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# Changes in Plasma Inhibin A Levels During Sexual Maturation in the Female Chicken and the Effects of Active Immunization Against Inhibin α-Subunit on Reproductive Hormone Profiles and Ovarian Function[1]

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## ABSTRACT

**Inhibins and activins are firmly implicated in the control of pituitary FSH secretion and ovarian follicular development in mammals. As in mammals, inhibin A and activin A are expressed in the preovulatory follicles of birds, and a defined ovulation cycle for inhibin A has recently been demonstrated in the laying hen. To investigate further the role of inhibin-related proteins in developing pullets, circulating concentrations of inhibin A, inhibin B, total immunoreactive inhibin α-subunit (ir-α), activin A, LH, FSH, and progesterone were measured from the juvenile state through to sexual maturity in 22 birds. In the 11 birds assigned to control groups, plasma inhibin A levels were low from 7 to 13 wk of age rising about threefold to a peak at Week 19 after which levels fell slightly to a plateau level characteristic of adult hens. Plasma inhibin A levels were negatively correlated with FSH ($r = -0.33$; $P < 0.001$) and positively correlated with progesterone ($r = 0.67$; $P < 0.001$) and ir-α ($r = 0.53$; $P < 0.001$). Plasma ir-α levels were much higher than inhibin A levels although the relative differences varied with age. Plasma levels of inhibin B and activin A were below assay detection limits at all times. The remaining group of 11 birds was actively immunized (IMM) against a synthetic chicken inhibin α-subunit peptide (amino acids 1–26). The IMM generated circulating antibodies that bound native bovine inhibin A but altered neither plasma FSH nor progesterone levels relative to control birds at any stage of development nor the timing of first oviposition in week 19. Apart from a transient decline 1 wk after primary IMM, plasma LH concentrations did not differ from controls. Comparison of the numbers and size-class distribution of ovarian follicles at 29 wk showed an approximate twofold increase in the number of 8- to 9.9-mm-diameter follicles (control; 1.82 ± 0.44 vs. IMM; 3.91 ± 0.89; $P < 0.05$), a size class that corresponds to follicles that have just joined the preovulatory hierarchy. The numbers of growing follicles in other size-classes and the sizes of hierarchical $F_1$–$F_7$ follicles were not altered by IMM. However, the number of postovulatory follicles increased (control 3.73 ± 0.20 vs. IMM 5.55 ± 0.28; $P < 0.01$), and significantly more ($P < 0.02$) immunized hens laid two eggs within a 24-h period on at least one occasion (control 1 of 11 vs. IMM 9 of 11). The IMM increased ($P < 0.05$) activin A content of $F_1$ and $F_2$ theca layers and decreased ($P < 0.05$) activin A content in $F_3$ and $F_4$ granulosa layers, raising the possibility of a local intraovarian role of activin in mediating the response to IMM. These findings support a role for inhibin A in regulating the entry of follicles into the preovulatory hierarchy in the chicken, although further studies are required to establish the mechanism by which inhibin IMM increases the rate of follicle selection and ovulation without raising plasma FSH.**

*activin, follicular development, FSH, inhibin, LH, progesterone*

## INTRODUCTION

The gonadal glycoprotein inhibin has been implicated as a negative regulator of anterior pituitary gland FSH production and secretion with little or no effect on LH [1, 2]. It was hypothesized that interference with this negative feedback action would result in a corresponding increase in FSH leading to enhanced follicular development and an increased ovulation rate. This has been demonstrated in sheep and cattle by passive or active immunization against inhibin [3, 4]. However, raised circulating FSH concentrations were not always observed following inhibin immunization (IMM) even when follicle development and/or ovulation rates were enhanced [5–10]. These observations have led to suggestions of a direct intraovarian autocrine-paracrine role for inhibin in follicular development that is perturbed by immunization against inhibin.

A potential endocrine role of dimeric inhibin(s) in the adult hen has been suggested by the recent observation of cyclic changes in the plasma concentrations of inhibin A ($\alpha$-$\beta_A$ dimer) during the ovulation cycle [11] with concentrations peaking around the time of the preovulatory LH surge, approximately 6 h before ovulation. In addition, plasma concentrations of inhibin A and FSH were negatively correlated during a period of ovarian regression induced by food restriction. Despite this, no correlation was demonstrated between circulating inhibin A and FSH levels throughout the ovulatory cycle [11]. Recently Moreau et al. [12] reported that active immunization of Japanese quail against the α-subunit of inhibin advanced the onset of lay and enhanced the rate of egg production, supporting a contribution of inhibin to the regulation of ovarian follicle development in birds. However, circulating and tissue concentrations of reproductive hormones were not measured in this study.

To investigate further the potential role of inhibin-related proteins in the modulation of the pituitary-ovarian axis in the domestic chicken, the aim of this study was to examine 1) the concentrations of inhibin A, inhibin B, activin A, total immunoreactive inhibin α-subunit (ir-α), FSH, LH, and progesterone in the circulation from juvenile status to sexual maturity; 2) the effects of active immunization of immature pullets against inhibin on reproductive hormone profiles throughout sexual maturation, time of onset, and pattern of egg laying and on the size distribution of ovarian follicles. In addition, 3) the inhibin A, inhibin B, and activin A contents of various extraovarian tissues were measured to assess their potential to contribute to circulating levels of inhibin-related peptides.

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## MATERIALS AND METHODS

### *Experimental Animals*

Female chicks (ISA; n = 22) were raised in a floor pen until the onset of the experiment (47 days old) when chicks were caged individually. Birds were maintained on a short-day photoschedule of 8L:16D throughout the entire experiment with an ambient temperature of 21–23°C. Food and water were freely available at all times. From 16 wk of age, birds were observed for onset of lay and daily oviposition records were kept.

