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EFFECTS OF ADMINISTRATION OF ANTIESTROGEN (TAMOXIFEN) *IN VIVO* ON THE METABOLISM OF 25-HYDROXYVITAMIN D_3 *IN VITRO* IN THE JAPANESE QUAIL

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Abstract—It has been reported that certain reproductive hormones exert a regulatory function on vitamin D metaboism in avian species. More specifically, we and others have demonstrated that administration of estrogen enhances renal 25-hydroxyvitamin D_3-1-hydroxylase activity. The present work is aimed at further elucidating this regulatory function of estrogen by studying the effects of an antiestrogen, tamoxifen, on renal 1-hydroxylase activity. Female Japanese quail, maintained on either a low calcium or a normal calcium diet, were injected with tamoxifen (10 and 30 mg/kg, i.m. three times weekly for 4 weeks) and renal 1-hydroxylase activity *in vitro* was determined. The higher dose of tamoxifen significantly inhibited 1-hydroxylase activity when compared with control birds injected with vehicle alone; this inhibitory effect was observed whether the birds were maintained on a normal or a low calcium diet. Tamoxifen at the lower dose significantly inhibited 1-hydroxylase only in those birds maintained on a low calcium diet. Plasma calcium concentrations and oviduct weights were significantly decreased by tamoxifen at both dose levels and under both dietary conditions. The 1-hydroxylase data, coupled with the previously reported stimulatory effects of exogenous estradiol and the inhibitory effects of ovariectomy, support the concept that estrogens can influence renal 1-hydroxylase activity in avian species. Nevertheless, when estradiol was administered (1 and 3 mg/kg, i.m., three weekly for 4 weeks) concurrently with tamoxifen, it did not reverse, but tended to add to, the blocking effect of tamoxifen. This indicates that the regulatory function of estrogen is not simple. Estradiol may have a latent inhibitory component with respect to renal 1-hydroxylase activity, a component which may be unmasked by the presence of an antiestrogen.

Vitamin D_3 (cholecalciferol) is metabolized in the animal body, first in the liver producing 25-hydroxyvitamin D_3 [25-(OH)D_3], which is then further hydroxylated at the 1-position in the kidney to 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]. This dihydroxy metabolite is the most active form and has been designated as a hormone. The kidney also hydroxylates 25-(OH)D_3 to a lesser active metabolite, 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$]. These two hydroxylations in the kidney are normally regulated so that only one product is predominantly formed, depending on the need. The exact physiological role of 24,25-$(OH)_2D_3$ is not clearly understood, but it is now largely accepted that 1,25-$(OH)_2D_3$ is the final active form of vitamin D_3 in the body [1].

The factors which regulate the synthesis of this metabolite in the kidney are not known with certainty. Various factors such as vitamin D status [2, 3], calcium [4, 5] and phosphate [6, 7] have been reported to be the regulator of 1,25-$(OH)_2D_3$ synthesis in the kidney. The role of parathyroid hormone has been investigated [3, 8, 9] but certain authors do not agree that parathyroid hormone is such a regulator [7, 10, 11]. Kenny *et al.* have reported that ovulation in Japanese quail [12–14] and chickens [15] results in enhanced 1,25-$(OH)_2D_3$ production in kidney homogenates *in vitro*. These studies indicated the possible existence of a physiological regulation of 1,25-$(OH)_2D_3$ production during increased need for calcium such as in egg laying.

The possibility that gonadal hormones, which are elevated during the egg-laying cycle, might also be a factor which can regulate the synthesis of 1,25-$(OH)_2D_3$ led us to study the effect of gonadal hormones on the regulation of vitamin D_3 metabolism. We have previously reported [16] that estradiol injection in male Japanese quail markedly increases renal production of 1,25-$(OH)_2D_3$ *in vitro*. Recently Tanaka *et al.* [17] have also reported similar findings. The present investigation was designed to study further this regulatory function of estrogen by investigating the effects of an estrogen antagonist (ICI, 46,474, tamoxifen citrate) on renal 25-hydroxyvitamin D_3-1-hydroxylase activity. In addition, the influence of calcium deprivation on these responses was also studied. Vitamin D metabolism was monitored *in vitro* using kidney homogenates and tritiated 25-(OH)D_3 as substrate. A preliminary report of this work has appeared elsewhere [18].

