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Honors Option Thesis

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GROWTH AND ENDOCRINE PATTERNS OF CLONED AND NONCLONED BRANGUS HEIFERS

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Abstract

This study monitored the growth and endocrine patterns of cloned (C; n=6), noncloned (NC; n=6) and single cloned (SC; n=2) Brangus females from 7 to 22 months of age. The cloned heifers were produced from the same embryo via a nuclear transfer procedure. Two clones were generated from the blastomeres from one embryo, and the four other clones were generated from the blastomeres of another single embryo. Accordingly, the cloned animals were divided into two sibling groups depending on their origin. Noncloned Brangus heifers were grouped with cloned heifers on the basis of similarity in age and birth weight to the cloned animals and served as controls. Single clone heifers were produced using the same nuclear transfer procedure as the aforementioned clones; however, these two individuals were produced from separate, unrelated embryos and served as nuclear transfer controls. Two sibling cloned heifers comprised Group I-A, and four sibling cloned heifers comprised Group I-B. Two unrelated noncloned heifers with similar age and birth weight to clones in Group I-A comprised Group II-A. Likewise, four unrelated noncloned heifers with similar age and birth weight to clones in Group I-B comprised Group II-B. The two single clone heifers comprised Group III-A.

All calves were weaned at 7 months of age and placed on the same bermudagrass pasture with access to minerals and salt. Post-weaning body weights and hip heights were recorded, and blood samples were collected via jugular venipuncture from each heifer once per month (30 days apart) for 15 months. Plasma samples were subjected to RIA procedures for insulin, thyroxine (T4) and insulin-like growth factor-I (IGF-I).

The variability in birth weight, hip height and three plasma hormone concentrations was determined by two methods of calculating the standard error of the mean (SEM). The average of the monthly SEM for insulin, T4 and IGF-I plasma concentrations for all cloned heifers (Groups I-A and I-B) was 1.08, 0.25 and 11.91, respectively. The average of the monthly SEM for insulin, T4 and IGF-I plasma concentrations for all noncloned control heifers (Groups II-A and II-B) was 1.36, 1.00 and 24.28, respectively.

Variability was also determined by calculating the overall SEM. The average of the overall SEM for insulin, T4 and IGF-I plasma concentrations for all cloned heifers (Groups I-A and I-B) was 0.55, 0.27 and 6.46, respectively. The average of the overall SEM for insulin, T4 and IGF-I plasma concentrations for all noncloned control heifers (Groups II-A and II-B) was 0.76, 0.43 and 10.24, respectively.

A divergence in weight was noted between clones in Group I-A between 9 and 22 months of age, with C1 out-gaining the second clone, C2, and the control heifers by nearly 90 kg. Body weights of C1, C2, and the average weight of the NC1 and NC2 were: 139, 239, 345, 427; 139, 216, 291, 341; and 190, 249, 298,

350 kg, respectively, at 7, 12, 16 and 20 months of age. No such divergence was noted in any animal in the second group of clones and nonclones, nor in any animal in the group of single clones.

In general, it was found that with respect to blood plasma hormone levels, there was less numeric variability between cloned heifers than noncloned control heifers for the post-weaning interval. In contrast, there was a marked divergence in post-weaning body weights (~90 kg) between one cloned heifer and its sibling clone; however, this difference in body weights could not be attributed to plasma hormone levels of the cloned animals.

Keywords: Body Weight, Cattle, Clone, Growth, Hormones, Variability

Introduction

The technique of cloning mammals is gaining interest in the scientific community. One method widely used to produce clones is embryonic cell nuclear transfer (Willadsen, 1986). This technique utilizes the genetic material from an embryonic cell to produce genetically identical copies of that embryo. The nucleus of the embryonic donor cell is microinjected into a mature recipient oocyte that has been enucleated. After electrofusion, the complex becomes a viable diploid oocyte (couplet) capable of embryonic development. To date, this procedure has been successful in several different species of animals. The process of embryonic or fetal cell nuclear transfer has been described for the frog (Briggs and King, 1952), mouse (McGrath and Salter, 1983; Robl *et al.,* 1986, Tsonda *et al.*,1987), rabbit (Stice and Robl, 1989), pig (Prather *et al.,* 1989),

sheep (Willadsen, 1986; Smith and Wilmut, 1989), goat (Baguisi, *et al.* 1999) and cow (Bondioli *et al.,* 1990; Robl *et al.,* 1986; Stice and Keefer, 1993). As techniques for cloning animals improve and greater efficiency is achieved, the implications of cloning on the future of research and livestock production will soon have to be addressed.

The future of cloning is being foreseen to have an impact on animal production and research. Cloning fully developed mammals would allow for the multiplication of phenotypically superior animals (Robl and Stice, 1989). In addition, the uniformity of a cloned population of production animals would be beneficial by introducing greater regularity to feeding patterns and other factors of production (Lamberson, 1994). The uniformity of carcass characteristics would reduce adjustments necessary in the packing procedure, and more efficient plant mechanization could be utilized.

At present, the research benefits of cloned animals seem to be the most promising. Robl and Stice (1989) discussed implications for cloned animals, which include the use of clones as research populations in order to reduce the number of animals necessary in experiments, while maintaining statistical validity. Nicholas and Smith (1983) suggest that in 1 year, a genetic response of 4 years can be realized in pigs by selecting the genetic material donor. After 3 years of growth and development, clones could be selected for superiority in the desired trait. The clones created from this select group would then be 13 years more genetically advanced than an animal population produced by artificial insemination. This acceleration in genetic advancement could continue further

with additional selection and cloning over generations of the population. In pigs, clones have the potential to reduce population size by a percentage that is related to the heritability of the trait (Lamberson, 1994). Data suggest that for experiments dealing with days to slaughter and backfat thickness, populations of pigs can be reduced by 67% and 65%, respectively. A population reduction of only 12% could be realized when studying litter size primarily because this trait is less heritable. Thus, for more lowly heritable traits, the potential benefits from cloning with respect to population reduction attenuate.

At present, monozygotic twins, nature's version clones, are highly prized as research subjects because of the identical nature of their genomes. Bigger (1986) has devised a formula to quantify this contribution of twins to research. The number of randomly chosen animals that can be replaced by an animal from an identical twin pair is given by the formula: $T_3 = 1/(1-P_I)$. P_I is the intraclass correlation coefficient; $2T_3$ is the number of random animals replaced by a monozygotic twin without a loss of statistical efficiency. Thus, benefits gained from the utilization of monozygotic twins could be exponentially enhanced by the production of greater numbers of genetically identical cloned animals.

Furthermore, animal clones would be highly desirable research populations because of the ability to explore interactions between treatment and phenotype of the individual while being able to minimize effects caused by genotypic variation (Robl and Stice, 1989). Because the genotypes of the population would all be identical, phenotypic variation could be attributed to treatment and environment with greater certainty. For this application of cloning

technology to be of real use, it must be determined that clones are indeed phenotypically less variable than their noncloned counterparts. Unfortunately, only a limited amount of data on somatic cell cloned animals is presently available. Monozygotic twins, however, have been studied and reported on extensively in the research literature.

Identical human twins have a higher intraclass correlation coefficient than do dizygotic twins (Biggers, 1986). In the following examples, if intraclass correlation coefficients were not calculated in the study cited, they are reported from calculations made by Biggers (1986). When variability of clinical blood chemical values was evaluated, intraclass correlation coefficients for serum urea nitrogen, bilirubin and phosphorus were 0.53, 0.57 and 0.63, respectively, for human monozygotic twins while values were 0.25, 0.33 and 0.28, respectively, for human dizygotic twins (Havlik *et al.,* 1977). When lipid variability data were calculated, it was found that total cholesterol, high density lipoproteins and very low density lipoproteins had coefficients of 0.81, 0.78 and 0.61, respectively, for monozygotic twins, whereas, dizygotic twin values were 0.65, 0.11 and 0.20, respectively (Milner *et al.,* 1980).

Melnick *et al.* (1979) reported similar findings were reported by with respect to IQ and twins. Intraclass correlational coefficients of 0.84 for white monozygotic twins compared with 0.53 for white dizygotic twins were calculated from reported data. Within-pair correlation of growth parameters from birth to 1 year of age are the greatest for monozygotic twins followed by dizygotic twins,

then regular siblings for the weight, height and head circumference (Livshits *et al.,* 2000).

Through twin studies, it has been found that growth hormone (GH) secretion in humans is largely dependent on genetic factors during wakefulness and, to a lesser extent, over a 24-hour period (Mendlewicz *et al.,* 1999). A higher intraclass correlation coefficient was reported for monozygotic twins than for dizygotic twins. Human monozygotic and dizygotic twins are excellent for comparative studies because with both groups being twins, the individuals experienced a relatively similar physical *in utero* environment prior to delivery.

Many twin experiments have also been conducted with different animals. Gartner and Baunack (1981) reported data from monozygotic and dizygotic mice that calculated into a higher correlational coefficient for body weight in artificially produced monozygotic male and female mice at four postnatal ages. After extensive studies in monozygotic twins in cattle, Hancock (1950) reported an intraclass correlation coefficient for monozygotic twins of 0.98 for both butterfat and casein in milk production. When comparing the growth of monozygotic cattle, Hancock (1951) reported a correlation coefficient of 0.96 and 0.93 for body weight and absolute growth rate, respectively. These data have higher intraclass correlation coefficients than most human studies presumably because animal research twins usually have a more controlled day-to-day environment than occurs with human twins (Biggers, 1986). Finally, it was found that for monozygotic twin cattle, variance for age at first estrus and length of pregnancy

within pairs was half that of dizygotic twins and half-siblings (Donald and Anderson, 1953).

Gartner and Baunack (1981) proposed that environmental factors account for some of the variation within sets of monozygotic twins. In the same study with monozygotic and dizygotic mice, it was postulated that, "each zygote is modified by a nongenetic mechanism before the third cell division in such a way that postnatal maturation times and body weights and possibly other characteristics are considerably different between individuals." It was hypothesized that if the separation of the developing embryo into a monozygotic twin occurs after this third cell division, then the modification would affect each individual in the same way (Gartner and Baunck, 1981).

As previously mentioned, the reduction in variation from monozygotic twins makes them often sought after for use as research subjects. However, it should be noted that there are limitations in the use of monozygotic twins in research studies. The first and most obvious is the limited number of individuals of a single genome that are available. In addition to small populations, it is well documented that twinning (e.g., cattle) can cause complications at parturition, and offspring are often developmentally compromised in their immediate extrauterine life (Gregory *et al.,* 1990; Davis *et al.,* 1989). These studies on beef cattle twins report increases in dystocia, retained placenta and days to first estrus with decreased conception rates and calf birth weights. Adams *et al.* (1993) reported that twins are at higher risk after birth than single calves and have lower rectal temperatures and arterial 0_2 tension.

The use of cloning to produce genetically identical research populations may one day provide a suitable alternative to twinning without the adverse affect on parturition and neonatal condition. Clone goat erythrocyte, lymphocyte, leukocyte and hematocrit counts have been found to be very similar to reported values of similar control goats (Keefer *et al.,* 2001). In the same study, all hematological and biochemical parameters of the clones were in range of those of a reference population of similar young goats.

Swine clones produced using a split-embryo technique have been analyzed for variation in days to slaughter, backfat thickness and litter size (Lamberson, 1994). It was determined that the range of expected values for clones was less variable for all traits than was the range for full-siblings and unrelated animals. In this study, the advantage of clones over unrelated animals and, to a lesser extent, over full siblings was most evident for days to slaughter and backfat thickness. Intraclass correlation coefficients for the clones in this experiment were 0.66, 0.64 and 0.15 for days to slaughter, backfat thickness and litter size, respectively.

However, some clones have been found to be more variable than monozygotic twins for some parameters. Gartner *et al.* (1998) reported a high degree of variation within nuclear transfer cattle clones aged 1 to 4 months for 12 body measurements. In this study, the clones had the same or larger variability percentages than did artificially produced monozygotic twins and even full siblings produced by embryo transfer. The reduced variation noted in artificially produced monozygotic twins implanted into different surrogate mothers was not

evident in the cloned calves. The authors conclude that the variation in animals with identical genotypes may be a biologically normal occurrence that is noted in inbred animals. It was theorized that the manipulations necessary in producing the clone may alter the interaction between nucleus and ooplasm or that DNA expression is affected by properties of the ooplasm or heteroplasmic mitochodrial DNA.

Although split-embryo clones demonstrated reduced variability for three traits in swine (Lamberson, 1994), cloning at this stage of technical development has its share of associated complications. It has been further observed that cloned animals have a high variability in birth weights, with many cloned calves having abnormally high birth weights and reduced neonatal survivability (Wilson, *et al.,* 1995; Garry *et al.,* 1996). In a comparison of clones produced by nuclear transfer to calves produced by embryo transfer and artificial insemination/natural mating, clones were 4 to 12 times more variable in birth weight (Wilson *et al.,* 1995). However, by day 205 and day 365, the body weights of the clones were similar in variability to embryo transfer and natural mating calves. In this study, birth weight variation was attributed to the cloning procedure. Analysis of plasma metabolite levels, both *in utero* and postpartum, indicate differences in metabolism of energy in the fetal development of clones relative to naturally produced offspring (Garry *et al.*, 1996). Bovine fetuses (late gestation) that were produced by nuclear transfer cloning had significant differences ($P < 0.10$) in blood plasma concentrations of insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II) and PO_2 when compared with nonclone control fetuses.

In this study, moderate differences were noted in plasma concentration of T3 ($P = 0.26$), insulin ($P = 0.21$) and glucose ($P = 0.34$). Blood plasma analysis of postnatal clones revealed T3 and T4 concentrations of clones to be greater ($P < 0.01$) and insulin concentration to be less ($P < 0.01$) than concentrations of a nonclone reference group (Garry *et al.,* 1996). The postnatal results suggest "an abnormality of *in utero* energy regulation, causing abnormal growth rate and poor preparation for extrauterine life." These limitations of clones must be overcome and corrected before clones will be suitable for large-scale use as research models.

For clones to be utilized as candidates to decrease necessary research populations, it must be established that their genotypic correspondence does, in fact, lead to phenotypic similarity, or at least, to a reduction in biological variation. This is the major objective of the present study. Three blood plasma hormones were chosen to serve as parameters for the measurement of phenotypic variability in cloned, noncloned and single cloned Brangus heifers. The hormones chosen were insulin, thyroxine and IGF-I. These three parameters are well documented for their relationship to the growth and body weight of livestock. Insulin has been found to be positively related to feed intake and body weight, and a greater insulin secreting ability has been linked to a greater degree of fat deposition (Verde and Trenkle, 1987). The authors also report that growth hormone regulation in cattle to be negatively related to insulin level. The same study reports that thyroxine is positively related to feed intake, and influences growth hormone function. In beef cattle, IGF-I may be an indicator of nutritional

status, and it links GH to anabolism, growth and production (Richards *et al.,* 1995). Serum levels of IGF-I are positively correlated with energy balance (Spicer *et al.,* 1990) and are correlated with skeletal growth and cell function (Richards *et al.,* 1995).

