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Bovine mammary gland development during gestation

Jeannette Isabel Poffenbarger

To the Graduate Council:

I am submitting herewith a thesis written by Jeannette Isabel Poffenbarger entitled "Bovine mammary gland development during gestation." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Eric W. Swanson, Major Professor

We have read this thesis and recommend its acceptance:

R. L. Murphree, M. C. Bell

Accepted for the Council:
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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council

I am submitting herewith a thesis written by Jeannette Isabel Poffenbarger entitled "Bovine Mammary Gland Development During Gestation." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Eric W. Swanson, Major Professor

We have read this thesis
and recommend its acceptance:

R L Murphree

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BOVINE MAMMARY GLAND DEVELOPMENT DURING GESTATION

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee

Jeannette Isabel Poffenbarger

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ABSTRACT

Ten pairs of identical twin heifers were slaughtered at successive two month gestation intervals to study mammary gland development during gestation. Gland composition showed trends of decreasing fat and DNA concentration and of increasing water, percent DFFT, RNA concentration and RNA/DNA ratio as gestation progressed into peak lactation. Udder, gland and DFFT weights, DNA g/gland and alveolar surface area all generally increased throughout gestation until parturition but at peak lactation they had decreased. These results were supported by histological and gross appearance of the glands. Data from six pairs of identical twins were used to plot semilogarithmic regression lines of the parameter vs. time. Parameters investigated were: full trimmed udder weight, glands only weight, DFFT weight, DNA (mg/kg body weight), DNA (g/gland) and alveolar surface area. Their rates of change per month were: 10.8 percent, 22.0 percent, 30.5 percent, 25.3 percent, 24.0 percent and 30.0 percent, respectively. All parameters measured indicated that bovine mammary gland growth during gestation was continuous from conception to parturition and that no further development occurred up to peak lactation.

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CHAPTER I

INTRODUCTION

Although mammary gland development during the prenatal, prepubertal and pubertal stages in the bovine has been thoroughly investigated, present knowledge of bovine mammary gland development during gestation is based on a few early studies of bovines and studies of other species. The earlier researchers were unable to use more recently developed techniques which more accurately measure mammary growth and development. Use of identical twin pairs would be an improvement over unrelated animals because within twin pair comparisons reduce inherent variation otherwise found between animals. Since mammary gland development is required for expression of lactation potential, it is important to consider how and when that development takes place. By measuring several parameters of mammary gland development and making successive identical twin gestation comparisons, it is expected that general trends and growth curves of mammary gland development can be established more accurately.

CHAPTER II

REVIEW OF LITERATURE

A. PARAMETERS OF MAMMARY GLAND DEVELOPMENT

The parameters reviewed here include old parameters which are considered now of limited accuracy or inapplicable to bovine mammary glands and modern parameters developed in recent years, which are used as indexes of mammary gland growth in this study. Other parameters used to measure mammary gland growth have been reviewed by Munford (1964).

Rating System

Cowie and Folley (1947) developed a semi-quantitative method of scoring rat mammary glands to determine the kind and degree of glandular development. The total score of a mammary gland was based on the numerical rating of four categories of development and the total mammary gland area; estimated by tracing the gland on cellophane and then measuring the area on graph paper.

Relative Growth Analysis

Relative growth analysis, which is the relationship of the growth of a particular part to the growth of the whole animal, was developed by Huxley (1924). Folley et al. (1949) used relative growth analysis to study the growth of rhesus monkey mammary glands and later, Cowie (1939) studied the relative growth of rat mammary glands. Both

studies showed that mammary gland growth followed the model, $Y = aX^b$, where Y is equal to total mammary area, X is the function of total body surface area, and a and b are constants (Silver, 1953). If the log of X and the log of Y are plotted the slope of the resulting line is b. When $b > 1$ the term allometry is used to indicate that growth of the part is proceeding at a faster rate than growth of the whole animal. When $b = 1$ the term isometry is used to indicate that the growth rates of the part and the whole animal are the same.

Udder Weight and Capacity

Matthews et al. (1949) measured bovine udder weight and udder capacity. Udder weight was determined by weighing excised glands which had been milked out before slaughter. Udder capacity was determined by the quantity of fluid retained after filling the glands. Various systems were used to fill the glands with water to a constant pressure.

Udder porosity or udder quality of a cow was indicated by the ratio of udder capacity to udder weight (udder capacity/udder weight). A higher ratio indicating greater capacity to weight represented a higher quality udder.

Palpation Grading System

Swett et al. (1955) developed a numerical grading system based on palpation of calves' udders at different ages to rate udder development in calves. The correlation coefficients between first lactation milk yield and the grading system were positive, but low. Legates et al. (1960), using a similar palpation-grading system, found that the

value of palpation-grading systems in predicting future production was no greater than the value of the dam's record.

Total Alveolar Surface Area

A method to estimate total alveolar surface area of the lung was adapted by Richardson (1953) to estimate the surface area of secretory tissue in the mammary gland. Fields chosen at random from histological sections of known magnification, were superimposed on a grid of lines of known total length (L). The number of intersections (n) which the lines make with alveolar epithelium were counted and multiplied by two. Thus, the total surface area of the alveolar epithelium (S) of a known total volume (V), obtained from the weight and density of the fixed tissue, was calculated by the formula, $S = 2nV/L$.

Weight of Trimmed Udder

Weight of the udder trimmed free of excess fat and connective tissue has been a useful index of mammary gland development. Benson et al. (1965) found high positive correlations between weight of the trimmed gland and milk yield.

Deoxyribonucleic Acid Content

Since the amount of DNA per cell is relatively constant, it was proposed that DNA could be used as a measure of cell numbers (Munford, 1964). Kirkham and Turner (1953) were the first to use DNA as a quantitative index of development of lactating mammary glands in the rat. Naito (1958) found a high positive correlation between cell numbers per field and DNA per milligram dry weight in all stages of the lactation

cycle. Sinha and Tucker (1966) reported that the relationship between DNA and total mammary gland area was high. Deoxyribonucleic acid was highly correlated with litter weight gain among rats within a stage of lactation (Tucker, 1966).

Ribonucleic Acid Content

The amount of RNA in several tissues has been associated with the intensity of protein synthesis (Munford, 1964). A high correlation was found between mammary RNA content and litter weight gain (Tucker, 1966, 1969). In the mammary gland, the rate of protein synthesis is related to milk secretion (Munford, 1964); therefore, RNA may be used as an index of functional activity of the mammary gland.

The RNA/DNA ratio is an index of protein syntehsis per cell in the mammary gland (Jones, 1969). Values which are in excess of one (RNA/DNA > 1) indicate rapid protein synthesis, i.e., rapid milk protein formation (Munford, 1964).

B. BOVINE MAMMARY GLAND DEVELOPMENT

Mammary gland development in the bovine has been thoroughly reviewed by Turner (1939, 1952), and briefly presented in textbooks by Smith (1959), Kon and Cowie (1961), Schmidt (1971) and Schalm et al. (1971). Following is a brief summary of these references.

Prenatal Development

The first anlage of the mammary gland is a thickened, raised area of the ectoderm appearing approximately 30 days after conception

on either side of the ventral midline of the embryo (Turner, 1952). The ectoderm proliferates and constricts to form the mammary line at four to five weeks of embryo age. Subsequently the mammary buds, which represent the future glandular portion of the gland, develop along the mammary line. Development continues with part of the mammary bud elongating into the mesenchyme and subsequently forming the primary sprout. From the primary sprout, several secondary sprouts arise which are anlagen of the future duct system. Before birth, tertiary sprouts will occasionally develop from the secondary sprouts.

Birth to Pregnancy

At birth the non-glandular structures, i.e., the skin and hair covering, vascular and lymphatic systems, morphological aspects of the teat, gland cistern, etc., are rapidly approaching mature form; whereas, the structures associated with the secretory processes are immature. The secondary sprouts are canalized, but have developed only slightly in comparison to their final development. The ducts and sprouts are confined to a very limited area around the gland cistern. The adipose and connective tissues are well organized with the connective tissue separating adipose cell aggregations of the distinctly formed fatty pad.

Before puberty the mammary gland increases in size with mainly a deposit of adipose and connective tissue in which the rudimentary duct system continues to extend itself (Reece, 1958).

Gross measurements of the developing mammary gland were conducted by early researchers trying to predict future lactational performance of young dairy animals. Matthews et al. (1949) detected a steady linear

increase in udder weight in three-to-thirty-month old unmated calves and heifers, but wide variation between animals was found. Fluid holding capacity of the udders of a zero to nine months age group definitely increased with age, and udder capacities of a nine to thirty months age group increased somewhat with age. However, the two groups could not be combined since different methods of determining capacity were used in each group.

Palpation of calf udders between birth and pregnancy indicated several distinct stages of mammary development. Briefly, Swett et al. (1955) found that the tissues of the quarters increased in size until the rear and front quarters approached each other and finally became joined at the base.

More recently, Sinha and Tucker (1969) studied the development of bovine mammary glands from heifers aged zero to twelve months. Total mammary DNA increased at accelerated rates between two and three months of age. By nine months of age total mammary DNA values reached a plateau. Thus, initiation of the allometric growth phase was well in advance of first observed estrus, which was at six to seven months of age. However, it was possible that ovarian secretions were influencing growth of heifer mammary glands before puberty, since ovariectomy in rats and mice abolished their usual prepubertal allometric growth phase (Cowie, 1949; Flux, 1954; Sinha and Tucker, 1966; Paape and Sinha, 1971). Furthermore, freemartins administered estrogen and progesterone (mammary gland growth stimulators) showed no or very slight response to the hormones (Turner, 1959; Turner et al., 1963). This

suggests that female hormones in the prepubertal stage is necessary for future mammary gland development.

Sinha and Tucker (1969) further reported that mammary RNA followed patterns very similar to mammary DNA from birth to twelve months of age. The mammary RNA/DNA ratio was highest between birth (1.10) and two months of age (1.28); it then declined to 0.60 at eight months and remained relatively stable to twelve months of age.

During the estrous cycle, mammary DNA increased greatly (118 percent) in the two days before estrus. The increase was followed by a gradual decrease during metestrus (Day 2 to Day 4) and diestrus (Day 7 to Day 18). Mammary RNA and RNA/DNA ratios followed a pattern similar to DNA. Sinha and Tucker (1969) suggested that at the time of estrus mammary cells proliferated but during subsequent metestrus and diestrus some of the cells were lost. This is supported by the histological appearance of the mammary gland. At estrus the lobules were large, the lumina of the alveolar ducts were filled with fluid and lined with cuboidal epithelium. During diestrus the lobules were relatively small and the lumina were shrunken, contained no secretion and were lined with columnar cells.

Total mammary DNA in sixteen month old heifers averaged 326 mg/100 kg body weight and was no greater than mammary DNA in nine months old heifers, which averaged 378 mg/100 kg body weight, as reported by Sinha and Tucker (1969). They concluded that the major portion of mammary growth before pregnancy takes place by nine months of age in the heifer. Turner et al. (1963) stated that recurring estrus periods

stimulate mammary gland development in heifers. Sinha and Tucker (1966) also stated the net effect of successive estrous cycles in rats was cumulative mammary gland growth. This apparent conflict can be resolved if it is remembered that DNA measurements are including connective tissue nuclei as well as secretory tissue nuclei, thus total DNA may remain approximately the same but secretory tissue may invade and replace connective tissue.

Pregnancy

Generally, two phases of mammary changes during pregnancy have been described. The first phase is hyperplasia of the mammary parenchyma and the second phase is increases in gland size due to cell hypertrophy and distention of the alveoli with secretion (Cowie and Folley, 1961).

Hammond (1927) reported the effects of gestation on mammary gland development in eight crossbred Shorthorn heifers obtained from the Cambridge Plant Breeding Farm and a dealer. Heifers whose age was estimated from their teeth were killed between two and three years of age at different stages of gestation. Each heifer represented one monthly stage of gestation from one to eight months. Body weights were not reported. Weights of the udders up to three months of gestation increased very little and were within the range of variation found in udder weights from virgin heifers (1420-2650 grams). After early pregnancy, the gains in weight "increased more and more each month" as pregnancy proceeded. Hammond's (1927) hypothesis was that the growth curve of mammary development was bimodal and was the same shape from

0 to 5 months as from 5 to 9 months of gestation. The udder weight data he presented, however, did not support this contention.

In the first few months of pregnancy, the heifers' udders were similar to virgin heifers' udders. Secretions from the mammary glands of heifers up to four months of pregnancy were similar in composition to that of virgin heifers. The ratio of gland tissue to fat tissue in heifers pregnant a few months was similar to that of virgin heifers. Ducts, which were present in and along the connective tissue bands separating the udder fat of the virgin heifer, increased very little in length during gestation. However, the duct tissue did become more organized during the first few months of pregnancy. The alveolar ducts had a double layer of epithelium, and formation of the lobules had just started by replacing the fatty tissue.

