Effects of Feeding Level and Diet Composition on Mammary Growth in Prepubertal Lambs and Mice

by

Thomas Bernard McFadden

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APPROVED:

R. Michael Akers, Chairman

A. V. Capuco

T. W. Keenan

F. D. McCarthy

D. R. Notter

R. G. Saacke

William E. Vinson, Department Head

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Blacksburg, Virginia

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(ABSTRACT)

Forty ewe lambs were grouped into four treatment groups: A) fed a standard, high-energy diet, ad libitum; G) fed as group A, but treated with GH (.1 mg/kg bodyweight/d); R) fed the standard diet in restricted amounts to a target weight gain of 120 g/d; S) fed a ration including 30% of a protected fat supplement, ad libitum. Rations were formulated to be isonitrogenous and isocaloric and were fed from approximately seven to 22 weeks of age. Growth rates differed in the order S > A = G > R, although final weights did not differ among ad libitum fed groups. Lambs in group S had heavier mammary glands, with greater amounts of parenchyma and fat pad and higher content of dry, fat-free parenchymal tissue compared to the mean of the remaining groups. Total gland weight was lower in group R, although weight of parenchyma was similar to groups A and G. Parenchyma made up a higher percent of total udder weight in lambs of group R compared to any other group. Parenchymal DNA content was not different by treatment, but glands from group G had twice the total DNA of groups A and R, and group S had 50% more than the latter groups. Volume of mammary glands occupied by parenchyma was increased by more than 50% in group S, compared to the other groups which were similar. Concentrations of prolactin receptors in mammary parenchyma and of GH receptors in liver were increased in lambs of group S. Percent linoleic acid in mammary parenchymal lipid of lambs in group S was increased relative

to other groups. Unsaturated acids also made up a greater percentage of total fatty acids in group S. Feeding the protected fat supplement resulted in increased unsaturated fatty acid, especially linoleic acid, percentage in mammary fat. This effect was associated with increased mammary growth compared to lambs fed a standard ration. Lambs treated with GH showed some indications of increased mammary growth, but groups A and R were similar except for the increase in percent of gland occupied by parenchyma in group R.

In a second study, mammary growth in prepubertal mice increased with increasing dietary energy intake. Differences in ductal growth persisted at 18 weeks of age, and effects of exogenous steroids at this time were not significant. Prepubertal mammary growth in mice is not sensitive to inhibition by high plane of nutrition as is the case in ruminants.

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Table of Contents

List of Tables

List of Illustrations

INTRODUCTION

It has long been recognized that rapid rearing of young livestock has great potential for increasing the economic efficiency of meat and milk production. Higher weaning weights, shorter time to market weight, increased numbers of offspring (due to earlier attainment of puberty), and more lactations per lifetime are among the potential benefits of such a management system. In addition, less capital and labor are invested in rearing animals that may ultimately prove to be unproductive as adults, and economic returns are realized more rapidly.

In practice, young animals raised for meat production are subjected to intensive feeding systems, in order to gain some of the advantages listed above. However, for animals in which milk production is of importance (dairy heifers, and beef heifers and ewe lambs to become brood stock), feeding to achieve elevated growth rates results in impairment of mammary growth and thus decreased future lifetime milk production. This is clearly unacceptable, since milk production is of paramount importance for its own sake in dairy animals, and for the survival and growth of offspring in beef cattle and sheep. Thus, rapid rearing has not been a viable management option for these animals.

Studies directed toward understanding this inhibitory effect on milk production have revealed that growth of the parenchymal portion of the mammary gland is retarded in females fed a high plane of nutrition during early development. More specifically, this effect occurs during a critical period of mammary development, limited to the prepubertal period of allometric mammary growth. During this period the gland is particularly sensitive to changes in amount or other characteristics of the fat pad which ultimately result in poorer growth of the epithelium. Administration of exogenous growth hormone can at least partially overcome the negative effect of overfeeding, although the mechanism of action is unknown.

The exact nature of the changes associated with rapid rearing that result in decreased subsequent milk yield are unclear. However, research in laboratory animals indicates that the fatty acid composition of mammary fat can regulate mammary parenchymal growth. In general, unsaturated fatty acids (especially 18:2) are associated with stimulation of parenchymal growth, while saturated fatty acids are inhibitory. A change in fatty acid composition of the mammary fat pad may not be responsible for dietary inhibition of mammary growth in production animals, but if the effect seen in lab animals applies also to ruminants then it could possibly negate the effects of overfeeding, and result in increased mammary growth.

In monogastrics, manipulation of fatty acid composition of depot fat is relatively simple, since depot fat reflects the composition of dietary fat. However, in ruminants, the situation is complicated by biohydrogenation of dietary fat in the rumen. Fortunately, technology exists to allow for protection of dietary fat from saturation in the rumen. This technique was developed originally to alter the fatty acid composition of food products derived from ruminants to better suit recommendations for human nutrition. It

should be equally applicable in altering the fatty acid composition of the mammary fat pad with the possibility of stimulating parenchymal growth.

Accordingly, the primary objective of the present study was to determine the effects of deposition of unsaturated fatty acids (especially 18:2) in mammary fat on prepubertal mammary growth in rapidly reared ewe lambs. A second objective was to investigate possible mechanisms by which elevated plane of nutrition retards mammary growth, and how treatment with growth hormone can overcome this inhibition. Finally, a study utilizing laboratory mice was designed to investigate whether mice would provide a suitable model for study of this phenomenon in ruminants, and to further study effects of fat pad character on mouse mammary growth.

REVIEW OF LITERATURE

Introduction

That understanding the regulation of mammary growth is of critical importance to the dairy industry is illustrated by the strong correlation between mammary cell numbers and milk production ($r = .50$-$.85$; Tucker, 1969). In fact, milk yield is ultimately a function of the number of epithelial cells and the secretory activity of each cell (Knight and Peaker, 1982). Thus cell numbers constitute a basic limitation to milk production, and ability to increase cell numbers has direct potential for increasing yields.

Growth of the mammary gland proceeds in a series of distinct stages, each subject to different physiological and endocrine environments within the animal. The glandular portion of the mammary gland develops upon a foundation of stromal tissues, established during fetal development (Mayer and Klein, 1961). At birth, the mammary rudiment consists primarily of the teat cistern, a compact gland cistern, and a limited number of primary ducts, extending a short distance into the stroma. At the same time,

the stromal elements are relatively well-developed. Vascular and lymph structures are present, and adipose and connective tissue compartments are evident (Turner, 1952).

The earliest phase of mammary ductal development involves proliferation and limited branching of a primary ductal network within the mammary fat pad. This development is achieved during a period of allometric growth, during which the mammary gland grows faster than the body as a whole (Folley, 1949; Mayer and Klein, 1961; Sinha and Tucker, 1969). This phase occurs during the prepubertal period and ends with the approach of puberty, with the result that the mammary gland exists as a compact gland cistern with an attached framework of major ducts, as the animal attains puberty (Hammond, 1927).

Pubertal growth involves secondary branching of the existing ducts, and continues until the fat pad is essentially filled with a finely branching network of ducts. Local-acting factors apparently maintain sufficient distance between ducts to allow for the eventual development of alveoli during pregnancy (Faulkin and DeOme, 1960).

Alveolar growth is limited primarily to the gestational period (Tucker, 1969). Under the influence of the hormones of pregnancy, alveoli bud from the sides and ends of smaller ducts within the gland, as aptly described by the much-used analogy with a cluster of grapes developing on a vine. The alveoli represent the basic milk-secreting unit of the mammary gland. Alveolar epithelial cells differentiate and attain full secretory capacity in concert with events leading to parturition. Thus, the milk-secretory apparatus reaches a functional status at the very time it is needed to provide nourishment for neonatal offspring.

It is clear from this very brief overview of mammary growth that the ultimate size of the gland (and therefore the number of secretory cells) is limited by the extent of the primary ductal framework established during the prepubertal period. For this reason, study of prepubertal growth is of particular significance, both as a means to better understand fundamental regulation of growth and as an especially promising interval for manipulation of milk production potential.

Mammary Growth in Laboratory Species

Much of the research on prepubertal mammary gland growth has been conducted in laboratory species. In mice, prepubertal ductular growth involves the orderly penetration of the fat pad by a highly specialized structure, the terminal end bud (Williams and Daniel, 1983). End buds are club-shaped structures, 0.1-0.8 mm in diameter, which represent the proliferating end of a duct. From 3-4 wks of age to approximately 7-8 wks end buds grow rapidly through the fat pad (up to 0.5 mm/day), occasionally bifurcating to form new growing ducts. Duct growth and extension continues in this manner until the end bud encounters the margin of the fat pad, at which time rapid growth ceases and the end bud regresses (Faulkin and DeOme, 1960; Williams and Daniel, 1983). Nandi (1958) found that the fat pad was not entirely filled with ducts by the 7th week, but the remaining portion was occupied during the next two weeks of pubertal growth. In either case, the majority of the ductular framework of the gland is established within the fat pad prior to the onset of puberty and ensuing pubertal growth.

The phase of end bud proliferation coincides with the period of allometric growth of the mammary gland. Flux (1954) reported that the rate of increase in mouse mammary

ductal area is allometric with respect to metabolic bodyweight from birth to 24 days. From the 24th day to approximately 8 weeks of age, mammary ductal area increased somewhat less rapidly. Likewise, Sinha and Tucker (1966) found that mammary area in rats increased 3.5 times faster than body surface area between 23 and 40 days of age. In each of these instances the rapid mammary growth occurs well in advance of puberty.

Among the early findings concerning mammary growth was the classic observation that an adipose tissue fat pad was absolutely essential for growth of transplanted mammary duct pieces (Hoshino, 1962). It has been suggested that mammary gland cells organize and grow normally only when transplanted into mammary fat pads (Dulbecco et al., 1982). The critical role of the fat pad is further supported by reports which indicate that the prepubertal phase of duct growth ends when proliferating ducts reach the limits of the fat pad (Faulkin and DeOme, 1960; Hoshino and Martin, 1974; Williams and Daniel, 1983). Mammary tumors have also been shown to exhibit preferential growth in mammary fat pads versus subcutaneous sites (Miller et al., 1981). Although several studies have described growth of dispersed mammary cells or organoids in collagen gels *in vitro* (Richards et al., 1982, 1983; Yang et al., 1979, 1980), structural organization has not been entirely typical. Thus the fat pad appears necessary for morphologically normal mammary growth and the size of the fat pad limits the ultimate size of the gland.

Mammary Growth in Ruminants

Early studies of prepubertal mammary growth in ruminants relied upon slicing the teat and mammary tissue into "steaks" and observing the apparent degree of parenchymal growth. In one such study Hammond (1927) described the mammary gland of a 6 month

old calf. The structures present included a gland cistern with a lumen, from which branching ducts extended. The duct ends were swollen and bulb-like, but were clearly not alveolar structures. Growth of the udder during this period was ascribed mainly to deposition of fat and connective tissue, with ductal growth postponed until the onset of ovarian activity of puberty (Mayer and Klein, 1961). However, other evidence suggested that glandular growth was stimulated well before puberty and in fact total gland area during this time was greater for calves from high-producing breeds (Chiodi, 1933, cf. Mayer and Klein, 1961).

Wallace (1953) reported on early development of mammary glands in lambs based upon examination of udder slices. From birth to 1 month ducts began to push their way between fat lobules of the fat pad. Duct extension was increased markedly at 2 months and even more so at 3 months. Growth apparently ceased between 4 and 8 months of age. These findings were supported by Anderson (1975) who reported that mammary growth in lambs based on gland weight, DNA content and dry fat free tissue weight, was slow to 3 months, increased dramatically during the fourth month and plateaued at about 5 months of age. From these studies the period between 3 and 5 months can be targeted as a critical period for prepubertal mammary growth in lambs.

A similar study supports the idea of a critical period of prepubertal mammary growth in cattle as well. Sinha and Tucker (1969) measured nucleic acid, hydroxyproline and lipid content of udders of Holstein heifers from birth to 12 months of age. Udder DNA content increased 1.6 times faster than body weight from birth to 2 months and 3.5 times faster from 5 to 9 months of age. Rates declined to 1.5 times faster between 9 and 12 months of age. Changes in the other gland components followed similar trends.

In addition to duct growth, the mammary fat pad increases in size as well. Extension of the fat pad is thought to proceed primarily through cell hypertrophy rather than cell hyperplasia (Knight and Peaker, 1982).

In summary, prepubertal mammary growth in the ruminant is similar in most respects to that in laboratory species. It is not clear whether the bulb-like duct ends in the ruminant fit the specific histological criteria set up for "end-buds" in mice by Williams and Daniel (1983). Nevertheless, these structures are similar in macroscopic appearance and seem to fulfill identical functions. The literature indicates that in ruminants, as in lab animals, allometric mammary ductal growth occurs only within a specific period of time. This growth is responsible for providing much of the framework for future growth. It is readily apparent that this period is critical to mammary development, and that derangements during this time could affect the ultimate extent of gland growth.

Dietary Studies In Laboratory Species

Nutritional effects on mammary growth in monogastrics can be quite profound. Early studies in laboratory species showed that undernutrition could retard mammary growth. Sykes et al. (1948) found that rats fed 70% of their usual intake from weaning had smaller glands than controls at the end of their first pregnancy. However, this effect could have been due to reduction in fat content in the restricted glands since the restricted group appeared to have more productive lactations than controls. Mammary growth responses to stimulatory hormones were reduced following reductions in food consumption to 50% of normal intake, although systemic effects could not be ruled out (Trentin and Turner, 1941; Srivastava and Turner, 1966).

More recent studies concerning the effects of manipulation of diet on mammary growth in laboratory species have concentrated primarily on mammary tumor growth and cancer. Incidence and/or spread of mammary cancer has been related to fat, protein, carbohydrate, or energy content of the diet (Gridley et al., 1983; Hoehn and Carroll, 1979; Sarkar et al., 1982). These factors appear to act primarily via systemic effects, i.e., changes in hormone secretion, but in several instances a local effect has also been implied. In each of these studies, growth of normal mammary tissues was not studied or was treated only cursorily. It is clear however, that fundamental understanding of normal dietary : mammary growth interactions must come from studies of normal tissue.

A few studies however, have also included findings related to normal mammary tissue. Such results indicate that the fatty acid composition of the fat pad may have profound effects on epithelial growth. Normal mammary gland growth is severely retarded when mice are fed a diet containing only saturated fatty acids (Abraham and Hillyard, 1983; Miyamoto-Tiaven et al., 1981). However, more recent evidence suggests that the effect is mediated through inhibited ovarian maturation, and thus indirectly impacts upon mammary growth (Faulkin et al., 1986). Nevertheless, a similar effect resulted from feeding indomethacin (an inhibitor of prostaglandin synthesis) at a level of 0.003% of the diet, suggesting that prostaglandins are involved in lipid regulation of mammary ductal growth (Miyamoto-Tiaven et al., 1981). Studies in vitro have shown stimulation of normal mammary growth upon addition of unsaturated fatty acids to culture medium, whereas addition of saturated fatty acids inhibited growth (Wicha et al., 1979). The culture work provides evidence that effects of dietary fat on mammary growth are not confined to indirect routes, but rather may also alter growth via local mechanisms.

