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Induction of Somatotroph Differentiation In Vivo by Corticosterone Administration During Chicken Embryonic Development

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Somatotroph differentiation in the embryonic pituitary of avian and mammalian species can be stimulated by glucocorticoids in vitro, and this effect can be augmented by concomitant treatment with growth hormone–releasing hormone (GHRH). Owing to its isolation from maternal influences, the chick embryo is a useful model for studying humoral regulation of pituitary cell differentiation. Somatotroph differentiation in chickens occurs between embryonic day (e-) 14 and e-16, and treatment of e-12 pituitary cells with e-16 serum or corticosterone induces growth hormone (GH) cell differentiation within 2 d in culture. The objective of the present study was to determine whether direct administration of embryonic serum and corticosterone to developing chick embryos was effective in vivo in inducing somatotroph differentiation prematurely. The albumen of fertile eggs was injected on e-11 with 300 μL of 0.9% saline or 150 μL of serum from e-12 or e-16 chick embryos diluted 1:1 with saline. The embryos were allowed to develop until e-14, when pituitaries were dispersed and the resulting pituitary cells were subjected to reverse hemolytic plaque assays (RHPA) and immunocytochemistry to detect GH-secreting and GH-containing cells, respectively. Injection of e-16 serum increased ($p < 0.01$) GH-secreting and GH-containing cells to 11.5 ± 1.0% and 17.4 ± 3.3% of all pituitary cells, compared to 5.0 ± 0.3% and 5.5 ± 0.9% for saline-injected controls, respectively. Day 12 serum increased GH-containing cells to 9.8 ± 0.9%, without changing percentages of GH-secreting cells. In experiment 2, saline, e-16 serum, and corticosterone were injected on e-11, and pituitary cells were subjected to GH RHPA on e-14. GH secretors were increased by e-16 serum and corticosterone. In experiment 3, we tested whether GHRH would magnify the effect of corticosterone, as we had seen in extended 6-d cultures previously. Saline, corticosterone, and corticosterone plus GHRH were injected on e-11, and pituitary cells were subjected to GH RHPA on e-18. Treatment with corticosterone alone and combined with GHRH increased the percentage of GH-secreting cells. However, combined treatment with corticosterone and GHRH was not more effective than corticosterone alone. The present findings demonstrate that glucocorticoid administration can stimulate somatotroph differentiation in living vertebrate embryos isolated from maternal interactions.

Key Words: Somatotropin; differentiation; pituitary; glucocorticoid; embryo; development.

Introduction

Differentiation of the anterior pituitary into its five cell types has been studied extensively. This research has resulted in a general model for the ontogenic appearance of the different cell types *(1)*. The first functional cell type to appear in the anterior pituitary is the ACTH-producing corticotroph. Corticotrophs are apparently the only anterior pituitary cells that differentiate in the absence of an extrapituitary signal, because they are readily detected in unstimulated cultures of pituitary anlagen *(2)*. The other cell types require some extracellular signal to stimulate their differentiation. Several studies have suggested that somatotroph and lactotroph differentiation in rats are stimulated by various peptides and steroids. Estradiol stimulated lactotroph differentiation in pituitary tissue transplanted under the rat renal capsule *(3)*, an effect dependent on the endocrine status of the host *(4)*. Differentiation of fetal rat somatotrophs has been induced with glucocorticoids in vitro *(5–7)*, and treatment of pregnant rats with dexamethasone induced premature appearance of growth hormone (GH) mRNA and immunoreactive GH cells in their fetuses *(8,9)*. However, in these latter studies

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the possibility that dexamethasone was indirectly stimulating somatotroph differentiation in vivo through maternal-fetal interactions could not be ruled out. For example, dexamethasone also increased water consumption by the pregnant females and decreased the body weight of their fetuses.

