Effects of Estradiol-17 β or Dihydrotestosterone on Cell Types in Equine Pituitaries
Staining for Prolactin, Growth Hormone, or Both

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ABSTRACT

Ovariectomized pony mares were treated with vehicle (controls), estradiol-17 β (estradiol), or dihydrotestosterone (DHT) daily for 21 days in summer, and the pituitary content of prolactin and growth hormone (GH) and the numbers of cells staining immunocytochemically for prolactin (lactotropes), GH (somatotropes), or both hormones (mammosomatotropes) were assessed. Treatments did not alter ($P > 0.1$) plasma prolactin concentrations over the 21 days. Estradiol treatment increased ($P < 0.05$) the pituitary content of prolactin several-fold relative to controls and DHT-treated ponies. The number of lactotropes was also increased ($P < 0.05$), as was the number of mammosomatotropes ($P < 0.05$). Treatment with DHT did not alter ($P > 0.1$) any prolactin characteristic. The number of somatotropes was not altered by either treatment ($P > 0.1$), nor was the pituitary content of GH ($P > 0.1$). Given that the mass of prolactin accumulation in the pituitary in response to estradiol was several-fold greater than the approximate doubling of the number of lactotropes and mammosomatotropes, it appears that estradiol administration to ovariectomized pony mares stimulates not only the number of prolactin-producing cells but also the amount of prolactin production per cell.

1. Introduction

We previously described [1] the morphological characteristics of cells in adenohypophyseal tissue of pony mares that stained immunocytochemically for prolactin (lactotropes) and those that stained for growth hormone (GH; somatotropes), as well as a third cell type that stained positive for both hormones (mammosomatotropes). In that report [1], we also described two distinct morphological forms of lactotropes, referred to as type I and II. Type I cells were larger and contained large, dense, polymorphic granules scattered throughout the

cytoplasm; type II cells were smaller and contained small granules that were predominantly found in peripheral areas of the cytoplasm. The relative importance of these two types of lactotropes to prolactin secretion is unknown.

Direct estrogenic stimulation of plasma prolactin concentrations and pituitary content in ovariectomized pony mares was previously reported by Thompson et al [2]. Several experiments since that first report have confirmed the stimulatory effect of estradiol on prolactin production and secretion in horses [3-5], and application of the results from those experiments led to the development of a estradiol cypionate-sulpiride (or domperidone) combination treatment for inducing follicular growth and ovulation in seasonally anovulatory mares in winter [6,7]. The stimulation of prolactin via the estradiol-dopaminergic antagonist combination is synergistic (reviewed by [8]), and is dependent on both factors being present at the same time; interestingly, other prolactin secretagogues, such as exercise or thyrotropin releasing hormone (TRH), do not synergize with estradiol pretreatment [5].

Testosterone, like estradiol, is known to stimulate prolactin secretion in other species [9,10], likely due to its ability to be aromatized to estradiol in peripheral tissues. Dihydrotestosterone (DHT), another potent androgen, cannot be aromatized to estrogen [11], thus serves as an androgen alternative to testosterone in experimental paradigms. The purpose of the present experiment was to examine the effects of estradiol and DHT treatment on the relative proportions of lactotropes, somatotropes, and mammosomatotropes in the adenohypophysis of ovariectomized pony mares.

2. Materials and Methods

2.1. Animals and Treatments

All procedures described herein were approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center. Twelve adult (6 to 12 years of age) long term (>2 years) ovariectomized mixed-breed pony mares weighing between 168 and 323 kg were kept on pasture and fed grass hay when needed to maintain good body condition throughout the year. The mares were randomly allotted to one of three treatment groups (n = 4/group) which were injected daily with: 1) 1 mL of corn oil (controls); 2) estradiol-17 β (estradiol; Sigma Chem. Co., St. Louis, MO), 35 μ g/kg bodyweight in 1 mL of corn oil; or 3) DHT (Sigma), 150 μ g/kg bodyweight in 1 mL of corn oil. All injections were administered subcutaneously in the neck region and were repeated at 0800 each day for 21 days. The doses of estradiol and DHT were derived from previous reports from this laboratory [2] and the effects on pituitary hormone characteristics were well documented.

