Thymosin-β4 Concentrations during the Estrous Cycle and after Hypophyseal Stalk Transection of Female Pigs (43023)

J. J. FORD,[1] D. D. VAKHARIA, L. L. ANDERSON, AND J. KLINDT

USDA-ARS, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska 68933 and Department of Animal Science, Iowa State University, Ames, Iowa 50011

Abstract. **Thymosin-β4 (ThB4) concentrations in the peripheral circulation of pigs were investigated during the first 30 days after weaning and after hypophyseal stalk transection of ovariectomized females. Significant increases in ThB4 were observed during the day of weaning, during follicular development, and during early luteal formation. During the first period of follicular development (Days 1 to 5 after weaning), ThB4 was uniformly elevated for 3 days whereas during the second period of follicular development (days 21 to 25 after weaning), the increase in ThB4 was bimodal. This period of bimodal secretion was closely associated with luteolysis. ThB4 concentrations were low during the luteal phase when progesterone concentrations were at their greatest. In ovariectomized pigs, ThB4 concentrations were not influenced acutely by a single intravenous injection (2 μg) of luteinizing hormone-releasing hormone in control (hypophyseal stalk intact) or hypophyseal stalk transected females. Both of these treatment groups responded to luteinizing hormone-releasing hormone with increased secretion of luteinizing hormone. These studies determined that ThB4 secretion changed dramatically throughout the estrous cycle of pigs but failed to identify an acute association between increased luteinizing hormone secretion and ThB4 in ovariectomized pigs. Our observations support the hypothesis that the thymus gland interacts with the hypothalamo-pituitary-ovarian axis primarily through changes in secretion of ovarian steroids.**

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It is well established that the thymus gland is influenced by, and has influence on, the hypothalamo-pituitary-gonadal axis (1, 2). Strich *et al.* (3) observed that pituitaries and hypothalami of athymic nude mice contained lower amounts of gonadotropins and luteinizing hormone-releasing hormone (LHRH), respectively, than those of heterozygous control mice. Absence of the pituitary gland is associated with atrophy of the thymus (4, 5) and results in impaired immune function (6, 7). Thymosin fraction 5 is a thymic extract that contains thymosin-α1 (ThA1), thymosin-β4 (ThB4), and other peptides. *In vivo* or *in vitro* infusion of TF5 or ThB4 into the hypothalamus induces release of LHRH and thereby stimulates release of luteinizing hormone (LH) from the pituitary gland (8–10). Furthermore, gonadal steroids act upon the thymus and modulate secretion of thymic peptides (1, 11). Hall *et al.* (12) implicated pituitary hormones and neuropeptides as modulators of thymic secretions. More recently, Dardenne *et al.* (13) reported that prolactin directly stimulated thymic epithelial cells and Marchetti *et al.* (14) observed specific binding of LHRH to thymic cells. To date, there are little data on ThB4 concentrations in swine, and no information is available on the secretion of ThA1 and ThB4 in response to acute changes in LHRH and LH concentrations. The potential for the hypothalamus to influence thymic function is apparent because hypothalamic extracts prepared from young mice increase thymic activity in old mice (15). In the present studies, we investigated changes in ThB4 during follicular growth after weaning and during the next estrous cycle. In addition, effects of hypophyseal stalk transections and LHRH injection on ThA1 and ThB4 were monitored in ovariectomized females. Ovariectomized animals were used to remove the variation in ovarian steroids that occurs during the estrous cycle and the influence of this variation on thymic secretions.

[1] To whom requests for reprints should be addressed at U.S. MARC, P.O. Box 166, Clay Center, Nebraska 68933.