Furthermore, research in laboratory animals has shown that diets containing high levels of unsaturated fatty acids (FA) can stimulate growth of normal and pre-cancerous mammary epithelium (Abraham and Hillyard, 1983; Miyamoto-Tiaven et al., 1981). Similarly, unsaturated FA have been shown to directly stimulate growth of rodent mammary tissue in vitro, and the ratio of unsaturated to saturated FA in the mammary gland is increased (by 45%) during periods of hormonally-induced mammary proliferation (Wicha et al., 1979). Hosick (1979) has also demonstrated increased (50%) growth of rodent mammary tissue in vitro, upon addition of unsaturated FA. Other results have shown that delipidized serum will not support growth of cultured mammary epithelium, and that saturated FA actually inhibit mammary growth (Kano-Sueoka, 1983). Carrington and Hosick (1985) found that a high-saturated-fat diet inhibited growth of normal epithelium in vivo. Conversely, growth was stimulated in pre-neoplastic cells co-cultured with explants of mammary fat pad from mice fed a high-polyunsaturated-fat diet but was not affected in the presence of adipose explants from saturated-fat fed mice. These results led to the hypothesis that dietary fat could affect mammary growth firstly through a systemic effect, and secondly through direct interaction between adipocytes and mammary epithelial cells. The local interaction has been proposed to result from changes in lipid composition of adipocyte plasma membranes which could lead to increased fluidity and greater ease of epithelial penetration. This hypothesis could also account for effects of prostaglandins on growth since prostaglandins are intimately associated with changes in membrane fluidity (Abraham and Hillyard, 1983). In addition, changes in plasma membrane fluidity could alter receptor numbers or availability, thus invoking a further potential regulatory step.

Other studies provide further evidence of the importance of diet in mammary growth. Welsch et al. (1985) fed mice diets varying in fat content, then observed growth re-

sponses to mammogenic hormones of glands in situ, as well as in glands removed and cultured in vitro. Level of dietary fat affected growth responses both in situ and in vitro, once again indicating a local effect on the mammary tissue. Graziano and Reece (1974) fed pregnant rats diets containing either highly unsaturated, or highly saturated fatty acids to study the effects of differing density of the fat pad on mammary growth. An additional aspect of this experiment involved increasing the size of the mammary fat pad through injections of insulin (previously shown to increase adipoctye size), and assessing the ability of the mammary gland to use the greater fat pad capacity for increased growth. Neither altered lipid composition nor increased fat pad size resulted in increased mammary nucleic acid content. This experiment was seemingly well-conceived, however it is quite possible that potential effects of these factors are limited to the prepubertal period of rapid ductal growth, and as a result no effects were observed during gestational growth.

In general, several points concerning the relationship between the mammary fat pad and mammary ductal growth seem clear: 1) growth of mammary epithelium in vivo requires presence of, and interaction with, white adipose tissue; 2) the extent of ductal growth within the mammary fat pad is limited by the size of the fat pad; 3) the composition of the mammary fat pad (or membrane fluidity) affects ductal growth, and in turn, fat composition may be altered by diet; and 4) changes in diet may also exert a systemic effect on mammary growth. These points, for the most part, are also likely true with regard to mammary growth in ruminants, however specific data are lacking.

Dietary Effects In Ruminants

Many studies in female ruminants have demonstrated that overfeeding during early development results in lower subsequent milk production and reduced mammary growth. Swanson (1960) grouped identical twin dairy heifers such that one of each pair was fed a normal ration while the other received a high concentrate diet to promote growth and fattening. Overfed calves had lower average milk yields through the first two lactations compared to controls. In addition, udder structure was abnormal in several of the fattened heifers. These results were confirmed by Little and Kay (1979), who also accounted for differences in age at breeding, and concluded that the dietary effect on yield was independent of breeding age. Similar observations have been made in beef heifers (Johnsson and Obst, 1984) and in ewe lambs (Umberger et al., 1985). In contrast, no difference in milk production due to differential feeding was noted in a study of dairy heifers in Australia (Bettanay, 1985).

Other recent studies on the effects of overfeeding and mammary growth focused on biochemical indices of udder growth, rather than simply upon milk production. Sejrsen followed up on his own finding that high planes of nutrition during the allometric growth phase inhibited subsequent milk production (Sejrsen, 1978) by measuring DNA content and composition of glands from heifers exposed to high or control planes of nutrition. The findings indicated that the negative effect of overfeeding was evident in reduced amount of secretory tissue and parenchymal DNA with no difference in mammary composition based on biochemical or histometric measures (Sejrsen et al., 1982). Feeding effects were confined to the prepubertal period, since plane of nutrition had no effect on growth during the postpubertal phase. These results were subsequently supported by Harrison et al., (1983). Johnsson and Hart (1985) demonstrated that a high level

of nutrition had essentially identical effects on mammary growth in ewe lambs. In accordance with findings in cattle, parenchymal growth was retarded by overfeeding during the prepubertal stage of allometric growth, while growth during later stages remained unaffected by diet.

Further research sought to define specific mechanisms which mediated this effect. In both sheep and cattle, growth hormone (GH) concentrations were significantly elevated in restricted-fed compared to overfed animals, suggesting an endocrine component to the dietary effect (Johnsson et al., 1985b; Sejrsen et al., 1983). Moreover, administration of GH stimulates prepubertal mammary growth (Sejrsen et al., 1986) and can override the negative effects of overfeeding (Johnsson et al., 1986). Thus, a convincing case may be made for GH as the primary mediator of diet-induced alterations in mammary growth. In spite of this, it would seem foolhardy to overlook the possibility of local effects on the mammary tissue itself, particularly in view of the evidence for such a route in laboratory species.

Other Factors Controlling Mammary Growth

Epithelial:Stromal Interaction: The interaction of mammary epithelium with the surrounding stroma is essential to the normal development of the gland (see Forsyth, 1971 for review). Early experiments (Kratochwil, 1969) demonstrated that mammary epithelium isolated from 12 to 14 day old mice failed to develop when cultured in the absence of mammary mesenchyme. Mammary rudiments co-cultured with salivary gland mesenchyme developed into glandular structures with the morphological appearance of salivary gland, while those exposed to mammary stroma developed in the typical

mammary pattern. Sakakura et al. (1976) demonstrated that while recombination of mammary epithelium with salivary stroma resulted in salivary morphology, the cytological appearance of the epithelium was that of normal mammary cells, and such explants were able to synthesize the milk protein alpha-lactalbumin. Thus, the mesenchyme is responsible for directing morphological development, but has no effect on cellular function.

The stromal compartment may also act as the target for endocrine influence on epithelial development. The androgen-directed destruction of the rudimentary epithelium in male mice requires the presence of mammary mesenchyme (Durnberger and Kratochwil, 1980). In addition, the mammary fat pad has been proposed as the initial site of estrogen influence on mammary growth (Shyamala and Ferenczy, 1984). It is apparent that the mammary stroma may function as a receiver and modulator of endocrine signals, which have their ultimate effects on the epithelial portion of the gland. Some evidence suggests that this modulatory effect operates in the opposite direction as well. Bartley et al. (1981) found that glycogen and lipid metabolism in mammary adipocytes from lactating glands is coordinated by the adjacent epithelial tissue. Fat depletion in adipocytes of lactating mice is also subject to local effects of neighboring epithelium (Elias et al., 1973). Thus communication between these compartments of the gland must be considerable, and serves an important integrative function.

The importance of a fat pad for supporting growth of transplanted mammary tissue has been described above (see Hoshino, 1962). Mammary tumors have been shown to exhibit preferential growth in mammary fat pads compared to subcutaneous sites (Miller et al., 1981). The general importance of the mammary fat pad in the etiology of breast cancer has been emphasized (Beer and Billingham, 1978). Furthermore, Levine and

Stockdale (1984) reported increased growth in cultures of dispersed mammary cells plated on a feeder layer of killed adipocytes compared to cultures plated on other cell layers or plastic.

Because the mammary fat pad plays a critical role in regulating mammary epithelial growth (Hoshino, 1962; DeOme et al., 1958; Mayer and Klein, 1961), it is logical that changes in the character of the fat pad might be reflected in altered epithelial growth patterns. At the simplest level, absolute amount of fat pad is thought to be limiting to the extent of ductal growth (Williams and Turner, 1961; DeOme et al., 1958; Richardson, 1953), which in turn limits the overall size of the parenchymal portion of the gland (Knight and Peaker, 1982). A more complex relationship is evident in the case of rapidly- reared ruminants, in which the amount of fat pad is in excess but the ductal epithelium fails to penetrate and does not entirely fill the fat pad (Johnsson and Hart, 1985; Sejrsen et al., 1982; Swanson, 1960). It seems likely that some characteristics of this "excess" fat make it a relatively poor substrate for ductal penetration.

Membrane fluidity of adipocytes has been postulated to have an influence on regulation of ductal growth (Carrington and Hosick, 1985; Wicha et al., 1979). This hypothesis, and the demonstrated stimulatory influence of unsaturated FAs on mammary growth suggest that high planes of nutrition may result in deposition of fat which is unsuitable in terms of membrane fluidity and/or FA composition, for ductal penetration. Following this reasoning, one might hypothesize that administration of GH overrides the negative effects of overfeeding by altering the character of the mammary fat pad. Thus, other methods of manipulating the properties of the fat pad might also have stimulatory effects on ductal growth.

It is clear that the role of the mammary fat pad is, in a positive sense, to provide a malleable substrate suitable for ductal penetration. From a negative standpoint, the fat pad margins somehow signal the end of duct extension, and thereby constitute a limitation to further growth.

The particular properties of the fat pad which allow it to fulfill these functions are unclear. As previously suggested (Abraham and Hillyard, 1983; Carrington and Hosick, 1985) membrane fluidity may play a role. Size of adipocytes, numbers of adipocytes per unit area, or lipid content of fat cells in the fat pad may also impact on ductal growth. Such characteristics would likely be affected by changes in diet, thus linking diet to growth regulation. This type of regulation would presumably constitute a local, cell-cell interaction type of control.

Endocrine Effects On Growth: The endocrine system is another possible regulator of growth, in this case, exerting systemic control. Certainly many studies have demonstrated hormonal impact on mammary growth, most notably the GH studies cited above (Johnsson et al., 1986; Sejrsen et al., 1986). Ovarian steroids, primarily estradiol, have also been shown to regulate ductal growth (Knight and Peaker, 1982). It seems probable however, that estrogen does not act directly upon the mammary epithelium (Trentin and Turner, 1941). Also, studies have failed to detect GH receptors in mammary epithelial cell membranes (Akers, 1985), although no trials using prepubertal tissues have been reported. It is more likely that GH acts first upon liver or other tissues, stimulating production of somatomedins or growth factors which in turn could interact directly with mammary epithelia or adipose cells. Estradiol may operate through a similar, indirect mode since experiments using mammary tissue from rodents in vitro have failed to

demonstrate a direct effect on epithelial growth (Sirbasku and Kirkland, 1976). Furthermore, estrogens have been shown to induce production of growth factors specific for mammary tissue from uterus, kidney and liver in rats (Sirbasku, 1978).

Manipulation Of Fat Pad Composition

The character of the fat pad, at least in terms of FA composition, is relatively simple to manipulate in monogastrics. Fatty acids are absorbed essentially as fed, and are subsequently deposited in adipose tissues. Thus, changes in dietary fatty acids are reflected in FA composition of depot fat. In the ruminant, however, the situation is complicated by the nearly complete hydrogenation of FAs by microorganisms in the rumen (Palmquist and Jenkins, 1980). This means that the FAs that are absorbed from the gut and subsequently deposited in adipocytes are almost entirely saturated. If unsaturated FAs could be made available for deposition in adipose tissues (especially the mammary fat pad) of the ruminant, the potential for greater mammary growth is easy to visualize.

Alteration of the FA composition of ruminant adipose tissues can be achieved by feeding protected fats or oils. Lipid droplets may be encapsulated with formaldehyde-treated protein, which protects the lipid from ruminal hydrogenation (Scott et al., 1971). Upon passage into the small intestine, acidic pH causes dissociation of the particles and release of the lipid. Unsaturated FA are then absorbed into the circulation as in the monogastric. This method has been widely exploited in studies attempting to alter the lipid composition of meat or milk in production animals. For example, Hood and Thornton (1976) reported that feeding a diet including 30% formaldehyde-treated sunflower seed to sheep increased the percentage of linoleic acid (18:2) in various adipose

tissues by about 10-fold. Other unsaturated FAs were also increased in adipose tissues to a similar degree. Although composition of the mammary fat pad was not determined per se, the pattern of 18:2 levels between different adipose tissues was similar, so qualitatively similar compositional changes would be expected in the mammary fat pad. In another study, feeder lambs were fed a protected lipid supplement as 40% of their diet (Garrett et al., 1976). Weight gains between control and supplement- fed lambs were similar, although feed intake was slightly (but significantly) depressed in the supplemented group.

The essential finding, in light of the present study, was the substantial increase in linoleic acid (18:2) concentration of subcutaneous fat of supplemented lambs. Fat of control lambs averaged 3 to 4% linoleic acid, while in supplemented lambs the concentration reached 20% after 10 wk of feeding. The major change in FA composition of lamb fat was an increase in linoleic, with decreases in palmitic (16:0), palmitoleic (16:1) and oleic (18:1) acids. These results suggest that use of such dietary manipulations should result in a marked increase in concentrations of unsaturated FAs in the mammary fat pad, which could promote ductal penetration and mammary growth. A particularly exciting aspect of this technique is that linoleic acid (18:2) percentage is dramatically increased in adipose tissue. This same FA was associated with maximal increases in mammary growth upon addition to cultures of rodent mammary tissue (Wicha et al., 1979). Thus the probability of obtaining increased mammary growth in response to feeding protected sunflower oil appears to be quite favorable.

Summary

Regulation of mammary growth during the prepubertal period is poorly understood, yet growth during this period is a critical determinant of mature gland size, epithelial cell population, and ultimately milk production (Sejrsen et al., 1986). The common denominator as far as growth control is concerned, appears to be the mammary fat pad, and the stromal:epithelial interactions in which it participates. For this reason, the present study is designed to investigate control of duct growth during the prepubertal period with particular emphasis on the effects of different characteristics of the fat pad or growth- matrix.

MATERIALS AND METHODS

Animals

Management: Forty early-weaned crossbred ewe lambs were purchased from a commercial farm at an average age of 45 days (range 30-53 days). Ten lambs were randomly assigned to each of four dietary treatment groups and groups were balanced for mean age and weight. The first group (R) was fed a high-energy total mixed ration (also referred to as standard diet), but intake was restricted to achieve approximately 120 grams per day liveweight gain. The second group (A) received the same ration, ad libitum, in order to reach weight gain of at least 240 g/d. The third group (G) was fed similar to AL, but lambs received daily injections (sc) of bovine growth hormone at a level of .1 mg/kg bodyweight. The final group (S) was fed a diet containing 30% formaldehyde-protected sunflower seed supplement, ad libitum. Composition of diets is summarized in Table 1. Diets were formulated to be approximately isonitrogenous and isocaloric. Digestible energy of each diet was estimated at 3.70 mcal/kg, and crude protein was 18.65% for the high-concentrate diet and 18.85% for the supplement diet.