In addition to the hormone parameters, variability was determined for body weight and hip height measurements for these three groups of animals for a period of 15 months. My null hypothesis then, is that a reduction in variability of clones produced by nuclear transfer can not be considered to be a foregone conclusion. Recent studies of mitochondrial DNA (mtDNA) suggest that the expression of mtDNA may make a contribution to the phenotype of an individual (Hutchin and Cortopassi, 1995) and, therefore, could lead to increased variation between clones.

Materials and Methods

Comparisons of animal body weight, hip height, plasma insulin, thyroxine and IGF-I levels were made after weaning between cloned, noncloned and single clone Brangus heifers. The comparisons were carried out for a period of 15 months post-weaning. Group I-A consisted of two clones produced from cells from the same embryo. Group I-B consisted of four clones produced from cells from another single embryo. Calves in Group I-A were born 1 day apart, and calves in Group I-B were born within a period of 11 days. The average birth weight of the cloned heifers in Group I-A was 43.4 ± 4.3 kg, and for Group I-B, the average birth weight of clones was 36.9 ± 3.4 kg. At weaning, the average

weight of clone calves in Group I-A was 138.6 ± 0.0 kg, while the average weight of Group I-B was 163.6 ± 8.9 kg. Average hip height at weaning for Groups I-A and I-B was 90.8 ± 0.8 cm and 89.1 ± 1.1 cm, respectively.

Group II-A consisted of two unrelated noncloned Brangus heifers of similar birth dates and birth weights to clones from Group I-A. Group II-B consisted of four unrelated noncloned Brangus heifers of similar birth dates and birth weights to clones in Group I-B. The calves in Group II-A were born 8 days apart, with calves in Group II-B being born within a period of 75 days. The average birth weight of Group II-A was 37.5 ± 3.4 kg. The average birth weight of Group II-B could not be determined due to a missing birth weight for one heifer. Average body weights at weaning were 189.8 ± 5.7 kg and 180.7 ± 13.9 kg for Group II-A and Group II-B, respectively. Average hip heights for Group II at weaning were 91.0 ± 0.5 cm for Group II-A and 88.5 ± 2.0 cm for Group II-B.

All calves, Groups I, II and III, were weaned in the same month and were in good body condition when placed on the same bermudagrass pasture with access to minerals and salt *ad libitum.* All calves were weighed and measured on a monthly basis. Along with monthly measurements, blood samples were collected via jugular venipuncture into heparinized tubes. Blood samples were centrifuged and plasma was isolated and stored at -20° C until assayed for concentrations of thyroxine, insulin and IGF-I. Hip height, body weight and blood samples were collected for a period of 15 months after the initial weaning interval.

Insulin concentrations were determined for each plasma sample. Following the procedure detailed in the Coat-A-Count kit purchased (Diagnostic Products Corporation, Los Angeles, CA), 200 μL of sample was diluted with 250 μL of saline solution using an ICN Digiflex pipettor. Then, 1.0 mL of ^{125}I Insulin was added to each tube, and the tubes were vortexed using a Baxter multi-tube vortexer. The samples were incubated for 3 hours at room temperature then decanted into a radioactive waste container. Samples remained inverted for ~3 minutes then visible moisture at the opening of the tube was blotted with a clean paper towel. The prepared tubes were then loaded into a Packard Auto-Gamma Counter where the level of radioactivity was detected. A group of 7 standard concentration tubes were prepared as per manufacturer specifications. All insulin samples were assayed in duplicate.

Blood plasma assays were carried out using a commercial standard radioimmunoassay (RIA) procedure for thyroxine (T_4 MAb; ICN Pharmaceuticals, Orangeburg, NY). An ICN Digiflex pipettor was used to dilute 25 μL of sample with 100 μL of saline. After the diluted plasma sample was pipetted into commercially prepared antibody-coated tubes, 1.0 mL of ^{125}I T_4 tracer was added to each tube. The solution was mixed gently using a Baxter multi-tube vortexer and incubated at room temperature for 60 minutes. At the end of the incubation period, the solution was decanted into a waste container and the tubes kept in an inverted position for ~3 minutes to allow for sufficient drainage. Any residual liquid around the mouth of the tube was blotted with a paper towel. The tubes were then placed into a Packard Auto-Gamma Counter which detected the level

of radioactivity in each tube. Samples were tested in duplicate, and again, 5 standard concentrations of T_4 were prepared and counted simultaneously alongside the plasma samples.

IGF-I concentrations were determined using the procedure described by Sticker, *et al.* (1995). Briefly, 300 μL of a standard glycine-HCl mixture was added to borosilicate glass tubes, and then 200 μL of each plasma sample was pipetted into the tube. Tubes were vortexed in a SMI Model 2600 multi-tube vortexer, covered, labeled and placed into a refrigerator at 4° C for 24 hours. After this incubation period, 2.5 mL of phosphate-buffered saline – ethylene diaminetetraacetate (PBS-EDTA) was to each tube and tubes were again vortexed. This first series of steps constitutes the sample extraction.

The next day, a second set of tubes was labeled and 150 μL of IGF-I Assay Buffer was pipetted into them. A 50 μL volume of each sample extraction tube was pipetted into the newly prepared assay tubes. This step is conducted twice for each sample extraction tube to create a duplicate assay tube set. To sample tubes, 200 μL of IGF-I Antiserum was then added. Samples were then vortexed and incubated at 4° C for a period of 48 hours. Upon removal from refrigeration for 48 hours, 200 μL of ^{125}I IGF-I solution was added to the sample tubes. Again, tubes were vortexed then refrigerated (4° C) for 48 hours.

After the incubation period, 200 μL of 8X normal rabbit serum (NRS) was added to tubes, followed by the addition of 8X antirabbit gamma globulin made in the pony (pARGG). Samples were once again vortexed then incubated under refrigeration for 24 hours. Finally, tubes were removed from refrigeration and

spun in a Beckman J6-HC centrifuge at 1,500 x *g* for 30 minutes. At the termination of centrifugation, tubes were removed and decanted, then inverted to allow the moisture to exit the tube. Tubes were then blotted with a paper towel, and 1 mL of cold PBS is added to all tubes. The process of centrifugation, decanting and blotting was repeated. These prepared tubes were then loaded into a Packard Auto-Gamma Counter and the level of radioactivity was detected.

Statistical Analysis

Variability in hip height, body weight and hormone concentration over the 15-month duration of the study was determined in two ways. The first measure of variability is referred to as monthly SEM and was calculated for all groups (Group I-A, I-B, II-A, II-B and III-A). In this first calculation of variability, standard error of the mean (SEM) was determined from each monthly data value within animal type (clone, control or single clone) over the 15-month duration of the project. This measure of variation expresses the variation between animals from clone, nonclone and single clone categories.

A second calculation of variability was used, and was again calculated for each group of heifers. It is referred to as overall SEM, and this SEM was calculated for each animal individually over the 15-month duration. The SEM values were averaged within animal type (clone, control or single clone). This measure of variation gives the variability of individuals within a group over time.

The two measures of variation were calculated and compared across classes (clone, control and single clone). Summary data was also calculated that relates the overall average variability of the three classes of animals.

Results

All calves were in good health and body condition at the onset of the study. The first measure of variability reported is the monthly SEM. The mean body weight of cloned heifers in Group I-A was numerically more variable (SEM = 21.06) (Figure 6) than that of noncloned control heifers in Group II-A (SEM = 9.24) (Figure 7). SEM was 11.24 for the mean body weight of cloned heifers in Group I-B (Figure 8). This variability was numerically smaller than that of the noncloned control heifers of Group II-B (SEM = 15.47) (Figure 9). Thus, cloned heifers in Group I-A were numerically more variable for body weight than noncloned control heifers in Group II-A, and clones in Group I-B were numerically less variable than their corresponding noncloned controls in Group II-B. Monthly SEM for single clones in Group III-A was 5.40.

Hip height was also recorded for heifers within each group. Clones in Group I-A had a SEM of 0.42 (Figure 10) while controls in Group II-A had a numerically greater SEM of 0.72 (Figure 11). Clones in Group I-B were also numerically less variable (SEM = 0.99) (Figure 12) for hip height than their corresponding controls in Group II-B (SEM = 1.54) (Figure 13). Thus, cloned heifers in Group I-A and Group I-B were numerically less variable for hip height than the respective control heifers (Group II-A and II-B). Single clone heifers in Group III-A had a monthly SEM of 1.34 for hip height.

Concentrations of three hormones in blood plasma were determined, and numeric variability between heifers within groups of clones and nonclones was

calculated. The numeric variability for insulin concentrations was less for clones in Group I-A (SEM = 0.50) (Figure 14) than for controls in Group II-A (SEM = 1.22) (Figure 15). Within Group B, variability was numerically greater between cloned heifers in Group I-B (SEM = 1.67) (Figure 16) than between the control heifers in Group II-B (SEM = 1.50) (Figure 17). Thus, the numeric indicator for variability of insulin concentration was greater for controls in Group A and greater for clones in Group B. For insulin concentration, single clone heifers in Group III-A had a monthly SEM of 1.11.

The same calculation was carried out on thyroxine concentration data. Numeric variability was less in clones in Group I-A (SEM = 0.04) (Figure 18) than nonclones in Group II-A (SEM = 1.23) (Figure 19). Similar to results from Group A, clones in Group I-B were numerically less variable (SEM = 0.46) (Figure 20) than were controls in Group II-B (SEM = 0.76) (Figure 21). Clones (Group I-A and I-B) were numerically less variable than the corresponding controls (Group II-A and II-B). Monthly SEM for single clones in Group III-A was 0.92 for thyroxine concentration.

IGF-I concentration data were analyzed in an identical manner. Cloned heifers in Group I-A were numerically less variable (SEM = 6.80) (Figure 22) than controls in Group II-A (SEM = 34.96) (Figure 23). However, cloned heifers in Group I-B were numerically more variable for IGF-I plasma concentration (SEM = 17.02) (Figure 24) than corresponding controls in Group II-B (SEM = 13.60) (Figure 25). Here again, clones in Group A were numerically less variable than their corresponding controls, but clones in Group B were numerically more

variable than their corresponding controls. Single clone heifers in Group III-A had a monthly SEM value of 16.65.

A second method for calculating the SEM was also used. In this method, SEM was determined for each individual animal over the 15-month duration of the project. The resulting SEM for each animal was averaged within the class to which the animal belonged (clone, control or single clone) and is referred to as the overall SEM.

Clones in Group I-A had a greater numeric variability (SEM = 22.81) (Figure 26) in body weight over time than did controls in Group II-A (SEM = 13.79) (Figure 27). Likewise, in Group I-B, clones were again more numerically variable (SEM = 14.56) (Figure 28) than the Group II-B nonclones (SEM = 13.38) (Figure 29) for body weight. With this measure of SEM, both groups of clones were numerically more variable over time than their respective group of controls. The overall SEM for single clone heifers in Group III-A was 14.09.

The overall SEM was calculated for hip height. Clones in Group I-A were numerically less variable for hip height (SEM = 3.06) (Figure 30) than nonclones in Group II-A (SEM = 3.18) (Figure 31). In Group I-B, clones were again numerically less variable (SEM = 2.94) (Figure 32) than their respective controls in Group II-B (SEM = 3.28) (Figure 33) for hip height. Both groups of controls were numerically more variable than their respective clones for measures of hip height. Single clones in Group III-A had an overall SEM of 2.90 for hip height.

With respect to insulin concentration, clones in Group I-A were again numerically less variable (SEM = 0.24) (Figure 34) than Group II-A nonclone

controls (SEM = 0.41) (Figure 35). The insulin concentration variability was numerically lower for clones in Group I-B (SEM = 0.86) (Figure 36) than for nonclones in Group II-B (SEM = 1.11) (Figure 37), as well. In both Groups A and B, nonclones were more numerically variable than clones for insulin concentration over time. For plasma insulin concentration, the overall SEM for single clone heifers in Group III-A was 0.72.

After plasma concentrations of thyroxine were analyzed, clones in Group I-A were numerically less variable than controls in Group II-A, with a SEM of 0.17 for cloned heifers (Figure 38) and a SEM of 0.48 for control heifers (Figure 39). In Group I-B, clones were again numerically less variable than controls in Group II-B, with a SEM of 0.36 for clones (Figure 40) and a SEM of 0.38 for controls (Figure 41). For thyroxine plasma concentration, clones in both groups were numerically less variable than their respective clones. Single clones in Group III-A had an overall SEM of 0.34 for plasma thyroxine concentration.

For IGF-I plasma concentration, clones in Group I-A were numerically less variable (SEM = 2.36) (Figure 42) than controls in Group II-A (SEM = 9.23) (Figure 43). Similarly, cloned heifer variability measures for IGF-I were numerically lower over time (SEM = 10.56) (Figure 44) than variability measures of control heifers in Group II-B (SEM = 11.25) (Figure 45). In both Groups A and B, clones were numerically less variable than their respective nonclones for plasma IGF-I level over time. The SEM of plasma IGF-I concentration for single clones in Group III-A was 14.31.

In the majority of parameters investigated, cloned animals had a numerically lower monthly and overall SEM when compared with their respective noncloned control groups (See Table 1). When calculating SEM between heifers within their respective class (clone, control or single clone), the two clones in Group I-A were numerically more variable than the two nonclone control heifers in Group II-A for body weight only (See Table 2). The four clones in Group I-B were numerically more variable than the four noncloned controls in Group II-B for plasma insulin and IGF-I concentrations only. For all other monthly measures of the SEM, the group of two cloned heifers and the group of four cloned heifers were each numerically less variable than the control groups of two noncloned heifers and four noncloned heifers, respectively. The monthly SEM of single clone heifers in Group III-A was numerically least variable in hip height and numerically most variable in IGF-I concentration for the three classes of heifers. For all other parameters, single clones in Group III-A had intermediate numerical variability.