### *Experimental Design*

At Day 47 (Week 6.7) the birds were weighed and blood samples were collected (2 ml) by venipuncture of a brachial vein into heparinized tubes. Samples were centrifuged (3000 × *g*, 20 min) and the plasma was stored at −20°C until required. Live weights were recorded and blood samples were taken at ~2-wk intervals at between 1400 and 1600 h until the end of the experiment (Week 29). At Day 70 (Week 10) pullets were randomly allocated into one of three groups: nonimmunized controls (n = 5), carrier-immunized controls (n = 6), and inhibin-immunized (IMM; n = 11). The IMM pullets were actively immunized against a synthetic peptide corresponding to the amino terminal region of the mature αC subunit of chicken inhibin (amino acids 1–26 inclusive) conjugated (1:3 mass ratio) to tuberculin-purified protein derivative (PPD) using the glutaraldehyde method [13]. The peptide sequence was based on the deduced sequence from the cloned chicken inhibin α-subunit cDNA [14] and was custom synthesized by Severn Biotech Ltd. (Kidderminster, Worcs, UK). The primary injection (Day 70) consisted of 1 ml of emulsified immunogen conjugate (1 volume [100 μg] of immunogen: 2.2 volumes of Freund's complete adjuvant) administered as 4 × 0.25 ml i.m. injections. Booster immunizations ($B^1$–$B^4$ given at 83, 98, 124, and 165 days of age) consisted of 0.5 ml of emulsified immunogen conjugate (1 volume [50 μg] of immunogen: 2.2 volumes of Freund's incomplete adjuvant) administered as 2 × 0.25 ml i.m. injections. Carrier-immunized pullets (n = 6) were actively immunized against PPD alone using the same schedule, while nonimmunized controls (n = 5) received no injection or treatment. Comparison between the untreated control group and the PPD-treated group were made as described in *Statistical Analysis.* In the absence of any significant differences, the results for the two groups were combined (n = 11), and for the remaining part of the study these birds are collectively termed control hens.

The age at first oviposition, first clutch of four or more eggs, and the frequency of double ovipositions were recorded. Eggs collected throughout the duration of the experiment were weighed and opened to check for the incidence of double yolks.

In Week 29 all hens were weighed and blood samples taken before being killed by an i.v. injection of 200 mg pentobarbitone sodium 4 h after the expected time of ovulation of a midsequence egg. The ovary was removed, weighed, and the four largest ($F_1$–$F_4$) preovulatory follicles dissected away. The remaining ovarian tissue was weighed, and all follicles with a diameter of 2–40 mm were sorted into distinct size categories (2–3.99 mm, 4–5.99 mm, 6–7.99 mm, 8–9.99 mm, 10–14.99 mm, 15–19.99 mm, 20–29.99 mm, and 30–39.99 mm) and counted. Postovulatory follicles with a clear rupture site were also counted. The $F_1$–$F_4$ preovulatory follicles were weighed and their diameters measured prior to dissection into granulosa and theca layers [15] that were snap frozen on dry ice and stored at −70°C. The brain, adrenal glands, and samples from liver, lung, heart ventricle muscle, and skeletal muscle were also removed, snap frozen on dry ice, and stored at −70°C.

A portion of each frozen tissue specimen was removed, weighed, and homogenized in a known volume of buffer A (PBS containing 1% [w/v] BSA and 0.1% [w/v] sodium azide) using an Ultra-Turrax T8 homogenizer (IKA, Staufen, Germany) to give the following volume:mass ratio homogenates: 1.5× brain and lung, 2× liver and heart ventricle muscle, 3× adrenal, 3.5× skeletal muscle, 4× theca, and 10× granulosa. Homogenates were centrifuged at 3500 × *g* for 15 min, and the supernatants were stored at −20°C until further analysis. A 20-μl aliquot of homogenate was removed before centrifugation for DNA estimation using a fluorometric assay [16].

### *Assessment of Inhibin Antibody Titer*

To provide a measure of antibody titer, plasma samples (1:200 dilution) were tested for their ability to bind $^{125}$I-labeled bovine inhibin A (32 kDa) as described previously [17]. The bovine inhibin A used was isolated and radiolabeled as described elsewhere [18]. After correction for nonspecific binding (binding in the absence of chicken serum-antiserum), results were expressed as percent binding relative to the total counts per tube.

### *Immunoassays*

Inhibin A, inhibin B, and activin A were determined using recently developed two-site ELISAs that employ monoclonal antibodies raised against synthetic peptide fragments of the human α-, $\beta_A$-, and $\beta_B$-subunits [19–21]. Recombinant inhibin A, inhibin B, and activin A were used as assay standards, respectively. The two-site ELISAs were validated for use in the domestic fowl as previously described [11, 22]. In brief, serial dilutions of pooled extragonadal (lung, adrenal gland, liver, skeletal muscle, heart ventricle muscle, brain) tissue extracts gave response curves in both inhibin A and activin A ELISAs that were parallel to the respective standard curve. Serial dilutions of inhibin-immunized hen theca and granulosa extracts also gave a parallel response curve in the activin A ELISA. The inhibin B content of extragonadal tissues was less than the minimum detection limit of the ELISA. The minimum detection level for the inhibin A, inhibin B, and activin A ELISAs were 2 pg/ml, 15 pg/ml, and 50 pg/ml, respectively. The cross-reactivities of a range of substances in the inhibin A, inhibin B, and activin A ELISAs have been reported previously [19–21] and were shown to be acceptably low. Within- and between-plate coefficients of variation for each assay were less than 10%.