Table 1. Composition of Lamb Diets[a]

Ingredient	Diet	
	Control	Protected Fat
Corn, ground (IFN 4-02-931)	69.0	40.0
Alfalfa hay, ground	10.0	25.0
Soybean meal (IFN 5-04-604)	21.0	5.0
Supplement[b]	0.0	30.0
Dicalcium phosphate	0.5	0.0

[a]percent dry matter basis
[b]formaldehyde-protected sunflower seed supplement

During the first week after arrival at the facility, all lambs were fed a pelleted calf-starter ration, with unlimited access to a mixture of alfalfa and orchard grass hay. In the second and third weeks, lambs were grouped according to diet, and fed only the appropriate total mixed ration, supplemented with hay as above. Beginning the fourth week, each group was switched to the complete ration, with no additional hay. Thus, diets were fed from about 7 to 22 weeks of age. Lambs had unlimited access to water through nipple-type automatic waterers. Animals in groups AL, GH and SS (ad libitum intake) were group-fed using self-feeders. Daily feed allowance was sufficient to assure at least 10% refusal. Refused feed was weighed-back and replaced with fresh feed daily. Animals in group R (restricted intake) were fed individually and feed allowance was adjusted weekly based on weekly weight gain.

The formaldehyde-protected fat supplement was mixed using the method of Palmquist and Moser (1981), but modified to include hammer-milled sunflower seed instead of milled soybeans. The supplement averaged 45% dry matter, and the complete ration was 67% dry matter. Dietary energy of the supplement mix was estimated from the analysis of Garrett et al. (1976), and this value (DE = 4.53 kcal/g) was used to calculate the final energy content of the complete diet.

Lambs were housed indoors in pens with expanded-metal mesh floors. Temperature was maintained at 25 C, and lighting was continuous. All lambs were fed once daily, at about 0900 hrs. Lambs were subjected to standard management practices, including vaccination, worming, vitamin E/selenium supplement etc.

Growth Hormone Injection: Lambs in the GH group received single daily injections (sc) of recombinant bovine somatotropin in saline (American Cynamid; lot # AC

4891-155-4). Dosage was .1 mg/kg bodyweight/day, and dose was adjusted weekly. Injections were administered immediately after feeding each morning (0900 hr).

Lamb Mortality: Two lambs died early in the study and did not contribute to final measurements other than blood sample and weight data which were included up to the week prior to death. Cause of death was diagnosed as polio encephalomalacia (thiamine deficiency) in the case of lamb #21 (AL group), and lamb #37 (GH group) died of bloat due to grain overload.

Four other lambs, all from the GH group, died near their expected slaughter dates. Age at death for these lambs was 126, 133, 131, and 144 days, respectively. Udders were removed from these lambs and mammary size and composition data were included in the data set, along with blood and weight data. No measurements requiring live tissue were performed on these lambs. Each of these lambs died suddenly, three of the four in a 4 day span and the fourth 3 days later, with little or no previous indication of poor health. The suspected cause of death in these lambs was calcium deficiency, based on subnormal serum calcium concentrations, and some improvement following i.v. calcium therapy in the final two lambs that died. Because of the sudden onset of symptoms, these lambs were treated too late for complete recovery, and were thus killed when symptoms recurred. Other lambs in this group were carefully observed for signs of distress but appeared perfectly healthy, and did not display any indication of physical problems.

Slaughter Protocol: The trial was terminated when lambs reached approximately 22 wk of age. Lambs were stunned with a blow to the head, and exsanguinated immediately. The mammary glands and liver were removed within 5 min of death. Udders were di-

vided into right and left halves by dissecting down the median suspensory ligament. Right halves were immediately frozen (-20 C) for later preparation of gland slices. Left halves were sliced open and parenchyma quickly removed and placed into warm (37 C) medium 199 (Gibco) while being diced into explants for thymidine incorporation studies. The remainder of these halves were trimmed of skin, and lymph tissue was removed. Parenchyma was separated from stroma, and each of these portions was frozen in .3 M sucrose at -70 C for later preparation of membranes, and nucleic acid analysis.

Approximately 20 g of liver was frozen in .3 M sucrose at -70 C for later preparation of liver membranes.

Measurements

Body Weight: All lambs were weighed weekly.

Blood Sampling: Beginning on the fifth week of the study, two blood samples (0800 hrs, and 1600 hrs) were collected on one day per week via puncture of the jugular vein. Lambs had been on experimental diets for one week when sampling began. Sera were harvested, and were frozen (-20 C) until assayed.

Hormone Assays: With the exceptions noted below, all pituitary hormones were obtained from the National Hormone and Pituitary Program (NHPP, Baltimore, MD). Hormones supplied by NHPP included: 1) bovine growth hormone (bGH-18) and ovine growth hormone (oGH-3) used in radioimmunoassay (RIA) for growth hormone in se-

rum, 2) ovine prolactin (oPRL-15) used as unlabeled competitor in assay of lactogenic hormone receptors in liver and adipose tissue membranes, and 3) human growth hormone (hGH-I-1) used as tracer in assay of lactogenic hormone receptors in all tissues measured.

Insulin concentrations were determined by double-antibody RIA using specific antisera purchased from Miles Laboratories (Lot GP20; Elkhart, IN). All samples were run in duplicate in a single assay. Intraassay coefficient of variation was 11.6%. Highly purified bovine insulin (lot No. 615-70N-80; 26.6 U/mg) was a gift from Lilly Research Laboratories (Indianapolis, IN 46285). Proinsulin and glucagon content were less than 0.001%. The insulin was iodinated using the procedure described by Bolt (1981) in his assay for FSH. Used at a dilution of 1:8000, the antiserum bound 50% of labeled insulin and approximately 90% when used at 1:500 dilution. Trichloroacetic acid precipitated 92 to 96% of radiolabeled insulin. The same highly purified insulin was used as a standard and reliable estimates of insulin activity were obtained over the range of 100 to 3200 pg/assay tube. Standard added to a pool of ovine serum was recoverable and inhibition curves parallel with the standard curve were obtained with assay volumes between 50 and 300 ul of serum. Highly purified bovine glucagon (Lot 258-25J-120; also obtained from Lilly Research Laboratories) showed no cross-reactivity in the assay. As a physiological test of the assay, four lactating ewes were given iv injections of glucose (0.1 g/kg bodyweight) after a 30 h fast (N = 2), or after control feeding (N = 2). Fasting decreased basal serum insulin concentrations (900 vs 1750 pg/ml) and glucose infusion increased insulin concentrations markedly in fasted ewes (peak 2850 pg/ml).

Concentrations of ovine growth hormone were determined by RIA as described (Barnes et al., 1985). Samples were run in duplicate in a single assay. Intraassay coefficient of

variation was 9.4%. The antiserum was essentially 100% cross-reactive with bovine or ovine growth hormone. Pools of both ovine and bovine serum produced inhibition curves parallel to the standard curve and added ovine or bovine growth hormone was recoverable (96-104%).

Serum calcium concentrations were determined using a Novaspec calcium analyzer.

Hormone Binding Assays: Insulin and growth hormone receptors in liver and mammary adipose tissue and lactogenic (prolactin-like) hormone receptors in liver and mammary parenchyma were assayed using crude membranes prepared by tissue homogenization and differential centrifugation as described by Akers and Keys (1984). Details of the assay for lactogenic hormone receptors were as described except the homogenization and incubation buffers included 100 KIU/ml aprotinin (Sigma Chemical Co., St. Louis, MO) and 1 mM PMSF (phenylmethylsulfonyl fluoride; Sigma). Protein content of membrane preparations was determined by dye-binding assay (BioRad) using IgG as standard. Lactogenic hormone receptor sites were quantified using human growth hormone as both iodinated tracer and unlabeled competitor in mammary membranes, but ovine prolactin was used as unlabeled competitor in assay of liver membranes. Recombinant methionyl human growth hormone (lot P-98492) was used as unlabeled competitor in assay of mammary lactogenic hormone receptors and was a gift from Lilly Research Laboratories.

Assays for insulin binding were conducted essentially as described by Oscar et al. (1986). Highly purified bovine insulin was a gift from Lilly Research Laboratories (lot # 615-70-80). Insulin was iodinated and separated from free iodine as described for hGH (Akers and Keys, 1984). Iodinated insulin (approximately 50,000 cpm) was incubated

with approximately 600 ug of membrane protein for 48 h, at 4 C, in a final volume of 0.6 ml of receptor buffer as described above.

Individual assays for all receptors were conducted in triplicate in the absence (total binding) or presence (non-specific binding) of 1 ug of competitor.

Measurement of growth hormone receptors was conducted as reported by Haro et al. (1984). Highly purified recombinant bovine somatotropin (a gift from American Cyanamid Company, Princeton, NJ; lot 6958C-42A) was used as both tracer and competitor.

Lipid Extraction: Duplicate samples of about 1 g of adipose tissue or mammary parenchyma were weighed and homogenized in 18 ml of chloroform:methanol (2:1;v/v) in 30 ml glass test tubes with teflon-lined screw caps. Homogenization was performed with a Brinkmann Polytron homogenizer, model PCU-2-110, using the PT 10/35 generator. Six milliliters of .7% NaCl were added and the tube was inverted several times to mix the contents. Tubes were centrifuged at 260xg for 10 min and the upper, aqueous layer was aspirated and discarded. Duplicate 5 ml aliquots of the organic layer were pipetted into tared 15 ml teflon-lined screw cap tubes and evaporated to dryness under a stream of N for fatty acid analysis, or under air for lipid content.

After determination of lipid weight, samples for fatty acid analysis were reconstituted with benzene. Methyl esters of fatty acids were prepared by adding 2 ml benzene (containing 8-20 mg of lipid) to 2 ml of methylation reagent (12% boron trifluoride in methanol) and boiling for 30 min, essentially as described (Morrison and Smith, 1964). Reactions were stopped by addition of 1 ml water and shaking. Organic and aqueous

layers were allowed to separate overnight, and fatty acids were sampled directly from the upper (benzene) layer. Lipid extracts were maintained under an atmosphere of N between and after each manipulation of the samples.

Gas Chromatographic Analysis: The fatty acid composition of each sample was determined by flame ionization detection on a Varian 6000 gas chromatograph. A 180 cm, coiled stainless steel column (approximately 3.2 mm ID) packed with 10% SP-2330 on 100/120 Chromosorb WAW (Supelco, Inc., Bellefonte, PA) was used. The carrier gas was N at a flow rate of 25 ml/min. The injection port temperature was 235 C, and the injection volume was .5 microliter. The column temperature was maintained at 200 C, isothermic, and the detector temperature was 250 C. Peak identities were determined from known reference standards (RM-6, Supelco). Areas under each peak were integrated using a Varian model 4270 integrator. Each fatty acid is reported as a percentage of the total area of quantified peaks.

Mammary Gland Measurements: Frozen udder-halves were cut into slices of about 5mm thickness using a meat slicer. Slices were made in a parasaggital plane, progressing from the medial aspect to the lateral-most portion of the gland. Slices were photographed, then trimmed of skin and lymph tissues removed. Gland slices were separated into parenchymal and extraparenchymal (fat) portions which were then pooled for each lamb. Each portion was minced and aliquots were used for determination of dry matter and lipid content in each compartment.

Negatives of photographs of each slice were projected onto preweighed sheets of paper using a photographic enlarger. Images of the entire gland surface and the area occupied

by parenchyma were traced on the paper. Gland tracings were cut out and weighed. Total area and area occupied by parenchyma for each slice were calculated and were corrected for apparent magnification. Briefly, weight of the traced area was converted to area, using a factor derived from the wt/unit area from the whole sheet. Total volume and parenchymal volume for each slice were calculated by averaging the area of each face of a slice and multiplying by the measured thickness of the slice. For slices in which parenchyma was visible in one face only (i.e., parenchymal border ended within the slice), parenchymal volume was estimated by averaging the area of the visible tissue with 1/2 of that area, and multiplying by 1/2 the slice thickness. Overall volume (total and parenchymal) of each gland was reconstructed by summing the volume of all slices.

Thymidine Incorporation: Thin tissue slices (approx. 2 cm^2) were obtained from the interface representing the furthest penetration of parenchyma into the fat pad and placed immediately into medium 199 (Gibco) at 37 C. Explants (approx. 2-4 mm^3) were prepared and approximately 200 mg of explants were placed into vials containing 2 ml medium 199 with 4 uCi/ml 3H-thymidine (Amersham; S.A. 45 Ci/mM). Vials were incubated for 2 h in a shaking water bath (GCA Precision) at 37 C. At the end of the incubation, medium was aspirated and explants were rinsed twice with .3 M sucrose, then frozen at -20 C. Approximately 4 explants were removed after rinsing, blotted briefly, and weighed, then transferred into fixative for subsequent preparation for autoradiographical analysis. The frozen explants were subsequently homogenized (about 200 mg of tissue in a total volume of 4 ml) in phosphate buffered saline (PBS) buffer, and 250 ul samples were precipitated with 1 ml ice-cold TCA (10%, trichloroacetic acid), centrifuged at 1800 x g for 30 min and the supernatant aspirated and discarded. Pellets were resuspended in 7 ml scintillation cocktail (ACS II,

Amersham) and radioactivity was determined in duplicate samples using a LKB model 1219 Rackbeta liquid scintillation counter.

Aliquots of explant homogenates were also diluted in PBS buffer, and DNA content was determined by the dye-binding (Hoechst 33258 dye) fluorometric procedure of Labarca and Paigen (1980), using a TKO 100 DNA mini-fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Udder Biochemistry: Remaining udder halves were divided into parenchymal and extra-parenchymal portions. Each portion was minced and representative aliquots (.3 to .5 grams) were homogenized in PBS buffer for determination of DNA content (Labarca and Paigen, 1980). In addition, RNA content was determined in parenchymal portions, using the orcinol method as described by Tucker (1964).

Dry fat-free tissue (DFFT) was estimated by calculation. Total weight of the parenchymal portion of the gland was corrected for lipid and water content, yielding indirect estimates of DFFT.

Density was similarly determined by calculation. Calculated volume of each compartment of the gland was divided into the weight of that compartment, resulting in wt/unit volume.

Histological Analysis: Tissue explants (removed from thymidine incorporation incubations) were fixed in modified Karnovsky's fixative (Karnovsky, 1965) for 4 h, then stored in .1 M phosphate buffer, pH 7.3. Tissues were dehydrated through a graded se-

ries of ethanol, then embedded in HistoResin (LKB 2218-500 Historesin) embedding medium (LKB, Bromma, Sweden). Sections (about 1.5 um thick) were cut on a Porter-Blum MT-1 ultramicrotome and four to five sections mounted per glass slide. Each tissue block contained 3 to 5 explants, and sections were taken such that at least 2 explants appear in each slice (for 4 lambs only 1 explant appeared in a section). Sections were taken serially from 3 sites, about 60 microns apart.

