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PREPUBERTAL MAMMARY DEVELOPMENT IN THE BOVINE: INFLUENCE OF NUTRITION AND AGE AT PUBERTY

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INTRODUCTION

In the bovine, prepubertal mammary development consists of branching and elongation of mammary ducts into the surrounding mammary fat pad. More extensive branching, elongation and ultimately the appearance of milk secreting alveoli occur only after conception and under the direction of the hormones of pregnancy. Early postnatal mammary growth has been shown to occur at an allometric rate prior to puberty and return to an isometric rate after puberty (Sinha and Tucker, 1969). Additionally, it has been shown that elevated nutrient intake during this period of allometric mammary growth reduces parenchymal weight and/or DNA at or prior to puberty (Pritchard et al., 1972; Sejrsen et al. 1982; Petitclerc et al., 1984; Capuco et al., 1995; Mäntysaari et al., 1995). This biological phenomenon has garnered considerable research attention however the mechanism remains elusive.

Both growth hormone (Sejrsen, 1978) and leptin (Silva et al., 2002) have been proposed as mediators of diet impaired prepubertal mammary development. Growth hormone is required for prepubertal mammary development (Cowie et al., 1966) and its secretion is reduced in animals on a high plane of nutrition (Breier et al. 1986). These facts have lead to the hypothesis that reduced circulating growth hormone in response to elevated nutrient intake impairs prepubertal mammary development (Sejrsen, 1978; Sejrsen et al. 1983).

Systemic levels of the adipocyte hormone leptin is increased in heifers raised on an elevated plane of nutrition (Block et al., 2002). Furthermore, leptin has been shown to retard both IGF-I, and serum stimulated MAC-T cell proliferation (Silva et al. 2002). Leptin has also been shown to mute basal and IGF-I stimulated prepubertal mammary epithelial cells in vivo (Silva et al., 2003). Silva et al. (2002) has proposed that increased circulating leptin caused by elevated nutrient intake directly inhibits growth factor stimulated mammary epithelial cell proliferation, which ultimately leads to impaired prepubertal mammary development.

The objectives of this study were to determine the impact of elevated nutrient intake from early in life on mammary development at multiple points between birth and puberty in the bovine. Specific variables of interest include the effect elevated verses restricted plane of nutrition has on parenchyma and fat pad weight and DNA, mammary epithelial cell proliferation, parenchyma DNA accretion rate, and the period of allometric mammary growth.

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MATERIALS AND METHODS

Animals and tissue collection

Seventy-eight Holstein heifers were purchased within one week of age from commercial dairy farms surrounding Ithaca, NY. Early in life (44.2 kg BW, 9.9 days) they were assigned to an elevated (E) or restricted (R) plane of nutrition. Heifers were fed to achieve 650 g (R) or 950 g (E) daily gain.

Prior to weaning, all heifers were fed twice daily at 0630 and 1800 h. E-heifers received a 28% CP, 20% fat milk replacer at 0.32 Mcal gross energy (GE) per kg of $BW^{0.75}$ while R-heifers received a 22% CP, 20% fat milk replacer at 0.20 Mcal GE per kg of $BW^{0.75}$. Heifers were weighted once weekly and the amount of milk replacer offered was adjusted accordingly. Weaning was initiated after approximately 6 weeks on treatment by reducing the amount of milk offered to 50% of the preweaning amount. The weaning period consisted of approximately 7 days. Calves had access to water throughout the entire milk-fed phase. A 26% CP textured starter was offered starting at approximately 3 weeks on study.

Throughout the entire length of the study, heifers were weighted once weekly and the amount of feed offered was adjusted to support the desired rate of gain. For two weeks following weaning, all heifers were offered a 26% CP textured starter. From 10 to 13 weeks of age, all heifers received TMR 1 (Table 1). After which they received TMR 2 (Table 1) until 200 kg. TMR 3 (Table 1) was offered from 200 to 350 kg. In all cases, the amount of feed offered was adjusted to achieve the desired daily gain. From initiation of treatment to 150 kg, all heifers were housed and fed in individual pens within a greenhouse structure. After 150 kg, heifers were group housed and individually fed via the Calan gate system.

Once heifers reached 225 kg, blood was collected twice weekly via jugular venipuncture and plasma progesterone concentration determined. Progesterone concentrations above 1 ng/ml were interpreted as the heifer possessing a functional corpus luteum and therefore pubertal.

Six heifers were slaughtered at 46 kg to determine mammary development prior to initiation of treatment. The remaining heifers (six per treatment per slaughter weight) were slaughtered at one of the following live body weights: 100, 150, 200, 250, 300, or 350 kg. Slaughter was conducted by stunning with a captive bolt and exsanguination at the Cornell University abattoir. Pubertal heifers were slaughtered only in the luteal phase of their reproductive cycle. At slaughter, the mammary gland was removed and weighted. The left half was immediately dissected and tissue from the mid parenchyma and fat pad were snap frozen in liquid nitrogen for RNA extraction. Additional tissue samples from these same regions were fixed overnight in 10% neutral buffered formalin at 4°C and then stored in 70% ethanol until further processing for BrdU localization.

Following collection of parenchyma and fat pad tissue from the left half, the gland was then skinned and separated into right and left halves at the medial suspensory ligament. The skin and teats from the whole gland were weighted together and the skinned right half was weighted separately. The weight of the skinned left half was determined by the difference between the weight of the whole gland and the sum of the whole gland skin and teats and the skinned right half.