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Materials and Methods

Animals. For the first study, jugular catheters were placed (16) in eight, first parity, Duroc sows (140 ± 4 kg) 6 days before their litters were weaned on Day 28.8 ± 0.5 postpartum. Daily blood samples were obtained each morning from five sows for 30 days after weaning. From the other three sows, blood samples were obtained at 0, 3, and 6 hr after weaning; then every 6 hr through Day 1 (Day 0 = day of weaning); every 12 hr on Days 2, 3, and 4; every 6 hr on Days 5, 6, and 7 (expected period of estrus); every 12 hr on Days 8 and 9; and each morning on Days 10, 11, and 12 after weaning. Sows were monitored for estrus by daily exposure to a sexually mature male on Days 1, 2, and 3, and by twice daily male exposure on Days 4–7 and 24–28 after weaning.

In the second study, sexually mature Yorkshire gilts (108 ± 4 kg) were ovariectomized 30 days before assignment to treatment; unoperated control (N) or hypophyseal stalk transection (HST). Jugular catheters were inserted when treatment was imposed. Two days later, LHRH (2 μg; Abbott Laboratories, North Chicago, IL) was injected via the catheter, and blood samples were collected at 0, 5, 10, 20, and 30 min after LHRH. On Day 9 after treatment, seven blood samples were obtained at 15-min intervals before animals were sacrificed for evaluation of prolactin and ThB4 concentrations. In addition, serum samples that were obtained on Day 2 after surgical treatment in a previous experiment (17, 18) were assayed for ThB4 concentrations. There were two samples collected 4 hr apart from each ovariectomized female; treatments were unoperated controls, sham-operated controls, and HST, five females per treatment.

For HST a modification of the transfrontal supraorbital approach for visualization of the hypophyseal stalk, developed for hypophysectomy of pigs (19), was used. A nylon disc (6.0 or 8.0 mm in diameter and 0.45-mm thickness) was inserted between severed ends of the stalk to prevent vascular regeneration between the hypothalamus and pituitary gland (20). Postmortem examination of each HST gilt on Day 9 after surgery revealed that the hypophyseal stalk had been completely severed. The nylon disc was in the proper location and had prevented regeneration of the stalk.

Reagents and Hormone Analysis. Synthetic ThA1 and ThB4 standards, tyrosin-modified synthetic ThA1 and ThB4 analogues for iodination, and the respective antibodies were purchased from Alpha 1 Biochemical, Inc., Washington, DC. Antibody to porcine prolactin was purchased from Research Products International, Mount Prospect, IL. Antibody to LH was obtained from Dr. G. D. Niswender and LH for iodination was a gift from Dr. L. E. Reichert, Jr. (LER 786-3). The porcine LH and prolactin reference preparations were obtained from NIH-USDA distribution program. Antibodies to ThA1 and ThB4 were prepared and characterized for specificity by Dr. A. L. Goldstein and colleagues (21, 22). LH, ThA1, ThB4, and prolactin were analyzed by radioimmunoassay using double antibody methods (21–23) and reported previously in our laboratory (24–27). Progesterone concentrations were determined by radioimmunoassay of heptane extracts of plasma with antiprogesterone-11-bovine serum albumin (Cambridge Medical, Billerica, MA). This assay was validated by quantitative recovery of added progesterone from serum of ovariectomized gilts and by evidence of parallelism between different volumes of gilt plasma and progesterone standard. All samples from each experiment were assayed for a specific hormone within a single assay. Changes in hormone concentrations after weaning were evaluated statistically by analysis of variance. Student's t test was used for comparison of treatment means and a paired t test was used for comparison of hormone concentrations before LHRH treatment to those after LHRH treatment.

