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Weaning and the Developmental Changes in Follicle-Stimulating Hormone, Pituitary Adenylate Cyclase-Activating Polypeptide, and Inhibin B in the Male Rat¹

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Abstract

Pituitary *Fshb* concentrations increase markedly and selectively beginning on Postnatal Day 20 in the male rat. To evaluate the factors potentially responsible for this rise in FSH, we adjusted the time of weaning, which is generally also on Day 20. Male rat pups were provided nutrients by suckling only and were weaned to laboratory chow earlier (Day 17) or later (Day 23) than normally performed in animal facilities (Day 20). Between ages 17 and 29 days, significant increases were seen in serum LH (1.4-fold) and FSH (2.4-fold) levels; pituitary expression of *Lhb* (5.4-fold), *Fshb* (21.3-fold), and inhibin beta B (*Inhbb*, 2.26-fold) mRNAs; and testicular expression of *Inhbb* (10-fold) mRNA. Concurrently, significant decreases occurred in serum inhibin B levels (1.8-fold); pituitary adenylate cyclase-activating polypeptide (*Adcyap1*, 4.2-fold), total follistatin (*Fst*, 3.5-fold), and *Fst* isoform 288 (5.6-fold) mRNAs; and testicular expression of inhibin beta A (8.2-fold) mRNA. Early weaning significantly increased serum FSH but not LH and increased pituitary expression of *Fshb* and GnRH receptor (*Gnrhr*) mRNAs but not *Lhb*. Early weaning also significantly decreased serum inhibin B but increased testicular expression of the *Inhbb* subunit. Early weaning also caused pituitary expression of *Fst* and *Adcyap1* to decline earlier than in the control group. Immediately after weaning, growth accelerated substantially, and the time of weaning produced significant and differential effects on circulating leptin levels that were not related to indices of FSH production. From these observations, we propose the novel hypothesis that the increase in growth rate subsequent to weaning signals circulating inhibin B levels to fall and pituitary *Adcyap1* and consequently *Fst* expression to decrease, and that these events together facilitate the rise in *Fshb* and *Gnrhr* expression by increasing pituitary activin signaling.

Keywords

follicle-stimulating hormone; gonadotropins; inhibin; luteinizing hormone; male reproduction; male sexual function; pituitary

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INTRODUCTION

Previous investigations in the male rat have demonstrated that circulating levels of FSH at birth are equivalent to levels in adult animals [1–3]. However, FSH concentrations decrease between the ages of 4 and 23 days and, beginning around Day 23, rise dramatically to peak levels at approximately 35 days that significantly exceed adult concentrations. On the other hand, there is very little change in LH throughout sexual maturation. Recent work in our laboratory and by others [4,5] demonstrated that *Fshb* subunit mRNA expression parallels the developmental pattern of circulating FSH with a significant rise between ages 20 and 30 days. Because of the presumed importance of these events to testicular development and fertility, it is essential to elucidate the factors that are responsible for the selective regulation of FSH.

It has become increasingly clear that the primary mechanism by which FSH is differentially regulated from LH is through the activin signaling pathway. Activin, a member of the TGF β superfamily of proteins, selectively stimulates *Fshb* synthesis with no direct effect on *Lhb* expression [6]. Activin also stimulates transcription of the gonadotropin-releasing hormone receptor (*Gnrhr*), however, and thereby indirectly increases LH by increasing GnRH responsiveness [7]. Conversely, *Fshb* expression levels are decreased when activin signaling is repressed [6,8,9]. The structurally similar dimeric protein inhibin is produced primarily within the gonads and, as part of a negative feedback control mechanism, competes with activin for binding to its receptor but fails to initiate activin signaling. A second regulator of activin is follistatin (FST), an activin-binding protein that is produced within the anterior pituitary and most peripheral tissues and neutralizes the actions of activin [10]. While there appears to be little change in pituitary activin, *Fst* expression in the pituitary is robustly regulated by GnRH, activin, testosterone, and pituitary adenylate cyclase-activating polypeptide (ADCYAP1) [11–13].

The single most dramatic event that occurs between the ages of 20 and 30 days in the life of the laboratory rat is weaning away from the nutritional environment of a lactating female. In the wild, rats will gradually wean themselves from nursing based on the availability of alternative food sources and the ability of the mother's milk to meet their nutritional needs. In many rodent research facilities, weaning occurs through separate caging, usually around Day 20, immediately prior to the time that FSH levels rise. Therefore, we developed the hypothesis that weaning and the ensuing acceleration in growth rate provide the physiological signal for the cessation of some factor(s) that repress maturation of the gonadotropin system.

In our experiments, we manipulated the timing of weaning of male rat pups and investigated the effects of early and late weaning on pituitary expression and serum concentrations of the gonadotropins. We also examined pituitary expression of *Fst*, inhibin beta B (*Inhbb*, also known as activin beta B), pituitary *Adcyap1* (also known as PACAP), and serum inhibin B levels, and testicular expression of inhibin alpha subunit (*Inha*), inhibin beta A (*Inhba*, also known as activin beta A), and *Inhbb* mRNAs in order to examine the potential role of these candidate regulators of FSH during this time period. We assessed pituitary expression of *Gnrhr* as its expression is also stimulated by activin signaling [7]. Finally, we recorded body weights and measured the serum concentrations of leptin in our weaning paradigm since leptin is a marker of adipose tissue mass and has been suggested to play a permissive role in pubertal maturation in mammals [14].

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* according to a protocol approved by the Animal Care and Use Committee

of the University of Louisville. Lactating Sprague-Dawley females and six male rat pups randomly assigned to the dam by the animal provider (Charles Rivers Laboratories) were housed together with free access to water. Once the pups were 10 days old, the lactating female was removed from the cage for 1 h twice daily and fed laboratory rat chow to ensure that suckling was the only source of nutrients to the pups. At the various times of weaning, pups were placed into cages without a lactating female, and one half of the cage was placed on a heating pad for 2 days to ensure adequate warmth. After weaning, water and laboratory rat chow were available ad libitum. Control rats were weaned from the lactating female at 20 days of age, and experimental pups were weaned on either Day 17 (early weaning) or Day 23 (late weaning). Body weight was determined daily starting at Postnatal (PN) Day 13. Pups from each group were killed on PN Days 17, 19, 21, 23, 25, and 29 (six per age). The pituitary gland from each animal was collected for the measurement of *Lhb* and *Fshb* subunits by Northern hybridization and mRNA levels of *Gnrhr*, *Adcyap1*, total *Fst*, and *Fst* isoform 288 by quantitative RT-PCR (qRT-PCR). To evaluate the peripheral effects of weaning, we processed the testes for qRT-PCR analysis of *Inhbb*, *Inhba*, and *Inha* subunit mRNA expression. Trunk blood was collected for analysis of circulating levels of LH, FSH, inhibin B, and leptin.

Serum Hormone Determinations

Enzyme immunoassays developed by Amersham Pharmacia Biotech were used to measure FSH and LH protein in serum. The within-assay coefficient of variation in the standard curves was 11.4% for FSH and 7.6% for LH. The range of the standards was 6.25 to 400 ng/ml for FSH and 0.41 to 100 ng/ml for LH.