The skinned right half was frozen in dry ice and stored at -20°C until further processing. At a later date, the right half was partially thawed at 4°C and cut into 5-mm thick slices using a meat slicer. From these slices, the parenchyma, fat pad, and supramammary lymph node were quantitatively dissected by color, collected and weighted. The total parenchyma and fat pad from each heifer were separately ground using a Hobart bowl chopper and subsampled for later proximate analysis and determination of DNA content. Ground parenchyma and fat pad subsamples were frozen in liquid nitrogen and further ground to a fine powder with a commercial blender.

Parenchyma and fat pad DNA content was determined using the fluorometric bisbenzimidazole technique (Labarca and Paigen, 1980). A subsample of the powdered tissue was freeze-dried and used for proximate analysis. Fat content was determined by ether extract (AOAC, 1981). Nitrogen content was analyzed by a Kjeldahl procedure (AOAC, 1990) that was modified to include the use of boric acid and steam distillation (Pierce and Haenisch, 1940). Crude protein content was calculated as N x 6.25. Hydroxyproline content of the parenchyma was determined by HPLC following acid hydrolysis (Gehrke et al., 1985) and hexane extraction of lipid (Keene, 1996). Amino adipic acid served as the internal standard.

Statistical Analysis

Data were analyzed by the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The sums of squares were partitioned to treatment, slaughter weight and treatment x slaughter weight interaction. To reduce the chances of overlooking a biologically significant interaction of the main effects, the interaction's means were separated using the PDIFF options for comparisons within slaughter weight groups when the F-test indicated a potential interaction ($P < 0.20$). Age and body weight at puberty and number of ovulations were analyzed using the PROC GLM procedure with sums of squares partitioned only to treatment. In all cases, when comparing means, an overall level of statistical significance was established at $P < 0.05$.

RESULTS

Body Growth

Preweaning and lifetime growth data are given in Table 2. Body weight, hip height, and age at initiation of treatment were not different between treatments. Daily gain during the milk fed phase averaged 960 and 640 g/d for E- and R-heifers, respectively,

with E- growing more rapidly than R-heifers ($P < 0.05$). R-heifers had a longer milk fed phase than E-heifers ($P < 0.05$). Despite this, E-heifers were significantly taller at the hips and heavier at weaning than R-heifers ($P < 0.05$). Both preweaning and lifetime daily hip height gains were also greater in E-heifers ($P < 0.05$). Lifetime ADG followed the same trend as preweaning ADG with E-heifers gaining 930 g/d and R-heifers gaining 640 g/d ($P < 0.05$).

A total of 17 heifers reached puberty before they were slaughtered; 10 E-heifers and 7 R-heifers. For these heifers that reached puberty, age at puberty was influenced by plane of nutrition with E-heifers reaching puberty at an average of 252 d and R-heifers at an average of 357 d of age ($P < 0.05$) (Table 3). Despite this dramatic difference in age at puberty, body weight at puberty was not significantly affected by treatment ($P = 0.09$). Mean body weight at puberty across all 17 heifers that reached puberty was 280 kg.

Mammary Development

Data for the right half of the mammary gland are present in Tables 3 and 4. In all cases, data presented represent the right hemigland. The weight of the parenchyma was negatively impacted by elevated nutrient intake ($P < 0.01$). Total parenchyma DNA followed the same pattern as parenchyma weight. Heifers on the elevated plane of nutrition had consistently less parenchyma DNA across all six slaughter points. Likewise, these heifers had significantly less parenchyma DNA per kg of live body weight than heifers on the restricted plane of nutrition ($P < 0.01$).

Despite diet having profound effects on parenchyma mass and parenchyma DNA, plane of nutrition did not influence the fat, protein, or hydroxyproline composition of the parenchyma (Table 4). There were significant developmental changes in the composition as the heifers matured. The content of both fat and protein increased linearly as the heifers matured ($P < 0.01$). Likewise, the hydroxyproline content tended to increase with maturity ($P = 0.10$).

Development of the mammary fat pad was also influenced by plane of nutrition (Table 4). Both elevated plane of nutrition and heavier slaughter weight significantly increased the weight of the mammary fat pad ($P < 0.01$). Fat pad DNA was also increased by both elevated plane of nutrition and greater slaughter weight ($P < 0.03$). Both plane of nutrition and slaughter weight significantly influenced both fat and protein content of the mammary fat pad ($P < 0.01$) (Table 4).

Epithelial cell proliferation

There was tendency for elevated plane of nutrition to increase mammary epithelial cell proliferation ($P = 0.08$, Figure 1). This effect was most pronounced at the 100 kg slaughter weight but was not sustained through the other 5 slaughter weights.

Parenchyma DNA Accretion Rate

Daily parenchyma DNA accretion rates between slaughter weights were also calculated (Figure 2). These values were calculated by taking the difference in the treatment by slaughter weight average parenchyma DNA at the previous slaughter point (150 kg, for example) from each individual heifer's parenchyma DNA at the current slaughter weight (200 kg, for example). This increase in parenchyma DNA between the 150 and 200 kg slaughter weights was then divided by the difference between the previous treatment by slaughter weight average number of days alive and the number of days alive at the current slaughter point individually for each heifer. Therefore, there were six accretion rates per treatment between each of the 7 slaughter points. Plane of nutrition had no effect on parenchyma DNA accretion rates between slaughter points ($P = 0.63$). The accretion rate was, however, developmentally regulated (slaughter weight $P < 0.01$) with the peak rate of 5.4 mg/d occurring between the 200 and 250 kg slaughter points.

