, BD) were added to samples to verify the size of cells and therefore help determine which events were debris and which were cells. Further confidence in determining where cell fragments were positioned on the histograms was obtained by using a cytokeratin stain. Cytokeratin binds to intact cells only and not debris or free nuclei (Shapiro, 1988). Using cytokeratin, debris was located on the Forward Scatter x Side Scatter plot and then this information could be gated out leaving information on intact cells only. Doublet discrimination was also implemented so that two cells each containing 1n amount of DNA that passed the laser beam simultaneously could be discerned from one cell containing 2n amounts of DNA. In addition, the resolution of DNA detection was improved by using a DNA Quality Control Particles Kit containing chicken erythrocyte nuclei (Becton Dickinson).

Using these procedures, cell debris and doublets were gated out leaving only data from single cells that was analysed. This information was presented on histograms of cell count versus FITC and cell count versus PI to yield information about cell

cycle and differentiation status (Figure 2.2). Calculations were done to determine the percentage of cells that survived the process of making a single cell suspension. The digestion and physical disruption procedure took approximately 4h from dissection of the gland to storage of single cells in ethanol at –15°C. The procedure was detrimental to the cells with only 1.2% of cells surviving to the storage stage. This figure was halved after the filte ing steps (only 50% of cells were singles) so the f nal proportion of the original cells in the mammary gland that were analysed by flow cytometry was about 0.5% which raises questions about representation of cells in the final sample. More than 95% of flow cytometers are used for diagnostic work in hospitals where blood components make up the large majority of samples. Of the flow cytometers used for research, most samples are from single cell solutions originally such as blood, sperm or cell cultures. Only a few research groups have had success with using cells from mammalian digested tissue (Dundas *et al.*, 1991; O'Hare *et al.*, 1991; Smalley *et al.*, 1995) as evident by the paucity of information in the literature and from the flow cytometer companies. Research groups that do have success have made significant investments in their flow cytometer and have a full time trained technician operating the machine in contrast to novices working infrequently on the analyses.

2.7.7 Outcomes of time invested in flow cytometry

Flow cytometry was an attractive method to include in the research project however in hindsight, it was unlikely to have provided useful results. This was because the digestion of tissues to single cells results in significant cell losses. Although the literature contains many reports of flow cytometry of primary mammary cells, these are all from non-lactating tissues. It became apparent during the work in this thesis that methods used to obtain single cell suspensions from non-lactating mammary tissues are too harsh for lactating tissues which results in only a very low percentage of cells remaining intact. Use of flow cytometry in the course of the work in this thesis therefore ceased but the methodological information is recorded here to assist with future efforts to use the technology.

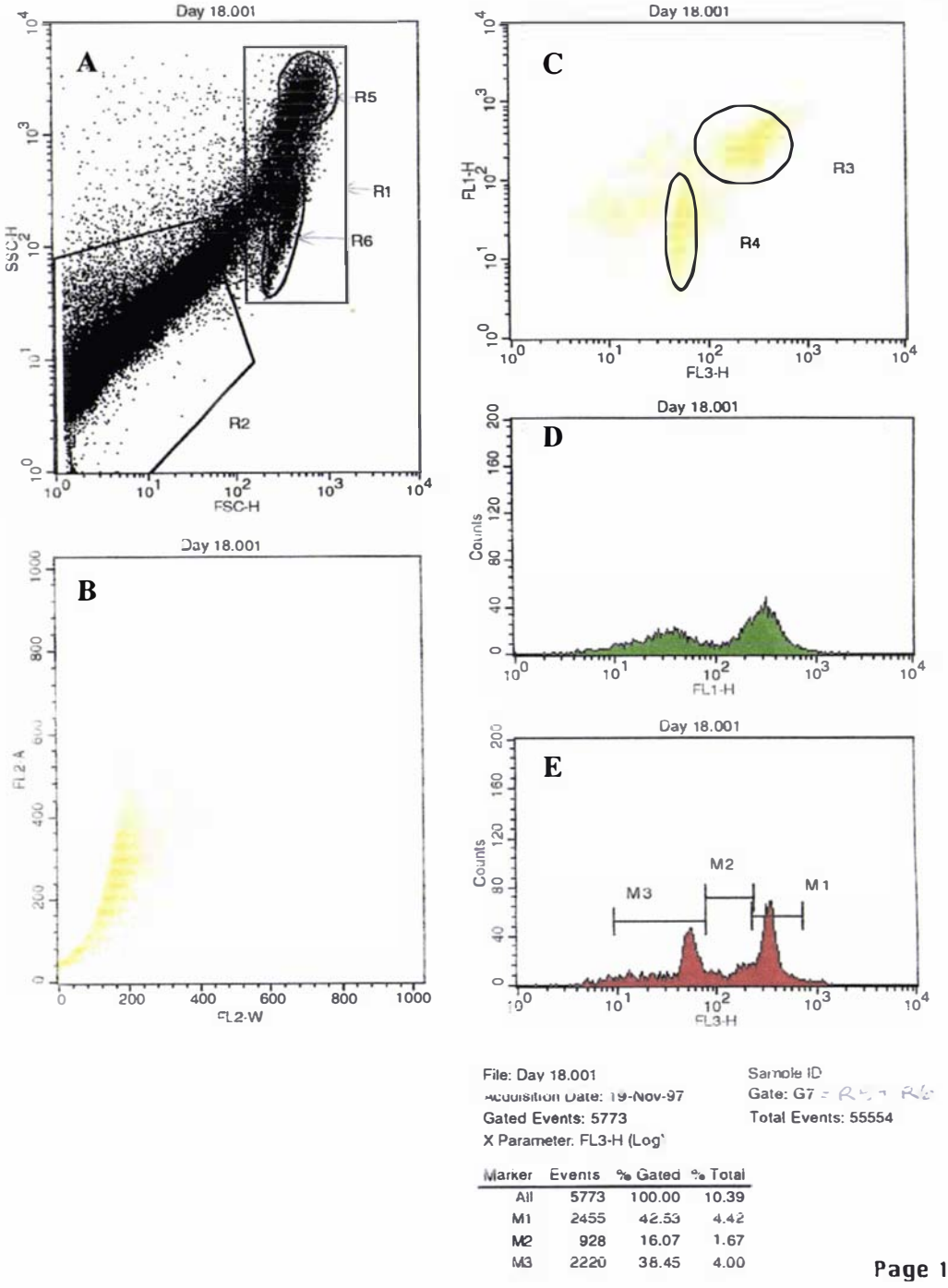


Figure 2.2: Example of flow cytometry analysis used to detect different populations of cells.

The single cells used in the analysis above were collected from the mammary glands of a mouse at D17 of lactation. Panel A: Forward scatter (cell size) x Side scatter (cell granularity and complexity). Panel B: Width x Area, used to discriminate single cells from doublets and debris. Panel C: PI (DNA content) x PNA. Panel D: PNA x count of events. Panel E: PI x count of events.

Chapter 3 Murine milk composition and milk yield during lactation

3.1 Summary

To enable comparisons among treatments in experiments reported in later chapters it was essential to know both the composition and the yield of the milk produced by Swiss mice during lactation. The composition of milk at different stages of lactation must be known so that mammary gland weights can be corrected for milk content. Knowing the milk yield of mice during lactation will permit calculations of milk produced per unit of gland weight or DNA. The small amount of information in the literature on murine milk composition and yield was inconsistent and therefore necessitated the measuring of these parameters. This chapter reports two studies done to determine the composition and the yield of milk produced by Swiss mice.

In the milk composition study, six mice in each of early, mid and late lactation, were sedated and milked manually after receiving an intraperitoneal injection of oxytocin. Milk samples were analysed for lactose, protein and fat concentration. Lactose content of the milk increased linearly ($P<0.001$) by an average of 1.2 g/l per day from 8.7 g/l on D3 to 25.9 g/l (SEM = 1.0) on D17 of lactation. In contrast, milk protein and milk fat did not alter significantly from 115 $\pm$ 8.3 g/l (mean $\pm$ SEM, $P>0.05$) and 25.3 $\pm$ 0.7% (mean $\pm$ SEM, $P>0.05$) respectively during the same interval of lactation.

In the milk yield study, three methods of calculating milk yield of mice over lactation with different litter sizes were investigated. The optimum method involved estimation of maintenance energy requirements by calculating metabolic weight. This was added to energy requirements for observed growth and the sum of these was converted to milk yield. The metabolic weight method is most reliable for D3 and D10 of lactation and more reliable than other methods for D17 data. Consequently, the metabolic weight method was used to estimate milk yield of mice in subsequent chapters, thereby enabling comparisons of milk output per unit gland weight, or unit DNA, between dams with different litter sizes throughout lactation.

3.2 Introduction

A study of the composition of mouse milk was initiated after it was observed during collection of dissected glands that some glands were very white in colour and leaked milk, while others were red and contained very little milk. It was necessary to know the amount of milk in the collected glands so it could be subtracted from gland weight to yield true mammary tissue weight, and milk protein could be subtracted from mammary gland protein. If it is assumed that all the lactose in the gland is present in milk, then milk volume can be calculated from the amount of lactose present provided the lactose concentration in the milk is known.

There are limited studies on the composition of milk from laboratory mice and the data reported for concentrations of milk constituents vary greatly. Lactose concentrations of milk reported in the literature vary threefold from 15 to 45 g/l (Allen, 1984; Knight *et al.*, 1986b) and fat content can vary twofold from 12 to 31% (Ben Shaul, 1962; Knight *et al.*, 1986b). Protein content is more consistent between different studies and usually ranges between 90 and 142 g/l (Ben Shaul, 1962; Ragueneau, 1987). The effect of stage of lactation on milk composition of mice is not well known and most papers report milk composition at only limited time points. For example, Ragueneau (1987) sampled milk on D6 and D9 after parturition in 5 strains of mice, and reported that protein and lactose concentrations increased while fat concentration did not change. In a comprehensive but contrasting study, Knight *et al.* (1986b) milked mice 5 times during lactation and found that fat concentration increased from 19.6 to 31.4% while protein concentration did not vary significantly over time. Considering this inconsistency in the data on murine milk composition, and the lack of information on potential changes in lactose concentration during lactation, it was necessary to analyse milk composition during the lactation cycle in the strain of mice that were used in the experiments described in this thesis. The aim of the milk composition study was to determine the concentrations of lactose, protein and fat in milk collected from Swiss mice on D3, D10 and D17 of lactation.

Estimation of milk yield of mice was the second study of this chapter. This was required so that milk production per gland, or per unit of DNA, could be compared between treatments. When all litters contain the same number of pups, daily weight gain can be used as a relative measure of milk production (Bruce, 1961). This method cannot be used however, when litter size varies as it will underestimate milk produced by dams with larger litters, which have larger maintenance energy requirements (Fiorotto *et al.*, 1991).

Milk production of mice has been measured previously using techniques that fall into four categories. These are (i) weigh-suckle-weigh, (ii) isotope dilution or transfer, (iii) direct milking and finally, (iv) calculation from published data on metabolic costs of growth and maintenance.

The use of the weigh-suckle-weigh technique in mice involves the separation of the pups from the dam for a period of approximately 6h, after which the dam and the litter are weighed separately, then the pups are allowed to suck for ~1.5h, and the dam and the litter are reweighed (Jara-Almonte and White, 1972; Lin *et al.*, 1977). The weight decrease of the dam is attributed to milk removed by the litter and the weight increase of the litter is attributed to milk consumed by the litter. Despite its apparent simplicity there are a number of factors that can cause errors. One is the stress caused by frequent handling of the animals and another is the physical interference with suckling that decreases suckling bouts and therefore milk consumption (Oftedal, 1984). Another potential error is the ability of the litter to empty the glands to the same degree as before the separation period, which may be difficult as pups are used to small, frequent meals (Bateman, 1957) rather than one large meal after 6h. Another inadequacy with the method is that a separation period of more than 4h can reduce milk production in mice (Hanwell and Linzell, 1972; Oftedal, 1984). Any weight difference measured in the dam or the litter would contain error owing to the dam licking the perineal area of altricial pups to evoke urination and defaecation, which she then consumes (Baverstock and Green, 1975; Walser, 1977). The method also requires scales that can accurately weigh the dam or litter even when they are moving about in the weighing vessel. In addition, diurnal variation of dam weight (see Figure 3.8 in Chapter 3 Appendices) will cause errors in any calculations involving weight change. These sources of error cast doubts on the

accuracy of results of studies of rodent milk production where the weigh-suckle-weigh technique is used. Indeed, Baverstock and Elhay (1981) reported that the weigh-suckle-weigh technique underestimated milk consumption of mice by at least 30%.

Isotopically labelled water has been used since the mid 1970s (Romero *et al.*, 1975) to estimate milk produced by rodent dams or ingested by their pups. Two isotope methods used are isotopic dilution and isotope transfer. Isotopic dilution is used to estimate the milk intake of young. Young are injected with a hydrogen isotope (tritiated water or deuterium oxide) and the labelled water equilibrates with body water (Holleman *et al.*, 1975). As milk is ingested, the water in milk will contribute to the turnover of body water in pups and cause the concentration of the isotope to decline exponentially. The degree of dilution is related to the amount of water consumed in the form of milk (Oftedal, 1984). The second isotope method, isotope transfer, relies on the transfer of the isotope from the mothers to the pups that consume her milk (Rath and Thenen, 1979). The amount of isotope transferred is proportional to the amount of milk consumed by the pups. Both isotope methods overcome some of the problems associated with the weigh-suckle-weigh technique, particularly the stress of handling, interference with suckling, small errors with weighing and loss of excreta. The isotope methods do, however, have sources of errors. The body water pool of growing, suckling young increases rapidly causing the biological half-life of the label to increase with time (Hendriks and Wamberg, 2000). This will cause errors as assumptions are made about equilibration time of the isotope and the size of the body water pool. Another error results from the incorporation of isotopes into non-exchangeable sites deposited during growth (Oftedal, 1984). A condition of the isotope dilution method is that milk is the only source of water available to the young and any water consumed by pups or created by metabolism of fat will alter the results (Oftedal, 1984). Recycling of the isotope will occur in pups that depend on maternal licking for urination and defaecation but this can be corrected for by using a non-labelled pup as a 'blank' measurement (Knight *et al.*, 1983). Despite the apparent sophistication of the isotope methods there is no evidence they are more valid than methods based on litter weight gains. For instance, the isotope study by Godbole *et al.* (1981) suggests the rate of milk production for Zucker rats increases threefold between D10 and 15 of lactation and

eightfold between D10 and 20 of lactation. Such increases are not supported by other milk production studies (Grigor *et al.*, 1987), food consumption studies (Grigor *et al.*, 1984) or lipogenic enzyme studies (Grigor *et al.*, 1982).

Direct milking of mice is another method used to estimate milk production. Mothers are separated from their litters for 8h (Hanrahan and Eisen, 1970) after which they receive exogenous oxytocin and the mammary glands are milked out manually. The volume of milk collected is an estimate of production during the time of separation. This method depends on enough oxytocin being administered so as to overcome the effects of adrenalin release in the non-anaesthetised mouse (Linzell, 1972). In addition, it depends on being able to milk out the glands to comparable degrees at each milking which is difficult for animals such as mice which store milk in mammary alveoli and ducts rather than cisterns (Cross, 1977). A concern with the method is that separation periods of more than 4h would lead to errors as the rate of milk production decreases after this time (Hanwell and Linzell, 1972). In addition, direct milking must be done very carefully to avoid spillage of milk onto fur, fingers or the collection vessel and to minimise evaporative loss when vacuum collection is employed (Oftedal, 1984).

The fourth category used to estimate rodent milk production is by calculation of the milk required to support growth and maintenance. Energy required for maintenance has been measured by either heat production of young mice (Lin *et al.*, 1979) or oxygen uptake of young rats (Brody and Nisbet, 1938) and guinea pigs (Hill, 1959). Milk required for maintenance has also been estimated by equating the weight loss of fasted litters to an equal weight of milk (Sampson and Jansen, 1984; Grigor *et al.*, 1987). Numerous metabolism experiments using many species have resulted in standard equations for calculating metabolic weight and energy required to support maintenance (Brody, 1945; McDonald *et al.*, 1988). Growth is measured by weight increase and this can also be converted to an energy requirement (McDonald *et al.*, 1988). Calculating milk production therefore requires measurements of litter weight, body composition and milk composition. These data are used with established figures for energy costs of tissue deposition and maintenance of body weight (McDonald *et al.*, 1988) to estimate milk production (Hendriks and Wamberg, 2000).

The size of the litters used in the experiments described in this thesis varied from 5 to 20 pups. A comparison of litter weight gain alone would lead to erroneous conclusions as it ignores milk produced and consumed to meet maintenance energy requirements. Given the inadequacies of weigh-suckle-weigh, isotopic dilution and direct milking methods, it was decided to measure milk production using the fourth category i.e. by calculations based on liveweight.

Three different calculations were tested and compared. The first was done by estimating maintenance energy requirements of fasted litters, adding these to energy requirements for observed growth rates, and then equating the energy to a quantity of milk. Previous studies have used this method to estimate milk yield of rats but have only studied one day of lactation, D14 (Grigor *et al.*, 1987), or different days of lactation, D3 to D13, (Sampson and Jansen, 1984) without variation in litter size. The results of the fasted weight loss method will be compared to two similar methods that differ as a) maintenance energy costs are calculated from body weight raised to the power of 0.83 (McDonald *et al.*, 1988), known as metabolic weight, and b) milk yield is calculated using litter weight gain data only (Cowie and Folley, 1945), known as the proportion method. The aim of the milk yield study described in this chapter was to use the three calculation methods to estimate mouse milk production on D3, D10 and D17 of lactation for dams with litters of 5, 10 or 15 pups. The best of the three calculation methods will be used in subsequent chapters to enable valid comparisons of milk production of mice suckling different litter sizes.

3.3 Experimental design

3.3.1 Milk composition study

Six virgin female Swiss mice were mated and within 12h after parturition litter size was adjusted to 11 pups. Mice were milked on D3 (early), D10 (mid) and D17 (late) of lactation. Mouse husbandry and management are detailed in Section 2.1 while methods for protein, fat and lactose analyses of milk are detailed in Section 2.2. The data were analysed using analysis of covariance (Minitab, Release 12.23, 1999) to

examine the change over time in the content of lactose, protein and fat in milk. Linear and quadratic components of day of lactation were tested for each animal and then these compared between all animals rather than fitting a line of best fit to the pooled data. This method was chosen because a time series analysis cannot be used when there are only three data points.

3.3.2 Milk yield study

Twenty five Swiss mice were mated and transferred to individual cages at D15 of pregnancy. By 12h after parturition litter sizes were adjusted to 5, 10 or 15 pups. Mouse husbandry and management are detailed in Section 2.1. Measurement of litter growth rate and the weight loss of fasted litters are detailed in Section 2.1.4. Means, standard error of the means (SEM) and standard error of differences between the means (SED) were calculated using Minitab (Release 12.23, 1999) for litter weights (and gains and losses) (Table 3.2). Minitab was also used to determine the linear regressions of the weight losses of fasted litters. Slopes and intercepts of the three litter sizes were compared and tested for non significant differences before the data were pooled to give a final regression equation, residual standard deviation (RSD) and R^2 value for each of D3, D10 and D17 (Table 3.3).

3.4 Results

3.4.1 Milk composition study

The concentrations of lactose, protein and fat in milk of Swiss mice in early, mid and late lactation are shown in Table 3.1. The lactose content increased linearly by 1.2 g/l per day from a concentration of 8.7 g/l on D3 to 25.9 g/l on D17 of lactation and was predicted by the regression;

$$Y = 2.211 + 1.23\ X \quad (\text{adjusted } R^2 = 87.5\ \%,\ P<0.001),$$

where Y = lactose g/l and X = day of lactation.

The concentration of protein in milk did not differ from 115 g/l (SED=11.8, P>0.1) between different stages of lactation. Similarly, fat content in milk did not change from 25.3% (SED=1.0, P>0.1). Mouse to mouse variation was greater for the protein and fat measurements than for the lactose measurement.

Table 3.1: Composition of milk collected from Swiss mice throughout lactation.

Mean concentrations of lactose (g/l), protein (g/l) and fat (%) in milk collected from Swiss mice on D3, D10 and D17 of lactation. Means are followed by SEM and number of observations are in brackets. Means adjusted for missing values.

Milk component	Day of lactation		
	3	10	17
Lactose	8.7 ± 1.2 (6)	15.7 ± 0.9 (6)	25.9 ± 1.4 (6)
Protein	110.5 ± 7.4 (5)	106.6 ± 4.2 (4)	129.0 ± 8.0 (6)
Fat	24.6 ± 1.5 (3)	26.0 ± 1.3 (5)	25.3 ± 1.7 (6)

3.4.2 Milk yield study

Milk yields of mice were calculated using three different methods which were called the fasted weight loss method, the metabolic weight method and the proportion method. All three methods used at least some of the data on litter weight changes in Table 3.2.

3.4.2.1 Fasted weight loss method

This method estimated the maintenance requirements of litters by measuring fasted weight losses of litters. This was added to the daily growth rates of litters and the sum was then converted to a quantity of milk.

Litter weights, daily gains and weight losses during fasting for litters of 5, 10 or 15 pups are shown in Table 3.2. Litter weight increased with litter size but not in proportion to pup number so that pups in small litters were heavier than pups in larger litters. Daily gain was greatest on D10 of lactation for litters of 5, 10 and 15 pups.

Table 3.2: Mean weights, daily gains and weight losses of fasted litters.

The means and (SEM) of weight (g), daily gain (g/d) and 24h fasted weight loss of litters of mice containing 5, 10 or 15 pups on D3, D10 and D17 of lactation where n>8 for each mean.

Day of lactation	Litter size (No. pups)	Litter weight (g)	Daily gain (g/d)	24h fasted weight loss (g/d)
3	5	15.21 (0.79)	2.58 (0.17)	0.64 (0.08)
	10	25.38 (0.86)	4.56 (0.33)	1.28 (0.14)
	15	32.36 (0.83)	3.77 (0.28)	1.23 (0.19)
10	5	36.64 (0.81)	3.27 (0.14)	2.01 (0.06)
	10	58.96 (1.06)	4.91 (0.11)	2.88 (0.18)
	15	68.61 (1.59)	4.18 (0.09)	2.57 (0.33)
17	5	52.71 (1.08)	2.33 (0.29)	1.61 (0.42)
	10	78.00 (1.67)	2.98 (0.12)	3.74 (0.71)
	15	87.03 (2.12)	2.53 (0.27)	6.07 (0.38)

Data for all litter sizes was pooled and linear regressions of litter weight loss versus litter weight of fasted litters were derived for each of D3, 10 and 17 of lactation (Table 3.3) and applied to a scatter plot of the raw data (Figure 3.1). The slopes of the regressions for three and 10 day old litters were not significantly different indicating that relative to initial litter weight, the amount of weight lost during fasting was similar at each age. The regression for D17 had a higher R^2 value which is partly a reflection of the greater spread of data along the X axis. The residual standard deviation (RSD) is a more relevant measure of the fit of a regression line, with a lower number meaning a better prediction of Y from X than a larger residual standard deviation (Draper and Smith, 1966). The RSDs were lower for D3 and 10 regression lines meaning they are better predictors of fasted weight loss from initial litter weight.

Table 3.3: Linear regressions of litter weight and weight loss of fasted litters.

Day of lactation	Linear regression *	RSD	R^2	P value
3	Y = 0.093 + 0.039X	0.39	37.6	0.001
10	Y = 0.863 + 0.030X	0.60	33.8	0.002
17	Y = -4.005 + 0.108X	1.60	54.3	0.000

* where Y = the weight loss of litters fasted for 24h (g) and X = the weight of the litter prior to fasting (g).

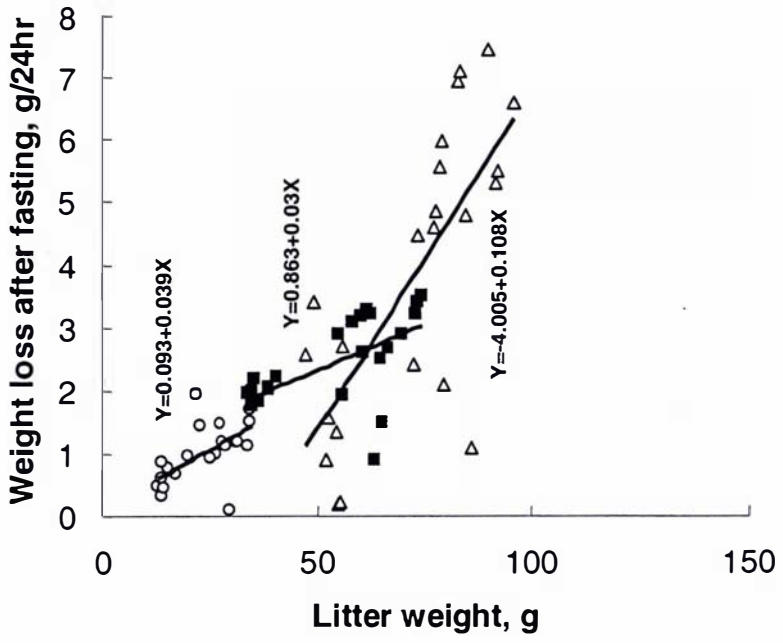


Figure 3.1: Scatter plot of litter weight versus weight loss after fasting.

The loss of weight of litters during fasting on D3 (○), D10 (■) and D17 (Δ) of lactation pooled for litters of 5, 10 and 15 pups and plotted against initial litter weight together with the within day regression lines.

The daily milk yield of mice was calculated from the maintenance costs of the litters and their weight gains. The volume of milk required to support the observed weight gain was calculated using the data on milk composition and energy content (Table 3.5 in Chapter 3 Appendices), pup body composition (Table 3.6 in Chapter 3 Appendices) and gross energy and energy costs for deposition of tissue (Table 3.7 in Chapter 3 Appendices). These data were used to construct Table 3.4 which was used to calculate the amount of milk consumed for each gram of weight gain for litters

aged 3, 10 and 17 days of age. The volumes of milk required to meet the maintenance requirements of the litter were estimated from the 24h fasted weight losses. The litter weight loss was assumed to be in proportion to body composition and converted to amounts of protein and fat tissue lost and then converted to energy lost. This was equated to a quantity of milk.

Using the information detailed above, the total daily milk production of Swiss mice for D3, D10 and D17 of lactation was calculated from the sum of milk required for litter growth and maintenance (Figure 3.2).

Table 3.4: Data used to calculate the milk required to support litter daily gain.

Day of lactation	Tissue deposited in 1 g litter weight gain		Energy required to deposit tissue		Energy in 1 g milk	Milk for 1 g litter weight gain
	Protein (mg)	Fat (mg)	Protein (kJ)	Fat (kJ)	(J)	(ml)
3	128	51	5.414	2.729	12.39	0.73
10	145	107	6.134	5.725	12.96	1.02
17	161	132	6.810	7.062	13.40	1.16

For example on D3 of lactation, a 1 g gain in litter weight contains 128 mg protein and 51 mg fat (Table 3.6). To deposit this much tissue, 5.4 and 2.7 kJ are required respectively (Table 3.7). Knowing the composition of milk and its energy content (Table 3.5), the milk required to support the litter weight gain is calculated, assuming that digestibility is 90%.

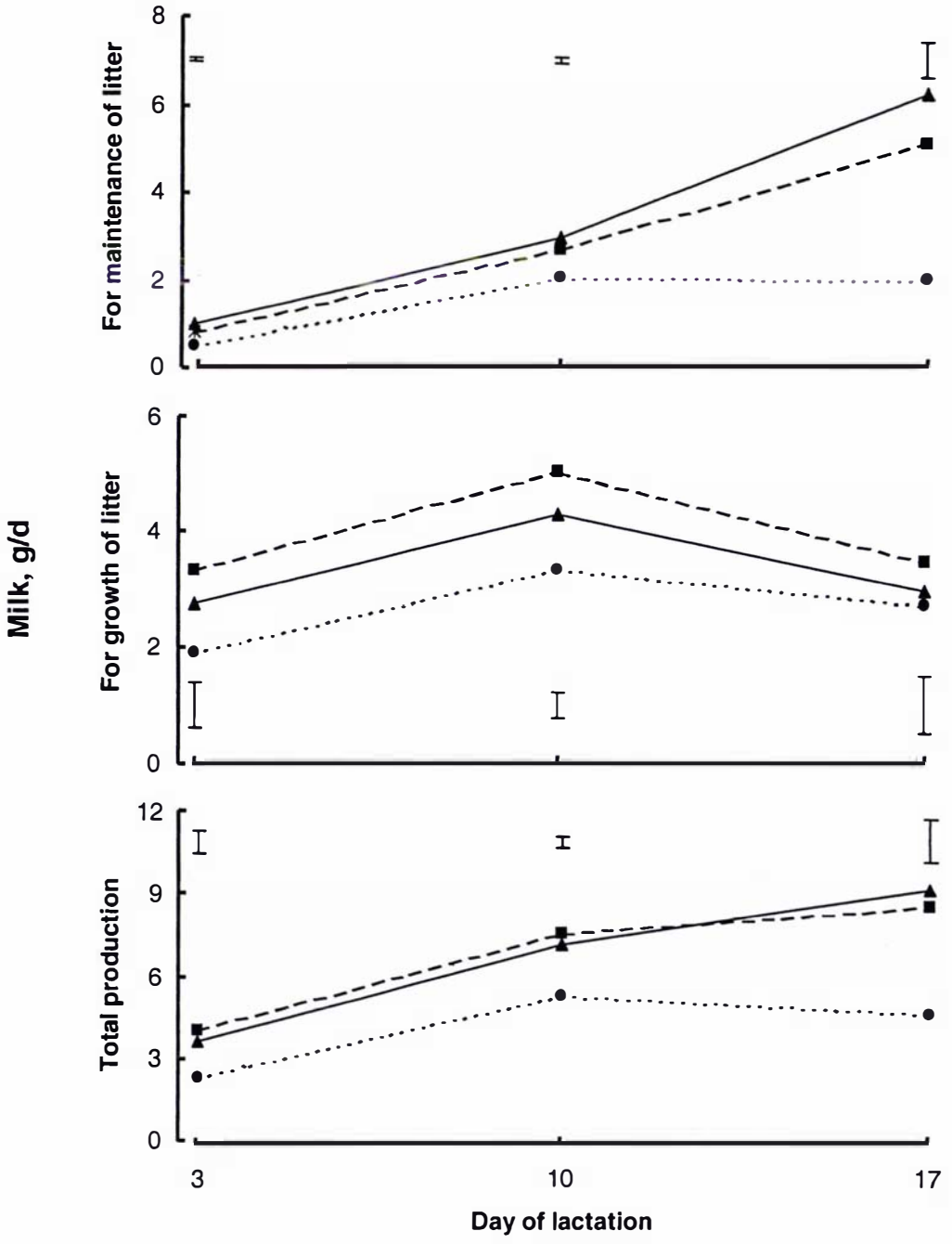


Figure 3.2: Milk production of mice estimated from fasted weight losses.

Milk production (g/d) (lower panel) of mice estimated from the requirements for growth (middle panel) and maintenance (upper panel) of litters of 5 (●), 10 (■) and 15 (▲) pups. The maintenance milk requirements were calculated from 24 h fasted weight losses, and milk required for growth was calculated from growth rates while total milk production was the sum of the two. Vertical brackets represent standard errors of differences among the means, n > 8 for each mean value.

3.4.2.2 Metabolic weight method

The metabolic weight method was the second method used to calculate milk yield. It was based on estimating maintenance energy requirements of litters by calculating metabolic weight and the energy required to support it. Metabolic mass of the litters was calculated from the equation:

$$\text{Metabolic mass of litter} = \text{litter size} \times W^{0.83}$$

where W is the average weight (kg) of the individual pups in the litter. The metabolic weight of young in litters is related to $W^{0.83}$ rather than $W^{0.75}$ which is a closer approximation for mature animals (Oftedal, 1984).

The gross energy requirement for maintenance of the litter was calculated assuming a gross energy requirement of 0.53 MJ/kg metabolic mass. This is the value calculated by Lin *et al.* (1979) for three to six week old mice fed a carbohydrate diet and is similar to that quoted by Deb *et al.* (1976) for six to 10 week old lean Zucker rats although higher than that reported by Pullar and Webster (1974) for lean Zucker rats (0.42 MJ/kg metabolic mass).

Estimates of milk production required to supply the maintenance energy requirements of litters of 5, 10 or 15 pups at the three stages of lactation are presented in Figure 3.3. The milk production required to support growth used the same data and assumptions as for Figure 3.2. Milk production to support growth and maintenance was summed to yield total daily milk production and is illustrated in Figure 3.6.

Milk for maintenance requirements, as shown in Figure 3.3, increased for all litter sizes over lactation and was greatest for the heaviest litters (15 pups). This was expected as yield was calculated using litter weight data that increased over time. Milk required for litter growth (Figure 3.2) reflected measured growth rates, which were highest for litters of 10 pups in mid lactation and lowest for litters of 5 in early lactation. Total milk yield (Figure 3.6) was curvilinear with milk yield being lowest for litters of 5 pups while yield for litters of 10 and 15 pups were similar suggesting increased yield does not occur when extra pups are added to a litter of 10.

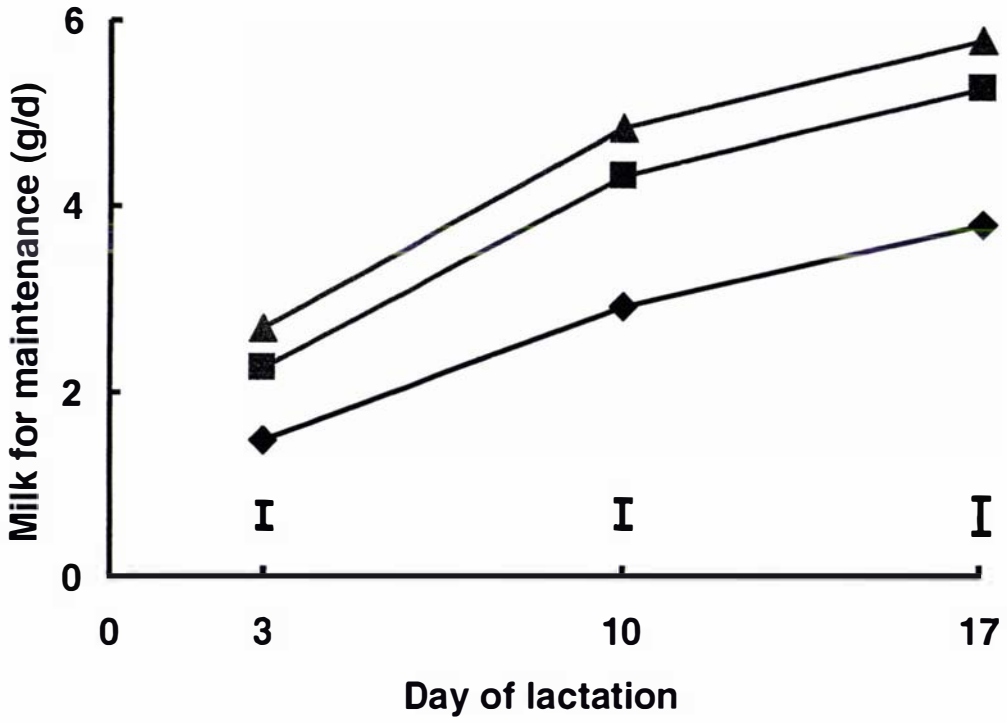


Figure 3.3: Milk production of mice estimated from the metabolic weight of the litters.

Milk production (g/d) of mice on D3, D10 and D17 of lactation estimated from the metabolic weight of litters of 5 (♦), 10 (■) and 15 (▲) pups and their growth rates. Vertical brackets represent standard errors of differences among the means.

3.4.2.3 Proportion method

The third method of calculating milk yield was the proportion method. This was based on a relationship between milk production and litter gain which was observed by Cowie and Folley (1945) in the data of Brody and Nisbet (1938). Cowie and Folley (1945) calculated that the average daily gain of rat litters was 50–60% of the milk produced, e.g., for a litter that grew at 1 g/d, the milk produced was 1.8 g/d. This relationship was applied to the mouse litter daily gains measured and reported in this chapter. For ease, it was assumed that litter daily gain was equivalent to 55% of the total milk produced. Total milk production of mice (Figure 3.4) was calculated from the litter growth rates.