The avian embryo has been used to study developmental events in areas such as the cardiovascular *(10)*, skeletal, and nervous systems *(11)*. One advantage of avian embryonic development as a model is the ease with which the embryos can be manipulated individually in the absence of maternal interactions. Recently, the ontogeny of functional lactotrophs and somatotrophs in the embryonic chicken has been determined *(12,13)*, and the role of extracellular factors in regulating differentiation of somatotrophs has been investigated in vitro *(14–16)*. The pattern of pituitary cell differentiation in chickens is comparable to that in mammals *(17,18)*. GH cells are occasionally detected as early as embryonic day (e-) 12, with significant somatotroph differentiation occurring between e-14 and e-16 of chick development *(12–14)*. Full differentiation of functional chicken somatotrophs continues gradually from e-16 through at least e-20 of the 21-d incubation period *(19)*, with somatotroph percentages in 4-wk-old male and female chickens reaching about 20 and 13%, respectively *(20)*. As in mammals, chicken GH cells do not differentiate in culture without an extrapituitary signal *(14)*. However, somatotroph differentiation in the chicken embryonic pituitary can be induced in vitro by embryonic serum or corticosterone within 2 d *(14,15)*. Longer exposure to corticosterone combined with GH-releasing hormone (GHRH) for 6 d increased the size of the somatotroph population even further *(16)*.

To date, the ability of embryonic serum and corticosterone to induce differentiation of chicken GH cells has only been evaluated in vitro. Corticosterone was identified as the GH cell–differentiating activity of e-16 chicken serum *(15)*, and the capacity of serum from chick embryos to induce GH cell differentiation in vitro correlated with the ontogeny of somatotrophs *(14)*. Others have reported that levels of adrenal steroids increase between d 12 and 16 of chick embryonic development *(21–24)*. The observation that GH cell differentiation on e-16 follows an increase in circulating corticosterone levels suggests that corticosterone could signal induction of somatotroph differentiation in vivo. In the present study, chicken embryos were used to study somatotroph differentiation in vivo, taking advantage of the relative ease with which the endocrine environment of individual embryos can be manipulated.

Results

Injection of serum from e-16 embryos into fertile eggs on e-11 increased ($p < 0.01$) the percentage of GH-secreting cells by e-14 to 11.5 ± 1.0%, compared to 5.0 ± 0.3% following injection of e-12 serum and 5.0 ± 0.4% for saline-injected controls (Fig. 1). Injection of e-16 serum also increased the proportion of GH-containing cells to 17.4 ± 3.3%, compared to 5.5 ± 0.9% for saline-injected controls. Although ineffective for increasing the percentage of GH-secreting cells, e-12 serum did increase the proportion of GH-containing cells to 9.8 ± 0.9%. The total number of cells recovered per pituitary was not different among treatment groups, and increased somatotroph proportions were not accompanied by increased levels of circulating GH in the embryos (mean GH level across treatments of 5.0 ± 0.5 ng/mL). The results from experi-