SEM was calculated for each individual heifer over the 15-month duration of the project, and this value was averaged to determine overall SEM within the respective class of the heifer (clone, control or single clone) (See Table 3). The two cloned heifers in Group I-A and the four cloned heifers in Group I-B were numerically more variable in body weight than the two nonclone control heifers in Group II-A and the four nonclone control heifers in Group II-B, respectively. In all other parameters, the clone groups of two and four cloned heifers were numerically less variable than their respective control heifer groups of two and

four noncloned heifers for the described overall SEM calculation. Group III-A was numerically the most variable class for hip height and numerically the least variable class for body weight. For all other parameters, single clones in Group III-A had intermediate variability when compared with the other two classes of heifers.

Discussion

When comparing the overall pattern of variability, it appears that cloned heifers were numerically less variable than noncloned heifers for most of the growth parameters measured in this study. The overall variability of the two groups of cloned heifers was numerically less than the average of the noncloned control groups and the single cloned control group for all three of the plasma hormone concentration measurements.

Being that the hormones assayed have been reported to be positively related with body weight and growth (Verde and Trenkle, 1987; Richards *et al.*, 1995; Spicer *et al.*, 1990), one might hypothesize that the body weight of the heifers would reflect the pattern of reduced variation. Because of a divergence in body weight of one cloned heifer (C1), this seems not to be the case for all cloned heifers. As previously stated, a marked divergence in body weight was detected, with one clone (C1) outgrowing its sibling clone (C2) by nearly 90 kg and outgrowing all other heifers in the study by over 50 kg. The cause of this accelerated growth, which began at 10 months of age (after weaning), was not explained by the pattern of growth related hormones evaluated in the present

study. C1 was 3.6 kg heavier at birth than the heaviest of all other subject calves at birth but had the same weaning weight as its sibling clone at 7 months of age. How one clone could outgrow a genetically identical sibling clone by nearly 90 kg remains unexplained at this time.

Recent scientific studies have begun to explore the role that mitochondrial DNA (mtDNA) may perform in the determination of the phenotype of an individual. Because mtDNA makes a crucial contribution to the process of cellular energy metabolism (Tanne, 1999), differences between the mtDNA of the two sibling clones in Group I-A could account for the divergence in body weight of C1. The potential for mtDNA heteroplasmy then becomes an issue worthy of consideration. Indeed, mtDNA dysfunction has been linked to severe phenotypic outcomes. Diseases and abnormalities positively correlated with mutations in mtDNA include Parkinson's disease (Shoffner *et al.*, 1994; Brown *et al.*, 1996), Alzheimer's disease (Brown *et al.*, 1996) and deafness (Shoffner *et al.*, 1994; Hutchin and Cortopassi, 1995).

A recent study by Hiendleder *et al.* (1998) reports that heteroplasmy in mtDNA of male cloned cattle produced by embryonic cell nuclear transfer (the method of cloning used in the present study) is a surprisingly common phenomenon. In this study, 29 clones were created from seven different embryos. The authors noted variation within 6 of the 7 groups of same-embryo cloned cattle and a total of 12 different haplotypes in the 29 cloned individuals. In a different study using 9 sheep produced by fetal cell nuclear transfer, all mtDNA was found to be derived exclusively from the recipient oocyte used in the

cloning procedure (Evans *et al.*, 1999). No mtDNA was found to be contributed by the fetal donor of the somatic cells. The degree of mtDNA variation between the cloned heifers was not determined in the present study.

One hormone suspected to influence *in utero* growth is IGF-II (D'Ercole, 1992). For *in vitro* derived bovine pregnancies, concentrations of IGF-II were increased in maternal blood plasma at 90 days of gestation and were associated with higher birth weights of calves (Bertolini, 2001). In mice, the developmental role of IGF-II was evaluated (DeChiara *et al.*, 1990). After disruption of one allele of the IGF-II gene in embryonic stem cells of mice, a 40% reduction from normal body weight was detected in the mutant mice. Mutants were otherwise normal, and it was concluded that the mutation was expressed during embryonic development. Differences in maternal or fetal IGF-II concentration could be a potential explanation for the slightly increased birth weight of C1; however, the divergence in body weight in the present study was not noted in clone C1 until 10 months of age.

One difficulty encountered in the efficient production of cloned animals via embryonic cell nuclear transfer is called megafauna. Also called "Big Calf" syndrome, the condition results in calves with abnormally large body weights at birth (Willadsen *et al.*, 1991). In this study, several calves weighing over 59 kg (130 lbs) at birth were reported including a few calves with birth weights exceeding 68 kg (150 lbs). This condition led to dystocia and decreased neonatal survivability. No explanation was offered as to the cause of megafauna. C1 in the present study was slightly larger at birth than the other heifers, but not

to such a degree as to be considered to exhibit Big Calf Syndrome. At weaning, C1 had the same weight as its sibling clone. Thus, megafauna is not likely to be a sufficient explanation for the rapid post weaning growth of C1.

No overriding consensus has been reached with respect to cloned animals and their level of intraclass variability. A study using swine reports that clones have a lower intraclass variability than normal siblings (Lamberson, 1994), while others report increased variability within classes of male and female calf clones (Wilson *et al.*, 1995; Garry *et al.*, 1996; Gartner, *et al.*, 1998). The report of decreased variability deals with backfat thickness and days to slaughter in pigs (Lamberson, 1994), whereas reports of increased variability are centered on birth weight and growth characteristics as primary parameters of investigation (Wilson *et al.*, 1995; Gartner, *et al.*, 1998; Garry *et al.*, 1996). The data set on which we report in the present study broaden the scope of observations of variability made within clone groups.

Though no consensus has yet to be established regarding the variability within groups of cloned animals, it is well established that monozygotic twins have phenotypic parameters that are less variable than normal siblings and even dizygotic twins (Gartner and Baunack, 1981). The authors report a significant difference between the components of variance for body weight in monozygotic and dizygotic mouse twins. These data are supported by a study from Mendlewictz *et al.* (1999), who report numerically higher intraclass correlation coefficients in human monozygotic twins for height, weight and pulsatile growth hormone (GH) secretion.

For cattle, a lower within-pair variance has been reported for pairs of monozygotic twins than for dizygotic twins and half-sibling calves (Donald and Anderson, 1953). This study compared the variance in age at first estrus and within groups of monozygotic twin female heifers (n = 16) to that of dizygotic twin females (n = 14) and half sibling females (n = 14) and found that within pair variance was lowest for the monozygotic twins. The study also compared the variance in length of first pregnancy within monozygotic twin heifers (n = 12), dizygotic twin heifers (n = 11) and half sibling heifers (n = 11) and again found the monozygotic twins to have the lowest mean square within pairs. The phenotypic similarities within pairs of monozygotic twins have been attributed to both genotype and environment (Gartner and Baunack, 1981; Guo, 2001). Gartner and Baunack (1981) report that even when monozygotic and dizygotic mouse twins are raised in an identical pre- and post-natal environment, monozygotic mice show fewer within-group differences than dizygotic mice. Guo (2001) reports that in humans, however, greater environmental similarity within groups of monozygotic twins over dizygotic twins can alone account for the decreased variation within monozygotic twin sets. Biggers (1986) concludes that the reliable reduction in variability between monozygotic twins can be capitalized upon with their use as research populations. In fact, Biggers (1986) proposed a formula that would allow for a reduction in the number of experimental animals necessary if monozygotic twins were used.

Further study should be undertaken before definitive conclusions can be drawn about the variability within sets of cloned cattle. In the present study, all

calves were raised on the same bermudagrass pasture environment following weaning. Even with this attempt to provide a constant environment across groups, environmental and other exogenous factors cannot be discounted as potential causes for variability in body weight and hip height between individuals. A larger number of cloned heifers would add to the results and allow for the application of more involved statistical evaluations. However, for this measure to be achieved, the efficiency of present cloning techniques must first be improved upon. Measurements of growth hormone (GH) and IGF-II levels and possibly mtRNA heteroplasmy would provide beneficial data for intensive studies of clone variation.

Further, utilization of a new technique for measuring the level of global genomic expression may provide interesting clues as to whether the level of expression of genes is conserved within groups of cloned animals. This analysis, called a DNA microarray (Lockhart and Winzeler, 2000), allows researchers to test the level of expression of the entire genome of an organism on one or two 1.6 cm^2 glass chips. DNA microarrays are the equivalent of running thousands of Northern blots simultaneously.

Intraclass correlation coefficients for expression of genes could be determined within groups of clones. It is this genetic expression level that ultimately results in the level of phenotypic variation. Any discordant activation of genes that may lead to phenotypic variation would be detected with such an analysis. For example, occasionally female human twins can exhibit Wiedemann-Beckwith Syndrome (WBS), which results in only one of the twins

expressing generalized overgrowth among other symptoms (Oestavik *et al.*, 1995). Nonconserved patterns of X chromosome inactivation have been implicated in the discordant presentation of symptoms within these twin pairs. With the data obtained from a DNA microarray, researchers could carry out more directed evaluations of variation in gene expression within cloned and noncloned subject animals.

Acknowledgment:

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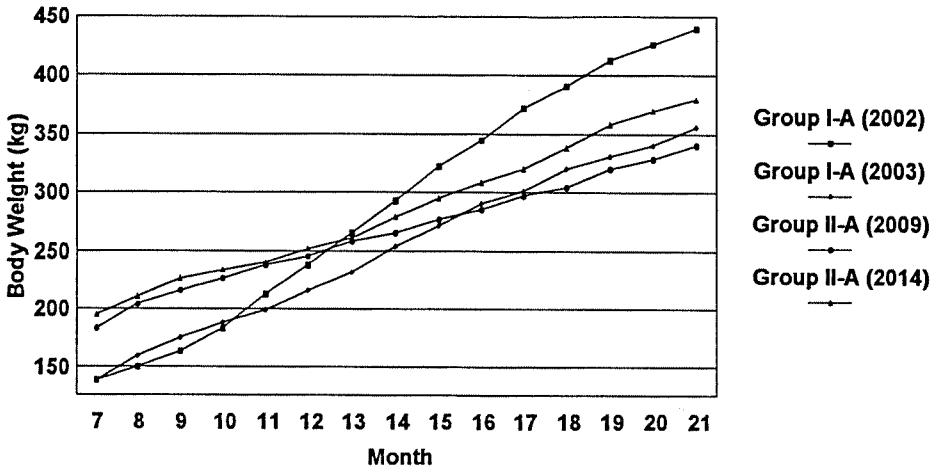

Figure 1. The individual monthly body weight of cloned heifers in Group I-A (n = 2) and noncloned heifers in Group II-A (n = 2).

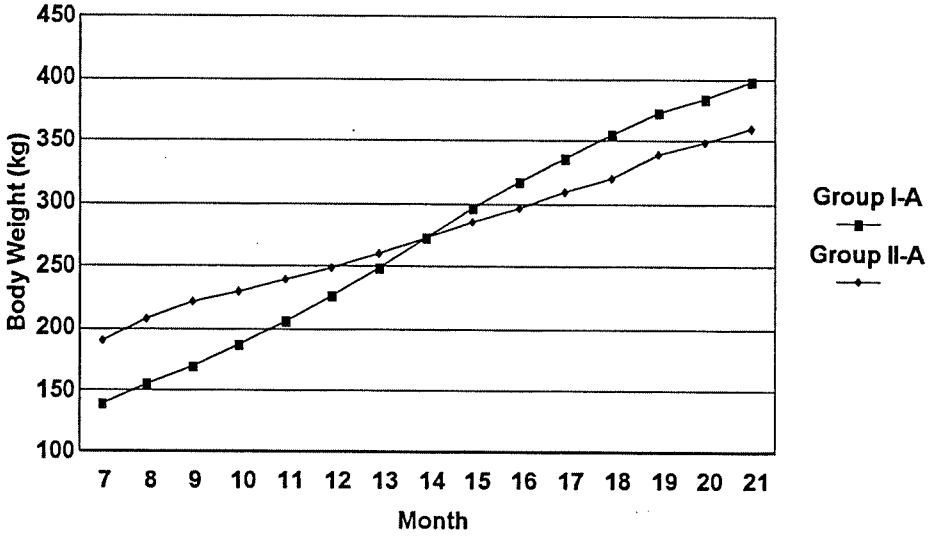

Figure 2. The average monthly body weight of cloned heifers in Group I-A (n = 2) and noncloned heifers in Group II-A (n=2).

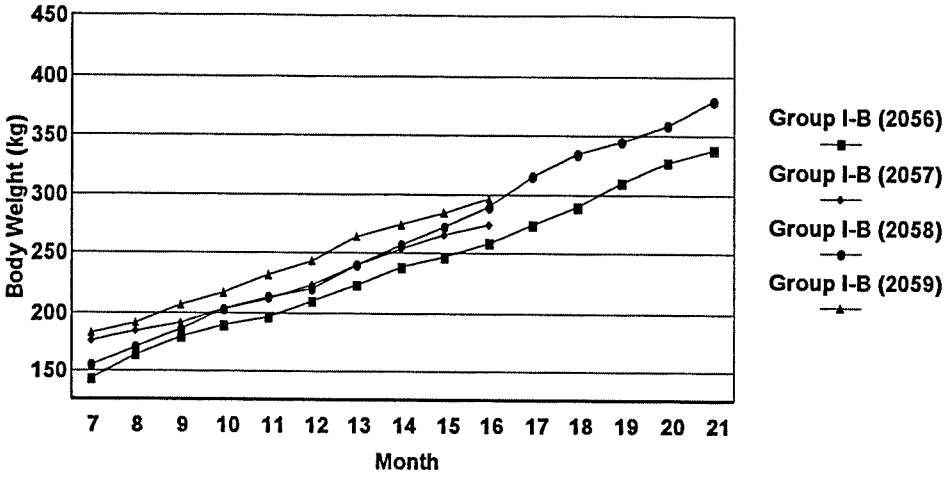

Figure 3. The individual monthly body weight of cloned heifers in Group I-B (n = 4).

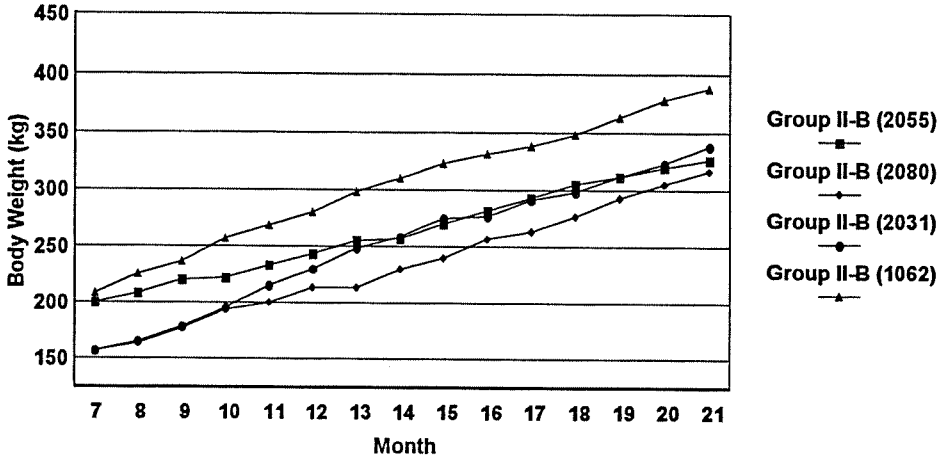

Figure 4. The individual monthly body weight of noncloned heifers in Group II-B (n = 4).