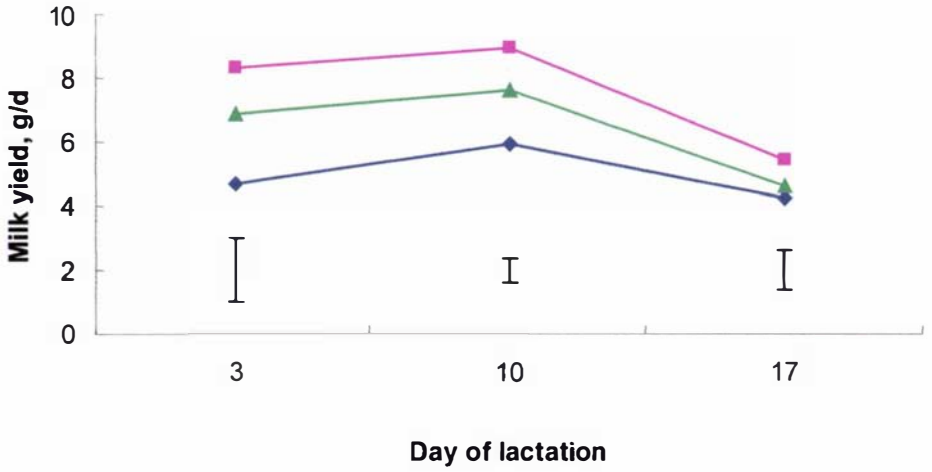


Figure 3.4: Milk production of mice estimated as a proportion of weight gain.

Milk production (g/d) of mice with litters of 5 (♦), 10 (■) or 15 (▲) pups on D3, D10 and D17 of lactation assuming litter gain (g/d) is 55% of total milk production (g/d). Vertical brackets represent standard errors of differences among the means.

Using the proportion method, the highest milk yield was produced for litters of 10 pups, the lowest being for litters of 5 pups with litters of 15 receiving an intermediate amount of milk in early and mid lactation. In late lactation milk yield decreased for all mice so that there were only small differences between mice suckling different sized litters.

The data in Figure 3.4 reflects the daily weight gains of the litters hence the estimated milk yields for litters of 10 are greater than those for litters of 15. Actual yield would be expected to be greater for litters of 15 compared to 10, or the same if peak yield is reached with 10 pups. Despite the relationship being observed from D1 to 18 of lactation for rat dams with litters varying from 7 to 10 pups, Cowie and Folley (1945) caution that it is an approximation only and ideally should be used only as a relative measure between litters of the same size at the same stage of lactation. The simplistic relationship does not take into account the different maintenance energy requirements of different sized litters or the increasing proportion of milk used for maintenance compared to growth as pup size increases.

3.5 Discussion

3.5.1 Milk composition study

This is the first report of the concentration of lactose in milk from Swiss mice and the first for early, mid and late lactation for any strain of mouse.

The average increase in lactose concentration in milk from Swiss mice was 1.2 g/l for every day from D3 until D17 of lactation. The results of the current study are compared with those obtained from other studies in Figure 3.5. The concentration of lactose in milk reported by Ragueneau (1987) and Nagasawa *et al.* (1989) for nine different strains of mice was similar to this study and it is noteworthy that the authors used the same method of lactose analysis as this study. In contrast, the lactose concentration fell after mid lactation in the study by Knight *et al.* (1986b), however, the authors did not actually measure the lactose concentration. Instead it was calculated from their data by the present author by subtracting the protein and fat concentrations from total solids and then by assuming that the remainder was comprised of approximately 55% lactose and 45% ash (Nagasawa *et al.*, 1989). Allen (1984) reported lactose concentrations of more than twice those measured in the present study. It is likely however, that this large difference is due to a different strain of mice or differences in the methods of lactose analysis. Allen (1984) used a glucose analyser converted to detect lactose with a galactose oxidase impregnated membrane. Analytical factors may also account for the differences in lactose concentrations reported here and by Ben Shaul (1962) who used an unspecified method to measure total carbohydrates rather than just lactose. The continual increase in lactose concentration measured in the present study goes against the dogma that lactose remains constant owing to its role as the main osmoregulator of milk. Similar results have been reported in rats where the concentration of lactose in milk increased more than two fold from D6 and 16 of lactation (Fiorotto *et al.*, 1991; Nicholas and Hartmann, 1991).

Protein concentration in milk from Swiss mice did not vary from 115 $\pm$ 8.3 (mean $\pm$ SEM) g/l over lactation. This was less than for the strains reported by Meier *et al.*

(1965), Allen (1984) and Ragueneau (1987), but greater than that reported by Ben Shaul (1962) (Figure 3.5). Knight *et al.* (1986b) and Nagasawa *et al.* (1989) reported similar values to this study. Milk fat concentration in Swiss mice also did not vary from 25.3% ± 1.0% (mean ± SEM) over lactation. The Swiss mice however, secreted milk with a higher fat content than those reported by Ben Shaul (1962), Meier (1965), Ragueneau (1987) and Nagasawa (1989) but less than those reported by Allen (1984) and by Knight *et al.* (1986b) after D6 of lactation (Figure 3.5). In contrast to the present study, the pattern of fat secretion reported by Knight *et al.* (1986b) showed a significant increase in fat concentration as lactation progressed.

This study provides detailed data on the composition of mouse milk over the whole of lactation and addressed some of the technical problems associated with previous compositional studies which may lead to less consistent results. For example, the experimental design for this study used 6 mice for repeated measures over lactation rather than different groups as Ragueneau (1987) did. The sampling procedure used a short separation period of 3h unlike Ragueneau (1987), Nagasawa (1989) and Meier (1965). Milk stasis occurs in the rat after a 4h separation period (Hanwell and Linzell, 1972) after which tight junctions between mammary epithelial cells are disrupted allowing two way movement of plasma and milk components which alter milk composition so that milk lactose concentration decreases (Yamauro *et al.*, 1993). In addition, the concentration of oxytocin used was reduced to 0.15 I.U. to avoid the effects of super pharmacological doses of oxytocin which affect lactose concentration (Linzell and Peaker, 1971; Linzell, 1972; Allen, 1984). Milk expressed from the gland was collected by capillary action rather than by the suction methods used by Meier (1965), Nagasawa (1989) and Allen (1984) which can increase evaporative losses (Oftedal, 1984). Sufficient milk was collected so that a subsample could be taken for analysis. Further proof of the glands being well milked out was the constant fat content which indicates that milk contained both fore and hind milk. Laboratory analyses of the milk components included lactose, which was an improvement on estimating it from total solids with protein and fat subtracted, as was necessary for the composition studies by Knight *et al.* (1986b) and Meier (1965). The other studies reported milk composition for many different strains of mice but not for Swiss mice. Calculation of milk content of murine mammary glands has been attempted previously (Knight and Peaker, 1982d) but the lactose value used was

neither from the same mice nor from the same stage of lactation and therefore yields erroneous results. This study was essential to complete and the results arising are considered as accurate and reliable as can be achieved using such a small amount of sample.

[1]Footnote to Figure 3.5:

Auldist Content of lactose, protein and fat in mouse milk collected on D3, D10 and D17 of lactation. Results reported in Chapter 3 of this thesis.

Allen (1984) Pooled milk analysed from collection between D12 to D15. Fat was calculated for the graph by multiplying the creamatocrit measurement by the correction factor of 0.868 as done by Knight *et al.* (1986b) who measured the packing volume of fat in mouse milk cream using a method first described by Fleet and Linzell (1964) for estimating fat concentration in goat milk.

Ben Shaul (1962) Collection day unspecified, carbohydrates measured not lactose.

Knight *et al.* (1986b) Lactose not measured in paper. Lactose for this graph was calculated as Total Solids – (Fat + Protein) = X, then assuming 55% of X is lactose according to Nagasawa *et al.* (1989).

Meier *et al.* (1965) Pooled milk analysed from collection between D8 to D14. Lactose not measured in paper. Lactose for this graph was calculated as Total Solids – (Fat + Protein) = X, then 55% of X is lactose according to Nagasawa *et al.* (1989).

Nagasawa *et al.* (1989) Only 1st lactation data used, four strains of mice.

Ragueneau (1987) Data from five strains of mice.

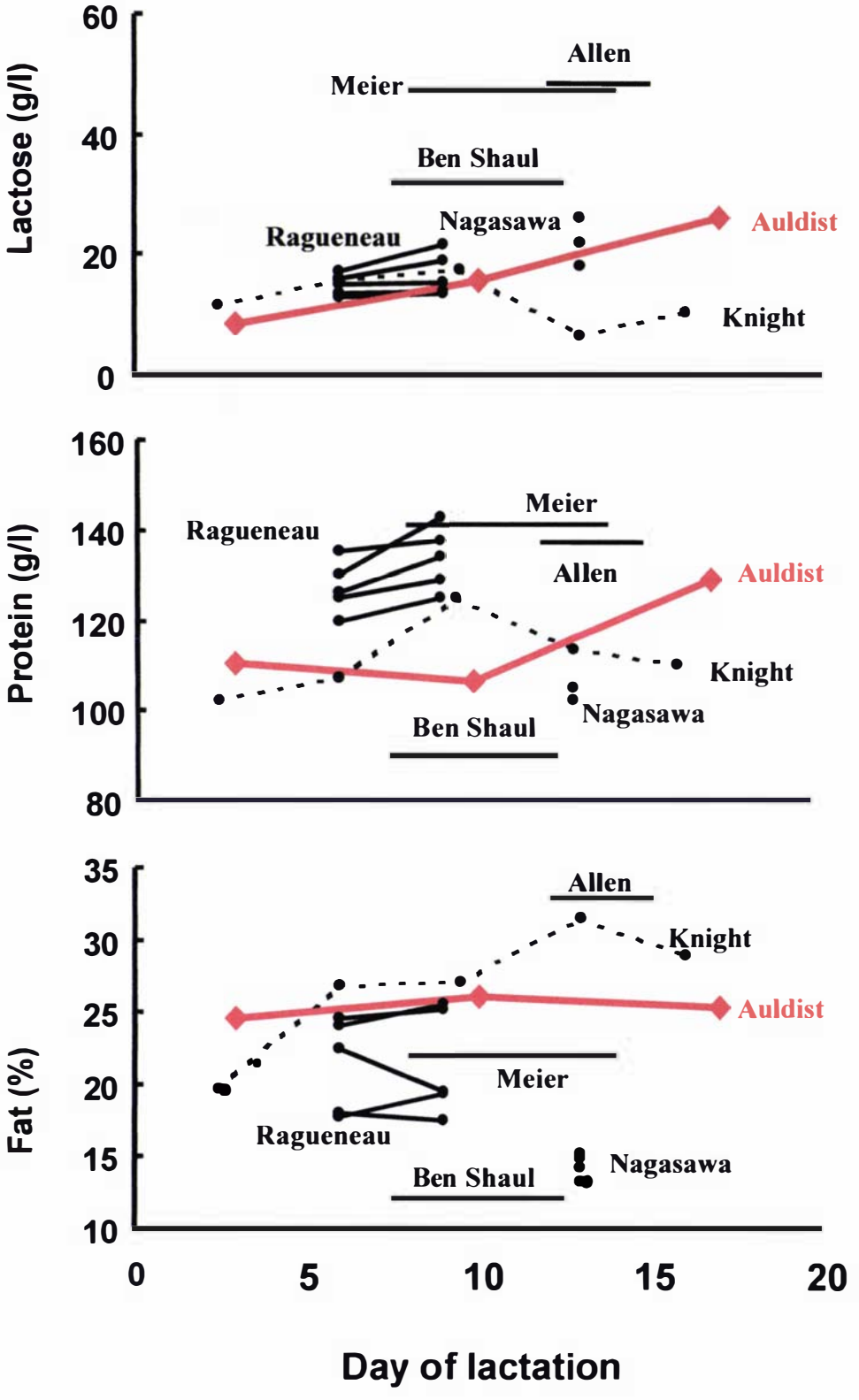


Figure 3.5: Content of lactose, protein and fat in mouse milk throughout lactation.[1]

3.5.2 Milk yield study

Milk production calculated by the fasted weight loss method resulted in lower yields for mice with litters of 5 pups compared to those with larger litters. Mice with litters of 5 pups produced a peak of 5.3 g of milk per day in mid lactation. Milk production of dams with litters of 10 or 15 pups were similar ($P>0.1$) in early (4.1, 3.7 g/d respectively) and mid (7.7, 7.3 g/d respectively) lactation (Figure 3.2). This suggests that milk yield of Swiss mice is at its maximum level once the litter contains 10 pups and extra pups do not have the effect of further increasing milk yield. The milk required for maintenance of the litters increased with litter size while the milk required for growth was greatest for litters of 10 pups.

Various estimates for milk production indicate that those for D17 differ significantly from those earlier in lactation. Thus milk required for maintenance increases dramatically at D17 for litters of 10 and 15 pups yet plateaus from D10 for litters of 5 pups (Figure 3.2). Also, milk yield is curvilinear during lactation for mice with litters of 5 pups yet it increases linearly for mice with litters of 10 and 15 pups. Closer examination of the regressions fitted to the raw data in Figure 3.1 also reveals cause for concern regarding the D17 data. The greater slope of the regression line of weight loss against litter weight on D17 in comparison to D3 and D10 (Figure 3.1) also indicates that there are significant differences in the underlying biology at different times. In addition, the RSDs of the regressions at D3 and D10 were low (0.4, 0.6 respectively) compared to that for D17 (1.6). This means that the initial litter weight is a poor predictor of weight loss after fasting for 17 day old litters yet it is a good predictor for the younger litters. These various differences between the data for D17 in comparison with those for D3 and D10 may reflect developmental and behavioural differences in 17 day old litters compared to younger litters. As pups mature, they grow fur and their eyes open. Behaviourally, they spend less time huddling for warmth and more time being active and investigating away from the nest. These changes mean that litters of 17 day old pups are not acting as one organism as is assumed for the 3 and 10 day old litters. In addition, pups are able to eat dry food from about D15. In the fasting experiment, pups were denied access to milk but the 17 day old pups had access to dry food so that at D17 the weight losses are confounded by an unknown intake of dry food. The large variation in weight loss

of 17 day old litters shows that removing the dams affected litters differently. The litters of 15 pups had bigger weight losses (6.1 ± 1.1, mean ± SEM) after fasting than did those in litters of 10 (3.7 ± 1.9, mean ± SEM) and 5 (1.6 ± 1.8, mean ± SEM) pups showing they are more dependent on milk than the smaller litters. This is probably due to pups in litters of 15 being smaller and therefore not able to reach dry food as easily as larger pups. In conclusion, the data on weight loss in 17 day old pups collected in this trial are not a reliable estimate of the maintenance costs of the pups.

The estimation of milk yield using growth rates and fasted weight losses involved several assumptions. To estimate maintenance requirements, litters were fasted for 4–6h and their weight loss was calculated up to a loss over 24h. It was assumed that the weight lost by the litter through the metabolism of body stores to meet maintenance energy requirements would be in proportion to body composition. During a fast however, monogastrics absorb and use any nutrients in the gut before body fat and body protein are used. Weight loss also occurs due to dehydration. Urination and defaecation by 3 and 10 day old pups would not contribute to weight loss during the fasting period as these functions only occur when stimulated by maternal licking of the pup perineal region (Baverstock and Green, 1975; Walser, 1977). Pups at D17 however would be able to urinate and defaecate without the assistance of their mother and therefore this would contribute to error. Perspiration is also another small source of error for pups of all ages (Fiorotto *et al.*, 1991). Reddy and Donker (1965) measured the weight loss attributed to respiration, urination and defaecation in rats as being approximately 8% of litter weight per day. These losses were measured by recording the weight loss of litters when separated from the dam and calculating this up to a loss over 24h. As this is essentially what has been done in the present study and there is no clear way to distinguish between insensible weight loss and basal metabolism, no correction was made to account for insensible weight loss.

The calculations of milk yield also required information and assumptions about body composition. Body composition data taken from the literature showed a consistent relationship with increasing age although none of the studies analysed body composition at more than two ages of pup (Table 3.6 in Chapter 3 Appendices). The

compiled mouse data followed similar patterns of change as suckling rats (Spray and Widdowson, 1950; Roberts and Coward, 1985; Fiorotto *et al.*, 1991) which are born very lean and then increase protein and especially fat content as they approached the age of weaning.

Information on the amount of milk required to suppor growth in litters of mouse pups was not found in the literature and therefore assumptions had to be made. Weight gain was assumed to consist of protein and fat only, whereas it also comprises carbohydrates and bone. The energy cost of tissue deposition therefore did not include all possible types of tissue, but it covered the major energy costs. Brody and Nisbet (1938) assumed rat litters required 6.2 kJ per gram of weight gain. This is similar for 3 day old mouse litters (Table 3.4) and less than for 10 and 17 day old litters, but the differences are due to the assumptions of Brody and Nisbet (1938) about body composition and no allowance for the increasing fat content of litters with age. Brody and Nisbet (1938) also calculated that rat milk provided 9.6 kJ/g which was lower than the present study (Table 3.4) but again the difference was due to a difference in milk composition. The amount of milk required to support a 1 g gain in litter weight ranges from 0.65 g (Brody and Nisbet, 1938) to 1 g (Sampson and Jansen, 1984; Grigor *et al.*, 1987) for rat litters. For the purposes of the present study, a 1 g gain in litter weight required 0.73, 1.02 and 1.16 g of milk for 3, 10 and 17 day old litters respectively (Table 3.4).

Sampson and Jansen (1984) and Grigor *et al.* (1987) estimated milk yield using a similar technique to that reported here. Sampson and Jansen (1984) fasted rat pups aged 3 to 13 days and the data on weightloss on fasting were used with other pup data to derive the equation: *milk yield, g/d = 0.0322 + 0.0667 pup weight + 0.877 pup gain.* Grigor *et al.* (1987) measured fasted weight loss of rat litters on D14 of lactation which, with litter daily gain, was used to derive the equation: *milk yield, g/d = (0.08 litter weight) + litter gain.* Sampson and Jansen (1984) and Grigor *et al.* (1987) used the assumption that 1 g of milk was required for 1 g gain whereas the present study found that 0.73, 1.02 and 1.16 g of milk was required for 1 g of gain in early, mid and late lactation respectively. The studies also made no attempt to measure the weight loss caused by respiration, urination, defaecation and perspiration of the litters but instead measured it as part of the fasted weight loss.

This was because the parameters contributing to weight loss are similar for all pups at a given age so only relative differences are estimated (Sampson and Jansen, 1984; Grigor *et al.*, 1987). The two equations shown above cannot be used directly with the data collected from the present study owing to the species difference and therefore the composition of milk and rate of development of young. It was more accurate to have measured the fasted weight loss of the mouse litters, adding this to daily gain and converting to an amount of milk, as done in this study, than to have used either of the published equations.

Comparisons of milk yields calculated using the fasted weight loss method; the metabolic weight method and the proportion method are presented in Figure 3.6. For each litter size at D3 and 10 of lactation, the milk yield estimates using the metabolic weight method was higher than for the linear regression method although the two were parallel to one another. The highest milk yield for D3 was predicted by the proportion method although by D10 it was more similar to the other two estimates. D17 milk yield data have errors associated with them that have been previously discussed.

The three methods each gave different estimates of milk yield of mice during lactation for different litter sizes (Figure 3.6). The proportion method, however, was not considered as accurate as the other two methods as it is only an approximation derived from data collected from rat litters of only 7 to 10 pups (Brody and Nisbet, 1938). The linear regression method has flaws such as the assumption that weight loss is in proportion to body composition and the unaccounted developmental differences between young and old pups, but the method still results in similar (although lower) yields to those derived using the metabolic weight method for D3 and D10 of lactation. The metabolic weight method is based on scientifically proven relationships of metabolic weight and maintenance energy requirements (Brody, 1945; McDonald *et al.*, 1988) and therefore should be considered a reliable predictor of milk yield for D3 and D10 of lactation. As pups only begin eating dry food on D15, estimates of milk yields up to D17 may still give results that are useful for comparison purposes. The metabolic weight method will be used in subsequent chapters because the milk yield curve during lactation is more consistent between litter sizes compared to the linear regression method.

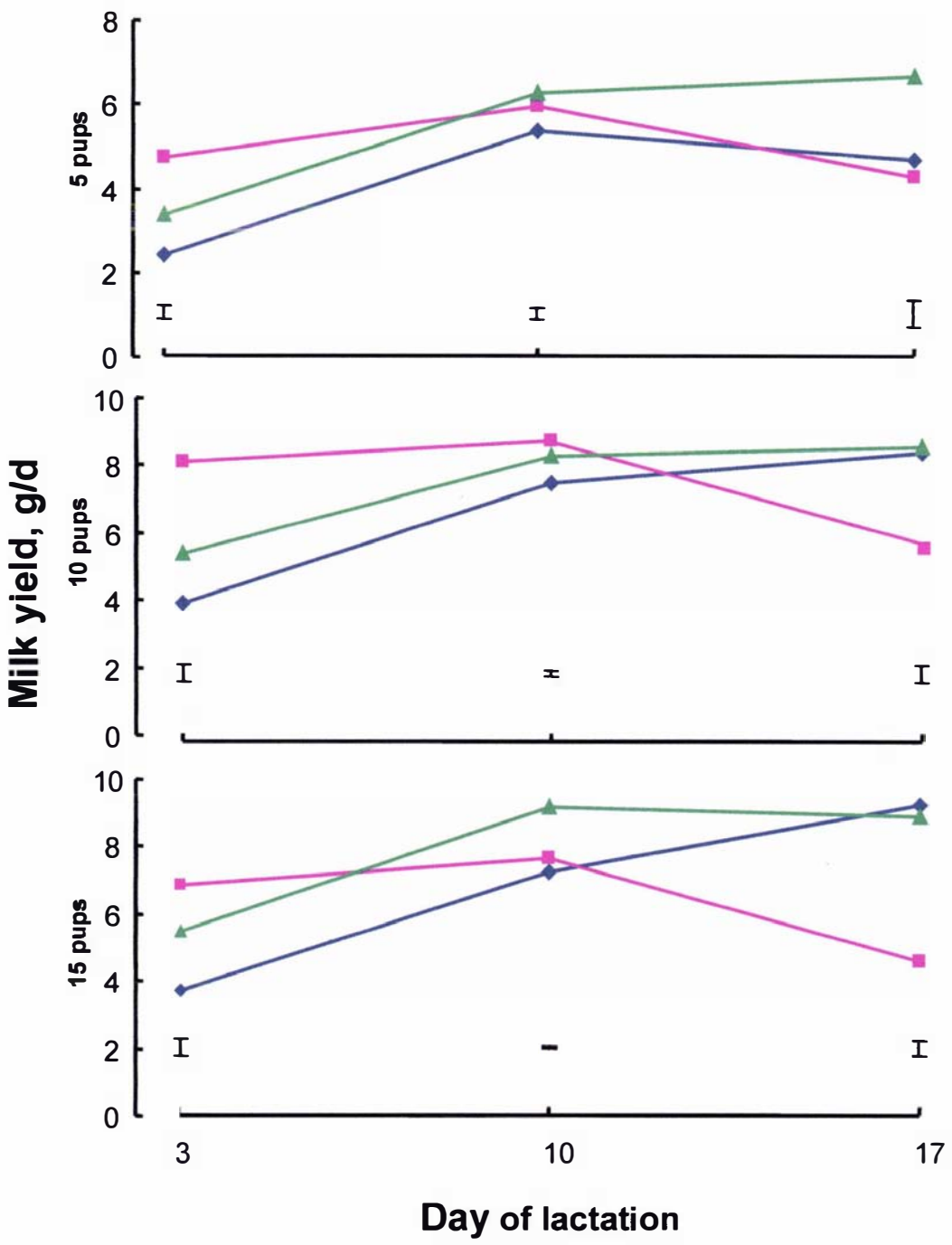


Figure 3.6: Comparison of milk production throughout lactation calculated using the linear regression, the metabolic and the proportion methods.

Milk production (g/d) on D3, D10 and D17 of lactation calculated using three methods for litters of 5 (upper panel), 10 (middle panel) and 15 (lower panel); ♦ = energy for maintenance requirements calculated using linear regression of fasting weight loss, added to energy for observed growth, summed and converted to yield total milk production. ▲ = energy for maintenance requirements calculated using metabolic weight, added to energy for observed growth, summed and converted to yield total milk production. ■= calculated using the proportion method

which uses the relationship of litter gain being 55% of milk produced. Vertical brackets represent standard errors of differences among the means.

A comparison of milk yields in the literature with those calculated using the metabolic weight method reveals some differences that are immediately noticeable (Figure 3.7). The differences may be due to factors such as the strain of the mouse and therefore different live weight, natural lactation potential and mothering ability as well as environmental factors such as nutritional quality of food provided. The high yields reported by Knight *et al.* (1986b) were from a strain of mouse that was heavier than the strain used in the present study. In addition, some variation in absolute milk yields of mice may be explained by the different measuring techniques and their associated errors. For instance, the very low yield reported by Hanrahan and Eisen (1970) illustrates the disadvantage of methods that rely on milk removal by humans rather than the more effective pups. Absolute milk yields were in agreement however, between the present study and those of Jara-Almonte and White (1972), Lin *et al.* (1977) and Rath and Thenen (1979).

Despite some differences in absolute milk yields there were similarities between some of the studies. Similar relative differences existed in milk yield for different litter sizes. Knight *et al.* (1986b) determined the milk yield of mice with 4 pups was approximately 60% that of mice with 10 pups. This was similar to the study by Rath and Thenen (1979) where milk yield of mice with 5 pups was about 55% of the milk yield of mice with 10 pups. In the present study, there were also similar relative differences in milk production between litters of different sizes as mice with 5 pups produced approximately 65% of that of mice with 10 pups.

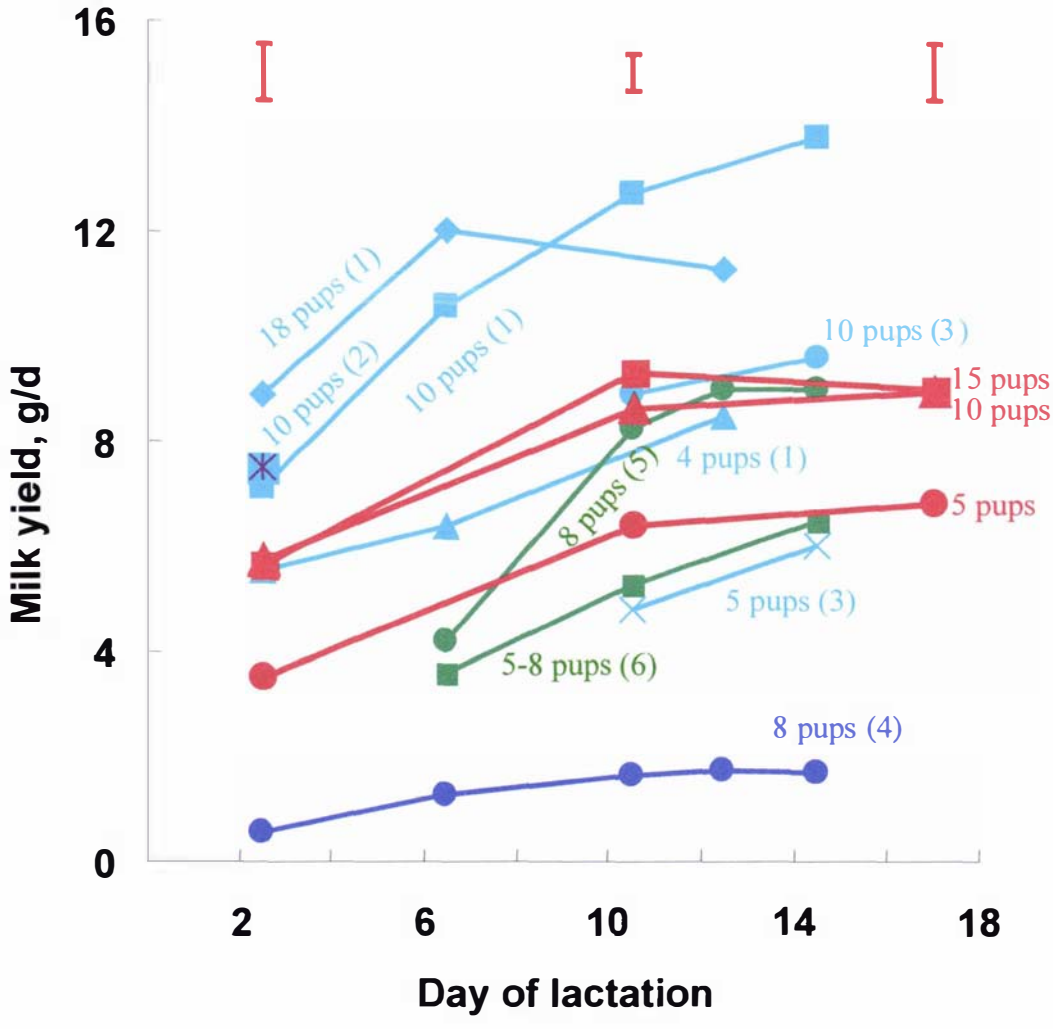


Figure 3.7: Comparison of milk production of Swiss mice with that reported in the literature.

Aqua lines represent data derived using isotope methods, green lines represent data derived using the weigh-suckle-weigh method, blue line represents data derived using direct milking and the red lines represent data derived using the metabolic weight method. Vertical red brackets represent pooled standard errors of differences for the data calculated using metabolic weight. The text on each series of data give the number of mouse pups in the experiment followed by a number which denotes the author of the publication.

Key to Figure 3.7;
(1) Knight *et al.* (1986b) 4, 10, 18 pups/litter, isotope dilution in pups
(2) Knight et al. (1983) 10 pups, isotope method in pups
(3) Rath and Thenen (1979) 5 and 10 pups/litter, isotopic transfer from dam to pups
(4) Hanrahan and Eisen (1970) 8 pups/litter, direct milking, separated for 8h then injected with oxytocin
(5) Jara-Almonte and White (1972) 8 pups/litter, weigh-suckle-weigh, dam and litter separated for 6h, reunited for 1.5h
(6) Lin *et al.* (1977) 5–8 pups/litter, weigh-suckle-weigh, dam and litter separated for 6h, reunited for 1h.

Another similarity between the literature and the present study was that milk yield was near maximal for litters of 10 pups and extra pups did not result in extra milk. Milk yield was greater for mice with 18 pups up to D6 of lactation after which yield was lower than for mice with 10 pups (Knight *et al.*, 1986b). In the present study, milk yield did not differ between litters of 10 and 15 pups. Any differences in these litter sizes in future experiments would therefore presumably be due to suckling intensity whereas differences between litters of 5 and 10 pups would also be due to increased milk removal.

Confidence is gained for the metabolic weight method of calculating milk yield when it is compared to other studies. The method produces results with relative differences between different litter sizes and reveals milk production to be maximal with litters of 10 pups, both of which are reported in the literature (Rath and Thenen, 1979; Knight *et al.*, 1986b). The method also produces absolute milk yields similar to some of those reported by other groups (Jara-Almonte and White, 1972; Lin *et al.*, 1977; Rath and Thenen, 1979).

In conclusion, the two studies presented in this chapter provided essential information for use in subsequent chapters. In the milk composition study, the Swiss mice produced milk in which the lactose concentration increased linearly over the lactation but protein and fat concentration did not alter significantly. These data will enable more accurate calculation of the milk content and milk protein content of homogenised mouse mammary glands dissected in experiments. In the milk yield study, the preferred method was that using the metabolic weight to determine maintenance requirements. This method is more accurate than the proportion method and less labour intensive than the linear regression method. Using the metabolic weight method, milk yield of mice with 5 pups was approximately 65% those with 10 pups, and milk yield was maximal when mice suckled 10 pups. Both these results are consistent with those of previous studies reported in the literature. In addition, the absolute milk yields estimated in the present study were in agreement with those from three other studies. The metabolic weight method was reliable for litters aged 3 and 10 days old and although not ideal for 17 day old litters it was preferable to both other methods. The metabolic weight method will be used in subsequent chapters to estimate milk production.

3.6 Appendices

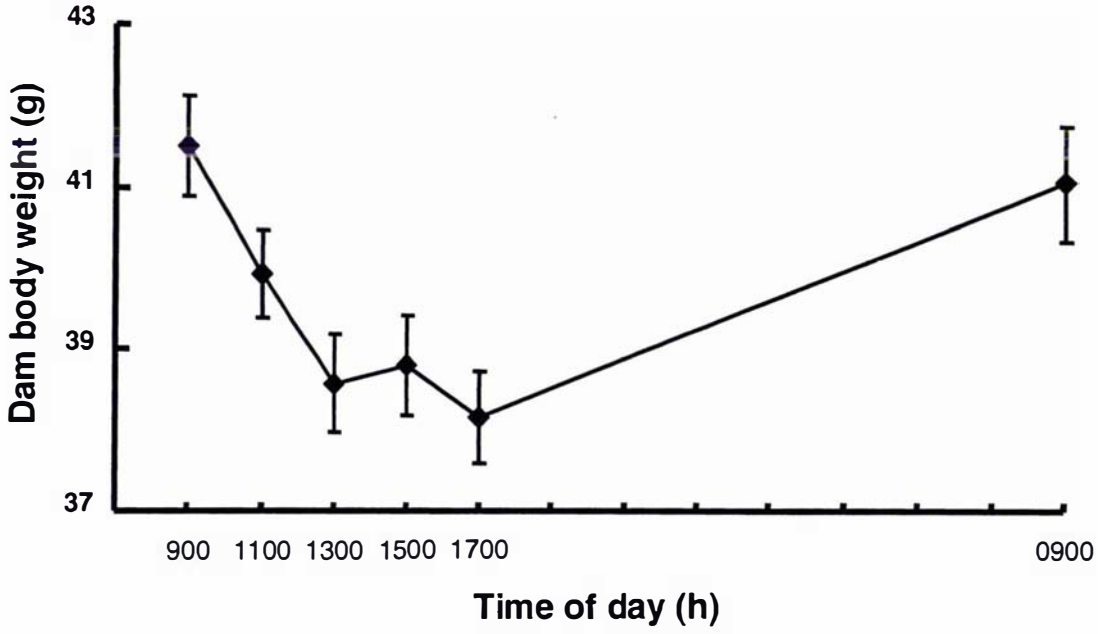


Figure 3.8: Diurnal variation of live weight of lactating Swiss mice.

Mean values (n=9) for dams with 5 to 15 pups per litter on D7 to D15 of lactation. Vertical brackets represent standard errors of means.

Table 3.5: Composition and energy content of mouse milk collected throughout lactation.

Mean concentrations of lactose (g/l), protein (g/l) and fat (%) in milk collected from Swiss mice on D3, D10 and D17 of lactation, and calculated energy content (kJ/g milk). Milk composition values are from Table 3.1.

	Day of lactation		
Milk component	3	10	17
Lactose (g/l)	8.7	15.7	25.9
Protein (g/l)	110.5	106.6	129.0
Fat (%)	24.6	26.0	25.3
Energy content* (kJ/g)	12.4	13.0	13.4

*The energy content of 1 g of milk is the sum of energy contained in the 3 individual components, where gross energy values of 17, 24 and 39 J/mg were used for carbohydrate, protein and fat respectively.

Data on the body composition of pups (Table 3.6), gross energy of tissue types and energy costs of tissue deposition (Table 3.7) used to calculate the energy requirements of litter growth were obtained from the literature.

Table 3.6: Body composition of rat and mouse pups.

Body composition of protein (%) and fat (%) of rat and mouse pups from D0 to D21 of age.

Age of pups, rodent type, reference source	Body Protein %	Body fat %
D0, (Widdowson, 1950)	12.5	2.1
D0, (Spray and Widdowson, 1950)	12.5	2.1
D7, mouse, (Boissonneault *et al.*, 1978)	---	6.4
D11-12, mouse, (Ghusain-Choueiri and Rath, 1995)	12.9	12.7
D14, mouse, (Boissonneault *et al.*, 1978)	---	13.5
D21, mouse, (Purchas *et al.*, 1985)	---	12.6
D21, mouse (Lin *et al.*, 1977)	18.1	13.3

The information in the above table was graphed and lines of best fit applied. The regression equations were:

Protein in body (%) = $(0.2367X) + 12.07$, $R^2 = 0.775$

Fat in body (%) = $(-0.0318X^2) + (1.2139X) + 1.7642$, $R^2 = 0.951$

where X = pup age in days.