Total ir-α levels were measured using a heterologous RIA employing a rabbit polyclonal antiserum against purified bovine inhibin [23] that has been validated previously for use in the domestic fowl [24]. This assay cross-reacts with dimeric inhibin forms and monomeric inhibin α-subunit forms including Pro-αC. Recombinant human inhibin A was used as an assay standard. Samples from inhibin-immunized hens could not be assayed for inhibin A, inhibin B, and ir-α due to interference from inhibin α-subunit-directed antibodies. Plasma samples were also assayed for LH

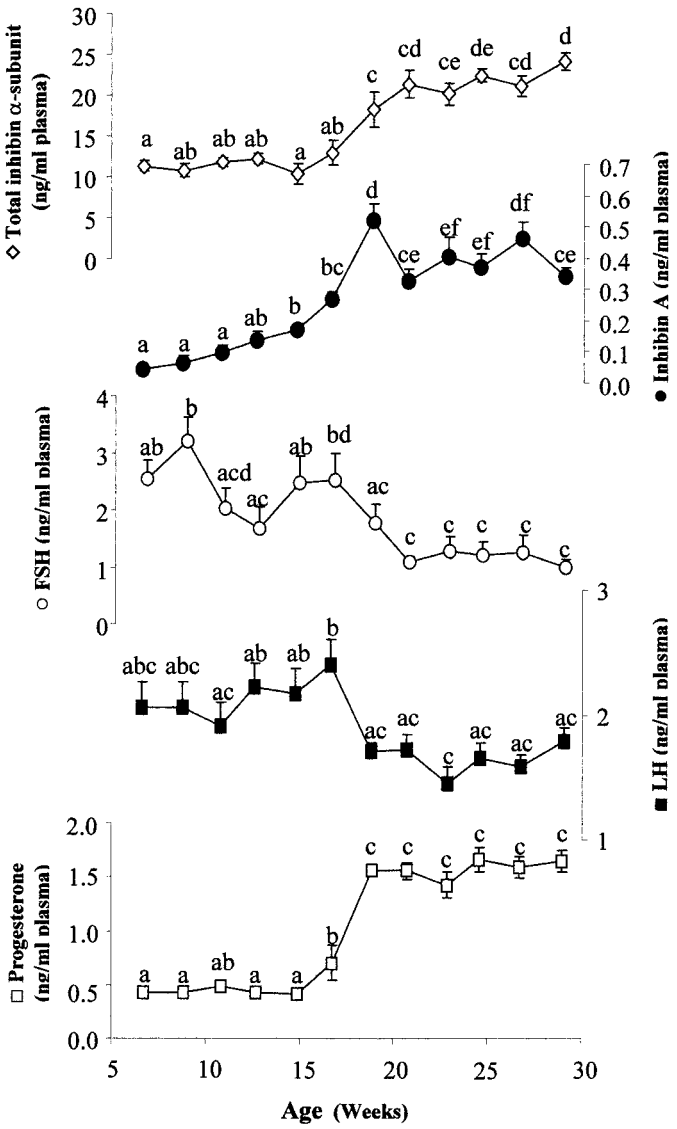


FIG. 1. Plasma concentrations of inhibin A (●), FSH (○), LH (■), progesterone (□), and total inhibin α-subunit (◇) during sexual development of the hen. Values are means ± SEM (n = 11). Significant differences ($P < 0.05$) in hormone concentrations at different time points are indicated by different letters.

[25], FSH [26], and progesterone [27], as previously described.

### *Statistical Analysis*

Confirmation of parallelism between dilution curves for assay standards and representative test samples was made using linear regression analysis of transformed data. The log-log transformation was used to linearize the ELISA dose-response curves. Comparison of the slopes (±95% confidence intervals) of the regression lines for standards and test samples indicated no significant departure from parallelism. This analysis was not possible when only the first two serial dilutions of a sample or extract gave a response above the detection limit.

One-way repeated-measures ANOVA was used in conjunction with post-hoc Fisher's protected least significant difference test to determine whether hormone concentrations differed between carrier-immunized and nonimmunized hens and between control and IMM hens, and whether inhibin antibody titers differed in the IMM hens over time. Two-way repeated-measures ANOVA was used to determine whether active immunization against inhibin α-subunit significantly altered plasma concentrations of progesterone, LH, and FSH with post-hoc one-way ANOVA used to determine variation between IMM and control plasma samples at individual time points. Post-hoc tests were only performed when the repeated-measures ANOVA yielded a significant $F$ ratio. $P < 0.05$ was considered to be significant. Comparisons between carrier-immunized and nonimmunized hens and between control and IMM hens with respect to a range of other end-points, e.g., preovulatory follicles (activin A concentration, diameter, and wet weight), subordinate follicles (diameter and number), ovary (wet weight), egg weight, and egg-laying characteristics (first oviposition and first clutch of four or more eggs), were made by one-way ANOVA. Comparison of the incidence of oviposition on zero, one, or two eggs per day in IMM and control hens were by means of a $\chi^2$ test on the frequency distribution. Unless otherwise stated, values are the means ± SEM.

## RESULTS

### *Developmental Changes in Plasma Inhibin-Related Proteins, LH, FSH, and Progesterone in Control Birds*

The mean plasma profiles of inhibin A, FSH, LH, progesterone, and ir-α during sexual development of the 11 control hens are shown in Figure 1. There was no significant increase in plasma inhibin A from 7 to 13 wk of age. Levels subsequently rose to a peak at 19 wk of age (onset of sexual maturity as indicated by the onset of oviposition), thereafter falling slightly to reach a plateau (~0.4 ng/ml) that was maintained until Week 29 when the birds were killed. Plasma ir-α levels were positively correlated with inhibin A ($r = 0.531$; $P < 0.001$). A low concentration of ir-α was present until 16 wk of age; the level then rose twofold over the next 5 wk, and this level was sustained until Week 29. In contrast to inhibin A no distinct peak of ir-α was demonstrated. Plasma levels of ir-α were significantly higher than inhibin A at all stages of development, although the ratio of ir-α to inhibin A fell about sevenfold between Week 7 and Week 19, reflecting a much more pronounced rise in inhibin A levels. Mean plasma LH concentrations rose slightly, yet significantly, from 11 to 17 wk of age (1.9 ± 0.2 to 2.4 ± 0.2 ng/ml). Plasma LH levels then fell significantly (~30% decrease; $P < 0.05$) over the following 2 wk to a concentration that was maintained until Week 29. There was no correlation between plasma LH and inhibin A levels. Plasma progesterone concentrations were very low until Week 15 followed by a progressive approximate fourfold increase over the subsequent 4 wk to a level that was maintained until Week 29. Overall plasma progesterone was positively correlated with inhibin A ($r = 0.670$; $P < 0.001$) and ir-α ($r = 0.792$; $P < 0.001$). Plasma FSH displayed a transient peak (3.2 ± 0.4 ng/ml) at 9 wk of age and a subsequent decline over the following 4 wk (1.7 ± 0.4 ng/ml). Thereafter, plasma FSH concentrations rose to a prepubertal peak at Week 17 (2.5 ± 0.4 ng/ml) with a progressive decline ensuing over the subsequent 2–4 wk to a level characteristic of the adult laying hen (1.2 ± 0.1 ng/ml). Overall, plasma FSH was negatively correlated with inhibin A ($r = -0.328$; $P < 0.001$), progesterone ($r = -0.403$; $P < 0.001$), and ir-α ($r = -0.497$; $P < 0.001$). Plasma activin A and inhibin B concentrations were below

the detection limit of the assays throughout the development of the domestic pullet.