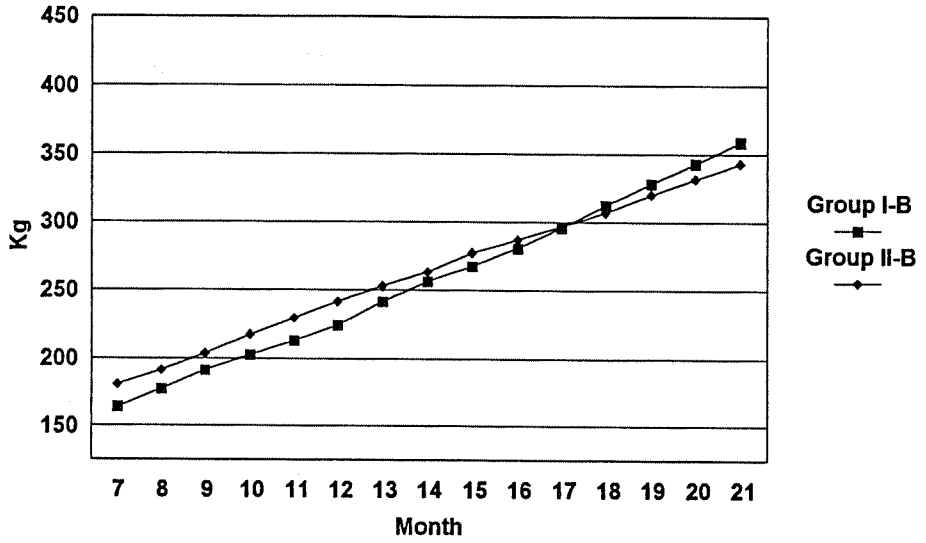

Figure 5. The average monthly body weight of cloned heifers in Group I-B (n = 4) and noncloned heifers in Group II-B (n = 4).

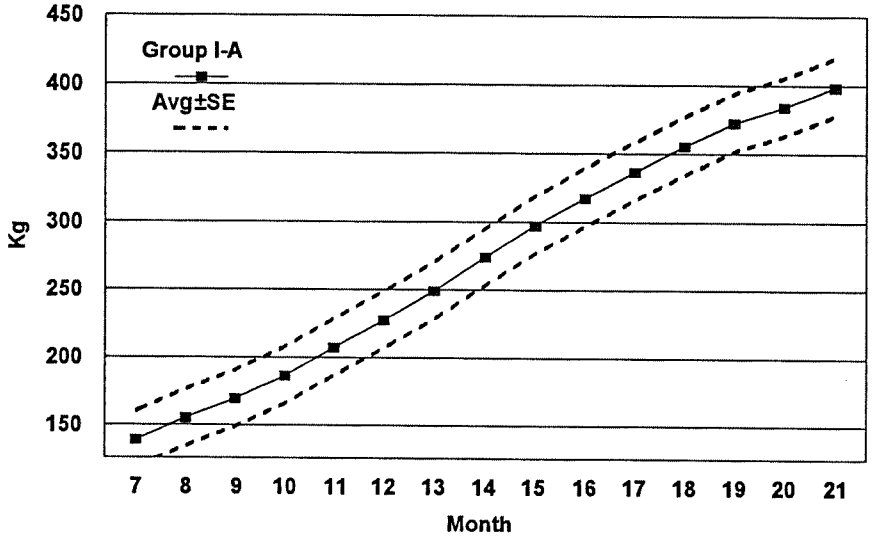

Figure 6. The average monthly body weight of cloned heifers in Group I-A (n = 2) with ± SE (21.06).

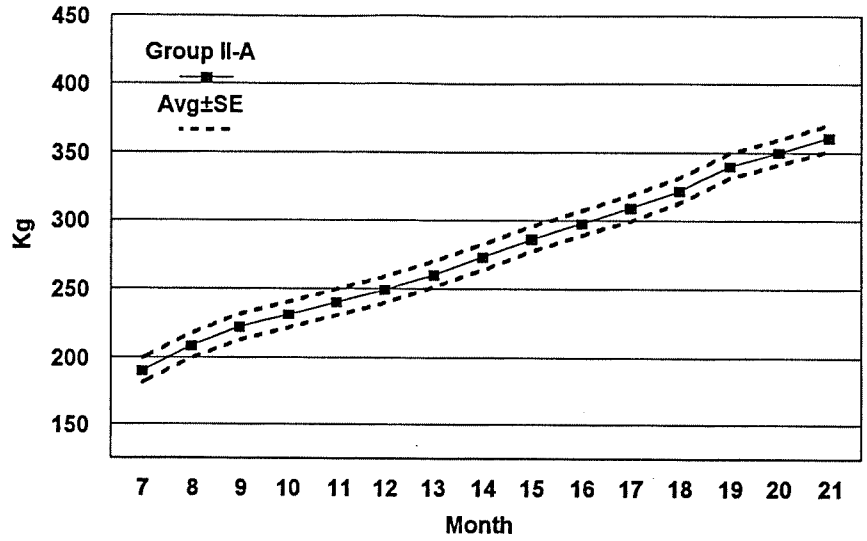

Figure 7. The average monthly body weight of noncloned heifers in Group II-A (n = 2) with ± SE (9.24).

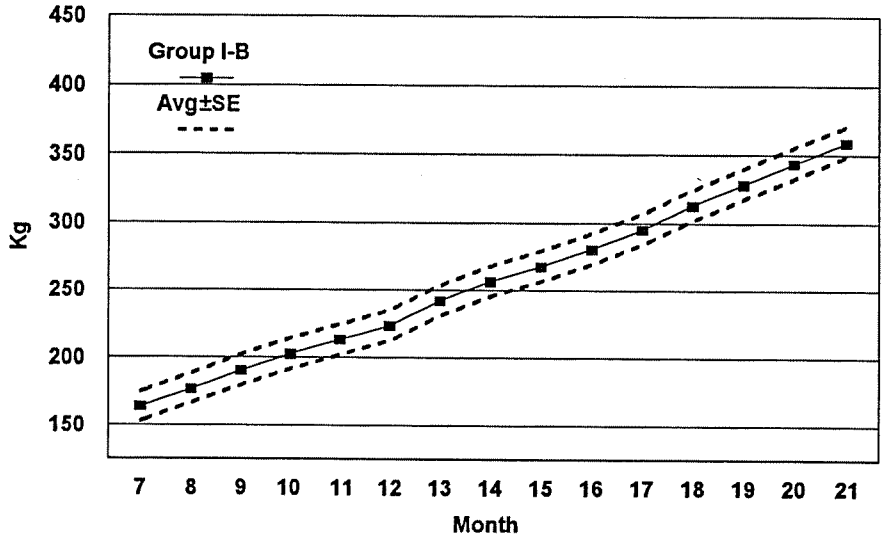

Figure 8. The average monthly body weight of cloned heifers in Group I-B (n = 4) with ± SE (11.24).

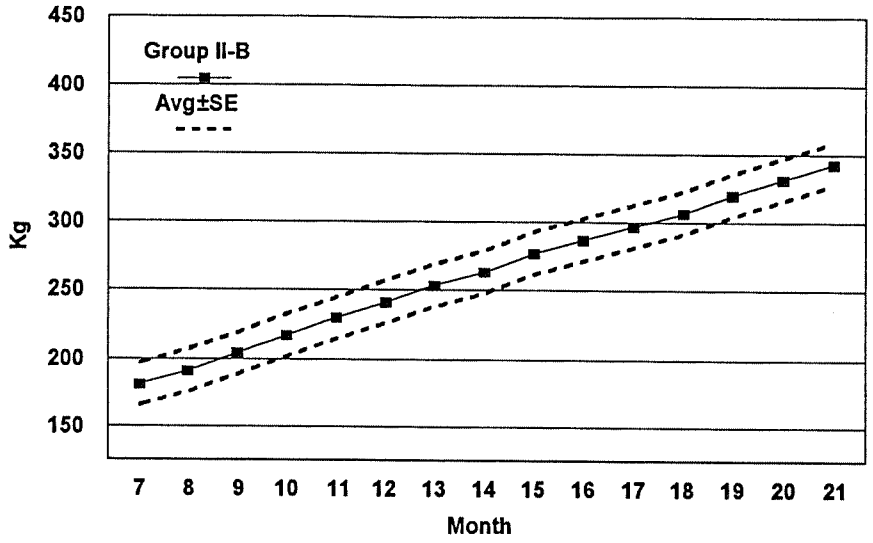

Figure 9. The average monthly body weight of noncloned heifers in Group II-B (n = 4) with ± SE (15.46).

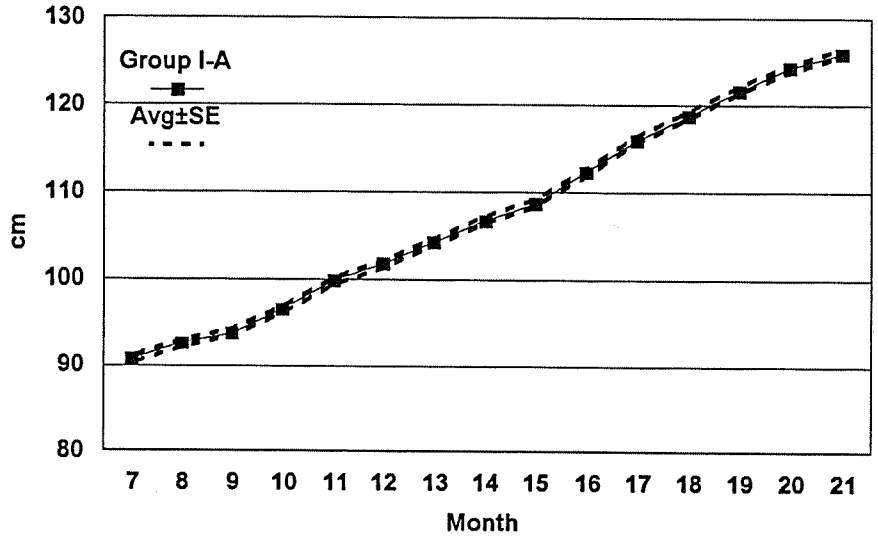

Figure 10. The average monthly hip height of cloned heifers in Group I-A (n = 2) with ± SE (0.42).

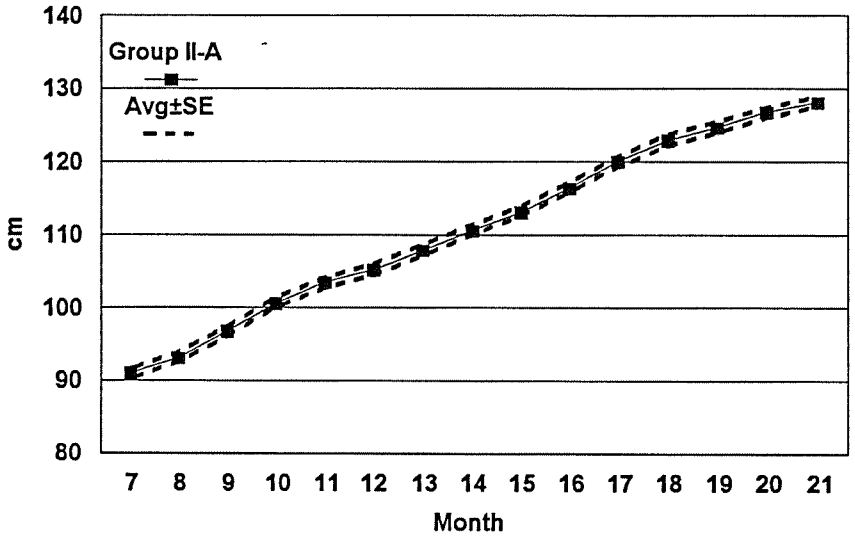

Figure 11. The average monthly hip height of noncloned heifers in Group II-A (n = 2) with ± SE (0.72).

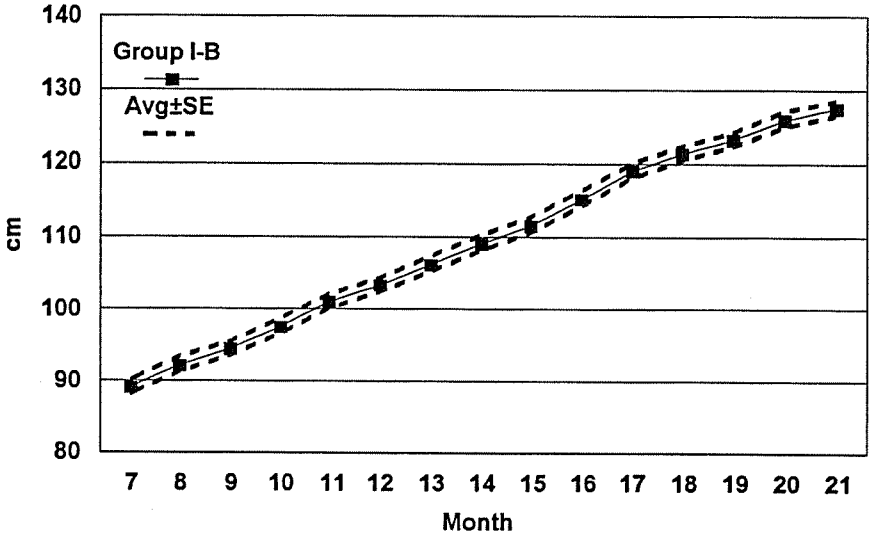

Figure 12. The average monthly hip height of cloned heifers in Group I-B (n = 4) with ± SE (0.99).