Because of the time needed for tissue processing after pituitary removal, one mare from each treatment group was started on treatment on one of four consecutive days and all mares were killed on the 22nd day of their treatment. The experiment was performed between July 11 and August 4. Daily blood samples were collected via jugular venipuncture immediately prior to the daily treatment or control injections. These samples were drawn into evacuated, heparinized tubes; plasma was harvested by centrifugation at 1200 x g and stored at -15°C for later analysis.

2.2. Pituitary Preparation for Hormone Analysis

Pituitaries were removed within 7 to 12 min following euthanasia (by electrocution and exsanguination, performed by the personnel of the LSU School of Veterinary Science), were rinsed with saline, and were cut in half on the mid-sagittal plane. One half was placed in ice-cold 0.01 M phosphate buffered saline (0.15 M NaCl; PBS) and was stripped of its surrounding

capsule. The neurohypophyseal tissue was separated from the adenohypophyseal tissue (AP) and the halved AP was weighed. This AP tissue was then homogenized in 100 mL of PBS in a Waring 7011 blender (Dynamics Corp. of America, New Hartford, CT) at maximum setting for 30 sec. Another 100 mL of PBS was used to rinse out the blender jar. The homogenate and rinse were mixed together and 10-mL aliquots were centrifuged at 1200 x g for 30 min at 4°C. The resulting supernatants were harvested and frozen for later analysis of hormone concentrations.

At the end of the experiment, each AP homogenate was diluted 1:500 in PBS containing 0.1% gelatin. Concentrations of prolactin and GH were estimated in two-fold serial dilutions (from 0.2 to 0.0125 mL) of each homogenate by radioimmunoassay as described previously [12,13]. Concentrations of prolactin in the stored plasma samples were estimated in duplicate 0.2-mL aliquots in the same assay. Concentrations of GH in daily samples were not determined due to the episodic nature of GH secretion in horses. The inter- and intra-assay coefficients of variation and limit of detection of the assays were 10%, 7%, and 0.2 ng/mL for prolactin and 11%, 6%, and 0.5 ng/mL for GH.

2.3. Tissue Preparation for Electron Microscopy

Following a rinse with saline, the remaining half of the pituitary was placed in freshly prepared 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) plus 0.1% glutaraldehyde (Polysciences, Warrington, PA) buffered with 0.1 M sodium cacodylate (pH 7.3; Electron Microscopy Sciences). While in the fixative, the tissues were freed of the surrounding capsule and the neural lobe was removed. The AP tissues were then cut into approximately 1-mm³ pieces and left in the fixative for another 2 h. Following fixation, ten randomly selected

pieces from each mare were removed and cut into smaller pieces, which were then used for embedding.

Tissue pieces were washed several times in 0.1 M sodium cacodylate buffer to remove any residual aldehyde, and were then dehydrated in 50% ethanol (EtOH) for 15 min, 70% EtOH for 20 min, and 90% EtOH for 30 min. Some of the tissue pieces were processed for ultrastructure study using routine Epon-Araldite embedding for transmission electron microscopy as previously described by Poolsawat [14]. Tissues used for immunocytochemistry were infiltrated and then embedded with 60% LR Gold (LRG; Electron Microscopy Sciences) monomer:40% EtOH for 30 min; 70% LRG:30% EtOH for 30 min; 100% LRG monomer for 2 h; 100% LRG monomer plus 0.1% benzil (initiator) for 2 h; and finally overnight in 100% LRG monomer plus initiator. All procedures were performed at room temperature. The tissues were then embedded in small gelatin or large BEEM capsules (Electron Microscopy Sciences). The LRG was polymerized under UV light at -15°C for 24 to 36 h. Ultrathin, 80-90 nm light gold-colored sections were cut on a Sorvall MT2-B ultramicrotome and sections were mounted on 400 mesh gold grids.