Using the equations above the estimates of protein content of pups aged 3, 10 and 17 days were 12.8, 14.5 and 16.1% respectively. The estimates of fat content of pups aged 3, 10 and 17 days were 5.1, 10.7 and 13.2% respectively.

Table 3.7: Gross energy content of fat, protein and carbohydrate and energy cost of tissue deposition.

Gross energy content of fat, protein and carbohydrates (J/mg) and energy cost of tissue deposition (J/mg), (McDonald *et al.*, 1988).

Tissue type	Gross energy (J/mg)	Energy cost of deposition (J/mg)
Fat	39	53.5
Protein	24	42.3
Carbohydrate	17	---

Chapter 4 Assessment of proliferation model I

The effect of exogenous steroids on proliferation of epithelial cells in the mammary glands of lactating mice

4.1 Summary

The literature indicates that exogenous steroids can increase milk production by increasing cell number when administered to lactating animals. However, the results are extremely variable and influenced by both the dose and stage of lactation. In the experiments described in this chapter, three different steroid treatments were administered for four days to mice in mid and late lactation, and cell proliferation measured by the incorporation of [^{3}H] thymidine into DNA. Treatments were a low dose of E, a high dose of E and a high dose of E+P. A control group received excipient only. In mid lactation the steroid treatments had no effect on the incorporation of [^{3}H] thymidine into DNA but in late lactation, increased doses of E caused increased incorporation of [^{3}H] thymidine into DNA. The addition of P to the high dose of E had no additional effect compared to estrogen alone. Mice treated with the high E dose in late lactation had the lowest content of DNA but the highest incorporation of [^{3}H] thymidine into DNA. This is consistent with gland remodelling in late lactation. These mice had litters with decreased growth rates and mammary glands that appeared to be in the early stages of involution as shown by histological sections. This led to the conclusion that exogenous E causes mammary glands to involute when administered in late lactation. In this study, steroid treatments did not stimulate mitosis in mammary epithelial cells during either mid or late lactation.

4.2 Introduction

Milk yield is determined by the number of mammary epithelial cells and the output of milk from each cell (Knight and Peaker, 1982b). Milk yield can therefore be increased by increasing the number of mammary epithelial cells, as well as increasing the milk output per cell. An increase in the population of mammary epithelial cells in pubertal and sexually mature mice can be stimulated by many

mammogenic hormones and growth factors including the ovarian hormones, E and P, which induce proliferation of epithelial cells in mature mammary glands (Topper and Freeman, 1980). Specifically, estrogen induces progesterone receptors in the epithelial cells, while progesterone stimulates ductal side-branching and alveolar development in mammary glands of sexually mature mice (Fendrick *et al.*, 1998). Much less is known about the effect of these hormones on mammary epithelial cells in lactating glands.

Exogenous E administered to lactating ruminants produces extremely variable results. Early studies reported milk yields of cows increased 4% after being fed diethystilboestrol (11 mg per 500 kg) with the increase being attributed to improved persistency (Browning *et al.*, 1957). Improved persistency was also recorded in a study of cows in late lactation fed diethylstilbestraol (10 mg/cow/d for 4 weeks) (Turner *et al.*, 1957). Conversely, milk yield of goats decreased severely after they were injected intra-muscularly with E (5 mg/d for 2 days) (Peaker and Linzell, 1974). A study in ewes suggested that some variation is accounted for by the dose of E given. A low dose of E (500 μg/d for a week) injected into ewes caused an increase in milk yield, yet a high dose (5000 μg/d for a week) caused a severe decline in milk yield (Fulkerson and McDowell, 1974). Variation in response to exogenous E can also be due to the stage of lactation. In late lactation, mammary glands of ewes responded to exogenous E (500 μg/d for a week) with an increase in milk yield yet they were unresponsive in early lactation (Fulkerson and McDowell, 1974). These early studies in ruminants determined that both the dose of E and the stage of lactation contribute to the variation in response to exogenous E, but all studies evaluated the response in terms of milk yield and none evaluated responses within the gland.

Later studies using mice measured cell proliferation, as indicated by [^{3}H] incorporation into DNA, to improve the understanding of responses of lactating animals to exogenous steroids (Knight and Peaker, 1982d; Shyamala and Ferenczy, 1982). They were extremely variable and were influenced by dose and stage of lactation. In early to mid lactation, a low dose of E and P (0.2 μg + 0.6 mg/d respectively) for 7 days caused a decrease in litter growth rate and no change in cell proliferation, yet in late lactation the same dose caused the opposite responses

(Knight and Peaker, 1982d). In mid lactation, mammary glands did not respond to either a low dose of E and P (0.2 μg + 0.6 mg/d respectively) for 7 d (Knight and Peaker, 1982d) or high E dose (3 μg/d) (Shyamala and Ferenczy, 1982) as neither treatment caused a change in cell proliferation. A response in mid lactation was however, caused by injection of an extremely high dose of E (10 μg/d) that stopped milk secretion and increased cell proliferation (Nagasawa and Yanai, 1978). A study that showed exogenous steroids increased milk yield was reported by Knight and McLelland (1988). Mice in the extended lactation study were injected from D 15 to 21 with a high dose of E+P (1 μg + 1 mg/d, respectively) which caused milk yield to decrease for 2 weeks after which yield increased above controls. Although cell proliferation was not measured, the authors speculated that yield was depressed during proliferation of new secretory cells and subsequently elevated when the new cells began to secrete milk. Injections of exogenous steroids to lactating mice cause varied responses that include cell proliferation as measured by increased incorporation of [^{3}H] thymidine into DNA.

Although little is understood about the effects of E on lactating glands, there are sufficient reports in the literature to hypothesise that exogenous E can cause proliferation of mammary epithelial cells during lactation. In order to examine the variation in responses of mammary glands to E, effects of both dose and stage of lactation were assessed in the present study. In addition, the potential additive effects of P+E were examined. The objective of this study was to determine the proliferative effects in mammary tissue of a low dose of E, and a high dose of E with and without P, when administered to mice during mid and late lactation.

4.3 Experimental design

Forty Swiss mice were mated and transferred to individual cages at D15 of pregnancy. Twelve hours after parturition litter sizes were adjusted to 10 pups. Five dams were assigned to each treatment or control group at each stage of lactation. Animal husbandry and management are detailed in Section 2.1.

There were three treatments consisting of daily subcutaneous injections (100 μl) containing either 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4-pregnen-3, 20 dione (E+P) whereas the control group received injections of the excipient in which the steroids were dissolved (C). The excipient was a mixture of 25 % corn oil in ethanol. Mice were injected daily for four days commencing on either D5 or D12 of lactation for the mid and late lactation groups, respectively. This treatment period was considered adequate for any detectable changes in DNA concentration and cell proliferation (Sutherland *et al.*, 1983). Twenty-four h after completing the hormonal treatments at either D10 (mid lactation) or D17 (late lactation), mice were labelled *in vivo* with [^{3}H] thymidine (0.5 μCi/g body weight) and euthanased (Section 2.1.7).

Litter weights were recorded daily (Section 2.1.4) for eight days starting from three days before treatment and ending on the day of euthanasia of the dam. The 3rd and 4th mammary glands on both sides were dissected (Section 2.1.7) and the sum of the four glands was designated wet gland weight. The wet gland weight was corrected for milk content as described in Chapter 3 to obtain the milk-free weight, which was called the 'corrected gland weight'. Each of the left 3rd glands was homogenised and aliquots analysed for concentrations of lactose, DNA, protein and [^{3}H] thymidine (Section 2.3).

Milk production was calculated using the metabolic weight method (Section 3.4.2.2). Milk production per unit of DNA was calculated by halving milk production and dividing by the content of DNA in the 4 glands. Milk production was halved as the weight of the four glands collected had previously been determined to be half the weight of all 10 glands.

A whole gland mount of right 4th glands was prepared (Section 2.4). Autoradiographs of sections of the mammary glands were developed (Section 2.4.2) and counter-stained with haemotoxylin and eosin (Section 2.4.5). Labelling indices in specific locations of the gland were determined in three arbitrary and equal parts designated as the proximal, mid and distal regions. Within each of the three regions, labelling indices were counted on the outer edge (from the edge to 2 mm inside the gland) and compared to the inner sites of each region. The combination of three

regions and two sites for each region gave a total of 6 locations per gland that were analysed for labelling indices. Microscope fields (x200 and x400) were randomly selected in the autoradiograph sections, images captured (Section 2.4.6) and all labelled and unlabelled epithelial cells were counted. Cells with more than five dark grains over the nucleus were considered to be labelled. An average of 115 cells were counted at each of the six locations. Data are presented as means of each of the six locations. The number of cells counted per gland ranged from 600–700.

Data were analysed by a two way ANOVA (fitting treatment, stage of lactation and their interactions) using the GLM procedure of Minitab (Release 12.23, 1999) where individual mean comparisons were performed by LSD. The litter growth rates were also analysed using this procedure with the pre-treatment growth rates as a covariate. The pooled standard deviation was used to calculate the standard errors of differences among the means which are represented on graphs by vertical brackets.

4.4 Results

4.4.1 Growth rates and milk production

In mid lactation, the litter growth rate was similar for all treatments ($P>0.05$, Figure 4.1) but in late lactation litters of mice receiving the steroid treatments had significantly lower ($P<0.05$, Figure 4.1) growth rates than those for the control mice. Milk production did not differ between treatments in mid lactation ($P>0.05$, Figure 4.2) but in late lactation, the milk production of C mice was greater than all mice receiving steroids ($P<0.05$). Milk production per unit of DNA did not alter at mid lactation (0.84 g/mg DNA $\pm$ 0.42, mean $\pm$ SEM, $P>0.05$) between any of the four groups but in late lactation, the milk production per unit of DNA of C mice was greater than all mice receiving steroids ($P<0.05$).

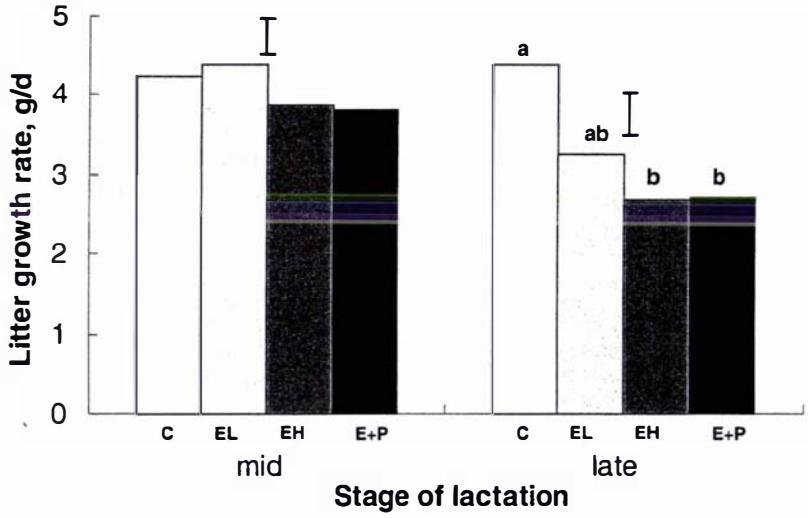


Figure 4.1: Effect of exogenous steroids administered to the dams on litter growth rates.

Litter growth rates (g/d) during the 4 day period when lactating mice were injected daily with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent standard errors of differences among the means, n=5. Where superscripts **a** and **b** differ within stage of lactation, the means differ (P<0.05).

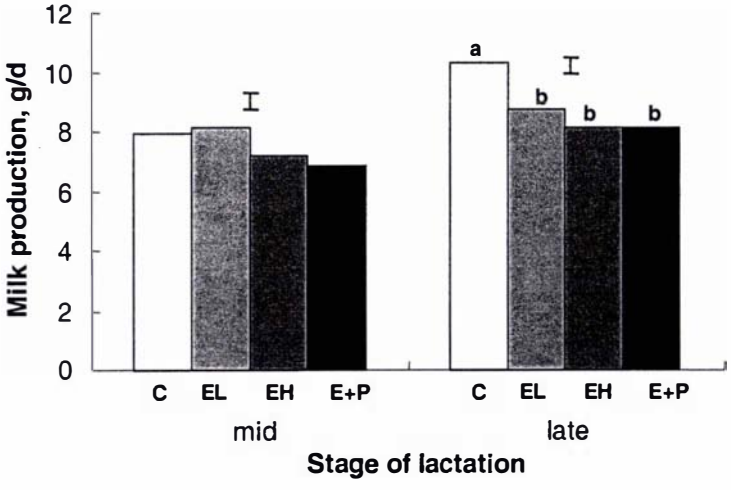


Figure 4.2: Effect of exogenous steroids administered to the dams on milk production.

Milk production (g/d) of lactating mice over last 24 h of a 4 day period when dams were injected daily with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent standard errors of differences among the means, n=5. Where superscripts **a** and **b** differ within stage of lactation, the means differ (P<0.05).

4.4.2 Milk content and total mammary gland weight

The milk content of mammary glands decreased from mid to late lactation (0.73 versus 0.26 g, P<0.01, Figure 4.3). Milk content of glands in mid lactation did not differ (P>0.1) between treatments but in late lactation the glands of mice allocated to EH and E+P contained less milk (P<0.01) than those allocated to C. The corrected gland weight decreased from mid to late lactation (1.17 versus 0.96 g, P<0.05, Figure 4.4) but there were no significant differences (P>0.05) in corrected gland weight among treatments within each stage of lactation.

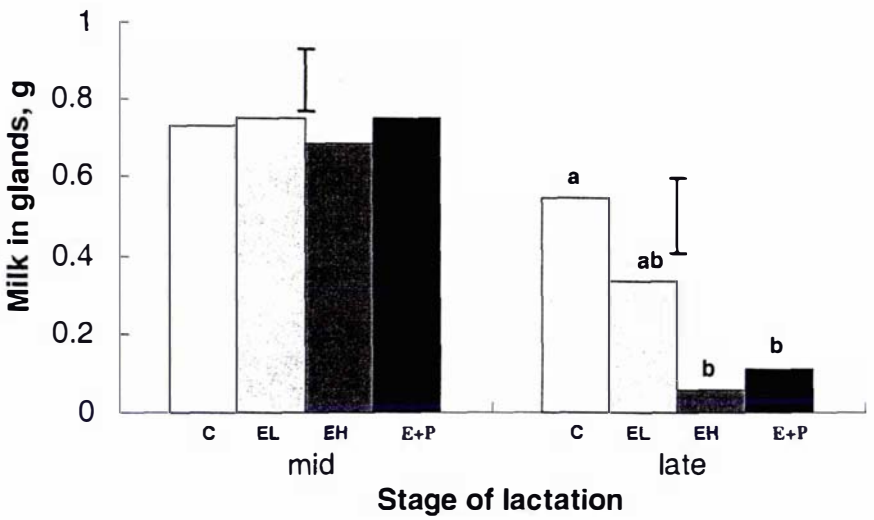


Figure 4.3: Effect of exogenous steroids administered to the dams on milk content of mammary glands.

Milk content of the mammary glands (g) of lactating mice that were injected for the 4 previous days with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent standard errors of differences among the means, n=5. Where superscripts **a** and **b** differ within stage of lactation, the means differ (P<0.05).

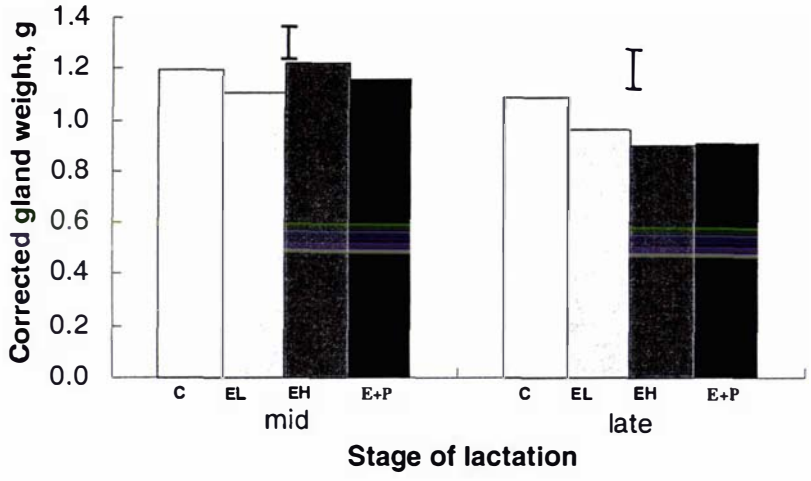


Figure 4.4: Effect of exogenous steroids administered to the dams on corrected weights of mammary glands.

Corrected mammary gland weight of 4 glands (g) of lactating mice that were injected for the 4 previous days with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent standard errors of differences among the means, n=5.

4.4.3 Mammary gland composition

4.4.3.1 DNA

The concentration of DNA in mammary gland tissue (Figure 4.5) and the content of DNA in the gland (Figure 4.6) followed similar patterns. There was a decrease in the concentration of DNA from mid to late lactation (4.45 versus 2.57 μg/mg gland, $P<0.01$) as well as content of DNA (5.07 versus 2.68 mg, $P<0.01$). In late lactation, mice treated with EH or E+P had lower DNA concentrations and contents compared to C mice.

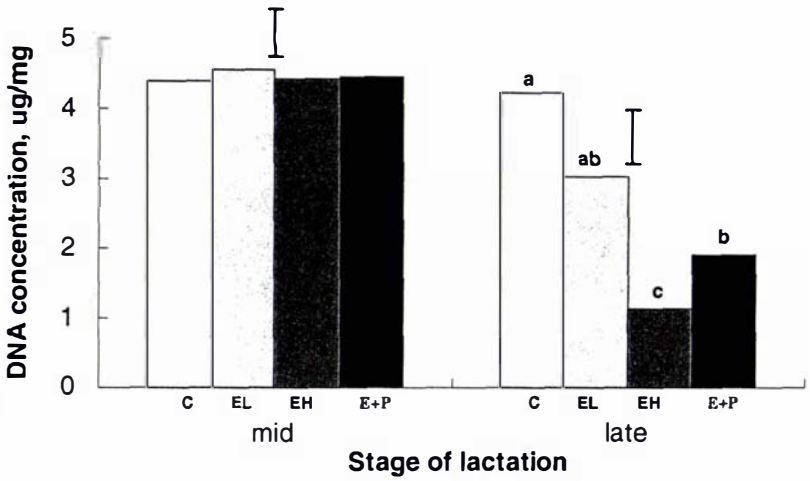


Figure 4.5: Effect of exogenous steroids administered to the dams on DNA concentration of mammary glands.

DNA concentration (μg/mg) in the mammary glands of lactating mice that were injected for the 4 previous days with either excipient (C),0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent standard errors of differences among the means, n=5. Where superscripts **a, b** and **c** differ within stage of lactation, the means differ (P<0.05).

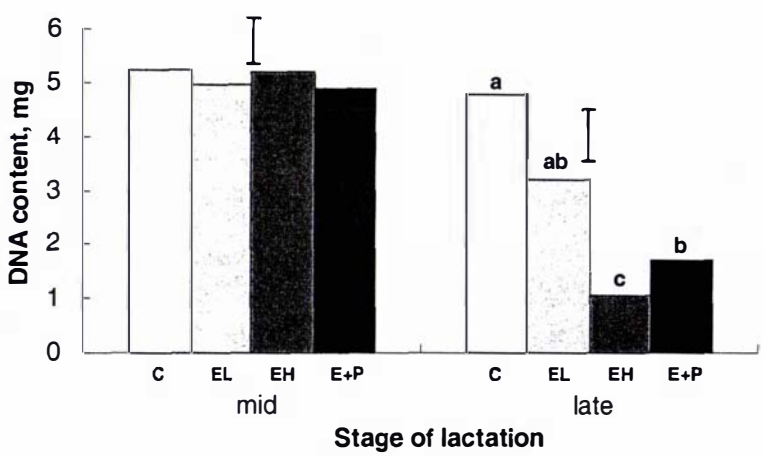


Figure 4.6: Effect of exogenous steroids administered to the dams on DNA content of mammary glands.

DNA content (mg) of milk-free mammary glands of lactating mice that were injected for the 4 previous days with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent

standard errors of differences among the means, n=5. Where superscripts **a**, **b** and **c** differ within stage of lactation, the means differ (P<0.05).

4.4.3.2 Protein

Overall, there was a decrease in both the protein concentration (Figure 4.7) and the protein content of the gland (Figure 4.8) from mid lactation to late lactation (P<0.05) in glands of mice treated with steroids. At mid lactation all treatment groups were similar to the control group for both parameters (P>0.05) but in late lactation the protein concentration and content in the glands of the mice receiving either EH or E+P was lower (P<0.05).

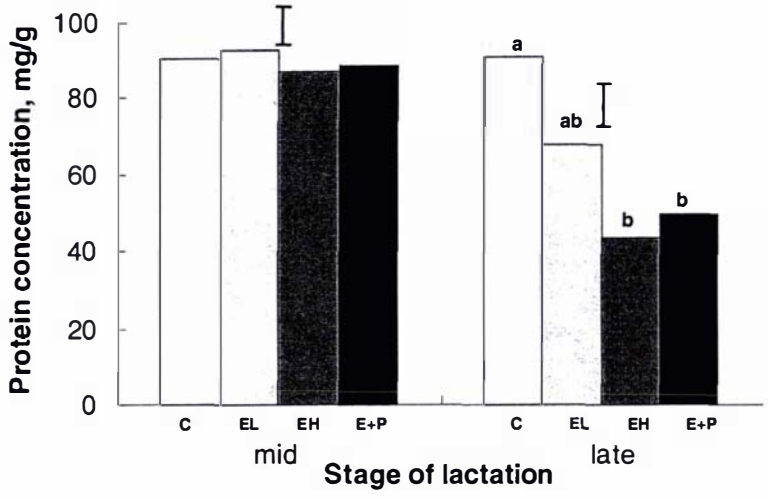


Figure 4.7: Effect of exogenous steroids administered to the dams on protein concentration of mammary glands.

Protein concentration (mg/g) of mammary glands of lactating mice that were injected for the 4 previous days with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent standard errors of differences among the means, n=5. Where superscripts **a** and **b** differ within stage of lactation, the means differ (P<0.05).

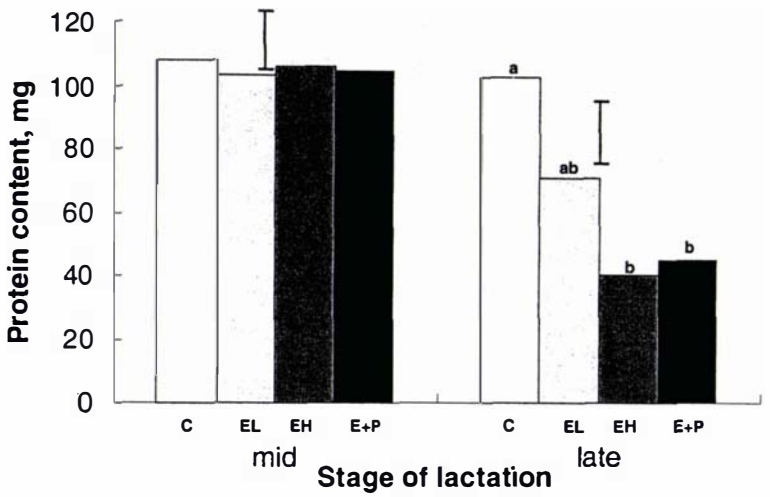


Figure 4.8: Effect of exogenous steroids administered to the dams on protein content of mammary glands.

Protein content (mg) of mammary glands of lactating mice that were injected for the 4 previous days with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent standard errors of differences among the means, n=5. Where superscripts **a** and **b** differ within stage of lactation, the means differ ($P<0.05$).

4.4.3.3 [^{3}H] thymidine incorporation

At mid lactation, [^{3}H] thymidine incorporation into DNA did not differ between C and the steroid treatment groups ($P>0.05$, Figure 4.9). In late lactation, however, there was greater incorporation of [^{3}H] thymidine into the DNA of mice injected with the two high estrogen treatments (EH and E+P) compared to mice in the C group ($P<0.05$), with mice in the EL group being intermediate.

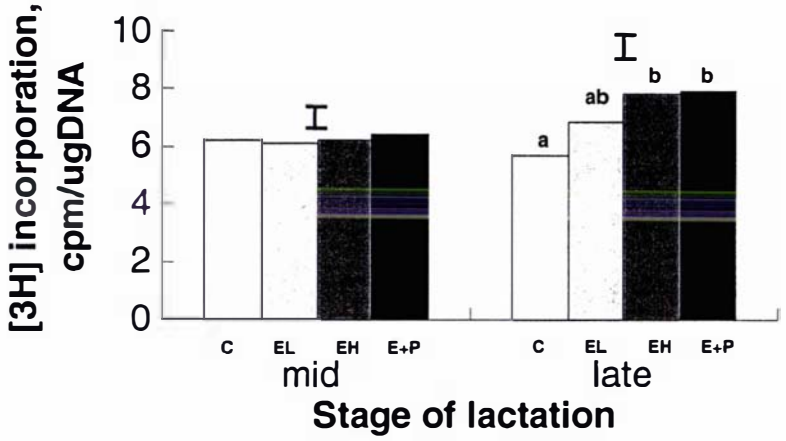
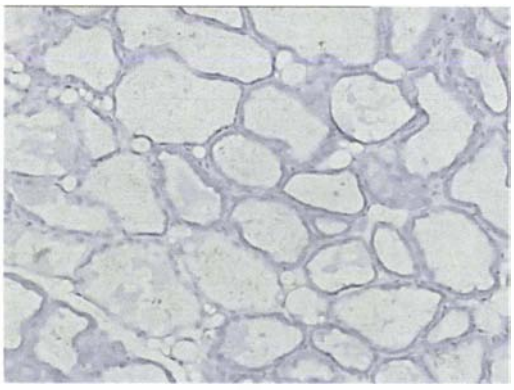


Figure 4.9: Effect of exogenous steroids administered to the dams on incorporation of [^{3}H] thymidine into DNA of mammary glands.

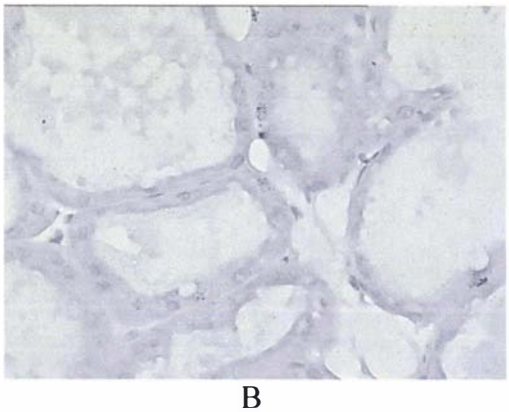
[^{3}H] thymidine incorporation (cpm/μg DNA) into the mammary glands of lactating mice that were injected for the 4 previous days with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Vertical brackets represent standard errors of differences among the means, n=5. Where superscripts **a** and **b** differ within stage of lactation, the means differ (P<0.05).

4.4.4 Histology

In mid lactation, there were no apparent histological differences among mammary glands from the three steroid treatments and the control treatment. All comprised of mainly alveolar units full of secretory epithelial cells (Figure 4.10). In late lactation, mammary glands from control mice contained secretory epithelial cells while the glands of mice receiving EH or E+P appeared to be in the early stages of involution as shown by the decrease in the proportion of secretory epithelial cells and increase in adipocytes (Figure 4.10).



A



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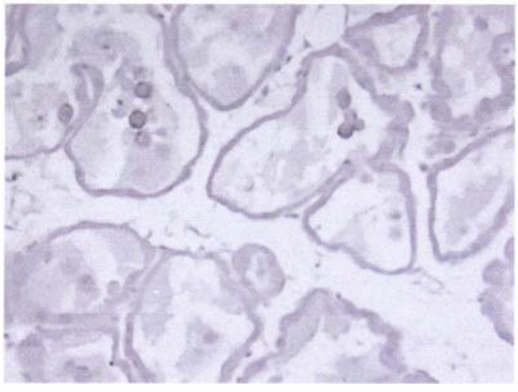

C

Figure 4.10: Effect of exogenous steroids administered to the dams on histology of the glands at mid and late after treatment with steroids and control.

- Panel A Mammary gland in mid lactation, alveolar units are full of secretory epithelial cells, x200. There were no apparent histological differences among glands from the control group and the steroid treated groups.
- Picture B Mammary gland from a mouse in the control group in late lactation, x400.
- Picture C Mammary glands from a mouse in the EH group in late lactation, x200, glands from E+P mice were similar.

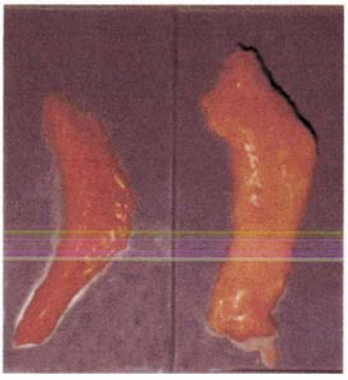

Figure 4.11: Effect of exogenous steroids administered to the dams on size and colour of mammary glands in late lactation.

Photograph of mammary glands collected from mice in late lactation, small gland on the left is from a mouse treated with EH, large gland on the right is from a control mouse.

4.4.5 Autoradiography

The labelling index of epithelial cells was 1.6% (SEM = 2.1%) and did not differ between stage of lactation ($P>0.05$) or location (region x site, $P>0.05$). Results are shown in Table 4.1.

Table 4.1: Effect of exogenous steroids administered to the dams on labelling index.

Labelling index (%) of mammary glands of lactating mice that were injected for the 4 previous days with either excipient (C), 0.1 μg β-estradiol 3-benzoate (EL), 1.0 μg β-estradiol 3-benzoate (EH), 1.0 μg β-estradiol 3-benzoate and 1.0 mg 4pregnen-3, 20 dione (E+P) in mid and late lactation. Values shown are means ± standard errors of the means, n = 2 mice for each location.

Stage of	Location		Treatment			
lactation	region	site	C	EL	EH	E+P
Mid	Proximal	inner	1.2 ± 1.6	1.0 ± 1.5	0.8 ± 1.9	1.1 ± 2.1
		outer	1.0 ± 2.0	0.9 ± 1.8	1.0 ± 2.1	1.2 ± 1.9
	Mid	inner	1.3 ± 2.2	1.1 ± 1.8	1.3 ± 1.6	1.0 ± 1.3
		outer	0.9 ± 1.6	0.8 ± 1.6	1.0 ± 2.3	0.9 ± 2.1
	Teat	inner	1.2 ± 1.4	0.8 ± 2.3	1.4 ± 1.4	1.2 ± 2.9
		outer	0.7 ± 2.3	1.2 ± 1.7	0.8 ± 1.5	1.0 ± 1.2
Late	Proximal	inner	1.6 ± 2.0	1.3 ± 1.9	1.5 ± 2.5	1.6 ± 2.5
		outer	1.5 ± 1.9	1.5 ± 2.0	2.2 ± 2.2	1.1 ± 2.0
	Mid	inner	1.6 ± 1.5	1.6 ± 2.2	2.3 ± 1.4	1.3 ± 2.4
		outer	1.7 ± 2.4	1.9 ± 1.8	1.5 ± 1.3	2.6 ± 1.3
	Teat	inner	1.6 ± 2.1	2.3 ± 2.1	1.2 ± 1.8	2.2 ± 2.1
		outer	1.7 ± 1.8	1.6 ± 1.7	2.4 ± 2.7	1.6 ± 1.8

4.5 Discussion

There was a large influence of stage of lactation on the response of mammary glands to exogenous steroids. Steroids did not affect either the incorporation of [^{3}H] thymidine into DNA or the milk production of mammary glands in mid lactation, but did in late lactation.

In mid lactation, the chemical composition of glands were similar to those for lactating mice reported in the literature for concentrations of protein (Alston-Mills *et al.*, 1995) and DNA (Knight and Peaker, 1982e; Shipman *et al.*, 1987), and uncorrected gland weights (Knight and Peaker, 1982d; Knight and Peaker, 1982c;

Shipman *et al.*, 1987). Studies presented in this thesis show that in mid lactation, exogenous steroids did not alter either the incorporation of [^{3}H] thymidine into DNA in mammary glands or the growth rate of dependent litters. This differs to a similar study by Knight and Peaker (1982d) in which there was no change in the incorporation of [^{3}H] thymidine per unit of tissue but there was a 20% decrease in litter growth rates, presumably due to decreased milk production. The difference between the current study and that by Knight and Peaker (1982d) may be due to the latter being conducted at an earlier stage of lactation (D2 to D9 versus D5 to D9), the different dose rate (0.2 μg estrogen plus 0.6 mg progesterone), the different ratio of E:P (1:3000 versus 1:1000) or treatment duration of seven days rather than four. The results of the present study do not support the hypothesis that exogenous steroids increase either the population of mammary epithelial cells or their milk production in mid lactation.

In late lactation, the effect of the ovarian steroids on the mammary glands can be explained by E alone as there were no major differences between mice treated with either EH or E+P. In addition, the effect of E was dose related with EH reducing growth rate, DNA and protein concentration, corrected gland weight and milk content significantly when compared to either EL or C. Knight and McLelland (1988) also reported decreased growth rates of litters after treating mice from D15–21 with the same dose of E and P used in the present study. When the length of lactation was extended beyond the steroid treatment period, the growth rate of litters increased above that of controls after D34 leading Knight and McLelland (1988) to suggest that milk production was reduced during proliferation of new secretory cells and was then increased when the new cells began to secrete. In contrast, the results of the present study imply that E induces early involution of mammary glands. Indeed, E decreased concentrations and total amounts of DNA and protein in mammary glands of mice injected with EH. This is consistent with the involution process during which secretory epithelial cells are replaced with adipocytes and stroma (Richert *et al.*, 2000) resulting in the gland having lower contents of DNA and protein (Cowie *et al.*, 1980). Glands of EH treated mice also had the greatest incorporation of [^{3}H] thymidine into DNA which can be interpreted as evidence of involution rather than growth. For example in cows, udder quarters that were sealed for 5 to 7 days, while contralateral quarters were suckled had the lowest DNA

contents and the greatest incorporations of [^{3}H] thymidine into DNA (Capuco and Akers, 1990). This shows that extensive cell turnover occurs during early mammary involution but the DNA content of glands decreases as cell mortality exceeds cell replication. Histological changes observed in the present study are similar to those during involution described by Richert *et al.* (2000) after four days of induced weaning. For example, the alveolar units of mice receiving EH or E+P in late lactation had lost some or all of the secretory epithelial cells (Figure 4.10) and litter growth rates had decreased which suggests milk synthesis had also decreased (Richert *et al.*, 2000). Administration of exogenous E in late lactation did not cause proliferation associated with growth of the mammary gland but it did cause the gland to undergo early involution.

The present study shows that the stage of lactation affects how the glands will respond to exogenous estrogen. Others studies have reported that exogenous estrogen causes milk stasis in ruminants (Hutton, 1958; Fulkerson and McDowell, 1974; Peaker and Linzell, 1974) and decreased milk production in mice (Knight and McLelland, 1988) but no particular mechanism has been shown to be responsible. In the present study, at mid lactation when milk production is near peak, the gland is refractory to exogenous steroids yet in late lactation when milk production is declining, the gland is sensitive. An examination of the hormonal difference between the stages of lactation shows elevated concentrations of Prl in mid lactation (Grosvenor and Mena, 1974). Prolactin is known to be both a mitogenic and a differentiating agent in mature glands depending on whether the hormone is phosphorylated and which of the different isoforms of receptor it binds to (Hynes *et al.*, 1997). It may be that the isoform of Prl and the number and type of receptors present during lactation cause differentiation into secretory cells only and override any potential mitotic signal such as that from exogenous steroids. There are however, other levels of control over cell responsiveness to exogenous steroids at the level of steroid receptors and signalling pathways (Fendrick *et al.*, 1998). E and P receptor number, location and isoforms change during the different developmental stages of mammary glands (Fendrick *et al.*, 1998). In comparison to nulliparous mammary glands, the number of E receptors in lactating glands declines by 55% mostly due to a loss of receptors in the stroma (Fendrick *et al.*, 1998). In addition, P receptors, previously detectable in both the stroma and epithelium become only

detectable in the stroma (Haslam and Shyamala, 1981). There is also a change in the ratios of α to β type E receptors in epithelium and stroma, and A to B type P receptors in the stroma during lactation (Fendrick *et al.*, 1998). As each receptor isoform has a different function (Fendrick *et al.*, 1998), any change in receptor numbers or ratios could alter the sensitivity of the gland to steroid challenge. For instance, a decrease in stromal E receptors will decrease mitogens produced by the stroma that target epithelial cells (Kleinberg, 1997). In late lactation, P receptors reappear in the epithelium along with an increase in the number of E receptors (Fendrick *et al.*, 1998). The increase and distribution of both E and P receptors may contribute to the sensitisation of the glands to exogenous steroids administered in late lactation.