Results

ThB4 concentrations were elevated above 50 ng/ml on Days 2–4 and on Days 9, 21, 23, 24, 28, and 29 after weaning (Fig. 1). Three of these periods were associated with early follicular development and two with the early luteal phase. First estrus was observed on the evening of Day 4 in four of five sows and on Day 5 after weaning in the other; duration of estrus was 2.2 ± 0.2 days. These sows were in estrus again on Day 24, 25, or 26. Progesterone concentrations were greater than 1 ng/ml from Days 7 to 22 and after Day 28; maximal progesterone concentrations were observed during Days 15–19 after weaning. During the first period of follicular development (Days 1–5), ThB4 was elevated on 3 consecutive days whereas during the second period of follicular development (Days 21–25) the increase in ThB4 was bimodal in each sow. During this second follicular phase, ThB4 concentrations were more closely associated with luteolysis than with onset of estrous behavior (Fig. 2). Sows had their first substantial increase in ThB4 after progesterone had de-

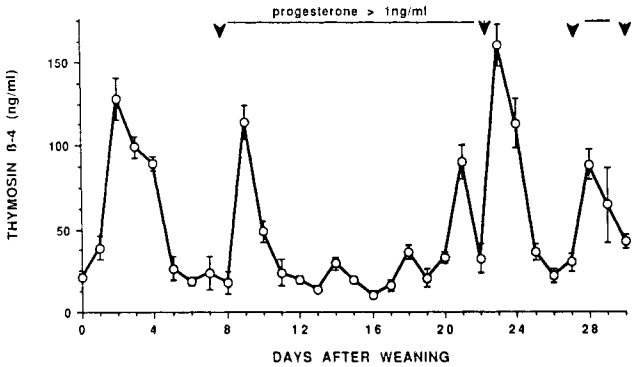

Figure 1. Peripheral plasma concentrations of ThB4 in sows during the first 30 days after their litters were weaned (n = 5). Values are mean ± SE. Periods when plasma progesterone concentrations were greater than 1 ng/ml are indicated by the solid line.

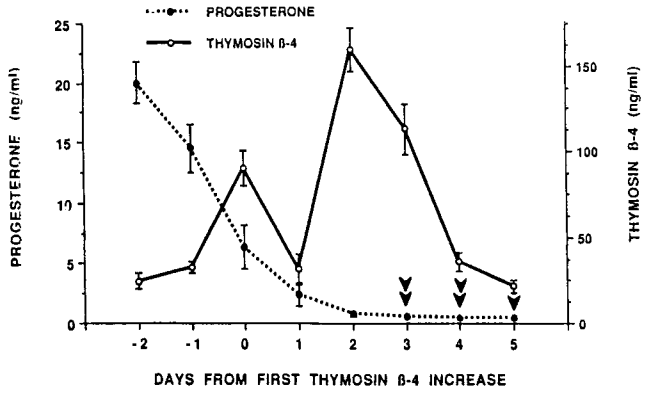

Figure 2. Peripheral plasma concentrations of ThB4 and progesterone during luteal regression and early follicular development ($n = 5$). Arrows indicate first day of estrous behavior for each sow. Values are mean ± SE. Day 0 corresponds to Day 21 after weaning.

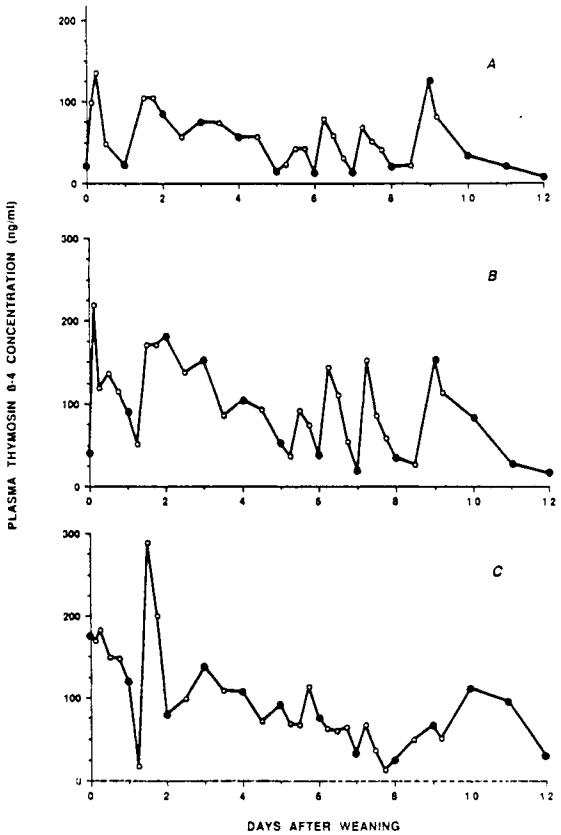

Figure 3. Peripheral concentrations of ThB4 in individual sows during the first 12 days after their litters were weaned. Early morning samples are indicated by solid circles.