2.4. Immunogold Labeling

Sections were first incubated for 10 min on a drop of 0.05 M Tris (pH 7.3) containing 0.02 M glycine to quench any aldehyde left in the tissue. They were then incubated for 30 min on a drop of Tris buffer containing 1% bovine serum albumin plus 10% normal goat serum (blocking solution) to reduce non-specific binding. The grids were transferred to a 35-μL drop of the specific (anti-prolactin or anti-GH; described below) primary antibody (diluted 1:20 in the blocking solution) and were incubated for 2 h. Next, grids were blotted and rinsed four times on

a drop of Tris buffer for 5 min each. The sections were then blotted and transferred to a drop of the blocking solution for 5 min followed by incubation for 50 min on a 35- μ L drop of a 20% solution (as recommended by the supplier) of goat anti-rabbit IgG-gold conjugate (Electron Microscopy Sciences). Two sizes of gold particles were used: 6 or 10 nm. Single labeling was done with either 6 or 10 nm gold conjugated IgG. The sections were washed in Tris buffer with 2.5 M NaCl for 10 min and rinsed in distilled water. The final step involved incubating the sections on a drop of 0.5 % formaldehyde for 10 min and rinsing with distilled water followed by complete drying.

Double-labeling was done following a protocol described previously by Bendayan [15]. Briefly, following the above steps for one side of the grid, the grid was turned over and the same procedure was followed except that the primary antibody and the size of the gold particle on the secondary antibody were changed. The sections were counter- stained with uranyl acetate and Sato lead before examination with a Philips 410 Electron Microscope.

Antibody specificities and cross-reactivities with various AP hormones have been reported for the anti-prolactin serum [12]. The rabbit anti-bovine GH serum was prepared by the late C. H. Li and colleagues (University of California, San Francisco) and was provided to us by H. Papkoff (University of California, Davis). The peptides used in pre-adsorption tests for specificity of immunocytochemical staining were bovine LH (USDA-bLH-B-6), human FSH (hFSH ; FSH-I-SIAFD-1, Lot # AFP-5720D), porcine prolactin (pPRL; USDA-pPRL-B-1), porcine growth hormone (pGH ; USDA-pGH-B-1), bovine thyroid stimulating hormone (bTSH; USDA-bTSH-I-1), and human ACTH (hACTH-I-SIAFD-1, Lot# AFP-2938C); all were obtained from the National Hormone and Pituitary Program (Harbor-UCLA Medical Center, Torrance, CA). In addition to the cross-reactivity procedures used to prepare the primary antisera, other

tests for specificity consisted of 1) omitting the primary antiserum step to test for nonspecific binding of gold-conjugates to the tissue and 2) testing for binding of primary antibodies after pre-absorption with either homologous or heterologous pituitary hormones. Those results are presented in our earlier report [1].

2.5. Cell Counting and Statistical Analyses

A total of 10 grids were examined per mare resulting in a total of 40 grids per treatment. The number of each cell type was recorded for each grid, the average number of cells per grid for each mare was calculated, and the analyses were performed on these data. Mean percentages were calculated from the overall cell counts and the total number of specific cell type in the 40 grids and were not statistically analyzed.

All data were analyzed via SAS software (SAS Institute, Cary, NC). Data consisting of repeated measurements taken over time were analyzed by analysis of variance that accounted for the repetitive nature of blood sampling [16]. Due to the large variation in pre-treatment plasma prolactin concentrations, the percent change from pretreatment was calculated from the raw data for each pony and used for analysis; the least significant difference test [16] was used to compare post-treatment means to 100%. Non-repetitive data were analyzed by one-way analysis of variance; means for each treatment were compared to controls by the least significant difference test.

3. Results

3.1. Plasma Hormone Concentrations

Plasma prolactin concentrations before initiation of treatments (mean $\pm$ standard error) were: Group 1/controls, 24.8 ± 20.7 ng/ml, Group 2/estradiol, 35.8 ± 34 ng/ml, and Group 3/DHT, 41.7 ± 22.6 ng/ml. Daily prolactin secretion was very variable among the three treatment groups (Fig. 1). There was no overall treatment affect ($P > 0.1$) on plasma prolactin concentrations. Due to the episodic nature of GH secretion [13], one daily blood sample would not have adequately tested any increase or decrease in GH secretion; therefore, blood samples were not analyzed for GH concentrations.

3.2. Hormone Content in the Pituitary

Estradiol treatment resulted in an eight-fold increase ($P < 0.05$) in pituitary prolactin content compared to those in the control and DHT-treated ponies (Table 1). There was no difference ($P > 0.1$) in pituitary prolactin content for the control and DHT-treated groups. Content of GH in the AP tissue was not affected ($P > 0.1$) by either of the treatments relative to controls (Table 1).