In conclusion, it was determined that the response of glands to estrogen depends on both the stage of lactation and the dose of the steroids. Mammary glands in mid lactation were insensitive to estrogen and progesterone showing that differentiated and highly active mammary tissue is incapable of becoming proliferative in response to steroids. Glands in late lactation exhibited a dose response to estrogen. Instead of the gland becoming proliferative and increasing the epithelial cell population, the increased incorporation of [^{3}H] thymidine into DNA was associated with involution. Further evidence of involution were the decreases in growth rates of litters, milk content of the gland, DNA and protein concentration and histological images consistent with early involution. This study did not yield a model suitable for studying proliferating epithelial cells during established lactation.

Chapter 5 Assessment of proliferation model II

The effect of increased suckling demand on proliferation of epithelial cells in the mammary glands of lactating mice

5.1 Summary

Compensatory growth of lactating mammary glands of rodents, pigs and ruminants can occur when contralateral glands are surgically removed or covered so that suckling young are denied access. The present study examined protocols to experimentally induce increased cell proliferation during lactation. Half the mammary glands were sealed by tape to examine potential compensatory growth in contralateral mammary glands of lactating Swiss mice. The treatment consisted of taping the 5 left teats of mice so that suckling demand of the 5 right glands was increased. Mice suckled 5 or 10 pups from 5 glands only (5/5 and 10/5 respectively) from parturition. One group suckled 10 pups from 10 glands which were reduced to 5 glands three days prior to euthanasia (10/10:5). Control mice suckled 5 or 10 pups from 10 glands (C5 and C10). Cell proliferation, measured by incorporation of [^{3}H] thymidine into DNA, was not increased by any of the treatments in early, mid or late lactation. Therefore it was concluded that increased suckling demand did not provide a consistent model of mammary growth during lactation.

5.2 Introduction

The potential benefit of increasing proliferation of mammary epithelial cells during lactation is that the larger cell population could result in increased milk yields. The understanding of this phenomenon requires the study of mammary tissue in which increased proliferation during lactation can be induced. In the previous chapter, mice challenged with exogenous E and P failed to increase proliferation of epithelial cells during lactation. In the present chapter, experiments are described which assessed the effect of increased suckling intensity on the proliferation of mammary epithelial cells. Suckling intensity is defined as the ratio of sucking young to mammary glands

which affects the length of time glands are massaged, the frequency of suckling and the thoroughness of milk removal.

Compensatory growth is a recognized phenomenon in several tissues, including liver (Rodeck *et al.*, 2000) and lungs (Sakamaki *et al.*, 2002), and mammary glands (Davis *et al.*, 1983; Knight, 1987). The removal of portions of organs stimulates specially the growth of the remaining tissue (Bucher and Malt, 1971). Compensatory growth also occurs in mammary glands contralateral to sealed and non-functional glands. The mechanism responsible for this type of growth is probably linked to the demand placed on the remaining tissue or in the case of the mammary gland the suckling intensity (Knight, 1987).

Several studies have shown that mammary glands of ruminants are capable of compensatory growth. For example, hemimasectomy of goats in weeks 5–8 of lactation increased DNA content, gland volume and milk yield in the contralateral gland in late lactation (Knight, 1987). Furthermore, ewes responded to hemimasectomy performed in late pregnancy by increasing udder volume and milk yield such that they did not differ from control animals at peak lactation (Davis *et al.*, 1980). These hemimasectomy studies suggest there is a mechanism by which mammary gland size increases when udder size decreases suddenly or is incapable of meeting functional demands (Knight and Wilde, 1987). Compensatory growth was demonstrated in a study by Capuco and Akers (1990) in which beef cows in mid lactation, that were suckling calves, had half their udders covered so that suckling intensity increased for contralateral glands. Epithelial cell proliferation increased in the suckled halves after only 5–7 days of treatment (Capuco and Akers, 1990). Taken collectively, these studies illustrate that ruminant mammary glands have the capacity for growth during lactation.

Animals raising litters also demonstrate compensatory growth during lactation in response to ligation or covering of contralateral teats. Sows with a suckling intensity of 12 piglets per 12 glands were compared to sows suckling 12 piglets (in 2 litters of 6) from six glands. The latter group suckled more frequently and after three weeks of treatment, had mammary gland weights that were 90% heavier (Auldist *et al.*, 2000). In rats, the DNA content of glands contralateral to ligated teats increased

with litter size and suckling stimulus (Tucker, 1964). Similar results were also reported by Moon (1965b). In rats with some glands ligated, the decrease in DNA content of ligated glands was compensated for by an increase in the DNA content of contralateral non-ligated glands (Moon, 1965b). Therefore it can be concluded that restricting the number of available teats in laboratory and livestock animals with litters increased the suckling intensity of accessible glands and which resulted in compensatory mammary growth.

The compensatory growth experienced by these rodents and pigs is probably linked to suckling intensity and its effect on endocrine and autocrine control of mammary glands. Endocrine control is facilitated by Prl as well as other galactopoietic hormones (Bole-Feyston *et al.*, 1998). The concentration of Prl in plasma of rodents increases in response to increased suckling stimulus (Tucker *et al.*, 1967; Grosvenor and Mena, 1974; Grigor *et al.*, 1984). Prolactin interacts with its receptors to activate the JAK/STAT signal transduction pathway that can result in cell proliferation, cell differentiation and milk synthesis (Bole-Feyston *et al.*, 1998). It is possible that autocrine control of lactation is also involved in compensatory growth in mammary glands contralateral to taped or covered glands. A small whey protein, called feedback inhibitor of lactation (FIL) is secreted in milk by epithelial cells with high concentrations causing milk synthesis to decrease (Wilde *et al.*, 1995). Increased milk removal from available glands would remove local factors, such as FIL, from the mammary gland, which would therefore allow increased milk synthesis (Wilde and Peaker, 1990). More importantly, the removal of FIL leads to increased receptor numbers for endocrine hormones, such as Prl, which results in mammary glands being more sensitive to these hormones (Wilde *et al.*, 1990). To summarise, increased milk removal from available mammary glands makes these glands more sensitive to circulating Prl, which can result in increased cell proliferation.

The advantage of sealing or ligating some teats is that high suckling intensities can be applied to the remaining glands without increasing physiological demands on the dam. For instance, a mouse is capable of feeding 10 pups from 10 glands but when attempting to suckle 20 pups from 10 glands she will be unable to eat and metabolise enough food and mobilise enough body stores to produce the quantity of milk required. A better method to achieve a suckling intensity of 2 pups per gland would

be to suckle 10 pups from 5 mammary glands as there is no doubt that the dam can eat enough and therefore produce enough milk to feed this size of litter. Teat ligation or sealing was therefore an attractive method to use in an experiment designed to increase suckling intensity and stimulate compensatory growth of mammary glands of mice.

The objective of the study was to stimulate the growth of lactating mammary glands of mice by halving the number of glands available to the litter. Mice suckled either 5 or 10 pups from parturition from 10 glands (C5, C10) or only 5 glands (5/5, 10/5). A fifth treatment was the suckling of 10 pups from 10 glands until three days before termination when gland number was reduced to 5 (10/10:5). This treatment was to determine the effect of 3 days of increased suckling intensity. The suckling intensity of the five groups varied from 0.5 pups per gland to 2 pups per gland.

5.3 Experimental design

Seventy five Swiss mice were mated and transferred to individual cages at D15 of pregnancy. By 12h after parturition litter sizes were adjusted to allocated treatments. Animal husbandry and management are detailed in Section 2.1.

Prior to parturition mice were randomly allocated to one of 15 groups of 5 mice. At birth, only dams that gave birth to 8–12 pups were used. Three groups were allocated to each of five treatments and a group from each treatment was killed at each of three stages of lactation on days 3, 10 and 17 (D3, 10 and 17). Dams in control groups 1 and 2 suckled litters of 5 (C5) or 10 (C10) pups from parturition onwards. In the other three groups, the 5 left teats (/5) were taped over at parturition preventing milk removal, which meant that milk was available only from the mammary glands on the right side. The left and right sides will herein be referred to as the non-sucked side and sucked side respectively. Teats were taped over from parturition and dams suckled either 5 (5/5) or 10 pups (10/5). The teats were taped according to Section 2.1.5. The final group suckled 10 pups (10/) and had teats taped over for only the last 3 d of lactation (10/10:5). The latter group was therefore only

taped from D0-3, D7-10 or D14-17, depending on the stage of lactation to which the mice were allocated.

Litter weight was recorded daily (Section 2.1.4) until the day of euthanasia of the dam. Milk production was calculated using the metabolic weight method (Section 3.4.2.2).

On D3, D10 or D17 the lactating mice were injected with [^{3}H] thymidine (Section 2.1.7) and euthanased 1h later. All 10 mammary glands were dissected from each mouse, blotted and weighed (Section 2.1.8). The left 5th and right 5th glands were homogenised individually and aliquots from each were analysed for concentrations of lactose, DNA, protein and [^{3}H] thymidine (Section 2.3). Gland weights and compositions were corrected for milk content (Section 3.5.1).

Data were analysed by a two way ANOVA (fitting treatment, stage of lactation and their interactions) using the GLM procedure of Minitab (Release 12.23, 1999) where individual mean comparisons were performed by LSD. Litter growth rates were analysed similarly but with the difference that pre-treatment growth rates were used as a covariate. The pooled standard deviation was used to calculate the standard errors of differences among the means, which are represented on graphs by vertical brackets.

5.4 Results

5.4.1 Litter growth rate, milk production and milk production per unit DNA

Litter growth rates (Figure 5.1) were highest in mid lactation ($P<0.01$), with litters of the C10 treatments having the greatest growth rate at each stage of lactation ($P<0.01$). This is explained in part by the C10 group having the highest milk production (Figure 5.2, $P<0.01$).

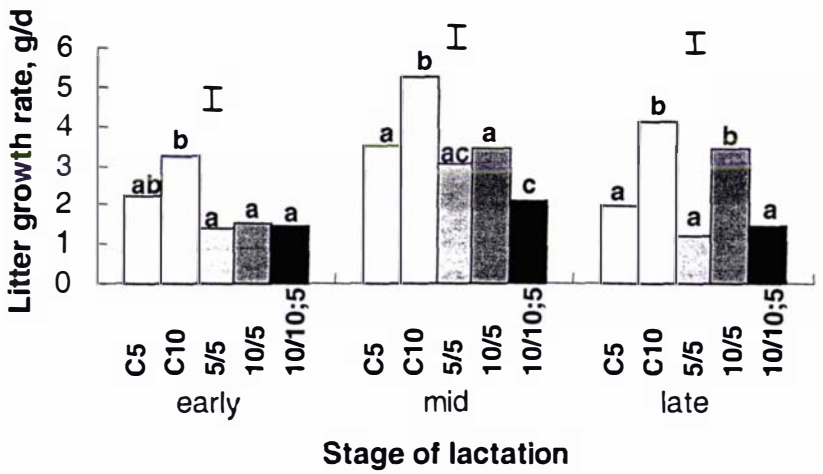

Figure 5.1: Effect of reducing the number of sucked mammary glands on litter growth rates.

Litter growth rates (g/d) during 3 days in early (D0-3), mid (D7-10) and late lactation (D14-17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a**, **b** and **c** differ within a stage of lactation, the means differ ($P<0.05$).

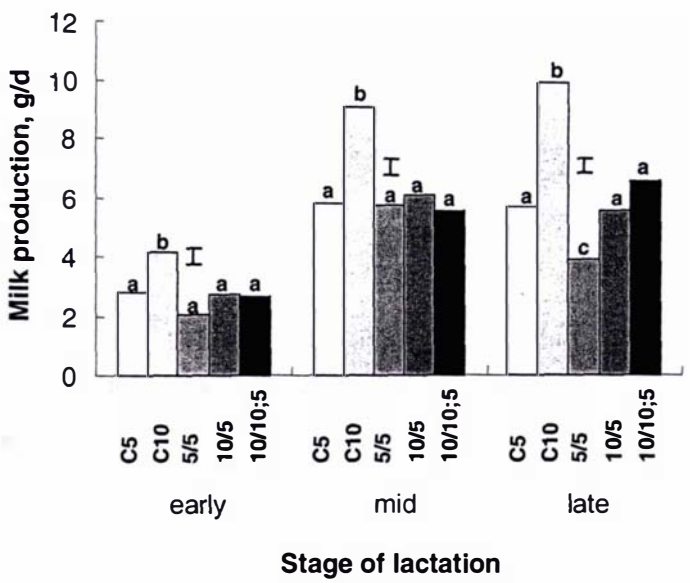

Figure 5.2: Effect of reducing the number of sucked mammary glands on milk production from sucked glands.

Milk production (g/d) in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences

between the means within a stage of lactation, n=5. Where superscripts **a**, **b** and **c** differ within a stage of lactation, the means differ (P<0.05).

Milk production per unit of DNA was calculated by dividing the milk produced by the DNA content of available glands. For mice in control groups, the milk production was divided by the DNA content of 10 glands whereas for the treatment groups, milk production was divided by the DNA content of only 5 glands. Milk production per unit of DNA (Figure 5.3) was lowest in mid lactation compared to early and late lactation (0.46 versus 0.58 and 0.71 g milk/mg DNA respectively, P<0.05). The large animal variation and SEDs within a stage of lactation resulted in no differences in milk production per unit of DNA among treatments (P>0.05), despite a 1.5 fold increase in all treatments compared to C5 in early lactation.

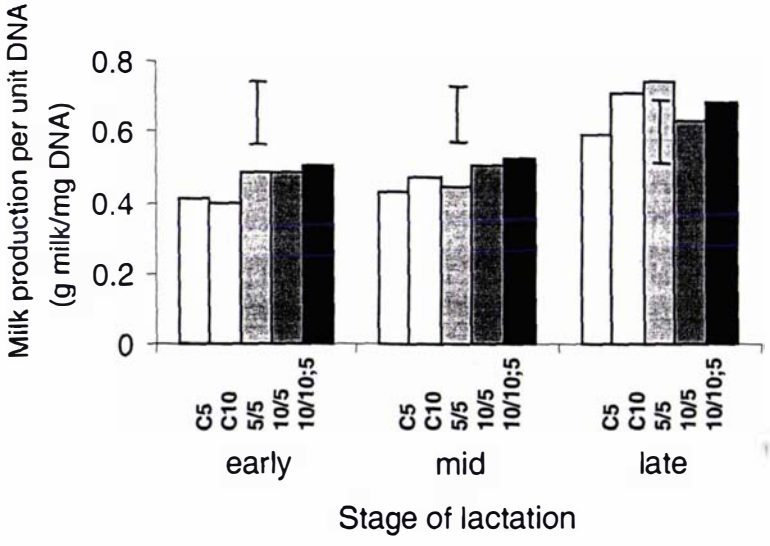

Figure 5.3: Effect of reducing the number of sucked mammary glands on milk production per unit of DNA.

Milk production per unit of DNA (g milk/mg DNA) of sucked glands in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5.

5.4.2 Total mammary gland weight and milk content

The milk content of the sucked glands (Figure 5.4) one hour after removal of the pups was highest in mid lactation followed by late and then early lactation (1.46 versus 0.84 versus 0.63 g milk respectively, $P<0.05$). Milk content of glands did not differ between treatments in early or late lactation ($P>0.05$) but in mid lactation, glands of C5 mice contained less milk than those in the other treatments ($P<0.05$).

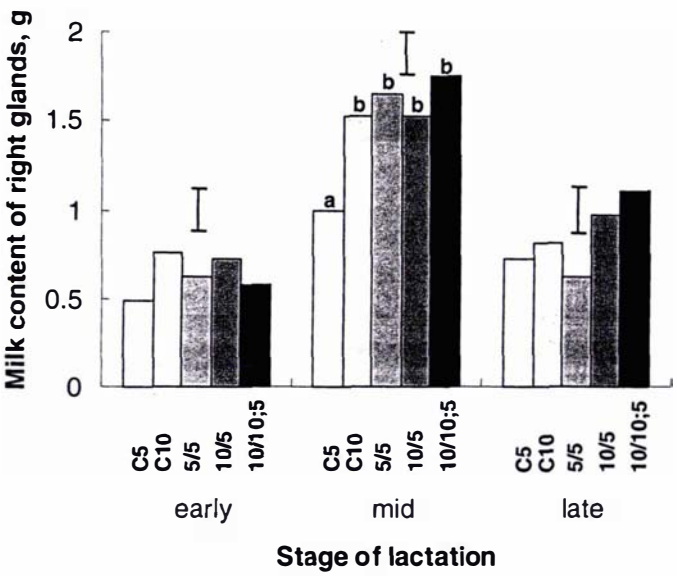

Figure 5.4: Effect of reducing the number of sucked mammary glands on milk content of sucked glands.

Milk content of sucked glands (g) in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a** and **b** differ within a stage of lactation, the means differ ($P<0.05$).

The corrected weight of sucked mammary glands (Figure 5.5) was greatest in mid and late lactation followed by early lactation (1.58, 1.42 and 0.91 g respectively, $P<0.01$). At mid and late lactation, the corrected gland weights for 10/5 and 10/10:5 were heavier than C5 ($P<0.01$).

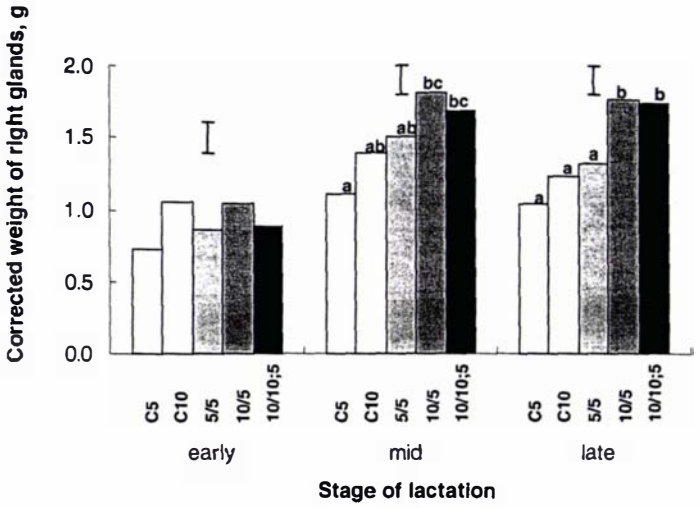

Figure 5.5: Effect of reducing the number of sucked mammary glands on corrected weights of sucked mammary glands.

Corrected weight of sucked mammary glands (g) in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a**, **b** and **c** differ within a stage of lactation, the means differ ($P<0.05$).

The ratio of DNA content of non sucked mammary glands to sucked mammary glands were compared to the controls, C5 and C10 (Figure 5.6). In early lactation, the ratio was lower for all three taped treatments compared to C5 and C10 ($P<0.01$). In mid and late lactation, the treatments that required taping of teats from D0 (5/5 and 10/5) had lower ratios than C5 and C10 ($P<0.01$) while the ratio for the treatment requiring only three days of taping was intermediate ($P>0.05$).

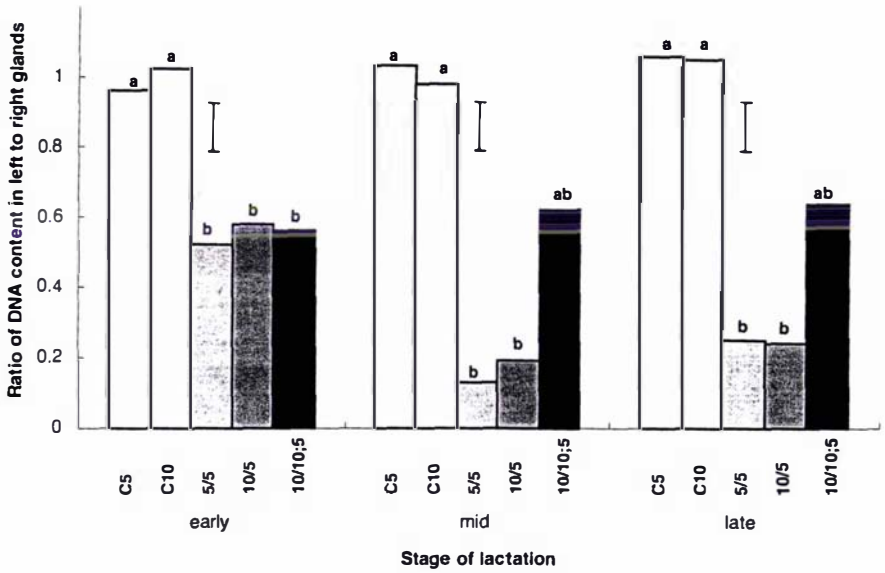

Figure 5.6: Effect of reducing the number of sucked mammary glands on the ratio of DNA content of left mammary glands to right mammary glands.

Ratio of DNA content in non-sucked mammary glands to sucked mammary glands in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a** and **b** differ within a stage of lactation, the means differ ($P<0.05$).

5.4.3 Gland composition

5.4.3.1 DNA

The concentration of DNA in sucked mammary glands (Figure 5.7) was higher in mid lactation than early and late lactation (6.9 versus 4.8 and 4.7 μg/mg respectively, $P<0.05$). Within a stage of lactation, there were no differences among treatments in concentration of DNA ($P>0.05$).

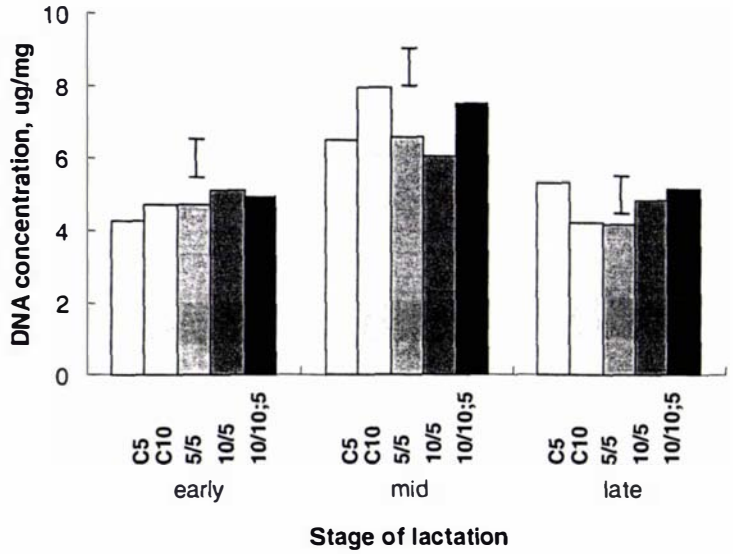

Figure 5.7: Effect of reducing the number of sucked mammary glands on DNA concentration of sucked glands.

DNA concentration of DNA (μg/mg) of sucked mammary glands corrected for milk content in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5.

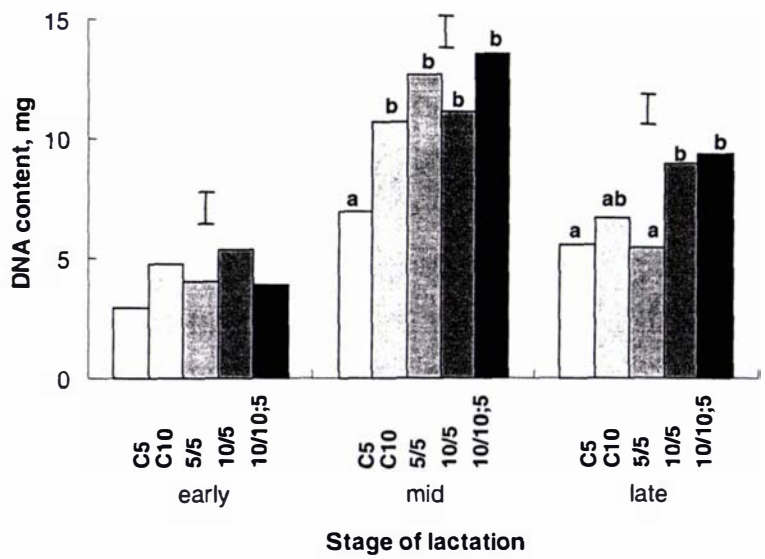

Figure 5.8: Effect of reducing the number of sucked mammary glands on the DNA content of sucked glands.

DNA content (mg) of sucked mammary glands corrected for milk content in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a

stage of lactation, n=5. Where superscripts **a** and **b** differ within a stage of lactation, the means differ (P<0.05).

The DNA content of the sucked mammary glands (Figure 5.8) was highest in mid lactation, followed by late and then early lactation (10.7 versus 7.3 versus 4.2 mg respectively, P<0.05). In early lactation there were no significant differences in DNA content between treatments (P>0.05) but in mid lactation the C5 treatment resulted in the lowest DNA content (P<0.05). In late lactation, 10/10 and 10/10:5 treatments resulted in greater DNA contents compared to C5 and 5/5 (P<0.05) but they were not significantly greater than for the C10 treatment (P>0.05).

5.4.3.2 Protein

The concentration of protein in sucked mammary glands (Figure 5.9) was greater in mid lactation than early and late lactation (133, 101 and 106 mg/g respectively, P<0.01) and did not differ among treatments within any stage of lactation (P>0.05).

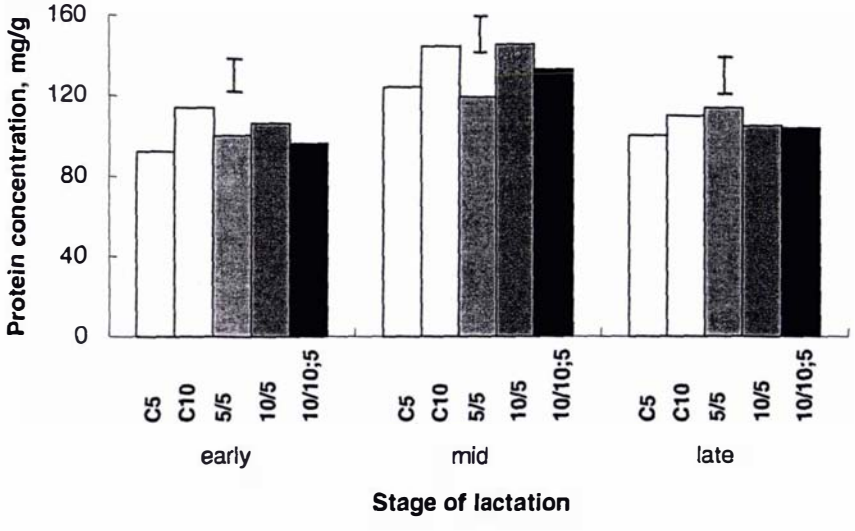

Figure 5.9: Effect of reducing the number of sucked mammary glands on on the protein concentration of sucked glands.

Protein concentration (mg/g) of sucked mammary glands corrected for milk content in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5).

Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5.

The protein content of sucked mammary glands (Figure 5.10) of mice was greatest in mid lactation followed by late and then early lactation (207, 145 and 86 mg respectively, P<0.01). In early lactation, there was no difference in protein content of glands among treatments (P>0.05). Glands of the C5 mice contained less protein than all other treatments in mid lactation (P<0.01). In late lactation, protein content of sucked mammary glands was higher for the 10/10 and 10/10:5 treatments compared to the C5 (P<0.05).

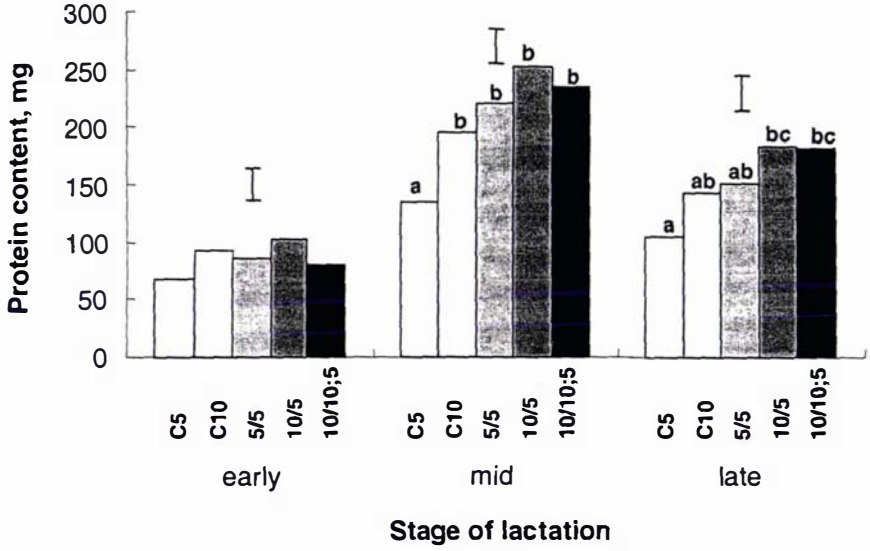

Figure 5.10: Effect of reducing the number of sucked mammary glands on the protein content of sucked glands.

Protein content (mg) of sucked mammary glands corrected for milk content in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a**, **b** and **c** differ within a stage of lactation, the means differ (P<0.05).

5.4.3.3 [^{3}H] thymidine incorporation

Incorporation of [^{3}H] thymidine into DNA of sucked mammary glands (Figure 5.11) was greatest in early lactation compared to either mid or late lactation (36 versus 23

and 27 cpm/μg respectively, P<0.01). Within any stage of lactation, there were no differences in incorporation of [^{3}H] thymidine into DNA among any of the treatments (P>0.05).

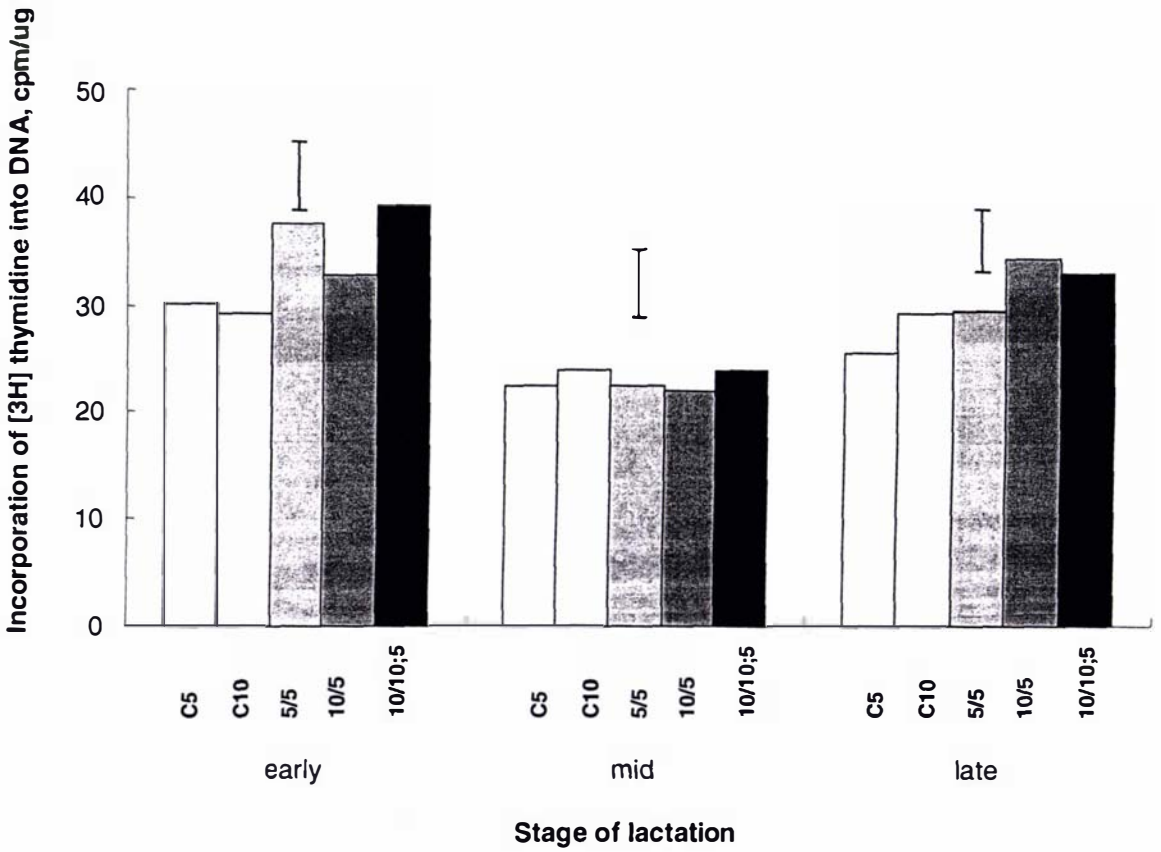

Figure 5.11: Effect of reducing the number of sucked mammary glands on incorporation of [^{3}H] thymidine into DNA of sucked glands.

Incorporation of [^{3}H] thymidine into DNA (cpm/ug) of sucked mammary glands, corrected for milk content, of mice in early (D3), mid (D10) and late lactation (D17) when mice suckled either 5 pups (C5), 10 pups (C10), 5 pups from 5 glands (5/5), 10 pups from 5 glands (10/5) or 10 pups from 10 glands which were reduced to 5 glands for the last 3 days (10/10:5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5.

5.5 Discussion

In early lactation, mammary gland growth was at a maximum and therefore the response to increased suckling intensity was limited. Consequently, there were no differences among the five groups in terms of [^{3}H] thymidine incorporation (Figure 5.11; P>0.05), weight (Figure 5.5; P>0.05), DNA content (Figure 5.8; P>0.05) and

protein content (Figure 5.10; P>0.05) of sucked mammary glands. These results concur with the report of Tucker (1966) that suckling intensity did not affect mammary gland size or DNA content in rats by D4 of lactation, although in that study the pup/gland ratio did not exceed 1. In a metabolic study where pup/gland ratio varied from 0.5 to 1.4, there were no differences in dam food intake, gut weight or faecal output (Hammond and Diamond, 1992) indicating that suckling intensity does not affect these parameters in early lactation.

In mid lactation, mammary gland size was related to suckling intensity when the pup/gland ratio was ≤1, but higher ratios did not result in any extra mammary growth or milk production. When pup/gland ratio increased from 0.5 (C5) to 1 (C10 and 5/5), the DNA and protein contents per gland increased significantly (Figure 5.8, P<0.05; Figure 5.10, P<0.05) which is in agreement with many other studies reporting correlations between litter size and mammary gland size for suckling intensities of ≤1 pup per gland at mid lactation (Tucker, 1964; Moon, 1965b; Tucker, 1966; Moon, 1969; Knight *et al.*, 1986b). Such increases have been attributed to the effects of increased levels of serum prolactin and increased removal of local inhibitory factors that make the mammary glands more sensitive and responsive to the requirements of the litters (Grosvenor and Mena, 1974; Wilde and Peaker, 1990). Once the suckling intensity exceeded 1 pup/gland in the present study, however, there is no increase in mammary gland size or milk production. Mammary gland weight, [^{3}H] thymidine incorporation, DNA and protein contents did not differ (Figure 5.5; Figure 5.11; Figure 5.8; Figure 5.10; P>0.05 for all) when suckling intensity increased from 1 (C10, 5/5) to 2 pups/gland (10/5, 10/10:5). In similar studies, increasing the number of pups per gland to >1 did not increase milk production (Knight and Peaker, 1982d; Knight *et al.*, 1986b). The limitations of mammary glands was not due to inadequate nutrition as milk production and therefore the total energy output of the dams with 2 pups/gland (10/5, 10/5;5) was actually less than C10 with 1 pup per gland (4.9, 4.7 and 7.7 g milk/d respectively, P<0.01; Figure 5.2). This study shows that the murine mammary gland is unable to respond to a suckling intensity of ≥1 pup/gland possibly due to an inherent limitation on its size and output which over rides the usual responses to suckling stimuli.