Slides were processed for autoradiography by a method adapted from Kopriwa and Leblond (1962), and Traurig (1967). Briefly, slides were dipped in photographic emulsion (NTB2 nuclear track emulsion; Eastman Kodak), diluted 1:1 with water. After drying, exposure proceeded for 8 days, at 4 C. Autoradiograms were developed for 5 min using Kodak D-19 developer (Kodak), fixed for 5 min (Kodak fixer), rinsed, and allowed to air dry. Sections were stained by immersing slides in Azure II stain for 10 s, followed by 4 rinses in water. After drying, slides were evaluated by light microscopy.

Histological sections were evaluated by classifying 100 (or all available) labeled cells in each of 2 or 3 explants on one to 3 slides representative of each lamb. Cells were classified as epithelial, adipose, endothelial, fibroblast, or other (usually myoepithelial).

Further analysis included determining the percentage of labeled epithelial cells occurring in a band occupying the outer 2/3 of selected growing lobules. This measurement was intended to test the hypothesis that the majority of cell proliferation in treatments that stimulate expansion of parenchyma into the fat pad should occur at the peripheral margin of existing epithelial structures.

Statistical Methods

Dependent variables were analyzed using the general linear models (GLM) procedure of SAS (SAS, 1982). Linear regression of lamb bodyweight on time was used to estimate mean daily gain for each lamb. Regression coefficients were used to analyze treatment (diet) differences in rate of gain. Other measurements were analyzed for treatment effects by univariate analysis of variance. Orthogonal contrasts were used as a mean separation procedure. Contrast 1 compared group S mean to the average of means of groups A, G, and R, i.e., comparing effect of protected fat supplement to all other diets. Contrast 2 compared the mean of group R, to the average mean of groups A and G, i.e., comparing effect of restricted intake to ad libitum intake among lambs fed the standard ration. Contrast 3 compared the means of treatments A and G, i.e., evaluating the effect of growth hormone administration among lambs fed the standard ration, ad libitum.

Because the set of defined contrasts failed to compare some treatment effects that proved to be of interest, the Tukey procedure was used as an after test, to separate all possible pairs of treatment means. Level of significance was set at $P < .05$. The Tukey procedure is a conservative test, with much less power than orthgonal contrasts. For this reason, larger differences between means were required to achieve significance with the Tukey test, compared to the contrasts.

Hormone concentrations were analyzed separately for each weekly sample. Treatment means were separated by the Tukey procedure.

All measurements involving mammary gland size or composition are reported for the single gland analyzed, i.e., values for one udder-half.

RESULTS

Lamb Growth and Feed Intake

Least squares mean initial age and weight of lambs did not differ by treatment group (Table 2). Overall, lambs averaged 15.6 kg and 44.5 days of age at the initiation of the trial. Rates of gain for treatments A and G approached the target rate of 240 g/d. Likewise, group R gained only slightly less than the target of 120 g/d. By contrast, lambs in group S grew at a faster rate than planned, gaining 314 g/d, compared to the expected 240 g/d. Growth rates differed in the order S>A = G>R, as indicated by mean separation procedures (Tukey test). Growth patterns portrayed as the regression of bodyweight on age (week 1 on diet equals week 7 of age) confirm that the desired differ ences in rate of gain were achieved (Figure 1.).

Final age and weight are also shown in Table 2. Mean age at slaughter did not differ between groups A, R, and S, (P>.05), but treatment G final age was significantly less than both A and R. Growth hormone-treated lambs were actually slaughtered at a similar age to the other groups, however including the 4 lambs that died early (see

Table 2. Growth and Feed Intake of Lambs.

Measurement	Treatment group A	G	R	S	SE	Contrast[1] 1	2	3
Initial age (days)	43.5	44.6	47.4	42.5	1.8			
Initial weight (kg)	15.7	15.7	15.6	15.5	2.1			
Mean daily gain (g/d)	256[a]	264[a]	114[b]	314[a]	11	C	C	-
Final age (days)	157[a]	148[b]	160[a]	153[a,b]	2.5-2.7	-	A	A
Final weight (kg)	41.2[a]	42.8[a]	27.3[b]	46.0[a]	1.7-2.4	C	C	-
Total feed consumption (kg DM)	104.2	97.9	54.9	116.5				
Feed conversion ratio (kg DM per kg gain)	4.09	3.61	4.69	3.81				

[1]Contrast 1: S vs mean of A,G, and R.
2: R vs mean of A and G.
3: A vs G.

A,B,C Significance of contrasts:A=P<.05; B=P<.01; C=P<.001.

[a,b,c]Means bearing different superscripts within a row differ (P<.05).

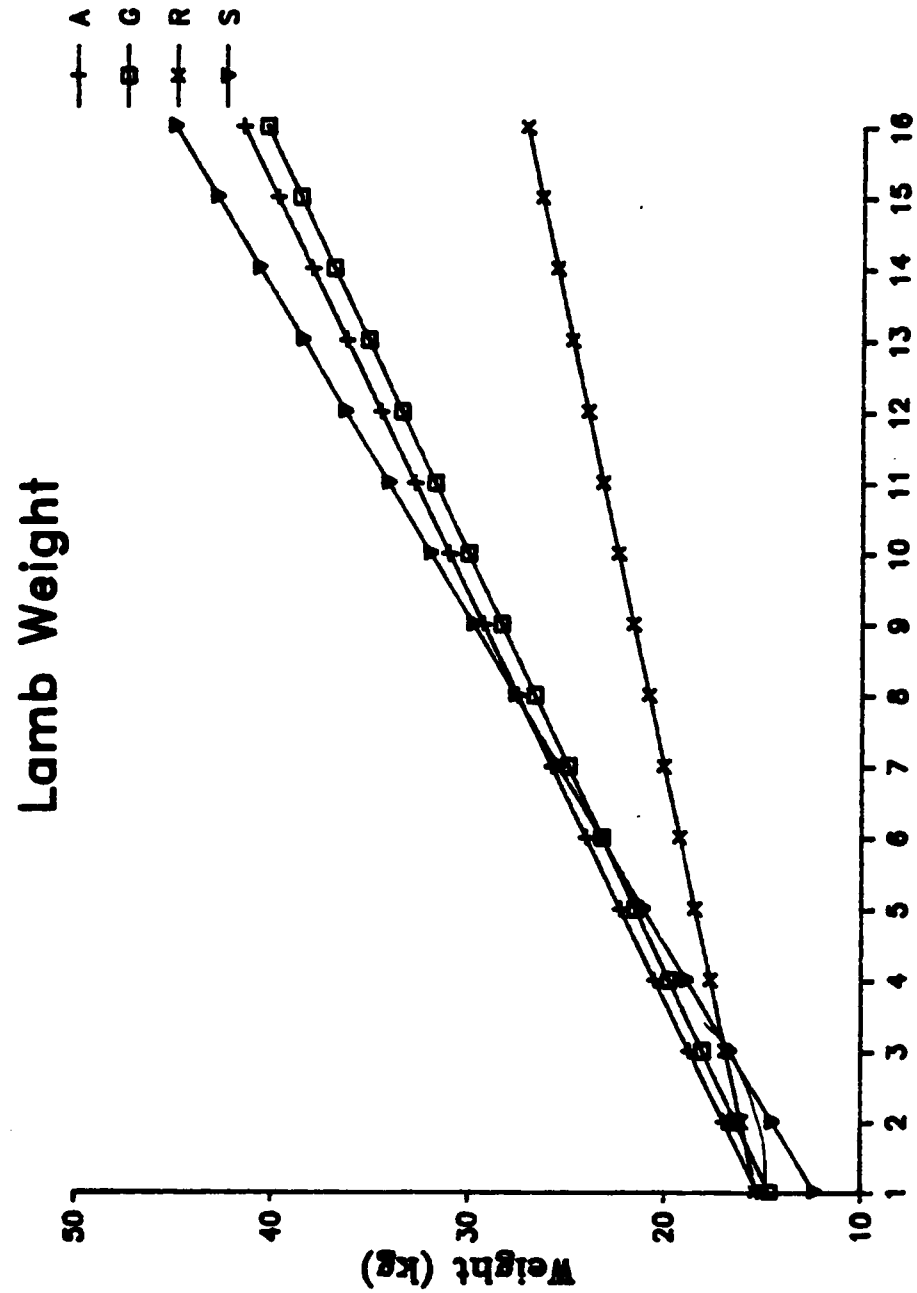

Figure 1. Regression of Lamb Weight on Age.

methods), reduced the mean age significantly. Mean weight at slaughter was similar for groups A, G, and S, each being greater than R ($P<.05$). The apparent lack of correspondence between final age and weight in the GH-treated group is due to excluding those lambs which died early, from the calculation of mean final weight.

Lambs receiving ad libitum feeding (A,G,S), were group-fed, thus total feed consumption per lamb was calculated as a group average rather than determined individually. This precluded statistical analysis of this measurement. However, feed consumption should logically follow the trends seen for rate of gain and final weight, (i.e., $S>A=G>R$), and absolute values appear to support this (Table 2). Because calculation of feed conversion ratio utilized group feed consumption means, statistical analysis was again impossible. Absolute values for feed conversion ratio are presented in Table 2. Treatments G and S would appear to favor more efficient utilization of feed, although firm conclusions are not possible without an estimate of variance.

Mammary Gland Composition

Least squares mean weight of the parenchymal portion of lamb mammary glands differed by treatment ($P<.05$; Table 3). Lambs from group S had significantly more parenchyma compared to the mean of the other 3 groups (25.6 vs 14.9 g). Comparisons of individual group means are depicted in Table 3. Mean weight of extraparenchymal (fat pad) mammary tissue was greater in S lambs than the average of the other 3 groups (97.7 vs 51.7 g; $P<.0001$), and group R had less fat pad than the mean of A and G (30.3 vs 62.4 g; $P<.01$). Growth hormone caused a reduction in weight of fat pad relative to

Table 3. Mammary gland composition in prepubertal ewe lambs.

Measurement	Treatment group A	G	R	S	SE	Contrast[1] 1	2	3
Parenchymal weight (g)	15.3^{ab}	15.1^{ab}	14.2^{b}	25.6^{a}	2.8-3.0	B	-	-
Fat pad weight (g)	76.4^{ac}	48.3^{ab}	30.3^{b}	97.7^{c}	7.1-7.5	C	B	B
Total gland weight (g)	91.7^{ac}	63.4^{ab}	44.5^{b}	123.4^{c}	9.0-9.5	C	B	A
%Parenchyma (by weight)	16.7^{a}	23.6^{a}	34.9^{b}	20.0^{a}	2.8-2.9	-	C	-
%Fat pad (by weight)	83.3^{a}	76.4^{a}	65.1^{b}	80.0^{a}	2.8-2.9	-	C	-
Parenchymal lipid (%)	51.4^{a}	38.9^{b}	49.6^{ab}	53.7^{a}	2.8-3.0	A	-	B
Fat pad lipid (%)	76.3^{a}	73.7^{ab}	69.0^{b}	76.7^{a}	1.6-1.7	-	B	-
Parenchymal DFFT[2] (g)	1.72^{a}	2.32^{ab}	1.32^{a}	3.10^{b}	.31-.33	C	-	-

[1]Definition and probability level of contrasts are described in Table 2.
[2]DFFT= dry, fat-free tissue.
^{a,b,c}Means bearing different superscripts within a row differ (P<.05).

lambs fed the same diet (G vs A; 48.3 vs 76.4 g; P<.01). Similar relationships were observed for total gland weight in each of the respective groups (Table 3).

Weight of parenchyma and fat expressed as a percent of total gland weight is also summarized in Table 3. Parenchymal tissue made up 16.7-23.6% of total gland weight in groups A,G, and S, but comprised nearly 35% of total in R lambs (P<.05 vs each other group).

Among lambs fed ad libitum (A,G,and S) lipid content (per cent) of parenchyma was significantly reduced by administration of growth hormone. Restricted lambs had an intermediate level of lipid, but did not differ from other groups. By contrast, per cent lipid of mammary fat pad did not differ significantly among high-intake groups, although lambs treated with growth hormone tended to have lower lipid percent. In addition, lipid content of extraparenchymal fat was depressed in R lambs relative to each other group (69.0% vs 73.7-76.7%; P<.05).

Mean parenchymal DFFT was greater in S lambs compared to the mean of the other 3 groups (3.10 vs 1.79 g; P<.001). Among lambs fed the standard diet (A,G,and R), restricted intake tended to reduce DFFT (R vs mean of A and G, 1.32 vs 2.02 g; P=.08; Table3).

Total nucleic acid content in each compartment of the udder is summarized in Table 4. Parenchymal DNA and RNA content did not differ significantly by treatment. In each case, however, lambs from groups A and R had a similar content of nucleic acids less than that of S, which in turn, was less than G. Although these differences did not reach significance, this may be more a reflection of the low number of animals in group G (due to mortality), and the choice of orthogonal contrasts, (which did not include a compar-

Table 4. Mammary gland nucleic acid content in prepubertal ewe lambs.

Measurement	Treatment group A	G	R	S	SE	Contrast[1] 1 2 3
Parenchymal DNA (mg)	12.6	24.1	10.4	18.8	4.1-5.7	
Fat pad DNA (mg)	38.6[a]	35.0[a]	15.6[b]	39.4[a]	3.4-3.6	A C -
Total gland DNA (mg)	51.2[a]	65.0[a]	26.0[b]	58.2[a]	5.6-7.9	- C -
Parenchymal RNA (mg)	25.8	44.4	24.0	36.4	6.9-9.8	
Parenchymal RNA:DNA ratio	2.05	2.01	2.46	2.15	.14-.21	- A -

[1]Definition and probability level of contrasts are described in Table 2.
[a,b]Means bearing different superscripts within a row differ (P<.05).

ison of S to the mean of A and R groups), than a lack of true difference between group means.

Total DNA present in mammary fat pad of restricted-fed lambs was approximately half of that measured in lambs fed ad libitum ($P<.05$; R vs each other group). High-intake groups had similar DNA content in fat pad (35.0 - 39.4 mg; $P>.05$). Total DNA in entire glands followed a pattern similar to that observed for the extraparenchymal portion of the gland (Table 4). The ratio of RNA to DNA in parenchymal tissue was elevated in group R lambs relative to lambs receiving the same diet at higher intake (2.46 vs 2.03 (mean of A and G); $P<.05$).

Concentrations of nucleic acids in mammary fat and parenchyma are presented in Table 5. Lambs receiving the protected-fat supplement (S) tended to have lower concentrations of DNA in parenchyma than those fed the standard diet (S vs mean of A,G, and R; .66 vs .87 ug/mg; $P<.10$). This relationship was also evident for fat pad DNA (.42 vs .69 ug/mg; $P<.05$). In addition, mean DNA concentration in G lambs was greater than in A, indicating an effect of GH administration (.82 vs .53 ug/mg; $P<.06$). Concentration of RNA in parenchyma was also lower in S lambs relative to the mean of all other groups (1.35 vs 1.85 ug/mg; $P<.05$).

Least squares mean volume of parenchyma (Table 6) was greater in mammary glands from supplement-fed lambs in comparison to the average of groups A,G, and R (22.3 vs 13.8 cm^3; $P<.01$). The same was true for fat pad volume (S vs A,G, and R; 141.7 vs 79.5; $P<.0001$), although individual group means for S and A were not different. Furthermore, volume of fat pad in glands from R lambs was lower than that of lambs fed the same diet ad libitum (R vs A and G; 49.3 vs 94.6 cm^3; $P=.001$), and GH reduced fat

Table 5. Mammary nucleic acid concentrations in ewe lambs[1].