Serum leptin levels were determined by a rat leptin ELISA kit (Crystal Chem, Inc., Downers Grove, IL). The within-assay coefficient of variation was 5.4%. The interassay coefficient of variation was 6.9%. The range of the standard curve was 200 to 12,800 pg/ml. Serum inhibin B levels were determined with an ultrasensitive ELISA kit (Serotec, Washington, DC). The range of the standards was 15.6 to 1000 pg/ml. The intraassay coefficient of variation was 4.7%.

RNA Extraction and Northern Analysis

RNA was extracted with the Perfect RNA Total RNA Isolation Kit (5 Prime-3 Prime, Boulder, CO). We determined the concentration of total RNA by reading the optical density at 260 nm. We determined sample purity by calculating the ratio of sample absorbance at 260:280 nm; samples were rejected if it was below 1.8. For Northern analysis, aliquots of pituitary RNA samples (2.5 µg) were subjected to electrophoresis on 1.2% agaroseformaldehyde gels. RNAs were transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) and cross-linked to the membranes by baking for 2 h at 80° to 90°C followed by irradiation for 2 min with UV light. Purified cDNAs for rat *Fshb* (Dr. Richard Maurer) and *Lhb* (Dr. James Roberts) were labeled by the random primer method with [α -³²P]dCTP (3000 Ci/mmol; New England Nuclear Research Products) to a specific activity of 6×10^8 to 8×10^8 dpm/µg as described elsewhere [15]. Labeled probes were added to the hybridization solutions at a concentration of approximately 5 ng/ml for 48 to 72 h. Membranes were washed, autoradiographed, and analyzed using a BioRad GS-700 imaging densitometer. Membranes were rehybridized without stripping for normalization to cyclophilin cDNA.

RT-PCR for Pituitary and Testis mRNAs

Construction of cRNA standard curves. For qRT-PCR, a standard curve of known amounts of target mRNA was prepared by the method of Fronhoffs et al. [16]. Briefly, target-specific primers (Table 1) were synthesized with and without a T7 promoter sequence (5'-GGATCCTAATACGACTCACTATAGGGAGG-3') at the 5' end of the forward primer and with an oligo(dT)₁₅ at the 5' end of the reverse primer. PCR was performed to produce cDNA

containing the T7 promoter sequence. The PCR product (1 μ g) was then used as template in an in vitro transcription reaction (MAXIscript T7 kit; Ambion, Austin, TX). The subsequent cRNA was quantified with a spectrophotometer and serially diluted to make standards containing 10^{11} to 10^4 molecules of cRNA. These standards were processed in parallel with experimental RNA samples for qRT-PCR with the primers lacking the T7 and dT sequences.

Quantitative RT-PCR. RNA (2 μ g) isolated from pituitary and testis samples was reverse transcribed in parallel with cRNA standards using an oligo(dT)₁₂₋₂₄ as the primer. Reverse-transcribed cRNA standards and samples were amplified in parallel by PCR on a Stratagene Mx4000 multiplex qPCR system (Stratagene, La Jolla, CA) using the Brilliant SYBR Green qPCR master mix (Stratagene) and the primers designed for use in the cRNA standard preparation but without the T7 and oligo(dT) sequences. Accumulation of PCR product was monitored in real time (Mx4000), and the crossover threshold (Ct) was determined with the Mx4000 software. For each set of primers, a no-template control and a no-reverse amplification control were included. Postamplification dissociation curves were performed to verify the presence of a single amplification product in the absence of DNA contamination. Concentrations of mRNAs were determined by means of the change in crossover time method with normalization to *Gapdh* mRNA and interpolation using a standard curve of known starting mRNA concentrations. Briefly, the Ct cycle defined as the fractional cycle at which the fluorescence signal becomes significantly different from the baseline signal, was determined by the fit-point method provided by the Mx4000 software. The Mx4000 software system generated the cRNA standard curve by plotting the Cts against the logarithm of the calculated initial copy numbers. The sample copy numbers were calculated from their Cts based on the cRNA standard curve.

Statistical Analysis

It was necessary to perform multiple serum protein and tissue RNA assays to analyze the large number of samples in these investigations. We controlled for interassay variations by analyzing a pooled sample (serum or tissue RNA) from PN Day 17 control rats in each assay. In addition, samples from each of the groups at each time point were included in every assay.

Data for each assay were normalized to the Day 17 control values and analyzed by two-way ANOVA with age and weaning group as the independent variables. This analysis determined whether there was a statistically significant overall effect of age or weaning group on the end points of interest. To determine whether weaning affected the timing of the end points, however, it was necessary to perform one-way ANOVA within each group and between groups at each time point in order to detect the age at which significant changes occurred.

RESULTS

Effects of Weaning Manipulations on Serum Gonadotropins

Serum FSH and LH concentrations increased significantly ($P < 0.0001$, two-way ANOVA) between PN Day 17 and PN Day 29 in each of the weaning groups (Fig. 1). Two-way ANOVA revealed a significant ($P < 0.01$) effect of weaning on serum FSH concentrations. Male rats in the early weaning group had higher levels of FSH ($P = 0.01$, post-hoc, Fisher PLSD) than the control weaning group, whereas the values in control and late weaning groups were not significantly different from each other. Altering the day of weaning had no significant effect on the concentration of LH. Thus, the experimental model modified the developmental rise in FSH secretion and allowed us to probe potential mechanisms for this change.

Effects of Weaning Manipulations on Gonadotropin Subunit Gene Expression

As demonstrated in Figure 2, a significant effect of age on pituitary expression of *Lhb* and *Fshb* was observed in each experimental group ($P < 0.0001$, two-way ANOVA). In control rats, there was a 5-fold increase in *Lhb* and a 21-fold increase in *Fshb* expression between Days 17 and 29. Manipulation of the day of weaning had a slight but nonsignificant effect on pituitary expression of *Lhb*. Conversely, altering the day of weaning significantly ($P = 0.0008$, two-way ANOVA) changed pituitary expression of *Fshb* mRNA. Early weaning induced a significantly ($P = 0.03$, Fisher PLSD) greater rise in *Fshb* mRNA expression compared to male pups in the control group. In the early weaning group, the level of *Fshb* mRNA on Day 21 was equivalent to the value in the control group on Day 29. Late weaning resulted in a modest, nonsignificant reduction in pituitary *Fshb* mRNA levels compared to control rats.

Effects of Weaning Manipulations on Pituitary Gnhr Expression

Analysis of the relative expression levels of pituitary *Gnrhr* mRNA revealed a significant effect of age and day of weaning ($P < 0.001$, two-way ANOVA). Pituitary *Gnrhr* mRNA levels increased significantly between Days 17 and 29 in controls, with an accelerated increase in the early weaning group (Fig. 3). Subsequent to weaning, the levels of pituitary *Gnrhr* mRNA were significantly higher ($P = 0.01$, Fisher PLSD) in the early weaning group compared to the control group. Pituitary *Gnrhr* mRNA levels were moderately but not significantly lower in the late weaned animals until Day 29, at which time there was a significant difference between the late and control groups. At the beginning of the experiment on Day 17, the pituitary *Gnrhr* mRNA concentration was 5.25 pg per microgram of total pituitary RNA.