In late lactation, the high suckling intensity of 2 pups per gland delayed the regression of mammary glands that is typical of late lactation. The stimulus of only 1 pup/gland (C10, 5/5) did not stop glands from reducing in weight and DNA content compared to mice with only 0.5 pup/gland (Figure 5.5, Figure 5.8; $P>0.05$) whereas suckling 2 pups/gland did (10/5, 10/10:5). Tucker (1964) showed that increasing the suckling stimulus from 0.5 to 1 rat pup per gland was not sufficient to cause any difference in mammary gland weight and DNA content in late lactation. In the present study, [^{3}H] incorporation did not differ between groups (Figure 5.11. $P>0.05$) suggesting that the larger gland sizes of mice in groups with 2 pups/gland (10/5 and 10/10:5) was due to increased cell persistency rather than proliferation. Increased cell persistency may have been caused by the action of the increased sucking stimulus on the modulation of both the endocrine and autocrine systems. A strong suckling stimulus increases the release of the anti-apoptotic factor, prolactin which then works to stimulate the cell survival factor, IGF1 and inhibit one of its binding proteins, IGFBP-5 (Sell *et al.*, 1995; Flint and Knight, 1997). The increased milk removal from glands suckling 2 pups each would also remove FIL which would otherwise increase in concentration to cause milk stasis and decrease gland sensitivity to the endocrine system (Wilde *et al.*, 1995).

Taping of the left mammary glands induced involution although the extent of regression was influenced by endocrine stimulation and the period of taping. By mid and late lactation, glands that had been taped since parturition (5/5 and 10/5) had regressed so that the DNA ratio of non-sucked to sucked mammary glands was 0.20 (Figure 5.6). When taping was only done for three days, such as in early lactation or for 10/10:5 in mid and late lactation, the regression of glands on the non-sucked side was not as complete with the DNA ratio being 0.55. This was probably due to both systemic prolactin slowing apoptosis and the shorter time frame for regression, which even in a forced weaning does not peak until D4 of involution (Quarrie *et al.*, 1995).

Total gland weights that included milk content, DNA and protein concentrations and contents of mammary tissue plus milk from right mammary glands were comparable to others reported in the literature (Knight and Peaker, 1982d; Shipman *et al.*, 1987; Alston-Mills *et al.*, 1995). The milk content of glands varies with stage of lactation

and suckling intensity (Figure 5.4) and there is more error associated with using tissue weights that include milk rather than milk free tissue units. Therefore the present study, which reports analysis of mammary glands corrected for residual milk, provides more accurate data on the weight and composition of mammary glands in lactating mice than other studies in the literature.

Milk production per unit of DNA is high in early lactation when cell number is low but it decreases in mid lactation when the cell population has increased. This relationship concurs with that documented by Knight and Peaker (1982d). In the present study milk production per unit of DNA in right glands was 0.58 and 0.46 milk/mg DNA (which is equivalent to 5.1 and 4.1 g milk/g wet mammary tissue) in early and mid lactation respectively. These levels are comparable to those in rodents reported by others (Linzell, 1972; Knight *et al.*, 1984). The highest level of milk production per unit of DNA calculated in the present study was in late lactation.

The rate of cell proliferation in mammary glands did not increase in response to suckling stimuli when more than 1 pup per gland had been reached. In early lactation, mammary gland size and growth (weight, DNA content, [^{3}H] thymidine incorporation) did not differ among groups even though suckling intensities varied from 0.5 to 2 pups per gland. In mid lactation, mammary gland size was correlated with suckling stimulus when there was $\leq$1 pup per gland but once this ratio was exceeded there was no additional gland growth. In late lactation, a high suckling intensity of 2 pups per gland increased cell persistency but not proliferation as [^{3}H] thymidine incorporation concentrations did not differ.

The suckling intensity of C10 resulted in approximately 30% more mammary DNA than for C5 in mid lactation. However, the elevated proliferation of cells and the time this occurred was not detected by measuring the incorporation of [^{3}H] thymidine. The increase in DNA may have increased gradually due to a small increase in proliferation or a decrease in apoptosis. The lack of a large increase in mammary growth contrasts to reports of significant compensatory growth of udders during lactation in ruminants (Davis *et al.*, 1980; Knight, 1987; Knight and Wilde, 1987; Capuco and Akers, 1990), and suggests major species differences between mice and ruminants. Pigs are also capable of increasing mammary gland size by

90% during lactation in response to suckling two litters for amost an entire lactation (Auldist *et al.*, 2000), a very intensive treatment that was not able to be reproduced in this experiment. Previous work on rodents reports compensatory growth of mammary glands during lactation (Tucker, 1964; Moon, 1965b) but the studies did not extend suckling intensities to >1 pup per gland. When this was done in the present study there was no further increase in gland size above that measured for suckling intensities of 1 pup per gland. This may be due to inherent limitations on the size and output of individual mammary glands. Alternatively, it may be related to systemic levels of hormones that are influenced by litter size, i.e., even though there was a high suckling intensity treatment of 2 pups per gland, the total litter size of this treatment was only 10 pups. There may have been too few pups to cause an increase in circulating prolactin that may have otherwise permitted larger gland growth.

Chapter 6 Assessment of proliferation model III

The effect of increased litter size on proliferation of epithelial cells in the mammary glands of lactating mice

6.1 Summary

In the previous chapter, suckling intensity was increased to 2 pups per gland but milk production and cell proliferation did not increase above that for mammary glands suckling 1 pup each. This may have been due to the litter size of 10 pups not being large enough to stimulate increased amounts of circulating mammogens. A larger litter and therefore increased amounts of systemic mammogens such as prolactin may lead to cell proliferation during lactation. Experiments in this chapter examine Swiss mice suckling 5 (C5) or 10 pups (C10) from parturition, with and without the addition of 5 extra pups to each litter size for three days prior to euthanasia (P5+5, P10+5). Cell proliferation was measured by incorporation of [^{3}H] thymidine into DNA in early, mid and late lactation. Only the P10+5 treatment in early lactation responded with increased cell proliferation. This demonstrates that suckling large litters of 15 pups leads to increased proliferation of cells in the mammary gland in early lactation.

6.2 Introduction

Reports in the literature show that milking frequency or suckling stimulus can increase the DNA content of the mammary gland. In Chapter 5, suckling intensity was increased to 2 pups/gland by reducing the number of available mammary glands but no increase in [^{3}H] thymidine incorporation into DNA was measured. This may have been due to a limit at the individual gland level which restricts the total size and milk production from each gland. Alternatively it may have been due to a limit on the whole animal. The largest litter size in Chapter 5 was only 10 pups which is not unusually large for Swiss mice. Mammogens such as circulating prolactin (Grosvenor and Mena, 1974) are known to increase with litter size. It is possible that 10 pups did not cause sufficiently large releases of systemic mammogens, and

therefore the mammary glands did not increase proliferation. Thus another approach to increasing suckling intensity to cause mammary cell proliferation may be to increase litter size even further.

Research on ruminants has shown that increased milking frequency stimulates DNA synthesis in the lactating mammary gland. In cows for example, four times a day milking of quarters from week 11 to 15 resulted in 10% more milk than quarters milked only twice a day (Hillerton *et al.*, 1990). The higher milking frequency increased DNA synthesis (measured in biopsy explants) and increased the population of epithelial cells involved in milk synthesis (Hillerton *et al.*, 1990). Increasing the frequency of milking of cows to six times per day for the first six weeks of lactation resulted in 25% more milk than cows milked three times per day (Bar-Peled *et al.*, 1995). Milk yield remained elevated after the treatment period, which was attributed to increased mammary mass accumulated during the treatment period. Increased suckling intensity on ewes bearing single lambs given a second lamb after parturition, caused an increase in milk yield and gland volume that approximated those of ewes bearing twins (Davis *et al.*, 1980). Mammary glands of lactating goats also responded to increased milking frequency. Milk yield was 47% greater and [^{3}H] thymidine incorporation was 26% greater after 37 weeks of three times a day milking compared to contralateral glands milked only twice a day (Knight and Wilde, 1987).

Reports have shown the mammary glands of rodents are responsive to litter size (Bateman, 1957; Davis et al., 1979; Moon, 1969; Tucker, 1966). In guinea pigs, the weight of their two mammary glands measured on D5 of lactation increased as litters increased from two to five pups (Davis *et al.*, 1979). In rats, the mammary glands of those with 12 pups contained 39% more DNA by D13 of lactation than those with only six pups (Moon, 1969). Tucker *et al.* (1966) demonstrated that mammary growth during lactation in rats was related to the number of pups suckled. For example, on D16 of lactation, mammary DNA content of rats with six pups per six glands increased 108% greater relative to D1 compared with a 30% increase in rats with two pups per six glands. These results supported his earlier study in which mammary gland DNA content was related to litter size (Tucker, 1964).

Both endocrine and autocrine mechanisms are implicated in the response of mammary glands to an increase in milking frequency or suckling stimulus (Wilde and Peaker, 1990). Endocrine control is by Prl as well as hormones that affect digestion and metabolism. Stimulation of Prl receptors in mammary glands activates the JAK/STAT signal transduction pathway resulting in cell proliferation and milk synthesis (Hynes *et al.*, 1997). The concentration of Prl in plasma is related to the suckling stimulus (Tucker *et al.*, 1967; Grosvenor and Mena, 1974) as is expression of mRNA of the Prl receptor (Kim *et al.*, 1997). Suckling also increases plasma concentrations of gut hormones, such as gastrin, and pancreatic hormones such as insulin and glucagon (Algers, 1993). These increase the size of the gastrointestinal tract to accommodate a larger intake of food, and optimise digestion and metabolism so that the increased nutritive needs of lactation are met (Algers, 1993; Hammond *et al.*, 1996). Prolactin also redirects energy towards lactation by increasing in the number of insulin receptors in the mammary gland and decreasing the number in maternal fat stores (Uvnas-Moberg, 1989).

Autocrine control of lactation is affected by increased milking frequency, as discussed in Chapter 5. A small whey protein, called feedback inhibitor of lactation (FIL), is secreted in milk by epithelial cells (Wilde *et al.*, 1995). If milk is not removed from the gland, the effect of FIL increases and it acts on secretory cells to decrease milk synthesis (Wilde *et al.*, 1995). Frequent and complete removal of milk from the gland also removes FIL thereby allowing milk synthesis to occur at a higher rate (Wilde and Peaker, 1990). Autocrine control is also exerted by altering the sensitivity of mammary glands to endocrine hormones as shown by *in vivo* studies in which FIL decreased number of epithelial cell receptors for prolactin (unpublished studies by C. J. Wilde and K. O'Reilly cited by Wilde and Peaker, 1990) and decreased key mammary enzymes involved in milk synthesis (Wilde *et al.*, 1990).

Autocrine control is conditional on nutritional status. An increase in milking frequency would usually be associated with an increase in milk yield but this does not occur in frequently milked goats that are underfed (Blatchford and Peaker, 1983). This is attributed to the negative energy balance over-riding the effect of low level of FIL which would otherwise be associated with increased production (Wilde and Peaker, 1990). Short term challenges of increased milking frequency typically

caused a temporary increase in the milk produced from each cell while longer challenges cause an increase in secretory cell population and therefore permanent increase in milk production (Wilde and Peaker, 1990). The endocrine and autocrine systems of the dam are both affected by milking frequency or suckling stimulus so that digestion, metabolism, mammary gland size, secretory epithelial cell population size and milk output are adjusted in order to meet the requirements of her young. It is therefore reasonable to suggest that increased litter size would be expected to increase milk production and cell proliferation in mammary glands of mice.

In order to investigate the physiological basis of the increased yield and growth observed in the experiments reported above, mice were used as a model because they are cheaper and their gestation times shorter. An experimental approach equivalent to changing the milking frequency of ruminants was to change suckling intensity of mice by altering litter size.

The objective of the present study was to cause proliferation in the lactating mammary glands of Swiss mice by adjusting litter sizes to 5 or 10 pups on the day of parturition. Additional suckling intensity was applied to another two groups by fostering 5 extra pups for the last three days of lactation to increase litter size to 10 or 15 pups. As mammary RNA and RNA/DNA changes occur within 24h of a change in litter size (Tucker, 1966) and the cell cycle can occur within 8h (Hall and Levison, 1990), three days was considered an adequate period for change to occur within the mammary glands. The effects of varying suckling intensity on proliferation of mammary cells were studied in early (D3), mid (D10) and late (D17) lactation.

6.3 Experimental design

Eighty virgin female Swiss mice were mated and pregnancy was confirmed by observation of a vaginal plug. Pregnant mice were transferred to individual cages at D15 of pregnancy. Animal husbandry and management are detailed in Section 2.1.

Prior to parturition mice were randomly allocated to one of 12 groups. Sufficient mice were mated so that 5 mice, which had each given birth to 8 to 12 pups, were

allocated to each treatment. Three groups were allocated to each of four treatments and a group from each treatment was killed at each of early, mid and late lactation on days 3, 10 and 17 (D3, D10 and D17). Dams in control groups suckled litters of 5 (C5) or 10 (C10) pups from parturition onwards. Dams in treatment groups also suckled litters of 5 or 10 pups from parturition but then had 5 extra pups added for the last 3 days of lactation to increase litter size from 10 to 15 pups respectively (P5+5, P10+5). Litter sizes were adjusted within 12h of parturition.

Litter weights were recorded daily (Section 2.1.4) until the day of euthanasia of the dam and her litter. Milk production was calculated using the metabolic weight method (Section 3.4.2.2).

On D3, D10 or D17 the lactating mice were injected with [^{3}H] thymidine (Section 2.1.7) and euthanased 1h later. All 10 mammary glands were dissected from each mouse, blotted and weighed (Section 2.1.8). The weight of the 10 glands was summed to obtain the wet gland weight which was corrected for milk content (Section 3.5.1). The left 5th and right 5th glands were homogenised individually and aliquots from each were analysed for concentrations of lactose, DNA, protein and [^{3}H] thymidine (Section 2.3).

A whole gland mount of the right 4th gland was made (Section 2.4.1). Autoradiographs were prepared from sections of the mammary glands (Section 2.4.2) and then counter-stained with haemotoxylin and eosin (Section 2.4.5). One replicate from the group was not assessed owing to nearly negligible labelling. The lumen area, lumen perimeter and labelling index of epithelial cells were measured in 6 locations (700-1000 cells per site) being the outer 2 mm of the gland (called outer) and inside area (called inner) of the proximal, mid and distal thirds of each gland. Lumen area and lumen perimeter were measured by capturing the microscope image and analysing using image software (Section 2.4.6). An eyepiece micrometer was used to calibrate the image software and enable conversion of pixels to microns (1.42 pixels equivalent to 1 μm). The labelling index was measured at x400 magnification by counting the number of epithelial cells surrounding lumens and the number of these labelled with [^{3}H] thymidine. Cells with more than five dark grains in the nucleus were considered to have been labelled.

Data were analysed by a two way ANOVA (fitting treatment, stage of lactation and their interactions) using the GLM procedure of Minitab (Release 12.23, 1999) where individual mean comparisons were performed by LSD. Litter growth rates were analysed similarly but with the difference that pre-treatment growth rates were used as a covariate. The pooled standard deviation was used to calculate the standard errors of differences among the means which are represented on graphs by vertical brackets. To compare the labelling indices of epithelial cells, and lumen areas and perimeters, Genstat (5th edition, Release 4.2, 2000) was used to fit a GLM with a binomial distribution with logit link (Nelder and Wedderburn, 1972) to test between inner and outer areas as well as among the different thirds of glands and to test the between mouse variation.

6.4 Results

6.4.1 Litter growth rates, milk production and milk production per unit DNA

In early lactation, litter growth rate did not differ between the litters (P>0.05) (Figure 6.1). Peak growth rates occurred in mid lactation (P<0.05) with the highest (P<0.05) growth rate of 5.2 g/d recorded for the C10 litters. In late lactation, C10 litters grew the most rapidly (P>0.05) with the C5 litters growing the slowest.

Milk production (Figure 6.2) was lowest for mice with C5 litters at all stages of lactation (P<0.05). In early lactation, milk production did not differ between the other three groups (P>0.05). In mid and late lactation, mice with C10 and P10+5 had similar milk production (P>0.05).

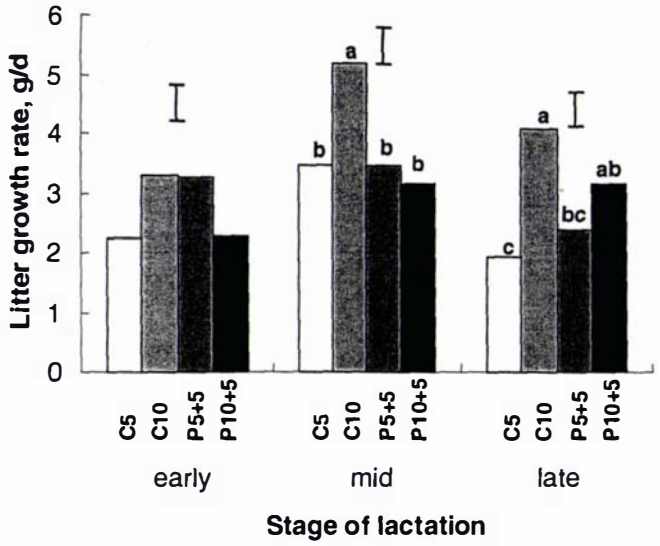

Figure 6.1: Effect of litter size on litter growth rates.

Litter growth rates (g/d) during 3 days in early (D0-3), mid (D7-10) and late (D14-17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a**, **b** and **c** differ within a stage of lactation, the means differ ($P<0.05$).

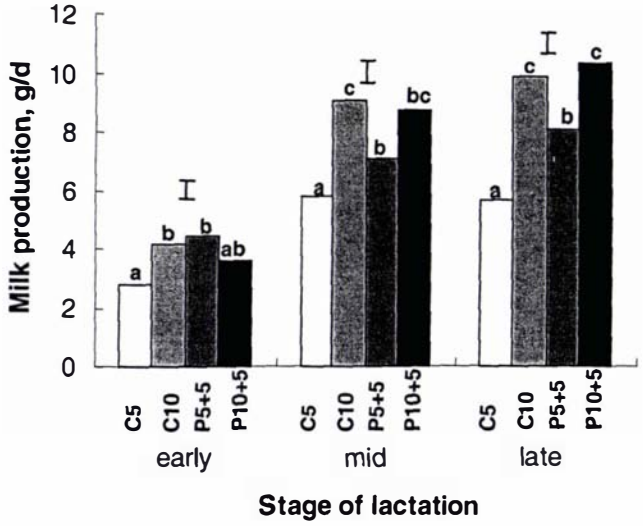

Figure 6.2: Effect of litter size on milk production.

Milk production (g/d) in early (D3), mid (D10) and late (D17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a**, **b** and **c** differ within a stage of lactation, the means differ ($P<0.05$).

Milk production per unit of DNA (Figure 6.3) which was estimated by dividing milk production by the total DNA content of the ten glands, did not differ between treatments in either early or mid lactation (P>0.05). In late lactation, the milk production per unit of DNA was lowest for mice with C5 litters (P<0.05).

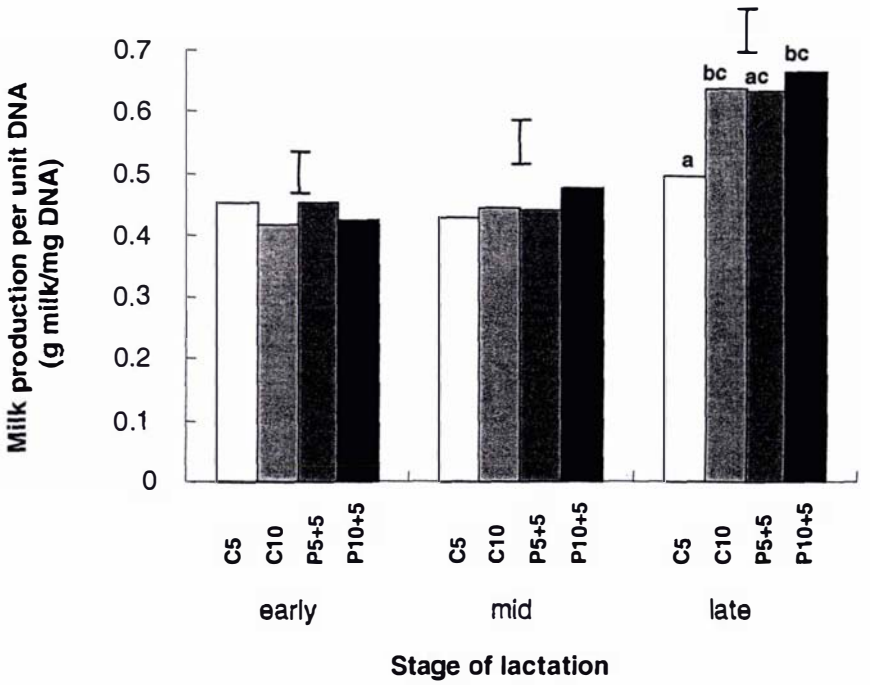

Figure 6.3: Effect of litter size and DNA content of the mammary glands on milk production.

Milk production per unit of DNA (g milk/mg DNA) in early (D3), mid (D10) and late (D17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a**, **b** and **c** differ within a stage of lactation, the means differ (P<0.05).

6.4.2 Mammary gland weight and milk content

The amount of milk in the glands one hour after the pups were removed (Figure 6.4) was significantly higher in mid compared to late lactation, which in turn was significantly higher than early lactation (2.2 versus 1.8 versus 1.2 g for mid, late and early lactation respectively, P<0.05). There was no difference in milk content of the

glands in early or late lactation (P>0.05). In mid lactation, the glands of C5 mice contained less milk compared to other treatments (P<0.05).

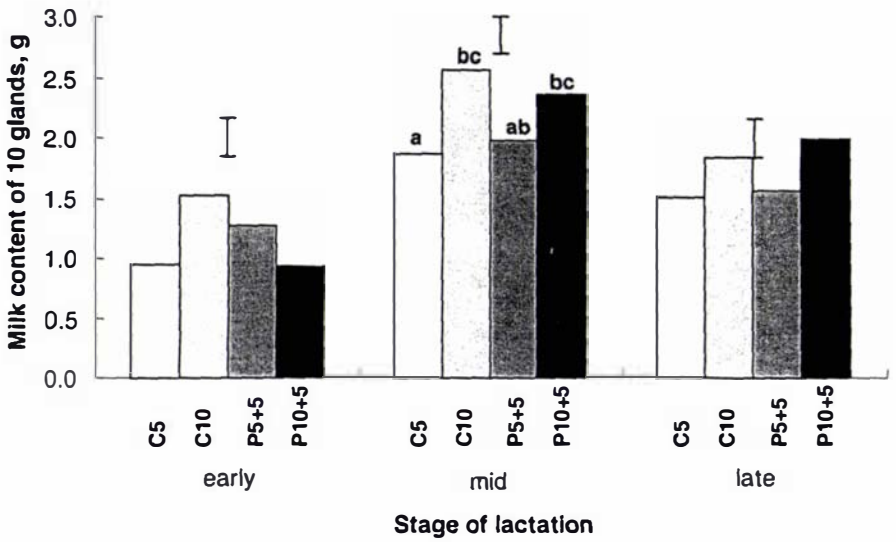

Figure 6.4: Effect of litter size on the milk content of mammary glands.

Milk content (g) of the mammary glands of lactating mice in early (D3), mid (D10) and late (D17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a**, **b** and **c** differ within a stage of lactation, the means differ (P<0.05).

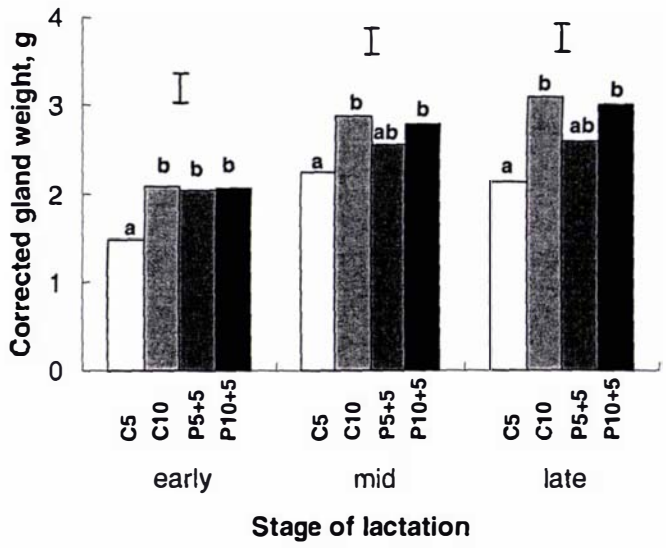

Figure 6.5: Effect of litter size on corrected weights of mammary glands.

Corrected weights of ten mammary glands (g) of lactating mice in early (D3), mid (D10) and late (D17) when lactating mice suckled either 5 pups from parturition

(C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a** and **b** differ within a stage of lactation, the means differ ($P<0.05$).

Corrected gland weight (Figure 6.5) was lowest in early lactation (2.1, $P<0.05$) but did not differ between mid and late lactation (2.7 and 2.8 g, respectively, $P>0.05$). Corrected gland weights of mice allocated to C5 were lighter than for C10 and P10+5 at all three stages of lactation ($P<0.05$).

6.4.3 Gland composition

6.4.3.1 DNA

The concentration of DNA (Figure 6.6) in corrected gland weight did not differ between treatments within a stage of lactation ($P<0.05$). The concentration was highest in mid lactation, followed by late and then early lactation (6.5 versus 5.0 versus 4.1 μg/mg respectively, $P<0.05$ between each). Similarly, the content of DNA (Figure 6.7) in the glands was highest in mid lactation followed by late lactation and finally early lactation (17.4 versus 14.2 versus 8.8 mg, respectively, $P<0.05$ between each). The content of DNA did not differ between treatments ($P>0.05$) except in mid lactation when glands of mice allocated to C10 and P10+5 contained more DNA than those in C5 ($P<0.05$).

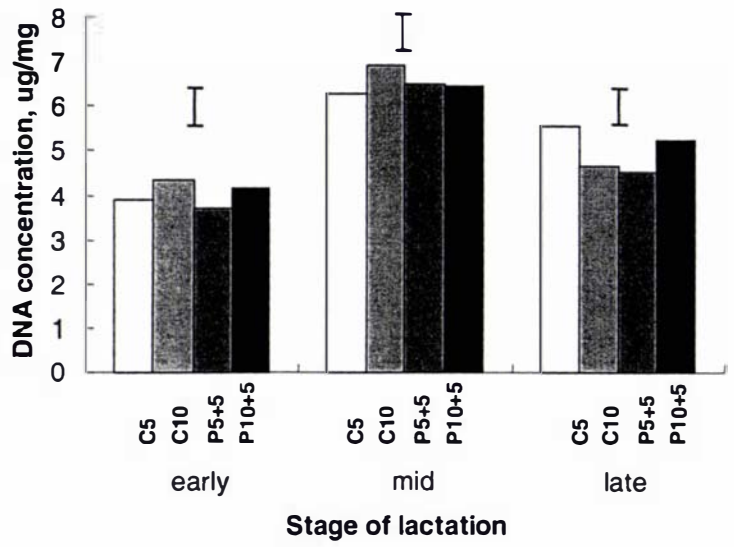

Figure 6.6: Effect of litter size on concentration of DNA in mammary glands.

DNA concentration (μg/mg) of mammary glands corrected for milk content of lactating mice in early (D3), mid (D10) and late (D17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5.

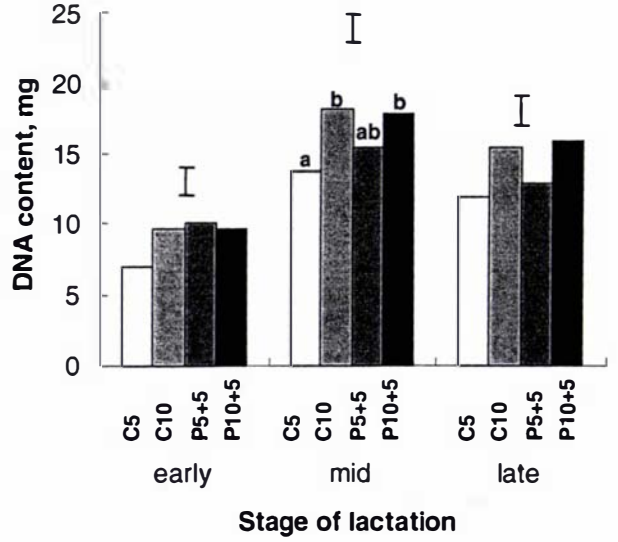

Figure 6.7: Effect of litter size on content of DNA in mammary glands.

DNA content (mg) of the ten mammary glands of lactating mice on the last day of 3 day periods in early (D0-3), mid (D7-10) and late (D14-17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of

differences between the means within a stage of lactation, n=5. Where superscripts **a** and **b** differ within a stage of lactation, the means differ (P<0.05).

6.4.3.2 Protein

The concentration of protein (Figure 6.8) was highest in mid lactation followed by late and then early lactation (131.9 versus 112.9 versus 93.3 mg/g respectively, P<0.05 between each) with there being no difference between treatments within any stage of lactation (P>0.05). The content of protein (Figure 6.9) in milk corrected mammary glands was also greatest in mid lactation followed by late lactation and then early lactation (355 versus 324 versus 189 respectively, P>0.05 between each). In early lactation, the protein content of glands did not differ (P>0.05). In mid and late lactation, the glands of mice allocated to C5 contained less protein than those of mice in C10 and P10+5 while P5+5 was intermediate.

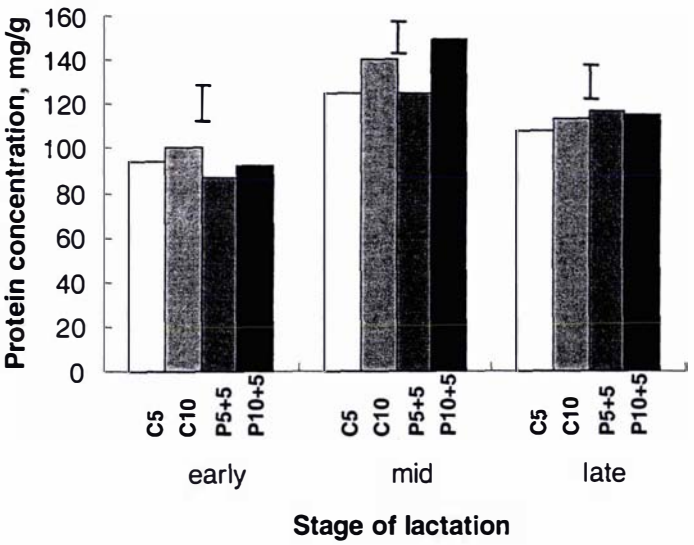

Figure 6.8: Effect of litter size on concentration of protein in mammary glands.

Protein concentration (mg/g) of mammary glands of lactating mice in early (D3), mid (D10) and late (D17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5.

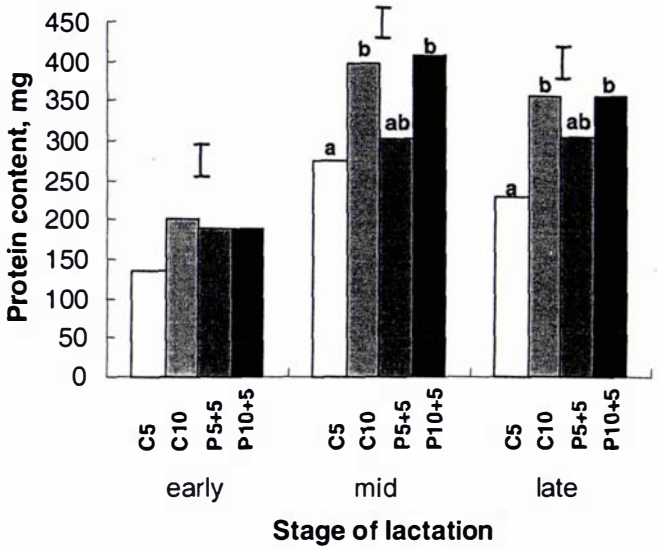

Figure 6.9: Effect of litter size on content of protein in mammary glands.

Protein content (mg) of the ten mammary glands of lactating mice in early (D3), mid (D10) and late (D17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a** and **b** differ within a stage of lactation, the means differ ($P<0.05$).

6.4.3.3 [^{3}H] thymidine incorporation

Incorporation of [^{3}H] thymidine (Figure 6.10) was highest in early lactation ($P<0.05$). There was no difference in levels of incorporation between mid and late lactation ($P>0.05$). In early lactation, P10+5 mice incorporated more [^{3}H] thymidine into DNA than mice in the other treatments ($P<0.05$).

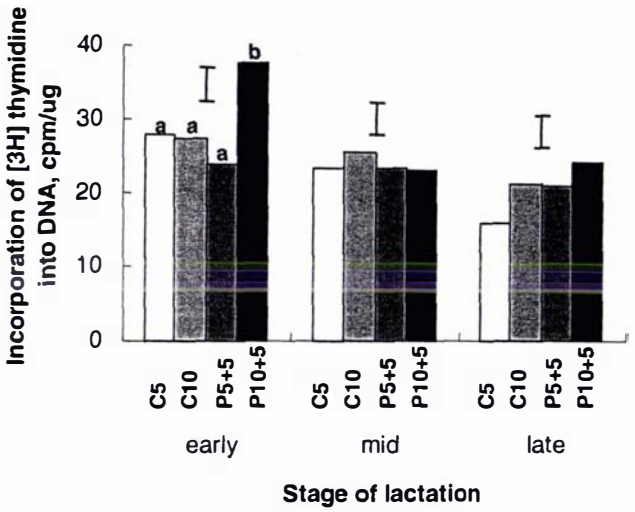

Figure 6.10: Effect of litter size on incorporation of [^{3}H] thymidine into DNA of mammary glands.

Incorporation of [^{3}H] thymidine into DNA (cpm/μg DNA) of mammary glands of lactating mice in early (D3), mid (D10) and late (D17) when lactating mice suckled either 5 pups from parturition (C5), 10 pups from parturition (C10), 5 pups from parturition plus 5 pups for the last 3 days (P5+5) or 10 pups from parturition plus 5 pups for the last 3 days (P5+5). Vertical brackets represent standard errors of differences between the means within a stage of lactation, n=5. Where superscripts **a** and **b** differ within a stage of lactation, the means differ ($P<0.05$).

6.4.4 Autoradiography and histology

Labelling indices were recorded for both the inner areas and outer edges of the proximal, mid and distal thirds of the glands of the P10+5 mice (four of the five replicates only) from early lactation (Table 6.1). Proliferation rates among the different thirds did not differ ($P>0.05$). No difference was measured in the labelling indices of inner areas compared to outer edges ($P>0.05$). Lumen area and perimeter were also measured in the same glands but did not differ among any of the 6 sites ($P>0.05$).

Table 6.1: Effect of litter size on labelling index, lumen area and perimeter of mammary glands.

Labelling index of mammary epithelial cells (%), lumen area (μm^2) and lumen perimeter (μm) in six locations (3 regions x 2 sites) of mammary glands of lactating mice that suckled 15 pups (P10+5) from D0-3. Means $\pm$ SEM, n = 4 mice.

Location	labelling index, %		lumen area, μm^2		lumen perimeter, μm	
	mean	SEM	mean	SEM	mean	SEM
distal	4.2	0.9	4165	487	253	17
mid	3.9	0.9	3553	438	237	15
proximal	4.3	0.9	3557	438	247	15
inner	4.7	0.7	3784	365	246	13
outer	3.5	0.7	3732	365	245	13

Proliferation within the gland was very heterogeneous and mosaic in nature rather than being even across a field of view (Figure 6.11).

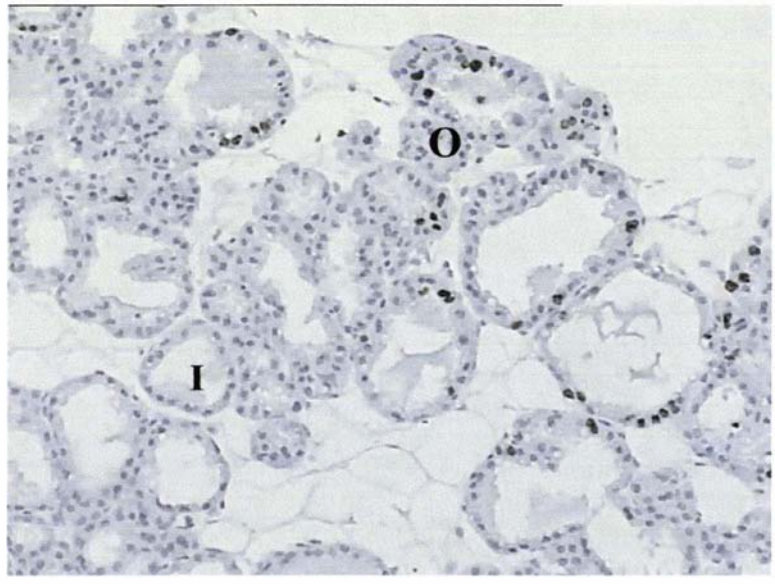

Figure 6.11: Heterogeneous labelling of epithelial cells in the mammary gland of a mouse.