Covariate analysis of mammary development

As demonstrated above, daily DNA accretion rate was not affected by plane of nutrition. Similarly, mammary epithelial cell proliferation was also unaffected by level of nutrient intake. Despite this, heifers on the elevated plane of nutrition had dramatically less total parenchyma DNA across all slaughter weights. That DNA accretion was not influenced by plane of nutrition suggests that treatment differences in time between birth and slaughter ($P < 0.01$) (Table 4) is at least partially responsible for the variation in total parenchyma DNA at slaughter. To test this hypothesis, all variation in total parenchyma DNA that is associated with the number of days alive was removed by covariate analysis (Table 4). Number of days alive was a highly significant covariate term when used in this analysis ($P < 0.01$). Furthermore, it resulted in plane of nutrition no longer having a significant impact on parenchyma DNA ($P = 0.75$). Using days alive as a covariate in the analysis of parenchyma DNA / kg body weight had the similar result by removing all significance from the treatment term ($P = 0.68$) (Table 4). Days alive was not a significant covariate term ($P = 0.70$) when used in the analysis of total fat pad DNA or fat pad DNA per kg of BW (data not shown).

Plane of nutrition effects on allometric mammary growth

To evaluate the effect of plane of nutrition on the timing into and out of the prepubertal allometric growth phase, the log(10) of parenchyma DNA was plotted against the log(10) body weight at slaughter (Figure 3). Heifers from both treatment groups were already in the allometric growth phase at the 100 kg slaughter weight. Furthermore, both treatment groups exited the allometric growth phase between the 250 and 300 kg slaughter weights. Interestingly, this range of body weight also encompasses the overall average weight at puberty (280 kg), which is indicated by the arrow in Figure 3. Furthermore, even the amount of parenchymal DNA in prepubertal heifers slaughtered after 280 kg nicely fit the new isometric curve. Not only did plane of nutrition appear to have no influence on the timing of exit from allometric growth, but

also the slopes of the allometric parenchyma DNA growth were similar between the two planes of nutrition (3.11 and 3.29 for R- and E-heifers, respectively). The slopes of the DNA growth curves in the subsequent isometric growth phase were also similar (1.44 and 1.28 for R- and E-heifers, respectively).

Predicting normal and diet impaired prepubertal mammary development

Taken together, the data presented above support the idea that parenchyma DNA accretion rate is regulated by physiological age (i.e. age relative to puberty). Furthermore, the only apparent influence plane of nutrition has on prepubertal mammary development is to hasten puberty and by default hasten the exit from the allometric growth phase in chronological age. This results in a reduced length of time between birth and puberty and ultimately less parenchymal DNA at puberty. This suggests that the length of time between birth and slaughter is the greatest influencer of prepubertal mammary development.

If our theory is correct, one should be able to use our DNA accretion rate to effectively predict the amount of parenchymal DNA present in the prepubertal mammary gland at any point between birth and approximately 350 kg as long as age and body weight at slaughter are known. This was done using data presented in five previously published studies that described “diet impaired” prepubertal mammary development. Those studies included Pritchard et al. (1972), Sejrsen et al. (1982), Petitclerc et al. (1984), Capuco et al. (1995), and Mäntysaari et al. (1995).

In order to standardize parenchyma DNA data across studies and to minimize the effect of between-study variation in DNA assay procedures, it was decided to work in fractions of 100 rather than absolute amounts of parenchyma DNA. For each study, parenchyma DNA in the low ADG group was designated as 100% and parenchyma DNA in the high ADG group was designated as some fraction of the low rate of gain group. For example, in the case of Sejrsen et al. (1982), the low rate of gain group had 1562 mg parenchyma DNA. This was set to 100%. The high rate of gain group had 1061 mg DNA so this was set to 67.9% (1062 / 1562 * 100).

The average DNA accretion rate from our study was also converted to a fractional basis. This fractional accretion rate represents the average daily accretion of parenchyma DNA as a fraction of the maximum DNA. Since physiological age (i.e. body weight) influences the daily parenchyma DNA accretion rate (Figure 2), the weight at slaughter will influence the average daily DNA accretion rate a heifer has experienced in her lifetime. For example, a heifer slaughtered at 250 kg will have a lifetime average DNA daily accretion rate that is greater than a heifer slaughtered at 350 kg. This is because the heifer slaughtered at 350 kg will have spent a considerable amount of time in the post-pubertal isometric growth phase, while a heifer slaughtered at 250 kg will not have. This will result the heifer slaughtered at 350 kg having a lower lifetime DNA accretion rate. Because of this, when calculating the fractional accretion rate from our data for use in predicting mammary development across studies, it was

necessary to calculate the average lifetime accretion rate up to the weight in which heifers in the study of interest were slaughtered.

When predicting parenchyma DNA mass in heifers slaughtered around 350 kg, the lifetime parenchyma DNA accretion rate was calculated using all of our data including the 350 kg slaughter weight. This was the case for the following studies: Pritchard et al. (1972), Sejrsen et al. (1982), Petitclerc et al. (1984), and Capuco et al. (1995). Mäntysaari et al. (1995), however, slaughtered their heifers at 250 kg. Therefore, the lifetime average parenchyma DNA accretion rate was calculated using data from our slaughter weights up to and including the 250 kg slaughter weights.