EXPERIMENTAL

Animals and diets

Four-week-old female Japanese quail (*Coturnix coturnix japonica*), hatched and bred in the animal quarters of either the University of Texas Medical

Table 1. Experimental groups, diets and treatments in female Japanese quail

Group	Treatment*	No. of Birds	Age (weeks) Start	End
Normal calcium diet†				
1	Control (corn oil)	7 (immature)	4	8
2	ICI (10 mg/kg)	8 (immature)	4	8
3	ICI (10 mg/kg) + estradiol (1 mg/kg)	7 (immature)	4	8
4	ICI (30 mg/kg)	6 (immature)	4	8
5	ICI (30 mg/kg) + estradiol (3 mg/kg)	5 (immature)	4	8
6	ICI (30 mg/kg)	5 (laying)	12‡	16
Low calcium diet§				
7	Control (corn oil)	7 (immature)	4	8
8	ICI (10 mg/kg)	5 (immature)	4	8
9	ICI (10 mg/kg) + estradiol (1 mg/kg)	5 (immature)	4	8
10	ICI (30 mg/kg)	6 (immature)	4	8
11	ICI (30 mg/kg) + estradiol (3 mg/kg)	5 (immature)	4	8
12	ICI (30 mg/kg)	5 (laying)	12‡	16

* Injections were given intramuscularly three times weekly in corn oil; ICI: tamoxifen citrate (ICI 46,474); estradiol: estradiol benzoate.
† Normal Ca diet: Purina Game Bird Breeder Layena (2.3 to 3.3% Ca).
‡ Mature egg-laying quail; normal females start laying around 6 weeks.
§ Low Ca diet: Teklad modified Special Quail Test Diet (0.2% Ca).

Branch or Texas Tech University School of Medicine, were used. The birds were maintained on a 14-hr light and 10-hr dark cycle, and were fed Purina Game Bird Startena (Ralston Purina, St. Louis, MO) from hatching until 4 weeks of age. Other husbandry procedures have been described elsewhere [14]. The birds had free access to tap water at all times. Six groups of birds (five to eight per group) were placed on a low calcium diet containing 0.2% calcium. This low calcium diet was obtained from Teklad Mills (Chagrin Falls, OH) and is a modification of their Special Quail Test Diet T-109 (Cat. No. 170630) by omission of the $CaHPO_4$ and $CaCO_3$ ingredients. Six more groups were placed on Purina Game Bird Breeder Layena (Ralston Purina, St. Louis, MO) beginning at 4 weeks of age and continuing until the time of sacrifice. The Layena diet consists of 2.3 to 3.3% calcium and is designated for the purpose of this report as a normal calcium diet. Two groups of mature layers were also used and were placed either on low or normal calcium diets for 4 weeks. The birds were caged individually during the experimental periods. All birds were weighed before and after the experiment. Their oviduct weights were recorded at the time of sacrifice.

Drugs

The antiestrogen, tamoxifen citrate (ICI 46,474 and kindly supplied by the Stuart Pharmaceuticals Division of ICI United States, Wilmington, Del.), used in this study is a derivative of triphenylethylene, of which clomiphene is another analog. The compound was dissolved in stock alcoholic solution and appropriate aliquots were dissolved in the required volume of corn oil after evaporation of the alcohol. Estradiol benzoate (β-estradiol-3-benzoate, U.S.P., General Biochemicals, Chagrin Falls, OH) was dissolved in the required volume of corn oil. Two dose levels of antiestrogen and estradiol were used. Antiestrogen and estradiol injections were given intramuscularly three times each week. Control groups received equal volumes of corn oil. The drugs were given for 4 weeks. The experimental groups and dose schedules are given in Table 1.