Measurement	Treatment group A	G	R	S	SE	Contrast[2] 1	2	3
Parenchymal DNA	0.80	1.07	0.74	0.66	.10-.14			
Fat pad DNA	0.53[a,b]	0.82[a]	0.72[a,b]	0.42[b]	.10-.11	A	-	-
Parenchymal RNA	1.64	2.10	1.80	1.35	.19-.27	A	-	-

[1]Values reported in ug DNA per mg wet tissue weight.
[2]Definition and probability level of contrasts are described in Table 2
[a,b]Means bearing different superscripts within a row differ (P<.05).

Table 6. Mammary gland volume in prepubertal ewe lambs.

Measurement	Treatment group A	G	R	S	SE	Contrast[1] 1	2	3
Parenchymal volume (cm^3)	14.1[ab]	14.9[ab]	12.4[a]	22.3[b]	2.5-2.7	B		
Fat pad volume (cm^3)	114.9[a]	74.2[b]	49.3[b]	141.7[a]	9.3-9.8	C	C	A
Total gland volume (cm^3)	129.0[ac]	89.1[ab]	61.7[b]	164.0[c]	10.9-11.5	C	C	B

[1]Definition and probability level of contrasts are described in Table 2.
[a,b,c]Means bearing different superscripts within a row differ ($P<.05$).

volume compared to ad libitum without hormone (114.9 vs 74.2 cm^3; $P<.05$). Similar results were evident for mean volume of the entire gland (Table 6).

Density of tissues comprising the different compartments of the mammary gland were calculated by dividing the weight of a given portion by the volume of that portion. Mean parenchymal density (Table 7) tended to be greater in lambs receiving a limited intake compared to lambs on the same ration, ad libitum (R vs A and G; 1.17 vs 1.04 g/cm^3; $P=.07$). No effects due to feeding unsaturated fat (group S) or treatment with GH were apparent. Mean fat pad density, however, was increased by feeding protected fat compared to the average of the remaining 3 treatments (S vs A,G, and R; .69 vs .63 g/cm^3; $P<.05$). The decrease in mean density for R versus the average of A and G (Table 7) approached significance as well (.59 vs .65 g/cm^3; $P=.06$). Treatments had no effect upon least squares mean density of the entire gland.

Density of DNA in the parenchymal part of the gland did not differ by treatment ($P>.10$). Fat pad DNA density was reduced in S versus the mean of the other 3 diets (.29 vs .42 ug/cm^3; $P<.05$) in direct contrast to fat pad density by weight. Furthermore, lambs from group A had less DNA per unit volume of fat pad than G lambs (.35 vs .52 ug/cm^3; $P<.05$). Mean DNA density in the entire gland was again lower for group S versus the other 3 groups (.36 vs .50 ug/cm^3; $P=.05$), and tended to be increased in G over A ($P=.09$). Density of DFFT was not affected by feeding unsaturated fat. However, dietary restriction decreased DFFT density (R vs A and G; .11 vs .16 g/cm^3; $P<.05$), while administration of GH increased mean density of DFFT relative to A lambs (.20 vs .12 g/cm^3; $P=.01$).

Incorporation of ^{3}H-thymidine by explants of mammary parenchyma (Table 8) was highly variable among animals within treatment groups. Thus, no differences were ap-

Table 7. Mammary gland density in prepubertal ewe lambs.

Measurement	Treatment group A	G	R	S	SE	Contrast[1] 1	2	3
Parenchymal density (g/cm^3)	1.07	1.00	1.17	1.15	.06			
Fat pad density (g/cm^3)	.66[ab]	.64[ab]	.59[a]	.69[b]	.02-.03	A	-	-
Total gland density (g/cm^3)	.70	.70	.72	.75	.02			
Par. DNA density (mg/cm^3)	.85	1.11	.86	.77	.12-.17			
Fat DNA density (mg/cm^3)	.35[ab]	.52[a]	.40[ab]	.29[b]	.05-.06	A	-	A
Gland DNA density (mg/cm^3)	.41	.58	.50	.36	.06-.08	A	-	-
DFFT DNA density (g/cm^3)	.12[ab]	.20[a]	.11[b]	.15[ab]	.02	-	A	B

[1]Definition and probability level of contrasts are described in Table 2.
[a,b]Means bearing different superscripts within a row differ (P<.05).

Table 8. Tritiated thymidine incorporation by mammary explants.

Measurement	Treatment group A	G	R	S	SE	Contrast[1] 1	2	3
DNA concentration (ug/mg)	2.01	2.16	1.48	1.57	.18-.25	-	A	-
cpm per mg tissue	2149	2007	2169	2174	445-629			
cpm per ug DNA	1050	1112	1572	1441	314-444			

[1]Definition and probability level of contrasts are described in Table 2.

parent in mean incorporation expressed per unit of tissue or per ug of DNA (P>.10). Concentration of DNA in explants did not differ among the 3 high- intake groups, but was lower in the restricted group compared to the mean of A and G (1.48 vs 2.09 ug/mg; P<.05).

Hormone Concentrations

Profiles of serum GH concentrations during weeks 6-14 of the experiment are depicted in Figure 2. Concentrations did not differ between am and pm sampling periods except for lambs in group G (GH elevated in pm samples, injections made immediately after am bleeding), thus means for each sampling day are reported. Injection of GH increased GH concentrations significantly at each sample period (P<.05). Restriction of intake caused a non-significant increase in mean GH concentrations during weeks 6-9 (P>.05), but thereafter, had no effect on serum GH. Lambs from treatments A and S had lower GH concentrations throughout the study, and did not differ from one another (P>.05).

Mean serum insulin concentrations (Figure 3) were elevated in G lambs compared to groups R and S during the entire trial (P<.05). Lambs on treatment A displayed elevated insulin levels relative to groups R and S at weeks 6, 8, 9, 13 and 14 of the trial (P<.05). Animals in groups R and S had consistently lower circulating concentrations of insulin, and did not differ from each other at any sample period (P>.05).

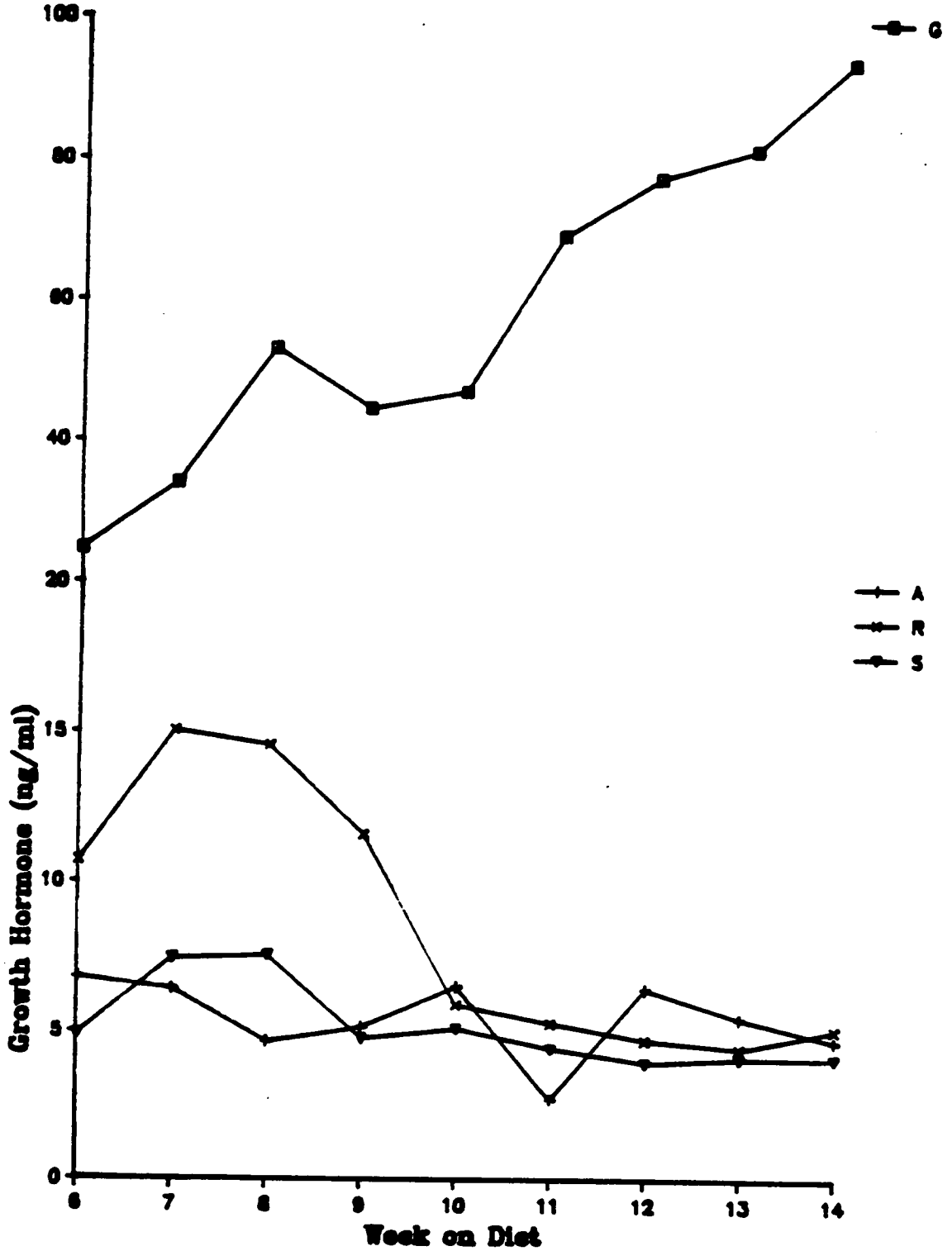

Figure 2. Serum Growth Hormone Concentrations in Lambs.

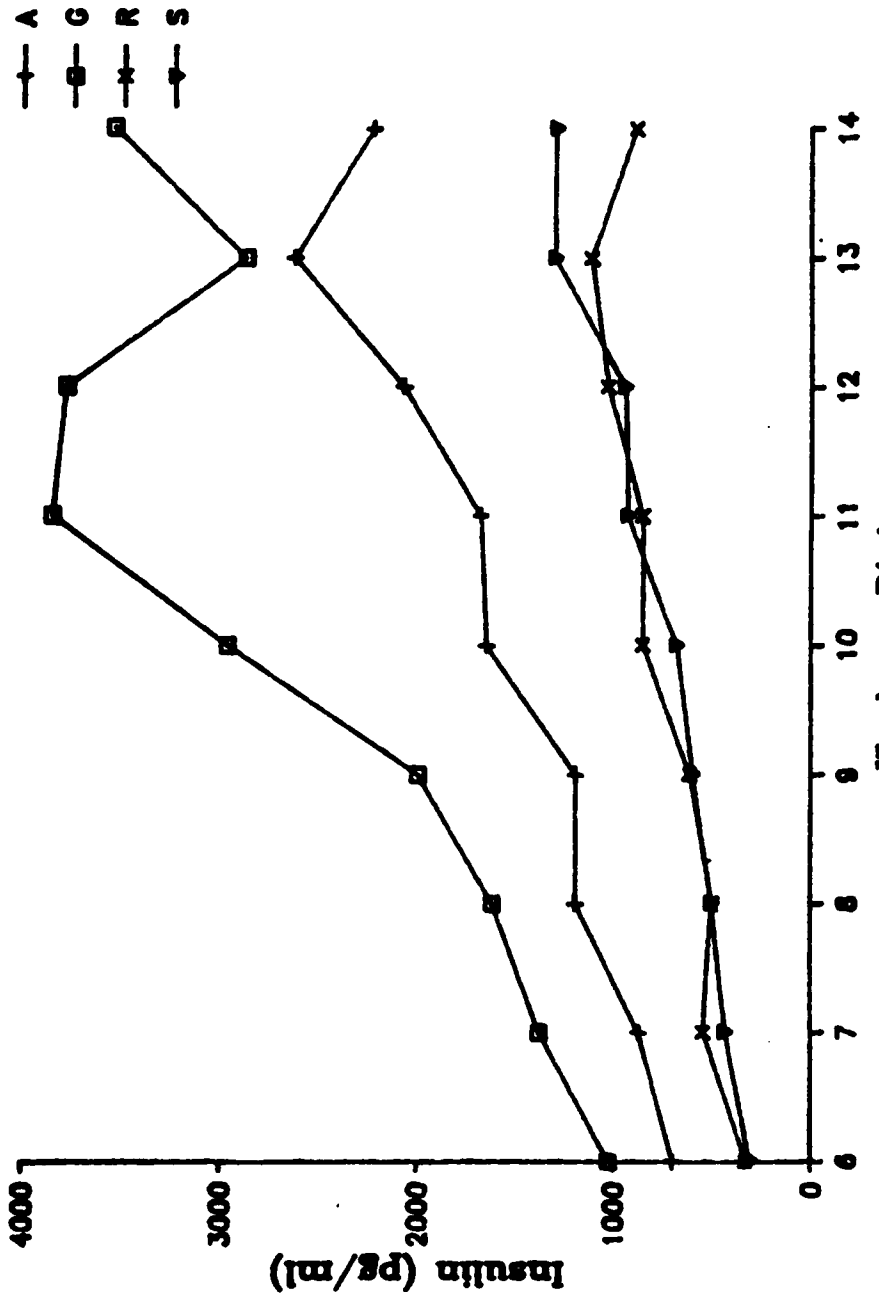

Figure 3. Serum Insulin Concentrations in Lambs.

Hormone-receptor Binding

Mean concentrations of prolactin receptors in crude membranes of mammary parenchymal tissue (Table 9) were increased by feeding the protected fat supplement (S vs average of A,G, and R; 29.9 vs 22.9 fmol/mg; P = .01). Prolactin receptor concentrations were virtually identical in each of the groups fed the standard ration. Insulin binding was not affected by treatment (Table 9).

In membranes prepared from mammary fat pad tissue, mean insulin receptor concentrations were increased nearly two-fold in R lambs compared to the mean of groups A and G (51.7 vs 28.3 fmol/mg; P = .01). Insulin binding was quantitatively similar among all groups fed ad libitum (A,G, and S). Mean number of GH receptors in extraparenchymal mammary tissue was not different among treatment groups (P>.10; Table 9).

Concentrations of receptor sites in liver membranes are also summarized in Table 9. Prolactin binding was similar across treatments (P>.10), but similar to mammary fat pad, insulin receptor numbers were elevated in lambs on restricted intake (R vs average of A and G; 40.3 vs 28.4 fmol/mg; P = .01). In contrast to fat pad, GH binding was elevated in hepatocyte membranes of S lambs compared to the average of lambs on standard diet (S vs A,G, and R; 11.2 vs 6.3 fmol/mg; P<.01), indicating an effect of unsaturated fat diet on liver, but not mammary fat pad GH binding.

Table 9. Hormone receptor concentrations in ewe lambs[1].