The lifetime average fractional DNA accretion rates were calculated by setting the parenchyma DNA for our R-heifers at the heaviest desired slaughter weight equal to 100%. For example, when calculating the lifetime average fractional DNA accretion rate for heifers slaughtered at 350 kg, the parenchyma DNA in our 350 kg R-heifers was set to 100% and this was considered the maximum amount of parenchyma DNA achievable. Parenchyma DNA values from all other heifers were set to some fraction of 100%. The fractional DNA accretion rate between consecutive weights was then calculated as the change in percent max DNA between two consecutive slaughter points divided by the time between the two slaughter points. This fractional accretion rate was calculated on an individual heifer basis and averaged by treatment in the same manner as the absolute DNA accretion rates discussed above. The average of all six slaughter weights was taken to represent the lifetime average fractional DNA accretion rate. This average rate for a heifer slaughtered at 350 kg was calculated to be 0.221% per d with no difference between treatments ($P = 0.61$). The lifetime average fractional DNA accretion rate for a 250 kg heifer was calculated by setting the parenchyma DNA in the 250 kg R-heifers to 100% and this was considered the maximum amount of parenchyma DNA achievable. This average rate for a heifer slaughtered at 250 kg was calculated to be 0.343% per d with no treatment effect ($P = 0.49$).

We predicted the normal and diet impaired prepubertal mammary development using the fractional DNA accretion rates described above and the age at slaughter published in the paper. For example, Sejrsen et al. (1982) slaughtered their high rate of gain heifers at 332.5 d and their low rate of gain heifers at 454.5 d. Both groups of heifers were slaughtered at approximately 320 kg so our lifetime average parenchyma DNA fractional accretion rate of 0.221% per d was used. This predicts that parenchyma DNA at slaughter for the high rate of gain heifers will be 73.4% (332.5 d x 0.221% / d) of the low rate of gain heifers. The predicted parenchyma DNA for these high rate of gain heifers is 1146 mg (1562 mg [the amount of parenchyma DNA in the low rate of gain heifers] x 73.4%). Using this same approach, the low rate of gain heifers were predicted to be 100.4% (454.5 d x 0.221% / d) and have a predicted parenchyma DNA of 1569 mg (1562 mg x 100.4%). The actual observed parenchyma DNA at slaughter for the low and high rate of gain heifers in the paper by Sejrsen et al. (1982) were 1562 and 1061 mg, respectively. This same approach was taken with the other four papers. A plot of the predicted verses observed parenchyma DNA at slaughter is presented in Figure 4. The slope of the regression is 0.92 and $r^2 = 83\%$ ($P < 0.01$).

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DISCUSSION

The negative effect of elevated prepubertal nutrient intake on prepubertal mammary development was once again demonstrated in this study. Our data are consistent with most other data in the literature (Pritchard et al., 1972; Sejrsen et al. 1982; Petitclerc et al., 1984; Capuco et al., 1995; Mäntysaari et al., 1995) in finding that elevated prepubertal nutrient intake results in a reduction in parenchyma weight and/or DNA. Despite more than 4 decades of work, a causative relationship between prepubertal mammary development and first lactation milk yield has yet to be proven. While a connection between prepubertal mammary development and first lactation milk yield seem logical (see review by Sejrsen and Purup, 1997), not all data support this conclusion. For example, Capuco et al. (1995) observed a 52% reduction in parenchyma DNA in 350 kg Holstein heifers as a result of elevated nutrient intake, however, there was no effect on first lactation milk yield in cohorts taken to lactation (Waldo et al., 1995). Similarly, a 48% increase in parenchyma DNA brought about by prepubertal growth hormone injections over non-injected controls yielded no improvement in first lactation milk yield (Radcliff et al., 1997 and 2000). Indeed, Tucker et al. (1973) observed only weak, non-significant correlations between parenchymal DNA at 5 months of age and milk yield in the first 60 days of lactation in Holstein heifers.

Heifers on the elevated plane of nutrition had heavier mammary fat pads already at the 100 kg slaughter weight and this continued throughout the other slaughter points with the exception of the 200 kg weight. Others have observed increased fat pad weight in response to elevated prepubertal nutrient intake (Sejersen et al., 1982 and Capuco et al., 1995).

Mammary parenchyma wet weight and DNA content were significantly reduced in heifers on the elevated plane of nutrition. The lipid and protein composition of the parenchyma was, however, unaffected by nutrient intake. Similarly, Sejrsen et al. (1982) observed no effect of prepubertal nutrient intake on lipid composition of the parenchyma. In the current study, the fraction of lipid increased and protein decreased within the parenchyma as the heifers mature. This is presumably the result of mammary ducts extending into the surrounding mammary fat without completely displacing all of the adipocytes.

There was no difference in daily parenchyma DNA accretion rates between heifers on the elevated verses restricted plane of nutrition. This fact is in agreement with our observed lack of effect by plane of nutrition on long-term mammary epithelial cell proliferation. This lack of effect on mammary cell proliferation and accumulation rate appear not to support the most popular schools of thought on the cause of diet impaired prepubertal mammary development. Silva et al. (2002), has proposed that elevated nutrient intake and the resultant increased circulating leptin impairs prepubertal mammary development by muting IGF-I stimulated epithelial cell proliferation. Based on this hypothesis, one would expect to observe reduced BrdU incorporation by mammary epithelial cells of our heifers on the elevated plane of nutrition and ultimately

a reduction in daily parenchyma DNA accretion rate. Instead, we observed a numerical tendency for heifers reared on the elevated plane of nutrition to have greater BrdU incorporation than heifers on the restricted plane of nutrition. Additionally, the daily DNA accretion rate was not affected by plane of nutrition. These data, therefore, do not appear to support the hypothesis of Silva et al. (2002).