The heifer representing the fourth month of gestation showed that the ratio of gland tissue to fat tissue had increased slightly, especially in the area of the large ducts at the gland cistern. The lobules were similar to their status at one to three months of gestation.

By the fifth month of gestation, the gland tissue had continued to develop, especially at the large ducts and cistern areas, and was spreading to all parts of the udder. The true alveoli were formed with their characteristic one layer of columnar shaped epithelial cells. The lobules were formed but were small in size and the connective tissue contained numerous capillaries which were needed to nourish the growing gland. Secretions of the gland had become more globulin in nature.

At the sixth month of gestation, the gland tissue filled most

areas of the udder to form a dense mass. At the cistern and large ducts, the gland tissue crowded out almost all of the fatty tissue. The lobules were greatly increased in size and were densely packed. The proportion of alveoli to dense connective tissue in lobules was increased and the alveoli were distended with homogeneous secretions. The gland cistern had increased slightly in size.

By the seventh month, the extent of the dense gland mass had increased and only thin strands of connective tissue separated the lobules. The alveoli were filled with granular secretion which was true milk with a high globulin content similar to colostrum. The epithelial cells were one layer deep and the gland cistern was comparatively large at this stage of gestation. At the eighth month of gestation, there was a slight increase in the proportion of gland tissue to fatty tissue. The connective tissue strands were thinner and the alveoli slightly larger.

Hammond (1927) concluded that in the virgin animal very little true alveolar development takes place; the main development was of duct tissue. Up to the fourth and fifth month of gestation, the granular connective tissue, vascular basis and alveolar duct elements developed. At the fifth month of gestation, the true alveoli were formed and started their growth in diameter. A honey-like secretion, high in globulin content, was associated with the fifth month of development of alveoli. At seven months gestation, true glandular activity and formation of milk could be initiated by removal of this secretion. If the secretion was not removed, the secretory activity did not begin until parturition. Instead, the alveoli continued their growth.

Kwong (1940) conducted a histological study of pregnant bovine udders which were obtained from a packing plant. Seven pregnant heifers were used to represent the first, third, sixth, seventh and ninth months of pregnancy stages and eleven pregnant cows, six of which represented the fourth, fifth and eighth months of pregnancy stages were also used in this study. Udders of four non-pregnant cows and heifers were also studied. The non-pregnant heifers had no secretory or alveolar tissue in the udder. The ductules were composed of two layers of epithelial cells and a basement membrane and were in groups among the fat tissue. Connective tissue bands separated the abundant fatty areas of the udder.

Up to three months of gestation, the mammary gland was concerned with ductal proliferation. The fundamental duct system was well laid down but the secretory tissue was not developed. From the fourth to seventh month of gestation the alveolar tissue was formed and began functioning. In the early part of this stage, the alveoli proliferated and expanded from the end buds of the ductules. The number of layers of epithelial cells varied and the lobes and lobules were well differentiated. By the eighth and ninth months of gestation, the alveoli were greatly distended with secretion and resembled the alveoli found in lactating udders. The ductules were lined with one layer of epithelial cells.

Jakobsen (1956) measured nitrogen content of the udders of three pairs of monozygotic twin heifers. One member of each pair served as a non-pregnant control and the other pair members were slaughtered after 280 days, 273 days and 259 days of gestation, respectively. He

calculated that after 175 days gestation, mammary gland nitrogen increased 2.36 percent per day according to the equation $VN = 25.2e^{0.0236(t-175)}$. Since Jakobsen's pregnant heifers were all within 21 days of parturition, his formula represented the slope between non-pregnant gland weights and near parturient weights all calculated as a linear log function from 175 to 280 days of gestation. His assumption that the entire increase in nitrogen content increased exponentially after 175 days gestation was based on Hammond's (1927) study of eight crossbred Shorthorn heifers which varied in age, size and condition when slaughtered, as well as previous Danish reports.

Hammond (1927) and Kwong (1940) indicated on the basis of their histological studies of udders that alveolar or secretory epithelium formation is first evident around the fifth month (140 days) of gestation with probable beginning and ending phases at the fourth and seventh months gestation, respectively. They agreed that the first months of gestation are periods of ductal growth and the last two months of gestation are periods of increasing secretion formation and enlargement of alveoli. Jakobsen (1956) on the basis of three animals, all near term, indicated that most of the increase in udder nitrogen occurred after 175 days gestation at the rate of 2.36 percent per day.

It is apparent that the subject of progressive bovine mammary development during gestation has not been very carefully studied in previous research. Hammond (1927) and Kwong (1940) used mainly histological parameters of development in unrelated animals of questionable uniformity. Although Jakobsen (1956) used identical twins, he did not

have a sufficient range of developing stages in his three pregnant heifers. It is likely that conclusions which have been drawn from such studies are not very accurate.

C. HORMONAL INFLUENCES OF MAMMARY GLAND GROWTH

Estrogen and Progesterone

It is generally accepted that both estrogen and progesterone are necessary for full mammary gland development in most species. Estrogen alone generally stimulates only ductal growth and progesterone plus estrogen is responsible for stimulating lobulo-alveolar growth of the mammary glands in numerous species including bovines.

Cowie et al. (1966) reported that administration of estrogen to goats caused extensive duct growth and considerable lobulo-alveolar growth. However, the lobulo-aveolar tissue had histological abnormalities, which included cystic alveoli, folded epithelium, immature lobules in addition to a great deficiency of alveolar surface area. Administration of progesterone eliminated the histological abnormalities and increased the alveolar surface area.

Sykes and Wrenn (1951) found that administration of stilbestrol in heifers resulted in abnormal development which included very porous udders and markedly distended ducts and alveoli. Administration of progesterone with stilbestrol resulted in many areas of normal tissue development.

Turner (1959) injected heifers daily for 180 days with 100 µg estradiol benzoate and 100 mg progesterone and then with 3 mg daily of

estradiol benzoate the following 14 days to initiate milk secretion. The heifers showed an external increase in mammary tissue growth and milk secretion was initiated although it was of low yield.

Sud et al. (1968) reported that the optimal combinations of estrogen and progesterone to stimulate maximal udder development in ovariectomized heifers was 200 mg progesterone and 800 µg estrogen, which was only slightly better than 100 mg protesterone and 400 µg estrogen.

Pituitary Hormones

Hormones of the anterior pituitary have been shown to be necessary for mammary development (Jacobsohn, 1961). In rats, anterior pituitary hormones alone will induce full lobulo-alveolar development in the absence of pituitaries, ovaries and adrenals; whereas, ovarian or adrenal steroids have little or no effect on mammary growth in the absence of the pituitary (Tucker, 1969). The adverse effects of hypophysectomy, i.e., mammary parenchyma atrophy, can only be reversed by the administration of anterior pituitary hormones. However, maximal development of the mammary gland requires the synergistic effect of the steroid sex hormones and anterior pituitary hormones (Lyons, 1958).

Placental Hormones

In rats, the placenta secretes a mammogen which acts synergistically with the pituitary or ovary to stimulate mammary growth during the second half of pregnancy (Leonard, 1945). Hypophysectomized-ovariectomized rats injected with estrogen, progesterone and placental extracts

responded by developing considerable lobulo-alveolar growth in their mammary glands; but this observation has not been confirmed (Tucker, 1969). In the bovine it has not been established that secretion of placental hormones may stimulate mammary gland development.

Jacobsohn (1961) and Tucker (1969) have reviewed the endocrine aspects of mammary gland development.

D. MAMMARY GLAND DEVELOPMENT IN OTHER SPECIES

Rats and Mice

Mammary gland growth relative to body growth entered the allometric growth phase at 22 to 23 days of age in rats (Sinha and Tucker, 1966; Cowie, 1949) and at 24 days of age in mice (Flux, 1954). During this rapid growing phase, mammary gland surface area increased between three and four times faster than body surface area in rats (Sinha and Tucker, 1966; Cowie, 1949; Silver, 1953) and five times faster than body surface area in mice (Flux, 1954). In rats, the allometric growth phase is well in advance of puberty (45 to 54 days of age) (Sinha and Tucker, 1966; Cowie, 1949; Silver, 1953). In mice the allometric growth phase occurs only a few days before puberty (27 to 33 days of age) (Flux, 1954). In both rats and mice the allometric growth phase can be abolished by ovariectomy at approximately 20 days of age (Flux, 1954; Cowie, 1949; Sinha and Tucker, 1966; Paape and Sinha, 1971). At 50 to 60 days of age in rats and mice the allometric growth phase tapered off to insignificant increases in relation to body surface area growth.

Recurring estrous cycles resulted in cumulative mammary growth

in rats (Sinha and Tucker, 1969). Throughout pregnancy, the mammary gland DNA of rats and mice increased rapidly and at a relatively constant rate from conception to parturition (Tucker and Reece, 1963a; Griffith and Turner, 1961; Greenbaum and Slater, 1951; Wada and Turner, 1959; Brookreson and Turner, 1959). Further increases in mammary gland DNA also occurred in the first half of the lactation period (Brookreson and Turner, 1959; Griffith and Turner, 1961; Tucker and Reece, 1963b).

Goats

Cowie (1971) described the histological development of fourteen primiparous goats during gestation. The greater part of proliferation of the mammary gland took place during the second half of pregnancy. Variation in the extent of the parenchymal growth between individuals and within the same gland was observed, particularly in goats exhibiting precocious cistern and teat development. Secretions accumulated in the larger ducts and cistern during the first half of pregnancy, but not until 80 to 100 days of gestation did secretion containing fat globules regularly appear in the alveoli. Normal gestation period of the goat is 151 days so secretion started in the last one-half to one-third.

Swine

Hacker and Hill (1972) investigated the mammary gland growth of gilts at three stages of gestation (Day 25, Day 50 and Day 100) and at estrus. There was no change in RNA and DNA concentration per gland when virgin gilts were compared to Day 25 and Day 50 gestation gilts. From Day 50 to Day 100 there was a significant increase in RNA and an

insignificant decrease in DNA concentrations. Little or no growth of the mammary gland parenchyma occurred during the first 50 days gestation.

Between 50 and 100 days gestation lypholized fat-free tissue (LFFT), DNA and RNA increased 6.5, 5.0 and 7.5 times ($P < 0.01$). The protein synthetic activity per cell (RNA/DNA ratios of 1.65, 1.78, 1.36, and 2.03 for estrus, 25, 50 and 100 days gestation) increased as mammary development progressed. Similar trends were found for values of total LFFT, DNA, and RNA per mammary system. Swine gestation periods average 114 days; therefore, the majority of swine mammary development took place in the second half of pregnancy.

Sheep

Wallace (1948) reported on udder development of multiparous, Border-Leicester X Cheviot ewes during pregnancy. As gestation progressed udder weights increased. In the early months, the gain in weight was comparatively small but in later pregnancy the gains per month of gestation became greater. Udder weight doubled between the end of the first month and the end of the fourth month of gestation. Normal gestation period in ewes is 147 days. During the last month of pregnancy the udder weights almost quadrupled. Much individual variation in udder weights at each stage of gestation was observed. Gland tissue distribution in the fatty pad did not change to any appreciable extent during the first three months gestation (first 60 percent). By the fourth and fifth month, gland tissue had become much denser, and during the last stage little fat remained within the gland area itself.

CHAPTER III

METHODS

A. GENERAL

Ten pairs of three to twelve month old identical twin dairy type heifers were purchased from Tennessee farmers as they could be located over a two year period. Identical twins were used because their mammary glands are expected to develop identically and thus reduce inherited variation in comparing within twin gestation stages (Turner, 1953). Monozygosity of twins was established on the basis of unusual similarities in hair types and color, body and head conformation and udder and teat conformation. All twins and available parents were also blood-typed for red cell antigenic groups, and were considered probable monozygous when both twins had identical blood types with no evidence of mixtures or mosaicism. Identification and characteristics of the identical twin pairs are shown in Table I. Blood testing revealed that one pair of twin heifers (Nos. 105-106) were probably not identical. Another pair (Nos. 93-94) appeared identical as calves and had identical blood types with no admixture; however, their body conformation differed enough to question their monozygosity after about 12 to 15 months of age.