creased to below 15 ng/ml, then ThB4 decreased until a second and greater rise occurred after progesterone had decreased to below 2 ng/ml.

Observations in sows in which plasma samples were assayed at more frequent intervals (Fig. 3) revealed that ThB4 concentrations were elevated during Day 0, then decreased markedly. Female C differed from all other sows because ThB4 was elevated at weaning. During Day 1, ThB4 was elevated again and then declined through Day 8. This decrease was interrupted by periods of increased concentrations that were characteristic of daily periods of secretory activity in two of three females. ThB4 increased again on Day 9 or 10 followed by a decrease. These sows were in estrus on the evening of Day 4. The pattern of change of ThB4 in early morning samples was similar to those observed for the previous five sows (Fig. 1).

Figure 4 illustrates the patterns of hormone (LH, ThA1, ThB4) concentrations in control and HST pigs after LHRH injection (2 days after HST). Both control and HST animals responded to LHRH injection by releasing increased amounts of LH ($P < 0.05$). Concentrations of LH in HST animals, both pre- and post-LHRH, were lower ($P < 0.05$) than in control animals. Concentrations of neither ThA1 nor ThB4 were changed in response to LHRH injection in either treatment group. Concentration of ThA1 was similar in control and HST animals, but HST females had higher concentrations of ThB4 ($P < 0.05$) than controls. On Day 9 after treatment, mean ThB4 concentrations increased in control females to 668 ± 37 ng/ml and were similar to concentrations in HST females (662 ± 40 ng/ml). Prolactin concentrations were 7.8 ± 0.3 and 9.1 ± 1.6 ng/ml for control and HST females. In samples from the previous experiment, serum ThB4 concentrations were 435 ± 80 for unoperated controls, 594 ± 103 for sham-operated females, and 428 ± 109 for HST females 2 days after treatments were imposed.

Discussion

Results from these studies indicate that changes in ThB4 secretion are associated with changes in ovarian function in pigs. Concentrations of ThB4 were consistently lower in gonadally intact females in the first study compared with ovariectomized females in the second study. At weaning, ThB4 concentrations were low in seven of eight sows. This is surprising because prolactin secretion is elevated in sows during lactation (28) and prolactin enhances thymic activity in mice (13). In the three sows that were sampled at 3 and 6 hr after weaning, ThB4 concentrations were elevated. At these times, peripheral concentrations of ovarian steroids

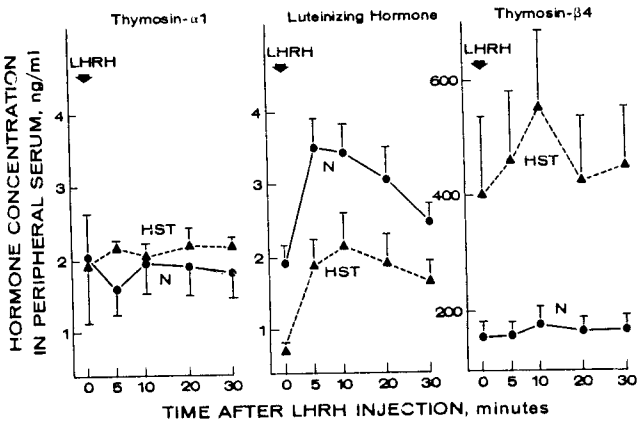

Figure 4. Peripheral serum concentrations of ThA1, ThB4, and LH in intact (N [●], $n = 5$) and HST ([▲], $n = 5$) pigs at various times after LHRH injection at time 0. Values are mean ± SE.