Altered secretion of growth hormone in the face of elevated nutrient intake was the first proposed and perhaps most intensively studied hypothesis explaining diet impaired prepubertal mammary development (Sejrsen, 1978). In a classical pituitary ablation study, Cowie et al. (1966) demonstrated that mammary development in the ruminant is dependent upon growth hormone. It is well established that elevated nutrient intake reduces circulating growth hormone (Breier et al., 1986). These facts led to development of the hypothesis linking reduced circulating growth hormone to diet impaired prepubertal mammary development (Sejrsen, 1978; Sejrsen et al., 1983). If reduced circulating growth hormone in heifers raised on an elevated plane of nutrition does indeed retard prepubertal mammary development, one would expect to observe a comparable reduction in both mammary epithelial cell proliferation and parenchyma DNA accretion rate. Despite a 37% reduction in prepubertal mammary development in the current study, there was no plane of nutrition effect on either epithelial cell proliferation or parenchyma DNA accretion rate. Therefore, our data do not appear to support the growth hormone theory.

Since the depression in prepubertal mammary development associated with elevated nutrient intake was not caused by an impairment of epithelial proliferation or parenchyma DNA accretion rate, it becomes obvious that the length of time between birth and slaughter is responsible for the variation in parenchyma DNA at slaughter. Indeed, the number of days alive is a highly significant covariate term when used in the analysis of total parenchyma DNA as well as parenchyma DNA per kg of bodyweight. Furthermore, when the number of days alive is used as a covariate term in this analysis, plane of nutrition no longer has a significant effect on prepubertal parenchyma DNA. This implies that all of the variation in parenchyma DNA thought to be associated with level of nutrient intake is actually the result of the fact that heifers on the elevated plane of nutrition were slaughtered at a younger age than heifers on the restricted plane of nutrition. Therefore, the effect of plane of nutrition on the circulating levels of hormones responsible for coordinating nutrient partitioning appear to have no direct effect on the developing parenchyma. We hypothesize that the amount of time between birth and slaughter is the single greatest determinant of prepubertal mammary development.

Additionally, the consistent lack of effect of dietary protein levels on prepubertal mammary development (Dobos et al., 2000 and Whitlock, et al., 2002) also appears to support our hypothesis. In both studies, body weight and age at slaughter was not different across the treatment groups. Therefore, based on our new understanding of prepubertal mammary development, the fact that they did not observed any treatment effects on parenchyma DNA at slaughter is not surprising.

The fact that one can accurately predict both normal and diet impaired prepubertal mammary development using the parenchyma DNA accretion rates discussed herein and the age at slaughter is an important one (Figure 4). This is perhaps the most convincing evidence that the number of days between birth and slaughter is the greatest influencer of prepubertal mammary development. There are two studies in which number of days alive did not accurately predict the observed amount of parenchyma DNA. The observed parenchyma DNA for heifers on an elevated plane of nutrition receiving a corn silage TMR was 1056 mg (Capuco et al., 1995). The predicted value for this group of heifers was 1681 mg. Additionally, the observed average parenchyma DNA for heifers on two elevated planes of nutrition was 1032 mg (Mäntysaari et al., 1995). The predicted value for this group of heifers was 1422 mg. In both cases, number of days alive fails to accurately predict actual parenchyma DNA. This may suggest that factors other than the number of days alive may be influencing the amount of parenchyma DNA at slaughter in these two instances.

Slopes of the allometric and isometric mammary growth phases were similar between the two treatments (3.11 and 1.44 for R- and 3.29 and 1.28 for E-heifers). Additionally, these slopes were remarkably similar to the prepubertal allometric (3.5) and post-pubertal isometric (1.5) slopes first reported by Sinha and Tucker (1969). The fact that neither the allometric and isometric slopes or the timing of the exit from allometric growth were affected by plane of nutrition (Figure 3) strongly suggests that the mammary parenchyma is receiving developmental cues from signals other than those directly related to nutrient status. These facts combined with the fact that all heifers reached puberty at the same BW suggests that prepubertal mammary development is driven by a heifer's physiological age just as the onset of puberty.

Mammary epithelial cell proliferation was markedly greater in heifers on the elevated plane of nutrition than those on the restricted plane of nutrition at the 100 kg slaughter weight. At this slaughter weight, E-heifers were 2.7 months old while R-heifers were 3.7 months old. Data from the literature suggests that basal mammary epithelial cell proliferation decreases as heifers mature. Ellis and Capuco (2002) reported mammary epithelial proliferation to be 6.83, 2.83, and 2.30% in heifers at 2, 5 and 8 months of age. The fact that basal proliferation drops between 2 and 5 months of age suggests that this effect of age may also be responsible for the 44% difference in proliferation we observed between the E- and R-heifers at the 100 kg slaughter weight. Unfortunately, we can only hypothesize as to the true cause of this effect.

CONCLUSIONS

While the mammary fat pad appears responsive to nutrient partitioning hormone concentrations that change in response to an elevated plane of nutrition, the mammary parenchyma does not. Plane of nutrition has no influence on parenchyma DNA accretion rates or long-term mammary epithelial cell proliferation. Instead the time between birth and slaughter appears to have a substantial influence on prepubertal mammary development.

Historically, experiments evaluating the effect of elevated nutrient intake on prepubertal mammary development have evaluated mammary development at puberty. Since body weight at puberty is typically not affected by plane of nutrition, assessment of mammary development has been done at a common body weight but substantially different ages. Our data demonstrates that it is this effect of nutrient intake on age at a common body weight that most greatly influences the amount of parenchyma DNA.

We postulate that the single most important variable affecting total parenchyma DNA is the length of time between birth and a given body weight and that plane of nutrition affects the time necessary to reach this given body weight but has no direct influence on local or systemic control over the rate of parenchyma DNA accretion, mammary epithelial cell proliferation, or ultimately total parenchyma DNA.

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Table 1. Diet dry matter and nutrient composition.