The heifers were reared at a moderate nutritional level, with both pair-mates together when possible. It was planned to artifically inseminate the members of each pair at ages two months apart so they would be the same age when sacrificed. Gestation stages of pairs when

TABLE I

IDENTIFICATION AND CHARACTERISTICS OF TWIN PAIRS FROM WHICH MAMMARY GLANDS WERE ANALYZED

Twin No.	Breed	At Time of Slaughter		
		Gestation Stage (mo.)	Age (mo.)	Body Weight (kg)
79	Holstein	9	67	431
80	Holstein	L-2*	65	417
91	Jersey	6	36	400
92	Jersey	4	35	356
93	Holstein	0	31	527
94	Holstein	3	31	572
95	Guernsey	5	27	363
96	Guernsey	0	32	400
97	Guernsey	L-2*	31	370
98	Guernsey	9	37	443
99	Jersey-Angus	P	18	290
100	Jersey-Angus	8	18	320
101	Holstein	6	35	606
102	Holstein	8	27	518
103	Gurnsey	9	34	413
104	Guernsey	7	33	347
105	Holstein	5	29	499
106	Holstein	7	27	508
107	Guernsey	8	29	420
108	Guernsey	6	29	393
109	Holstein	5	26	438
110	Holstein	7	23	424

*L-2 is second month of lactation.

slaughtered were: 0 vs. 5 months, 0 vs. 3 months, 4 vs. 6 months, 5 vs. 7 months, 6 vs. 8 months, 7 vs. 9 months, 8 months vs. parturition, and 9 months vs. 2 months in lactation. This array of two-month comparisons was designed to provide a progressive determination of udder development from conception to parturition and on to peak lactation.

The heifers were slaughtered at the appropriate stage of gestation as planned. The udders were excised to include the fatty pad and skin up to the junction of the mammary gland and abdominal wall. Fetuses were checked to confirm that expected stages of gestation were accurate. Udders were immediately transferred to the laboratory where the following procedures occurred:

1. The two halves of the udder were carefully separated at the median suspensory ligaments.
2. Each half was weighed as it came from the abattoir.
3. Each half was milked out if fluid could be expressed from the teats, and it was then trimmed of excess skin and fatty tissues so that both halves were comparable.
4. Each half was weighed and combined weights gave gross udder weight.
5. (a) One half was selected, usually because it had been cut in excision and would not hold injected formalin for histological and chemical analyses.

   (b) Skin, teats and excessive connective tissue were removed and weighed.

(c) Supramammary lymph nodes and adipose tissue which was easily separable outside the gland parenchyma were removed and weighed. The remaining gland area included all of the developing mammary gland and the fatty tissue intimately connected with it.

(d) Blocks of tissue for histological analysis were taken from four representative areas of the half and weighed.

(e) The remaining trimmed gland was weighed, placed in a plastic bag and frozen at about -20°C.

6. (a) The other half of the udder was placed in a tray so that the teats were on top.

(b) A solution of 10 percent formalin colored with crystal violet was injected into the udder via the streak canals to capacity with slight pressure, using a 100 ml glass syringe.

(c) After 5 to 7 days the formalin-dye injected halves were frozen at -20°C.

(d) The frozen halves were sawed into strips to demonstrate the gross development of mammary gland parenchyma within the udder.

B. LIPID AND WATER ANALYSIS

The frozen trimmed mammary gland was cut into strips while still frozen and ground through a meat grinder with 2 mm holes in the plates. The total ground tissue was mixed by kneading in a plastic bag and a

representative subsample was mixed for analyses. Duplicate 10 gram samples of ground mammary tissue were extracted with 100 ml methanol and 200 ml chloroform for 24 hours by shaking in a 500 ml Florence flask on a Burrell wrist-action shaker. Extraction was repeated with 100 ml chloroform and 50 ml methanol. The residue was extracted twice by periodic hand shaking with 100 ml anhydrous ethyl ether. Fine residue from all solvents and residue from the last extraction flask contents were collected by filtration with a Buchner funnel. The combined residues were washed two to three times with ether. The tissue was transferred to a weighed 100 ml beaker and placed in a 40°C drying oven for about 2 hours to drive off most of the ether. This tissue which was fat-free and nearly dry was then placed in a vacuum desiccator where it remained until a constant weight was attained (three to four weeks). The final weight was recorded as the dry, fat-free tissue (DFFT) weight. The DFFT was ground through a fine screen in a laboratory size Wiley mill to a fine powder and transferred to stoppered test tubes which were placed in a desiccator for storage.

One to two grams of freshly ground tissue were weighed into aluminum drying pans to the nearest milligram and placed in a vacuum oven at 100°C for 24 to 48 hours. After cooling in a desiccator the pans and samples were weighed and their loss of weight was recorded as water.

Fat percentage was estimated by subtracting the moisture and DFFT percentages from 100. This difference was expressed as "fat" although it may have included a minor quantity of other substances dissolved out of the tissue by extraction processes.

C. ANALYSIS FOR DEOXYRIBONUCLEIC ACID (DNA)

DNA was determined by the method of Martin et al. (1972) except that the final chromogen solution was diluted ten times with 1 M perchloric acid (PCA) because about 20 mg DFFT produced too much color for accurate reading in the Beckman DU spectrophotometer at the original dilution. It was desired to retain the sample size, so dilution of the chromogen was used to give accurate readings.

Martin et al. (1972) modified the Webb-Levy procedure and used phenylhydrazine (p-NPH) as the colorimetric reagent. Improved yield, increased stability and better reproducibility were obtained with this new method. Major modifications included combination of the chromogenic reaction and the extraction of DNA from the tissue (to minimize destruction of DNA-deoxyribose), addition of sulfite to the reaction mixture, use of acetylated p-NPH as the reagent, removal of excess reagent by reaction with acetylacetone and extraction of the chromophore into butanol.

There was a problem with some of the fine tissue particles still floating in the screw cap tubes after centrifugation and also loss of some tissue during decanting. Three alternate methods to combat the floating of tissue were proposed: (1) mixing of tissue with PCA, centrifuging and allowing to settle overnight; (2) mixing tissue with water and heating to hydrate it before proceeding; and (3) eliminating the cold PCA washing. The results showed that the four methods were comparable so the method of eliminating the cold step was used.

The following procedure was used:

1. About 20 mg DFFT was weighed into 16 X 100 mm screw cap tubes.
2. Suspend in 2 ml water (start reagent blank also).
3. Add 0.2 ml 0.01 M potassium metabisulfite (freshly prepared) and mix.
4. Add 0.15 ml acetylated p-NPH reagent solution and mix (equivalent to 1.5 mg p-NPH).
5. Add 2 ml 2 M PCA, cap tubes and shake well.
6. Heat tubes in 90°C water bath for 2 hours.
7. Terminate heating period by chilling in ice bath.
8. Add 0.2 ml 5 percent aqueous acetylacetone and mix.
9. Recap the tubes and heat in 90°C water bath for 30 to 60 minutes, then chill in ice water.
10. Centrifuge in Size 1 IEC centrifuge at 1500 rpm for 5 minutes.
11. Transfer supernatant to 100 ml volumetric flask.
12. Wash the precipitates twice with 2 ml 0.1 M PCA and add to supernatant in step 11.
13. Add 1 M PCA to make mammary supernatants up to 100 ml.
14. Mix well and transfer 10 ml 1 M PCA for spectrophotometer blank.
15. Add 5 ml of water saturated butanol and mix by inverting the tubes.
16. Add 2 ml of 10 M NaOH; stopper the tubes and shake well.
17. Let stand a few minutes for phases to separate. Top phase may be cloudy.

18. Transfer 3 ml of the upper organic phase to another test tube and add 0.5 ml of 1 M NaOH in 90 percent methanol (prepared daily) to clarify the solution.
19. Let stand 15 minutes (5 to 30) then pour colored solution into a cuvette.
20. Determine absorbance at both 580 mμ and 680 mμ, using the 1 M PCA blank as the blank solution.
21. Subtract readings of reagent blank and record the difference between the 580 and 680 mμ absorbance as a measure of DNA.
22. DNA standards were prepared from highly polymerized calf thymus DNA.

The standard curve of DNA plotted against corrected Density was nearly a perfect straight line with the formula: DNA (μg) = 196.72 X Density - 0.679. It was found that the standard curve must be made with freshly prepared water solutions of DNA.

D. ANALYSIS OF RIBONUCLEIC ACID (RNA)

Since DFFT was found to mix relatively well with trichloroacetic acid (TCA), analysis of RNA contents was based on the method of Schmidt and Thannhauser (1945).

The following procedure was used:

1. Weigh 15 to 25 mg DFFT into a 16 X 125 mm screw top culture tube.
2. Move to cold room.
3. Add 5 ml ice-cold 10 percent TCA and mix well by shaking

long enough to cause all tissue to settle.

4. Centrifuge in cold room at 3500 rpm for 15 minutes, then discard supernatant.

5. Resuspend residue in 5 ml TCA, centrifuge for 15 minutes and discard supernatant.

6. Repeat step 5.

7. Resuspend residue in 5 ml ice-cold ethanol which has been saturated with sodium acetate. Mix by shaking.

8. Centrifuge in cold room, as before, for 15 minutes and discard supernatant. Keep tubes in refrigerator (5°C) until ready to add KOH.

9. Add 2 ml of 1 N KOH to the precipitate, stopper, mix well, and place in oven at 37°C for exactly 15 hours.

10. Cool the tubes in ice water and move to cold room.

11. Add 0.3 ml of ice-cold 5 N HCl and 5 ml of ice-cold 1 M PCA and mix well.

12. Centrifuge for 15 minutes and decant supernatant into a cold screw-top test tube calibrated at 20 ml.

13. Add 5 ml 0.5 M cold PCA to residue, mix well, centrifuge 15 minutes and add supernatant to that in step 12.

14. Repeat step 13.

15. Bring volume of combined supernatants to 20 ml with 0.5 M PCA and shake thoroughly to mix.

16. Prepare orcinol reagent just before use by dissolving 0.5 g orcinol in 50 ml ferric chloride solution (1.6 g ferric chloride in 1 liter of concentrated HCl).

17. Mix 3 ml of the combined supernatant from step 15 with 3 ml of orcinol reagent in a test tube. (If necessary to dilute, use 0.5 M PCA.) Make solution for spectrophotometer blank by mixing 3 ml PCA and 3 ml orcinol reagent together and heat for 30 minutes at 100°C in a boiling water bath, then cool.
18. Read optical density at 670 mμ in a spectrophotometer.
19. Standard curve for RNA should be made with highly purified RNA (yeast). This curve may vary with different sources of orcinol reagent.

The standard curve of RNA plotted against Density was nearly a perfect straight line with the formula: RNA (μg) = 207.8 X Density.

E. HISTOLOGICAL ANALYSIS

Blocks of tissue from representative areas of the udders were fixed in Bouin's fluid and infiltrated with paraffin by standard histological procedures. Paraffin mounts were sliced at 10 microns thickness with a microtome. Sections were selected for mounting on slides and were stained with hematoxylin and eosin according to the method of Humason (1962). Stained sections were mounted in Canada balsam.

Total alveolar surface area of the mammary glands was determined by Richardson's (1953) method of random lines intersecting with alveolar epithelium. Magnification was 157x and the random lines' length totaled 157 mm. Volume of the gland was determined by dividing specific gravity (estimated as 1.04) into the whole gland equivalent weight which was

determined by multiplying by two the trimmed portion of the half udder (glandular portion) as used in chemical analysis. Total surface area was expressed as square meters per udder.

F. GROSS ANATOMY

Representative slices through the median portion of the formalin-stained half udders were photographed to show the extent of duct and lobule alveolar development in the fatty tissue surrounding the glands.

CHAPTER IV

RESULTS AND DISCUSSION

A. COMPOSITION OF MAMMARY GLANDS

Average changes in composition of the mammary glands during gestation stages are summarized in Table II. Average gland values showed increasing trends in percent water, percent DFFT and RNA/DNA ratio and decreasing trends in percent fat and DNA concentration (mg/g DFFT) as gestation progressed. Ribonucleic acid concentration (mg/g DFFT) increased rapidly from the three-to-four-months gestation stage to the five-months gestation stage. It then stabilized through the eight-months gestation stage and increased rapidly at the ninth month-parturition and second month of lactation stage. Gland composition had changed distinctly by the fifth month of gestation. Compared to the non-pregnant state, from the fifth month of gestation mammary glands contained less fat and more water and the concentrations of DNA and RNA also changed. The changes indicated that udder fat was being replaced by glandular tissue, and the lobule alveolar tissue became more active in protein synthesis as gestation proceeded.

B. MAMMARY GLAND MASS

Average values of body weight, udder weight (fill trimmed udder), gland weight (full glands equivalent of the portion used in chemical analyses), DFFT weight and DNA (g/gland) are presented in Table III.