Autoradiograph showing the incorporation of [^{3}H] into mitotic DNA (dark grains over nuclei in inner (I) and outer (O) areas of mammary gland tissue from a mouse that suckled 15 pups for 3 days after parturition (x100).

6.5 Discussion

Increased proliferation of cells in lactating mammary glands was observed in early lactation in dams suckling 15 pups (P10+5) (Figure 6.10). Sufficient proliferation had not occurred by D3, however, for a detectable rise in gland weight, DNA content or concentration compared to mice with 10 pups (C10, P5+5). It was expected that given a longer time period prior to sampling, the elevated proliferation would result in increased mammary gland weight and DNA content so that these parameters would be related to litter size (Tucker, 1966; Moon, 1969; Knight and Peaker, 1982e).

Results from early lactation were attributed to suckling intensity and its effect on endocrine and autocrine control of lactation. In the present study, litters of 15 pups removed a similar volume of milk as litters of 10 pups (Figure 6.2) so the increased proliferation of cells (Figure 6.10) was potentially a response to greater concentrations of plasma prolactin induced by the increased suckling intensity of 15 pups (Tucker *et al.*, 1967; Grosvenor and Mena, 1974). Experiments in which differing doses of bromocriptine (an agonist that suppresses secretion of Prl) were administered to mice showed that in early lactation the proliferation of secretory cells is directly related to the concentration of Prl in plasma (Knight *et al.*, 1986a). The difference between litters of 10 and 5 pups in the present study would have been both increased milk removal and suckling intensity in dams with litters of 10 pups. In addition to increased Prl in plasma there would be a more efficient removal of FIL with the result of increased gland weight and milk production of mice with 10 pups compared to C5 mice by D3 of lactation. Although there was no difference in DNA contents of glands of C5 mice compared to mice with 10 pups, it is likely there was a difference in the number of active, secretory cells. The lower amount of milk removed from C5 mice would lead to an accumulation of FIL that would slow milk synthesis (Knight *et al.*, 1986a) and slow development of cells into secretory epithelial cells (Knight *et al.*, 1986a), resulting in a proportion of the mammary epithelial cell population that was non secretory.

In mid and late lactation, mammary glands did not respond to the P10+5 challenge. There were no differences between mice nursing C10 and P10+5 in terms of gland weight, DNA and protein content and concentration, and amount of [^{3}H] thymidine incorporated into DNA. Although the larger litter would have been expected to increase the amount of prolactin released (Grosvenor and Mena, 1974), this did not increase proliferation of cells, possibly due to physiological limitations of the mothers. In early lactation when milk yield is low, the food intake and nutrient absorption of the mothers do not limit milk production (Hammond and Diamond, 1994) and, therefore, extra energy requirements of the gland, such as growth, can be supported. Mid lactation, however, is a time of high milk yield and therefore high physiological demands on the mothers (Hammond and Diamond, 1994). It is possible that conversion of substrates to milk is maximised for litters of 10 pups and cannot be increased further; therefore no extra response to the P10+5 challenge could be made in mid lactation. In the present study, an inadequate nutrient status may not have permitted actions of the extra prolactin in the endocrine system or removal of FIL in the autocrine system. Nutrient status can limit yield as demonstrated in a study by Roberts and Coward (1985) in which milk yield of rats suckling eight pups increased in response to feeding of dietary supplements. Another possibility for the absence of a response of mice to P10+5 is the physical limitations of the gland. The gland may have an upper limit for size and output which may have been reached when the litter size was 10 pups.

The lesser challenge of P5+5 did have an effect in mid and late lactation. Compared to C5 mice, milk production from P5+5 mice was greater ($P<0.05$; Figure 6.2) and rather than being due to increase production per unit of DNA (Figure 6.3) it appeared to be due to an increase in gland size. Mammary gland weight, DNA and protein content of P5+5 mice were numerically intermediate between those of C5 and C10, mice although none were statistically greater than C5 in mid lactation. Increased proliferation of cells in the glands of P5+5 mice was not detected with the 1h label of [^{3}H] thymidine possibly because proliferation had occurred prior to sampling. The increase in gland size implies that the physiological limitations present in mothers suckling litters of 15 pups, or the limitations of the mammary glands, were not present in mothers suckling P5+5 pups.

The mammary gland weights, DNA concentration and content, protein concentration and content of mammary tissue containing residual milk were comparable to others reported in the literature (Knight and Peaker, 1982d; Shipman *et al.*, 1987; Alston-Mills *et al.*, 1995). However, the present study corrected for milk content since the milk content of glands changes with stage of lactation and litter size. The present study therefore provides the most accurate data to date on the weight and composition of mammary glands in lactating mice.

Milk production per unit of DNA was similar to that reported in the literature. In the present study it was estimated that 3.8 to 4.0 g milk was produced from each gram of mammary tissue containing residual milk (equivalent to 0.43 to 0.45 g milk/mg DNA) from D3 to 10 of lactation. Knight *et al.* (1984) reported milk production per unit of DNA in rats to increase from 2.7 to 3.7 g milk/g mammary tissue containing residual milk from D1 to 13 but no such increase was measured in the present study (Figure 6.3; $P>0.05$) possibly because lactation was more established and therefore milk production per unit of DNA had increased above the low levels found closer to parturition. The present study using mice also gave results similar to those of Linzell (1972) who reported that milk production per unit weight of mammary tissue to be relatively constant at approximately 5 g/g tissue across livestock species.

The location of epithelial cell proliferation in mammary tissue was studied in the mammary glands with the highest cell proliferation which were from mice in early lactation that suckled 15 pups. Mitosis in the mammary glands from these four mice was observed to be very heterogeneous (Figure 6.11). In some fields of view, highly labelled alveolus were neighbouring non-labelled alveoli. Heterogeneity similar to these observations has been reported for mitosis in secretory epithelial cells in rats (Franke and Keenan, 1979) and the expression of protein genes in ruminant mammary tissue (Molenaar *et al.*, 1992). The present study attempted to quantify differences in labelling indices in the inner and outer areas of the proximal, mid and distal thirds. No significant differences were measured (Table 6.1; $P>0.05$) however this may have been due to the technique for identification of the orientation of the gland not being sufficiently rigorous. The proximal third of the gland was identified by the presence of the primary milk duct but this was not easily found in some of the mammary gland sections with the result that proximal thirds may have been counted

as distal thirds and vice versa. Lumen area and perimeters were also measured to determine differences between highly labelled and non-labelled alveoli. An increase in alveolar diameter has been previously measured in rats as epithelial cells change from being mitotic to differentiated and secretory (Munford, 1963) but no differences were detected in the present study.

Cell proliferation was stimulated by the highest suckling intensity treatment of P10+5 pups in early lactation. This was probably because the mammary glands responded to an increase in prolactin arising from the more intense suckling pressure. This response could only occur if permitted by a nutritional plane sufficient to meet the metabolic requirements of the gland, and autocrine conditions, such as low FIL levels, which increased gland sensitivity. The absence of elevated proliferation in mid and late lactation could be explained by a potential physiological limitation in either the energy balance of the dam, or an inherent limit to gland size. Mice responded in mid and late lactation with a slight increase in gland weight as a result of increasing litter size from 5 to 10 pups. Proliferation in the glands of mice in early lactation suckling 15 pups showed marked heterogeneity although this was not supported by labelling index data. The treatment of P10+5 in early lactation showed great promise for use as a model of proliferation during lactation and was used in further experiments.

Chapter 7 Validation of model of proliferation of epithelial cells in mammary glands during lactation

7.1 Summary

In a previous study (Chapter 6), the mammary glands of mice suckling 15 pups incorporated significantly more [^{3}H] thymidine than those with only 10 pups. The experimental design was improved by increasing the number of replicates and selecting mice based on live weight, natural litter size and natural litter weight. The study (Experiment 7A) did not confirm earlier results necessitating a further repeat study (Experiment 7B) that also failed to confirm the study in Chapter 6. It was concluded that incorporation of [^{3}H] thymidine into mammary DNA on D3 of lactation was inversely related to litter size for suckling intensities of $\geq$1 pup per gland (Experiment 7C). This result was most likely due to larger litters providing greater stimulation of endocrine and autocrine systems that accelerate mammary development in terms of cell number and differentiation into cells capable of milk synthesis. Mammary glands were found to have inherent limits on their size and output (Experiment 7A and 7B) and these limits were achieved with a suckling intensity of 1 pup per gland (or 10 pups). Extra pups may bring forward the time at which this limit is reached but they do not result in the limit being exceeded (Experiment 7C). It was concluded that increasing suckling intensity did not increase mammary cell proliferation to the magnitude required for molecular biology studies.

7.2 Experiment 7A

7.2.1 Introduction

A principle aim of the experiments described in this thesis was to induce experimentally an increase in proliferation of mammary epithelial cells during lactation. Such an outcome would improve the biological understanding of

proliferation of secretory mammary tissue and the knowledge may be used strategically to increase mammary growth in lactating dairy species.

Two challenges reported in this thesis, the administration of exogenous steroids and taping of mammary glands to increase the suckling intensity of available glands, did not increase cell proliferation in lactating mice. In contrast, a third challenge, increasing litter size, did increase cell proliferation successfully. In the latter study, incorporation of [^{3}H] thymidine into DNA was elevated in early lactation by increasing litter size to 15 pups (Chapter 6, Figure 6.10, $P<0.05$).

The experimental approach described in Chapter 6 successfully generated mammary tissue that incorporated high levels of [^{3}H] thymidine but it required confirmation. In addition it was considered that the experimental design could be improved and the experiment was therefore repeated. For example, there were large variances within groups and between groups and these could be reduced by increasing replicate numbers and by selection of dams with similar weights within similarly sized natural litters. The post partum weight of dams has a significant effect on mammary gland weights and lactational performance (Bateman, 1957) and therefore using only dams of similar live weight would reduce variation. In Chapter 6, mice were mated at seven weeks of age when they were sexually mature but still gaining weight. Delaying mating until mice are 10 weeks old would ensure all growth is complete so that future mammary gland size and lactational performance are maximised. Another source of variation is fetal litter size which is correlated with the concentration of placental lactogen released into the maternal circulation that acts as a mammogen. As a consequence, mammary weight at parturition is related to litter size (Nagasawa and Yanai, 1971; Nagai, 1978; Markoff and Talamantes, 1981). Some studies report that the effect of fetal litter size is not permanent (Bateman, 1957) as weight gain of fostered litters at D12 was related to the number of pups fostered and not the natural litter size (Eisen *et al.*, 1980). This result is attributed to growth of mammary glands post partum in response to the changed litter size (Knight and Peaker, 1982e). However, the effect of fetal litter size on mammary tissue closer to parturition (Markoff and Talamantes, 1981; Knight and Peaker, 1982e) is greater and therefore any study of early lactation should correct for fetal litter size. A third source of variation addressed in the current experiments was the unexplained

variation between groups. In particular, mice with 15 pups had more varied responses than control mice with 10 pups and therefore the number of replicates was increased in order to reduce the standard errors.

The objective of the present study was to confirm the conclusions drawn in Chapter 6 in which proliferation was increased on D3 of lactation in the mammary glands of Swiss mice suckling 15 pups. An attempt was made to reduce the variances measured in that experiment by the use of dams of similar live weights and natural litter sizes and natural litter weights as well as increasing the number of replicates.

7.2.2 Experimental design

The procedures followed were the same as for Chapter 6 (Section 6.3) except where indicated below.

Thirty female Swiss mice were mated at 10 weeks of age, rather than seven weeks, to ensure they were sexually mature and all growing was complete. Sufficient mice were mated to enable selection of 20 mice that were similar in weight and had given birth to litters of similar size and weight. The treatments were adjustment of litter size on D0 of lactation to either 10 or 15 pups. Five mice were allocated to the 10 pup treatment and 15 were allocated to the 15 pup treatment. Mice suckled litters until D3 of lactation when they were euthanased. Litter growth rates (Section 2.1.4) and milk production (Section 3.4.2.2) were measured. Lactating mice were injected with [^{3}H] thymidine on D3 (Section 2.1.7). One hour later they were killed and the 4th and 5th left and right mammary glands were dissected from each mouse, blotted and weighed (Section 2.1.8). Gland weights were corrected for content of milk to give corrected gland weights (Section 3.5.1). The left 5th and right 5th glands were analysed for concentrations of lactose, DNA and [^{3}H] thymidine incorporation (Section 2.3).

7.2.3 Results

Mice with very similar characteristics were selected for this trial in order to reduce variation among replicates. On D0 of lactation, mice in both groups had similar live weights and had given birth to litters of similar sizes and weights (Table 7.1; P>0.1 for all). After three days of lactation there were no differences between the groups in dam live weight (P>0.1) and weight gain (P>0.1).

Table 7.1: Experiment 7A; dam and litter weight and litter size.

Weights of dams on D0 and D3 of lactation, dam weight gains, natural litter sizes and natural litter weights of mice allocated to treatments in which litters were adjusted to either 10 or 15 pups on the day of parturition. Means are followed by SEM. n=5 for mice with 10 pups and n=15 for mice with 15 pups.

	Litter size		
	10	15	P value
Dam wt on D0, g	35.3 ± 1.53	35.1 ± 0.88	0.911
Dam wt on D3, g	38.3 ± 1.09	38.0 ± 0.63	0.836
Dam wt gain, g/d	2.9 ± 0.87	2.9 ± 0.50	0.950
Natural litter size, pups	12.0 ± 0.56	11.9 ± 0.32	0.838
Natural litter wt, g	17.9 ± 0.70	18.1 ± 0.42	0.880

Similarly, there were no differences between the two treatments (P>0.1) for litter growth rate, milk content of glands, DNA concentration and protein concentration (Table 7.6 in Appendices at the end of this chapter). Milk production, corrected gland weights and DNA content of glands did not differ between either treatment (Table 7.2; P>0.1). Incorporation of [^{3}H] thymidine into DNA, however, was greater in glands of mice suckling 10 pups than those with 15 pups (P<0.05).

Table 7.2: Experiment 7A; milk production and weights, DNA content and incorporation of [^{3}H] thymidine of mammary glands.

Milk production, weights and DNA contents of four glands, and incorporation of [^{3}H] thymidine into DNA (all corrected for milk content) of mammary glands collected on D3 of lactation after mice had suckled either 10 or 15 pups for three days. Means are followed by SEM. n=5 for mice with 10 pups and n=15 for mice with 15 pups.

	Litter size		
	10	15	P value
Milk production, g/d	4.21 ± 0.167	4.50 ± 0.097	0.148
Weight of 4 glands, g	0.94 ± 0.087	1.09 ± 0.050	0.160
DNA content of 4 glands, mg	3.40 ± 0.342	3.90 ± 0.182	0.187
[^{3}H] incorporation, cpm/μg DNA	34.9 ± 3.47	24.7 ± 1.86	0.020

7.2.4 Discussion

Incorporation of [^{3}H] thymidine into DNA was higher for mice with 10 pups compared to those with 15 pups (Table 7.2; $P<0.05$) on D3 of lactation in contrast to the results in Chapter 6 (Figure 6.10; $P<0.05$). In addition, an ANOVA of the two studies revealed a significant interaction ($P<0.05$) such that between studies the milk yield did not differ for mice with 10 pups (4.1 versus 4.2 g/d for Chapter 6 and Experiment 7A, respectively, $P>0.05$) but it differed by 1 g/d for mice with 15 pups (3.5 versus 4.5 g/d for Chapter 6 and Experiment 7A, respectively, $P<0.05$). In Chapter 6, the higher incorporation of [^{3}H] thymidine (Figure 6.10; $P<0.05$) and tendency for lower milk production (Figure 6.2, $P=0.2$) of mice with 15 pups compared to those with 10 pups on D3 of lactation is consistent with the theory of Knight and McLelland (1988). They suggested that during synthesis of new cells, milk yield is temporarily depressed but eventually increases to yields above that of control animals resulting from the increase in population of secretory cells. It should be noted that Knight and McLelland (1988) did not include a measure of DNA content or incorporation of [^{3}H] thymidine to confirm their theory of reduced milk yield during mitosis. However, the discrepancy in the results for incorporation of

[^{3}H] thymidine between the experiment in Chapter 6 (Figure 6.10) and in Table 7.2 suggests there may be an alternative explanation for the results.

Three of the five replicates in the treatment with 15 pups reported in Chapter 6 had a large influence on the mean of that treatment. The three mice had lower milk yields and higher incorporations of [^{3}H] thymidine. This may be consistent with involution as uptake of [^{3}H] thymidine is higher in involuting glands owing to remodelling of mammary tissue (Capuco and Akers, 1990). However, it is unexpected that mammary glands should be regressing on D3 of lactation which is typically a time of growth and development of secretory cells (Knight and Peaker, 1982b). There are reports of mice that are unable to lactate effectively in early lactation (Udy *et al.*, 1997) but this too would be unusual for a wild type mouse such as the Swiss mice used. An alternative explanation is that these three mice had mammary infections and invading macrophages were labelled *in vivo* with [^{3}H] thymidine (Ryu *et al.*, 1990) however histological observations did not indicate the presence of large numbers of macrophages. The results may also be due to a type 1 statistical error or the chance occurrence of allocating the three atypical mice to one treatment.

In the present study, mice suckling 10 pups incorporated more [^{3}H] thymidine into mammary DNA than mice suckling 15 pups. This could be interpreted as higher mammary cell proliferation in glands of mice with 10 pups. Compared to the mammary glands of mice with 10 pups, the glands of mice with 15 pups may have contained a greater proportion of differentiated cells capable of milk synthesis and a lesser proportion of cells undergoing mitosis, and therefore less [^{3}H] thymidine was incorporated into mammary tissue. In support of increased milk removal causing increased cell differentiation is the report by Wilde *et al.* (1990) who measured enzymes indicative of milk secretory cells to be greater in udders of goats milked thrice daily compared to goats milked twice daily.

Variation in most parameters measured in the current study was reduced by selection of mice of similar live weights and with similar natural litter weights and sizes. For instance, the SEM of milk production by mice with 10 pups was more than halved in the present experiment compared to that in Chapter 6 (0.17 versus 0.44 g/d, n=5 for each). Similar reductions in SEM were obtained for all parameters except the

incorporation of [^{3}H] thymidine in DNA. Further reductions in SEM were also achieved for the group suckling 15 pups by increasing replicate numbers from 5 in Chapter 6 to 15 in the present study as evident by a near halving of the SEM for [^{3}H] thymidine incorporation results (0.18 versus 0.31 cpm/μg DNA, n=5 and 15 respectively).

Methods to improve the sensitivity of labelling procedure were evaluated after the present study. This was because the use of mice selected using the criteria discussed above reduced variation in most measurements but not for incorporation of [^{3}H] thymidine. In the present study, the label was injected at a dose of 2 μCi/gm live weight and left to incubate for 1h, which is the same as the experimental design reported in the literature (Haslam, 1988). It was decided not to increase the dose as this would require volumes of ≥80 μl that may take too long to clear from the perineal region of the mice. Increasing the incubation time to 2h would perhaps allow labelling of more mitotic cells to occur to increase the between group variation with a concomitant decrease for within group variation and was therefore considered for future experiments.

Cell proliferation, measured by incorporation of [^{3}H] thymidine into DNA, was higher on D3 of lactation for mice with 10 pups compared to mice suckling 15 pups. This is the opposite result to a previous experiment (Chapter 6) which necessitated repeating the experiment a third time. Selection of mice with similar traits reduced variation of measurements in the present study and the procedure was used in subsequent experiments. Increasing the incubation time of the [^{3}H] thymidine label was implemented to improve the sensitivity of the experiment and reduce error.

7.3 Experiment 7B

7.3.1 Introduction

Two previous studies yielded conflicting results on cell proliferation in mammary glands of mice in response to litter size. In Chapter 6, mice with 15 pups

incorporated more [^{3}H] thymidine into DNA than mice with 10 pups while in Experiment 7A, the opposite result was obtained. Therefore the experiment was repeated.

Additional treatments were proposed to extend the degree of suckling stimulus, including a litter size of 20 pups and imposing large litters on small mice that have small natural litters and therefore smaller mammary glands at parturition (Knight and Peaker, 1982e).

The objective of the present study was to determine the differences in proliferation in lactating mammary glands of Swiss mice on D3 of lactation that had litter sizes adjusted to 10, 15 or 20 pups post partum. A fourth treatment was applied by selecting small dams with small natural litters and adjusting their litters to 15 pups. A final objective was to improve sensitivity of labelling with [^{3}H] thymidine by increasing the incubation time to 2h. Again, within group variation was reduced by selection of mice based on live weight, litter weight and size and by increasing the numbers of replicates.

7.3.2 Experimental design

The procedures followed were the same as for Chapter 6 and Experiment 7A except where indicated below.

Forty female Swiss mice were mated at 10 weeks of age and after parturition 21 were selected on the basis of similar live weight, natural litter size and natural litter weight. An additional 5 mice were selected based on their low live weights and small litter sizes. The 21 mice of similar characteristics were randomly allocated to one of three treatments. Treatments required adjustment of litter size on D0 of lactation to 10, 15 or 20 pups. The 5 small mice had their litter sizes adjusted to 15 pups on D0 of lactation. Mice suckled litters until D3 of lactation when they were euthanased. Litter growth rates (Section 2.1.4) and milk production (Section 3.4.2.2) were measured. On D3 lactating mice were injected with 2 μCi [^{3}H] thymidine/g body weight (Section 2.1.7) and incubated for 2h rather than only 1h as in

Experiment 7A and Chapter 6. One hour later they were killed and the 4th and 5th left and right mammary glands were dissected from each mouse, blotted and weighed (Section 2.1.8). Gland weights were corrected for content of milk to give corrected gland weights (Section 3.5.1). The left 5th and right 5th glands were analysed for concentrations of lactose, DNA and [^{3}H] thymidine incorporation (Section 2.3).

7.3.3 Results

Differences in dam weight on D0 or D3, dam weight gain or natural litter size and weight among groups of average sized mice allocated to litter sizes of 10, 15 or 20 were not significant (Table 7.3; P>0.1 for all results). The smaller mice allocated to the 15 pup treatment were lighter on D0 and D3, and their natural litters were smaller and weighed less than the others (P<0.05). Dam live weight gains did not differ among the four groups (P>0.1).

There were no significant differences for litter growth rate, DNA concentration and protein concentration among the 3 treatments which used mice of similar characteristics (Table 7.7 in Chapter 7 Appendices, P>0.1). Measurements of these parameters for the small mice were also not significantly different to the other three treatments (P>0.1) but litter growth rate tended to be lower (P=0.12).

Overall, there were no differences in milk production, corrected gland weights and DNA content of glands among the four treatments (Table 7.4; P>0.1 for all results). Incorporation of [^{3}H] thymidine into DNA was greatest in mammary glands of mice suckling 10 pups compared to the three other treatments that suckled either 15 or 20 pups (P<0.05).

The longer incubation time did not affect the absolute values of [^{3}H] incorporated into DNA when compared to Experiment 7A and a slight reduction in the SEM was attributed to increased replicate numbers in the present study. The SEM for other parameters was at least two fold smaller for the present experiment compared to those in Chapter 6.

Table 7.3: Experiment 7B; dam and litter weights and litter sizes.

Weights of dams on D0 and D3 of lactation, dam weight gains, natural litter sizes and natural litter weights of average sized mice allocated to treatments in which litters were adjusted to 10, 15 or 20 pups on the day of parturition. Parameters are also listed for small mice with litters adjusted to 15 pups. Means are followed by SEM. n=7 for average sized mice with 10, 15 or 20 pups and n=5 for small mice with 15 pups.

	Litter size that 'average' mice were allocated to:				Litter size of small mice	
	10	15	20	P value*	15	P value**
Dam wt on D0, g	36.3 ± 1.51	35.2 ± 1.51	36.2 ± 1.51	0.847	30.4 ± 1.82	0.080
Dam wt on D3, g	38.6 ± 0.97	38.5 ± 0.89	39.7 ± 1.04	0.666	34.7 ± 1.21	0.006
Dam wt gain, g/d	0.8 ± 0.29	1.8 ± 0.31	1.2 ± 0.29	0.146	1.4 ± 0.34	0.230
Natural litter size, pups	11.0 ± 0.74	11.3 ± 0.79	12.3 ± 0.79	0.481	4.4 ± 0.90	0.000
Natural litter wt, g	18.2 ± 0.66	17.8 ± 0.70	18.8 ± 0.70	0.574	7.7 ± 0.83	0.000

* denotes P value for comparison among three treatments using average sized mice

* * denotes P value for comparison among small dam treatment and other three treatments

Table 7.4: Experiment 7B; milk production and weights, DNA content and incorporation of [^{3}H] thymidine of mammary glands.

Milk production, weights and DNA contents of 4 glands, and incorporation of [3H] thymidine into 4 glands and into DNA (all corrected for milk content) of mammary glands collected on D3 of lactation after mice had suckled either 10, 15 or 20 pups for 3 days. Means are followed by SEM. n=7 for mice with 10, 15 or 20 pups and n=5 for small dams with 15 pups.

	Litter size				
	10	15 (small dam)	15	20	P value
Milk production, g/d	4.1 ± 0.21	3.4 ± 0.32	4.4 ± 0.23	4.2 ± 0.23	0.157
Weight of 4 glands, g	1.0 ± 0.09	0.9 ± 0.11	1.1 ± 0.10	1.0 ± 0.10	0.541
DNA content of 4 glands, mg	3.1 ± 0.15	3.4 ± 0.26	3.7 ± 0.17	3.8 ± 0.22	0.184
DNA concentration, total DNA mg/total weight of glands, g	3.0 ± 0.21 ^{a}	3.7 ± 0.25 ^{b}	3.4 ± 0.22 ^{ab}	3.8 ± 0.22 ^{b}	0.050
[^{3}H] incorporation, cpm x 1000/glands	107 ± 13.3	69 ± 15.7	88 ± 14.3	94 ± 15.7	0.359
[^{3}H] incorporation, cpm/µg DNA	34.9 ± 2.58 ^{c}	17.9 ± 3.26 ^{d}	25.3 ± 2.98 ^{d}	23.7 ± 2.98 ^{d}	0.004

Where superscripts a and b differ, the means differ (P<0.05), and where superscripts c and d differ, the means differ (P<0.01).

The DNA content of mammary glands did not differ between mice with different litter sizes when analysed by ANOVA (Table 7.4, P>0.1). However, when the data were analysed by linear regression, it was found that the DNA content of mammary glands was influenced by litter size, according to Equation 7.1:

Equation 7.1 $Y = 2.52 + 0.061 X$ $R^2 = 24\%$ $P = 0.024$

where X = number of pups in the litter

and Y = the DNA content (mg) of 4 mammary glands.

7.3.4 Discussion

The results of this experiment duplicate those of Experiment 7A and contrast with those reported in Chapter 6. Interpretation of the results therefore supports the hypothesis that on D3 of lactation, mice suckling large litters have mammary glands that contain fewer cells undergoing mitosis and more cells capable of milk synthesis compared to mice with smaller litters (Experiment 7A). In the present study, mice with only 10 pups incorporated the most [^{3}H] thymidine indicating more proliferation occurring in mammary glands on D3. As proliferation requires the synthesis of pre-mitotic DNA to increase the DNA content of the cell by 100% (Zwierzchowski *et al.*, 1984), it is associated with large uptakes of the thymidine analogue [^{3}H] thymidine. DNA synthesis also occurs when mammary epithelial cells are differentiating into milk secretory cells (Smith and Vonderhaar, 1981) but this results in much less incorporation of [^{3}H] thymidine (Zwierzchowski *et al.*, 1984). The lower incorporation of [^{3}H] thymidine in mice with litters of 15 or 20 pups indicated there were less mitotic cells and therefore more cells that had differentiated into milk synthesising cells.

In the present study it appears that the suckling intensities of larger litters stimulate both the autocrine and endocrine systems to accelerate the rates of cell proliferation and differentiation into secretory cells within mammary glands. This is evident as mice with larger litters have higher DNA contents (Equation 7.1, $R^2 = 24\%$, P<0.02) and concentrations (Table 7.4, P=0.05) in their mammary glands indicating more cell

proliferation had occurred prior to D3 than in glands of mice with only 10 pups. Furthermore, incorporation of [^{3}H] thymidine into DNA (cpm/μg DNA) was less for mice with 15 or 20 pups on D3 (Table 7.4, P>0.01). The phenomenon of high suckling intensity affecting endocrine and autocrine systems to promote mammary gland differentiation and development has been reported by others (Tucker, 1964; Wilde *et al.*, 1990).

It is apparent that the mammary glands of Swiss mice have inherent limitations that do not alter when suckling intensity increases from 1 to 2 pups per gland. There were no differences in milk production and gland weight among the groups (Table 7.4, P>0.1). Incorporation of [^{3}H] thymidine per μg of DNA differed (Table 7.4, P<0.01) but on a whole gland basis there was no difference between treatments (P>0.05), indicating the amount of total DNA synthesis in glands did not differ between groups. The content (Equation 7.1, R^2 = 24%, P<0.02) and concentrations (Table 7.4, P<0.05) of DNA in mammary glands was related to litter size on D3. However, there may be a ceiling on the content of DNA and that mice with larger suckling intensities achieve this level more rapidly. Future experiments should address whether mice suckling >1 pup per gland have more DNA at peak lactation than those with 1 pup per gland. The results of the present study are in agreement with Knight *et al.* (1986b), who did not measure but did propose, that mammary gland weights of primiparous mice suckling 1 pup per gland do not increase when litter size increases above 1 pup per gland. Such inherent limitations of mammary glands apply to many mammals as there are strong relationships among gland size, milk yield, energy output and body weight that are consistent across many species (Linzell, 1972).

The suckling of 15 pups on small dams appeared to apply a similar level of stimulus to 20 pups. The small dams had litters of few pups and therefore small mammary glands at parturition (Nagasawa and Yanai, 1971; Markoff and Talamantes, 1981). Yet after suckling a litter of 15 pups for 3 days, the DNA concentration (total DNA/total gland weights, Table 7.4) increased above that for the mice of normal weight with 10 pups and was as high as for mice of normal weight suckling 20 pups (3.7, 3.0, 3.8 mg/g respectively, P<0.05). Thus, the increased stimulation of the autocrine and endocrine systems (Tucker, 1964; Wilde *et al.*, 1990) of mice suckling

20 pups appeared to be as high for small mice with 15 pups since both treatments resulted in significantly higher DNA concentrations in mammary glands.

Increasing the incubation time of *in vivo* labelling of [^{3}H] thymidine from 1 to 2h, did not alter the absolute amounts of [^{3}H] thymidine incorporated, nor reduce the SEM when compared to results in Experiment 7A. This is in agreement with Traurig (1967a) who states that incorporation of [^{3}H] thymidine begins within 5 min of injection and unincorporated precursor is removed from the circulation within 30 to 60 min.

The present study confirmed the results reported in Experiment 7A. It was therefore concluded that increasing litter size did not increase incorporation of [^{3}H] thymidine on D3 of lactation above that for mice with 10 pups and therefore the high suckling intensity challenge did not result in the desired model for further study. Mammary glands of the mice were resistant to change in weight and milk output in response to suckling intensities of >1 pup per gland but larger litters did accelerate mammary development in terms of DNA content and differentiation into milk secretory cells.

7.4 Experiment 7C

7.4.1 Introduction

In the previous study (Experiment 7B), the DNA content (Equation 7.1, R^2 = 24%, P=0.02) and concentration (Table 7.4, P<0.05) in mammary glands on D3 of lactation increased as litters increased from 10 to 20 pups. Other parameters did not increase when compared to levels associated with suckling litters of 10 pups; extra pups did not increase mammary gland weights, total [^{3}H] thymidine incorporation, milk production (Table 7.4, P>0.1 for all). While there are many reports on the relationship of mammary gland DNA content related to suckling intensities of <1 pup per gland (Munford, 1964; Tucker, 1964; Moon, 1965b; Tucker, 1966; Moon, 1969; Grigor *et al.*, 1984), the effect of increasing suckling intensity to >1 pup per gland is less clear, although Knight *et al.* (1986b) suggest there is no change in

experiments with >1 pup per gland. As Experiment 7B only measured DNA content after three days of treatment, it was unclear whether the mice with 15 or 20 pups had reached the limit of DNA content earlier than those with 10 pups owing to accelerated development and if the treatment would result in a permanent increase in mammary DNA content. Therefore it was proposed to determine if suckling intensities of ≥1 pup per gland results in mammary glands with similar DNA contents at mid lactation when DNA content is at a peak.

The objective of the present study was to determine the DNA content of mammary glands of mice on D10 of lactation after they had suckled either 10 or 15 pups from parturition. The treatment of 20 pups was not included as dams are unable to sustain such large litters for extended periods of time.

7.4.2 Experimental design

The procedures were the same as for Chapter 6, Experiments 7A and 7B except where indicated below.

Thirty female Swiss mice were mated at 10 weeks of age and after parturition, 16 were selected on the basis of similar live weight, natural litter size and natural litter weight. All mice were sisters or first cousins from five different litters. The treatments required adjustment of litter size on D0 of lactation to either 10 or 15 pups. Eight mice were allocated to each treatment. Mice suckled litters until D10 of lactation when they were euthanased. Lactating mice were injected with [^{3}H] thymidine on D10 (Section 2.1.7). One hour later they were killed and the 4th and 5th left and right mammary glands were dissected from each mouse, blotted and weighed (Section 2.1.8). Gland weights were corrected for content of milk to give corrected gland weights (Section 3.5.1). The left 5th and right 5th glands were analysed for concentrations of lactose, DNA and [^{3}H] thymidine incorporation (Section 2.3).

7.4.3 Results

Mice allocated to either treatment had similar live weights on the day of parturition (36.1 ± 1.2 g, P>0.05), natural litter weights (16.5 ± 0.7 g, P>0.05) and sizes (10.9 ± 0.4 pups, P>0.05). Dam live weight gain during lactation did not differ between groups (0.6 ± 0.06 g/d, P>0.05).

Results were also collected for litter growth rates, DNA concentration, and protein concentration but there were no differences between the two treatments (Table 7.8 in Chapter 7 Appendices, P>0.1 for all).

Milk production, corrected gland weights, DNA content and concentration of glands and incorporation of [^{3}H] thymidine into DNA did not differ between treatment groups (Table 7.5; P>0.1 for all).

Table 7.5: Experiment 7C; milk production and composition of glands collected on D10.

Milk production, mammary gland weights and DNA contents of four glands, and incorporation of [^{3}H] thymidine into DNA (all corrected for milk content) and total incorporation of [^{3}H] thymidine into mammary glands collected on D10 of lactation after mice had suckled either 10 or 15 pups for 10 days. Means are followed by SEM, n=8.

	Litter size			
	10	15	SED	P value
Milk production, g/d	7.3 ± 0.61	7.7 ± 0.57	0.83	0.665
Corrected wt of 4 glands, g	1.11 ± 0.11	1.27 ± 0.09	0.14	0.493
[^{3}H] incorporation/DNA, cpm/μg	28.0 ± 3.4	25.3 ± 3.4	4.75	0.121
[^{3}H] incorporation/glands, cpm*1000	112.6 ± 6.4	116.9 ± 5.4	8.4	0.616
DNA content of 4 glands, mg	4.03 ± 0.28	4.62 ± 0.24	0.36	0.139
DNA concentration, total DNA /total weight of glands, mg/g	3.62 ± 0.12	3.69 ± 0.11	0.16	0.249

7.4.4 Discussion

In the present experiment, increasing suckling intensity from 10 to 15 pups did not cause an increase in DNA content and concentration, protein content, gland weight, [^{3}H] thymidine incorporation and milk production on D10 of lactation. However, in Experiment 7B, DNA content of glands on D3 increased as litter size increased from 10 to 20 pups. The results from D3 of lactation may have been due to either the increased suckling intensity causing a permanent increase in DNA content or due to accelerated development of mammary glands so that cell number and differentiation into secretory cells was achieved earlier than for mice with only 10 pups. The results of the present study suggest the latter reason is correct and therefore the content of DNA in mammary glands is similar to other parameters such as gland weight and milk production which have a limit that is attained at a suckling intensity of 1 pup per gland and while increased suckling intensities may cause the limits to be reached earlier, they will not alter the inherent levels.