Effects of Weaning Manipulations on Pituitary Fst Expression

The mRNA species encoding *Fst* isoform 288 has a 10-fold greater FSH-suppressing activity than the more abundant *Fst-315* isoform [17]. We showed previously that the rise in *Fshb* and *Gnrhr* mRNA levels in the male rat pituitary between Days 20 and 30 occurs together with a decline in the level of pituitary *Fst* isoform 288 expression [5]. Because early weaning increased both *Fshb* and *Gnrhr* expression, *Fst* isoform 288 as well as total pituitary *Fst* mRNA levels were evaluated. Analysis of the relative expression levels of total pituitary *Fst* mRNAs revealed a significant effect of age ($P < 0.001$; Fig. 4A). Pituitary *Fst* mRNA levels declined significantly between Days 17 and 29, and the decline was accelerated in the early weaning group. Analysis of the individual weaning groups by one-way ANOVA revealed a significant decline in *Fst* expression by Day 23 in the control group and by Days 19 and 25 in the early and late weaning groups, respectively. A similar pattern to total *Fst* was observed for *Fst* isoform 288 mRNA expression except that, in the control group, pituitary expression of *Fst* isoform 288 was significantly lower by Day 21 rather than Day 23 (Fig. 4B). Therefore, we conclude that early weaning advances the decline in pituitary expression of *Fst*. Animals on Day 17 had pituitary mRNA concentrations of 1.2×10^6 molecules of total *Fst* and 3.7×10^5 molecules of *Fst* isoform 288 mRNA per microgram of total pituitary RNA. *Fst* isoform 288 accounted for 20% to 25% of total pituitary *Fst* mRNAs during the various stages of development studied.

Effects of Weaning Manipulations on Pituitary Adcyap1 Expression

Insofar as ADCYAP1 stimulates *Fst* expression in vitro [15,18], the decline in pituitary *Fst* between ages 17 and 29 days could result from a decrease in pituitary *Adcyap1* expression. To evaluate whether ADCYAP1 plays an autocrine/paracrine role in the developmental rise in FSH, we measured pituitary *Adcyap1* mRNA levels in this model of FSH regulation by early and late weaning. Analysis of the relative expression levels of pituitary *Adcyap1* mRNA by two-way ANOVA revealed a significant effect of age ($P = 0.008$) but no difference in overall levels associated with the time of weaning (Fig. 4C). Analysis of the individual weaning groups

by one-way ANOVA revealed a significant decline in *Adcyap1* expression by Day 21 in the control and late weaning groups, whereas the value on Day 19 was significantly less than the Day 17 value in the early group. Therefore, we conclude that early weaning may advance the decline in pituitary expression of *Adcyap1*. Furthermore, the timing of the significant changes in pituitary *Adcyap1* mRNA was exactly reciprocal to that of the changes observed in *Fshb* mRNA expression in each group and paralleled the changes observed in *Fst* isoform 288 mRNA expression levels in the control and early weaning groups. The pituitary *Adcyap1* mRNA concentration of Day 17 animals was 3.85×10^5 molecules of *Adcyap1* mRNA per microgram of total pituitary RNA.

Effects of Weaning Manipulations on Pituitary *Inhbb* Expression

Previous studies have shown that *Inhbb* is the predominant isoform of activin expressed within the pituitary gland [19]. To evaluate a possible role of activin expression in this model of selective regulation of FSH, we measured pituitary *Inhbb* mRNA by quantitative RT-PCR. Two-way ANOVA revealed a significant effect of age ($P = 0.0001$) but no effect of time of weaning (Fig. 4D). Moreover, the temporal pattern of expression, with higher levels at weaning and on Day 29 and lower levels on Days 23 and 25, differed from the pattern of *Fshb* and *Gnrhr* mRNA expression. On Day 17, the pituitary *Inhbb* mRNA concentration was 3.1×10^4 molecules per microgram of total pituitary RNA.

Effects of Weaning Manipulations on Serum Inhibin B

Because inhibin B levels were reported to decline in male rats between ages 20 to 30 days [20], we sought to determine whether circulating inhibin B, like *Fshb*, is influenced by weaning. Analysis of serum inhibin B concentrations by two-way ANOVA revealed a significant effect of age ($P < 0.001$) and day of weaning ($P < 0.001$). Inhibin B concentrations declined significantly in the control group between Days 17 and 29, with an accelerated decline in the early weaning group. Moreover, the decline in inhibin B levels was delayed in the late weaning group (Fig. 5A).

Effects of Weaning Manipulations on Testicular Activin/*Inhbb* Subunit Expression

Inhibin subunit mRNA levels in testis were measured to determine whether the decline in circulating inhibin B during sexual maturation and weaning was explained by reduced inhibin production. Testicular levels of *Inhbb* mRNA (Fig. 5B) were significantly affected by age ($P < 0.001$) and by day of weaning ($P < 0.001$). Unlike the fall in serum inhibin B levels, however, *Inhbb* mRNA levels in testis increased significantly between Days 17 and 29, with an accelerated increase in male rats in the early weaning group. Levels of testicular *Inhbb* mRNAs were significantly higher ($P = 0.04$, Fisher PLSD) in the early weaning group compared to the control group. Testicular *Inhbb* mRNA levels were moderately but not significantly lower in the late weaned rats compared to control animals. Two-way ANOVA also revealed a significant effect of age ($P < 0.01$) on the expression levels of *Inha* and *Inhba* mRNAs but no effect of the time of weaning (Fig. 6). *Inhba* expression levels fell dramatically between Days 19 and 29, while *Inha* expression levels rose following weaning and then declined. On Day 17, animals had testicular mRNA concentrations of 4.3×10^6 molecules of *Inhbb*, 2.4×10^8 molecules *Inhba*, and 2.9×10^8 molecules *Inha* mRNA per microgram of total testicular RNA. However, by Day 29, the concentration of *Inhbb* mRNA was sixfold higher than that of *Inhba* mRNA.

Effects of Weaning Manipulations on Growth and Serum Leptin Concentrations

Body weight increased in each group of rat pups immediately following weaning (Fig. 7A), and the rate of increase was linear and comparable for each group. Consequently, the early weaned rats were heavier throughout the course of the experiment, whereas the control and late weaned pups were similar in weight between PN Days 13 and 29.

There was a significant effect of age ($P = 0.0002$) and day of weaning ($P = 0.012$) on serum leptin concentrations. The results in Figure 7B reveal that serum leptin levels in control rats declined on Day 21 subsequent to weaning on Day 20 but returned to Day 19 values on Day 23. A decrease in leptin was also observed on Day 21 in the late weaning group; however, the subsequent rise was delayed until Day 25, 2 days after weaning on Day 23. Serum leptin levels in the early weaning group declined slightly but not significantly on Day 19 following weaning on Day 17 and increased on Day 23, as in controls. Thus, early weaning prevented the decline in serum leptin concentrations that occurred in the final days of suckling in control rats. On the other hand, late weaning prolonged the period of decreased leptin concentrations. Finally, the pattern of circulating leptin concentrations was unrelated to FSH, which rose continuously from Day 17 to Day 29 (Fig. 1).

DISCUSSION

The data in this report imply that the increase in growth that follows weaning provides a developmental cue for *Fshb* gene expression to increase from Day 17 to Day 29 in male rats. Furthermore, the mechanism for repressed FSH during infancy appears to involve inhibition of pituitary activin signaling by inhibin and FST since, subsequent to weaning, there were reciprocal changes in *Fshb* mRNA and plasma inhibin B and pituitary *Fst* levels.