Item	Diet		
	TMR 1	TMR 2	TMR 3
Ingredient, % of diet DM			
Alfalfa hay	10.3	…	…
Alfalfa silage	15.1	31.8	31.8
Corn silage	13.8	31.8	31.8
Soybean meal	…	…	4.2
Heat treated SBM	10.3	18.0	8.4
Starter grain mix[1]	50.3	…	…
High moisture corn	…	15.9	23.1
Rumensin 800	…	1.6	1.7
Vit. E supplement	0.12	0.10	…
Mineral mix	0.12	0.9	1.4
Nutrient, % of diet DM			
CP	24.3	19.0	17.2
NEm, Mcal/kg	1.72	1.62	1.65
NEg, Mcal/kg	1.08	1.03	1.06
ADF	18.1	21.1	20.2
NDF	35.5	34.0	31.4
NFC	34.3	35.9	40.0
EE	1.91	3.93	3.45

[1]27% CP textured starter.

Table 2. Least square means of pre-treatment and growth data.

Item	Treatment[1]		SEM[2]
	E	R	
Initial BW, kg	43.9	44.4	0.74
Initial age, d	10.3	9.4	0.70
Initial HH[3], cm	82.8	82.9	0.51
Weight at weaning, kg	85.9[a]	75.7[b]	0.66
Length of milk fed phase, d	43.3[a]	49.7[b]	1.2
HH at weaning, cm	94.3[a]	93.1[b]	0.33
Preweaning weigh gain, kg/d	0.96[a]	0.64[b]	0.015
Preweaning HH gain, cm/d	0.36[a]	0.27[b]	0.017
Lifetime weight gain, kg/d	0.93[a]	0.66[b]	0.009
Lifetime HH gain, cm/d	0.18[a]	0.14[b]	0.004

[a,b]Means with uncommon superscripts are different at $P < 0.05$.
[1]Treatment R – restricted nutrient intake; Treatment E – elevated nutrient intake.
[2]Standard error of the mean.
[3]Hip height.

Table 3. Least square means of puberty data.

Item	Treatment[1]				F-Test
	E		R		
	Mean	SEM[2]	Mean	SEM	*P* Value
Number reaching puberty	10	-	7	-	-
Age at puberty, d	245[a]	7.6	353[b]	9.1	< 0.01
Weight at puberty, kg	270	6.6	289	10.4	0.09
Average number of ovulations	3.4	0.73	4.6	0.87	0.32

[a,b]Means with uncommon superscripts are different at $P < 0.05$.
[1]Treatment R – restricted nutrient intake; Treatment E – elevated nutrient intake.
[2]Standard error of the mean.

Table 4. Effect of prepubertal nutrient intake on mammary parenchyma development from birth to 350 kg. All values represent the right hemigland.

	Slaughter weight, kg														
	100		150		200		250		300		350				
	Treatment[1]												F-test *P* Value		
	E	R	E	R	E	R	E	R	E	R	E	R	TRT[2]	BW[3]	Int.[4]
BW, kg	102.7	104.8	152.0	151.8	205.0	204.7	255.0	255.8	303.8	299.8	359.7	361.2	1.00	< 0.01	< 0.71
Days alive	81.3[a]	113.2[b]	133.7[a]	187.8[b]	182.8[a]	264.5[b]	231.2[a]	316.8[b]	280.0[a]	378.0[b]	338.2[a]	466.7[b]	< 0.01	< 0.01	< 0.01
PAR[5] wt., g	17.5	30.3	62.7	122.2	158.0	275.1	315.3	393.6	367.3	535.7	551.7	715.1	< 0.01	< 0.01	0.27
DNA, mg	33.5	63.0	129.5	276.5	354.3[a]	708.7[b]	623.5[a]	985.7[b]	710.7[a]	1121.0[b]	848.9[a]	1351.5[b]	< 0.01	< 0.01	0.10
DNA[6], mg	751.7	644.6	623.2	537.8	637.2	641.2	698.9	693.7	576.7	566.6	465.4	416.9	0.75	0.27	0.99
DNA, mg/kg BW	0.33	0.60	0.85	1.82	1.73	3.44	2.44	3.85	2.34	3.72	2.35	3.74	< 0.01	< 0.01	0.32
DNA[7], mg/kg BW	3.22	2.94	2.84	2.87	2.87	3.17	2.74	2.67	1.80	1.49	0.80	-0.03	0.68	< 0.01	0.64
Fat[8], %	12.48	15.07	19.77	19.64	27.09	32.10	39.61	40.83	44.91	44.75	52.52	47.55	0.18	< 0.01	0.47
CP[8], %	10.23	10.06	9.75	10.36	9.36	9.12	8.48	8.85	9.03	8.68	7.06	8.34	0.40	< 0.01	0.58
OH-Pro[9], mg/g tissue	2.81	2.84	3.11	3.45	3.15	2.52	2.02	3.07	3.19	3.80	3.21	4.92	0.12	0.10	0.42

[a,b]Means with uncommon superscripts within a common slaughter weight are different at $P < 0.05$.
[1]Treatment R - restricted nutrient intake; Treatment E - elevated nutrient intake.
[2]Treatment.
[3]Slaughter weight.
[4]Interation of main effects. Interaction means were separated using the PDIFF options for comparisons within slaughter weight groups when the *F*-test indicated a potential interaction ($P < 0.20$).
[5]Parenchyma.
[6]Total DNA analyzed with days alive as a covariate. Significance of days alive as a covariate term, $P < 0.01$.
[7]DNA / kg body weight analyzed with days alive as a covariate. Significance of days alive as a covariate term, $P < 0.01$.
[8]As is basis.
[9]Hydroxyproline on an as is basis.

Table 5. Effect of prepubertal nutrient intake on mammary fat pad development from birth to 350 kg. All values represent the right hemigland.