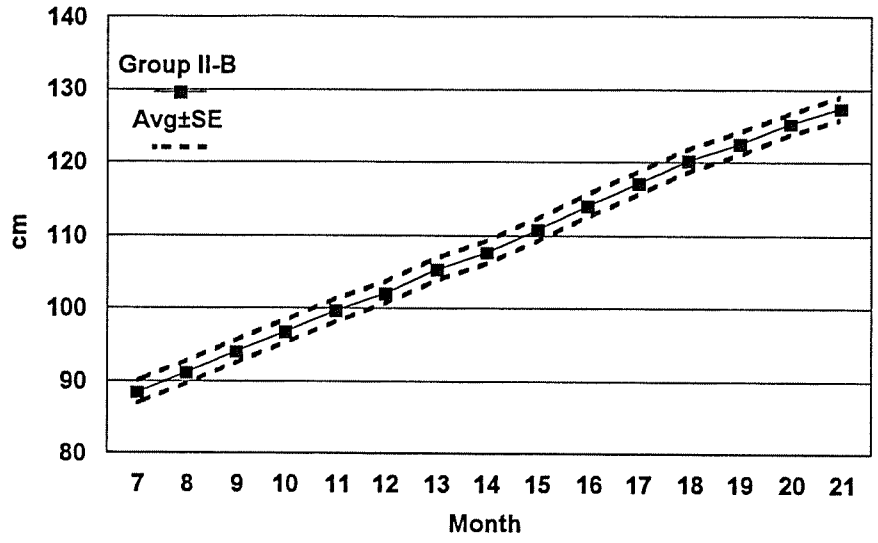

Figure 13. The average monthly hip height of noncloned heifers in Group II-B (n = 4) with ± SE (1.54).

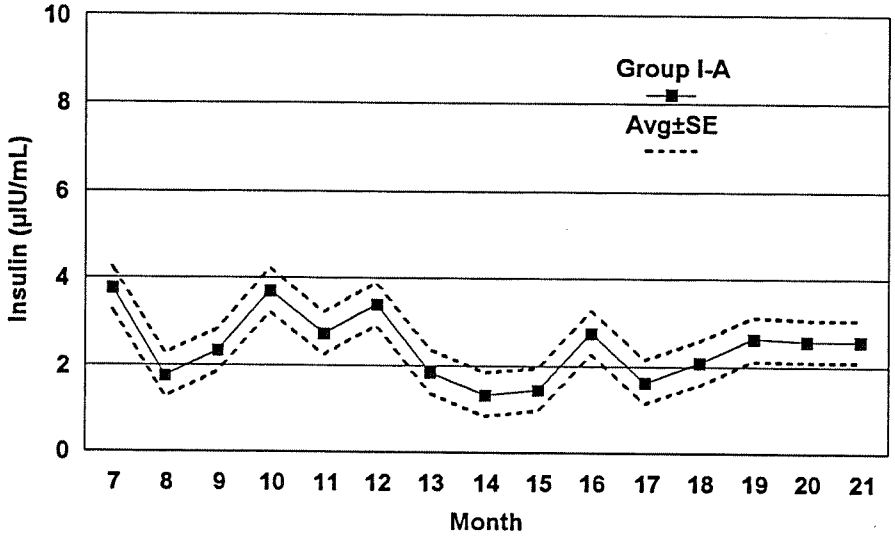

Figure 14. The average monthly insulin level of cloned heifers in Group I-A (n = 2) with ± SE (0.50).

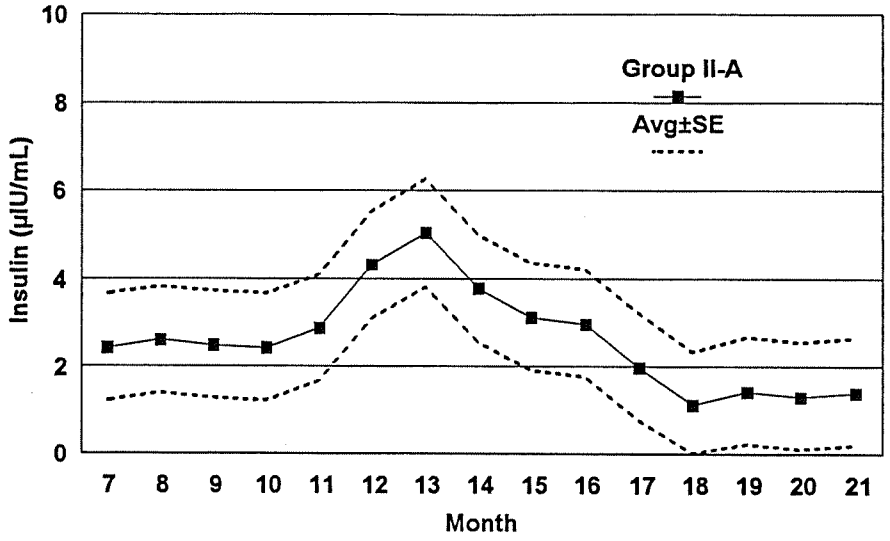

Figure 15. The average monthly insulin level of noncloned heifers in Group II-A (n = 2) with ± SE (1.22).

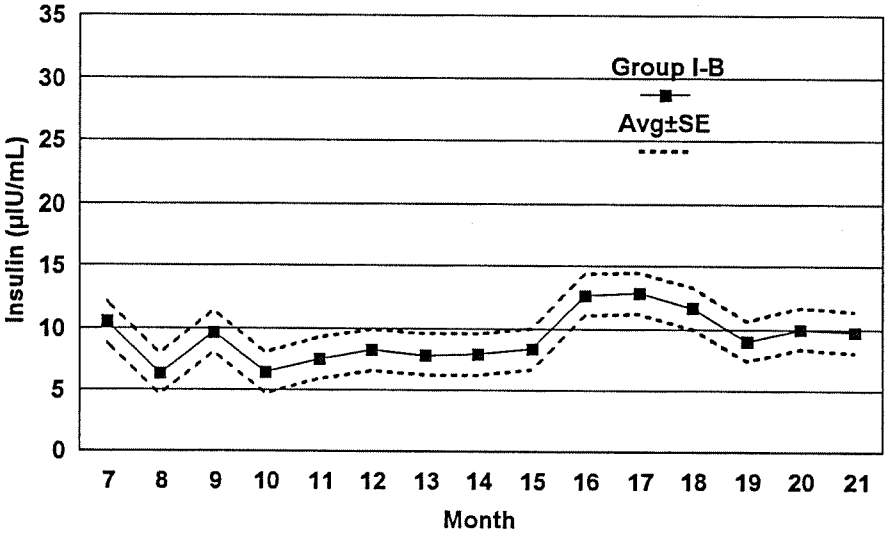

Figure 16. The average monthly insulin level of cloned heifers in Group I-B (n = 4) with ± SE (1.67).

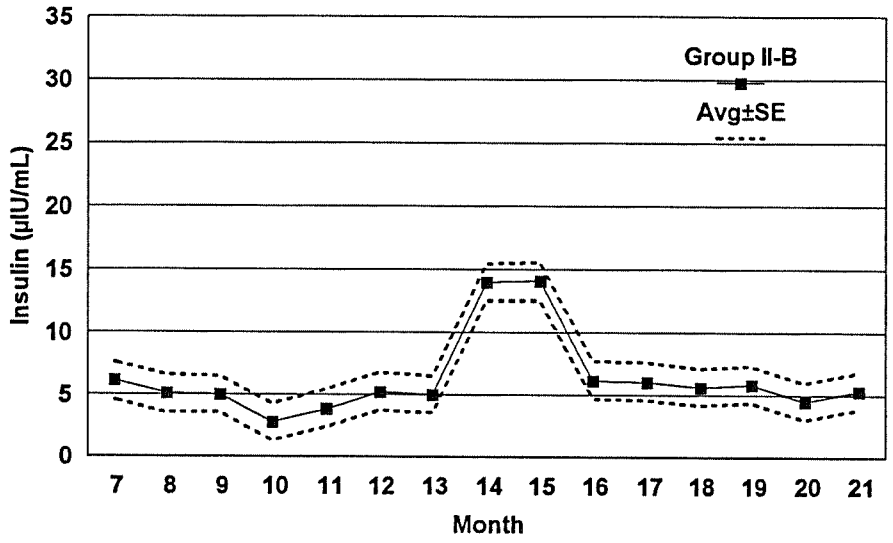

Figure 17. The average monthly insulin level of noncloned heifers in Group II-B (n = 4) with ± SE (1.50).

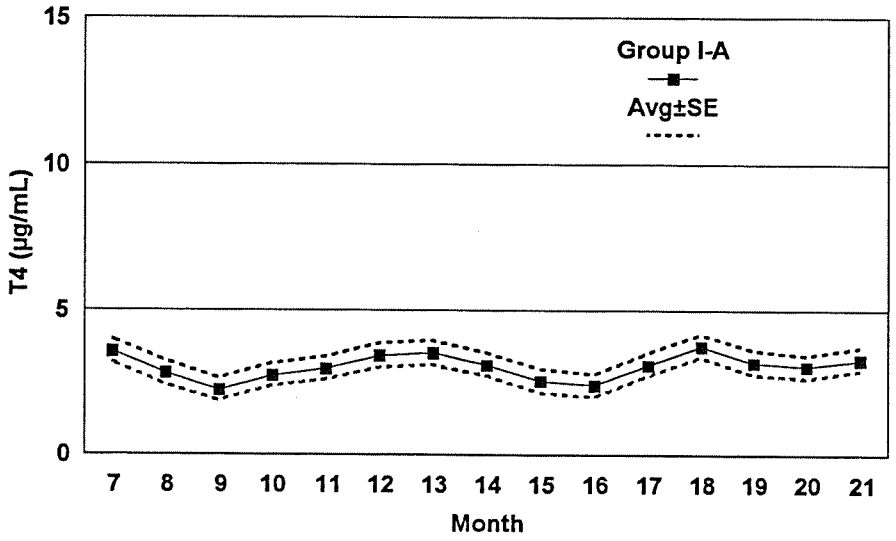

Figure 18. The average monthly T4 level of cloned heifers in Group I-A (n = 2) with ± SE (0.04).

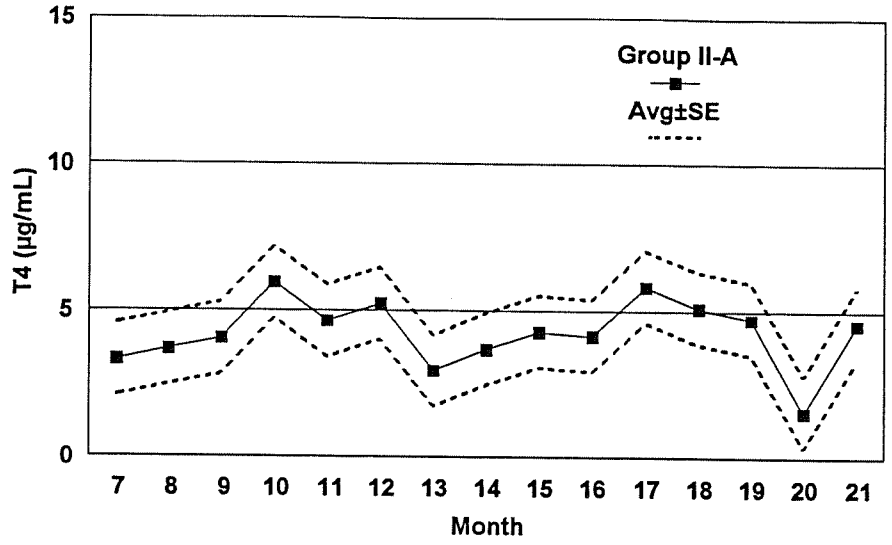

Figure 19. The average monthly T4 level of noncloned heifers in Group II-A (n = 2) with ± SE (1.23).

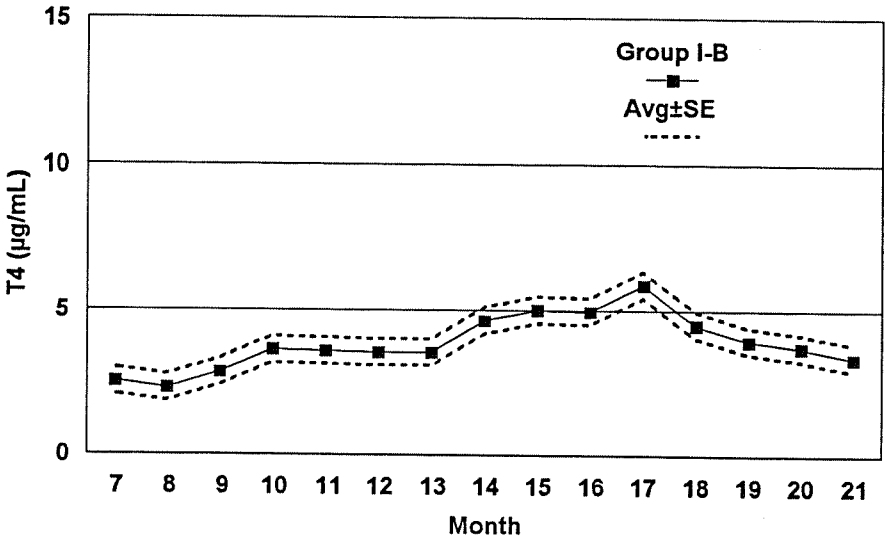

Figure 20. The average monthly T4 level of cloned heifers in Group I-B (n = 4) with ± SE (0.46).

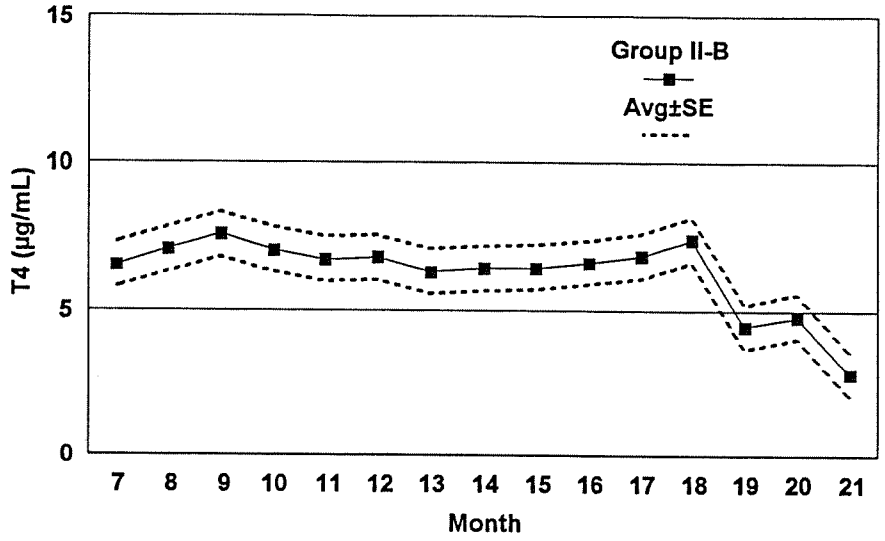

Figure 21. The average monthly T4 level of noncloned heifers in Group II-B (n = 4) with ± SE (0.76).