Specific two-site immunoassays have shown that inhibin B is the predominant circulating isoform in males [21,22]. Our results confirm those of Sharpe et al. [20], who reported that circulating inhibin B levels decrease from Day 20 to Day 30 in male Sprague-Dawley rats, suggesting that postweaning somatic growth may regulate this decline. A shift in the day of weaning by 3 days produced a marked advance or delay in the timing of the decline in circulating inhibin B concentrations. This decline permits enhanced activin signaling due to less competition by inhibin for the activin receptor. The end result, as demonstrated in this investigation, is a significant increase in expression of activin-responsive mRNAs, including *Fshb* and *Gnrhr*. Another study [23] did not observe a decrease in circulating concentrations of inhibin B until after Day 40, well after the peak in circulating FSH; the reason for these dissimilar results is uncertain. However, those results suggest that a decline in inhibin is not solely responsible for increased *Fshb* expression.

Inhibin B is primarily produced in and released from the testes [21,23]. Inhibin B is a dimer of the *Inha* and inhibin beta B subunit proteins. In this investigation, we observed a rise in the concentration of testicular *Inha* subunit mRNA following weaning that was presumably a consequence of the rise in FSH [21] followed by a decrease by Day 29, which may have been partly due to germ cell expansion diluting the *Inha* from Sertoli cells. When adjusted for total RNA content, our results are similar to those of Klaij et al. [24], revealing an increase in total testicular *Inha* mRNA levels between the ages of 23 and 25 days. However, there was no effect of weaning on testicular *Inha* expression levels. By contrast, the concentration of testicular *Inhbb* mRNA increased substantially from Day 17 to Day 29, perhaps because this gene is expressed in germ cells as well as Sertoli cells [23].

Thus, the developmental decrease in circulating inhibin B concentrations is unrelated to changes in testicular *Inhbb* or *Inha* gene expression. There are several potential explanations for decreased circulating inhibin levels. First, it is possible that weaning influences the posttranscriptional processing of the inhibin subunit mRNAs. Second, the rat blood-testis barrier becomes functional at this time [25] and may limit the release of inhibin into the circulation. Third, the metabolic clearance of inhibin appears to be greater in adult than in immature male rats [26], and the change in diet with weaning and the associated increase in somatic growth may affect peptide clearance rates.

We also observed a significant increase in pituitary *Inhbb* expression from Day 17 to Day 29. Activin stimulates and inhibin decreases expression of the activin/inhibin subunit genes [27]. Therefore, decreased circulating inhibin B concentrations during development might allow for increased activin signaling to increase pituitary activin expression and thereby stimulate FSH.

In this investigation, we confirmed our previous finding [5] that the expression level of the *Fst* isoform 288 mRNA isoform in the male rat pituitary declines significantly between Days 20 and 30. RT-PCR analysis also revealed a significant decline in total pituitary *Fst* mRNA not previously observed by semiquantitative PCR. Furthermore, early weaning accelerated the age at which *Fst* isoform 288 and total *Fst* mRNA levels were significantly lowered. Inhibin B is thought to suppress *Fst* expression by blocking activin signaling [28]; therefore, some other signal consequent to weaning must be responsible for the fall in pituitary *Fst* expression that occurs simultaneously with a fall in inhibin B.

ADCYAP1 (PACAP) administration causes a robust increase in *Fst* expression within primary cultures of rat pituitary cells [15,18]. Moreover, we previously reported a significant decrease in *Adcyap1* mRNA levels within the paraventricular nucleus of the hypothalamus between the ages of 20 and 30 days in male rats [5]. In this study, we observed a significant decrease in pituitary *Adcyap1* mRNA levels between 20 and 30 days. Furthermore, the timing of decreased pituitary *Adcyap1* expression was advanced in the subjects that were weaned early compared to the developmental pattern in the control group. Thus, these findings further strengthen the hypothesis that a developmental decrease in ADCYAP1 contributes to the increase in *Fshb* expression by reducing *Fst* expression, which increases activin signaling.

The reciprocal relationship between pituitary *Adcyap1* and *Fshb* mRNA levels is an especially novel discovery. Until recently, ADCYAP1 has been considered a hypophysiotropic hormone as high concentrations of ADCYAP1 peptide and *Adcyap1* mRNA are found within the hypothalamus [29]. In a previous report, we determined that hypothalamic expression of *Adcyap1* is reciprocal to pituitary *Fshb* expression between ages 20 and 30 days in the male rat [5]. The results of this study suggest that an autocrine/paracrine mechanism links ADCYAP1 to *Fshb* expression in the male rat during peripubertal sexual maturation. ADCYAP1 has been demonstrated to have an autoregulatory effect to stimulate *Adcyap1* mRNA accumulation in cultures of tilapia astrocytes [30]. Thus far, only cAMP signaling has been reported to regulate mammalian *Adcyap1* expression [31]. Therefore, due to potent activation of cAMP signaling, changes in hypothalamic expression of *Adcyap1* during development may influence pituitary expression of *Adcyap1* and further strengthen its effects on gonadotrophs.

ADCYAP1 has been demonstrated to selectively inhibit *Fshb* mRNA expression, and this effect is thought to occur through its potent stimulatory action on *Fst* expression (e.g. [18]). Increased *Fst* expression in response to ADCYAP1 stimulation is thought to diminish activin signaling and thus selectively repress expression of *Fshb*. In this model of weaning-associated regulation of gonadotropin expression, the levels of pituitary *Adcyap1* and *Fst* mRNAs declined substantially at the time when *Fshb* mRNA levels were increasing. Thus, this relationship appears to provide an additional mechanism by which LH and FSH are differentially regulated during development in the male rat.

While less pronounced than the rise in FSH, serum LH levels and pituitary expression of *Lhb* also increased significantly between Days 20 and 30. This rise in LH may partially reflect increased GnRH secretion [32,33]; however, *Gnrhr* mRNA levels also increased following weaning, and increased *Gnrhr* will enhance gonadotroph responsiveness to GnRH to increase secretion of both gonadotropins. The rise in *Gnrhr* mRNA levels is likely to result from

increased activin signaling due to suppressed inhibin B and *Fst* expression as well as to increased GnRH.

Repression of FSH production during early development is thought to prevent premature maturation of the testes. In previous investigations in which FSH was administered to immature male rats, there were multiple changes to testis morphology that persisted into adulthood [34, 35]. Administration of recombinant human FSH to immature rats accelerated testicular growth, including increases in seminiferous tubule volume and length with proportionate increases in Sertoli and germ cell numbers as well as increased numbers of spermatogonia and serum levels of testosterone. Human FSH also stimulated spermatogonia differentiation, which resulted in a fivefold increase in the number of spermatocytes. The male rats in the early weaning group in this investigation had significantly elevated circulating FSH levels compared to those in the control group. Further studies are required to determine the long-term effects of early and elevated serum FSH levels in our weaning paradigm.