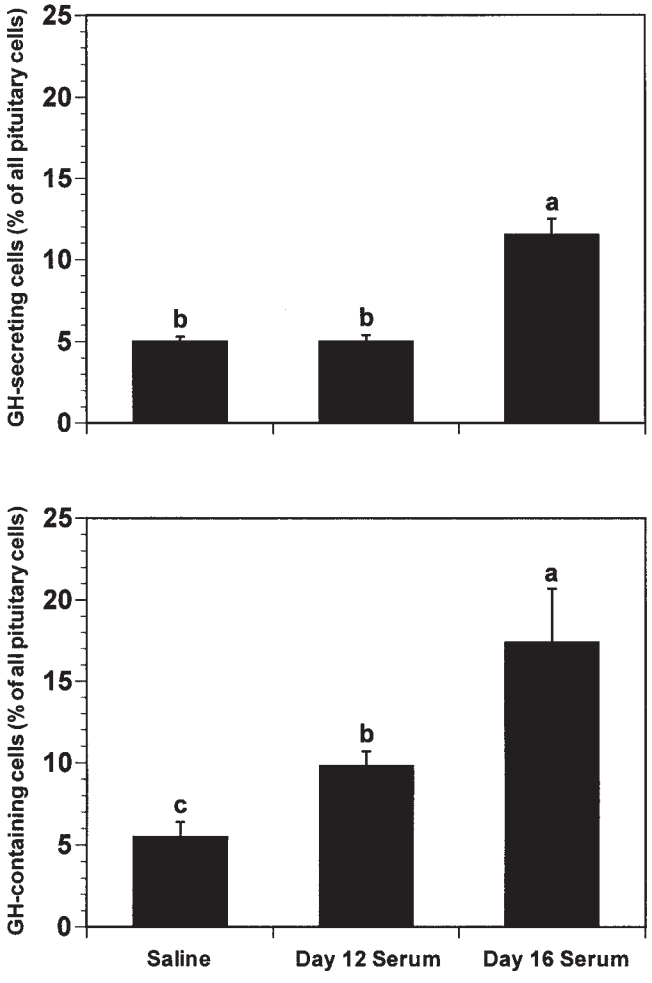

Fig. 1. The effects of *in ovo* injection of embryonic chicken serum on proportions of GH-secreting **(upper)** and GH-containing **(lower)** pituitary cells. The albumen of fertile chicken eggs was injected on e-11 with 300 μL of 0.9% saline (controls) or 150 μL of e-12 or e-16 chicken serum diluted 1:1 with saline. Anterior pituitary glands were isolated and dispersed on e-14, and pituitary cells were subjected to RHPA to detect GH-secreting cells and immunocytochemistry to detect GH-containing cells. RHPA chambers were incubated for 8 h in the presence of GHRH (10^{-7} *M*). These results are the means and SEM from three independent experiments. Values with no letters in common are significantly different ($p < 0.05$). Nearly identical results were found in RHPAs performed for 20 h.

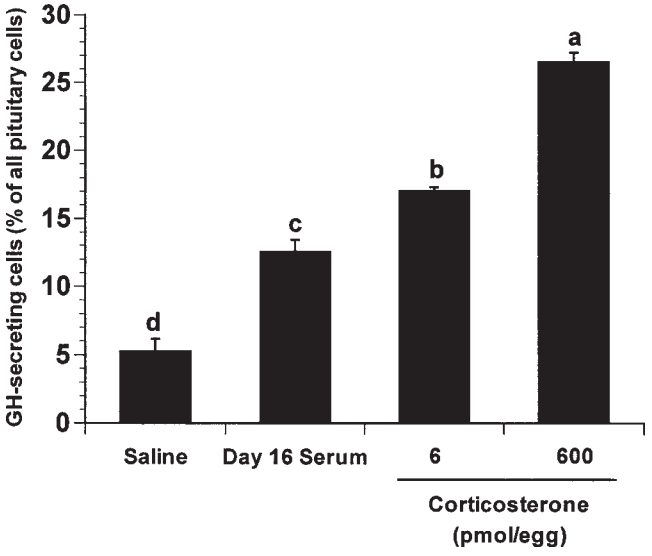

Fig. 2. Effect of *in ovo* injection of embryonic chicken serum and corticosterone on proportions of GH-secreting cells. The albumen of fertile chicken eggs was injected on e-11 with 120 μL of 0.9% saline (control), 60 μL of e-16 chicken serum diluted 1:1 with saline, or corticosterone (6 or 600 pmol in 120 μL). Anterior pituitary cells were subjected to GH RHPA on e-14. These results are the means and SEM from three independent trials. Values with no letters in common are significantly different ($p < 0.05$). See legend to Fig. 1 for additional details.

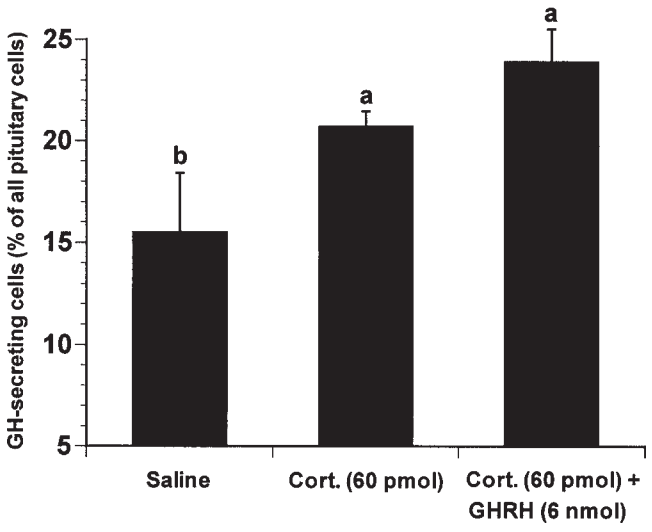

Fig. 3. Effect of *in ovo* injection of corticosterone alone and combined with GHRH on proportions of GH-secreting cells. The albumen of fertile chicken eggs was injected on e-11 with 120 μL of 0.9% saline, corticosterone alone (60 pmol), or corticosterone combined with GHRH (6 nmol). Anterior pituitary cells were subjected to GH RHPA on e-18. These results are the means and SEM from three independent experiments. Values with no letters in common are significantly different ($p < 0.05$). See legend to Fig. 1 for additional details.

ment 1 indicate that administration of serum from a later stage of embryonic development can induce premature differentiation of GH-secreting cells in intact embryos.