were low; however, cortisol secretion would likely be elevated in sows in response to separation from their piglets. (28). During the follicular phase of the estrous cycle (Days 1–5 and 21–25 after weaning) when ThB4 concentrations were elevated, estrogen secretion is increasing (28, 29). Pattern of ThB4 secretion differed during these two periods of follicular development. ThB4 secretion was uniformly elevated during the first follicular phase when ovarian luteal tissue was absent and bimodal during the follicular phase in association with luteolysis. Throughout estrus, ThB4 concentrations were decreasing. The rapid increase in ThB4 during early luteal formation is of interest but difficult to explain because progesterone secretion was increasing, and during the midluteal phase when progesterone secretion was elevated, ThB4 concentrations were low. Our understanding of regulation of thymic activity in pigs is limited and prevents us from developing full appreciation of the present observations. These findings do, however, identify the pig as a species in which distinct changes in ThB4 secretion occur during the estrous cycle. In cattle, ThB4 concentrations were elevated at estrus, but frequent samples were not obtained during the estrous cycle to evaluate temporal changes with ovarian steroids (30).

The changes in ThB4 concentrations that we observed in pigs do not agree with previous observations (27). We attribute these differences to method of blood collection and accuracy of determination of stage of the estrous cycle. In the current study, sows were sampled from indwelling jugular catheters. In the earlier report (27), blood samples were obtained by jugular venipuncture shortly after restraint and induction of anesthesia in females that were being prepared for ovariectomy and stage of the estrous cycle was assigned on basis of ovarian morphology.

Serum concentrations of ThA1 were not affected by HST, whereas ThB4 concentrations were greater in HST females than in controls in one study but not in the other. In the first of these, ThB4 increased in controls from Day 2 to Day 9 after treatment such that by Day 9, ThB4 concentrations were similar to those in HST females. An explanation for this change in control females is not obvious, but a response to surgical stress seems unlikely because in the later study no difference was observed between sham-operated and unoperated controls. It is readily apparent from studies with rodents that ThB4 or extracts rich in ThB4 cause acute increases in LH secretion via increased secretion of LHRH (8–10) and that prolonged treatment with an LHRH agonist enhances thymic weight (14). The present observations indicate that ThB4 secretion is not altered by an acute increase in LH secretion in ovariectomized female pigs. This provides additional support for the idea that gonadal steroids are the primary components of the hypophyseal-pituitary-gonadal axis that modulate thymic secretions. For comparison, ThB4 concentrations were 17.3 ± 2.8 ng/ml in serum from seven pregnant sows (Days 105–109 of gestation); a time when both estrogen and progesterone concentrations are elevated. Because LH and ThB4 are chronically elevated in ovariectomized female pigs, we cannot rule out the possibility that ThB4 might respond to increased LH if LHRH was administered to ovarian intact females.

Serum concentrations of LH were 72% lower in HST females that in controls before LHRH was given. The maximal increase in LH concentration after LHRH was 307% in HST females compared with 83% in controls. Thus, 2 days without hypothalamic input did not eliminate pituitary responsiveness to LHRH. This confirms the observations of Kesner *et al.* (31).

Studies *in vitro* showed that thymosin fraction 5 (thymic extract that contains ThA1 and ThB4) caused release of prolactin from pituitary cells (32) and that prolactin directly stimulated thymic epithelial cells (13). Serum prolactin concentrations were elevated in HST pigs during the first few days after surgery (17, 31), but by Day 9 in the present study, this was not observed. Results from the current studies do not support a relationship between ThA1 or ThB4 and prolactin under the *in vivo* conditions that were investigated. Because these females were ovariectomized, ThB4 secretion may be maximal and unable to respond to increased prolactin secretion after HST.