Fst and *Adcyap* mRNAs as well as serum inhibin B levels were not significantly different between groups at the time when serum FSH and pituitary *Fshb* mRNAs differed maximally (Days 25 and 29). Increased *Fshb* expression in the early weaning rats at these later time points could be due to the earlier increases in activin signaling. Alternatively, ADCYAP1 and inhibin modulation of activin signaling may not be the sole mechanism by which FSH is regulated during development. Extending our studies with the weaning paradigm should readily determine whether the control and late weaning groups ultimately achieve similar levels of FSH.

In designing this study, we were concerned that the animals in the early weaning group would experience malnutrition due to premature removal of suckling nutrients and an inability to consume sufficient amounts of rat chow. However, animals in each weaning group displayed a marked increase in growth rate after being switched to laboratory chow as their exclusive dietary source. Thus, animals in each group had adequate nourishment after weaning, as reflected by a dramatic increase in growth.

Our data reveal a relationship between serum leptin concentrations and weaning. Leptin is produced within adipocytes and is secreted into the circulation to affect multiple tissues, including hypothalamic neurons important for reproductive function [36]. Leptin is thought to play a permissive role in sexual maturation, and animals deficient in leptin peptide or its receptors do not develop to reproductive maturity [37,38]. In this investigation, animals in the control and late weaning groups displayed a significant, albeit transient, decrease in circulating concentrations of leptin toward the end of the suckling period. However, animals that were weaned early did not experience this decline. Several previous studies in which suckling pups were provided laboratory chow ad libitum also demonstrated a significant decline in serum concentrations of leptin at or near the time of weaning [39–41]. Circulating levels of leptin decrease with fasting in rodents [42] and are an indication of a change in metabolic status. Therefore, we hypothesize that a fall in leptin levels may provide a metabolic signal for rat pups to seek novel food sources; however, further studies are required to delineate the role of leptin in the cessation of suckling.

Other data from this investigation and previously published studies imply, however, that leptin plays an insignificant role in the delay in *Fshb* and *Gnrhr* maturation in control and late weaning groups. First, the decline in serum leptin that occurred at approximately Day 19 appears to be the normal pattern of circulating leptin in immature male rats [39–41]. Second, in a recent study that employed severe nutritional deprivation in preweaning male rats, a 38% decrease in body weight and a 70% decrease in serum leptin levels produced no significant change in serum gonadotropins at the time of weaning [43]. Finally, in this investigation, at the time when serum

leptin levels were decreasing, pituitary expression of *Fshb* was rising, and serum leptin levels were comparable in each weaning group by Day 25.

In conclusion, the results of this investigation suggest that weaning and the ensuing acceleration in growth provide a signal for the rise in *Fshb* and *Gnrhr* expression that occurs in the male rat between Days 20 and 30. Weaning and somatic growth are associated with a decrease in circulating inhibin B and pituitary *Fst* mRNA levels and thereby enhanced activin signaling. The decline in serum inhibin B while testicular *Inhbb* expression levels rise suggests that weaning may accelerate inhibin clearance. In addition, the weaning-related decline in pituitary *Adcyap1* expression is likely to contribute to decreased pituitary *Fst* expression. Finally, controlled alterations in weaning may represent a valuable new model to study sexual maturation in the male rat.

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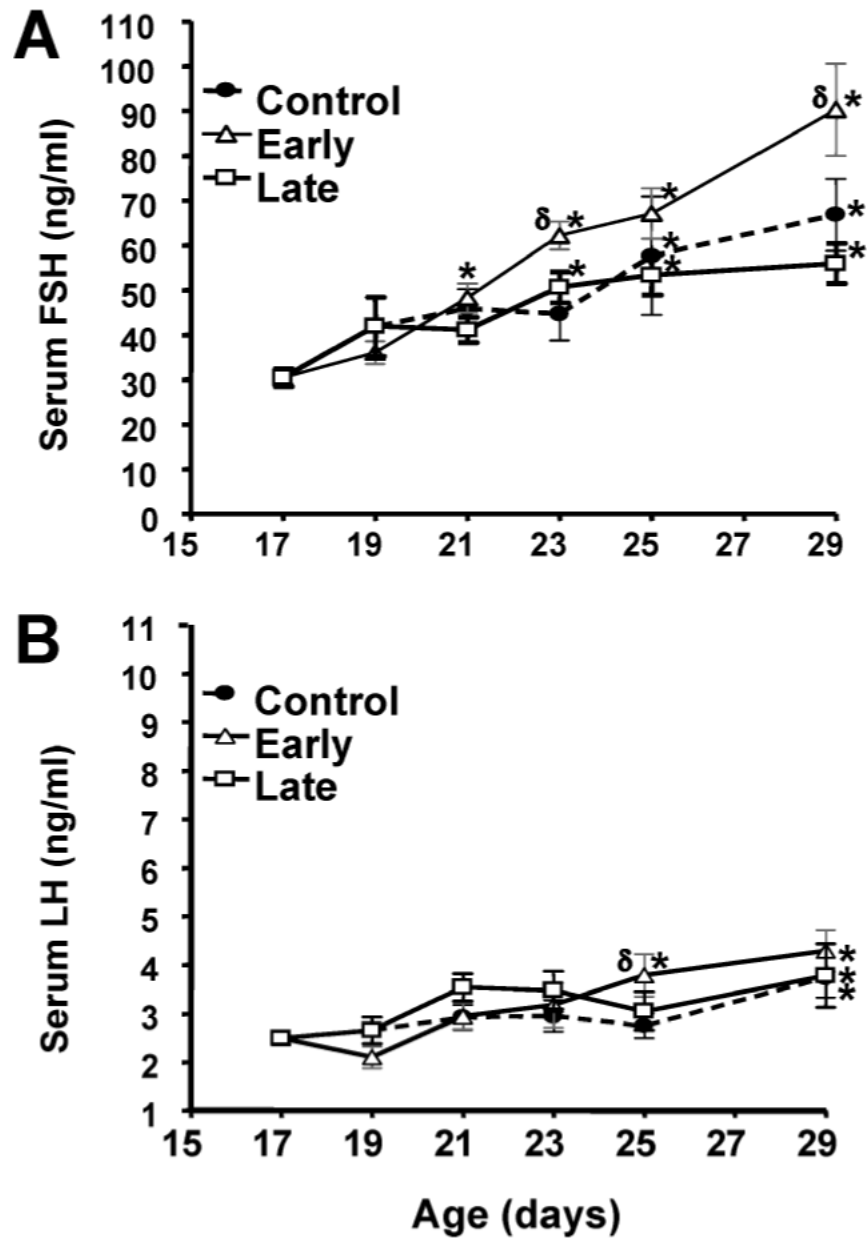


FIG. 1. Effects of weaning manipulations on serum FSH and LH concentrations. Serum FSH (**A**) and LH (**B**) concentrations were determined at the indicated ages by ELISA. Each value represents the mean \pm SEM of 6 animals. *, significantly different (ANOVA, $P < 0.05$) from Day 17 values within groups; δ , significantly greater (ANOVA, $P < 0.05$) than control values on the same day.