TABLE II

COMPOSITION OF GLANDS

Average of All Glands (mos. gestation)	No.	Water (%)	Fat (%)	DFFT (%)	DNA (mg/g DFFT)	RNA (mg/g DFFT)	Ratio RNA/DNA
0 - 1	2	38.94	53.31	7.76	50.20	18.55	0.370
3 - 4	2	27.71	66.19	6.11	41.54	14.04	0.338
5	3	59.63	26.65	13.72	33.13	22.49	0.679
6	3	54.24	32.79	12.97	29.25	23.79	0.813
7	3	64.20	22.28	13.52	28.27	23.32	0.825
8	3	62.20	23.14	14.66	27.35	23.63	0.864
9 - P - L	4	73.12	15.55	11.33	26.35	35.08	1.331

TABLE III

MAMMARY GLAND MASS[a]

Average of All Glands (mos. gestation)	No.	Body Weight (kg)	Udder Weight (kg)	Gland Weight (kg)	DFFT Weight (g/gland)	DNA (g/gland)
0 - 1	2	464	4.399	1.593	132	6.958
3 - 4	2	464	4.641	1.063	64	2.675
5	3	433	5.708	3.428	473	15.295
6	3	466	7.492	4.523	598	17.301
7	3	426	7.742	5.111	656	18.916
8	3	419	6.899	4.455	680	18.419
9 - P	3	382	11.364	7.820	896	24.196
L	1	370	9.153	5.741	646	15.963

[a]All data.

Body weights of the groups ranged from 370 kg (one cow) at the second month of lactation to 466 kg at the sixth month of gestation with an overall average of 428 kg. General trends of average gland and tissue mass showed gradual increases up to and including the eighth month of gestation in all measurements. Between the eighth and ninth month of gestation or parturition stage, the heifers sampled decreased in udder weight, gland weight and body weight. This was probably due to the small size of heifer number 100, an 18-month old crossbred Jersey-Angus, which had a smaller udder than was found on older, dairy type heifers. Deoxyribonucleic acid (g/gland) progressively increased throughout gestation except at eight months which was due to unusual development in one pair of heifers (Nos. 101-102) which had suckled each other.

When fraternal twins (Nos. 105-106, 93-94) and suckled twins (Nos. 101-102) were omitted, the most reliable twin comparisons for mammary gland development were calculated as shown in Table IV. Generally, the identical twin data (Table IV) is very similar to all twin data (Table III) with some exceptions. Data from identical twins showed more gradual increases at the early stages of gestation than all twin data. Deoxyribonucleic acid (g/gland) increased throughout gestation and decreased during lactation. The eighth month gestation stage was still low in udder weight and gland weight which again was due to the younger, less dairy type heifer, No. 100.

When the udder parameters were expressed as percentages of body weight, shown in Table V, udder weight increased gradually up to eight

TABLE IV

MAMMARY GLAND MASS - MOST RELIABLE TWIN COMPARISONS[a]

Average of All Glands (mos. gestation)	No.	Body Weight (kg)	Udder Weight (kg)	Gland Weight (kg)	DFFT Weight (g/gland)	DNA (g/gland)
0 - 1	1	400	3.982	0.964	63	2.835
3 - 4	1	350	3.992	0.904	60	2.202
5	2	401	4.208	2.407	315	10.931
6	2	397	6.378	4.073	505	15.852
7	2	386	8.016	5.267	601	16.839
8	2	370	6.938	4.294	745	19.969
9 - P	3	382	11.364	7.820	896	24.196
L	1	370	9.153	5.741	646	15.963

[a]Identical twins data only.

TABLE V

MAMMARY GLAND MASS[a] AS A PERCENT OF BODY WEIGHT OR MG/KG B.W.

Average of All Glands (mos. gestation)	No.	Udder Weight (kg)	Gland Weight (kg)	DFFT Weight (g/gland)	DNA (mg/kg B.W.)
0 - 1	2	0.96	0.33	0.03	14.1
3 - 4	2	1.02	0.23	0.02	5.9
5	2	1.29	0.77	0.11	35.5
6	3	1.61	0.99	0.13	38.4
7	3	1.92	1.28	0.16	45.7
8	3	1.64	1.01	0.16	42.4
9 - P	3	2.92	1.98	0.23	60.8
L	1	2.47	1.55	0.18	43.1

[a]All data.

months of gestation, decreased at eight months (due to No. 100), then increased dramatically by the ninth month of gestation and the second month of lactation. Gland weight increased less dramatically than udder weight at nine months of gestation and the second month of lactation. Dry fat-free tissue increased gradually after three to four months of gestation, then leveled off at seven and eight months gestation and increased rapidly at nine months gestation. Deoxyribonucleic acid (mg/kg body weight) increased rapidly from three to four months gestation to five months gestation, then increased gradually to eight months gestation, at which time it decreased slightly. At nine months gestation and at parturition DNA (mg/kg body weight) increased greatly but it had decreased at two months of lactation to a level similar to that of seven and eight months gestation glands.

C. GROSS APPEARANCE

Photographs of the gross structures of mammary gland slices from twin pairs are shown in Figures 1 through 5. The gross gland development of 0 vs. 3 month gestation stage was similar in appearance to heifer udders studied by Hammond (1927). There was an abundance of fat in both the open and three month gestation udders. Gland tissue was present in both of them in the form of small areas of ducts spreading outward and upward from the cistern area. The glandular tissue itself appeared quite porous with only a framework, i.e., duct system, of the future whole gland. The three-month udder was larger in overall size than the zero-month udder, but it appeared that the three-month udder

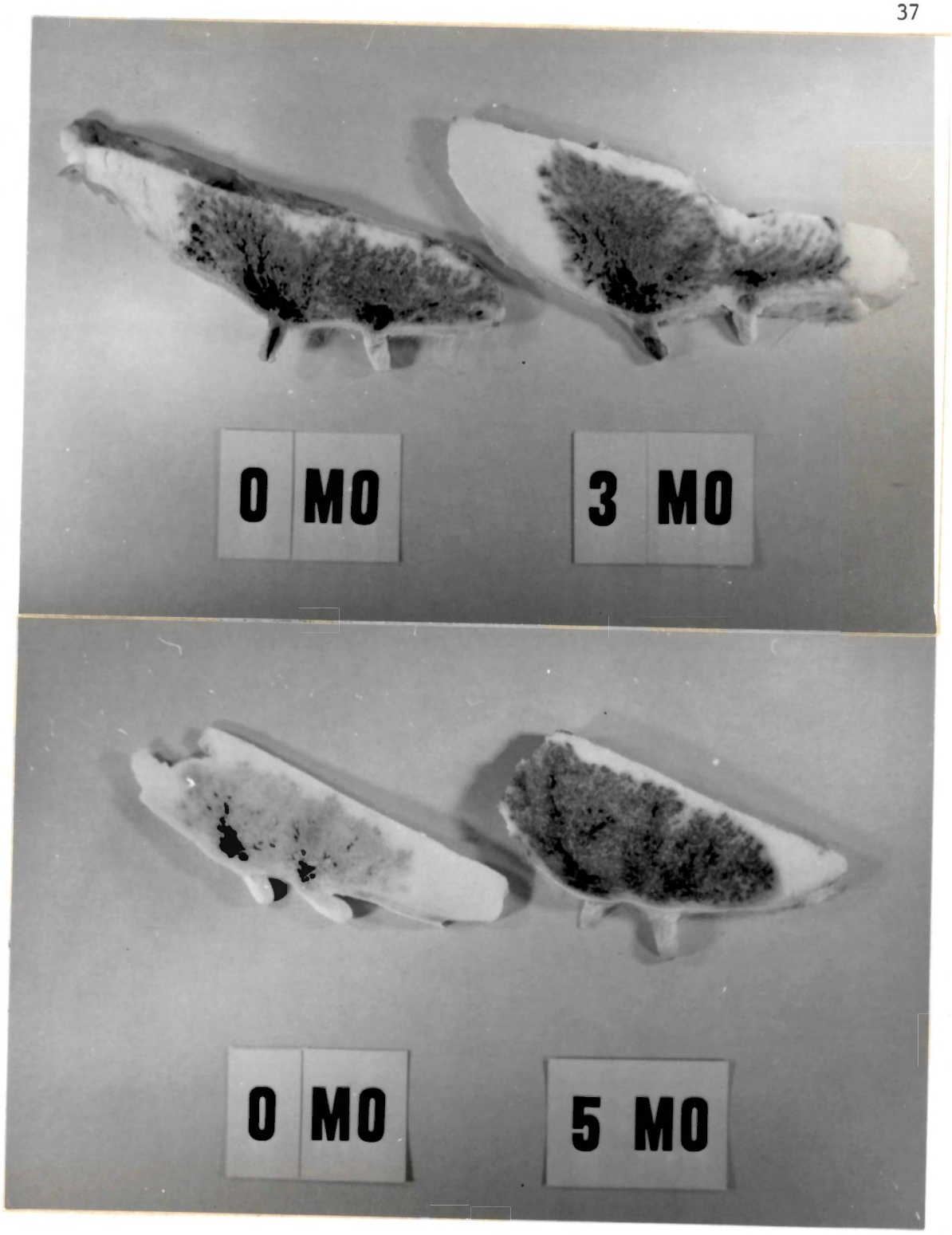

Figure 1. Gross appearance of mammary gland sections of fraternal twins, numbers 93, 94 (top) and identical twins, numbers 96, 95 (bottom) at designated months of gestation.

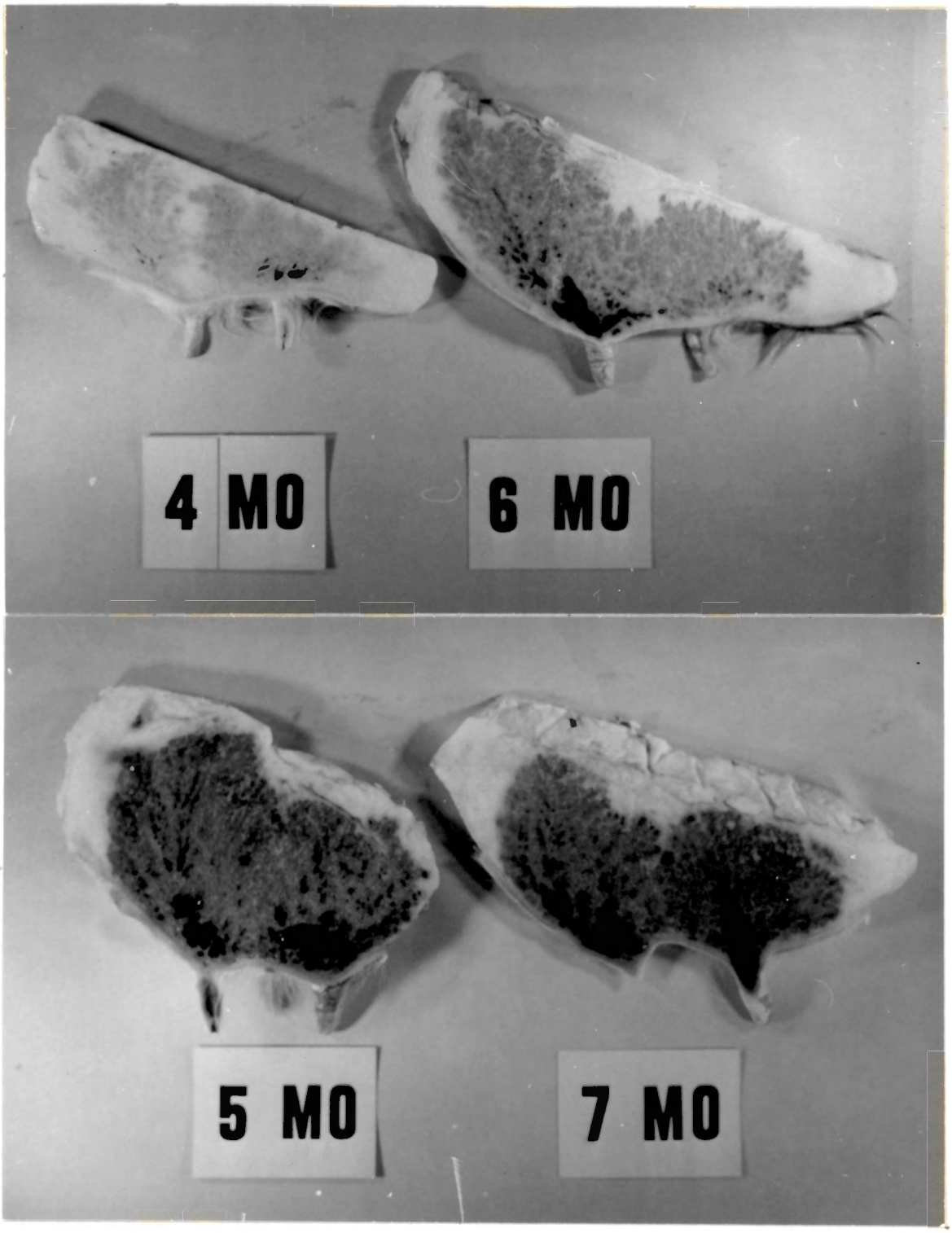

Figure 2. Gross appearance of mammary gland sections of identical twins, numbers 92, 91 (top) and numbers 109, 110 (bottom) at designated months of gestation.