Collectively, our observations in ovariectomized female pigs fail to show direct associations between serum LH or prolactin concentrations and ThA1 or ThB4. Therefore, if ThB4 directly affects LH secretion during the estrous cycle, ThB4 secretion must first be influenced by changes in ovarian steroids that are associated with the demise of corpora lutea and growth of follicles. Now, it appears that elevated progesterone inhibits and estrogen may acutely enhance ThB4 secretion. Equally possible is that estrogen and ThB4 secretion increase concurrently but independent from each other. Further studies must address acute and chronic influences of exogenous ovarian steroids on ThB4 secretion in ovariectomized females.

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1. Grossman CJ. Regulation of the immune system by sex steroids. Endocr Rev **5**:435–455, 1984.
2. Wise TH. The thymus: Old gland, new perspectives. Dom Anim Endocrinol **5**:109–128, 1988.
3. Strich G, Petze JE, Silva de Sa MF, Rebar RW. The effects of thymus-derived peptides on hypothalamic LRF and pituitary

gonadotropin content in prepubertal congenitally athymic nude mice and their normal heterozygous littermates. J Reprod Immunol 7:351–359, 1985.
4. Smith PE. The effect of hypophysectomy upon the involution of the thymus in the rat. Anat Rec **47**:119–129, 1930.
5. Duquesnoy RJ, Christensen K, Pedersen GM. Development of immunodeficiency of pituitary dwarf mice. Am Zool **15**:167–176, 1975.
6. Berczi I, Nagy E, Dovacs K. Regulation of humoral immunity in rats by pituitary hormones. Acta Endocrinol **28**:506–513, 1981.
7. Saxena QB, Saxena RK, Adler WH. Regulation of natural killer activity in vivo. III. Effect of hypophysectomy and growth hormone treatment on the natural killer activity of the mouse spleen cell population. Int Arch Allergy Appl Immunol **67**:169–174, 1982.
8. Rebar RW, Miyake A, Low TLK, Goldstein AL. Thymosin stimulates secretion of luteinizing hormone-releasing factor. Science **214**:669–671, 1981.
9. Rebar RW, Miyake A, Erickson GF, Low TLK, Goldstein AL. The influence of the thymus gland on reproductive function: A hypothalamic site of action. In: Greenwald GS, Terranova PF, Eds. Factors Regulating Ovarian Function. New York: Raven Press, pp465–469, 1983.
10. Hall NR, McGillis JP, Spangelo BL, Healy DL, Goldstein AL. Immunoregulatory peptides and the antral nervous system. Springer Semin Immunopathol **8**:153–164, 1985.
11. Suh BY, Naylor PH, Goldstein AL, Rebar RW. Modulation of thymosin $\beta 4$ by estrogen. Am J Obstet Gynecol **151**:544–549, 1985.
12. Hall NR, McGillis JP, Spangelo BL, Goldstein AL. Evidence that thymosins and other biologic response modifiers can function as neuroactive immuno-transmitters. J Immunol 135(suppl 2):806S–811S, 1985.
13. Dardenne M, Savino W, Gagnerault M, Itoh T, Bach J. Neuroendocrine control of thymic hormonal production. I. Prolactin stimulates *in vivo* and *in vitro* the production of thymulin by human and murine thymic epithelial cells. Endocrinology **125**:3–12, 1989.
14. Marchetti B, Guarcello V, Morale MC, Bartoloni G, Raiti F, Palumbo G, Farinella Z, Cordaro S, Scapagnini U. Luteinizing hormone-releasing hormone (LHRH) agonist restoration of age-associated decline of thymic weight, thymic LHRH receptors, and thymocyte proliferative capacity. Endocrinology **125**:1037–1045, 1989.