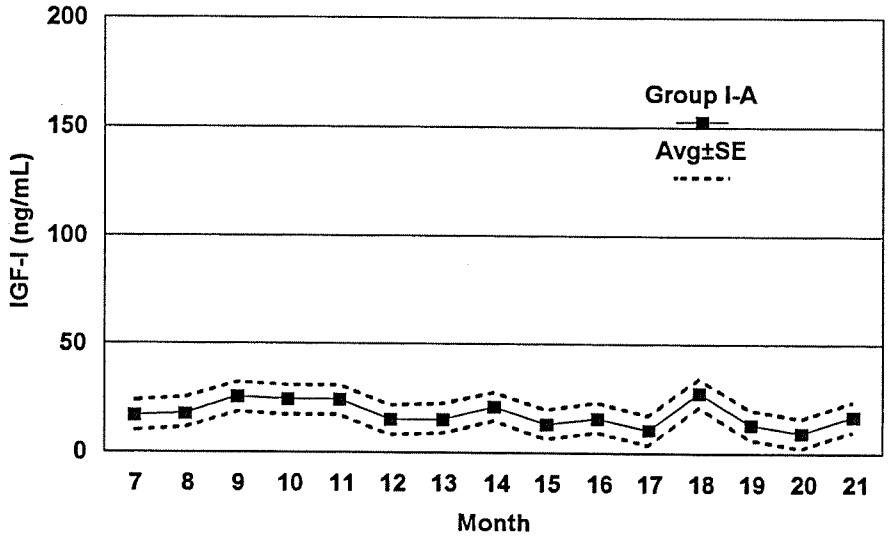

Figure 22. The average monthly IGF-I level of cloned heifers in Group I-A (n = 2) with ± SE (6.80).

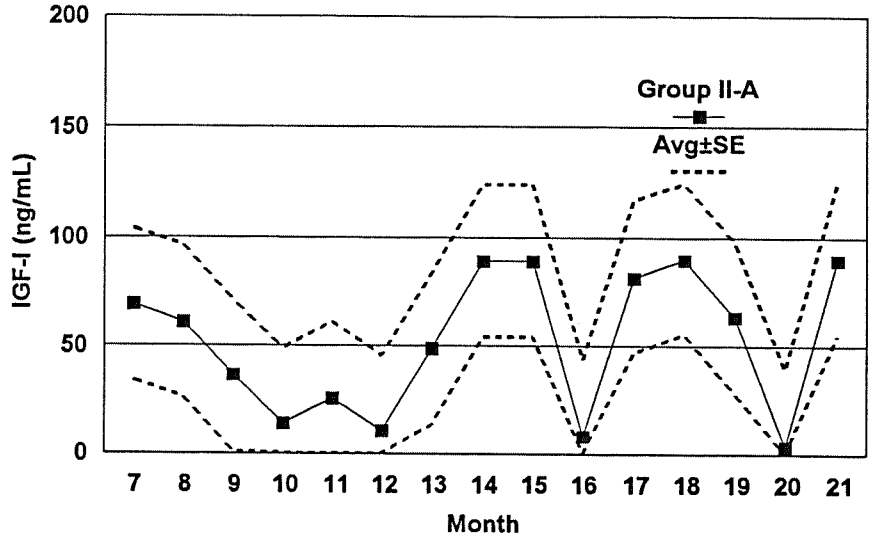

Figure 23. The average monthly IGF-I level of noncloned heifers in Group II-A (n = 2) with ± SE (34.96).

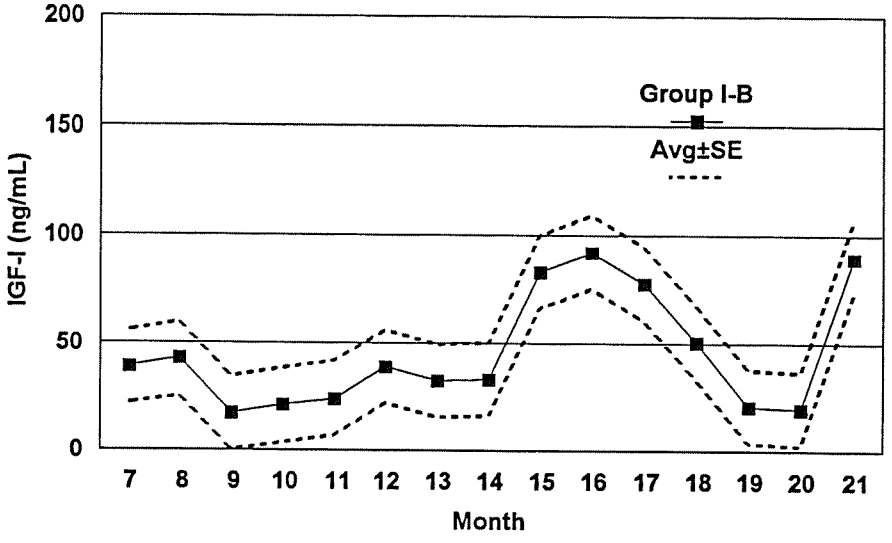

Figure 24. The average monthly IGF-I level of cloned heifers in Group I-B (n = 4) with ± SE (17.02).

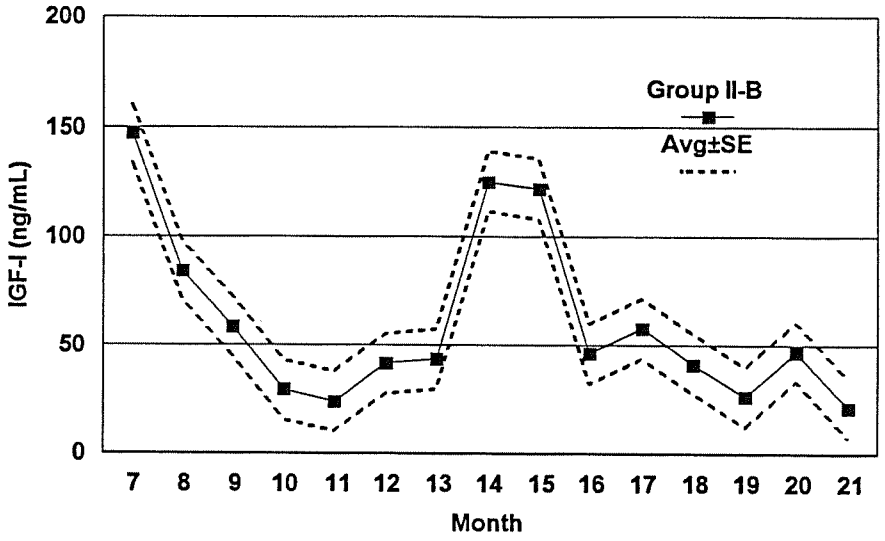

Figure 25. The average monthly IGF-I level of noncloned heifers in Group II-B (n = 4) with ± SE (13.60).

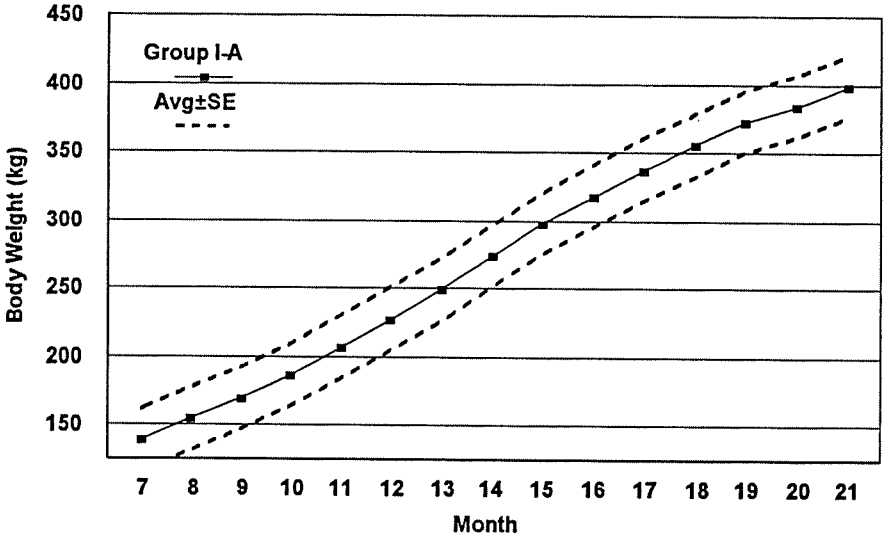

Figure 26. The average monthly body weight of cloned heifers in Group I-A (n = 2) with ± overall SE (22.81).

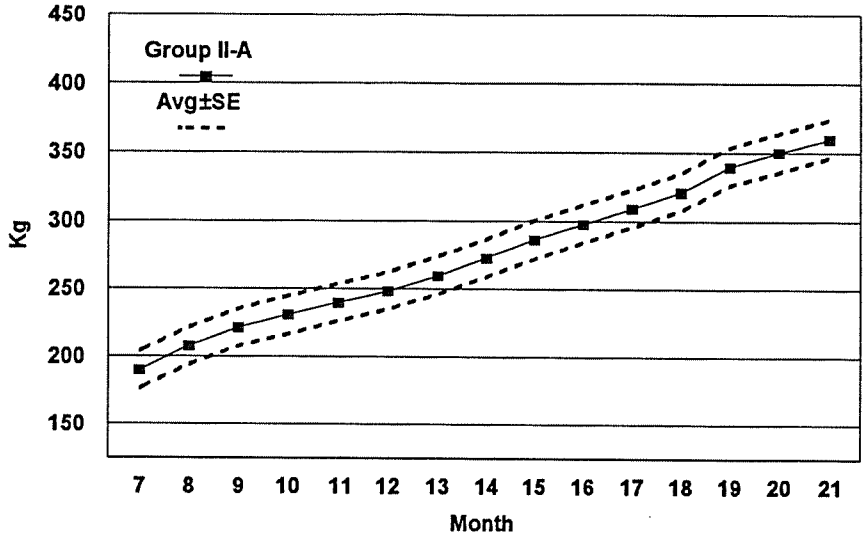

Figure 27. The average monthly body weight of noncloned heifers in Group II-A (n = 2) with ± overall SE (13.79).

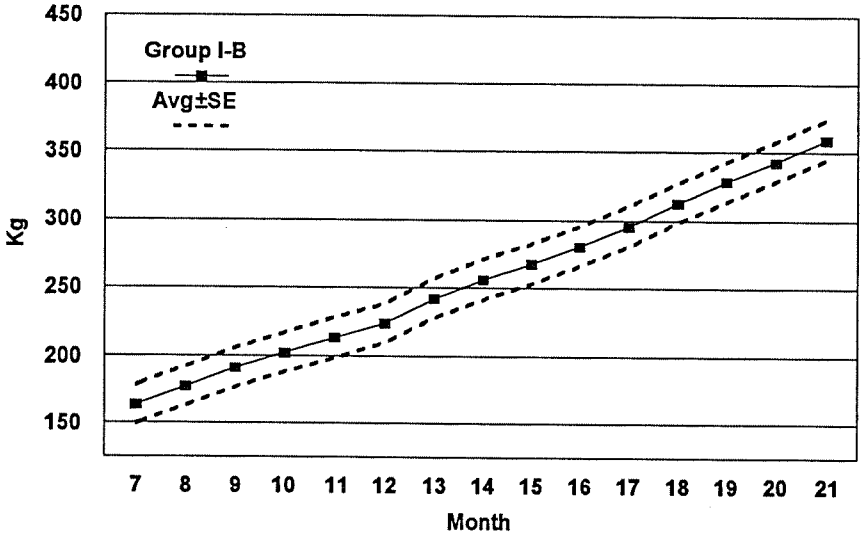

Figure 28. The average monthly body weight of cloned heifers in Group I-B (n = 4) with ± overall SE (14.56).

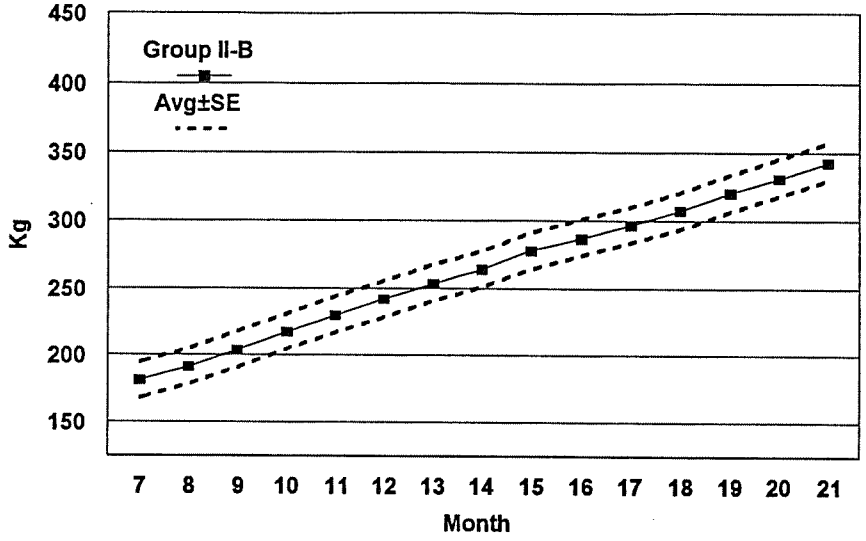

Figure 29. The average monthly body weight of noncloned heifers in Group II-B (n = 4) with ± overall SE (13.38).

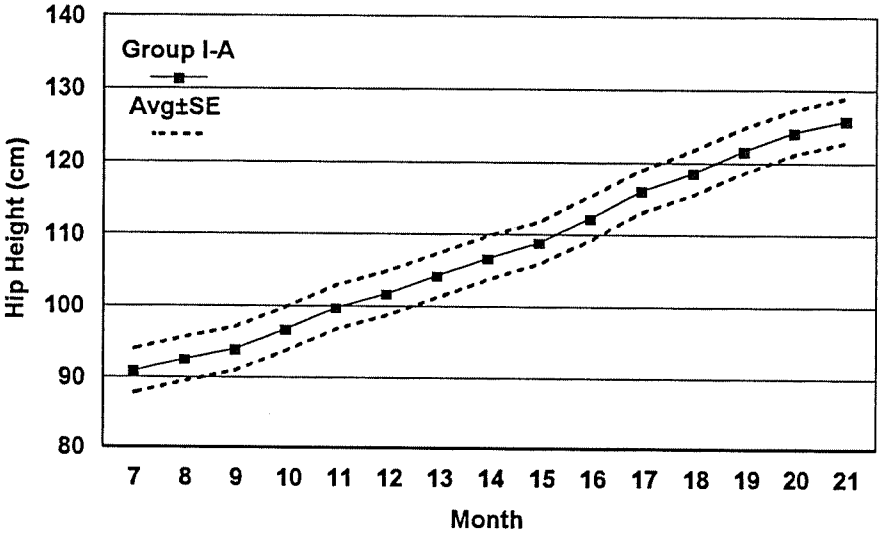

Figure 30. The average monthly hip height of cloned heifers in Group I-A (n = 2) with ± overall SE (3.06).