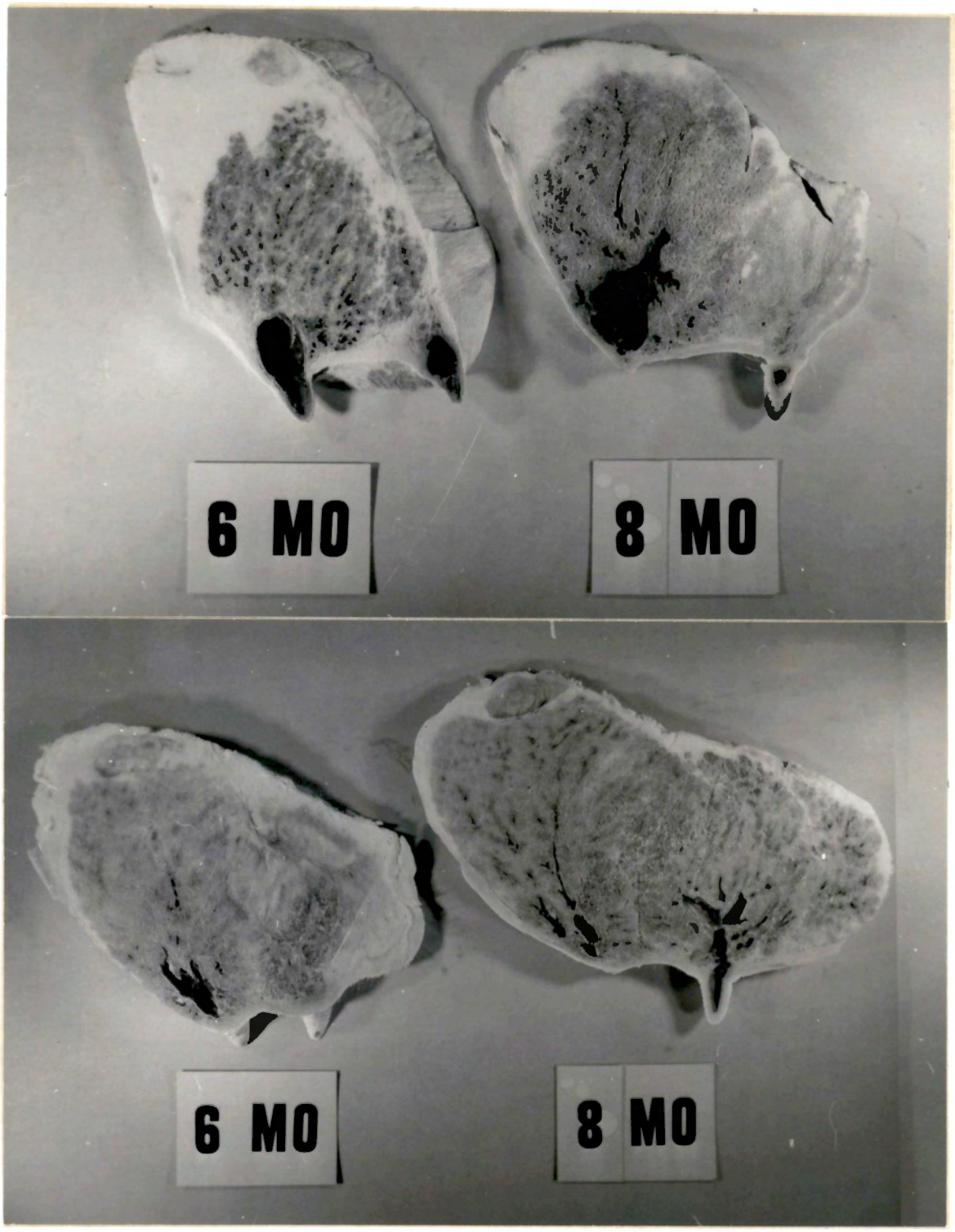

Figure 3. Gross appearance of mammary gland sections of identical twins, numbers 101, 102 (top) and numbers 108, 107 (bottom) at designated months of gestation.

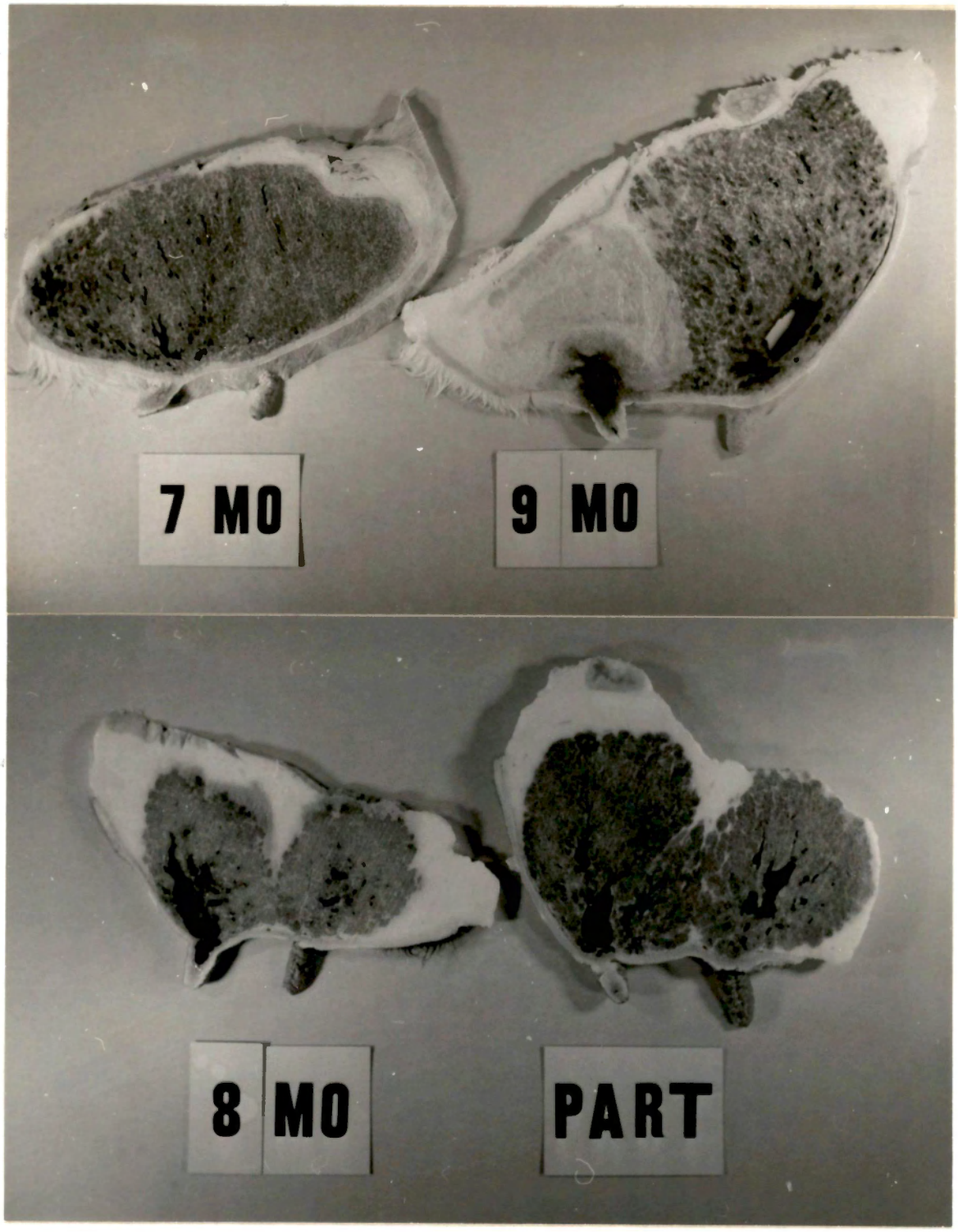

Figure 4. Gross appearance of mammary gland sections of identical twins, numbers 104, 103 (top) and numbers 100, 99 (bottom) at designated months of gestation (PART - parturition).

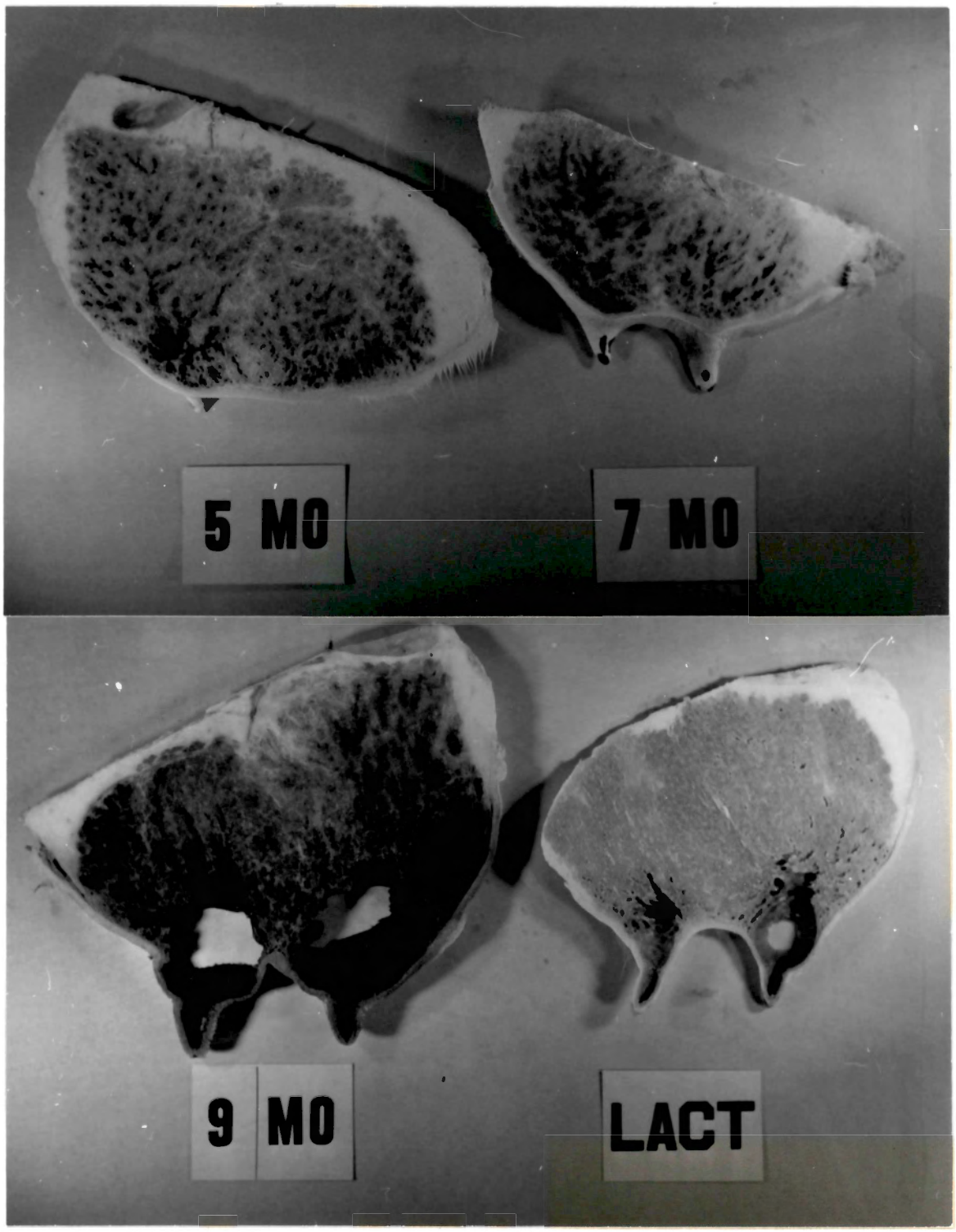

Figure 5. Gross appearance of mammary gland sections of fraternal twins, numbers 105, 106 (top) and identical twins, numbers 98, 97 (bottom) at designated months of gestation and lactation (LACT).

had much more fat and about the same amount of glandular tissue as the zero-month udder. This pair of twins (Nos. 93-94) had identical blood types but failed to develop identical body conformation, so they were assumed to be fraternal twins.

The zero-month udder of the 0 vs. 5-month gestation stage (Nos. 95-96) was also similar to Hammond's (1927) early gestation udders as well as to the 0 vs. 3-month gestation stage. The ratio of fat to gland tissue was high and the gland tissue appeared porous. The five-month udder of the 0 vs. 5-month gestation stage was larger in size than the zero-month udder. The ratio of fat to gland tissue had definitely decreased. Glandular tissue had spread upward and outward from the initial small areas of gland tissue centered around the gland cistern. The glandular tissue at the fifth month also appeared more dense than in the zero-month udder and in the 0 vs. 3-month gestation stage udders of the previous pair.