It can be assumed that the mechanism limiting mammary gland size and output in the present study is not due to a negative energy balance of the dams as they all gained weight during lactation. The limit seems to be inherent within the gland which confirms the results reported in Chapter 6, showing that when suckling intensity was increased to 2 pups per gland without the milk energy output being increased (taping of half of the glands) gland weight, DNA content and output did not increase above that for mice suckling 1 pup per gland.

The present study confirms that there is a limit to the content of DNA in mammary glands. This limit is reached when mice suckle 10 pups (1 pup per gland) and while a greater suckling intensity may accelerate the attainment of the limits of glands, it will not result in an increase of these limits.

7.5 Conclusions

The series of experiments in Chapter 7 show that the main effect of suckling intensities of >1 pup per gland is to accelerate mammary development so that the rate of cell proliferation and differentiation into secretory cells during early lactation is faster than for mice with only 1 pup per gland. The increased rate of DNA synthesis led to mice with 20 pups having 22% more DNA in glands by D3 of lactation compared to mice with only 10 pups. This averages to an increase in the rate of DNA synthesis of 7% per day. However, this is not large enough for studies comparing normal to highly proliferative tissues which require ≥2 fold differences in tissue. No model suitable for the investigation of proliferation of epithelial cells during lactation was found in the present study.

Appendices

Table 7.6: Experiment 7A; litter growth rate, milk content, DNA concentration and protein concentration of mammary glands.

Litter growth rate, milk content, DNA concentration, protein concentration of mammary glands collected on D3 of lactation after mice had suckled either 10 or 15 pups for three days. Means are followed by SEM. n=5 for mice with 10 pups and n=15 for mice with 15 pups.

	Litter size		
	10	15	P value
Litter growth rate, g/d	3.2 ± 0.28	3.1 ± 0.11	0.450
Milk content, g	0.82 ± 0.218	0.92 ± 0.109	0.338
DNA concentration, μg/mg	3.71 ± 0.55	3.61 ± 0.21	0.447
Protein concentration, mg/g	99.4 ± 10.79	96.0 ± 4.68	0.656

Table 7.7: Experiment 7B; litter growth rate, DNA concentration and protein concentration of mammary glands.

Litter growth rate, DNA concentration, protein concentration of mammary glands collected on D3 of lactation after mice had suckled either 10, 15 or 20 pups for 3 days. Means are followed by SEM. n=7 for mice with 10, 15 or 20 pups and n=5 for small dams with 15 pups.

	Litter size				
	10	15 (small dam)	15	20	P value
Litter growth rate, g/d	2.9 ± 0.32	2.0 ± 0.59	2.8 ± 0.15	2.1 ± 0.27	0.120
DNA concentration, μg/mg	3.3 ± 0.31	3.7 ± 0.49	3.5 ± 0.34	3.8 ± 0.49	0.791
Protein concentration, mg/g	90.3 ± 7.25	104.8 ± 9.04	87.0 ± 7.25	93.8 ± 8.05	0.466

Table 7.8: Experiment 7C; litter growth rates, DNA concentration and protein concentration of glands collected on D10.

Litter growth rates, milk content, DNA concentration and protein concentration of glands collected on D10 of lactation after mice had suckled either 10 or 15 pups for 10 days. Means are followed by SEM, n=8.

	Litter size			
	10	15	SED	P value
Litter growth rate, g/d	4.2	3.3	0.58	0.221
DNA concentration, μg/g	3.8	3.8	0.18	0.260
Protein concentration, mg/g	139.4	147.2	16.21	0.174

Chapter 8 Location of mammary epithelial cells undergoing DNA synthesis during lactation

8.1 Summary

The pattern and extent of DNA synthesis in epithelial cells in the mammary gland was determined on the day after parturition. Mice injected with BrdU on D1 had ≥1.5 times the labelling index on the edges of glands compared to inner areas. This important finding means that edges of mammary glands are potentially useful sources of material for use in future studies of mitosis in lactating tissue. There were no differences in labelling indices between the teat, mid and distal thirds of the mammary glands on D1 of lactation. Cells labelled for DNA synthesis were observed to occur in a non-random pattern within each of the six locations within glands suggesting local stimulation or synchrony. The labelling index of cells labelled on D1 decreased at a constant rate of 4% throughout lactation and into involution and did not differ among the edges and inner areas and the three thirds of glands.

8.2 Introduction

Parenchymal growth is heterogeneous in the mammary glands of pubertal and post pubertal mice (Richert *et al.*, 2000). At about three weeks of age, terminal end buds (TEBs) appear at the ends of the ducts and begin the process of ductal elongation which continues until about three months of age when the TEBs reach the edge of the fat pad (Richert *et al.*, 2000). TEBs are highly mitotic and therefore heterogeneity exists between areas of parenchyma that contain TEBs and those that do not. A wave of proliferation of alveolar buds and ductal branching occurs during each estrous cycle (Dulbecco *et al.*, 1982) that results in areas of both high and low rates of DNA synthesis within developing glands. During late pregnancy and early lactation, mammary glands of rodents continue to fill with ductal tissue and lobules of alveolar units containing epithelial cells (Richert *et al.*, 2000). It is conceivable that mammogenesis and lactogenesis also occur in a heterogeneous fashion within mammary glands. Identification of areas of high rates of DNA synthesis would

assist in collection of tissue for further studies on factors associated with DNA synthesis.

It was hypothesised in Chapters 4 and 6 that the distribution of cells undergoing mitosis may show a gradient from the inner to outer parts of the glands. However, the results of the experiments in which the mitotic figures counted in 3 sections of the glands were reported (Table 4.1 and Table 6.1), were inconclusive. This result may have been due either to the stage of lactation of the mice from which the tissue was collected or to the number of cells counted in each location. It was therefore decided to repeat the experiment with improvements to the design that would increase the sensitivity. These new approaches included assessing the labelling index of epithelial cells in six locations of mammary glands and counting 1000 cells per location. Significant gains in sensitivity are made by counting up to 1000 cells but thereafter the gain in significant results is minimal (Traurig, 1967b). The results were expected to yield new information about the pattern of DNA synthesis during lactation.

In addition to the location of DNA synthesis in the mammary gland, it was of interest to determine the lifespan of epithelial cells and determine if there is a pattern to their loss throughout lactation. There is a paucity of information in the literature about cell lifespan during lactation. It is generally accepted that cell loss occurs during lactation and that an imbalance of cell loss with cell mitosis is responsible for the decline in milk yield after peak (Knight *et al.*, 1984; Knight and Wilde, 1993). It is also known that challenges such as increased milking frequency can extend the lifespan of epithelial cells so that the cell population and milk yield are maintained at high levels (Wilde *et al.*, 1987; Knight and Wilde, 1993). The lifespan of mammary epithelial cells is unknown but some are known to carry over into the next lactation (Pitkow *et al.*, 1972). A study was therefore designed to determine the lifespan of the secretory epithelial cells throughout lactation and their pattern of disappearance. It was proposed to do this by labelling cells undergoing DNA synthesis in early lactation and then quantifying the loss in labelling indices over time at different locations within glands. Since the label was required to remain in mammary tissue for over three weeks, it was decided to use BrdU which in contrast to [^{3}H] thymidine, does not alter cell health and function (Hyatt and Beebe, 1992).

The objective of the present study was to determine the distribution of DNA synthesis in epithelial cells in early lactation within six locations of mammary glands of mice. The six locations were the edge and inner areas of the teat, mid and distal thirds of the mammary glands. In addition, the decline in labelled cells throughout lactation and into involution would be quantified in order to ascertain epithelial cell lifespan.

8.3 Experimental design

Thirty-five virgin female Swiss mice were mated at 10 weeks of age and pregnancy confirmed by presence of a vaginal plug. Pregnant mice were transferred to individual cages at D15 of pregnancy. Animal husbandry and management are detailed in Section 2.1.

Prior to parturition, mice were randomly allocated to one of five groups. Sufficient mice were mated so that five mice, which had each given birth to 8 to 12 pups, were allocated to each treatment. All mice had litters adjusted to 10 pups on the day of parturition (D0). On D1 all dams received an intra-peritoneal injection of BrdU (Section 2.1.7). Dams were killed on D2, D3, D10, D17 or D24 after parturition. Mice were lactating on D2, D3, D10 and D17 whereas mice allocated to D24 group were weaned on D17. After being euthanased, the left 5^{th} and 4^{th} mammary glands were dissected from each mouse, blotted and weighed (Section 2.1.8) and analysed for concentrations of lactose and DNA (Section 2.3). The left 5^{th} mammary glands were homogenised individually and aliquots were analysed for concentrations of lactose and DNA (Section 2.3).

A whole mount of the 4^{th} mammary gland on the right side was made for each mouse as detailed in Section 2.4. The presence of BrdU in epithelial cells was detected by immunocytochemistry (Section 2.4.3) and sections were counter-stained with haemotoxylin or methyl green, as described in Section 2.4.5. Care was taken to note the orientation of mammary glands when making whole gland mounts and cutting sections such that the teat end was always at the top of the slide. Orientation was

confirmed using microscopy to identify the primary duct leading to the teat or by the location of the lymph node that is closer to the teat end than the distal end of the gland (Rasmussen *et al.*, 2000). Lines were drawn on the cover slips to arbitrarily divide the whole gland mounts into thirds that were designated teat third, mid third and distal third. The outer 1mm edge of each region was designated 'the edge' with all the remaining interior area termed 'inner area'. The combination of the edge and inner areas for each of the three thirds (teat, mid and distal) meant that six different locations were identified for each gland. Microscopic fields (x200) were randomly selected with an ocular grid. Only alveoli that had been cross sectioned to reveal cells surrounding a lumen were studied. The number of labelled and unlabelled epithelial cells in each alveolar unit were counted. The procedure was continued until over a total of 1000 cells had been counted for each of the six different locations of the mammary glands. The labelling index was expressed as a percentage of the number of labelled cells divided by the total number of cells counted.

To compare the labelling indices of epithelial cells, Genstat (5^{th} edition, Release 4.2, 2000) was used to fit a GLM with a binomial distribution with logit link (Nelder and Wedderburn, 1972) to test between the edge and inner areas and the three thirds and to test the between mouse variation.

Minitab (Minitab, Release 12.23, 1999) was used to determine the linear regressions of labelling indices of mammary epithelial cells and the polynomial equation that described the changing DNA content of mammary glands post partum.

8.4 Results

For each of the five days sampled, the labelling index did not differ between the teat, mid and distal thirds of the glands ($P>0.1$, Table 8.1) but it was higher for the edges than the inner areas ($P<0.05$, Table 8.2). When the data were pooled (for thirds of the glands and day) the labelling index of the edges was nearly twice that of inner areas (14.0 versus 7.9% respectively, $P<0.001$).

Table 8.1: Labelling indices of epithelial cells in teat, mid and distal thirds of mammary glands.

Labelling indices (%) of epithelial cells on D2, D3, D10, D17 and D24 in the teat, mid and distal thirds of mammary glands of mice injected with BrdU the day after parturition. Values shown are means followed by SEM, n=3 for D2 and D24, n=4 for D3, D10 and D17.

Days	Third of mammary glands				
postpartum	Teat	Mid	Distal	SED	P value
2	17.5 ± 2.9	19.4 ± 2.9	19.7 ± 2.9	4.92	0.886
3	16.8 ± 2.4	14.9 ± 2.1	16.8 ± 2.4	4.40	0.872
10	12.0 ± 2.9	11.5 ± 2.1	13.0 ± 2.9	4.57	0.940
17	7.1 ± 2.4	7.5 ± 2.4	5.8 ± 2.4	2.90	0.839
24	0.9 ± 1.9	0.6 ± 1.9	0.9 ± 1.9	0.63	0.842

Table 8.2: Labelling indices of mammary epithelial cells in inner areas and edges.

Labelling indices (%) of epithelial cells on D2, D3, D10, D17 and D24 in the inner areas and edges of mammary glands of mice injected with BrdU the day after parturition. Values shown are means followed by SEM, n=3 for D2 and D24, n=4 for D3, D10 and D17.

Days	Location in mammary glands			
post partum	Inner area	Edge	SED	P value
2	15.1 ± 2.4	23.6 ± 2.4	3.06	0.033
3	12.0 ± 1.9	20.3 ± 1.9	2.61	0.011
10	7.5 ± 2.2	16.5 ± 2.2	2.57	0.004
17	4.2 ± 1.9	9.3 ± 1.9	1.94	0.018
24	0.1 ± 2.4	1.5 ± 2.4	0.21	0.000

The labelling index of epithelial cells in mammary glands decreased by 0.9% and 0.6% for the edge and inner areas respectively, each day after D1 (Figure 8.1, Equations 8.1 and 8.2, P<0.01).

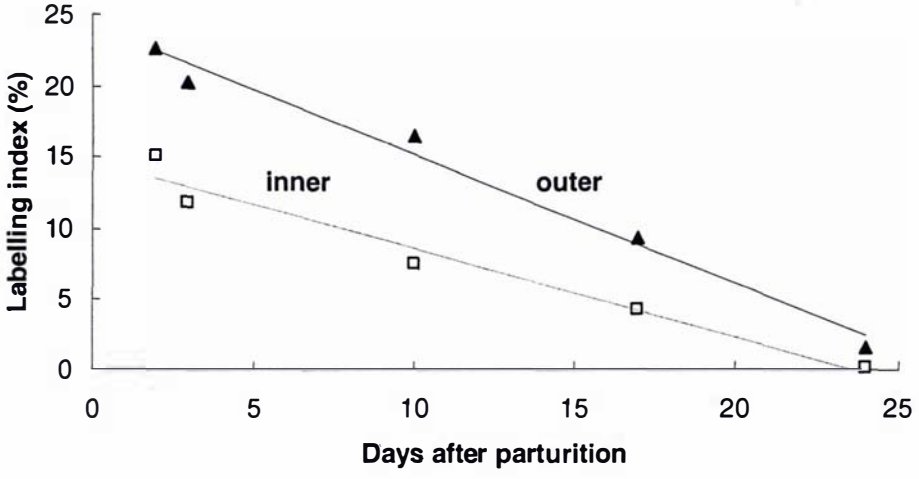

Figure 8.1: Decline in labelling indices of epithelial cells.

The decline in labelling indices, %, of epithelial cells from D2 to D24 post partum in the edges (▲) and inner areas (□) of the mammary glands of mice injected with BrdU on the day after parturition.

Equation 8.1 $Y = 24.3 - 0.91\ X$ $R^2 = 60.2\%$ $P<0.01$

where X = days after BrdU injection and Y = the percentage of cells labelled on D1 that are still present, for edges only.

Equation 8.2 $Y = 14.7 - 0.62\ X$ $R^2 = 65.6\%$ $P<0.01$

where X = days after BrdU injection and Y = the percentage of cells labelled on D1 that are still present, for inner areas only.

The relationship between the DNA content of the left 4th and 5th mammary glands and day of lactation was curvilinear in that it increased from D2 to D13 of lactation after which it declined to the lowest amount measured at D24 (Figure 8.2, Equation 8.3).

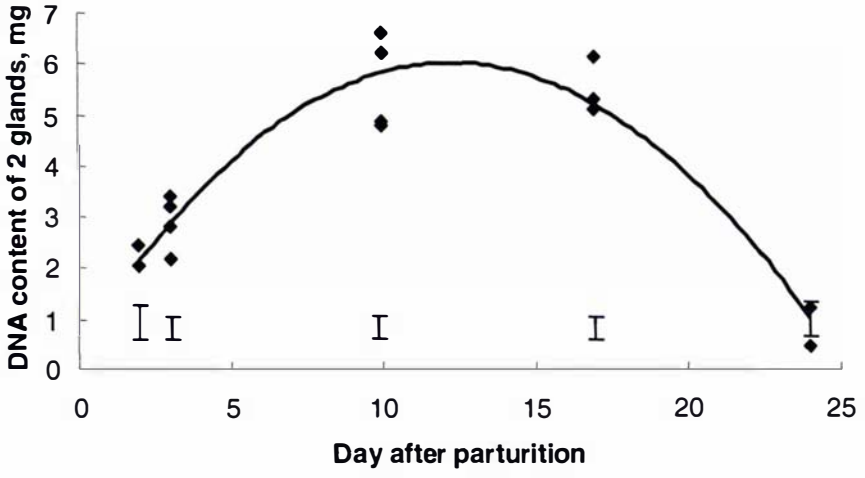

Figure 8.2: DNA content, mg, of the two inguinal glands of lactating mice on D2 to D24 post partum.

Vertical brackets represent standard errors of differences among the means. n=3 for D2 and D24, n=4 for D3, D10 and D17.

Equation 8.3 $Y = -0.04 X^2 + 0.90 X + 0.51$ $R^2 = 90.5\%$ $P<0.01$

where X = days post partum and Y = the content of DNA in two mammary glands.

Heterogeneity of the distribution of labelled cells was observed repeatedly while counting cells. In addition to the difference in labelling indices of alveoli between the inner areas and edges (Figure 8.3) there was considerable quantitative variation in labelling of epithelial cells within individual alveoli (Figure 8.4). Similar patterns of distributions were observed in sections of mammary glands collected from all five time periods.

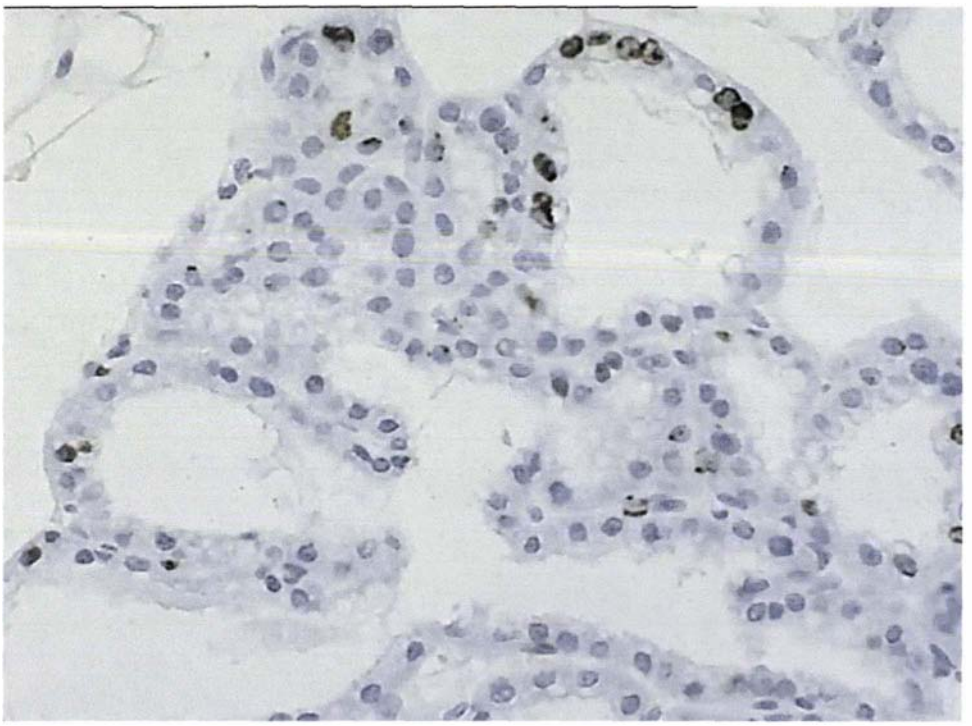

Figure 8.3: Heterogeneity of BrdU labelled cells; an alveolus with nearly half of all epithelial cells staining positive for content of BrdU, x200.

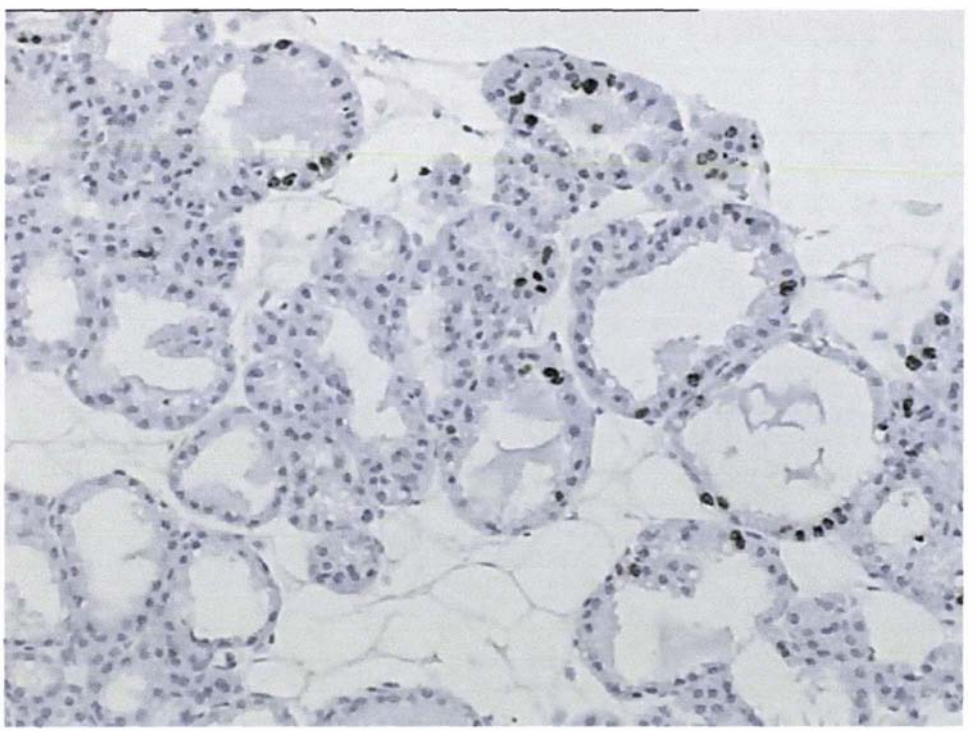

Figure 8.4: Heterogeneity of BrdU labelled cells; heavy labelling of alveolus on the edge of the mammary gland, x100.

8.5 Discussion

A useful model for the investigation of DNA synthesis during lactation was found in the present study. The labelling indices of epithelial cells labelled on D1 of lactation were found to vary between mice, but within each mouse the edges of mammary glands were labelled at 1½ to 2 times that of the inner areas (Table 8.2). This makes an attractive model as the result was consistent for all mice and the areas of different rates of DNA synthesis were easily located. Another appealing feature is that the tissue is from the same gland and therefore the edges and inner areas have the same exposure to potential variables such as systemic mitogens.

The present study is the first to quantify DNA synthesis in six locations in mammary glands. The labelling indices of D1 are higher than the 11.1% and 10.9% reported by Traurig (1967a) and Pitkow (1972) respectively but neither quantified labelling indices at different locations in glands. In addition, the latter experiment used rats that were labelled on D3 of lactation, two days later than the present study. The teat, mid and distal thirds of mammary glands were at similar stages of development on D1 of lactation as evident by similar rates of DNA synthesis. This contrasts to the differences in proliferation of epithelial cells between the basal, mid and upper areas of beef cow udders, however, the latter study was conducted in mid lactation (Capuco and Akers, 1990). A high rate of DNA synthesis on the edge of glands appears to be one of the last stages of development as mammary glands already contain 85% of peak DNA content by D1 of lactation (Knight and Peaker, 1982d). Proliferation on the edges of glands is probably due to available fat pad as this is a requirement for the development of mammary alveoli (Faulkin and DeOme, 1960; Topper and Freeman, 1980).

DNA synthesis occurred in a heterogeneous pattern in mammary glands. Such heterogeneity of DNA synthesis has been reported previously in lactating mammary glands (Franke and Keenan, 1979), non lactating mammary glands (Bresciani, 1968; Purnell and Stowers, 1977) and in human epidermis (Rowe and Dixon, 1975). This may be due to mitosis in one cell causing a local increase or decrease of a cell product that stimulates division in other cells in the immediate vicinity (Purnell and

Stowers, 1977) or it may be due to a high degree of synchrony in individual lobules so that cells of these units undergo mitosis in unison (Bresciani, 1968). The mammary gland of ruminants at least exhibits heterogeneity not only with DNA synthesis but also with the expression of milk genes. These vary dramatically within and between alveoli in the udders of sheep and cows during pregnancy, lactation and involution (Molenaar *et al.*, 1992).

The decrease in the proportion of labelled epithelial cells over time (Figure 8.1) could be interpreted as a constant rate of loss of labelled cells which implies that cell life span can vary from <1 to >22 days. This interpretation would be incorrect, however, as cell number is a function of both mitosis and apoptosis (Knight and Wilde, 1993). The decrease in labelling index from 19.4% on D2 to 12% on D10 is attributed to a dilution effect caused by a >2.5 fold increase in the DNA content of mammary glands (2.2 versus 5.6 mg, $P<0.01$, Figure 8.2). In contrast, the decrease in labelling index from D10 to D24 was attributed to apoptosis of mammary epithelial cells (Knight *et al.*, 1984). In support of this, there were dramatic decreases in both the labelling index and the DNA content of mammary glands during this period (decreases of 93% and 85% respectively).

There is disagreement regarding the proportion of mammary epithelial cells carried over into the subsequent lactation in rodents. In the present study, a labellin index of approximately only 5% of the cells labelled on D1 were observed on D24 which is much less than that reported by Pitkow *et al.* (1972). Pitkow *et al.* (1972) observed 73% of epithelial cells labelled on D3 of lactation were present four days into the next lactation of rats. The difference between the two studies may be explained by more of the labelled cells undergoing division and giving rise to labelled daughter cells. Furthermore, the difference may be due to the type of label used. Pitkow *et al.* (1972) determined that the [^{3}H] thymidine used did not affect cell health as milk production of labelled rats did not differ from unlabelled rats. However, subsequent studies have reported that the label does cause damage to cellular DNA and use of [^{3}H] thymidine as a long term label is detrimental to cell health (Schutte *et al.*, 1987). However, it is difficult to explain why so many damaged cells remained in the rat mammary glands for >70 days after incorporating [^{3}H] thymidine. The results by Pitkow *et al.* (1972) imply that many alveoli are present for more than one lactation

but this contrasts to many reports showing that DNA content decreases dramatically during involution between lactations (Knight and Peaker, 1982d). Therefore the DNA content of mammary glands and the labelling indices should be very well correlated. The DNA content of mammary glands during lactation and involution are consistent with many other studies (Knight and Peaker, 1982d) and therefore the labelling index data of the present study are considered more reliable than those reported by Pitkow *et al.* (1972).

8.6 Conclusion

DNA synthesis on D1 was >1.5 times greater in the edges compared to the inner areas of mammary glands with no differences occurring in labelling indices between the teat, mid and distal thirds. Such differences between edges and inner areas may provide tissue useful for future study of mitosis during lactation. The labelling indices of the edges and inner areas decreased by 0.9% and 0.6% respectively per day. This was attributed to new cells having the effect of diluting labelled cells up to D10 of lactation while from D10 to D24, the decline was attributed to apoptosis of mammary epithelial cells. Heterogeneity of DNA synthesis was observed within each location suggesting synchrony and the presence of local mitogenic factors.

Chapter 9 General Discussion and Future Directions

Technology for enhancing growth of mammary glands during lactation could benefit many agricultural industries ranging from dairying to production of weaner piglets, through the provision of increased milk yield, increased persistency and possibly improved milk quality. Some manipulations during the lactation of both ruminants (Knight *et al.*, 1987; Bar-Peled *et al.*, 1995) and monogastrics (Auldist *et al.*, 2000) have caused increases in the DNA content and milk yield of mammary glands, but little is understood about the mechanisms involved. The phenomenon could be further studied and understood if lactating mammary tissue that is undergoing increased growth could be generated consistently. The primary objective of the research reported in this thesis was to examine mechanisms to produce increased mitosis in mammary tissue during lactation, so that the population of epithelial cells is increased. Mice were chosen for experimental work as their high milk production per unit of bodyweight (Linzell, 1972) made them a suitable model for farmed livestock. In addition, their small size along with their short gestation and lactation periods meant that sufficient numbers could be produced to supply many replicates in a variety of different challenges.

During early work in this thesis, it was noticed that mammary glands dissected from mice varied in their milk content. In order to accurately compare the chemical compositions and weights of mammary glands, it was necessary to correct for milk content; this required information about milk composition. Murine milk composition reported in the literature varied between strains of mice and stages of lactation (Ben Shaul, 1962; Meier *et al.*, 1965; Allen, 1984; Knight *et al.*, 1986; Ragueneau, 1987; Nagasawa *et al.*, 1989) so a study was designed to measure the lactose, fat and protein content of milk collected from Swiss mice during early, mid and late lactation. Although the concentration of protein and fat did not vary through lactation, the concentration of lactose was found to increase from early to late lactation (Chapter 3). This is the first report of the concentration of lactose in milk from Swiss mice and the first to cover the range of early, mid and late lactation for any strain of mouse. The knowledge of changing lactose concentration during lactation enabled the weight of mammary glands to be corrected for milk content so

that measurements of chemical composition reflected only the mammary tissue. This is a significant improvement to the methods used previously where either milk content was not corrected for at all (Tucker, 1964; Alston-Mills *et al.*, 1995) or it was corrected by using values from other strains of mice and not from the relevant stage of lactation (Knight and Peaker, 1982d).

Another capability required in order to compare mice given different challenges was the measurement of milk yield. Three methods of calculating milk yield were investigated. In the best one, the maintenance energy requirements of the litters were added to the energy required to support the measured growth rates and the sum was converted to a quantity of milk (Chapter 3). It yielded sensible comparative results for litters of different sizes and results that were similar to absolute milk yields reported in the literature (Jara-Almonte and White, 1972; Lin *et al.*, 1977; Rath and Thenen, 1979; Knight *et al.*, 1986b). It provides an accurate and inexpensive means of estimating milk production so that comparisons can be made in early and mid lactation among mice with different sized litters.

The first experimental challenge tested in this thesis to promote growth of epithelial cells in lactating tissue was the administration of exogenous estrogen and progesterone (Chapter 4). Some reports in the literature show that exogenous estrogen causes proliferation of mammary epithelial cells during lactation in mice (Nagasawa and Yanai, 1978; Knight and Peaker, 1982d). However, the results reported in this thesis indicate that the response of mammary glands to exogenous estrogen and progesterone depended on the stage of lactation and the dose of estrogen. In mid lactation, the steroid treatments did not alter cell proliferation or milk production in glands. In late lactation, a dose response to estrogen was measured with progesterone having no apparent effect. As the dose of estrogen increased in late lactation, milk production decreased and histological examination of the tissue showed early signs of involution. In addition, in late lactation, increasing doses of estrogen resulted in increased incorporation of [^{3}H] thymidine into DNA. However, as cell death was much greater than cell proliferation, the increased incorporation of [^{3}H] thymidine did not result in a net increase in epithelial cell population and was instead associated with involution. Increased incorporation of [^{3}H] thymidine into DNA has been reported in involuting udders (Capuco and Akers,

1990; Capuco *et al.*, 1997) where it is described as part of the remodelling process. While no further explanation was offered by those authors, it is possible that these cells that proliferate during involution, which are positioned throughout the mammary gland, have the role of lying nearly dormant until the next gestation and lactation, when they become responsible for the repopulation of mammary glands (Smith, 1996). Further research is warranted on these cells using markers of stem cells and progenitor cells such as keratin-19 and keratin-14 (Bartek *et al.*, 1990; Smith and Chepko, 2001).

The effect of suckling intensity on growth of mammary glands during lactation was investigated because many reports in the literature concluded that rodent mammary gland size increases in response to increased litter size and suckling intensity (Tucker, 1964; Tucker, 1966; Moon, 1969) but these reports do not examine suckling intensities of >1 pup per gland. In the experiments reported in this thesis, suckling intensities were increased up to 2 pups/gland (Chapters 5, 6 and 7) which accelerated mammary gland development, in terms of cell number and milk synthesis (Experiment 7C), but there was little or no additive effect on the size of mammary glands at peak lactation compared to mice with a ratio of 1 pup/gland. This limited response was not nutritional (Chapter 6), which implies mammary glands have an inherent limit to their maximum size and output. Increasing suckling intensity does not generate a suitable model for the study of DNA synthesis in mammary epithelial cells during lactation.

In retrospect, mice have some features that were not conducive to achieving the objectives of the research. Besides the obvious body size differences, mice differ from livestock in other ways. There is no report in the literature that the important autocrine protein, FIL, has been isolated from mouse milk or mouse mammary tissue but it has been found in cattle (Wilde *et al.*, 1995), goats (Wilde *et al.*, 1995), humans (Prentice *et al.*, 1989), wallaby (Hendry *et al.*, 1998) and pigs (Mailer, unpublished data). Future work should examine whether FIL exists in mouse milk, and whether autocrine control is a mechanism through which mammary glands and milk production can be altered in mice. In some species the frequent removal of FIL sensitises the epithelial cells to endocrine hormones, such as prolactin, and therefore increase milk synthesis (Wilde *et al.*, 1990). Increased milk synthesis can be

followed by growth of the mammary gland in livestock species (Knight and Peaker, 1984; Bar-Peled *et al.*, 1995; Auldist *et al.*, 2000). The potential difference in autocrine systems between livestock and mice may be responsible for the lack of detectable growth of mouse mammary glands in response to increasing the suckling intensities of >1 pup per gland. Another difference between mice and livestock species is in the lactose content of milk. The percentage of lactose in the milk of cattle (Auldist *et al.*, 1995), goats (Linzell, 1973) and pigs (Auldist *et al.*, 1998) typically decreases or remains constant over the lactation but in mouse milk it increases over lactation (Chapter 3). The reason for this is not immediately obvious. A hypothesis for the differences between mice and livestock in terms of mammary growth and output is that it is dependent on species survival. The survival of livestock species, which lactate for long periods, may benefit from mammary glands that can increase in size and output so that offspring are well reared. There appears to be no natural advantage for mice to increase mammary size and output above that required to rear 10 pups. Litters of >10 pups have poorer growth rates and mortality rates, and sustained high milk production may compromise the energy balance and reproductive ability of the dam.

The use of mice is sometimes restricted in that treatments that were reported to increase mammary growth during lactation of livestock could not be duplicated using mice. For instance, mammary growth occurred when milk removal was increased by milking cows six times per day (Bar-Peled *et al.*, 1995) or swapping litters of piglets every 30 mins for three weeks (Auldist *et al.*, 2000). These treatments cannot be readily repeated for mice as manual milking is an ineffective measure of milk production and frequent handling to swap litters would cause stress and decrease milk production (Oftedal, 1984). Where it is important to increase the frequency of milk removal, dairy goats or sheep are probably better models.

Methodological changes should also be considered in future work. The labels of DNA synthesis used in this project were [^{3}H] thymidine and BrdU. The incorporation of [^{3}H] thymidine correlates closely with proliferation (Meyer *et al.*, 1986) as does BrdU (DeFazio *et al.*, 1987). However, both labels are used by DNA polymerases α and β and therefore fail to distinguish between DNA synthesis for mitosis, differentiation or DNA repair (Weissbach, 1979; Zwierzchowski *et al.*,

1984). A better indicator of cell proliferation would be a metabolite or enzyme exclusively related to mitosis, such as DNA polymerase α, Ki-67 or cyclin D1. Another method that deserves re-evaluation in future work is the use of flow cytometry to quantify cells that are both mitotic and secretory. The work in this thesis established the techniques for the digestion of lactating mammary glands to single cells, staining of cells with markers and their analysis by flow cytometry (Chapter 2). This information represents a significant step towards optimising the technology for use with lactating mammary tissue and has not been reported before. However in this thesis, flow cytometry was abandoned because the digestion and sample preparation steps yielded only a low percentage of viable cells. This was considered inadequate to be representative of cell types in the original mammary gland. Others groups have successfully used flow cytometry to analyse cells from non lactating mammary glands (O'Hare *et al.*, 1991; Kim and Clifton, 1993; Keys *et al.*, 1997; Smalley *et al.*, 1998) but there are no reports for lactating mammary glands. It is possible that cells in lactating glands are more easily damaged than those in non lactating glands and further work is required to optimise the digestion and preparation of fragile tissues such as lactating mammary glands. A broader concern for the interpretation of data reported from experiments using flow cytometry is that while research groups concede there is a significant loss of cells during the digestion and preparation of the tissue for analysis (Geoff Lindeman, personal communication; Ionna O'Brien, personal communication), the percentage of cells that are intact ready for analysis are not routinely reported in the literature. Until this calculation is done, doubt remains over whether these methods provide cells that are representative of the mammary tissue from which they were derived.