3.3. Pituitary Cell Counts

Number of cells per grid staining for GH, prolactin, and both hormones are presented in Table 1. Total number of cells counted in each treatment group (40 grids) were: controls, 466; estradiol-treated, 628; and DHT-treated, 599. The number of labeled prolactin cells/grid was higher ($P < 0.05$) in the estradiol-treated group relative to the other two groups. Mammosomatotropes per grid were also more prevalent ($P < .05$) in the estradiol-treated group. There was no effect ($P > 0.1$) of treatment on the cells/grid labeled for GH. In the control group, the percentages of cells staining for prolactin and GH cells were almost equal. These cell types

collectively accounted for about 41% to 57% of the total number of pituitary cells counted. Mammosomatotropes comprised about 3 to 6% of the total number of pituitary cells in control and DHT-treated mares and 10% of the pituitary cells in estradiol-treated mares.

3.4. Ultrastructure of Somatotropes and Lactotropes

We previously detailed the morphological characteristics of reactive somatotropes (Fig. 2), type I lactotropes (Fig. 3), type II lactotropes (Fig. 4 and 5) and mammosomatotropes (Fig. 6) in our previous report [1]; figures 2, 3, and 4 are reprinted from that publication for ease of reference. Results from the present experiment indicated that the ultrastructure of various cell types in these ovariectomized pony mares is similar to that reported for gonadally intact mares in the previous study [1]. Based on visual observations there seemed to be a higher number of type II lactotropes in estradiol-treated ponies compared to the other two treatment groups, sometimes appearing in clusters (Fig. 7). However, type I and type II lactotropes were not specifically counted as distinct subsets in this experiment and our impression will need verification in the future.

4. Discussion

Similar to the results of Thompson et al [2], treatment of these ovariectomized ponies with estradiol increased pituitary content of prolactin several-fold, even though there was no change in daily plasma prolactin concentrations over the 21-day period. Thompson et al [2] did report an approximate 25% increase in plasma prolactin concentrations due to estradiol treatment, but it was transient. Not until Kelley et al [3] combined estradiol pretreatment with subsequent administration of the dopamine antagonist, sulpiride, was it known how the two

229 treatments synergized to produce large increases in not only pituitary content but also day-to-day
230 secretion. Since that original report [3], the synergistic effect of estradiol coupled with a
231 dopamine antagonist (either sulpiride or domperidone) on prolactin secretion has been shown
232 multiple times [4-7]. Interestingly, other secretory stimuli for prolactin (exercise, $\text{PGF}_{2\alpha}$
233 administration, TRH administration) do not synergize with estradiol treatment in the same
234 manner [5], indicating that those stimuli must act on lactotropes via a different mechanism than
235 dopaminergic antagonists. Given that no sulpiride or domperidone was given in the present
236 experiment, only pituitary content of prolactin was stimulated, but not secretion. Note also that
237 pituitary GH was not affected by either estradiol or DHT treatment, a fact that has not been
238 reported previously.

239 Whenever an increase in hormonal content of a gland of origin is observed, it is not
240 necessarily clear whether the increase is due to a greater number of cells or to a greater
241 production rate per cell (or both). Our data confirm for the first time in ovariectomized pony
242 mares that the stimulatory effect of estradiol on prolactin content in the pituitary is at least in part
243 due to an increase in the number of the two cell types immunostaining as lactotropes and
244 mammosomatotropes. In fact, the number of cells per grid was about double in estradiol treated
245 ponies relative to controls in both cases. Given that the actual content, in mg per gland, in
246 estradiol-treated ponies was about eight-fold that in control ponies, we would conclude that the
247 amount of prolactin contained in each cell was increased as well. In contrast, the
248 nonaromatizable androgen, DHT, had no effect on either pituitary content or the number of cells
249 staining for prolactin. Typically, in other species, the aromatizable androgens, such as
250 testosterone and androstenedione, also stimulate prolactin production and secretion
251 characteristics [9,10,17], which may explain why stallions (and mares) both have greater plasma

prolactin than geldings in summer [18] and why stallions also respond to sulpiride in winter with a large increase in prolactin secretion [19]. Aromatization of testosterone to estradiol in the brain is likely what maintains normal libido in stallions, given that Thompson et al [20] demonstrated that both testosterone and estradiol separately could restore sex drive in recently castrated geldings.