### *Effects of Inhibin Immunization on Plasma LH, FSH, Progesterone, and Activin A in Developing Pullets*

All IMM hens showed a significant increase in plasma $^{125}$I-labeled 32-kDa bovine inhibin binding capacity after the first booster that was maintained for the duration of the study (Fig. 2). No $^{125}$I-labeled 32-kDa inhibin binding was detected in plasma from either nonimmunized controls or carrier-immunized controls. Although the mean plasma FSH concentrations were numerically higher in IMM than in control hens at all stages of development, this difference was evident before commencing immunization and analysis revealed no significant effect of treatment ($P = 0.134$) and no significant interaction between immunization and time. The plasma progesterone profiles of control and IMM hens also showed no significant effect of treatment, and no interaction between immunization and time. Overall, plasma LH concentrations were not significantly influenced by immunization, but there was a significant treatment × time interaction ($P < 0.01$). Plasma LH concentrations were significantly depressed in IMM birds at 13 and at 29 wk of age by 46% and 20%, respectively, when compared with corresponding values in control birds, at all other sampling times the plasma LH concentrations in IMM birds were not different to those in controls. Plasma activin A concentrations were below the detection limit of the assay at all times during development of both IMM and control birds.

### *Effects of Inhibin Immunization on Pullet Growth Rates, Ovarian Follicle Characteristics, and Egg-Laying Profiles*

Comparison of pullet growth profiles (data not shown) showed no significant effect of IMM, with mean weights increasing from ~0.7 kg at Week 7 to ~2.0 kg at Week 29 in both groups. However, there was a significant interaction ($P < 0.05$) between the rate of weight gain and treatment. The growth rate was transiently reduced 1–3 wk after the primary IMM, corresponding to a 64% decrease in weight gain between 11 and 13 wk of age (IMM increase 44.6 ± 16.4 g vs. control increase 125.2 ± 18.8 g). No further significant variation in bird weight or weight gain occurred between control and IMM groups during the remainder of the experiment.

Active immunization against inhibin α-subunit had no effect on the age of first oviposition (control 19.10 ± 0.25 wk vs. IMM 19.52 ± 0.21 wk) or the age at which the first clutch was four or more eggs (control 20.79 ± 0.61 wk vs. IMM 21.38 ± 0.53 wk; ages referred to based on lay of the first egg of the clutch). Comparison of ovaries recovered from birds at 29 wk showed that immunization had no significant effect on the diameters of $F_7$–$F_1$ preovulatory follicles (Fig. 3), the wet weight of the $F_4$–$F_1$ preovulatory follicles, the total wet weight of the ovary (control 47.0 ± 2.9 g vs. IMM 47.2 ± 2.9 g), or the residual weight of the ovary after removing the $F_4$–$F_1$ preovulatory follicles (control 12.0 ± 1.2 g vs. IMM 11.9 ± 1.1 g) (Fig. 3). The numbers of follicles in discrete size classes (diameters ranging from 2 to 40 mm) and the numbers of postovulatory follicles displaying a clear rupture site are shown in Figure 4. The ovaries of immunized hens contained significantly ($P < 0.05$) more 8- to 9.99-mm follicles (control 1.82 ± 0.44 vs. IMM 3.91 ± 0.89) and more postovulatory follicles (control 3.73 ± 0.20 vs. IMM 5.55 ± 0.28) compared with control hens. The numbers of follicles in all other size