Kidney incubation in vitro

All birds were anesthetized with halothane (kindly supplied by Ayerst Laboratories through the courtesy of Dr. J. B. Jewell) after 4 weeks on a specific diet and treatment schedule and were bled by cardiac puncture to obtain heparinized plasma for calcium and inorganic phosphate analysis. The preparation of the kidney homogenates, the incubation with tritiated 25-(OH)D_3 and subsequent extraction and separation of the metabolites were performed according to the method described by Kenny [14], with one exception: 0.25-ml aliquots of kidney homogenate were added to 4.75 ml of incubation mixture containing 50 pmoles 25[26,27-^{3}H]hydroxyvitamin D_3 (dissolved in 25 μl of 95% ethanol), and incubated for 10 min only. The concentration of substrate used in this study is not saturating [2], so that maximal enzymatic activity is not being compared between the groups. Metabolite production is expressed in terms of pmoles min^{-1} g^{-1} kidney as the mean value together with the standard error of the mean.

Analytical methods

Plasma calcium and phosphate were determined by the automated methods of Kessler and Wolfman [19] and Fiske and Subbarow [20] respectively. Protein was determined by the method of Lowry *et al.* [21] as automated by Waite [22].

Medullary bone

Femurs were removed at the end of the experiment, cleaned of soft tissue, and defatted with ethanol and ether as described by Kenny *et al.* [23]. Two-cm sections of the diaphysis were cut from the dry bones with a fine saw and weighed as an index of the development of medullary bone.

RESULTS

Normal calcium diet experiments

Immature birds. The birds were treated beginning at 4 weeks of age until they were 8 weeks old. The data are summarized in Fig. 1. Control birds (group 1)

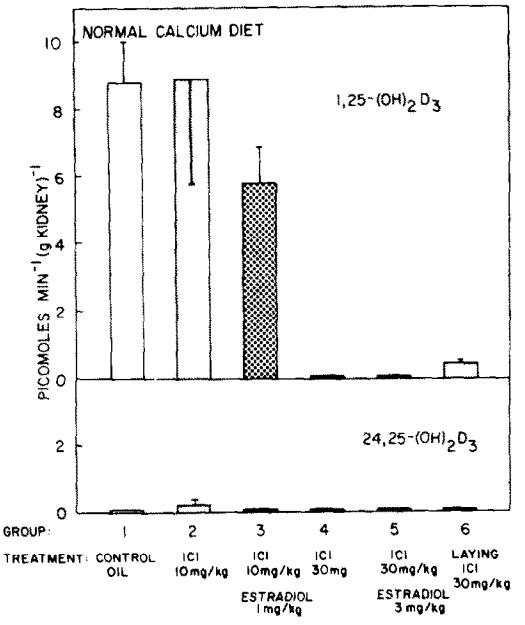

Fig. 1. Effect of an antiestrogen (ICI 46,474) with and without estradiol treatment on vitamin D metabolism in female Japanese quail on a *normal* calcium diet. Drug treatments were initiated in 4-week (groups 1–5) and 12-week (group 6) birds and continued for 4 weeks. Both 1,25-$(OH)_2D_3$ (upper panel) and 24,25-$(OH)_2D_3$ (lower panel) production were determined after incubation of kidney homogenates with [^{3}H-25]-(OH)D_3 and were expressed in terms of pmoles min^{-1} g^{-1} kidney. Antiestrogen treatment significantly inhibited 1,25-$(OH)_2D_3$ production but only at the 30 mg/kg dose. Very little 24,25-$(OH)_2D_3$ was produced in any of the groups.

all of which had an egg in the oviduct at the time of sacrifice, produced only 1,25-$(OH)_2D_3$ (8.8 ± 1.2 pmoles min^{-1} g^{-1} kidney) and none of the 24,25-$(OH)_2D_3$ metabolite. Those birds which received the antiestrogen at 10 mg/kg (group 2) also produced 1,25-$(OH)_2D_3$ predominantly (8.9 ± 3.1) with little 24,25-$(OH)_2D_3$ metabolite (0.54 ± 0.50). The higher dose of antiestrogen (30 mg/kg) completely inhibited the production of either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ (group 4). When the antiestrogen-treated birds were injected concomitantly with estradiol (groups 3 and 5), the 3 mg/kg dose of estradiol failed to break through the blockade induced by the antiestrogen at 30 mg/kg. In fact, in group 3 (birds treated with the lower doses of agonist and antagonist), the production of 1,25-$(OH)_2D_3$ metabolite (5.8 ± 1.1) was slightly less than, although not significantly so, that produced by the birds (group 2) receiving the lower dose of the antagonist alone (8.9 ± 3.1). Mature laying birds receiving the higher dose of the antagonist (group 6) produced predominantly 1,25-$(OH)_2D_3$ but at a markedly reduced rate (1.1 ± 0.09 pmoles min^{-1} g^{-1} kidney).