Stratmann et al. [21] reported that following 21-day treatment of rats with estradiol there was a true transformation of somatotropes to lactotropes. Later, Nogami [22] also showed that stimulation of newborn and adult rats with estradiol resulted in an increase in lactotrope populations. Finally, Frawley et al. [23] described the presence of a distinct cell population in the pituitary that is capable of secreting both GH and prolactin concomitantly; hence, these cells were referred to as mammosomatotropes. Others [24-26] subsequently reported that the fluctuation in number of lactotropes and somatotropes was influenced by gonadal steroids and that there was a bi-directional interconversion of these two cell types that depended upon hormonal cues [27]. Given that the number of somatotropes did not differ among the three groups of ponies in the current experiment, we cannot speculate on whether there was any interconversion of cell types, or whether there was an increase in cell numbers due to mitotic division of existing cell types or some precursor cell type.

In our previous report [1] describing the morphological characteristics of lactotropes and somatotropes in pony pituitaries, two distinct populations of lactotropes in the equine pituitary, type I and type II, were easily differentiated once they were immunologically stained for prolactin. Type I cells were characterized as being larger than type II cells and they contained numerous large polymorphic secretory granules. Type II cells were smaller in size and had a large nucleus-to-cytoplasm ratio, and relatively small peripherally located secretory granules. In

the current experiment, based on visual observation, it appeared that there were more type II lactotropes than type I lactotropes in the estradiol-treated group whereas the proportion of these subsets of cells was approximately equal in control ponies. The relative contribution of these two lactotrope cell types to the overall secretion of prolactin to various stimuli is unknown, although they certainly may help explain the dichotomy in secretion rates in response to dopaminergic antagonists as opposed to other stimuli, as described by Thompson and Oberhaus [8].

Reports on estradiol effects on somatotropes have been inconsistent and seem to be species-specific. Simard et al. [28] reported a 2- to 3-fold increase in GH release as well as in the cellular GH content in estradiol-treated rat pituitary cell cultures. In contrast, Bethea and Freesh [29] and Copeland et al. [30] reported that physiological doses of estradiol did not stimulate GH release in primates. Kineman et al. [31] reported that the relative proportion of somatotropes remained unchanged throughout the bovine estrous cycle, in spite of the fact that prolactin-secreting cell numbers fluctuated significantly. Although we observed no change in somatotropes in the current experiment in response to estradiol treatment, one might speculate that the increase in mammosomatotrope numbers could result in a greater GH response to secretagogue(s). However, we have unpublished data on the GH responses to exercise, $\text{PGF}_{2\alpha}$, epinephrine, and sulpiride injection either with or without estradiol pretreatment from the experiment of Thompson et al [5]; no alteration in GH response was observed for any secretagogue.

Treatment of these pony mares with DHT did not alter plasma prolactin concentrations nor the pituitary content of prolactin, which is similar to the lack of DHT effect on plasma prolactin concentrations reported previously [2]. Interestingly, DHT (and testosterone) administration did increase the prolactin response to TRH injection in that experiment [2], even though pituitary prolactin content was not altered. Apparently, androgens enhance the sensitivity

of equine lactotropes to TRH stimulation. In this study, DHT treatment did not affect pituitary content of GH or the relative proportion of pituitary cells that stained for GH. Testosterone has been shown to promote storage of GH in the pituitary of rats [32,33] and increase the pituitary responsiveness to GHRH [34]; however, it is possible that those effects were a result of the aromatization to estradiol in peripheral tissues.

In conclusion, the results of the present experiment are consistent with the model of prolactin production and secretion in the horse in which estradiol, in the absence of other (possible) competing steroids, increases both the number of prolactin-secreting cells in the adenohypophysis as well as the amount of prolactin produced per cell. The nonaromatizable androgen, DHT, had no effect on prolactin or GH characteristics.