15. Folch H, Eller G, Mena M, Esquivel P. Neuroendocrine regulation of thymus hormones: Hypothalamic dependence of "facteur thymique serique" level. Cell Immunol **102**:211–216, 1986.
16. Ford JJ, Maurer RR. Simple technique for chronic venous catheterization of swine. Lab Anim Sci **28**:615–618, 1978.
17. Anderson LL, Berardinelli JG, Malven PV, Ford JJ. Prolactin secretion after hypophyseal stalk transection in pigs. Endocrinology **111**:380–384, 1982.
18. Klindt J, Ford JJ, Berardinelli JG, Anderson LL. Growth hormone secretion after hypophyseal stalk transection in pigs. Proc Soc Exp Biol Med **172**:508–513, 1983.
19. du Mensil du Buisson F, Léglise PC, Chodkiewicz JP. Techniques de l'hypophpectomie par voie transfrontale sus-orbitaire chez la porc. Ann Biol Anim Biochim Biophys **4**:229–237, 1974.
20. Anderson LL, Dyck GW, Mori H, Henricks DM, Melampy RM. Ovarian function in pigs following hypophyseal stalk transection or hypophysectomy. Am J Physiol **212**:1188–1194, 1967.
21. McClure JE, Lameri SN, Wara DW, Goldstein AL. Immunochemical studies in thymosin: Radioimmunoassay of thymosin-$\alpha 1$. J Immunol **128**:368–375, 1981.
22. Naylor PH, McClure JE, Spangelo BL, Low TLK, Goldstein AL. Immunochemical studies on thymosin: Radioimmunoassasy of thymosin-$\beta 4$. Immunopharmacology **7**:9–16, 1984.
23. Niswender GD, Reichert LE Jr, Zimmerman DR. Radioimmunoassay of serum levels of luteinizing hormone throughout the estrous cycle in pigs. Endocrinology **87**:576–580, 1970.
24. Ford JJ, Schanbacher BD. Luteinizing hormone secretion and female lordosis behavior in male pigs. Endocrinology **100**:1033–1038, 1977.
25. Vakharia DD, Kinder JE, Wise TH. Thymosin-α_1 in the ovary and the levels of thymosin-α_1 and T lymphocytes during estrous cycle in swine. Tumbleson M, Ed. Proc Swine in Biomedical Research. New York: Plenum, pp1925–1936, 1986.
26. Klindt J, Stone RT. Porcine growth hormone and prolactin: Concentrations in the fetus and secretory patterns in the growing pig. Growth **48**:1–5, 1984.
27. Vakharia DD, Kinder JE, Wise TH. Thymosin-beta-4 (ThB4) levels in serum and follicular fluid during estrous cycle (EC) in swine. Fed Proc **45**:405, 1986.
28. Britt JH, Armstrong JD, Cox NM, Esbenshade KL. Control of follicular development during and after lactation in sows. Control of pig reproduction II. J Reprod Fert Suppl **33**:37–54, 1985.
29. Foxcroft GR, Van de Wiel DFM. Endocrine control of the estrous cycle. In: Cole DJA, Foxcroft GR, Eds. Control of Pig Reproduction. London: Butterworths, pp161–177, 1982.
30. Wolfe MW, Vakharia DD, Wise TH, Kinder JE. Characterization of secretion of thymosin alpha 1 and thymosin beta 4 during prepuberty, estrus and pregnancy in the bovine female. Dom Anim Endocrinol **6**:71–78, 1989.
31. Kesner JS, Estienne MJ, Kraeling RR, Rampacek GB. Luteinizing hormone and prolactin secretion in hypophyseal-stalk-transected pigs given estradiol and pulsatile gonadotropin-releasing hormone. Neuroendocrinology **49**:502–508, 1989.
32. Spangelo BL, Judd AM, Rosa PC, Login IS, Jarvis WD, Badamchiam M, Goldstein AL, MacLeod RM. Thymosin fraction 5 stimulates prolactin and growth release from anterior pituitary cells in vitro. Endocrinology **121**:2035–2043, 1987.