Measurement	Treatment group A	G	R	S	SE	Contrast[2] 1	2	3
Mammary parenchyma[3]								
Prolactin	22.9	22.8	22.9	29.9	2.2-3.1	B	-	-
Insulin	32.9	24.1	28.4	22.5	3.9-5.5			
Mammary fat								
Insulin	27.9	28.7	51.7	31.5	6.3-8.8	-	B	-
Growth Hormone	2.27	2.28	2.36	1.62	.46-.73			
Liver								
Prolactin	8.6	8.9	9.0	10.0	.8-1.0			
Insulin	27.4[a]	29.3[ab]	40.3[b]	30.8[ab]	3.2-4.1	-	B	-
Growth Hormone	5.7[a]	7.2[ab]	6.1[ab]	11.2[b]	1.4-1.7	B	-	-

[1]Values are reported in fmol/mg membrane protein.
[2]Definition and probability level of contrasts are described in Table 2.
[3]Growth hormone binding was undetectable in mammary parenchyma.
[a,b]Means bearing different superscripts within a row differ (P<.05).

Fatty Acid Composition of Mammary Parenchymal Lipid

Mean percentages of each class of fatty acid (FA) in lipid extracted from mammary parenchyma are shown in Table 10. Dietary-treatment had significant effects on each class of FA. Particularly dramatic effects were noted in groups R and S. Lipid from S lambs contained a lower percent of each FA in classes C14 - C16 compared to lambs fed the standard ration (S vs mean of other groups; $P<.001$). Of primary importance is the approximately two-fold increase in percent of linoleic (C18:2) acid in lambs fed the protected fat diet ($P<.0001$ vs other groups). This result confirms that the dietary manipulation caused the desired change in FA composition of mammary fat. Supplement-fed lambs also had a much higher proportion of unsaturated FA (of all classes) in lipid than the other treatment groups ($P<.0001$). Average FA percent in classes C18:0 - C18:2 was different in lambs from group R in contrast to those from A and G ($P<.001$). Restricted lambs also had greater mean total saturated FA percent compared to A and G ($P<.0001$). Growth hormone had no effect on the distribution of FA in parenchymal lipid (G vs A; $P>.05$).

The major shifts in distribution of FA in lambs fed S appeared to be from saturated to unsaturated FA in general, and in particular, the increase in linoleic acid was compensated by decreased percent of myristic, palmitic and palmitoleic acids. Lambs on restricted intake had FA profiles favoring deposition of saturated FA. Especially evident was the increase in percent of FA C14 - C18:0, with compensatory decreases in the unsaturated FA of the C18 class.

Table 10. Fatty acid composition of mammary parenchymal lipid[1].

Fatty Acid	Treatment group					Contrast[2]		
	A	G	R	S	SE	1	2	3
Myristic (C14:0)	4.0[ab]	4.9[a]	5.3[a]	3.3[b]	.3-.4	C	-	-
Palmitic (C16:0)	24.5[a]	23.8[a]	25.8[a]	18.7[b]	.6	C	A	-
Palmitoleic (C16:1)	2.5[ab]	3.2[a]	3.3[a]	1.6[b]	.2-.3	C	-	-
Stearic (C18:0)	17.5[a]	17.1[a]	21.4[b]	16.9[a]	.8	-	C	-
Oleic (C18:1)	45.9[a]	45.9[a]	40.5[b]	48.2[a]	.7	C	C	-
Linoleic (C18:2)	5.2[a]	5.0[a]	3.6[b]	11.3[c]	.3	C	C	-
Linolenic (C18:3)	0.4[a]	0.1[ab]	0.1[ab]	0.0[b]	.09			
Total Saturated FA	46.1[a]	45.8[a]	52.5[b]	38.9[c]	.8-.9	C	C	-
Total Unsaturated FA	53.9[a]	54.1[a]	47.5[b]	61.1[c]	.8-.9	C	C	-

[1]Values reported are percent of total fatty acid.
[2]Definition and probability level of contrasts are described in Table 2.
[a,b,c]Means bearing different superscripts within a row differ (P<.05).

Serum Calcium Concentrations

Least squares mean concentrations of calcium in serum from lambs of each treatment group are summarized in Table 11. Values presented represent the mean of blood samples taken during weeks 6-14 on diets (weeks 12-20 of age). Lambs fed ration S had the highest concentrations of calcium, while R lambs had higher mean levels compared to A and G ($P<.05$). Normal levels for serum calcium concentrations in sheep range from 8-12 mg/dl (Merck Veterinary Manual), thus groups S and R were in the normal range, while groups A and G exhibited subnormal concentrations. As previously discussed (see methods) lamb mortality in group G may have been related to calcium deficiency. It is curious that group A had similar calcium levels to group G, but did not incur an increased mortality rate, implying an increased sensitivity to low blood calcium in GH-treated lambs.

Autoradiographical Analysis

The distribution of labeling in cells classified as epithelial, fibroblast, endothelial, or other, is shown in Table 12. Variation attributed to treatment groups was quite low, with only 1-4% of the total variance accounted for by treatment. As a result, percent labeling of each cell type did not differ by treatment. It is clear, however, that dividing cells were predominantly of the epithelial class.

Proportions of labeled epithelial cells observed to be in the outer margin or interior mass of defined mammary lobules did not differ by treatment ($P>.05$; Table 13). In all cases

Table 11. Serum Calcium concentrations in prepubertal ewe lambs.

Measurement	Treatment group A	G	R	S	SE	Contrast[1] 1	2	3
Calcium (mg/dl)	7.19[a]	7.12[a]	8.64[b]	10.05[c]	.14-.15	C	C	-

[1]Definition and probability level of contrasts are described in Table 2.
[a,b,c]Means bearing different superscripts within a row differ (P<.05).

Table 12. Distribution of labeled cells in mammary explants[1].

Cell type	Treatment group A	G	R	S	SE
Epithelial	73.7	74.8	75.5	75.6	2.8-3.9
Fibroblast (CT)	18.4	17.6	16.1	15.6	2.1-3.0
Endothelial	6.2	6.0	6.1	7.2	1.0-1.4
Other (myoepithelial)	1.7	2.0	2.0	2.1	.6-.8

[1]Values reported as percent of all labeled cells counted per section. Treatment means did not differ (P>.05) for any listed variable.

Table 13. Location of labeled epithelial cells in growing lobules[1].

Location	Treatment group A	G	R	S	SE
Outside (%)	77.5	81.2	79.2	79.0	2.1-2.9
Inside (%)	22.5	18.8	20.8	21.0	2.1-2.9

[1]Values reported as percent of all labeled cells counted per lobule. Treatment means did not differ (P>.05) for any listed variable.

approximately 80% of labeled epithelium occurred in a circular band encompassing the outer 2/3 of a cross-section of a lobule.

DISCUSSION

It is well-known that overfeeding during the prepubertal period results in impairment of mammary growth in heifers. This reduction in growth is reflected in reduced milk yields in each subsequent lactation (Little and Kay, 1979; Sejrsen, 1978; Swanson, 1960). A similar relationship has been recently observed in ewe lambs (Umberger et al., 1985). A number of studies have been directed toward understanding this phenomenon, using biochemical analyses of mammary composition to estimate effects of diet on growth of specific tissue components in heifers (Harrison et al., 1983; Sjersen et al., 1982), and lambs (Johnsson and Hart, 1985). Effects of exogenous GH and/or relation with alterations in circulating concentrations of GH (Sjersen et al., 1986; Johnsson et al., 1985a,b; 1986), and photoperiod (Petitclerc et al., 1984) have also been studied. These latter studies showed that GH treatment could compensate for the inhibition of growth typically associated with high planes of nutrition, and that nutritional effects were not altered by photoperiod. However, little attention has been focused on the potential for local effects due to the ability of the fat pad to regulate mammary growth and morphogenesis. The primary objective of the present study was to determine effects of deposition of unsaturated fatty acids (UFA) in mammary fat pad on growth of

mammary parenchyma in prepubertal lambs. A second objective was to examine differences in mammary growth and morphogenesis, and specific endocrine measures in an attempt to gain insight into mechanisms by which restricted feeding or GH administration overcome the inhibition of prepubertal mammary growth characteristic of feeding high planes of nutrition. The treatments employed provided the desired differences in growth rate and FA composition to allow comparison of effects on mammary growth.

Lambs fed the protected UFA diet achieved the rapid rates of gain typical of those associated with retardation of prepubertal parenchymal growth. Although growth rate was higher for S than the other ad libitum fed groups (A_1 and G) final weight did not differ among groups (Table 2). This effect is explained by noting that rate of gain is expressed as the linear regression of weight on time. Because S lambs apparently took slightly longer to become accustomed to the diet, the increased rate of gain did not result in significantly higher final weight. Rate of gain and final weight of groups A, G and R were similar to values obtained in previous studies on dietary effects on mammary growth in sheep (Johnsson et al., 1985a,b; 1986).

Indices of mammary growth support the contention that feeding UFA promotes parenchymal growth in lambs fed a high plane of nutrition. Lambs from group S had the largest udders, containing the greatest amounts of tissue in both parenchymal and fat pad compartments (Table 3). In addition, glands from S lambs contained the most parenchymal DFFT, and were next to highest in parenchymal DNA content (Tables 3 and 4). Although much research has been directed toward feeding UFA to ruminants in an effort to tailor the polyunsaturated fat content of meat to demands of human nutrition, the potential for stimulating mammary growth in ruminants by feeding UFA has not been previously investigated. Many studies in laboratory species suggest a

stimulatory role for UFA in mammary epithelial cell proliferation (Carrington and Hosick, 1985; Hosick, 1979; Wicha et al., 1979). The present findings support these results and extend them to at least one ruminant species.

Mean weight of the parenchymal component was similar among the 3 groups fed a standard diet, although large differences in extraparenchymal weight resulted in different whole gland weights (Table 3). Mean parenchymal content of DFFT and DNA were not statistically different among standard-fed lambs, though absolute values were considerably elevated in G lambs. These results are at odds with the general perception that high planes of nutrition inhibit prepubertal mammary growth. In considering groups A and R, however, these data are supported by two separate studies in lambs (Johnsson and Hart, 1985; Johnsson et al.,1986). In particular, whole gland weights were increased in overfed, relative to restricted intake lambs, but no differences were detected in parenchymal mass, DFFT, DNA concentration or total DNA. Apparent differences in parenchymal growth emerged only when data was expressed as percent of fat pad occupied (Johnsson and Hart, 1985; Johnsson et al., 1986), or when corrected for bodyweight (observed only in one of the two studies). The present data are not expressed on a bodyweight basis, but such an expression would increase values for R (by 50%) relative to A. This would result in absolute values being greater in R vs A, but would not achieve statistical significance. The relative occupancy of fat pad by parenchyma in R lambs was roughly twice that of A lambs (Johnsson and Hart, 1985; Johnsson et al., 1986), in agreement with the doubling observed in the current study (Table 3). It should be noted however, that in Johnsson's reports, absolute values for these measurements uniformly favored the restricted diet. This is in contrast with the present report.

Johnsson et al. (1986) also reported increased parenchymal growth in lambs fed a high plane of nutrition and treated with GH, compared to contemporaries not receiving hormone. Parenchymal weight and DFFT, and fat pad occupancy were elevated, while total DNA and DNA concentration did not differ significantly. In the present study, mass of parenchyma was nearly equal in A and G. Evidence of a growth-stimulatory effect of GH was seen in absolute increases in occupancy, DFFT, DNA and DNA concentration (Tables 3, 4, 5). As alluded to previously, statistical differences were likely precluded by the lower number of lambs in group G, caused by early mortality. The increase in total parenchymal DNA (absolute value) among lambs treated with GH in the current study was largely due to greater concentrations of DNA within the parenchyma, accompanied by less lipid in this compartment. Weight of parenchymal tissue did not differ between A and G. By contrast, Johnsson et al. (1986) observed that GH apparently acted to increase peripheral expansion of parenchyma (greater mass of parenchymal compartment in GH-treated lambs), without increased DNA concentration. The contrast between these studies suggests two different modes of action by GH in increasing parenchymal DNA.

Measurement of volume and density of different tissue compartments within the mammary gland (Tables 6 and 7) were intended to provide insight into possible differences in spatial architecture of the gland resulting from the various treatments. Volume of mammary tissue has been estimated by Sjersen et al. (1986) using computer-assisted tomography (CT). Results from this methodology are not strictly compatible with the present findings, since CT scans distinguish between parenchyma and stroma within the parenchymal compartment, whereas the dissection techniques employed in the current study cannot account for stroma embedded within the parenchymal component. Differences in volume occupied by parenchyma, fat pad, and entire gland (Table 6) largely

reflected differences in weight of these compartments. This implies that determination of weight gives a reasonable estimate of volume as well, as has been assumed in other studies.

Despite the close relationship between volume and weight, several inferences were changed by expressing weight on a volume basis (density, Table 7). The lack of difference in parenchymal density suggests that epithelial ducts penetrated fat pad to a similar extent in each group, assuming that greater penetration would by indicated by lower density values (more fat surrounding the epithelium). In addition, the similarity of parenchymal DNA density between groups suggests similar numbers of cells per unit volume. The increase in absolute value for DNA density in G lambs reflects the greater concentration of DNA in parenchyma for this group (Table 5); the inference being that G lambs had greater numbers of smaller epithelial cells than other lambs. This supposition is supported by the observation of increased mean DFFT density as well.

Also in support, Sjersen et al. (1982) found no difference in the composition of parenchyma based on biochemical and morphological measurements in overfed vs restricted heifers. By contrast, Johnsson et al. (1986) found lower proportions of epithelium to fat in restricted-fed lambs, although a similar, earlier study failed to demonstrate this effect (Johnsson and Hart, 1985). Abnormal duct enlargement in response to exogenous bovine GH in young lambs has been observed by Johnsson and Hart (1984). And the general impression of more dense ductular epithelium with less lumenal area in GH-treated vs control glands in prepubertal dairy heifers is given by the micrographs published by Sjersen et al. (1986), although their discussion downplayed any such effect.

Along similar lines, incubation of mammary explants in (^{3}H)-thymidine provided estimates of relative rates of cell division in tissue obtained from the periphery of the ductular penetration (interface of epithelium with fat pad; Table 8). This protocol also allowed for autoradiographical analysis to distinguish which cell types were most actively dividing (Table 12), and whether duct extension was indeed occurring by end-wise appositional growth (Table 13). Rates of DNA synthesis as estimated by (^{3}H)-thymidine incorporation did not differ between treatment groups. This result fails to support the hypothesis that treatments historically associated with increased mammary growth accomplish greater penetration of fat pad through greater rates of cell division in the peripheral ducts or terminal structures. It should be noted however, that extreme variation existed among lambs within each group. For instance, incorporation ranged from 74-2292, 399-2543, 53-4053, and 371-2526 cpm/ug DNA in groups A, G, R, and S, respectively. Reasons for this excessive variation are unclear, but one clear limitation of the measurement is that inferences are limited only to the time of incubation. Thus timing of the incubation relative to endogenous rhythyms controlling cell division, or relative to age at slaughter could explain such differences.