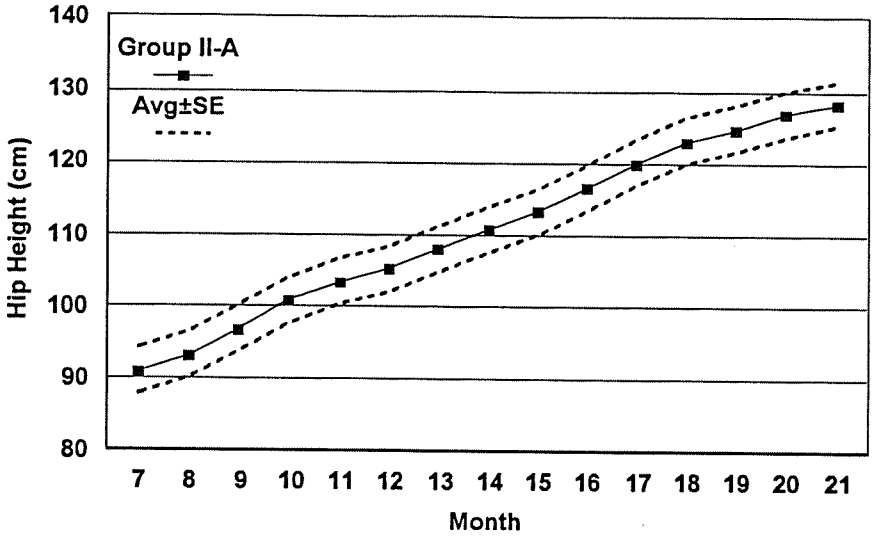

Figure 31. The average monthly hip height of noncloned heifers in Group II-A (n = 2) with ± overall SE (3.18).

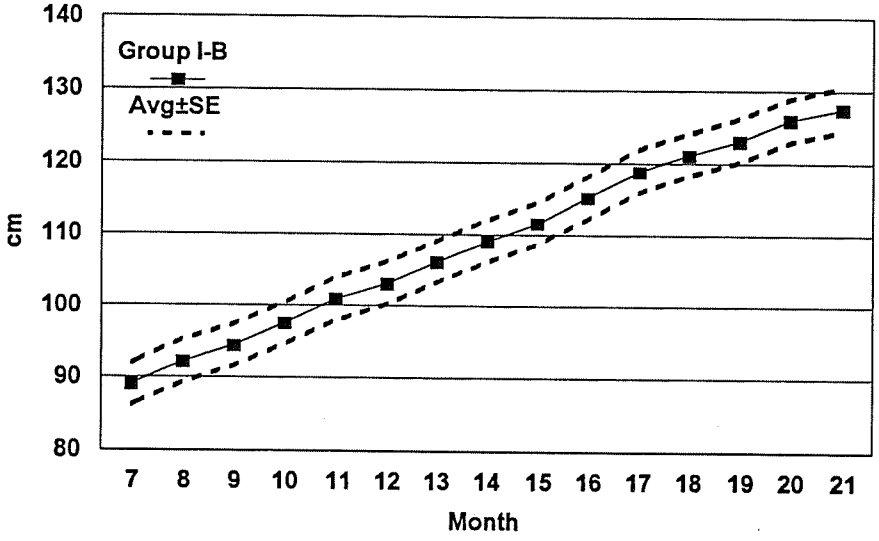

Figure 32. The average monthly hip height of cloned heifers in Group I-B (n = 4) with ± overall SE (2.94).

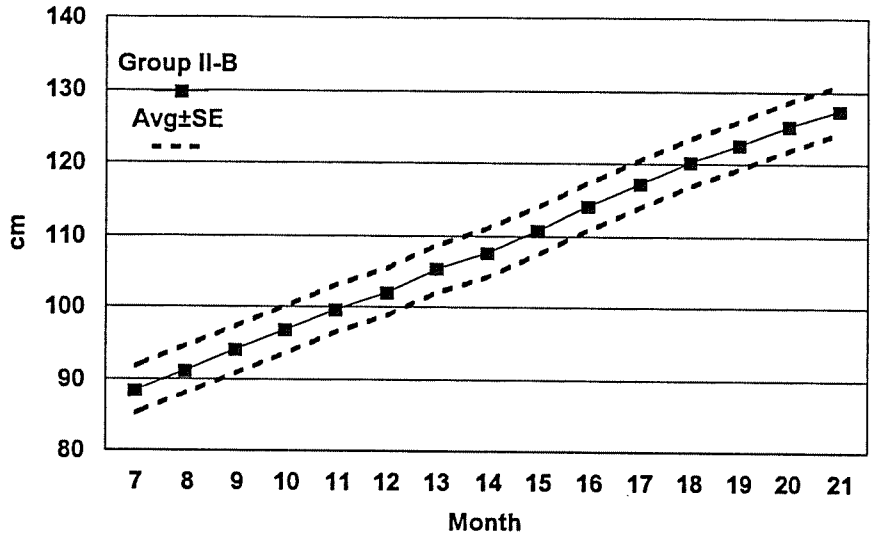

Figure 33. The average monthly hip height of noncloned heifers in Group II-B (n = 4) with ± overall SE (3.28).

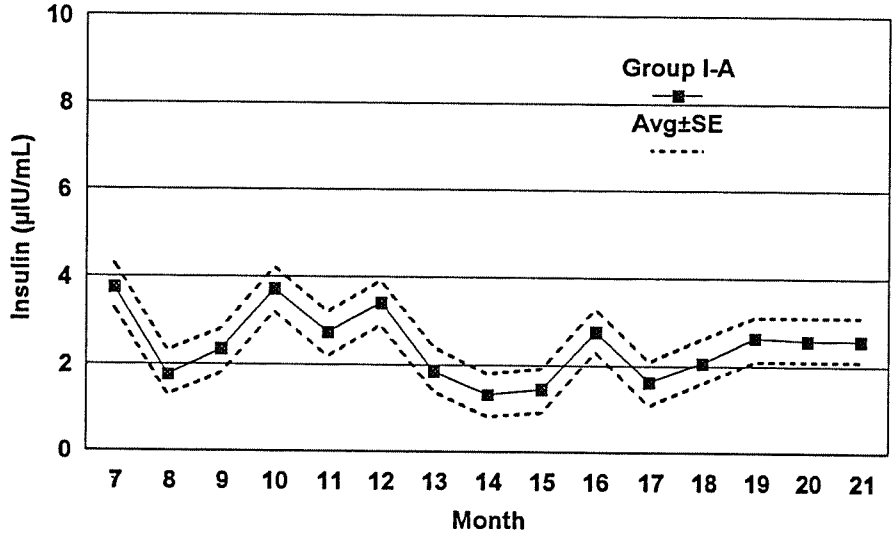

Figure 34. The average monthly insulin level of cloned heifers in Group I-A (n = 2) with ± overall SE (0.24).

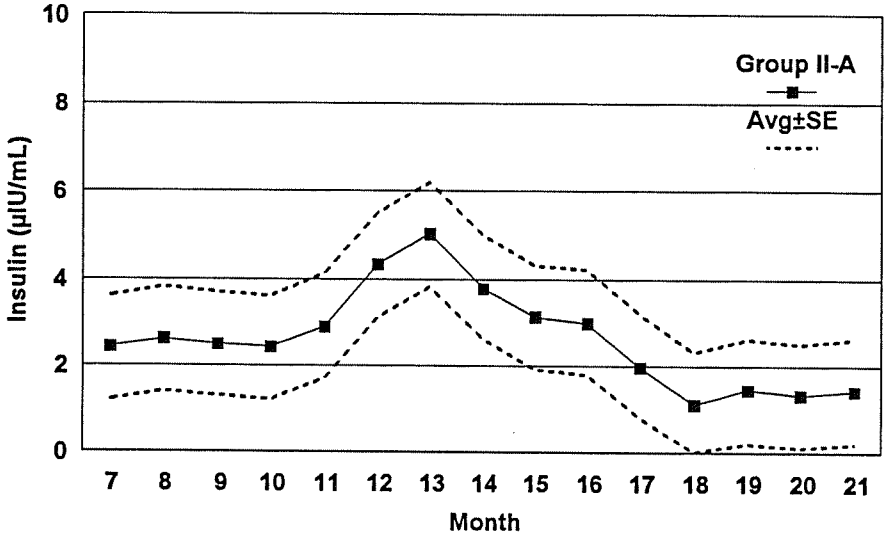

Figure 35. The average monthly insulin level of noncloned heifers in Group II-A (n = 2) with ± overall SE (0.41).

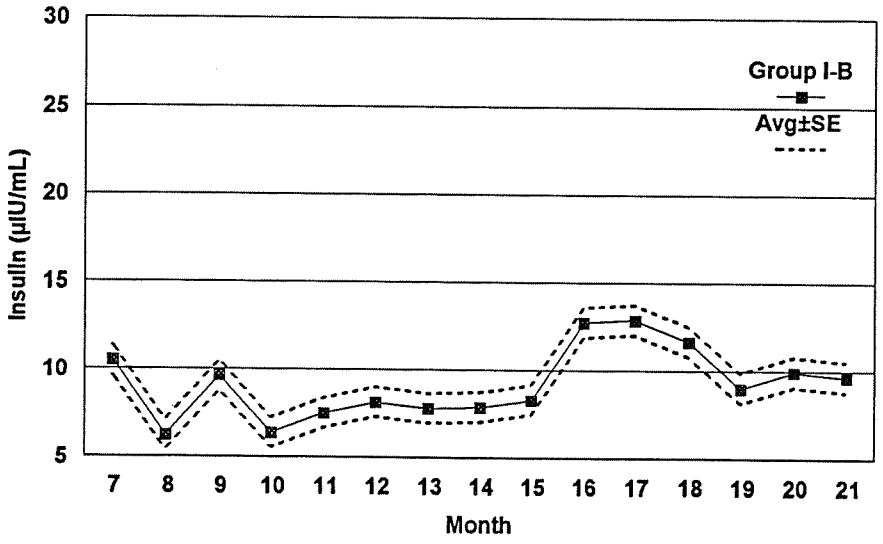

Figure 36. The average monthly insulin level of cloned heifers in Group I-B (n = 4) with ± overall SE (0.86).

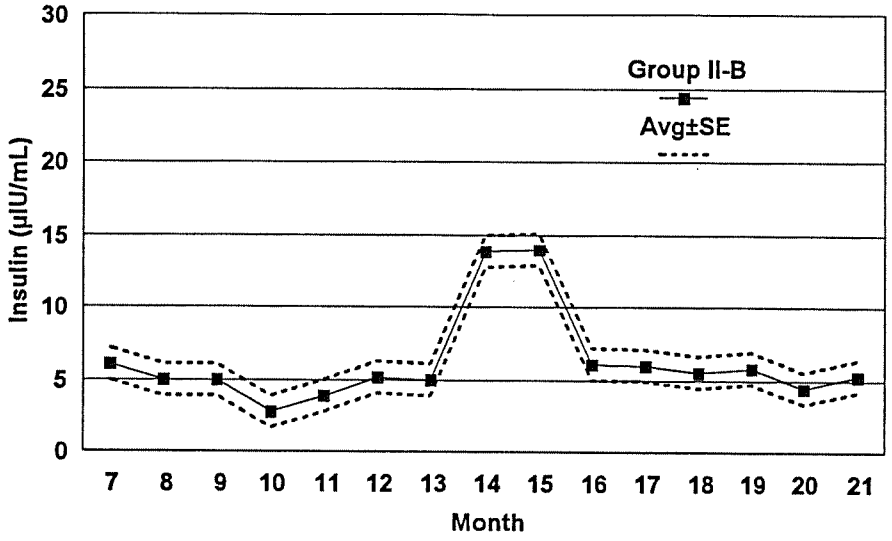

Figure 37. The average monthly insulin level of noncloned heifers in Group II-B (n = 4) with ± overall SE (1.11).

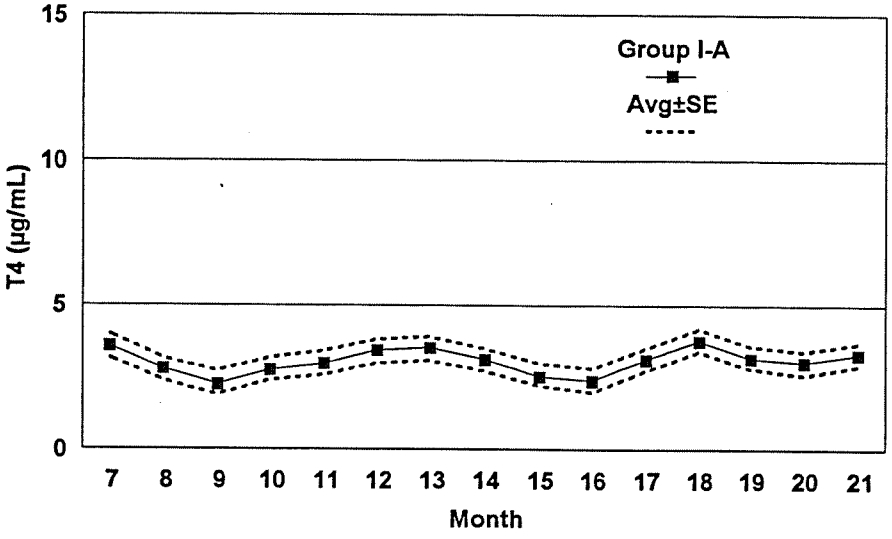

Figure 38. The average monthly T4 level of cloned heifers in Group I-A (n = 2) with ± overall SE (0.17).