	Slaughter weight, kg														
	100		150		200		250		300		350				
	Treatment[1]												F-test P Value		
	E	R	E	R	E	R	E	R	E	R	E	R	TRT[2]	BW[3]	Int.[4]
FP[5] wt., g	116.9	88.9	214.0	147.2	381.7	370.3	691.7[a]	379.3[b]	920.0[a]	580.2[b]	1125.8[a]	710.0[b]	< 0.01	< 0.01	< 0.01
DNA, mg/g tissue	0.63	0.87	0.56	0.70	0.40	0.46	0.35	0.50	0.34	0.38	0.39	0.51	< 0.01	< 0.01	0.76
DNA, mg	73.0	65.6	116.6	96.8	149.8	160.8	236.6	192.0	298.9	215.9	539.0[a]	323.7[b]	0.03	< 0.01	0.19
Fat[6], %	55.15[a]	35.98[b]	60.82[a]	47.66[b]	66.35	66.75	71.39	66.88	74.06	66.75	77.81	71.35	< 0.01	< 0.01	0.02
CP[6], %	8.95[a]	12.32[b]	7.70[a]	10.11[b]	6.88	6.55	5.58	6.58	5.43	6.40	4.49[a]	6.21[b]	< 0.01	< 0.01	0.04

[a,b]Means with uncommon superscripts within a common slaughter weight are different at $P < 0.05$.
[1]Treatment R - restricted nutrient intake; Treatment E - elevated nutrient intake.
[2]Treatment.
[3]Slaughter weight.
[4]Interation of main effects. Interaction means were separated using the PDIFF options for comparisons within slaughter weight groups when the F-test indicated a potential interaction ($P < 0.20$).
[5]Fat Pad.
[5]As is basis.

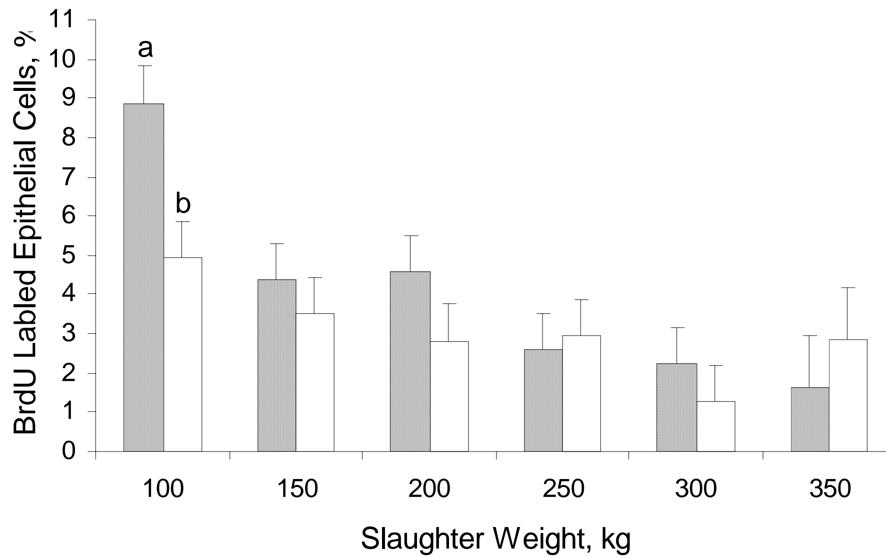

Figure 1. Least square means of BrdU labeling by mammary epithelial cells among prepubertal heifers across six slaughter weights raised on an elevated (hashed bars) or restricted (open bars) plane of nutrition. Significance of main effects and their interaction: treatment, $P = 0.08$; slaughter weight, $P < 0.01$; interaction, $P = 0.16$. Within slaughter weight, a and b are different at $P < 0.05$.

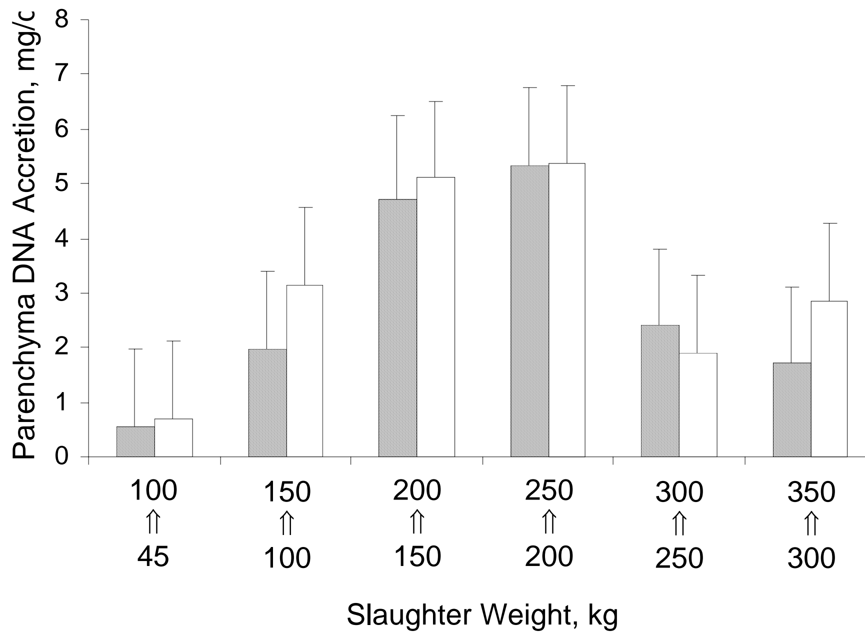

Figure 2. Least square means of parenchyma DNA accretion rates between slaughter weights among prepubertal heifers raised on an elevated (hashed bars) or restricted (open bars) plane of nutrition. Significance of main effects and their interaction: treatment, $P = 0.63$; slaughter weight, $P < 0.01$; interaction, $P = 0.99$.