Morphometric analysis of the distribution of labeled cell types (Table 12) indicated no difference by treatment. Likewise, the proportion of labeled epithelial cells occurring in the outer 2/3 of defined growing epithelial lobules (Table 13) was similar across treatments. In fact, the degree of similarity across treatments in these characteristics is quite remarkable. The apparent conclusion is that the mode and qualitative characteristics of parenchymal growth patterns are not altered by dietary effects, even though these effects promote differences in total parenchymal growth. If growth proceeds in a similar manner within proliferative structures in all lambs, then one is forced to accept that differential growth is accomplished through increased numbers of these structures. An

alternative hypothesis would suggest that growth differences occur not by differences in end-wise appositional growth, but rather by changes in growth within the interior of long ducts which would then force the peripheral cells to penetrate the fat pad.

The essential features of hormonal profiles in the present experiment are the markedly elevated concentrations of GH in G lambs, and increases in insulin concentrations in G lambs, and occasionally in A lambs (Figures 2 and 3). Increases in GH concentration in injected lambs are consistent with those reported previously in a similar experiment (Johnsson et al., 1986), although the relative increases in GH (up to 20-fold) are larger in magnitude than previously reported (about 5-fold; Johnsson et al., 1986). Further, the continual increase in GH over progressive weeks of trial may indicate that metabolic clearance of GH does not increase as rapidly as bodyweight. By contrast, lambs receiving the same dose of GH (.1 mg/kg bodyweight/day) had similar GH concentrations at 12 and 18 weeks of age (Johnsson et al., 1986). The temporary increase in absolute concentrations of GH in R lambs (weeks 6-9 on diet) is in accord with similar studies in restricted vs ad libitum-fed lambs (Johnsson et al., 1985a), and with the elevation of GH in prepubertal, but not postpubertal heifers fed a restricted diet (Sejrsen et al., 1983).

Higher concentrations of insulin in serum following exogenous GH administration have been documented previously in sheep (Davis et al., 1970), and lambs (Johnsson et al., 1985b). Otherwise, insulin concentrations are generally positively associated with level of feed intake in ruminants (Bassett et al., 1974a,b), supporting the present findings with respect to groups A and R. In contrast, lambs fed S did not exhibit increased insulin profiles (figure 3), despite a high level of intake and dietary energy. Perhaps insulin is more responsive to dietary carbohydrate than fat. This could explain the difference in endocrine profiles, because delivery of energy in S lambs was in large part through pro-

tected lipid, whereas lambs fed the standard diet would presumably have absorbed higher levels of carbohydrates and VFA. In support, Palmquist and Moser (1981) found decreased concentrations of insulin in one of two experiments in lactating cows fed a protected fat supplement. The increase in insulin binding capacity in membranes from liver and mammary fat in R lambs compared to treatments A and G is consistent with the inverse relationship between circulating insulin concentrations and receptor numbers (Cowie et al., 1980). It is not clear why insulin binding remained relatively low in S lambs, in the presence of low insulin in blood. It is possible that receptor numbers change with energy balance of the animal (which would in most cases be reflected by blood insulin levels), rather than strictly with insulin concentrations.

Lambs fed the protected-UFA diet had higher mean concentrations of prolactin receptors in mammary parenchyma and GH receptors in liver compared to the average of lambs fed a standard ration (Table 9). It is tempting to speculate that these differences constitute some of the foundation for the positive effects on growth associated with this diet. In the case of prolactin, however, considerable evidence exists to show prolactin has little effect on mammary growth in ruminants (Johnsson et al., 1986; Sejrsen et al., 1983). On the other hand, the ability of prolactin to induce differentiation of mammary epithelial cells has been well-documented (Akers et al., 1981a,b; Goodman et al., 1983). It is possible that increased parenchymal binding of prolactin in S lambs may be related to the observation that epithelial cells from this group had a more "secretory" appearance (lipid droplets in cells and lumena, increased secretion in lumena) than cells from lambs on standard diets.

The increase in GH binding sites in liver of S lambs represents a more plausible pathway by which UFA diets might stimulate mammary growth. Growth hormone receptors are

undetectable in mammary parenchyma (present data and Akers, 1985). The general perception is that GH effects on the mammary gland are indirect, mediated through production of somatomedin C in the liver (Akers, 1985). Thus, increased hepatic GH binding could result in stimulation of mammary growth. A similar mechanism may explain the growth-promoting effects of exogenous GH. In one case, receptor numbers are increased, while in the other concentrations of hormone are elevated, both resulting in enhanced mammary growth.

Increases in numbers of receptor sites may result from increased membrane fluidity caused by increased UFA content as proposed by Carrington and Hosick (1985) and Abraham and Hillyard (1983). Several studies have shown that modification of membrane fluidity can indeed increase hormone binding (Bhattacharya and Vonderhaar, 1979, 1981; Dave et al., 1983). Such a mechanism may be responsible for increased binding in S lambs, but does not explain the lack of difference in GH binding in mammary fat (Table 9). Organ-specific differences in receptor numbers have been shown (Cowie et al., 1980), however, one might expect the effects of UFA to be rather ubiquitous, although differences in deposition of dietary UFA among different organs has been shown (Hood and Thornton, 1976).

Feeding protected UFA diets resulted in significant increases in UFA content of lipid in lambs (Garrett et al., 1976). The present results (Table 10) confirm this. Percent of linoleic acid in mammary parenchymal lipid was doubled in S lambs relative to lambs fed the standard ration. Nearly identical shifts were reported in mammary fat of lambs fed a protected fat supplement (Astrup and Nedkvitne, 1975). Among lambs fed control diets, ad libitum, FA profiles were similar those previously reported in lambs (Garrett et al., 1976). By contrast, FA profiles in R lambs differed from reported values. The

major shift in R was toward saturated FA, with lower values for UFA in the 18 carbon class, and an increase in percent of 18:0. Reasons for this shift, and possible implications for mammary growth are unclear, but if saturated FA are inhibitory to parenchymal growth in lambs as has been reported in rat mammary epithelial cell cultures (Wicha et al., 1979) then a possible explanation for the lower than expected growth in group R emerges. Previous studies on effects of plane of nutrition on early mammary growth in ruminants have not quantified FA, thus comparisons between studies are not possible.

The low calcium levels in lambs of groups A and G remain somewhat enigmatic. The calcium content of the standard ration was .38% (dry matter basis). This value falls short of the NRC recommendation for early weaned lambs (about .50%, dry matter basis), but is well within the range listed (.20 to .82%). Furthermore, at least one recent study on lambs receiving a similar ration reported no negative effects, although calcium content of the rations were .36 to .40% (Turgeon et al., 1986). The only speculation is that the combination of early weaning and rapid rate of growth left the lambs deficient in calcium. As noted earlier, the only evidence for low calcium was from the blood samples. Thus, if not for the mortality in group G, this effect would likely have gone unnoticed. The observation that the increase in mortality was confined to GH-treated lambs, although another group had similar blood concentrations of calcium, suggests that GH may have heightened the sensitivity to low serum calcium levels in these lambs.

In summary, lambs fed a ration containing 30% protected-UFA exhibited the most desirable mammary development, in terms of parenchymal mass, DFFT, and total parenchymal DNA, of the 3 treatments compared in this study. Results are consistent with the hypothesis that UFA are stimulatory to mammary growth, acting perhaps

through increased membrane fluidity. A more fluid membrane may allow for unmasking of hormone receptors, elaboration of lipid-derived stimulatory factors (e.g., prostaglandins), or increased penetrability of mammary fat pad. These and other postulated actions have been reviewed recently (Welsch, 1987). A further point in favor of treatment S is the increased amount of fat pad, and thus total gland. Although excessive fattening during the prepubertal period may limit parenchymal growth during this period, some evidence suggests that amount of available fat pad may be limiting to the ultimate size of the gland (Williams and Turner, 1961; Faulkin and DeOme, 1960). In the case of S lambs, not only is prepubertal growth maximized, but more unfilled fat pad remains for potential future expansion of the parenchymal component. A further point is that without the negative effects of rapid-rearing on mammary development, management practices to promote rapid growth may be adopted without endangering future production. Rapid-rearing of livestock has long been recognized as economically desirable, since females may be bred sooner and thus become productive at an earlier age, and with lesser investment in feed and housing costs.

These projections are certainly exciting, yet effects of this type of diet on milk production and reproductive traits must be evaluated before the utility of such feeding regimes to the animal production industry can be fully evaluated.

Mouse Study

Introduction

In ruminants, excessive energy intake during the prepubertal period leads to reduced future milk production (Swanson, 1960; Little and Kay, 1979; Umberger et al., 1985). This phenomenon is not well-understood, however alterations in circulating concentrations of growth hormone have been implicated as a potential factor (Sejrsen et al., 1983, 1986; Johnsson et al., 1985a, 1986). In laboratory species, effects of dietary fat levels and composition of dietary fat has been linked to regulation of mammary epithelial growth (Welsch, 1987; Abraham and Hillyard, 1983). Studies in rodents have led to the suggestion that local regulation of mammary duct growth, mediated by the mammary fat pad, acts in concert with a systemic, endocrine component to regulate mammary ductal growth (Carrington and Hosick, 1985; Welsch et al., 1985). One aspect of this local regulation appears to be the physical presence of a fat pad, since transplanted mammary epithelium requires the presence of white adipose tissue for survival (Hoshino, 1962), and growing ducts change their direction of growth when they en-

counter the margin of the mammary fat pad (Faulkin and DeOme, 1960). However, no studies have attempted to quantify growth of mammary epithelium transplanted into non-mammary fat pads. Thus, qualitative differences in ability of various fat pads to support growth of mammary ducts are unknown. Furthermore, the only attempt to determine directly whether the size of the mammary fat pad is limiting to parenchymal growth was conducted in sexually mature rats from just prior to, to day 20 of gestation (Graziano and Reece, 1974). By analogy to the situation in ruminants, the major effects of diet (and presumably size of fat pad) are exerted early on in mammary development, during the prepubertal period of allometric growth, whereas pubertal and gestational parenchymal growth are relatively insensitive to these effects.

Clearly, the size and qualitative characteristics of the mammary fat pad could exert regulatory effects on mammary ductal growth. Accordingly, the objectives of the present study were 1) to investigate the effects of size of fat pad on prepubertal mammary ductal growth in mice, and 2) to study growth patterns of mammary duct segments transplanted into ovarian or mammary (gland-free) fat pads. The primary goal of the study was to determine whether laboratory mice could provide a useful model for studying effects of diet on prepubertal mammary growth in ruminants.

Methods

Forty-five female mice (ICR albino white mice) were obtained from the colony maintained by the Department of Dairy Science, VPI&SU. Mice were housed, three or four per cage, under controlled temperature and lighting (22 C and 12 h light). Mice were weaned at 18 days of age, and were provided with standard laboratory chow in addition

to the powdered diets. This was to ease the transition to experimental diets since many mice had begun to consume the lab chow diet intended for their lactating mothers. After 21 days of age, mice were fed experimental diets exclusively. Mice were weighed weekly throughout the study.

Mice were divided into groups of 15 to be fed one of three powdered diets (Table 14). Diet C (control) was balanced to contain the standard caloric intake for mice, at normal intakes. The remaining diets contained roughly 20% increase (diet H) or 20% decrease (diet L) in caloric density. Diets contained identical proportions of protein, and differences in caloric density were accomplished by varying proportions of starch and sucrose to cellulose filler. Predetermined content of fat (5, 10 or 20%, for L, C, and H diets, respectively), also affected caloric density of diets. All components of diets were obtained from ICN (ICN Nutrition Biochemicals, Cleveland, OH). Diets were presented in stainless steel group-feeders (one per cage) designed to minimize fouling or loss of food. Mice were initially fed 2.9 grams/mouse/day of each respective diet. Food was replenished every 2 days, and any remaining food was weighed for correction of intake. Water was provided ad libitum. During the first 5 weeks of the trial, food refusals were negligible (< 2% refused). Thereafter, however, mice fed the high-energy diet refused food more frequently. In an attempt to maintain established weight differences between groups, feeding level was reduced to 2.75 grams/mouse/day for all diets. Mice in group H occassionally failed to consume all of their bi-daily allotment, but no further adjustments in feeding level were made.

Table 1. Composition of Mouse Diets[1]

	Diet		
Ingredient	Control[2]	High-energy[3]	Low-energy[4]
Casein	25	25	25
Starch/Sucrose	49	54	37.5
Corn Oil	10	15	5
Cellulose	10	0	26.5
Vitamin mix	2	2	2
Mineral mix	4	4	4
	100	100	100

[1]grams per 100 grams of diet, dry basis.
[2]3.86 calories per gram.
[3]4.51 calories per gram.
[4]2.95 calories per gram.

Surgeries

All surgical procedures were carried out using Metofane (methoxyflurane; Pitman-Moore, Inc., Washington Crossing, NJ.) as anesthetic. At 20-21 days of age, mice were anesthetized and the left 4th mammary fat pad was cleared of epithelium as described (DeOme et al., 1959). On the day prior to surgery, all mice were injected i.p. with .2 ml of .5% trypan blue in aqueous solution to aid in visualizing epithelium to be cleared (Hoshino, 1962). Following a one week recovery period, (at 28 days of age) mice received transplants of approximately 2 mm of primary lactiferous duct obtained from selected donors, essentially as described by Hoshino (1962). Donors were female siblings of the original group of experimental subjects. Donors were injected with a trypan blue solution on the day before surgery (as above). Approximately 8 mm of primary duct was removed from each of 2 donors, dissected into 3 to 4, 2 mm segments (about 2x2 mm total, since duct was accompanied by associated stroma), and placed into sterile holding medium (Medium 199, Gibco; Grand Island Biological Co., Grand Island, NY) at 37 C. Tissue was transplanted into cleared mammary fat pad and right ovarian fat pad of one mouse from each group, before the process was repeated for the next subset of mice. There was no mortality associated with surgical procedures.

Mice were killed in groups of 5 from each treatment at 8 and 18 weeks of age (two groups killed on the latter date). Of those killed at 18 weeks, half of the mice in each treatment group received daily injections sc of estradiol 17-B (1 ug) and progesterone (1 mg; both hormones from Sigma) in corn oil solution (.1 ml injection volume) over the 5 days prior to sacrifice.

At sacrifice, right and left ovarian fat pads and 4th mammary glands (right control gland; left implanted gland) were dissected out, weighed, and placed into modified Karnovsky fixative (Karnovsky, 1965). Mammary glands were whole-mounted on glass microscope slides prior to fixation, in the method of Strum (1979). All tissues were stained, dehydrated through a graded series of ethanol (to 100%) and stored in 100% ethanol (Strum, 1979).

Tissues were evaluated for presence of epithelium by viewing under a dissecting microscope. No mammary epithelium was apparent in any ovarian fat pad. Mammary gland whole-mounts were evaluated and photographed in 100% ethanol. Evaluation included overlaying whole-mounts on a grid (3mm divisions; under 7.5X magnification) and estimating area of the fat pad and percentage of area occupied by epithelium. The extent of parenchymal growth was estimated by visualizing an imaginary line connecting the tips of the furthest penetrating ducts and a second line around the entire fat pad. In addition, ductular growth was scored on a subjective scale from 1 (little branching or extension of ducts compared to 21 days) to 6 (gland essentially filled with multiple-branching ducts, and infilling with fine ducts between major ducts) as described by Wrenn et al. (1966) and Meites (1965). Cleared mammary glands (CFP) were evaluated for recovery of recognizable implants, and where recovered, implant growth was scored from 1 (indistinguishable from initial implanted mass) to 6 (growth to fill fat pad, and infilling with minor ducts).