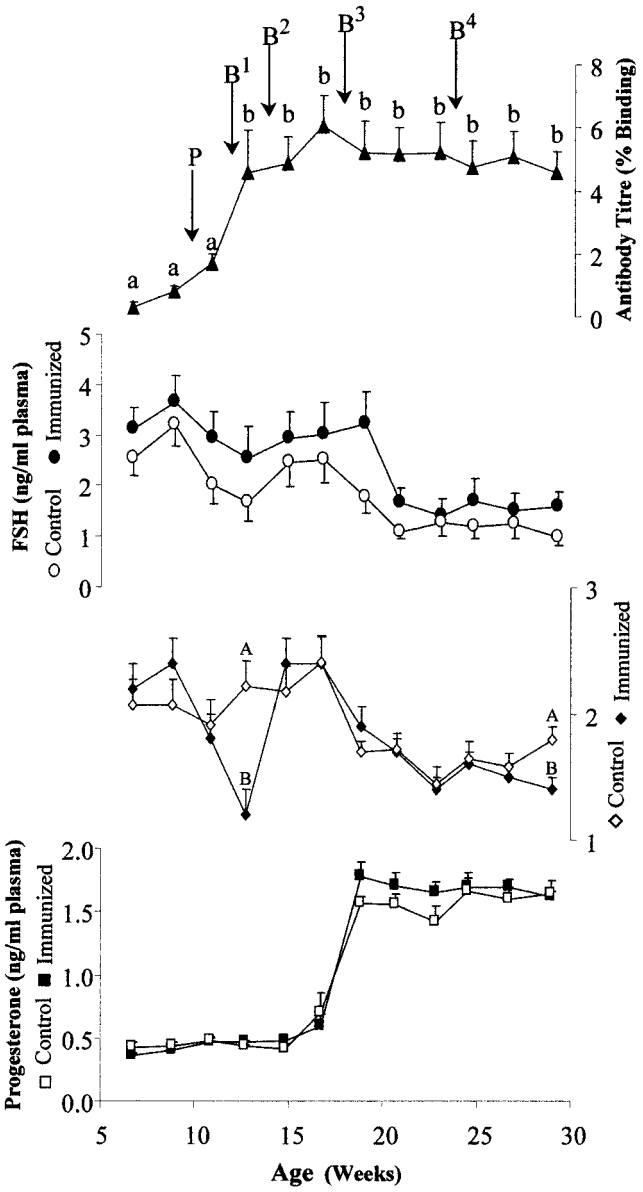


FIG. 2. Effect of immunization against inhibin α-subunit on plasma concentrations of FSH (○, control; ●, immunized), LH (◇, control; ◆, immunized), and progesterone (□, control; ■, immunized) during sexual development of the hen. Plasma anti-inhibin antibody titers (▲) are also shown. Values are means ± SEM (n = 11). Significant differences ($P < 0.05$) between antibody titers at different time points are indicated by different lowercase letters and between treatment groups by different capital letters. The timing of primary (P) and booster ($B^1$–$B^4$) immunizations is indicated.

categories did not differ significantly between control and IMM hens. The oviposition record of each bird was analyzed from the time that all hens were laying regularly at 24 wk to the end of the experiment at 29 wk of age (42 days). During this period, the mean number of days on which control (36.82 ± 1.39) and IMM (38.45 ± 0.76) hens laid was not significantly different, but several of the IMM hens had double ovipositions. Double ovipositions were said to occur when more than one egg was laid within a single 24-h period, the second a soft-shelled egg laid less than 24 h after an expected oviposition. In the control group of 11 hens, only a single bird had a double oviposition (on two separate occasions). In the immunized group 9 of the 11 hens had double ovipositions, a total of 16 extra ovi-

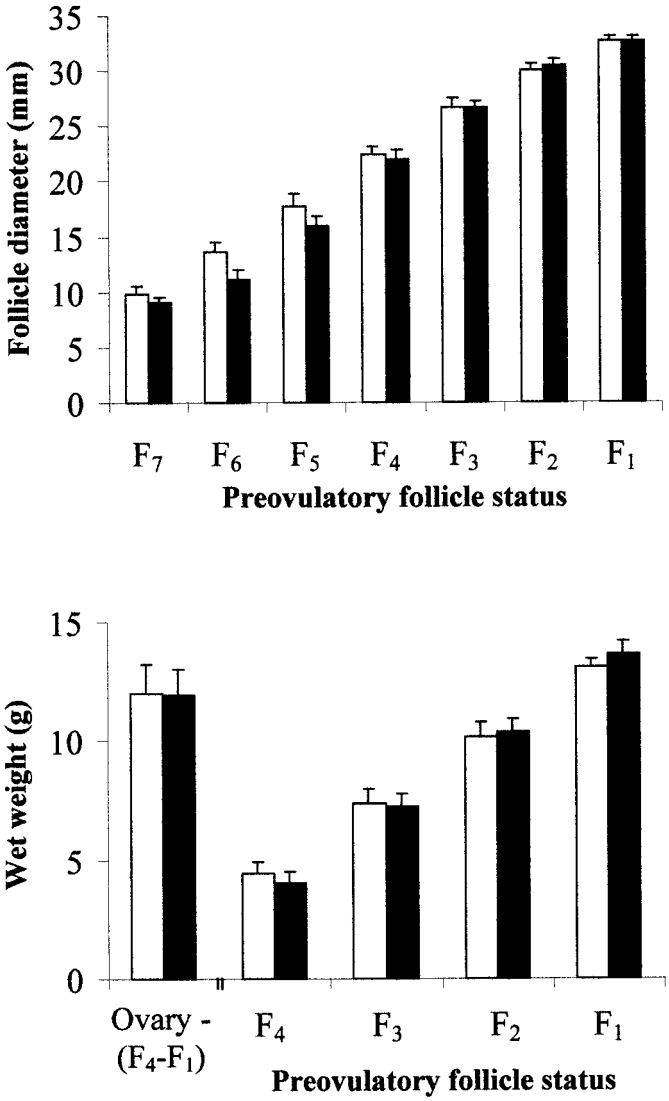


FIG. 3. Characteristics of the preovulatory follicle hierarchy ($F_1$–$F_n$) on ovaries collected from control (□; n = 11) and inhibin α-subunit immunized (■; n = 11) 29-wk-old hens. The upper graph illustrates preovulatory $F_1$–$F_7$ follicle diameters, and the lower graph illustrates the wet weight of the corresponding preovulatory $F_1$–$F_4$ follicles and of the remaining ovarian tissue. Values are means ± SEM. Significant differences ($P < 0.05$) between control (□) and immunized (■) groups are indicated by different letters.

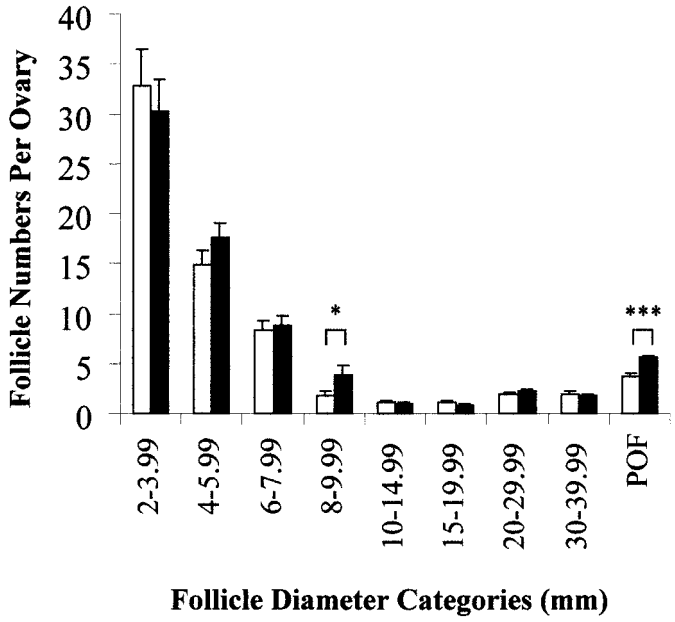


FIG. 4. Numbers of follicles on ovaries collected from control (□; n = 11) and inhibin α-subunit immunized (■; n = 11) 29-wk-old hens. All follicles with a diameter of 2–40 mm were assessed and divided into eight distinct size categories. Numbers of postovulatory follicles displaying a clear rupture site were also recorded. Values are means ± SEM. Asterisks denote statistically significant differences (*$P < 0.05$; ***$P < 0.001$) between control and immunized groups.