In experiment 2, the effects of e-16 serum and corticosterone administration in vivo on GH cell differentiation were compared. In agreement with the results of experiment 1, e-16 serum increased the proportion of GH-secreting cells to 12.5 ± 0.9%, relative to the control (saline-injected) level of 5.2 ± 0.9%. Corticosterone further increased the proportion of GH secretors in a dose-related manner to 17.0 ± 0.3% and 26.5 ± 0.7% at 6 and 600 pmol, respectively (Fig. 2). These results demonstrate that the ability of corticosterone to induce somatotroph differentiation is not restricted to cell culture conditions.

In the final experiment, somatotroph proportions were determined on e-18 following a single *in ovo* injection of corticosterone alone and combined with GHRH on e-11. This interval was chosen because previous results in vitro indicated that 6 d were required to observe any enhanced effect of GHRH on corticosterone-induced somatotroph differentiation *(16)*. Figure 3 summarizes the results from the current in vivo experiments. Treatment with corticosterone alone and combined with GHRH increased the percentage of GH-secreting cells from control (saline-injected) levels of 15.5 ± 2.9% to 20.7 ± 0.7% and 23.9 ± 1.6%, respectively. Combined treatment with corticosterone and GHRH was not significantly more effective ($p > 0.05$) than corticosterone alone for increasing somatotroph proportions in this experiment. In subsequent experiments, *in ovo* injection of GHRH alone on e-11 failed to increase percentages of GH cells detected by e-14 and e-18.

Discussion

In the present study, we demonstrated that somatotroph differentiation can be induced in vivo by a single injection of corticosterone or serum from d 16 chicken embryos. In previous studies, the somatotroph-differentiating activity of embryonic serum in vitro increased between e-12 and e-16, and this effect was observed after 2 d in culture *(14)*. Interestingly, corticosteroid secretion from the adrenal gland of the embryonic chick becomes regulated by the pituitary by e-14.5 *(21)*, which is about the time when somatotroph differentiation begins during normal development *(12)*. In the present study, we found that administration of e-16 serum or corticosterone directly into e-11 eggs resulted in premature somatotroph differentiation by e-14. Nogami et al. *(8,9)* showed that treatment of pregnant rats with dexamethasone induced premature expression of GH in the pituitaries of their fetuses. In their studies, the possibility that the effects of the treatment on the fetuses were indirect and mediated by responses in the mothers could not be ruled out. Because the embryonic chick develops in the isolated environment of the egg, the results of the present study show that glucocorticoids can directly influence somatotroph differentiation during development. Similar results across avian and mammalian classes suggest that a common extracellular signal, acting through the glucocorticoid receptor, is involved in

somatotroph differentiation. The present results are the first to demonstrate this in individual vertebrate embryos, removed from maternal influences, and support the hypothesis that somatotroph differentiation during normal development is stimulated by an increase in adrenal glucocorticoid production.

The finding that e-16 serum, but not e-12 serum, increased proportions of GH-secreting cells was not surprising. The same results were obtained in vitro *(14)* and likely reflect the differences in corticosterone levels between e-12 and e-16 serum. Circulating levels of corticosterone increase between d 12 and 16 of chicken embryonic development *(24)*. Interestingly, e-12 serum increased the proportion of GH-containing cells, but not that of GH-secreting cells, suggesting that the e-12 serum treatment induced differentiation of somatotrophs that contained but failed to secrete GH. One possible explanation for the incongruity between GH-containing and GH-secreting cells is that differences exist in the level of corticosterone necessary to stimulate GH synthesis vs release. Another is that other compounds present in embryonic serum are affecting the two parameters individually. This finding could also reflect a lack of hypothalamic control of GH secretion at that stage of development. Others have failed to observe GHRH-stimulated GH release from the chicken pituitary until after e-14 *(25)*, when the adenohypophyseal vascular sinusoids attain their adult structure *(26)* and normal somatotroph differentiation has begun *(12)*.