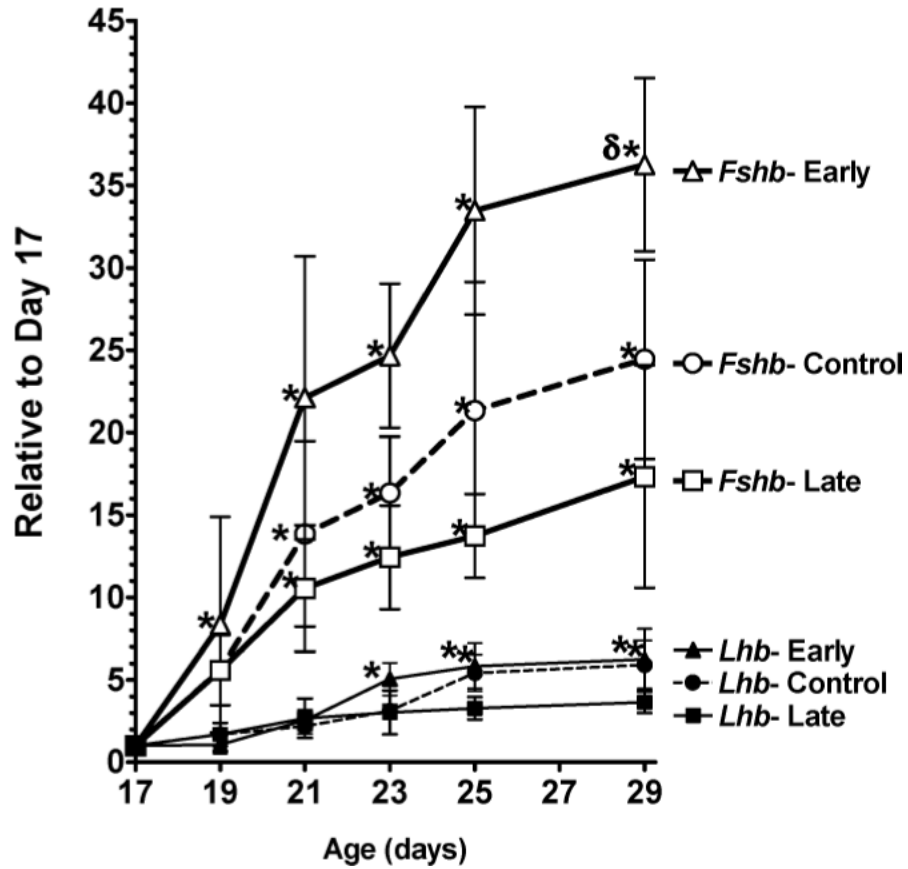


FIG. 2.

Effects of weaning manipulations on pituitary *Fshb* and *Lhb* mRNA concentrations. *Lhb* and *Fshb* mRNA levels were determined by Northern hybridization with normalization to cyclophilin mRNA levels. Data are expressed as mRNA expression levels relative to Day 17 values. Each value represents the mean \pm SEM of 6 animals. *, significantly different ($P < 0.05$) from Day 17 values within groups; δ , significantly greater ($P < 0.05$) than control values on the same day.

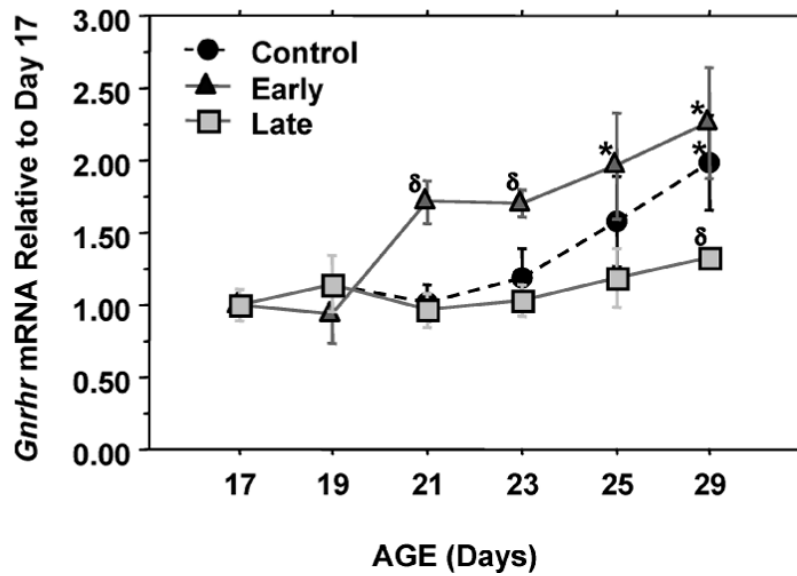
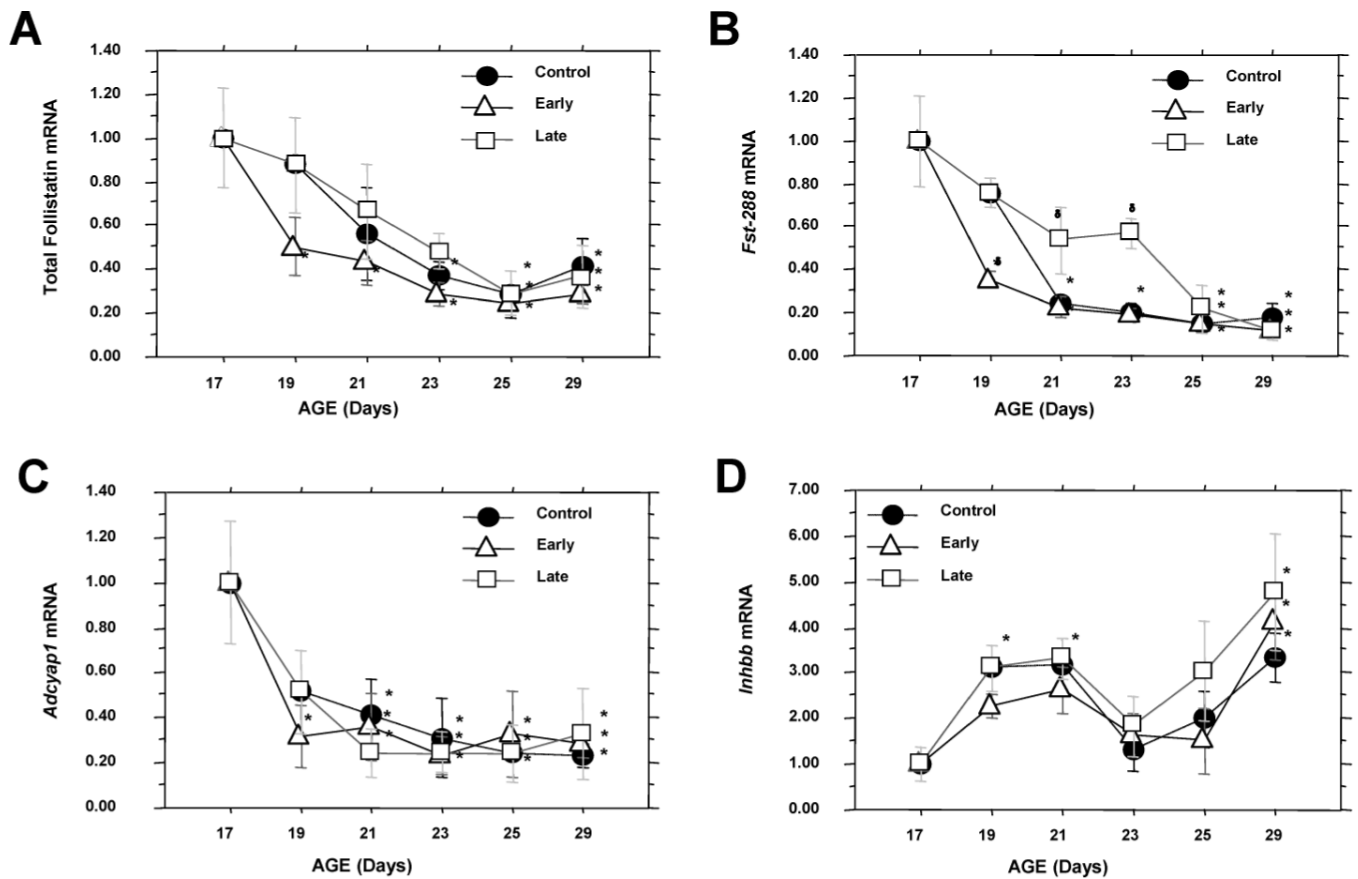