positions being observed within the group. The timing of these double ovipositions was distributed more or less evenly throughout the observation period of 24–29 wk of age. The mean number of eggs laid during the 42 days was thus higher in the immunized hens (IMM = 39.91 ± 0.91) than in the controls (control = 37.00 ± 1.41), a difference approaching statistical significance ($P = 0.09$, two-tailed Student's *t*-test).

### *Concentrations of Inhibin A and Activin A in Ovarian and Extraovarian Tissues*

Analysis of tissue extracts from control hens killed at 29 wk (Fig. 5) showed that inhibin A was most abundant in the granulosa layer of preovulatory ($F_4$–$F_1$) follicles, with the highest levels ($P < 0.05$) in the $F_1$ follicle. Levels of inhibin A measured in brain tissue approached those in $F_4$ granulosa while all other tissues examined including $F_4$–$F_1$ theca tissue, heart ventricle muscle, skeletal muscle, liver, adrenal gland, and lung tissue had lower levels that were not significantly different from each other. Heart ventricle muscle contained markedly higher activin A concentration than any other ovarian or extraovarian tissue examined. Arranged in rank order, activin A concentrations decreased from heart ventricle to theca, liver, skeletal muscle, brain, granulosa, adrenal gland, and lung (Fig. 5). While it was not possible to quantify inhibin A levels in tissues from IMM birds, the activin A levels in $F_2$ and $F_1$ theca layers were significantly ($P < 0.05$) greater (37% and 26%, respectively) in IMM birds relative to controls (Fig. 6). In contrast, IMM was associated with a significant decrease ($P < 0.05$) in the activin A content of $F_4$ and $F_3$ granulosa layers (33% and 31%, respectively).

## DISCUSSION

This study provides new information on plasma concentrations of inhibin A during sexual maturation in the female chicken and their relationship with other reproductive hormones. It also reports the effects of active immunization of juvenile birds against inhibin α subunit on circulating concentrations of LH, FSH, and progesterone and on several aspects of ovarian function in the adult hen. Attempts to measure plasma concentrations of inhibin B and of activin A were unsuccessful, levels being below assay detection limits in all samples. Recent reports have documented plasma concentrations of inhibin A and inhibin B in developing cockerels and of inhibin A during the ovulatory cycle of adult hens [11, 17]. Inhibin A and activin A contents of chicken ovarian and testicular tissue have also been reported [17, 22]. These studies have extended earlier information from several groups who, in the absence of specific two-site inhibin assays, used conventional inhibin RIAs of limited specificity to measure total ir-α in chicken plasma and tissue extracts [28, 29].

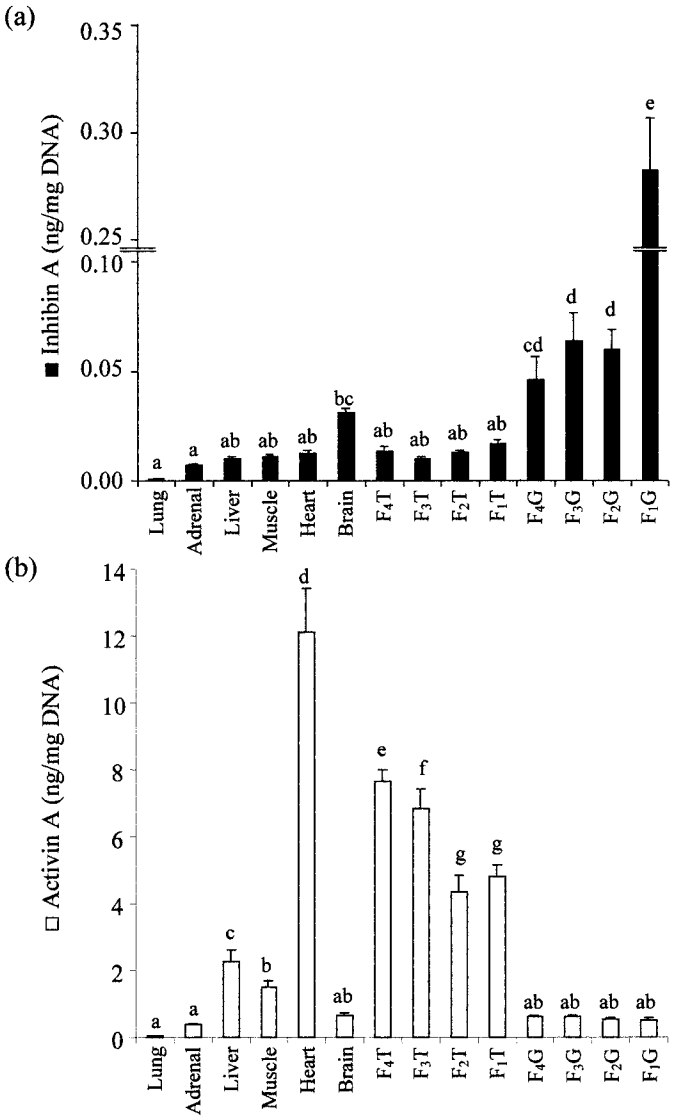


FIG. 5. Concentrations (ng per mg DNA) of **a**) inhibin A and **b**) activin A in ovarian tissues (theca, T and granulosa, G layers of $F_1$–$F_4$ preovulatory follicles) and extraovarian tissues (lung, adrenal, liver, muscle, heart, and brain) of 29-wk-old control hens. Values are means ± SEM (n = 11). Significant differences ($P < 0.05$) between tissues are indicated by different letters.