In the final experiment, GH cell proportions following injection of corticosterone alone were compared with those attained by injecting corticosterone combined with GHRH. Treatments were administered in a single injection on e-11, and GH cell proportions were evaluated on e-18. The rationale for evaluating the effect of the e-11 treatments on e-18 was based on our previous report that synergistic stimulation of somatotroph differentiation by corticosterone and GHRH in vitro required as many as 6 d of treatment *(16)*. A single *in ovo* injection of corticosterone alone, or combined with GHRH, on e-11 increased the percentage of GH-secreting cells by e-18. In contrast to the previous findings in vitro, combined treatment with corticosterone and GHRH was not significantly more effective than corticosterone alone for increasing somatotroph differentiation in vivo. One explanation for this discrepancy may be that the effect of exogenous GHRH was obscured by the maximal effect of endogenous GHRH between e-11 and e-18. Another explanation may be that combined treatment of d 11 embryos with corticosterone and GHRH in the present study may have resulted in a transient increase in GH cell proportions that was no longer apparent by e-18. In our previous in vitro study, we observed synergistic induction of somatotroph differentiation by corticosterone and GHRH on e-12, but this synergistic effect was lost by e-14 *(16)*. Finally, GHRH may have been rapidly cleared in vivo following the single injection so that a synergistic effect with corticosterone could not develop. The potential for interactions of corticosterone and GHRH on somatotroph differentiation in vivo deserves further study.

In conclusion, premature somatotroph differentiation in the chicken embryonic pituitary can be induced in vivo by a single injection of e-16 chicken serum or corticosterone. Taken together with previous observations from our own *(14–16)* and other laboratories *(5–9)*, these findings provide strong evidence that glucocorticoids are directly involved in somatotroph differentiation during vertebrate embryonic development.

Materials and Methods

Animals and Injections

Unless stated otherwise, all cell culture reagents were obtained from Life Technologies (Grand Island, NY), and hormones and other chemicals were purchased from Sigma (St. Louis, MO). All animals used in this study were Single Comb White Leghorn (SCWL) chicken embryos. All experiments described were approved by Animal Care and Use Committees at Texas A&M University or the University of Maryland. Fertile eggs were placed in a humidified incubator (G.Q.F. Manufacturing, Savannah, GA) at 37.5°C.

The normal duration of incubation for chickens is 21 d. Eggs were removed from the incubator on e-11 and candled to determine whether they contained live embryos. Fertile eggs were placed in an inverted position with the smaller apical ends up. The ends of the eggs were swabbed with a small amount of 70% ethanol, and then a tiny hole was made in the apical surface of each egg using the tip of an 18-gage needle. Then, treatments were injected into the albumen at a depth of approx 8 mm using a tuberculin syringe with a 25-gage needle. The injection site was sealed with melted paraffin, and the eggs were returned to the incubator until the appropriate developmental stage. In the first experiment, the albumen was injected with 300 μL of 0.9% saline or 150 μL of serum from d 12 or d 16 chick embryos diluted 1:1 with saline (three eggs per treatment). This dose of serum was selected to achieve a concentration of 0.25% treatment serum by volume in the egg, assuming that the eggs (60 g) were composed solely of water. Serum treatment pools (one per embryonic age per trial) were collected from chorioallantoic blood vessels of d 12 or d 16 SCWL embryos, 10 embryos per pool, as described previously *(12)*. Throughout the rest of the experiments, an injection volume of 120 μL was utilized. In the second experiment, fertile eggs (three per treatment) were injected with saline, 60 μL of e-16 serum diluted 1:1 with saline (to yield approx 0.10% treatment serum by volume), or corticosterone. Corticosterone was initially dissolved in 100% ethanol to 1×10^{-3} *M*. This solution was further diluted with saline to make 5×10^{-6} and 5×10^{-8} *M* treatment solutions. Then, 120 μL of each treatment solution were injected into the albumen of d 11 fertile eggs to deliver

6- or 600-pmol doses of corticosterone per egg. In the third and final experiment, fertile eggs (three per treatment) were injected with saline, 60 pmol of corticosterone, or 60 pmol corticosterone combined with 6 nmol of GHRH. The amounts of hormones injected in experiments 2 and 3 were chosen based on those previously shown to induce somatotroph differentiation in vitro *(15,16)*.