All groups which received the estrogen antagonist, whether they were treated with estradiol or not, did not lay eggs, whereas the control (group 1) birds layed regularly and had an egg in the oviduct at the time of sacrifice. All antiestrogen-treated birds (groups 2–6), whether they received estradiol treatment or not, showed a significant ($P < 0.05$) reduction in body weight, plasma calcium, oviduct and femur weights. Plasma phosphate levels, however, were not significantly different except in the birds (group 4) treated with 30 mg/kg of antiestrogen along (Table 2).

Laying birds. A 4-week treatment with antiestrogen alone at a dose of 30 mg/kg (group 6) stopped egg laying within 1 week after start of the treatment. Renal production of 1,25-$(OH)_2D_3$ (1.1 ± 0.09 pmoles min^{-1} g^{-1} kidney) was significantly ($P < 0.05$) less in these birds than in (group 1) egg-laying birds (8.8 ± 1.2) which reached maturity at the end of the experiment. Production of 24,25-$(OH)_2D_3$ was undetectable in both groups. Treatment of mature birds with the antiestrogen at the 30 mg/kg dose level significantly ($P < 0.05$) reduced plasma calcium, oviduct and femur weights (Table 2).

Low calcium diet experiments.

Immature birds. Control birds, when placed on a low calcium diet (group 7) exhibited, as expected, a greater production of 1,25-$(OH)_2D_2$ (25.4 ± 3.80 pmoles min^{-1} g^{-1} kidney) than similar birds (Fig. 1, group 1) maintained on a normal calcium diet (8.8 ± 1.20). Under these circumstances, antiestrogen

Table 2. Effects of antiestrogen (ICI 46,474) and estradiol in immature and laying female Japanese quail*

Group	Treatment	Body weights Initial (g)	Body weights Final (g)	Plasma concs Ca (mg/dl)	Plasma concs P (mg/dl)	Tissue weights Oviduct (g/100 g)	Tissue weights Femur (mg/cm)
Normal calcium diet							
1	Control	94 ± 4	135 ± 5	22.5 ± 2.0	7.6 ± 1.1	4.9 ± 0.4	101 ± 2.3
2	ICI (10 mg/kg)	94 ± 3	115 ± 4†	13.2 ± 1.3†	8.5 ± 1.3	1.3 ± 0.5†	85.9 ± 5.6†
3	ICI (10 mg/kg) + E(1 mg/kg)	93 ± 3	121 ± 4†	14.5 ± 1.7†	7.4 ± 0.7	3.0 ± 0.3†	93.7 ± 5.4†
4	ICI (30 mg/kg)	90 ± 2	106 ± 3†	8.9 ± 0.3†	6.0 ± 0.3†	0.01 ± 0.001‡	83.5 ± 3.4†
5	ICI (30 mg/kg) + E(3 mg/kg)	83 ± 5	116 ± 4†	8.6 ± 0.3†	5.8 ± 0.7†	0.01 ± 0.001‡	81.9 ± 3.0†
6	ICI (30 mg/kg)	133 ± 6	107 ± 3	10.0 ± 0.4	5.7 ± 0.9	0.08 ± 0.002	88.6 ± 4.0
Low calcium diet							
7	Control	89 ± 2	118 ± 3	11.0 ± 1.3	5.8 ± 0.6	3.9 ± 0.4	74.6 ± 2.4
8	ICI (10 mg/kg)	82 ± 2	109 ± 3	8.5 ± 1.4	5.1 ± 0.6	0.4 ± 0.1†	80.6 ± 2.1
9	ICI (10 mg/kg) + E(1 mg/kg)	83 ± 2	118 ± 4	9.7 ± 1.4	7.2 ± 0.9	1.1 ± 0.6†	81.9 ± 4.0
10	ICI (30 mg/kg)	80 ± 3	111 ± 3	7.5 ± 0.8†	5.8 ± 0.5	0.01 ± 0.001‡	75.9 ± 3.3
11	ICI (30 mg/kg) + E(3 mg/kg)	85 ± 4	116 ± 2	6.7 ± 1.0†	7.9 ± 0.9	0.01 ± 0.001‡	76.9 ± 1.7
12	ICI (30 mg/kg)	141 ± 3	122 ± 3	8.4 ± 0.9	5.6 ± 1.0	0.7 ± 0.2	88.9 ± 2.1

* Results shown are expressed as mean ± standard error of mean. See Table 1 for details of experimental groups; groups 1–5 and 7–11 were immature females, while groups 6 and 12 were laying birds at the start of the experiment.