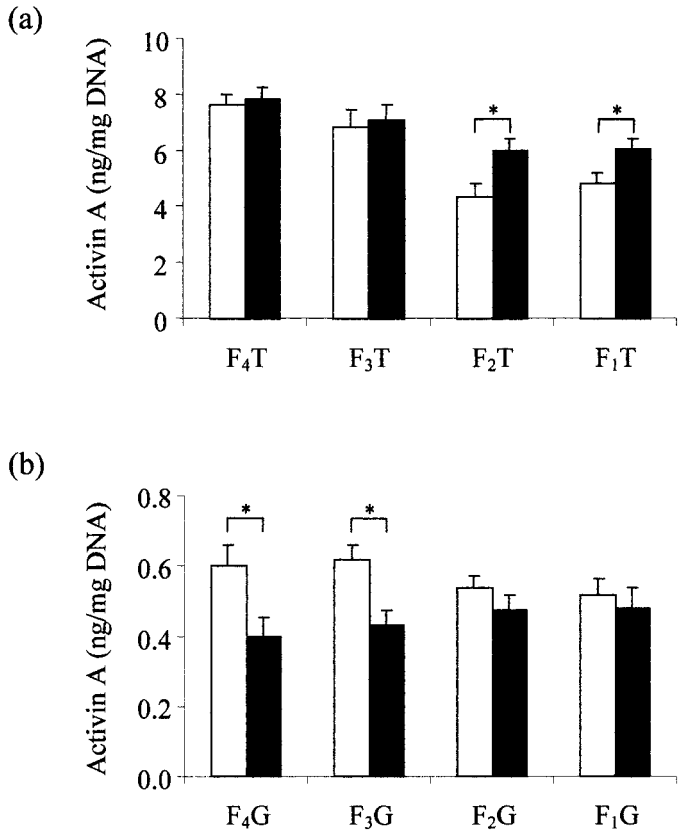


FIG. 6. Effects of immunization against inhibin α-subunit on the activin A content of **a**) theca (T) and **b**) granulosa (G) layers of preovulatory $F_1$–$F_4$ follicles from 29-wk-old hens. Asterisks denote statistically significant differences (*$P < 0.05$) between control (□) and immunized (■) groups. Values are means ± SEM (n = 11).

Concentrations of ir-α in plasma were significantly higher than those for inhibin A in all samples, but there was a positive correlation between the two hormones throughout development. Developmental profiles for ir-α [30, 31], progesterone [32], and LH [32–34] reported previously correspond to those observed in the present study. Circulating progesterone concentrations were also positively correlated with those for plasma inhibin A and for ir-α, and the concentrations of all three hormones increased progressively prior to the onset of lay. This suggests that the maturing ovary and, in particular, the large preovulatory follicles that are more steroidogenically active [35], are a primary source of inhibin A, ir-α, and progesterone. This result is in agreement with the significant decrease in plasma inhibin A, ir-α, and progesterone concentrations after induced ovarian regression, a process that results in atrophy of ovarian preovulatory follicles [11]. It has also been demonstrated that the $F_1$ granulosa layer contained the highest level of inhibin A [22], $\beta_A$ subunit mRNA [36, 37], and progesterone [38, 39] in the preovulatory follicle hierarchy.

The developmental profile for circulating FSH was negatively correlated with that for inhibin A. Plasma FSH concentrations decreased over the 2 wk prior to the onset of lay, coincident with the time that inhibin A concentration peaked. This supports the possibility of a negative feedback loop between inhibin A and FSH that may be established by the increase in inhibin A during ovarian development. However, given that the concentrations of progesterone and estradiol [40] are also rising at this time, a negative feedback action of these hormones in the regulation of circulating FSH concentrations cannot be discounted. Indeed plasma FSH was negatively correlated with progesterone, and the timing of the FSH decrease corresponds to the time that plasma estradiol concentrations increase [41].

The granulosa cell layers of the preovulatory follicles contain significantly higher concentrations of inhibin A than the corresponding theca layers. The inhibin A concentration in the $F_1$ granulosa layer was significantly higher than in $F_2$–$F_4$ granulosa layers as demonstrated in a previous study [22]. Detectable amounts of inhibin A were also found in all extragonadal tissues investigated, although values were lower than in $F_1$–$F_3$ follicle granulosa tissue. This suggests that the granulosa layer, especially that of the $F_1$ follicle, may be the primary source of inhibin A in the plasma. Further work is required to ascertain whether extragonadal tissues contribute significantly to circulating inhibin A concentrations or if inhibin A is acting as a paracrine-autocrine regulatory factor in any of these tissues.

As previously demonstrated [22], activin A concentrations in the theca layer of preovulatory follicles were significantly higher than in the corresponding granulosa layer. The highest concentration of activin A was in cardiac ventricular muscle, confirming a similar observation in the cockerel [17] and suggesting a possible role for activin in cardiac function that warrants further investigation.