In the 4 vs. 6-month gestation stage (Nos. 91-92) the six month udder was much more developed than the fourth month udder. The sixth month udder was larger in overall size with a marked increase in the amount of glandular tissue. The ratio of fat to glandular tissue in the sixth month udder appeared to have decreased as compared to the fourth month udder. The glandular tissue still appeared porous, especially on the outer edges. The fourth month udder contained considerable fat but it appeared to be a slight improvement in development compared to the three and zero months udders (Nos. 93-94, 96) of the previous pairs.

The 5 vs. 7-month gestation stage (Nos. 109-110) showed

considerable development compared to the earlier stages of gestation. The ratio of fat to gland had decreased because more glandular tissue had developed spreading in the outward and upward regions of the udder. The fifth month udder, with more fat, appears slightly larger in overall size than the seventh month udder. The amount and distribution of glandular tissue was about the same in this pair of udders. The fifth month udder of this pair was larger than the fifth month udder of the 0 vs. 5-month gestation comparison due to inherent variation between the different sets of twins. However, the distribution of the glandular tissue and the amount of fat relative to glandular tissue was similar in both 5-month udders.

The 6 vs. 8-month gestation comparisons (Nos. 107-108, 101-102) showed a more definite increase in development as gestation progressed. Glandular tissue at eight months of gestation had spread to all portions of the udder leaving fatty tissue only around the periphery of the udder. The ratio of fat to gland had decreased in the eighth month udders and the overall size had increased. The glandular tissue of the eighth month udders appeared more densely packed than the more porous-looking sixth month udders. The gland cisterns had become larger in the eighth month stage than the sixth month stage. Both sixth month udders were comparable to each other in size and likewise the eighth month udders were comparable to each other in size. Compared to the sixth month udder (No. 91) of the 4 vs. 6-month gestation udder, these sixth month udders (Nos. 101, 108) were more completely developed. Numbers 101 and 108 also had the front and rear gland areas merged whereas number 91 had two separate gland areas.

In the fraternal twins of the 5 vs. 7-month gestation comparison (Nos. 106-105), total udder size and total glandular tissue of the seven month udder was less than that at the fifth month of gestation. This is an example of individual variation found between even closely related but non-identical animals. The relative amount of glandular tissue was approximately the same and the gland tissue itself appears quite porous in both udders.

The ninth month gestation udder of the 7 vs. 9-month gestation comparison were larger in overall size and contained more glandular tissue as compared to the seven-month udder. The glandular tissue in both glands was a dense mass filling most of the udder. The ratio of fat to gland appeared to be very low and approximately the same in both udders. The front and rear gland areas of both udders had completely merged and formed one continuous gland mass. The overall size of the seven-month udder (No. 104) was comparable to the udder size of number 110, but there was more glandular tissue in number 104 than in number 110. The seven-month udder of 5 vs. 7-month gestation comparison (Nos. 105-106) was smaller in size and amount of glandular tissue than number 104.

The udders of the eight months gestation vs. parturition stage (Nos. 99-100) were smaller in overall size than the 6 vs. 8, 5 vs. 7 and 7 vs. 9 comparison udders. They were relatively as fat as the udders of the comparisons up to six months of gestation. The small size and fatness is probably due to the young age and breeding (Jersey X Angus) of numbers 100, 99. Within the pair there was considerable

increase in overall size and amount of glandular tissue in the parturition stage udder compared to the eight-month udder. The glandular tissue appeared as dense masses in both udders. The two gland areas centered around the gland cistern had become larger and had merged together more completely in the parturition stage udder than in the eight-month udder. The ratio of fat to gland decreased in the latter stage udder also.

In the nine month gestation vs. two months lactation comparison (Nos. 97-98), the lactation udder was smaller in overall size and glandular tissue amount than the nine-month udder, but the ratio of fat to gland, which was low, appeared to be the same in both udders. Glandular tissue was dense in both udders but the lactation udder appeared to have more of a brownish tinge, due to secretion mixed with crystal violet dye, indicating much secretion. The gland cistern was larger in the nine-month udder than in the lactation udder. The nine-month udder (No. 98) was similar in size to the nine-month udder (No. 103) of the 7 vs. 9-month gestation stage. Number 98 was six months older and 73 kilograms heavier than number 97 at slaughter; thus, part of the gross differences in udder development could be due to this age and size difference.

D. ALVEOLAR SURFACE AREA AND HISTOLOGY

Alveolar Intersections and Surface Area

Average alveolar intersections per field, shown in Table VI, generally increased throughout gestation and then decreased at

TABLE VI

ALVEOLAR SURFACE AREA[a]

Average of All Glands (mos. gestation)	No.	Intersections per Field (No.)	Alveolar Surface Area (m^2)
0 - 1	2	93.5	28.1
3 - 4	2	76.5	15.6
5	3	125.3	82.8
6	2	128.0	100.1
7	3	159.3	157.1
9 - P	3	166.0	244.0
L	1	155.0	115.4

[a]All data.

lactation. In the early gestation stages there were few alveoli per field to intersect the random lines. As gestation proceeded and the number of alveoli per field increased, the number of intersections increased. In late gestation and parturition stages, the alveoli were larger and folded together tightly which resulted in more intersections per field. Alveolar intersections per field increased as alveoli per field increased or as interalveolar spaces decreased.

Average values of alveolar surface area, summarized in Table VI, increased markedly from 0 to 5 months gestation, then increased gradually up to 8 months gestation. At eight months the average decreased (probably due to No. 100) and then peak surface area was reached at nine months to parturition stages. In one lactating heifer the alveolar surface area decreased below its nine-month gestation mate.

Histological Observations

Histological sections showed increases in development during gestation similar to those reported by Kwong (1940), Hammond (1927), and Schalm et al. (1971).

In the zero to four months gestation glands (Nos. 93, 94, 92, 96), there was an abundance of fatty tissue and connective tissues throughout the histological sections. Small, isolated areas of duct tissue were interspersed in the adipose and connective tissues. Ducts in number 96, zero months, had larger diameters than those in the other zero to four months gestation glands, but in all sections the ducts had several cell layers. In some areas of the three month (No. 93)

histological sections there was slightly more development of ductal tissue into lobules than in the other histological sections. The fourth month sections (No. 92) were extremely fat with isolated areas of large diameter ducts. The fourth month appeared no more developed than the zero months gestation sections. A few, small isolated areas contained what appeared as large alveoli filled with secretion.

At the fifth month of gestation (Nos. 105, 109, 95), alveoli lined with a single epithelial cell layer appeared. Most of these alveoli were filled with homogeneous secretions and were interspersed within the duct and connective tissue areas. Some of the histological sections contained large areas of adipose tissue as well as relatively large amounts of connective tissue. However, other sections, parimarily of number 109, had little fat and thinner connective tissue bands. Formation of lobes and lobules had definitely taken place by this stage. Some precocious alveoli, filled with colostral secretions, were present in a few sections. Sections from number 91, at six months gestation, were very fat with more duct tissue and less alveoli than the sections taken from the five months gestation udders. Number 108, at six months gestation, on the other hand had much less fat in the glandular tissue with more uniformly distributed alveoli than observed at five months gestation. The fat was between lobules and lobes and many alveoli were filled with purple staining secretion.

Sections from numbers 110 and 106, at seven months gestation, were similar to the six month sections; whereas number 104, at seven months gestation, appeared more developed than the six month sections.

In number 104, many large, folded alveoli were packed close together in the lobules. There was very little adipose tissue between lobules and the alveoli were filled with purple staining secretions. These alveoli were very similar to those seen in sections from eighth to ninth month gestation or parturition stage glands. Kwong (1940) found this type development in the latter part of the four to seven month pregnant stage.

The eight month gestation histological sections appeared very mature with very large alveoli distended with granular, purple staining secretion. The alveoli were packed closely together. Little connective tissue or adipose tissue was present. The nine months gestation sections were similar to the eight months sections. Both eight and nine months gestation sections were similar in appearance to sections from lactating udders.

Sections from the parturient glands (Nos. 98 and 99) were similar to the lactating gland sections (No. 97), with extremely large alveoli, filled with purple staining granular secretion. The alveoli were well packed together and were folded irregularly in groups. Small thin strands of connective tissue separated the lobules and thicker strands separated the lobes.

E. ANALYSIS OF RATE OF GROWTH

The mammary gland growth parameters were analyzed according to the standard growth formula, $W = Ae^{kt}$; in which W is the parameter and t is months of gestation. Average regression lines were plotted on

semi-logarithmic paper to express percentage change with time of gestation in the full trimmed udder weight (kg), glands only weight (kg), DFFT weight (g), DNA (mg/kg body weight), DNA (g/gland) and alveolar surface area (m^2) for six pairs of identical twins (Nos. 96-95, 91-92, 104-103, 107-108, 109-110, 99-100). Figures 6 through 11 show the average regression lines which were constructed from the general formula log Y = a + bX where Y is in weight units and X is in time units. The solid line of each figure represents the average regression line of the parameters measured and the dotted lines represent identical twin pairs' actual values of the parameters measured.

All parameters increased exponentially throughout gestation with no significant changes in rate. The average rate of change of the full trimmed udder weight, calculated from the formula log Y = 4.9378 + 0.04708X, was 10.8 percent per month. The weight of the glands only increased 22.0 percent per month and its regression formula was log Y = 2.90251 + 0.09564X. Dry fat-free tissue increased 30.5 percent per month and its regression formula was log Y = 1.73785 + 0.13265X. The regression formula for DNA (mg/kg body weight) which increased 25.3 percent per month was log Y = 0.78696 + 0.10996X and similarly DNA (g/gland) increased 24.0 percent per month with the formula log Y = 0.39994 + 0.10415X. Total alveolar surface area increased at the rate of 30.0 percent per month calculated from the regression formula log Y = 1.0991 + 0.1304X.

The glands only weight (devoid of teats, excessive connective tissue and extra gland fat) increased twice as fast (22.0 percent per month) as the full trimmed udder weight (10.8 percent per month). Dry

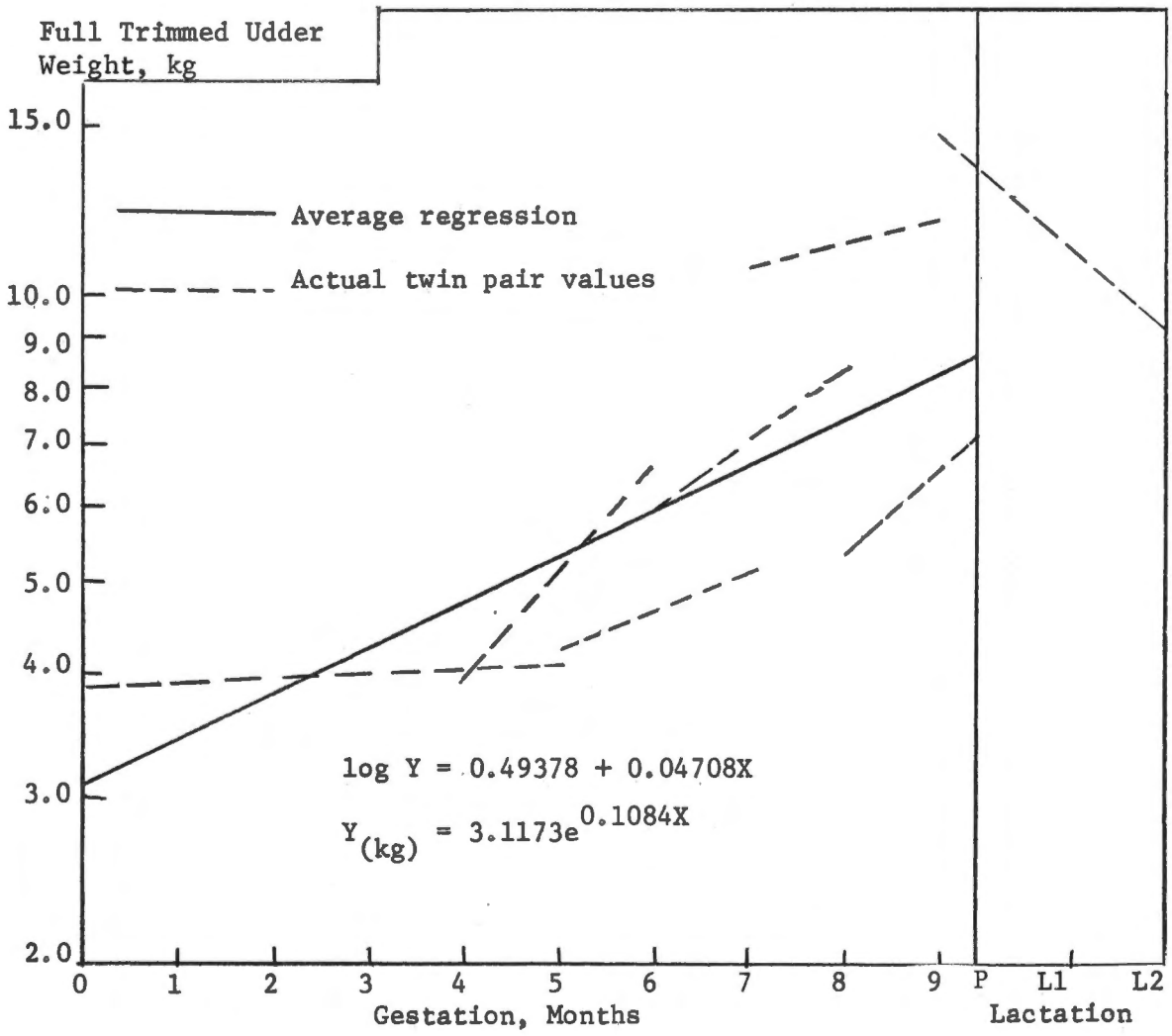

Figure 6. Rate of growth of full trimmed udder weight of identical twin pairs during gestation and peak lactation.