Statistical Analysis

Dependent variables were analyzed using the general linear models (GLM) procedure of SAS (SAS, 1982). The model accounted for fixed effects of diet and treatment (age at sacrifice or hormone-treatment in groups killed at the same age), and their interaction. Treatment means were compared using orthogonal contrasts, the set of contrasts included: 1) comparison of the mean of the group killed at 8 wk to the mean of the two groups killed at 18 wk, and, 2) comparison of hormone-treated vs control group at 18 wk. For dependent variables that were significantly affected by diet, dietary group means were compared using Tukey pairwise comparisons (an option of the GLM procedure in SAS; probability level set at $P = .05$).

Results and Discussion

Visual evaluation of ovarian fat pads failed to detect the existence of implanted mammary duct segments in any instance. Thus the second objective of this experiment, relating to qualitative differences between different fat pads, could not be met. Explanations for this result are not readily forthcoming. Although it is possible that implants floated free from their site of placement in the fat pad, this seems unlikely since the placements appeared secure after implantation, and transplants into the mammary CFP were often successful. Hoshino (1962) successfully transplanted similar segments of mammary duct into various fat pads, including pararenal, retroauricular, and CFP, thus, a systematic defect in the transplantation technique is doubtful. A more plausible explanation is that some characteristic of the ovarian fat pad itself caused this site to be

hostile to implant survival. Or, perhaps the proximity of the transplant site to the ovary subjected the implants to an unfavorable endocrine environment and resulted in degeneration of the mammary tissue.

Characteristics of food intake and growth of mice are summarized in Table 15. Equivalent intake of diets differing in caloric density resulted in the desired changes in mean bodyweight of each diet group at 8 wk of age. Final bodyweight was increased by 7% in mice fed a high-energy diet, and mice receiving low-energy food were lighter by 21% on average, relative to control mice. Differences in calculated rate of weight gain were even more marked (18% above, and 45% below control rate for H and L, respectively).

However, between 8 and 18 wk of age the difference in bodyweight between groups H and C was no longer maintained, as mice fed the high-energy diet apparently compensated for caloric intake by decreasing their voluntary dietary intake. An attempt to counter this development by slightly reducing daily food allotment in all groups was unsuccessful as mice in group H continued to refuse a portion of food. Mice fed the low-energy diet continued to have lower average bodyweight (from 8 to 18 wk of age) relative to groups C and H.

Dietary-induced changes in bodyweight were of secondary importance in the present study, however. The primary goal was to induce changes in the size of mammary and ovarian fat pads, although such alterations would logically (but not necessarily) follow change in bodyweight. Effects of diet on mean weight of whole-body, implanted cleared mammary fat pad (CFP), intact mammary fat pad (MFP), implanted ovarian fat pad (ROFP), and control ovarian fat pad (LOFP) were all highly significant ($P<.0002$; Tables 16, 17). Effects of treatment were also highly significant for each of these variables ($P<.001$), although differences in individual treatment means were limited to the ex-

Table 2. Growth and Feed Intake in Mice.

	AGE					
	8 weeks			18 weeks		
Treatment	C	H	L	C	H	L
Initial wt	12.1	11.9	12.0	22.1	23.9	17.4
food/day	2.9	2.9	2.9	2.75	2.55	2.75
cal/m/d	11.2	13.1	8.6	10.6	11.5	8.1
change vs C	-	+17%	-23%	-	+8%	-23%
Final wt	21.9	23.5	17.4	28.4	27.1	22.5
change vs C	-	+7%	-21%	-	-5%	-20%
gain (g/d)	.28	.33	.15	.093	.047	.075
change vs C	-	+18%	-45%	-	-50%	-19%

a,bMeans within a row bearing different superscripts differ (P<.05).

Table 3. Weight of Body, Mammary and Ovarian Fat Pads, and Measures of Mammary Growth in Mice at 8 Weeks of Age

Measurement	Diet C	Diet H	Diet L	SE
Final wt	21.2a	23.1a	17.0b	.67
CFP wt	94.8a	105.8a	58.4b	8.5
MFP wt	136.4ab	150.8a	92.2b	11.7
ROFP wt	55.8a	89.4b	43.4a	10.3
LOFP wt	63.2a	109.8b	32.8a	8.3
% Full	44.4ab	64.6a	22.8b	7.1-7.9
Score	1.3	2.1	1.0	.30-.34
Implant recovery	5/5	3/4	5/5	
Implant score	3.0	2.3	1.4	.66-.86

a,bMeans within a row bearing different superscripts differ (P<.05).

Table 4. Weight of Body, Mammary and Ovarian Fat Pads, and Measures of Mammary Growth in Mice at 18 Weeks of Age

	Treatment							
	Hormone				No Hormone			
	Diet				Diet			
Measurement	C	H	L	SE	C	H	L	SE
Final wt	29.0	27.2	23.5	1.5	27.8a	26.9a	21.4b	.9-1.0
CFP wt	161.6	149.4	118.2	18.9	172.3a	149.0a	95.0b	14.1-15.7
MFP wt	257.4	241.6	180.2	20.5	252.3a	226.0a	159.8b	14.8-16.6
ROFP wt	113.0a	106.6a	48.4b	10.8	137.8a	119.2a	43.6b	11.2-12.5
LOFP wt	128.0a	120.0ab	58.8b	18.0	181.0a	142.6a	46.4b	12.8-14.3
% Full	77.6a	91.4a	45.4b	6.2	67.0a	88.8a	40.4b	6.4-7.1
Score	5.2a	5.5a	2.5b	.34	3.3a	4.6b	2.8a	.22-.25
Implant recovery	3/5	1/5	3/5		2/5	1/5	2/5	
Implant score	5.0	5.5	1.7	1.1-1.9	3.0	4.5	5.3	1.4-2.0

a,bMeans within a row bearing different superscripts differ (P<.05).

pected effect of age at sacrifice (contrast 1, mean at 8 wk compared to average mean of both groups at 18 wk). No effect of hormone-treatment at wk 18 on body or organ weights was observed ($P>.05$).

Among mice killed at 8 wk of age, weight of each variable was directly related to dietary energy content, although these numerical differences were not statistically significant in all cases (see Table 16 for statistical inferences). In the two groups sacrificed at 18 wk of age (Table 17), differences in mean weights (body and fat pads) were due to lower averages for group L ($P<.05$) relative to groups H and C, which did not differ from each other ($P>.05$).

Others have attempted to increase the size of fat pads in laboratory rats for experimental purposes. In a study similar to the present one, Graziano and Reece (1974) injected insulin into rat mammary fat pads, and observed an increase in fat pad size. However, this technique leaves open the undesirable possibility that changes in mammary growth accompanying changes in fat pad size could be due to effects of insulin itself. A more attractive method is suggested by the work of Oscai and Brown (1983), who demonstrated that obesity could be induced without overeating in rats fed a high-fat diet. Carcass fat of rats fed the fat-rich diet averaged 51%, compared to 30% fat content of carcasses in rats on a control diet. In light of the latter study, the present finding of increased fat pad weights at 8 weeks of age in mice fed diets progressively higher in fat content (and caloric content) is not surprising. The lack of effect on fat pad size of diet H compared to diet C at 18 weeks of age may be a reflection of a decrease in growth rate (and accompanied decline in food intake) as the animals approach mature weight. Mice fed the lower energy diets had lower mean bodyweight, and thus may have remained in

a more rapid growth phase, allowing group C to catch-up to the mice in group H in terms of bodyweight.

Differences in diet were associated with significant differences in mammary ductal growth as assessed by percent of fat pad occupied by ducts, and by subjective developmental score (Tables 16, 17). Occupancy of fat pad increased with increasing energy (and fat content) of diets in each treatment group, although differences in diet group means did not reach levels of significance in all cases (see Tables 16 and 17, for statistical inferences). The observation that increased filling of fat pad in mice fed H relative to those fed C persisted even after body and mammary fat pad weights became similar (at 18 wks), suggests a systemic effect of dietary energy or fat content, rather than an effect of fat pad size. An alternative explanation might be that increases in ductal growth occurring during early growth gave mice in group H an advantage in fat pad occupancy that was maintained through week 18. The absolute increases in mammary fat pad weight and percent occupancy in group H at 8 weeks of age support the second explanation.

Mean mammary developmental score did not differ by diet at 8 weeks of age, although absolute score again favored group H (Table 16). The relatively small difference in development scores during this early period indicate that the primary mode of growth at this time was extension of existing major ducts (best estimated by percent occupancy measurement), rather than branching of existing ducts (indicated by development score). Development score was associated with increased dietary energy in both treatments at 18 weeks of age. Also evident at this time is a stimulatory effect of estrogen and progesterone on development score, particularly in group C. This result may reflect a change in mode of growth from extension to branching, when growth is stimulated after

the fat pad is essentially full. Alternatively, this combination of hormones may specify an increase in branching regardless of percent occupancy, although the data for mice in group L discourage this possibility. Recent evidence supports that mammary growth responses to exogenous estrogen and progesterone are stimulated by increased levels of dietary fat in mice (Welsch et al., 1985).

Evaluation of effects of diet and treatment on survivability and development score of mammary duct segments transplanted into CFP did not reveal significant differences. This lack of difference can be attributed to the relatively small differences in development score at week 8, when many explants were recovered, and the decline in numbers recovered at the later date, when dietary effects on score appeared more pronounced. An interesting feature of this study concerns recovery of implants. At week 8, when recovery was high, the original implant, with or without visible outgrowth, was usually present. However, at the later date original implants were rarely visible, with only the outgrowth of ducts apparent. This suggests that the original implant disintegrates (or is resorbed) over time, regardless of new proliferation of ducts. If this supposition is correct, then the lower recovery of implants indicates failure to grow, rather than unsuccessful transplantation procedures. A similar phenomenon may provide an explanation for the failure to recover transplants from ovarian fat pad sites. That transplants into the mammary CFP were usually successful is supported by the high recovery at week 8.

Reports of dietary effects on mammary growth in laboratory animals are numerous. In particular, recent attention has focused on the effects of dietary fat, and fatty acid composition on mammary growth (Welsch, 1987; and Abraham and Hillyard, 1983; for review). These effects are thought to be associated with changes in cell membrane fluidity,

and are at least in part, local-acting. Unfortunately, only the study of Graziano and Reece (1974) has treated the subject of the relationship between fat pad size and mammary growth. In their experiment, hormone-induced increases in fat pad size had no significant on content of nucleic acids in the mammary gland. This experiment was conducted in sexually mature female rats (that were subsequently mated), and mammary glands were evaluated on day 20 of pregnancy. The timing of this trial is unfortunate in view of the fact that in ruminants the period of critical sensitivity of mammary ductal growth to plane of nutrition or fat pad size occurs prior to puberty (Sejrsen et al., 1982; Johnsson and Hart, 1985). And overfeeding subsequent to this critical period has no effect on mammary growth.

The present experiment was intended to investigate the effects of increased caloric intake and greater fat pad size on prepubertal mammary ductal growth in mice. If elevated plane of nutrition resulted in retardation of mammary growth in mice, as in ruminants, a potentially valuable model would be provided for future studies. Results of the current study do not support this hypothesis, instead higher caloric intake and fat pad weights were associated with increased prepubertal mammary growth in mice. In fact the increased percent of fat pad occupied in mice fed a high-energy diet is all the more remarkable when one considers that there is proportionally more fat pad to be filled. In summary, mechanisms mediating the effects of diet on prepubertal mammary growth appear to differ between laboratory mice and ruminants. Therefore, studies of this type in mice may not provide an appropriate model for ruminant mammary growth.

SUMMARY

Feeding prepubertal ewe lambs a ration including 30% protected unsaturated fat supplement resulted in a rapid growth rate accompanied by deposition of fat markedly increased in percent unsaturated fatty acids (especially 18:2). Mammary growth in these lambs was superior to that of lambs fed a standard ration at ad libitum or restricted intake. In comparison to lambs fed the standard ration, ad libitum, and receiving exogenous growth hormone treatment, all indices of mammary growth except for parenchymal DNA concentration favored the supplement-fed group. These findings indicate that the supplement diet promoted mammary parenchymal development, and despite the high rate of gain achieved, mammary growth was maximal in this group.

Further analysis of properties of mammary fat pad or parenchyma, including volume and density of these tissue compartments, did not fully explain the observed differences in mammary growth. Similarly, morphometric analyses indicated similar patterns of parenchymal growth in all treatment groups, suggesting only that mammary growth associated with feeding of unsaturated fatty acids is essentially normal. In addition,

hormone profiles were not particularly insightful with respect to discerning mechanisms of action that might regulate mammary gland growth.

The most pronounced differences among all parameters measured in hopes of detecting regulatory mechanisms for prepubertal mammary growth were in numbers of hormone binding sites. Lambs fed the protected fat diet had increased numbers of membrane receptors for prolactin in mammary parenchymal tissue, and for growth hormone in liver tissue. Effects of these changes are not clear, but it is possible that alterations in hormone binding are mechanistically related to control of mammary growth.

Proliferation of ductal epithelium in mouse mammary glands increased with increasing dietary energy intake and greater fat pad size. Thus, mice apparently do not exhibit the inhibition of mammary growth associated with high plane of nutrition in prepubertal ruminants. For this reason, dietary effects on mammary growth in mice would not be a suitable model for similar studies in ruminants. However, the ease of alteration of depot fat composition in mice would make them an ideal model for further studies on effects of fatty acid composition (rather than plane of nutrition) on mammary growth in ruminants, and in general.

The observed increase in mammary parenchymal growth in rapidly growing, prepubertal ewe lambs on a protected, unsaturated fat ration has exciting implications for the animal production industry. The ability to manage heifers and replacement ewe lambs for high rates of gain, without suffering negative effects on future milk production would be of great benefit to producers. However, it remains to be seen if the increased parenchymal growth observed at this early stage of development will translate into increased milk yield in the adult animal. All related data suggest that it should. In fact, the pronounced impact of prepubertal parenchymal growth on future milk production is dispropor-

tionate to the relatively small amount of total parenchymal growth it represents. Thus, the outlook is promising.

Another factor to be considered in evaluating the potential for feeding unsaturated fat to young animals is the possibility of effects on reproduction. Because the need for successful reproduction outweighs the potential gains from increased milk yield, and rapid rearing, this aspect will require careful evaluation in future studies. The current study gave no suggestion of reproductive problems, however, and no reasons exist to expect that they might arise.

As a final note of optimism, it is encouraging to speculate that the stimulatory effects of unsaturated fatty acids may not be limited to the prepubertal phase of mammary growth. It is entirely possible that continued exposure to this altered lipid environment might stimulate parenchymal growth through puberty and gestation. Tremendous increases in numbers of secretory cells would be possible in this scenario. One may also envision feeding unsaturated fatty acids to dry cows, to promote regeneration of the secretory tissue of the udder leading into the next lactation. Thus, the potential applications of this technique to increase unsaturated fatty acid percent in mammary fat are widespread. Further research in this area should prove to be most interesting.

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