The aim of the project was to seek experimental approaches to increase proliferation of epithelial cells in lactating mammary tissue. This aim was met by the finding that DNA synthesis occurs in >1.5 times as many cells in the outer 1 mm of mammary glands compared to the inner areas on D1 of lactation (Chapter 8). An attractive feature of this observation is that the tissue comes from the same gland and is therefore exposed to the same variables including endocrine and nutritional factors. It will be possible in the future to study the control of mammogenesis in the lactating gland by investigating reasons for the increased proliferation on the edges of mammary glands. One explanation may relate to the availability of space in the fat

pad as proximity to adipocytes can directly influence cell proliferation through the release of lipids and growth factors (Hovey *et al.*, 1998; Hovey *et al.*, 1999). The finding of an area of increased DNA synthesis enables future work to focus on the comparison of tissues from edges and inner areas of lactating mammary glands using microarray technology (Master *et al.*, 2002). A transcript profile of these tissues may reveal expression of genes that regulate milk synthesis and growth of epithelial cell populations.

A consistent finding reported in this thesis was heterogeneity of labelling indices between inner and outer areas of glands, between neighbouring alveoli as well as within an individual alveolus (Chapters 5 and 8). This heterogeneity in labelled alveoli suggests there may be local control of DNA synthesis. Heterogeneity also occurs with regards to milk synthesis (Molenaar *et al.*, 1992). As mentioned previously, the heterogeneity between the edges and the inner areas of glands is probably related to the proximity of adipocytes and their mitogenic effects (Hovey *et al.*, 1999). However, the heterogeneity seen within an alveolus and between neighbouring alveoli may be similar to the mitotic clusters reported in mature virgin rat mammary glands (Purnell and Stowers, 1977). These authors suggested mitotic clusters were initiated by a local increase or decrease of a cell product that stimulated other cells in the immediate vicinity. Direct evidence for a spreading mitosis stimulus was reported by Cone (1968). His *in vitro* system of neoplastic mouse fibroblasts showed mitosis stimulus spreading from cell to neighbouring cell via intercellular connections. Further work on the heterogeneous labelling of mitotic cells should involve analysis of autocrine factors that may trigger mitosis in neighbouring cells.

Another previously unreported feature to arise from histological studies reported in this thesis was that epithelial cells labelled the day after parturition decrease at a constant rate over the next 23 days (Chapter 8). In addition, the remaining labelled cells were spread throughout the gland. It is conceivable that these are progenitor cells, daughter cells of stem cells, capable of repopulating the mammary glands in the next lactation. Future studies could use newly developed histology stains to determine if the remaining cells stain positive for stem cell or progenitor cell epitopes. Further studies also need to determine whether the constant loss of labelled

cells is due to a dilution effect caused by proliferation of new cells or a selective loss of labelled cells which may be connected to cell lifespan.

An experiment worth considering that extends the research conducted in this thesis involves the use of implants in mammary glands. Using a design similar to that described by Silberstein and Daniel (1987), the local effects of mitogens on lactating mammary tissue could be studied using implants containing mitogens. Implants could be placed at the edges of glands as well as in the centre of glands. Different mitogens could be placed in different implants, including prolactin, estrogen and growth factors. The RNA from the tissue surrounding the implants, along with control mammary tissue, would be analysed for expression of thousands of genes using microarray technology (Master *et al.*, 2002). With the use of bioinformatics, comparison of candidate regulatory genes across species is becoming increasingly valuable. Therefore comparison of results may reveal commonality between some mitotic samples and elucidate some genes involved early in the mitosis process in lactating mammary glands from a number of species. Once understanding of local control of mitosis is improved, this may lead to improved investigation of systemic control using dairy species such as cattle and goats.

List of publications resulting from this thesis

D.E. Auldist, T.B. McFadden, D.D.S. Mackenzie and K. Nicholas. (1997) The effect of stage of lactation and nursing demand on mammary gland weight in mice. *Proceedings of the New Zealand Society of Animal Production* **57,** 207-208.

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Examiners' Reports

Associate Professor Duncan Mackenzie
Institute of Food, Human Health and Nutrition
Massey University
Palmerston North
New Zealand

Examiner's Report on the Thesis entitled

'DNA synthesis in mammary epithelial cells of Swiss mice during lactation'

Submitted by

Danielle E. Croke-Auldist

for the degree of Doctor of Philosophy

General Comments:
This thesis contains a number of new observations, amongst the more important are that the lactose concentration of mouse milk is not constant through out lactation and that the rate of mitosis during lactation is greater in the periphery of the gland. The bulk of the original observations, however, refine our understanding of mammary gland development during lactation rather than present radically new perspectives. Nevertheless these data are useful and it is perhaps surprising that results of a similar nature have not been reported previously.

In research it is important to learn to be flexible and on several occasions the direction of the experimental programme had to reassessed as expectations of progress were not met. For example considerable time was spent trying to adapt flow cytometry for use with mammary gland tissue. That it was not successful is not a reflection on the technical competence of the candidate but it did mean that time was not spent on more productive projects. On the other hand, a reluctance to follow up on early results on the distribution of BrdU labelling may have meant that significant opportunities were missed.

The experimental programme did progress in a logical and orderly fashion. As the programme progressed, there was a considerable refinement in experimental design with the greater appreciation of the importance of statistical analysis. Consequently a feature of the thesis is the consistent use of appropriate statistical analysis and an appreciation of the value of taking this into account when interpreting the results.

The thesis is well written. The meaning is unambiguous, descriptions of the results are concise and the discussions evaluate the results in relation to the literature and to how they relate to the objectives of the experiments. The editorial standard is very high and there are remarkably few editorial oversights.

Specific Comments:
Chapter 1
The review of literature is well written and appropriately focussed in its overall structure. It provides a clear summary of the literature that is particularly relevant to the thesis. It is descriptive rather than critical but the topic of mammary gland development has been covered in such detail by previous reviews that it difficult to provide an original interpretation. There was greater scope open to evaluate in more

depth the control of the cell cycle, which would have been very relevant although at risk of an unacceptable expansion of the review.

Chapter 2
This chapter describes the methods and analytical techniques used in the thesis. Many of the analytical methods are well established and perhaps do not need detailed descriptions, nevertheless the descriptions presented are clear and demonstrate the candidate's skill at technical writing.

There were several instances where methods did not work as originally intended. Problems with the detection of BrdU were tackled systematically and a solution was found.

Attempts to develop flow cytometry for identifying and quantifying different cells types in the lactating mammary gland, however, were not successful. The concept is exciting and the method is worth exploring as it would provide a major technical impetus for studying mammary gland development. In retrospect this approach, proposed initially by the supervisors, was too risky for a PhD programme and after a considerable effort and a substantial period it was agreed to abandon it.

Chapter 3
The rationale for the experiments described in this chapter is outlined well in the introduction.

The results for lactose concentration in milk are novel and as pointed out unexpected in that lactose concentration of milk in dairy animals decreases as lactation progresses. The results suggest that further study on the mechanisms of milk secretion in the mouse might be profitable.

The exercise of estimating the milk production of the mouse is seemingly trivial but actually quite difficult. Surprisingly it has not been well addressed in the past. It provided a challenge that required a careful attention to detail and also a consideration of numerous factors that might impinge on the results from the various methods of estimating production. The descriptions in this chapter of the methods, their strength and weaknesses are clear and the comparisons with results from the literature are very relevant. Nevertheless in the final analysis the choice of the 'metabolic weight method' is perhaps more arbitrary than is acknowledged. Overall the discussion in this chapter was easily followed and relevant.

Chapter 4
The objectives and results of the experiment described in this chapter are clearly presented. The results clarify some apparent inconsistencies in the literature. It is concluded, however, that this particular line of investigation would not be useful in meeting the overall objectives of the experimental programme, which would have to be progressed in some other way.

Chapter 5
This was quite an ambitious experiment that required careful attention to detail. The chapter is clearly written, the appropriate data are presented in figures that are easily understood and the discussion puts the results in context of the literature. The

discussion also leads into proposals for further experiments including that described in the following chapter.

Figure 5.3
The derivations of the values in this figure are not clear as it does not appear that they can be derived from the data in Figures 5.2 and Figure 5.8, which would be the expectation.

Page 123, second paragraph, second last sentence
The origins of the data cited in this sentence are not clear as they do not appear to be from Figure 5.2.
The observation is made that when 10 pups had access to 5 glands milk production was significantly less than milk production when 10 pups had access to 10 glands so that it may be concluded that the dam has the capacity to supply the requisite nutrients but the glands were limiting. It would seem, however, that production per gland must have increased when 10 pups sucked 5 glands. This needs to be clarified.

Chapter 6
Figure 6.11
The distribution of silver grains would have been clearer if the figure had been larger.

Chapter 7
The experiments described in this chapter follow logically from those described in the previous chapter. The protocols used were tightened significantly in ways that would increase the robustness of the experiments. The first experiment was designed to confirm and further the overall objectives of the experimental programme but instead the results contradicted those reported in Chapter 6. This meant that the experiment had to be repeated and the results also did not confirm the observations made in Chapter 6, which meant that it was not appropriate to pursue that particular line of research further.

Table 7.3
Values for 'dam wt gain' appear to be for 3 days not g/d and the values for dams with litter sizes of 10 and 15 pups appear to be incorrect.

Chapter 8
The results in this chapter are novel and make a significant contribution to our understanding of cell turnover in the lactating mammary gland of the mouse. It is unfortunate that initial observations leading to these results were not followed up earlier.

Chapter 9
The general discussion is a very good and clear summary of the unique findings reported in the thesis. The importance of these findings is critically evaluated and related to those reported in the literature. A number of logical and exciting proposals for future experimental work are made arising from the results reported in the last chapter. It is frustrating that the opportunity to follow through on some of these during the course of the PhD programme was missed.

Editorial

Table 3.4
Units for energy in milk and volume of milk are not included.

Page 92, line 4 from bottom
'The were ...' requires attention.

Page 110, line 8
'.. growth probably..' should read '.. growth is probably ..'

page 138, line 12
'..intermediary..' should read '..intermediate..'

page 144, third paragraph
The meaning of the third sentence is not clear.

Page 172, line three from bottom
'..and..' should read '..than the..'

Potential Questions.

1. In light of your experience, do you think flow cell cytometry has a place in studying the physiology of the mammary gland? How might the technical difficulties that you encountered by overcome?

2. You have shown that lactose concentration of the milk increases over a lactation in the mouse. What is the pattern of sodium and potassium concentrations in the milk might you expect? What reservations would you have about any conclusion that you might draw?

3. Do you think that it would have been better to have measured milk production with an isotope dilution technique?

4. When milking the mice they were injected with a dose of 0.15 IU of oxytocin. What is the size of dose that you would consider physiological? What impact do you think a dose of 0.15 IU might have on milk yield and composition?

5. You suggest in the discussion on chapter 4 that the variation in responsiveness of the gland to oestrogen at different stages of lactation may be related to the presence or absence of receptors for oestrogen and progesterone. Could you elaborate on how this may work, especially on possible signals that would initiate the changes in receptor numbers or type?

6. In the introduction of chapter 5 and elsewhere mention is made of autocrine control of milk secretion. How do you envisage that this is mediate via FIL?

7. With reference to the conclusions in the second paragraph on page 125, could it be that the milk production is less when 10 pups had access to 5 glands than when 10 pups had access to 10 glands because of competition for teats so that the pups are not able to suck for as long as they need to become full?

8. On page 125, second paragraph it is suggested that highest level of production per unit of DNA was in late lactation. Is this a fair assessment or is it a reflection of an overestimate of milk production?

9. Milk production of mice with 10 pups in mid lactation is approximately 0.35 ml/hour (Figure 6.3) yet the amount of milk recovered in the gland 1 hour after removal of the pups is approximately 2.5 ml (Figure 6.4). This implies a residual milk of approximately 2 ml. Please comment on the significance of this observation especially in relation to the concept of the autocrine control of milk production where the presence of FIL in milk inhibits milk secretion.

10. In Chapter 8 there was a decline in labelling index from 19% to 12% from early to mid lactation. Yet DNA content of the glands increased 250% over the same period so that one may have expected the labelling indcx to decrease from 19% to 8%. Do feel this is a serious discrepancy?

11. In retrospect what was the most serious error you made?

12. What would be the next experiment you would do if you had access to the appropriate funding and facilities?

D D S Mackenzie 7 January 2003

Dr Peter Wynn
University of Sydney
New South Wales
Australia

PhD thesis: Examiners report
Title: DNA synthesis inn mammary epithelial cells of Swiss mice during lactation.
Candidate: Danielle E Croke Auldist
General comments: This thesis describes a series of experiments designed to establish a model for differential proliferation of mammary epithelial cells in vivo using the mouse as a model. The candidate has based these studies on a number of techniques that have proved over the years to be suitable for the characterization of both cellular proliferation and for the output of milk and its constituents. Of course there are two sides to net tissue growth involving both cell deletion and cell division. Both of these sides of the equation have been dealt with in some detail in these studies.

The literature review provides an adequate account of the literature associated with mammogenesis and lactogenesis in the mouse. While this is adequate in many ways the scope for a truly comparative physiological approach across species in this review has not been realized. Nor have the key elements of tissue dynamics been explored. For example molecular mechanisms regulating the phenomenon of apoptosis have not been addressed in any detail. This forms part of the core material for this thesis and should have been dealt with in more detail. Despite this the review is written in a lucid style and provides the basic information required to build the subsequent experimental program.

Although the studies included in chapter 3 are of basic nature they do provide valuable information upon which subsequent studies are based. The need to estimate milk output in a mouse is important given the likelihood of the use of this phenotypic measurement upon which to base subsequent molecular expression studies. It is clear that these have been conducted with great thoroughness and the implications of these results are discussed in some detail. Clearly the candidate has a close empathy with the subject matter she is dealing with. Given the fact that the whole thesis is based on Swiss mice, some information should be provided on the genetic background of the Swiss mice and their level of inbreeding.

Chapter 4 has yielded some interesting results that then become the focus for subsequent investigations in the thesis. Again most of the observations relate to changes in the morphology and milk output/composition. It would have been nice to include some cellular characterization of the sensitivity or functionality of the steroid activation pathway in order to explain the responses achieved.

The suckling studies described in chapter 5 are also comprehensively described and investigate the role of pup/teat ratios above 1 on proliferation at various stages of lactation. Again a measurement of the sensitivity of the prolactin signal transduction pathway would have added greatly to this study. However the experiments are well designed and provide answers to the hypothesis being investigated.

Chapter 6 investigates the hypothesis established in ch 5 in another way. In this study the role of nutrient balance in raised on occasion and yet little attempt has been made to establish the metabolic status of the mice in these studies. Partitioning nutrients within

the dam at different stages of lactation will play an important role in the functionality of the gland. Perhaps this could have been included in the discussion.
A number of these studies rely on the incorporation of brdU and ^{3}H thymidine into cells in the S phase of the cell cycle and yet in the general discussion the candidate states that these methods may not be appropriate for the quantitation of DNA synthesis for mitosis relative to cellular repair and differentiation. Both of these latter aspects of cellular metabolism are likely to be important in mammary tissue. Perhaps other methods should have been attempted to delineate between these functions.

The results from chapter 8 will have major implications for studies of the transcriptome in lactating mice, which has been duly recognized by the candidate.

The summary and conclusions are well written and the candidate identifies both the shortcomings and strengths of her own studies, while at the same time providing a useful framework for future work for this research program.

The manuscript is refreshingly devoid of typographical errors and errors in referencing for which the candidate deserves to be congratulated. For this reason just a few minor changes are required to the manuscript as detailed below. There is no doubt that the candidate has made a unique contribution to our understanding of lactational physiology and I hope she is provided with the opportunity to carry her work on as a postdoctoral fellow. I sympathize with the candidate over some of the methodological problems that were encountered throughout the studies, but this does not seem to have detracted greatly from the experimental outcome. Her comments relating this work to our understanding of lactation in commercially important species are particularly insightful as we strive to improve the efficiency of production from the commercial dairy cow.

Much of this material is unique and therefore publishable. I look forward to seeing the results appear in a reputable journal.

Detailed comments:

Page	Para	line	
III		3-7	These two sentences tend to contradict each other
1	3	5	mesenchymal
13	3	12	Glucocorticoids
14	3	14	shrink
19	1	3	--little or no activity---
46-47			The candidate has not explained why she did not attempt the BrdU labeling again rather than trying to explain the failure of the technique on the first attempt.
54	1	15	cytoplasm
56	3	10-13	Simply amazing!
58	2	8	intact
76	1	8	---concentration. Instead---

92	2	3	diethystilbocstrol
92	3	4	They were---
112	3		The rationale for taping over 5 glands for the last 3 days only is not well explained. The candidate should clarify for the reader.
158	1	2	-- data were –
183	2	12	---model for the study---

Prof Chris Knight
Hannah Research Institute
Ayr, Scotland
United Kingdom

General comments

This thesis is competent and thorough in what it does cover, but lacking in originality and conceptually outdated, in that measurements of apoptosis are not included.

Overall, I suggest that too much emphasis has been given to 'data trawling' and not enough to critical and discriminatory analysis. There is considerable repetition of rather similar data between the different results chapters, which in some cases detracts from the more important measurements. For instance, I am not convinced that litter weight gains and calculated milk production both need to be given for each experiment, nor am I convinced by the usefulness of mammary gland protein content.

The plethora of rather trivial data combined with the lack of apoptosis data mean that the main hypothesis (that lactating mammary tissue can grow) and objective (identification of stimuli causing such growth) have not been achieved.

Nevertheless, the candidate is to be commended for the rigour and thoroughness with which the experiments undertaken have been done, for the comprehensive and accurate account of the available literature on mammary cell proliferation and for her overall tenacity and determination. I am satisfied that the thesis as presented forms the basis of an acceptable PhD thesis and that, after consideration of points raised and some modification and expansion of discussion sections, the degree should be awarded.

Literature review

As a review of mammary cell proliferation this is a comprehensive and generally accurate account which reflects great credit on the candidate.

If there is a deficiency it relates to the almost complete concentration on proliferation, to the exclusion of any real consideration of cell death, or apoptosis. The point is made on p19 that cell population size is a function of proliferation and death, but this is not followed up in any way.

There has been an abundance of work on apoptosis in recent years, including a great many mammary studies. I would not expect the candidate to be totally familiar with the whole of the apoptosis field, which could easily be the subject of a thesis in its own right. Nevertheless, I do feel that there needs to be more awareness of the concept, of the basic factors that regulate mammary apoptosis and of the significance of apoptosis to overall mammary development. This could be added to the Literature review but might be better in Chapter 8 under Future Directions.

Two specific examples that make the point and which could be explored in discussion with the candidate are:

Whilst it is often assumed that terminally differentiated cells enter directly into apoptosis from G0 or GD, it has also been suggested that some may first revert to the cell cycle and complete S phase. This is the 'dual-signal' hypothesis proposed by Evan and Littlewood (1993) Curr. Opin. Genet. Dev. 3, 44-49. The salient point is that cells detected in S phase may not be committed to proliferation, rather, some may then 'choose' to die rather than proceeding through mitosis. How does this affect the way in which the candidate would interpret thymidine incorporation data?

There is good evidence to indicate that declining lactation is associated with loss of cells rather than loss of cellular activity. This is referred to in Fig 1.3. Given that the thesis objective is ultimately to achieve a larger lactating gland, one could argue that preventing this loss of cells (ie reducing apoptosis) would be the most effective method of achieving the objective. How does the candidate justify her concentration on proliferation?

Other points meriting some discussion:
Do terminal end buds 'regress soon after birth' (bottom of p5) or do they remain, to be the 'major sites of proliferation…' (bottom of p7). I don't know the answer! I suspect it is partly a terminology problem, but it is certainly the case that the literature is somewhat confused on this matter.

The factor that prevents neighbouring ducts from converging has been suggested to be TGFβ (Daniel, Robinson & Silberstein, 1996, J Mamm. Gland Biol. Neopl. 1, 331-341). Is the candidate aware of this?

Materials and methods

As with the literature review, a distinguishing feature of this chapter is thoroughness. Three histology based methods and two separate attempts at flow cytometry were all used to assess proliferation. This is commendable, although it does beg the question, Why? It would be sensible to explore with the candidate the decision processes that contributed to the design of the thesis. Was there an early, conscious decision to take a 'belt and braces' approach, or did the different methodologies evolve along the way?

It is evident that a number of problems were encountered with several of the techniques. This is not surprising, indeed, our own experiences of BrdU (for instance) have been somewhat similar. The tenacity with which these problems were faced and (mainly) overcome is, once again, commendable. With the benefit of hindsight, does the candidate feel that the effort put into making all of the techniques work as well as possible was worthwhile, or could more have been achieved if difficult techniques had been abandoned sooner? The flow cytometry is an obvious example, but I am also puzzled by the use of thymidine, BrdU and PCNA. To mind, the major advantage of BrdU is that it can be used for *in vivo* applications that preclude a radioactive isotope (eg large animals, lengthy treatments), but that was not the case here. Was anything substantive gained from having the different measures?

The 'positive control' for the BrdU staining was actively proliferating wool follicles. Not, I presume, from mice! Is it possible that part of the problem was species specificity of the antibody?

One specific question regarding the labelling experiments. Why were mother and young separated? One hour is a relatively short period and it is unlikely that much would have changed, but I do not understand the logic.

And one regarding the milk yield maintenance energy requirement correction method. Neonatal rodents are exothermic and will have a different (lower) maintenance requirement in the presence of their mother compared to without her, simply because she keeps them warm. Was this taken into account?

Why was a different protein method used for tissue samples as compared to milk?

Chapter 3

This experimental chapter reports data for murine milk composition and yield, the latter obtained by three different methods. The rationale is that subsequent study of mammary growth during lactation can only be properly interpreted if it can be related to the true size of the gland (ie corrected for retained milk) and its productive capacity.

The work has been conducted with very considerable thoroughness. It is more confirmative than innovative, although the candidate's suggestion that literature data are inconsistent is accurate.

There is one surprising finding, namely that lactose content varied considerably (increased) during the course of lactation. Current theories regarding the secretion of the aqueous components of milk predict that lactose content should be relatively constant, so this finding is potentially very significant. This aspect is not explored sufficiently in the discussion. On p65 the candidate criticises the 'milking out' method of measuring milk yield on the basis that it is difficult to remove all milk. I agree. The obvious concern arising out of this is that measurements of composition are compromised by the same issue, yet this is hardly discussed. Was care taken to ensure that the glands were always thoroughly milked out? Fat content increases during the course of milking, such that incomplete milking underestimates fat content and overestimates other components. It is noteworthy that fat content remained constant across the course of lactation, whereas an earlier study reported a gradual increase. I hesitate to claim that this study (mine) was necessarily correct, but we did take care to ensure thorough emptying.

Given the importance of the lactose observation I consider it imperative that the discussion be amended to include a better account of the methodological problems inherent in this type of work. If the candidate can provide reassurance that the mice were always thoroughly milked out this should be done. If not, then the lactose observation has to be regarded as tentative.

Similarly, whilst I am generally impressed by the thoroughness of the yield methodologies, their complexity has perhaps distracted the candidate from some simpler but nevertheless important observations. In Table 3.2 for example:

- Daily gain was lower for 15 pup litters at both day 5 and day 10. However, calculations from mean litter weight data show that between these two days 15 pup litters gained 36.25g overall, whilst 10 pup litters gained less (33.58g). How can these figures be reconciled?
- At d5 and d10 the 15 pup litters lost less weight during fasting than the 10 pup litters. How can this be? Is this perhaps evidence of the heat loss issue I alluded to above?
- By contrast, 15 pups lost considerably more at d17. Why? What is it about 17d-old pups that is different to younger ones? Can the candidate exclude the possibility that some pups were eating solid food by this stage? Actually, I consider that Fig 3.1 provides clear evidence that some were, and I suggest that one could predict which of the fitted points belonged to which litter size.

The statement at the bottom of p68 is wrong.

Some of the calculations depended on an assumed digestibility value (90%). How reliable is this figure? Does the candidate feel comfortable with the conclusion that young pups can deposit more than one gram of body tissue for each gram of milk consumed?

The calculated values for 10 and 15 pup milk yields (fig 3.2) are surprisingly close given the data presented in Table 3.2, where both daily gain and weight loss are said to be higher for 10 pups (although see my comments above).

In the metabolic weight method the maintenance energy requirement was calculated from a figure for older mice (p73). How reliable is this? Older pups are active but homeothermic, young pups are sedentary but exothermic.

It is confusing to present important data only in a later, summary figure (Fig 3.6, referred to on p73).

I am left with the conclusion that a lot of hard work has been done, but the conclusion (that the metabolic weight method is the most reliable) has been reached without any really clear justification. It is evident that the proportion method underestimates yield. The other two methods give similar results that are, nevertheless, only about two-thirds of our published isotope dilution data. Again, I hesitate to claim validity for this earlier report but I am not sure that strain differences can totally explain the differences, since our litter weights were similar to those reported here. Another method that has been used is gland weight difference (ie *post mortem* comparison of 'full' and 'empty' glands). Is the candidate aware of this method? It gave similar values to isotope dilution when we compared the methods in rats (Knight et al, 1984, J Dairy Res. 51, 29-35).

Chapter 4.

This experimental chapter reports effects of exogenous steroids on lactational mammary development.

It is reported (p91) that estrogen (with or without progesterone) increased thymidine incorporation in late lactation without any concomitant increase in mitosis, the net effect being one of involution and tissue remodelling. Whilst this conclusion may well be correct, it does raise two concerns in my mind:

- Was mitosis measured? I am not aware that it was.
- The statement hints at a knowledge of apoptosis-related DNA synthesis (see above) that is never referred to elsewhere.

Why was DNA synthesis measured commencing 24h after the last steroid injection (p94)? Is it possible that an effect was missed?

From Fig 4.2 and 4.3 it is evident that milk production and milk retained in the mammary gland are not necessarily correlated. How would the candidate interpret this? I suggest the older pups are keeping the glands very empty, especially those hungry pups of steroid-treated mothers. On the other hand, panel C of Fig 4.10 appears to show significant amounts of milk contained within involuting alveoli of estrogen treated glands. Comments?

The candidate suggests that estrogen has an effect on milk production only in late lactation. Fig 4.1 and 4.2 suggest to me that this is a question of degree, rather than an absolute difference.

Would the candidate expect progesterone to have any effect (milk production or mammary development) if given alone?

The main conclusion is that estrogen given in late lactation causes involution accompanied by DNA synthesis. The candidate needs to address:

- If they are not proliferating, what does happen to the cells that are synthesising DNA?
- Is it possible that involution is then followed by proliferation of new cells? Remember that DNA synthesis was measured last. Such an explanation is reminiscent of lactation rescue; see Knight & Sorensen (1998) J. Reprod. Fert. Abstr. Ser. 21, 43.
- Reduced prolactin is cited as a possible cause of the stage of lactation difference. Some of the observations suggest that the pups will be suckling most vigorously in late lactation, so why should prolactin be decreased?
- Is d17 really late lactation? In the sense that the pups are starting to eat solid food it may be, but on the other hand the data in Fig 4.2 indicate that milk yield is actually higher than in mid lactation!

Chapter 5.

This experimental chapter reports effects of suckling intensity on mammary development and milk yield. The objective was to develop a model of enhanced cell proliferation.

In the Introduction the argument is developed that compensatory mammary growth can be stimulated in early lactation by increased suckling intensity. I have two concerns with this:

- The 'compensatory' term is usually taken to mean restoration of normality following a deviation, so for instance in mice we inhibited gestational mammary growth (by reducing fetal number) and then observed compensation in early lactation when a strong suckling stimulus was applied (Knight & Peaker, 1982e in cited refs). If mammary size was normal at birth, any extra growth would not, strictly speaking, be 'compensatory'.
- An association between larger peak-lactation glands and bigger litters need not necessarily require additional growth; if there is 'spare' mammary capacity at parturition then the same result would be achieved from a reduction in post-partum mammary involution. Although I am not aware of published data showing postpartum apoptosis in mice, it is something we have recently observed (Ong, E (2002) Phd Thesis, Univ of Glasgow). The candidate should be able to discuss the implications of this observation.

Was there any difference between 5/5 and 10/10;5 at d3?As far as I can see they are treated identically.

The data for milk production per unit DNA (Fig 5.3) suggests a dip in mid lactation. Notwithstanding the lack of significant effects, I would wish the candidate to comment on these observations. Given that cells differentiate between early lactation and mid, an increased production per cell might be expected. In late lactation the size of the cell population has decreased (Fig 5.8), nevertheless increased production per cell is somewhat surprising. Is it possible that milk production is being overestimated at this time, perhaps because the young are starting to eat solid food?

The data reported in Fig 5.6 should not be described as sucked to non-sucked gland DNA ratio. In the case of C5 and C10 we have to assume that all glands were sucked.

In Fig 5.5 the y axis descriptor makes clear that the data refers only to right glands. Elsewhere (eg Fig 5.8) one is left to assume that the same is the case, since if all glands are included then the C5 and certainly C10 data are unexpectedly low. It must be made absolutely clear in Methods and in Figure legends and text exactly what was being measured.

The discussion starts by stating that mammary growth was maximal in early lactation. In what sense is it maximal? The growth rate is as high as at mid pregnancy? Or is 'growth' used here to mean size, ie the gland has reached its maximum size. Fig 5.8 clearly shows that it hasn't. The candidate must think carefully about what she means, and what she thinks is going on here. Thymidine incorporation did not differ between

C5 and C10 on d3. At this point DNA content did not differ either, but by d10 it had increased in both groups but was now significantly higher in C10. So, is thymidine incorporation too imprecise to detect a small but biologically meaningful difference, or has a proliferative phase been missed, or is there a different explanation?

The conclusion that a suckling stimulus of >1 increased cell survival is a nice explanation of the 10/5 data. However, d17 DNA content was identical for 10/5 and 10/10;5, although the latter had a stimulus of 1 until the last 3 days. How can this be reconciled? Is it possible that a sudden increase in suckling stimulus from 1 to 2 does stimulate a burst of cell proliferation, which has ended before the thymidine incorporation measurement is made 3 days later? If so, perhaps this has also happened in groups taped from parturition onwards.

Chapter 6

This experimental chapter is a variation of the previous, wherein suckling stimulus is increased by fostering extra pups rather than reducing gland number.

In addition to measuring total thymidine incorporation, the methods indicate that whole mounts were made for counting labelled cells (p131). Data are only reported for one group (p140). Please clarify what was actually done.

Here (p129) there is allusion to the fact that FIL might affect mammary development. FIL's main recognized action is on milk secretion. Is there evidence that FIL can alter proliferation? Or apoptosis? If so, is this is a direct action, or one mediated by changes in hormone sensitivity? I suggest the latter may be true, but there is no real evidence of the former.

As in Chapter 5 there appears to be duplication of treatment. Were C10 and P5+5 the same in early lactation, or different?

In Fig 6.1, why did P10+5 do so badly at mid-lactation?

In contrast to Fig 5.3, there is no evidence of a mid-lactation dip in milk production per unit DNA (Fig 6.3). Why the difference?

Given that the stimulus of 15 pups increased cell proliferation at d3, would it not have been interesting to apply this treatment and then follow the mothers through to later stages of lactation? Would a constant 15 pup stimulus create the same effects as 15 pups reduced (after d3) to 10?

The fact that the whole mount of one P10+5 mouse differed from the rest is dismissed rather lightly. I assume that her data are included in the other analyses, including that of total thymidine incorporation. Why was she different?

The candidate suggests that the lack of a proliferative response later in lactation may be due to energetic differences. What is the energy status of mid vs early lactation mice? Food consumption increases markedly, so energy status may not be much different!

Chapter 7.

This experimental chapter attempts to build on the observation of increased proliferation in early lactation glands stimulated by increased suckling. The observation is not confirmed.

Why was it felt necessary to confirm experiment 6? Was there some concern about the data?

Comparing experiment 6 and experiment 7a reveals that 15 pups had lower growth rates and stimulated more proliferation in the former but not in the latter. This could reflect inadequate mammary development at term, which is then rectified by proliferation. The younger age of the mice would be consistent with this explanation. In a way part of experiment 7b (small dams) follows-up this possibility. Had it been thought of?

In experiment 7b one could argue that the small dam 15 pup group do not have a proper control, although given the data I think it is reasonably obvious that proliferation would not have been enhanced had there been a small dam 10 pup group to compare with. So, does this negate the hypothesis advanced above, or are these mice fundamentally weaker, runts, almost?

The 'acceleration' hypothesis is fine for explaining the present data. But in that case, why was proliferation increased in experiment 6?

On page 161 it is stated that mice could not sustain 20 pup litters. Was it ever the case that individual pups died or failed to thrive in smaller litters? I think particularly of the small dam 15 pup group. Weak pups may not suck very vigorously!

Day 10 C10 mice were killed for analysis in chapters 4, 5, 6 and 7c. In the UK this might be regarded as contravening animal ethics legislation, in that no effort was made to limit the number of animals used. Was statistical advice sought on whether it was necessary to repeat these observations to this extent? Chapter 4 mice were adjuvant treated, and there may have been age differences with others, nevertheless it does seem extravagant.

Chapter 8.

This experimental chapter reports on the localisation and lifespan of BrdU labelled mammary epithelial cells arising in early lactation.

The comment (p170) that a proliferation gradient hypothesis was developed in earlier chapters is somewhat misleading. The data presented in Chapter 4 did not show such a gradient and were hardly discussed, those in Chapter 6 did not show a gradient and the explanation given related more to proximal versus lateral thirds of the gland than to inner and outer zones.

The labelling indices are very high compared to what was previously determined using thymidine (compare Table 8.2 and Table 6.1) and literature values. How confident is the candidate that false positives have been excluded? Is this very high proliferation perhaps a specific feature of d1 of lactation?

The observation of a gradient (outer>inner) is very exciting and potentially very significant, and the explanation offered is highly plausible. However, it has to be remembered that the thymidine data did not show such a difference. It is recognized that immunocytochemical methodologies can be compromised by incomplete penetration of antibodies. Can the candidate exclude the possibility that detection was better at the periphery simply because the antibody was able to access cell nuclei more easily there, perhaps by 'sideways' diffusion? How could this possibility be excluded? If the observation is genuine, tissue blocks cut retrospectively from the periphery and from the centre and then sectioned randomly should yield a between block difference but no within block difference. I do not suggest that this actually needs to be done for the thesis, rather, it should appear in the discussion as a potential validatory approach.

Since labelling occurred only on d1 and is then followed over 5 days, the analyses comparing inner and outer or teat vs mid vs distal should arguably be a repeated measures or time series analysis of some sort, even though different animals were used. Is this the case?

Why did the labelling index decrease at different rates in inner and outer areas? (p174). Is the difference between the rates statistically significant?

The Pitkow data referred to on p178-179 were from a different model, in that the rats were concurrently pregnant. The rate of new cell proliferation would almost certainly be increased as a result. A major disadvantage of the thymidine approach that the candidate should be aware of is recycling of label from cell to 'daughter' cells. In some studies grain counting has been used to discriminate between the original cells (eg 8 grains), daughter cells (4 grains in this case) and even granddaughter cells (2 grains).

General discusssion

It is concluded (p184) that mice would gain little selective advantage from being able to increase lactational mammary development. The candidate should be aware that mice are communal nesters and cross-suckle readily, so it is not at all unusual for mothers to 'share' the rearing burden. This may not be entirely altruistic: the mothers are often closely related and so are investing in their 'own' genetic material. It does mean that lactation can be extended for long periods of time by litter swopping (eg Bruce, HM (1958), Proc. Royal Soc. 149 421-423). How would the candidate interpret this type of observation.

Given her own experiences, does the candidate truly believe that flow cytometry is a useful approach for mammary tissue (p185)?

The statement on p186 regarding consistent heterogeneity between outer and inner areas is confusing. For one thing it was chapters 4, 6 and 8, not 5 and 8. For another, although heterogeneity was a common feature, the inner vs outer comparison differed greatly.

A major part of any consideration of future work should be to address the whole area of apoptosis. This is the main deficiency of the thesis and an opportunity exists to remedy this. I suggest that a section approximately equal in length to the present Chapter 8 be added, specifically concerned with apoptosis. A reasonable way into the literature might be through Stefanon et al (2002), J Dairy Res. 69, 37-52.