Active immunization of pullets against inhibin α-subunit generated circulating antibodies reactive with native bovine inhibin A but did not significantly increase plasma FSH concentrations. Although the mean FSH levels in IMM birds were numerically higher than in control birds at all times throughout the study, this included the preimmunization period, and overall, there was no significant effect of IMM. This result is in contrast to observations in sexually mature mammals that active immunization against inhibin α-subunit increased plasma FSH [2, 4]. However, active immunization of 3-wk-old lambs against inhibin α-subunit had no significant effect on plasma FSH concentrations [42]. The latter investigation is more comparable to this experiment because both studies involved prepubertal immunization protocols. Recently, Moreau et al. [12] actively immunized Japanese quail prior to the onset of lay with an immunogen that consisted of a bacterial maltose binding protein-inhibin α-subunit fusion protein. Immunization took place at approximately halfway between hatch and the time of onset of lay and led to a slight, yet significant advance in the onset of lay and an enhanced egg production over a 30-wk period. Anderson et al. [42] also demonstrated that active immunization of lambs against inhibin advanced puberty and increased ovulation rate. In the absence of any increase in circulating FSH, this suggested an alternative mechanism whereby inhibin immunization may increase follicular development and ovulation rate. Other studies [5–8, 10] also demonstrated increased ovulation rates in sheep without significant changes in plasma FSH concentrations. Another possible explanation for the failure of immunization to provoke an increase in plasma FSH concentrations is a negative feedback role of estradiol on FSH secretion. This hypothesis was proposed after studies on the effects of ovariectomy and food withdrawal on plasma FSH concentrations [43]. Ovariectomy increased FSH concentrations, demonstrating that an ovarian factor was involved, but food withdrawal that leads initially to a fall in circulating inhibins without reducing estradiol concentrations did not increase plasma FSH concentrations.

In this study, in contrast to the quail study of Moreau et al. [12] immunization against inhibin α-subunit did not advance the onset of lay but did enhance the rate of oviposition as reflected by the increased incidence of double ovipositions. The increase in the number of recent postovulatory follicles on their ovaries in immunized birds compared to controls also indicated an increased rate of ovulation. It may be deduced that many of the ova must have ovulated into the abdominal cavity and/or been reabsorbed in the female tract as they were not represented by ovipositions. Additional eggs derived from double oviposition events were incompletely calcified as the egg needs to be in the shell gland for 18–20 h for complete calcification [44]. Eggs from immunized hens in which a single oviposition occurred per ovulatory cycle had a fully formed calcareous hard shell and were not different in weight to eggs from control hens (data not shown). This indicates that the immunization process did not interfere directly with the ability to form a normal, calcified eggshell.

An important observation was that inhibin immunization was associated with a selective twofold increase in the number of follicles in the 8- to 9.99-mm-diameter size-class on the ovaries of hens. Follicles of this size-class are considered to be at the bottom of the preovulatory hierarchy having only recently been selected from the prehierarchical follicle pool. In the hen a single follicle is selected each day from the group of 6- to 8-mm follicles to join the preovulatory hierarchy, a change associated with the expression of chicken LH-receptor mRNA [45] and an active P450 side-chain cleavage enzyme ($P450_{scc}$) [46] first detected in granulosa cells of 9- to 12-mm follicles. In the absence of significant changes in circulating LH or FSH concentrations, the evidence we present for an increased ovulation rate in IMM birds coupled with finding of an increased number of 8- to 9.9-mm-diameter follicles suggest a role for inhibin in regulating the entry of follicles into the preovulatory hierarchy. Inhibin A is present in significant concentrations in the granulosa layers of preovulatory follicles $F_4$–$F_1$, predominating in the $F_1$ granulosa layer [22]. Plasma inhibin A concentrations follow a distinct pattern of change during the ovulatory cycle, peaking at around the time of the LH surge ~6 h before ovulation [11]. The expression of $\beta_A$-subunit mRNA (a prerequisite for inhibin A and/or activin A synthesis) could not be detected in 5- to 12-mm-diameter follicle granulosa cells [47]. Therefore, cyclic changes in inhibin A output from granulosa cells of the larger hierarchal follicles (mainly $F_1$) may be involved in the regulation of recruitment of 6- to 8-mm follicles into the preovulatory hierarchy. This hypothesis would suggest that inhibin A might exert a negative effect on the recruitment and/or survival of follicles to the preovulatory hierarchy, possibly through a direct paracrine effect at an intraovarian level, although an indirect endocrine or a combined endocrine and intraovarian response cannot be ruled out.

An alternative hypothesis to explain the increase in 8- to 9.99-mm follicle numbers in hens immunized against inhibin α-subunit is based on the finding by Schneyer et al. [48] that free inhibin α-subunit precursors are able to compete with FSH for FSH-receptor binding. The highest levels of chicken FSH-receptor mRNA in the granulosa layer were found in 6- to 8-mm-diameter follicles [49], which may imply a higher responsiveness to FSH in this size of follicle. In 6- to 8-mm follicles FSH increased granulosa cell responsiveness to LH [50], increased $P450_{scc}$ mRNA levels, and initiated progesterone production [51]. This is consistent with a role for FSH in maintaining the viability of prehierarchical follicles. Inhibin α immunization may reduce the competition of free inhibin α-subunit precursors with FSH for the binding to FSH receptors, thus enhancing follicular sensitivity to FSH and promoting their development in vivo. The effect would therefore be similar to that elicited by raising plasma FSH concentrations. However, further work is required to identify and characterize inhibin subunit precursor forms in hen plasma and to identify possible roles for high molecular weight inhibin α-subunit precursor forms in FSH receptor competition.

A recent study of long-term inhibin α-subunit-immunized sheep showed an enhanced ovulation rate without raised plasma FSH concentration [10]. The ovaries of inhibin-immunized ewes contained more ≥3-mm follicles that contained markedly higher (approximately sixfold) activin A concentrations. This led to the proposal that inhibin immunization might stimulate follicle development by increasing granulosa cell responsiveness to FSH, as a consequence of increased exposure to activin A [52]. An anal-

ogous mechanism could be invoked to explain the ovarian response to inhibin immunization in the hen, as we also observed raised follicular activin A levels in the $F_1$ and $F_2$ theca layers of IMM hens.

In conclusion, evidence has been presented that active immunization of immature pullets against inhibin α-subunit promotes increased recruitment of follicles to the preovulatory follicular hierarchy and an increased ovulation rate. The mechanism for this remains to be resolved, but it evidently does not depend on raised circulating levels of FSH. These results, together with the observation that plasma inhibin A concentrations peak around the time of the preovulatory LH surge in the hen [11] and that the inhibin A content of the $F_1$ granulosa layer is maximal around this time [22], lead us to propose that cyclic fluctuations in inhibin A play a significant role in recruitment of follicles to the preovulatory hierarchy.

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