Pituitary Dispersions and Assays

All culture media were supplemented with 0.1% bovine serum albumin, 100 U/mL of penicillin G, and 100 μg/mL of streptomycin sulfate. In experiments 1 and 2, injected eggs were removed from the incubator on e-14, and blood was collected from the chorioallantoic vessels as described previously *(12)*. Serum GH levels were determined in a single radioimmunoassay *(27)* with an interassay coefficient of variation of 5.6% and sensitivity of 2.5 ng/mL.

In experiment 3, eggs were removed from the incubator on e-18, and no blood samples were obtained. Anterior pituitaries from the embryos in each treatment group were isolated and pooled in Spinner's Minimum Essential Medium (SMEM). The pituitaries in each pool were then dissociated into individual cells by trypsin digestion and mechanical agitation as described previously *(12)*. Briefly, anterior pituitaries were placed in 10 mL of SMEM containing trypsin (1 mg/mL; Difco, Detroit, MI) and incubated at 37.0°C for 45 min under 95% O_2–5% CO_2 in a Spinner flask (Bellco, Vineland, NJ). Tissue dissociation was aided during the incubation with gentle trituration using a siliconized, flame-polished Pasteur pipet at 15-min intervals. The resulting monodispersed cells were washed twice with 10 mL of Dulbecco's modified Eagle's medium (DMEM) followed by centrifugation. The viability of the cells was assessed by the trypan blue dye-exclusion method and was consistently >90%. The dispersed cells were subjected to RHPA and ICC (only experiment 1) to detect GH-secreting and GH-containing cells, respectively. The RHPAs were conducted according to the method described previously *(28)*, using rabbit antiserum against chicken GH and modifications described earlier *(12)*.

Briefly, recovered anterior pituitary cells were mixed with protein A–coated ovine erythrocytes (Colorado Serum Co., Denver, CO) and infused into Cunningham chambers. After the cells attached for 45 min (37.5°C, 95% air-5% CO_2), the chambers were rinsed with DMEM to remove unattached cells. DMEM containing GH antiserum (1:40) and hGHRH1-40 (10^{-7} *M*) was then added to the resulting monolayer of cells, and replicate chambers were incubated for 8 or 20 h (three chambers per treatment per time point). Plaque formation was then induced by a 45-min incubation with guinea pig complement (1:40, in DMEM). The cells were then fixed with 2% glutaraldehyde in 0.9% saline and stained with methyl green. The chambers were analyzed using a light microscope to determine the percentage of all pituitary cells that formed plaques. At least 200 pituitary cells were counted per assay chamber. Cells containing GH were detected using immunocytochemistry as previously described *(14)*. Approximately 10,000 cells were attached to poly-L-lysine-coated microscope slides during a 45-min incubation period. The cells were then fixed with B-5 (4% formalin, 220 m*M* mercuric chloride, and 150 m*M* sodium acetate) for 10 min and treated sequentially (1 min each) with 100% ethanol, Lugol's iodide, water, 2% sodium thiosulfate, and Tris-buffered saline (TBS). Slides were then processed with the Vectastain ABC kit for rabbit immunoglobulin G (PK-4001) according to the directions supplied by the manufacturer (Vector, Burlingame, CA). After blocking with normal goat serum, replicate slides were incubated overnight at room temperature with chicken GH antiserum diluted 1:4000 or 1:8000 in TBS (three slides/group). After rinsing with TBS, slides were stained for GH using a peroxidase substrate kit (VECTOR-VIP, SK-4600; Vector). Triplicate slides were counted under a light microscope, and at least 400 cells were counted per slide. As a control for nonspecific binding, slides from each treatment were incubated with normal rabbit serum alone. No such binding was observed.

Statistical Analysis

The data reported are the mean ± SEM from three completely separate trials of each experiment. For each trial, percentages of GH-plaque-forming or GH-containing cells were determined for each combination of treatment and time point using three replicate chambers for that combination (for a total of at least 600 pituitary cells analyzed for each combination per trial). All data were analyzed using the general linear models procedure of SAS *(29)*. Differences among treatments were tested using orthogonal contrasts, and all possible contrasts were tested.

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