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Figure legends

Fig. 1. Mean plasma prolactin concentrations, expressed as percent of pretreatment values, for ponies injected daily with vehicle (controls), estradiol, or dihydrotestosterone (DHT) for 21 days. There was no effect of treatment ($P > 0.1$) on prolactin concentrations. Pooled standard error of the means was 48%. Concentrations of GH in daily samples were not determined due to the episodic nature of GH secretion in horses.

Fig. 2. A typical, well preserved example of a cell staining positively for growth hormone (i.e., a somatotrope) from an intact pony mare in summer. The small granules (arrow) in the adjacent cell were labeled for prolactin (6 nm immunogold). * Rough endoplasmic reticulum; N Nucleus. Bar = 3 μm . (Reproduced from [1] with permission of the American Society of Animal Science).

Fig. 3. A typical, well preserved example of a cell staining positively for prolactin (i.e., a lactotrope) from an intact pony mare in summer. The relatively large and often polymorphic secretion granules (arrows) throughout the abundant cytoplasm indicate that this is a type I lactotrope. N Nucleus. Bar = 2 μm . (Reproduced from [1] with permission of the American Society of Animal Science).

Fig. 4. Another cell type staining positively for prolactin (i.e., a lactotrope) from an intact pony mare in summer, distinctly different from that presented in Fig. 3, and classified as a type II lactotrope. Type II lactotropes have much less cytoplasmic volume (C) and smaller polymorphic granules, mostly on the periphery of the cell (arrow). The adjacent cell (*) is a somatotrope. N

Nucleus. Bar = 1 μ m. (Reproduced from [1] with permission of the American Society of Animal Science).

Fig. 5. Higher magnification of the cytoplasm of a type I lactotrope from an ovariectomized pony mare treated with vehicle (oil) in the present experiment. The section was incubated with anti-prolactin (10 nm immunogold) and anti-growth hormone antisera (6 nm immunogold).

Lactotropes sections retained only the 10-nm immunogold particles. Bar = 1 μ m.

Fig. 6. Example of an equine pituitary mammosomatotrope incubated with anti-prolactin (10 nm immunogold) and anti-growth hormone antisera (6 nm immunogold). The mammosomatotropes retained both the 6- and 10-nm immunogold particles (difficult to see at this lower magnification). Golgi body (arrow head). Polymorphic granules (small arrows). Centriole (large arrow). N Nucleus. Bar = 2 μ m.

Fig. 7. A cluster of type II lactotropes in the pituitary of an ovariectomized pony treated for 21 days with estradiol in the present experiment. Bar = 5 μ m.

Figure 1

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Figure 2

Figure 3

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Figure 4

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Figure 5

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Figure 6

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Figure 7

Table 1. Pituitary content of prolactin and growth hormone (GH) and number of cells counted staining for either prolactin, GH, or both in ponies injected daily with vehicle (controls), estradiol, or dihydrotestosterone (DHT) for 21 days.

	Controls				Estradiol-treated				DHT-treated		
	Prolactin	GH	Both		Prolactin	GH	Both		Prolactin	GH	Both
Pituitary content of hormone, mg	0.45 ^a	4.65 ^c			4.11 ^b	6.85 ^c			0.18 ^a	4.85 ^c	
Pooled standard error of pituitary hormone content, mg	0.81	1.9			0.81	1.9			0.81	1.9	
Total # of cells counted over 4 mares (40 grids)	466	466	466		628	628	628		599	599	599
Mean cells counted per grid	11.65	11.65	11.65		15.7	15.7	15.7		15.0	15.0	15.0
Total # cells counted stained for the specific hormone	105	103	27		215	85	62		83	147	18
Mean # cells per grid stained for specific hormone	2.63 ^a	2.57 ^c	0.68 ^d		5.38 ^b	2.12 ^c	1.55 ^e		2.08 ^a	3.68 ^c	0.45 ^d
Pooled standard error of # cells stained for specific hormone per grid	0.767	1.117	0.204		0.767	1.117	0.204		0.767	1.117	0.204
% of cells stained for specific hormone	22.5 ^f	22.1	5.8		34.2	13.5	9.9		13.8	24.5	3.0

^{a-b, c, d, e} Means across treatments for a specific hormone with different superscripts differ (P < 0.05).

^f Calculated from 105/466 x 100 = percent specifically stained of all cells visualized.