† $P < 0.05$, when compared with appropriate control group.

‡ $P < 0.01$, when compared with appropriate control group.

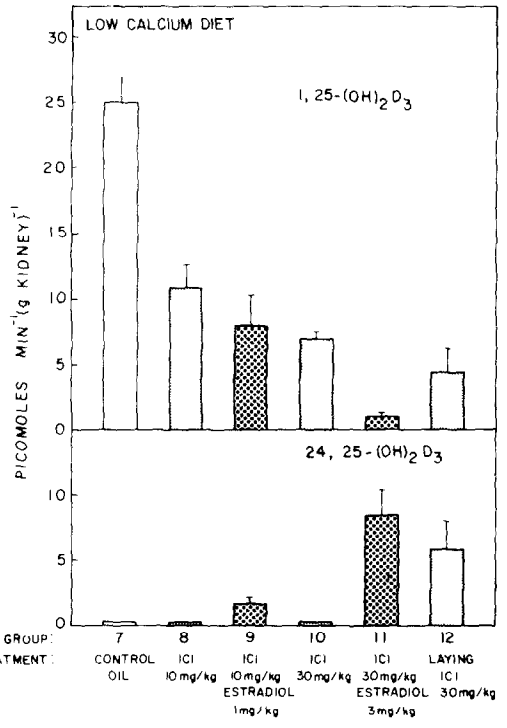

Fig. 2. Effect of an antiestrogen (ICI 46,474) with and without estradiol treatment on vitamin D metabolism in female Japanese quail on a *low* calcium diet. See legend to Fig. 1 for other details. Antiestrogen treatment significantly inhibited 1,25-$(OH)_2D_3$ production at both the 10 and 30 mg/kg dose levels. Estradiol treatment (groups 9 and 11) not only failed to reverse this inhibition but actually contributed to it.

treatment significantly inhibited 1,25-$(OH)_2D_3$ production at both the 10 and 30 mg/kg doses (Fig. 2, groups 8 and 10). Estradiol treatment (Fig. 2, groups 9 and 11) not only failed to reverse this inhibition but actually contributed further to it. Concomitant with this inhibition of 1,25-$(OH)_2D_3$ synthesis, estradiol increased the production of 24,25-$(OH)_2D_3$, so that, at the higher doses of combined antiestrogen and estradiol (group 11), the production of 24,25-$(OH)_2D_3$ (8.4 $\pm$ 1.9 pmoles min^{-1} g^{-1} kidney) was even greater than that of 1,25-$(OH)_2D_3$ (1.0 $\pm$ 0.13). This is interesting in view of the fact that those birds (group 10) which were treated with the high dose (30 mg/kg) of antiestrogen alone produced predominantly 1,25-$(OH)_2D_3$ (6.9 $\pm$ 0.48) with no detectable synthesis of 24,25-$(OH)_2D_3$. Treatment of the birds on a low calcium diet with either antiestrogen alone (groups 8 and 10) or with combined antiestrogen and estradiol (groups 9 and 11) significantly ($P < 0.05$) lowered both plasma calcium and oviduct weight. On the other hand, plasma phosphate and femur weight were not significantly different in any of the treated groups (Table 2). All birds placed on a low calcium diet (groups 8–11) which were treated failed to lay any eggs.

Laying birds. When laying birds were placed on a low calcium diet and injected with the high dose (30 mg/kg) of antiestrogen, they stopped laying within 1 week after the start of the treatment. Unlike the similar group of birds (Fig. 1, group 6) maintained on a normal calcium diet, these birds (Fig. 2, group 12) produced both 1,25-$(OH)_2D_3$ (4.6 $\pm$ 1.7 pmoles min^{-1} g^{-1} kidney) and 24,25-$(OH)_2D_3$ (5.9 $\pm$ 2.0), although the latter metabolite slightly predominated. When these birds are compared with the group receiving no treatment (Fig. 2, group 7), it is found that both the plasma calcium and oviduct weight were significantly reduced but the femur weight was significantly higher (Table 2). The groups receiving no treatment (Fig. 1, group 1, and Fig. 2, group 7) served as controls for the antiestrogen-treated laying birds (groups 6 and 12), which were otherwise similar in age and development.