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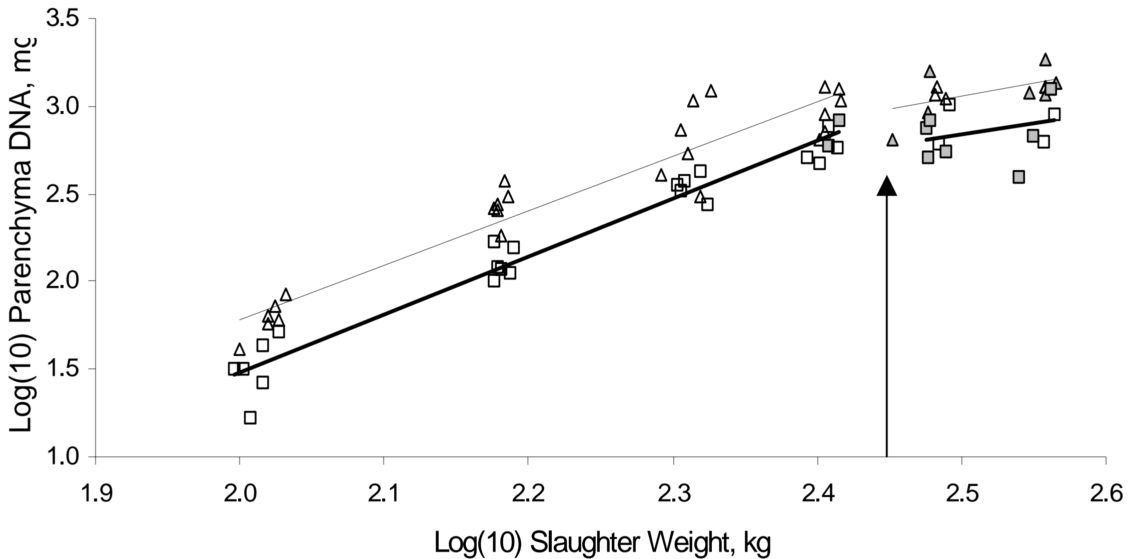

Figure 3. Prepubertal mammary parenchyma development in Holstein heifers on an elevated (□) or restricted plane of nutrition △). Heifers that were pubertal at slaughter are represented by filled square (■, elevated plane of nutrition) or triangle▲ , restricted plane of nutrition). Average body weight at puberty for all heifers is also indicated (arrow). Data are presented as log(10) body weight at slaughter versus log(10) parenchyma DNA at slaughter. Within treatment, data are grouped into the allometric and isometric phase of prepubertal mammary development. Within the allometric phase, regression for the restricted plane of nutrition heifers (light line) is $y = 3.113x - 4.4425$, $R^2 = 90.7\%$ and for the elevated plane of nutrition (heavy line) is $y = 3.2852x - 5.086$, $R^2 = 96.0\%$. Within the isometric phase, regression for the restricted plane of nutrition heifers is $y = 1.4425x - 0.5519$, $R^2 = 30.0\%$ and for the elevated plan of nutrition is $y = 1.2756x - 0.3469$, $R^2 = 10.1\%$. In all cases, x is log(10)body weight (kg) and y is log(10) parenchyma DNA (mg).

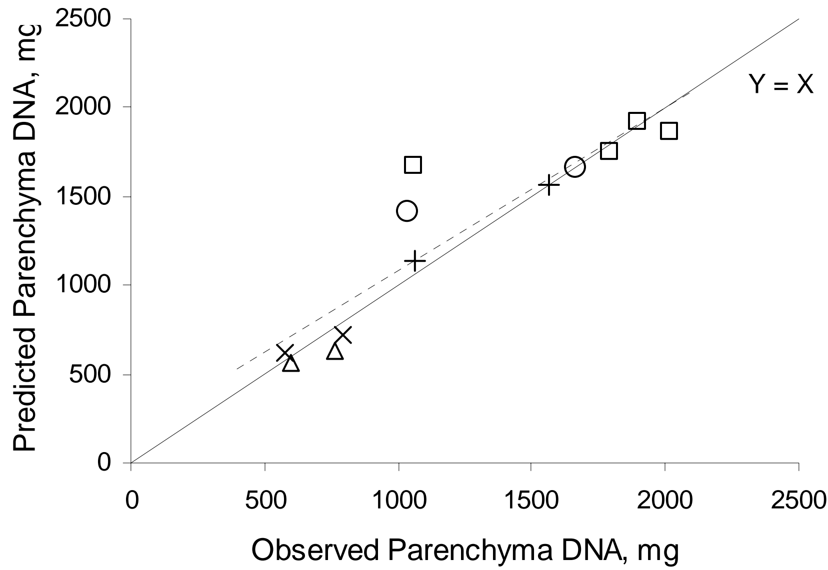

Figure 4. Evaluation of the prediction of "normal" and "diet impaired" prepubertal parenchyma development in Holstein heifers. The data points are predicted versus observed. Observed data are from previously published papers [Pritchard et al., 197△, ( ); Sejrsen et al., 1982, (+); Petitclerc et al., 1984, (X); Capuco et al., 199□( ); and Mäntysaari et al., (1995), (○)]. Predicted values were generated using the mean daily DNA accretion rate determined in the current study and the average age at slaughter as published in the respective papers. Slope of predicted verses observed (dashed line) is 0.92, $r^2 = 83\%$ ($P < 0.01$).