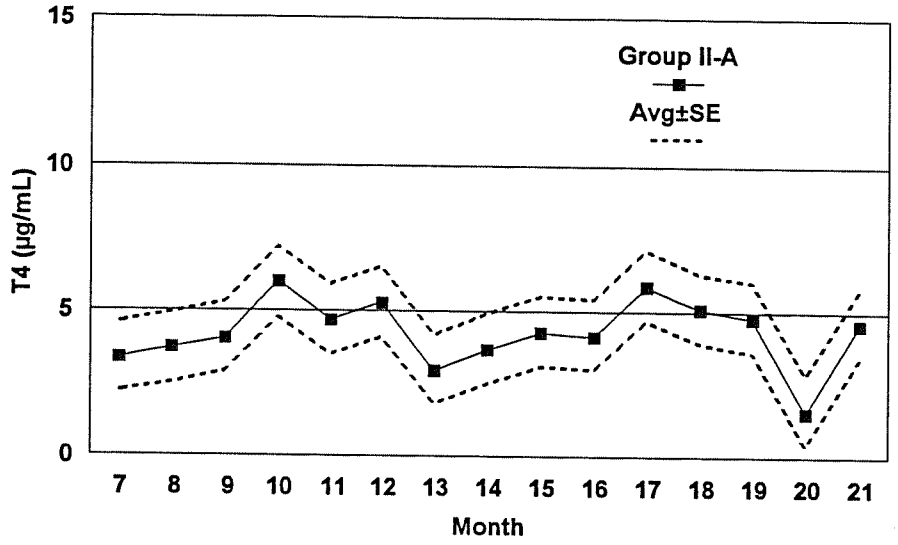

Figure 39. The average monthly T4 level of noncloned heifers in Group II-A (n = 2) with ± overall SE (0.48).

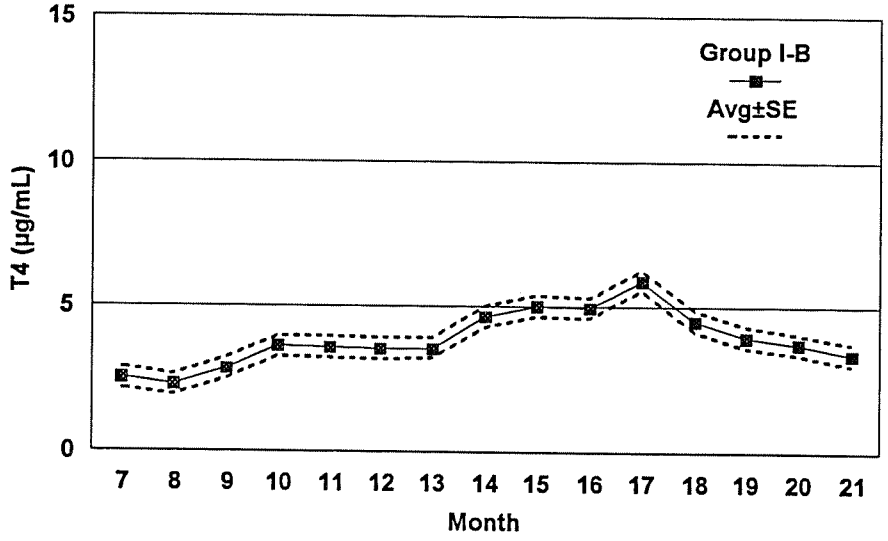

Figure 40. The average monthly T4 level of cloned heifers in Group I-B (n = 4) with ± overall SE (0.36).

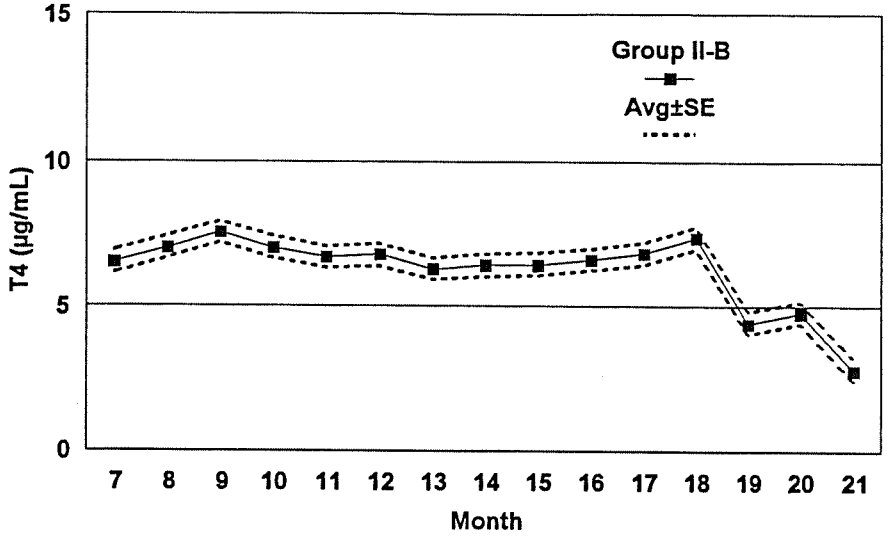

Figure 41. The average monthly T4 level of noncloned heifers in Group II-B (n = 4) with ± overall SE (0.38).

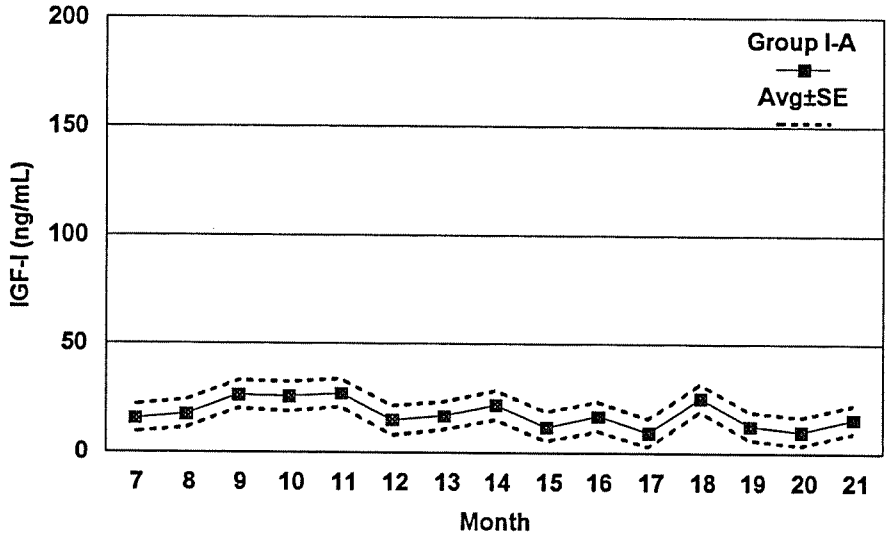

Figure 42. The average monthly IGF-I level of cloned heifers in Group I-A (n = 2) with ± overall SE (2.36).

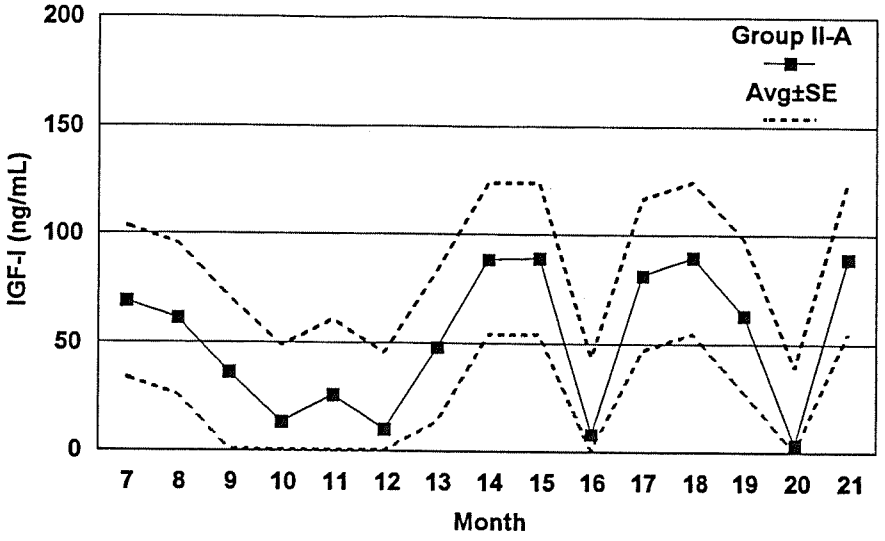

Figure 43. The average monthly IGF-I level of noncloned heifers in Group II-A (n = 2) with ± overall SE (9.23).

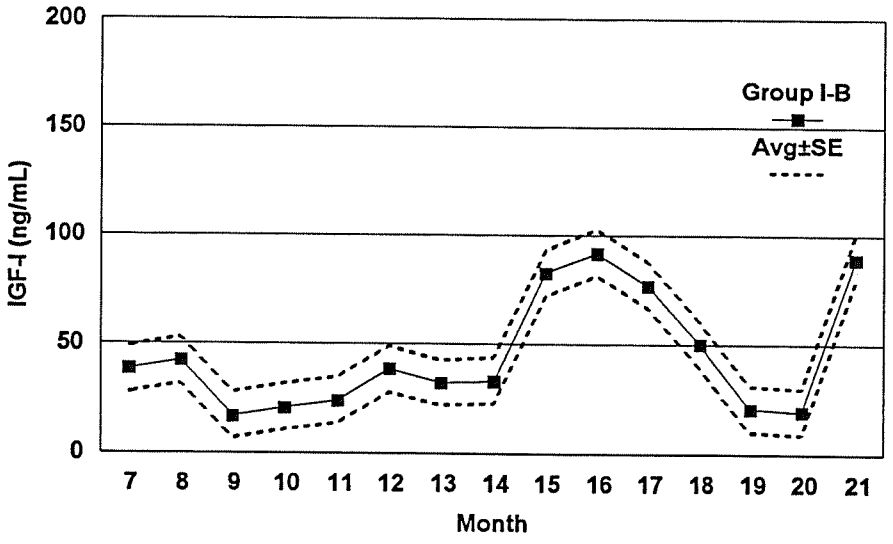

Figure 44. The average monthly IGF-I level of cloned heifers in Group I-B (n = 4) with ± overall SE (10.56).

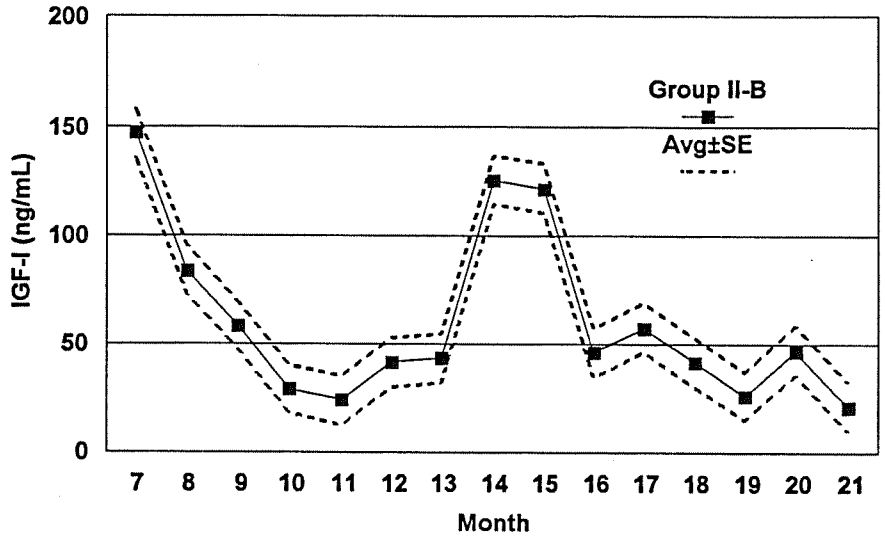

Figure 45. The average monthly IGF-I level of noncloned heifers in Group II-B (n = 4) with ± overall SE (11.25).

Table 1. Summary of intraclass numeric variability.

Parameter	Group Exhibiting the Least Numeric Variability			
	Overall Variability		Monthly Variability	
	Group A[a]	Group B[b]	Group A[a]	Group B[b]
Body Weight	Control	Clone	Control	Clone
Hip Height	Clone	Clone	Clone	Clone
Insulin Concentration	Clone	Clone	Clone	Control
Thyroxine Concentration	Clone	Clone	Clone	Clone
IGF-I Concentration	Clone	Clone	Clone	Control

[a] Group A is a comparison of Group I-A cloned heifers (n=2) and Group II-A noncloned control heifers (n=2).

[b] Group B is a comparison of Group I-B cloned heifers (n=4) and Group II-B noncloned control heifers (n=4).

Table 2. The monthly standard error pattern for parameters evaluated for Brangus cloned, noncloned and single cloned heifers in the study (monthly SEM)

Clone group	No./ group	Monthly Standard Error on Parameters Evaluated: Body Weight	Hip Height	Insulin	T_4	IGF-I
Clones[a] I-A & I-B	6	16.25 [d**]	0.71*	1.08*	0.25*	11.91*
Nonclones[b] II-A & II-B (control)	6	12.35	1.13	1.36**	1.00**	24.28**
Single Clones[c] III-A (control)	2	5.40*	1.34**	1.11	0.92	16.65

[a] I-A = 2 cloned Brangus heifers from the same embryo, II-B = 4 cloned heifers from a second embryo.

[b] II-B = 2 noncloned control Brangus heifers and II-B = 4 noncloned heifers all from different Brangus sires and different Brangus dams.

[c] III-A = 2 Brangus heifers born from the nuclear transfer procedure but were born as single clones from two different Brangus embryos.

[d] When the Brangus heifer exhibiting an abnormal growth pattern was removed, the overall standard error for the cones in Group I-B was 11.24.

* Lowest ranking of the three groups for variability of the parameter.

** Highest ranking of three groups for variability of the parameter.

Table 3. The overall standard error pattern for parameters evaluated for Brangus cloned, noncloned and single cloned heifers in the study (overall SEM)

Clone group	No./ group	Overall Standard Error on Parameters Evaluated: Body Weight	Hip Height	Insulin	T_4	IGF-I
Clones[a] I-A & I-B	6	18.69 [d**]	3.00	0.55*	0.27*	6.46*
Nonclones[b] II-A & II-B (control)	6	13.59*	3.23**	0.76**	0.43**	10.24
Single Clones[c] III-A (control)	2	14.09	2.90*	0.72	0.34	14.31**

[a] I-A = 2 cloned Brangus heifers from the same embryo, II-B = 4 cloned heifers from a second embryo.

[b] II-B = 2 noncloned control Brangus heifers and II-B = 4 noncloned heifers all from different Brangus sires and different Brangus dams.

[c] III-A = 2 Brangus heifers born from the nuclear transfer procedure but were born as single clones from two different Brangus embryos.

[d] When the Brangus heifer exhibiting an abnormal growth pattern was removed, the overall standard error for the cones in Group I-B was 14.56.

* Lowest ranking of the three groups for variability of the parameter.

** Highest ranking of three groups for variability of the parameter.