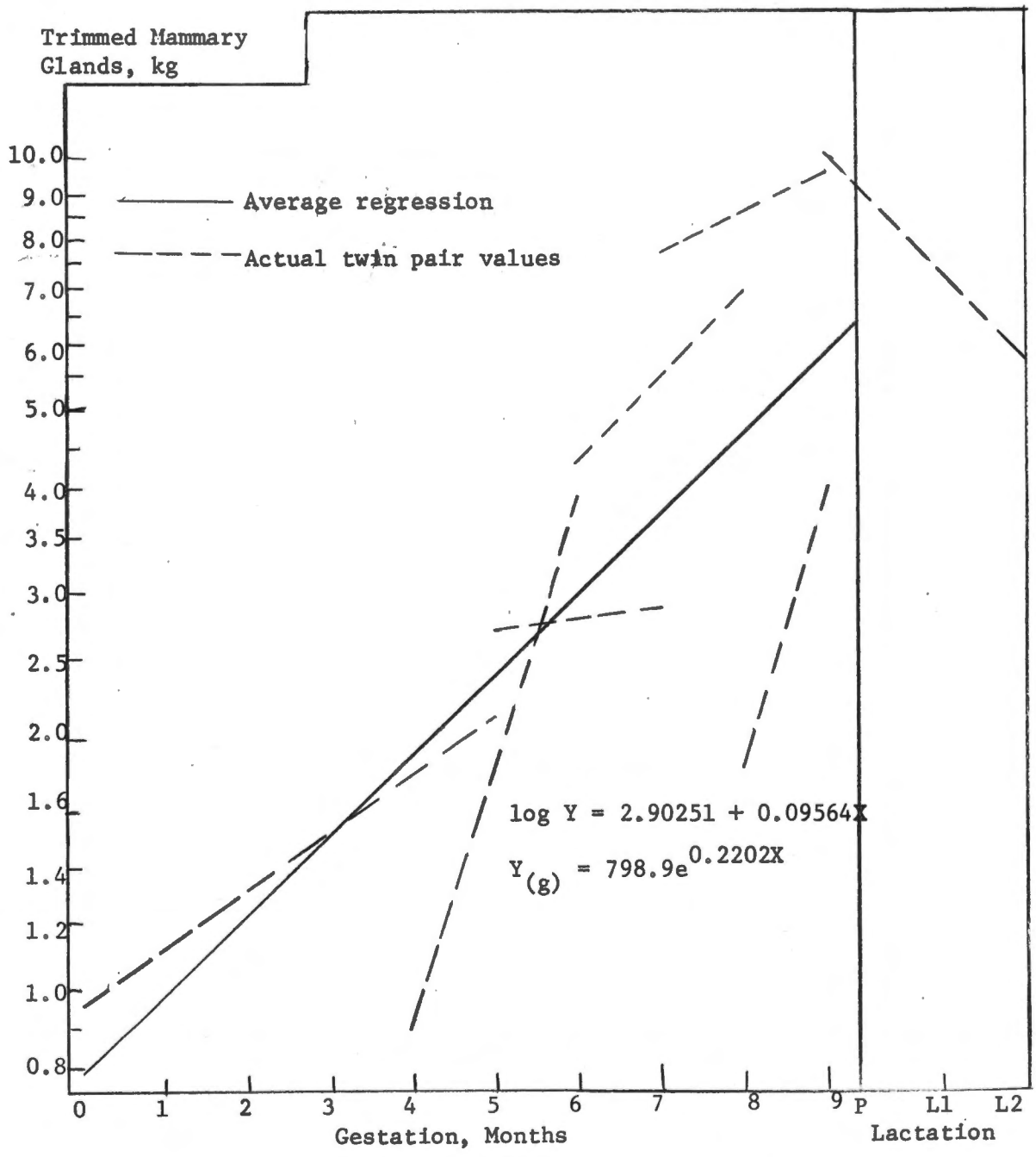

Figure 7. Rate of growth of trimmed mammary gland weight of identical twin pairs during gestation and peak lactation.

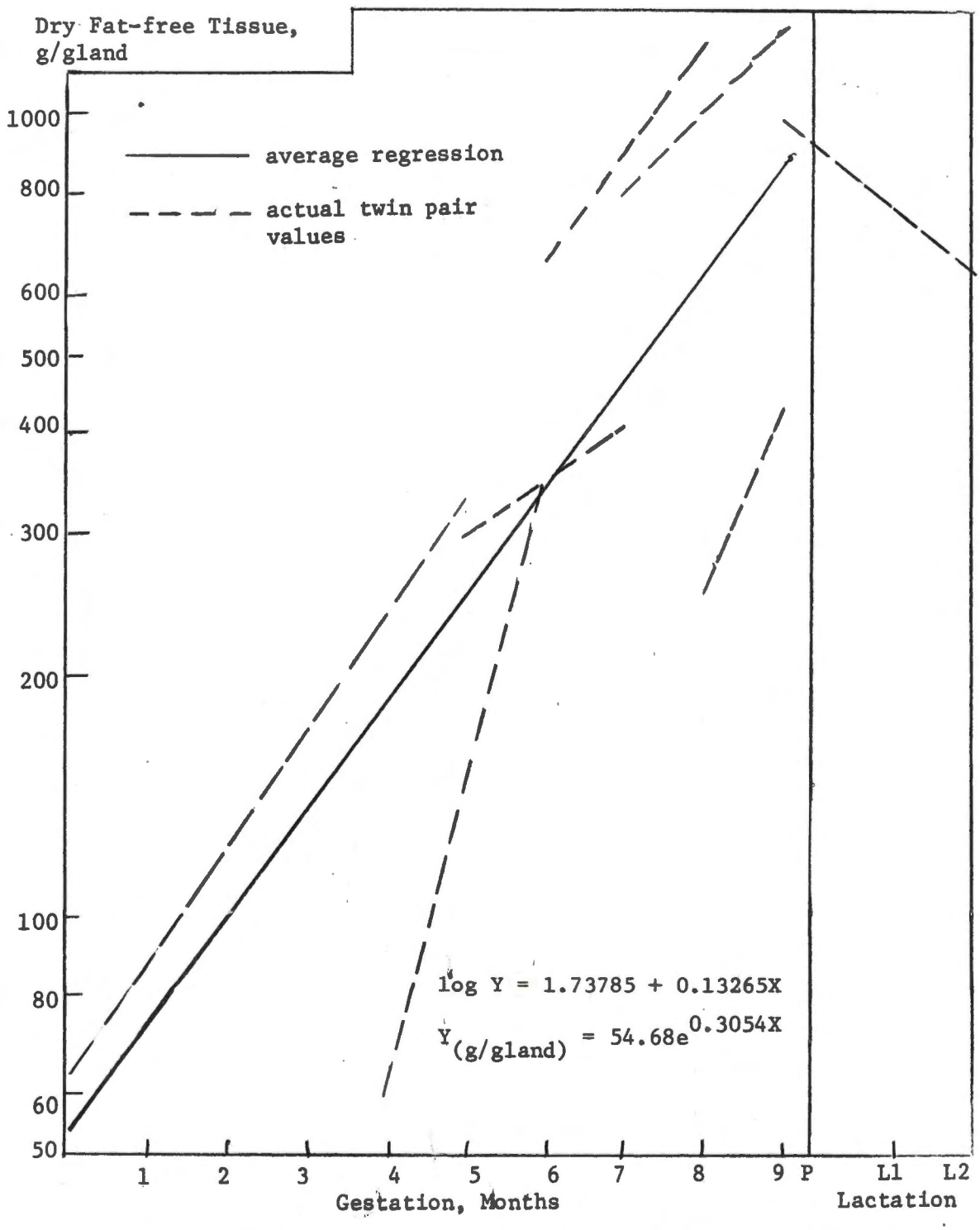

Figure 8. Rate of growth of dry fat-free tissue of identical twin pairs during gestation and peak lactation.

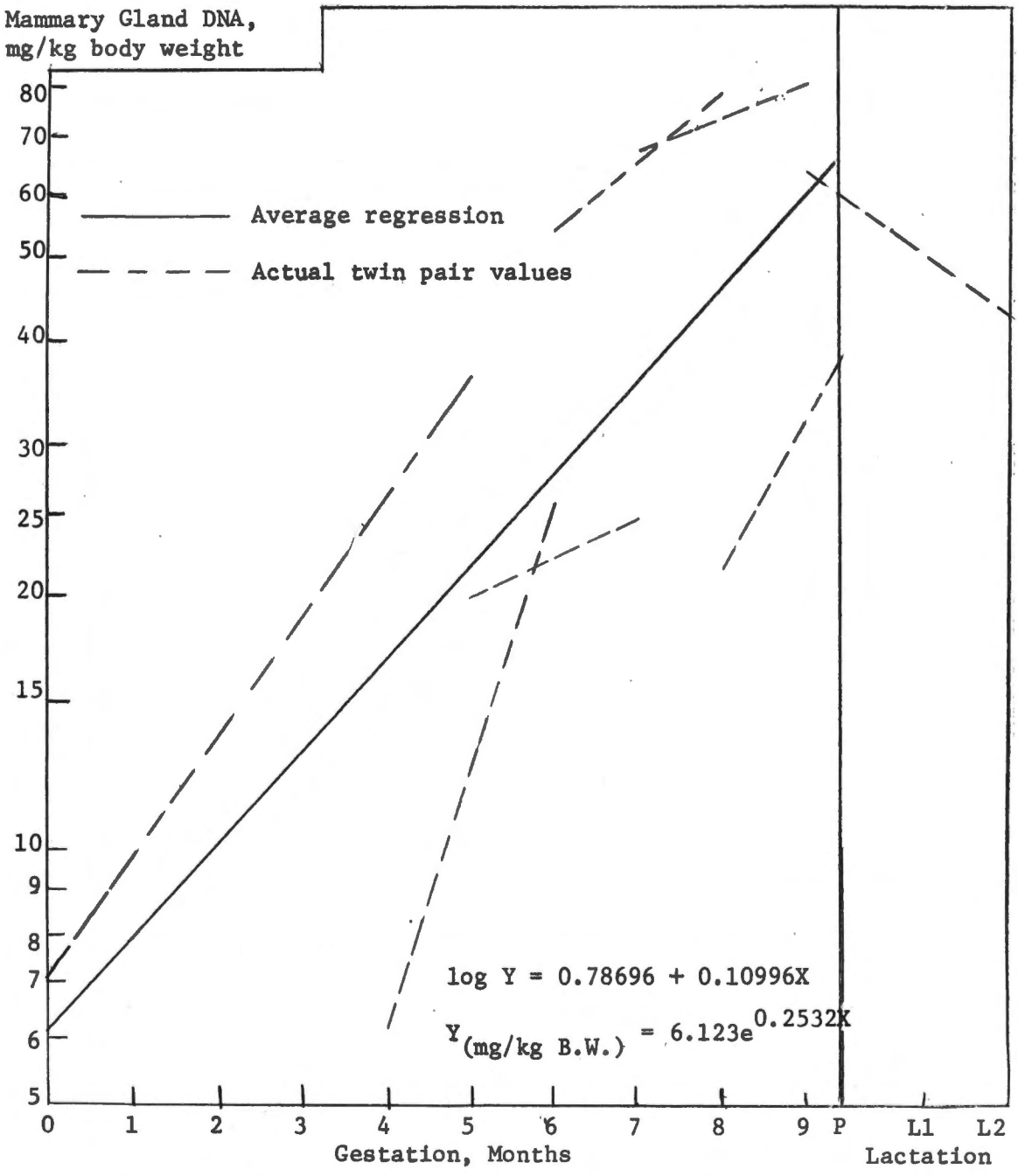

Figure 9. Rate of growth of mammary gland DNA (mg/kg body weight) of identical twin pairs during gestation and peak lactation.