DISCUSSION

It is well known that the gonadal hormones, particularly estrogens, play an important role in avian calcium metabolism [24]. Medullary bone, which is formed under the influence of estrogens and androgens prior to egg laying, has intrigued many physiologists since it was reported several decades ago [25]. However, the role of gonadal hormones in vitamin D metabolism and its relationship with calcium metabolism are not precisely known. That estrogen can stimulate the renal production of 1,25-$(OH)_2D_3$ *in vitro* in Japanese quail on a normal calcium diet was first reported by us [16, 26]. Tanaka *et al.* [17] have also reported similar findings. Prior to these studies, Kenny [14] had shown a physiological regulation of 1,25-$(OH)_2D_3$ synthesis in the kidney of reproductively active Japanese quail.

In the present study we have been able to reveal that, by blocking the action of both endogenous and exogenous estrogen, regulation of 1,25-$(OH)_2D_3$ production is thereby inhibited if the dietary calcium is adequate. However, this antagonism of 1,25-$(OH)_2D_3$ production by an antiestrogen is partially overridden if the dietary calcium is low. This indicates that if the estrogens, either directly or indirectly, influence the activity of the 25-hydroxyvitamin D_3-1-hydroxylase enzyme, this influence is not absolute. It is, however, also possible that the antiestrogen compound might have influenced the 25-hydroxyvitamin D_3-1-hydroxylase enzyme by some actions unrelated to its antiestrogenic effect. The present study does not clarify this. Imposition of a low calcium diet can partially override the inhibition of the 1-hydroxylase enzyme by an antiestrogen.

The inability of estradiol to overcome the inhibitory effect of the antiestrogen on the 1-hydroxylase enzyme deserves a comment. It is of course possible that a higher dose of estradiol, particularly in those birds on a normal calcium diet, would have broken through the blockade and restored the activity of the 1-hydroxylase enzyme. However, the oviduct response in the group 3 birds (antiestrogen 10 mg/kg; estradiol, 1 mg/kg) did show evidence of blockade breakthrough (in Table 2, groups 2 and 3 were significantly different); this was not accompanied by a similar response in 1,25-$(OH)_2D_3$ production (Fig. 1, group 3). In fact the contrary was noted. Whenever estrogen treatment of the antiestrogen-treated birds had a significant effect on 1,25-$(OH)_2D_3$ metabolism, it was always in the direction of an inhibition of production.

This can be readily seen if the comparison is made between groups 2 and 3 (Fig. 1), between groups 8 and 9 (Fig. 2), and between groups 10 and 11 (Fig. 2). In all three instances, 1,25-$(OH)_2D_3$ production was *inhibited* by the treatment with estradiol. This effect of estrogen under these circumstances is paradoxical when viewed in the context of our previous finding that estrogen enhances 1-hydroxylase activity in male [16] and female [26] quail and that ovariectomy inhibits 1-hydroxylase activity [27]. One explanation is that the influence of estrogen on 1-hydroxylase activity is indirect rather than direct. If, for example, estrogen is mediating its effect on vitamin D metabolism through a pituitary hormone such as prolactin, then it is understandable that the relationship would be much more complicated than if estrogen were acting directly on the 1-hydroxylase enzyme. Recently, prolactin has been reported to stimulate 1,25-$(OH)_2D_3$ production in the chicken [28, 29]; we have confirmed this finding in the immature Japanese quail (S. N. Baksi and A. D. Kenny, unpublished observations). Studies aimed at delineating the role of the pituitary in this relationship between estrogen and vitamin D metabolism have been initiated in our laboratory in Japanese quail. Other studies in amphibia and fishes are being done in collaboration with Dr. Peter K. T. Pang *et al.* [30], who have previously noted the hypercalcemic properties of prolactin in lower vertebrates.

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