FIG. 3. Effects of weaning manipulations on pituitary expression of *Gnrhr*. *Gnrhr* mRNA levels were determined by RT-PCR. Data are expressed as mRNA expression levels relative to Day 17 values. Each value represents the mean \pm SEM of 6 animals. *, significantly different ($P < 0.05$) from Day 17 values within groups; δ , significantly different ($P < 0.05$) from control values on the same day.

**FIG. 4.**

Effects of weaning on expression levels of pituitary-derived FSH regulating factors. Levels of pituitary *Fst* (A), *Fst* isoform 288 (labeled *Fst*-288)(B), *Adcyap1* (C), and *Inhbb* (D) mRNAs were determined by RT-PCR. Data are expressed as mRNA expression levels relative to Day 17 values. Each value represents the mean \pm SEM of 6 animals. *, significantly different ($P < 0.05$) from Day 17 values within groups; δ , significantly different ($P < 0.05$) from control values on the same day.

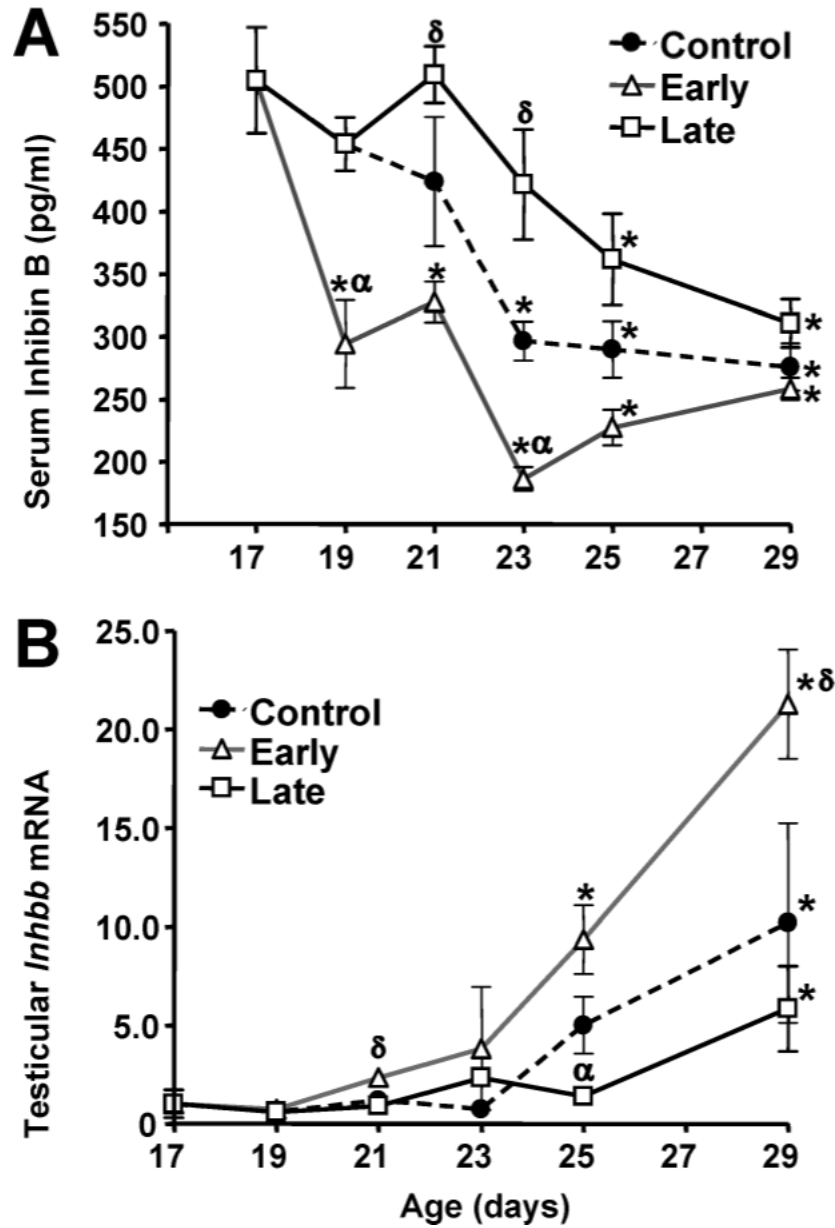


FIG. 5. Effects of weaning manipulations on serum inhibin B and testicular *Inhbb* mRNA levels. Serum inhibin B concentrations (**A**) were measured by ELISA. Each value represents the mean \pm SEM of 6 animals. Levels of testicular *Inhbb* mRNA (**B**) were determined by RT-PCR. Data are expressed as mRNA expression levels relative to Day 17 values. Each value represents the mean \pm SEM of 6 animals. *, significantly different ($P < 0.05$) from Day 17 values within groups; δ , significantly greater ($P < 0.05$) than control values on the same day; α , significantly less ($P < 0.05$) than control values on the same day.