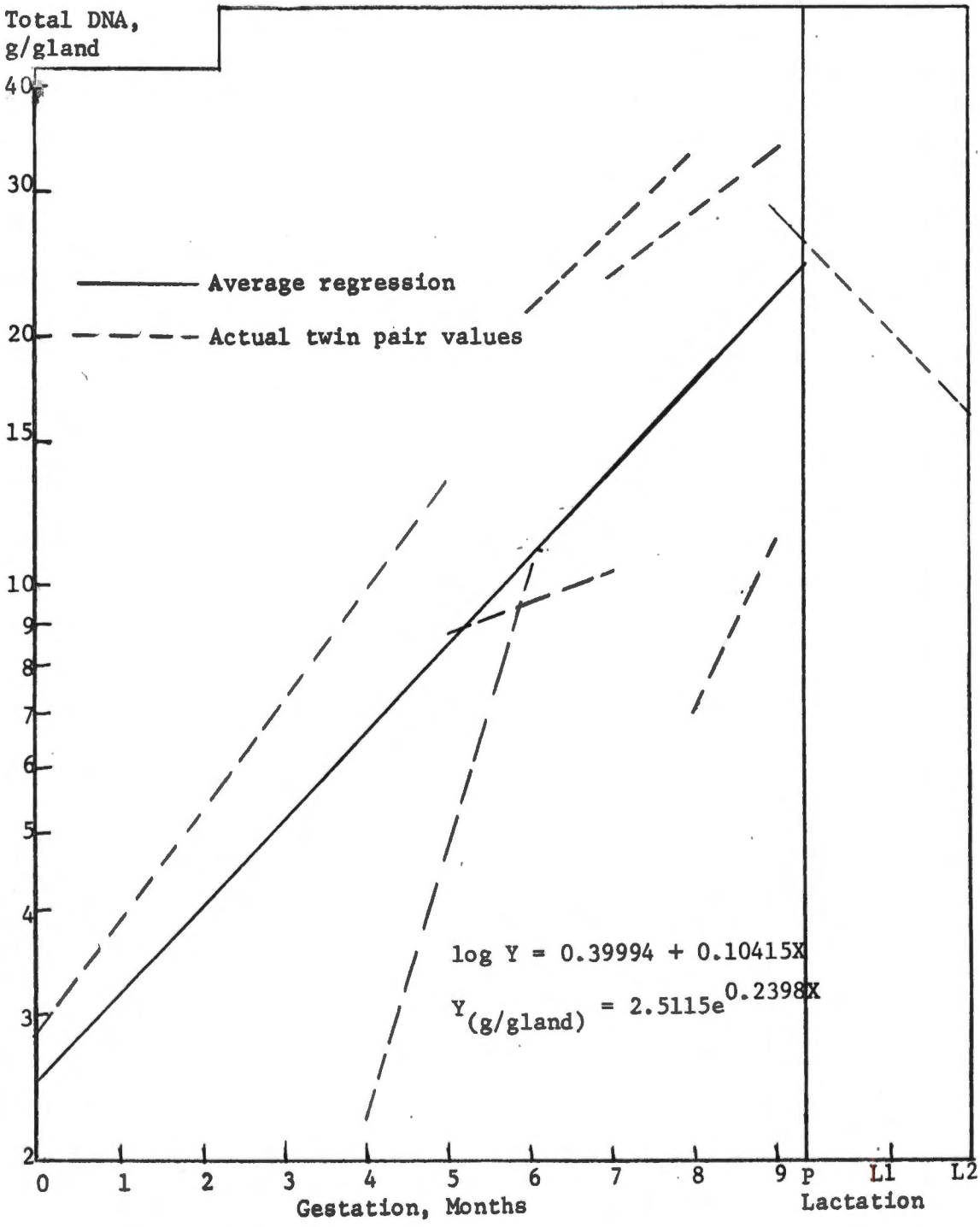

Figure 10. Rate of growth of total DNA (g/gland) of identical twin pairs during gestation and peak lactation.

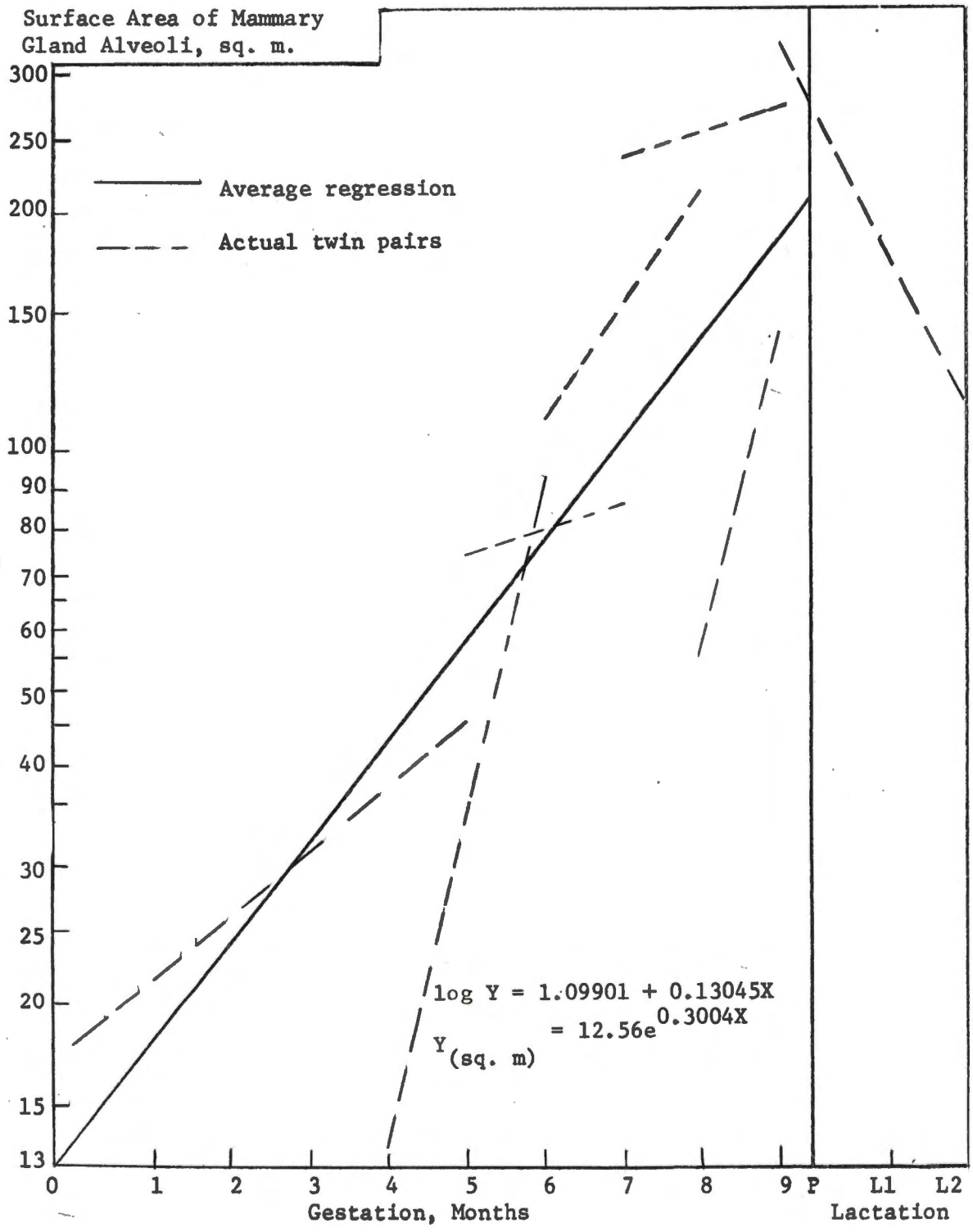

Figure 11. Rate of growth of alveolar surface area of identical twin pairs during gestation and peak lactation.

fat-free tissue derived from the gland tissue increased about three times as fast (30.5 percent per month) as full trimmed udder weight. The alveolar surface area change per month (30.0 percent) substantiates the three times as fast as full trimmed udder weight growth rate of DFFT. These identically high growth rates are logical because alveolar surface area is concerned with lobulo-alveolar growth and DFFT is measuring the changes in tissue devoid of fat and water which would be mostly secretory and associated connective tissue. However, DNA (mg/kg body weight and g/gland) increased at a rate half way between the rates of glands only weight and DFFT weight. This suggests that an appreciable amount of connective tissue persists in the gland from the beginning of gestation, and this framework provides a base level of cellular material from which later development occurs. The ratio of DNA to DFFT could be used as a measure of nuclear density of the developing gland. The full trimmed udder weights of the early gestation stages contain much fat; however, as the glands develop, protein-type tissue replaces the fat so that the total mass increases more slowly than the secretory tissue develops.

The results of this study suggest a different type of growth curve than do the data of past investigators (Hammond, 1927; Kwong, 1940; Jakobsen, 1956) of bovine mammary gland development during gestation. Bovine mammary gland development during gestation as indicated by this study is a continuous growth process throughout gestation. There is no critical starting month and growth does not plateau during the later months of gestation as suggested by Hammond (1927) and Kwong

(1940). There was no evidence of a non-growth period before 175 days of gestation as predicted by Jakobsen (1956). Jakobsen derived formulas for nitrogen deposition in mammary glands and udder weights during gestation by averaging the constants and exponents from individual formulas which were derived from each of three pairs of Red Danish Milk Breed identical twins. His assumption was that no development occurred until 175 days gestation, but he did not have any observations around that stage to verify this assumption. Mammary gland parameters studied in this investigation indicate that growth was continuous from conception throughout gestation.

From the ninth month of gestation to the second month of lactation (one pair of heifer twins), there was a decrease in all parameters measured except for RNA (g/gland, mg/kg body weight), RNA/DNA ratio and percent water. It was expected that RNA and the RNA/DNA ratio should increase since during the lactation the gland must increase its protein synthesis to produce milk. Unlike rats and mice, in which mammary gland development continued in lactation (Brookreson and Turner, 1959; Griffith and Turner, 1961; Tucker and Reece, 1963b), the bovine appeared to have completed development by parturition. But, differences in age and body weight must be considered since the parturition stage heifer was younger (by six months) and weighed less (by 73 kg) than the nine months gestation stage heifer. On the other hand, a pair of identical twin cows (Nos. 79, 80) which were also sacrificed at parturition vs. two months in lactation also decreased in gland weights and increased in the RNA/DNA ratio similar to the first calf heifers.

Mammary gland development may take place in the primiparous lactation period but it would seem to be a minor amount compared with that at parturition.

In other species, i.e., sheep, swine and goats, investigators (Wallace, 1948; Hacker and Hill, 1972; Cowie, 1971) have reported that the majority of the mammary gland development takes place in the second half of gestation. These studies were all based on progressive increases in weight or size or DNA content. If they had been expressed on a percentage basis, according to the relationship $Y = Ae^{kt}$, it is likely that they would not have stressed the lack of growth in the first half of gestation.

CHAPTER V

SUMMARY

Ten pairs of identical twin heifers were slaughtered at successive two month intervals to give a progressive picture of mammary gland development during gestation. Parameters investigated included: percent water, fat and DFFT; udder, gland and DFFT weights; DNA and RNA concentrations; total DNA and RNA contents; RNA/DNA ratio; total alveolar surface area; and gross appearance. Gland composition showed trends of decreasing percent fat and DNA concentration and increasing percent water, percent DFFT, RNA concentration and RNA/DNA ratio as gestation progressed into peak lactation. At the fifth month of gestation, the mammary gland composition became distinctly different from the non-pregnant state and remained so through peak lactation. Mammary gland mass parameters (udder, gland and DFFT weights and DNA g/gland) all increased throughout gestation until parturition but at peak lactation they had decreased. When parameters were expressed as percentages of body weight, they followed the same general trends as before except there was little decrease in udder and gland weight at peak lactation. Alveolar surface area also increased until parturition with a rapid increase between the eighth and ninth month of gestation, and a decrease at lactation. Gross appearance of the udders supported the results of the other parameters measured by indicating increasing development throughout gestation and some decreasing development at two months of lactation.

Full trimmed udder weight, glands only weight, DFFT weight, DNA (mg/kg body weight and g/gland) and alveolar surface area of six identical twin pairs were plotted against time on semi-logarithmic paper to determine average regression lines and their formulas. Average rates of change for all parameters measured increased throughout gestation with no significant changes in rate. Full trimmed udder weight increased 10.8 percent per month, glands only weight increased 22.0 percent per month, DFFT weight increased 30.5 percent per month, DNA (mg/kg body weight) increased 25.3 percent per month, DNA (g/gland) increased 24.0 percent per month and alveolar surface area increased 30.0 percent per month.

All parameters measured in this study indicate that bovine mammary gland growth during gestation was continuous from conception to parturition and that no further development occurred up to peak lactation.

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