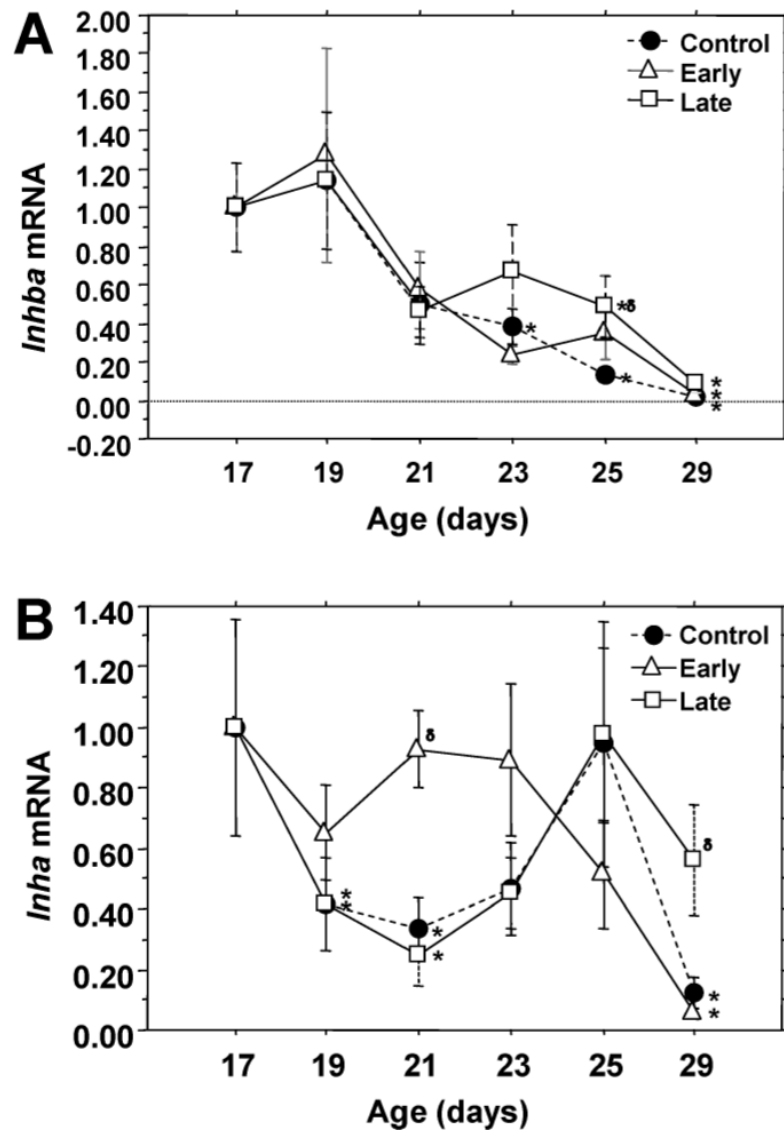


FIG. 6. Effects of weaning on *Inhba* and *Inha* levels in testis. Levels of testicular *Inhba* (**A**) and *Inha* (**B**) mRNAs were determined by RT-PCR. Data are expressed as mRNA expression levels relative to Day 17 values. Each value represents the mean \pm SEM of 6 animals. *, significantly different ($P < 0.05$) from Day 17 values within groups; δ , significantly different ($P < 0.05$) from control values on the same day.

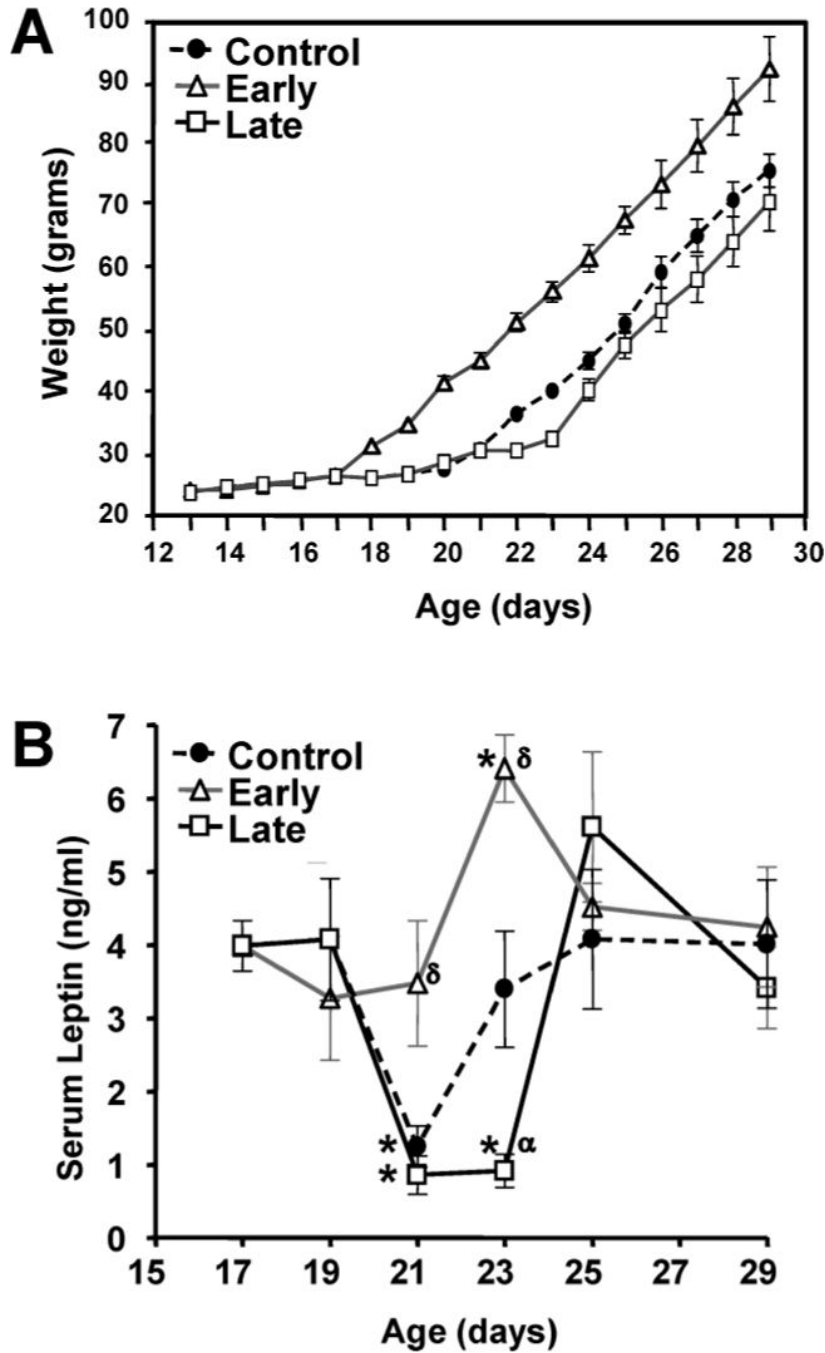


FIG. 7. Effects of weaning manipulations on body weight and serum concentrations of leptin. Body weights (**A**) of pups in the control, early weaning, and late weaning groups were recorded beginning on Day 13. There were no apparent differences between groups in the rate of growth before or after weaning. Values represent the mean \pm SEM of 12 to 18 animals at each time point. Serum leptin concentrations (**B**) were measured by specific ELISA. Each value represents the mean \pm SEM of 6 animals. *, significantly different ($P < 0.05$) from Day 17 values within groups; δ , significantly greater ($P < 0.05$) than control values on the same day; α , significantly less ($P < 0.05$) than control values on the same day.

TABLE 1

Primers utilized for real-time PCR analyses.

Gene	Forward	Reverse	GenBank accession no.
<i>Gapdh</i>	CTGGAAGATGGTGATGGGT	ATGATTCTACCCACGGCAAG	M17701
<i>Inha</i>	CTGAACCAGAGGAGGAGGAT	CCAGATGACAGCACCAGAAG	NM_012590
<i>Inhbb</i>	TGAGATCATCAGCTTTGCAG	CTGCACCACAAATAGTTCT	XM_344130
<i>Inhbb</i>	GCAGACCTCGGAGATCATCA	GGAAGATGTGCCAAGTGCTC	NM_017128
Total <i>Fst</i>	GCTCCACTTGTGTGGTGGA	CCGCACTGGATGTCTTCACA	NM_012561
<i>Fst</i> isoform 288	GAGGCCAAAAGACAAAACA	ATGGGGGAATACAGGGAGAG	AY280523
<i>Gnrhr</i>	TCACACGAGTCCTTCATCA	GCCACTGTCATCTTAGAGTTC	NM_031038
<i>Adcyap1</i>	CCTACCGCAAAGTCTTGGAC	TTGACAGCCATTGTTTTTCG	NM_016989