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Secretion of Inhibin in Male Japanese Quail (*Coturnix japonica*) from One Week of Age to Sexual Maturity

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Abstract. The objective of this study was to investigate the changes in secretion of inhibin and cellular localization of the inhibin α and inhibin/activin (β_A and β_B) subunits in male Japanese quail from 1 to 7 weeks after hatching. The post-hatch profile of plasma luteinizing hormone (LH), immunoreactive (ir) inhibin and testosterone were measured by radioimmunoassay. Testes were immunostained by the avidin-biotin-peroxidase complex method (ABC) using polyclonal antisera raised against inhibin α , inhibin/activin β_A and inhibin/activin β_B from one week of age to sexual maturity. Testicular weight increased gradually until 4 weeks and abruptly increased from 5 weeks of age onwards. The plasma concentrations of LH and ir-inhibin increased significantly at 5 weeks of age, and the plasma concentration of testosterone increased significantly at 6 weeks of age. Pituitary contents of LH showed a steady increase until 6 weeks of age and then abruptly increased at 7 weeks of age. Coincident to the increase in plasma testosterone, the testicular contents of testosterone significantly increased from 5 weeks through sexual maturity. Immunohistochemically, localization of the inhibin/activin α , β_A and β_B subunits was found in the Sertoli and Leydig cells at all ages of development from one week of age to sexual maturity. These results suggest that Sertoli and Leydig cells are the major source of inhibin secretion during development in male Japanese quail.

Key words: Inhibin, Japanese quail (*Coturnix japonica*), Luteinizing hormone (LH), Testes, Testosterone

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Inhibins and activins are growth and differentiation factors that have been localized in both the reproductive and non-reproductive organs. They exert their effects through both endocrine and local (autocrine/paracrine) mechanisms [1]. Inhibins and activins are structurally related dimeric gonadal proteins with the ability to regulate follicle-stimulating hormone (FSH) secretion from the pituitary glands [2, 3]. Inhibin consists of an α subunit linked by a disulfide bridge to one of the 2 highly homologous β subunits (β_A and β_B) to form inhibin A (α and β_A) or inhibin B (α and β_B) [4]. In mammalian species, apart from their action on FSH secretion, the inhibins and activins have been shown to exert paracrine/autocrine effects within the gonads [5–8] and other tissues [9] and have been proposed to have an important paracrine function during testes development [10, 11]. In the male, inhibin is produced in the testis, principally by Sertoli cells. There are temporal changes in inhibin expression and secretion with the changing role of the Sertoli cell in immature and adult testes. Variations in inhibin production among species reflect the different patterns of maturation [11–13].

In avian species, studies have confirmed a mammalian-type endocrine role for inhibins [14–17]; however, the autocrine/para-

crine roles of inhibins and activins in the gonads are still being defined. The profiles of plasma dimeric inhibin A and B have recently been reported for developing male chickens, but the activin A levels were undetectable [18]. Developmental changes in the inhibin α and inhibin/activin β_A and β_B mRNA levels have been identified in the gonads during post-hatch prepubertal development of male and female chickens [1]. The mRNA for inhibin/activin subunits is also detectable in the immature and mature testes of the rooster [19]. In our previous studies, secretion of inhibin and testicular expression of inhibin subunits were found in male duck embryos and newly hatched ducks [20]. To extend these observations and knowledge in male birds, the aim of the present study was to investigate the cellular localization of the inhibin α , β_A and β_B subunits from one week of age to sexual maturity in the male Japanese quail. Furthermore, developmental changes in the serum concentrations of immunoreactive (ir) inhibin, testosterone and LH were measured during sexual maturation in the male Japanese quail.

Materials and Methods

Animals

Male Japanese quail (*Coturnix japonica*) from low antibody response (L) selected lines were used in this study from one week

of age to sexual maturity [21, 22]. Fertilized eggs were incubated using a Showa Incubator Laboratory (Showa Furanki, Urawa, Japan) under normal conditions, including temperature (38.7°C), humidity ($55 \pm 10\%$) and turning (once every hour), controlled daily. One day before hatching (at days 16) of incubation candling was preformed. The birds were provided with food (Kanematsu quail diet; Kanematsu Agri-Tech Corporation, Ibaraki, Japan) and water and were allowed to feed *ad libitum*. They were housed in metal cages in a controlled environment (lights on, 0500–1900 h; temperature, $23 \pm 2^{\circ}\text{C}$; humidity, $55 \pm 10\%$; air exchanged 20 times hourly). This study was conducted in accordance with the Guiding Principles for the Use of Animals in Toxicology and was approved by the Animal Care and Use Committee of the Japanese National Institute for Environmental Studies.

Experimental design

Birds were decapitated weekly, and blood samples were collected into heparinized tubes. Blood samples were centrifuged for 15 min at 4°C and 1,700 g. The Plasma was separated and stored at -20°C until assay for ir-inhibin, LH and testosterone by specific radioimmunoassay (RIA). After dissection, the testes were removed, and weighed and fixed for 24 h in 4% paraformaldehyde (Sigma-Aldrich Chemical, St. Louis, MO, USA) in 0.05 M PBS (pH 7.4) for immunohistochemical examination.

Tissue preparation

The testes were dehydrated through a series of graded concentrations of ethanol and xylene, embedded in paraffin, sectioned serially at $4\text{ }\mu\text{m}$, mounted on glass slides coated with 3-aminopropyltriethoxysilane (APS; Sigma) and dried overnight at 37°C .

RIA procedure

A double-antibody RIA system was employed for measurement of plasma inhibin. The standard buffer for RIA was sodium phosphate (50 mmol/l, pH 7.5) containing NaCl (0.15 mol/l) and 0.1% (w/v) NaN_3 (PBS). Standards or samples (50–200 μl) obtained from adult female and male quails and homogenates of quail testes were pipetted into $10 \times 75\text{ mm}$ disposable glass culture tubes, and the volume was adjusted to 300 μl by addition of PBS containing 1% (w/v) BSA. Diluted antiserum against inhibin (100 μl) in 0.4% (v/v) normal rabbit serum in PBS containing EDTA (50 mmol/l) was added, and the tubes were mixed and incubated for 24 h at room temperature. Iodinated 32 kDa bovine inhibin (100 μl ; about 5,000 c.p.m.) in PBS containing 0.1% (w/v) bacitracin A (P-L Biochemicals, Milwaukee, WI, USA), L-methionine (5 mmol/l; Wako Pure Chemical Industries, Osaka, Japan) and 0.1% (w/v) 3[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS; Sigma) was then added. After brief vortex mixing, the samples were incubated for 24 h at room temperature. Thereafter, the second antibody [goat antiserum to rabbit γ globulin: 100 μl in PBS containing 6.0% (w/v) polyethylene glycol (M_r 7500; Wako)] was added. After brief mixing, the tubes were further incubated for 24 h at 4°C . Following centrifugation at 1,700 g for 30 min, the supernatant was aspirated, and the precipitate was counted in a gamma counter.

Bioactivity of quail inhibin

The bioactivity of quail inhibin was determined using the rat anterior pituitary cell culture system as reported previously [23]. Inhibin preparations from 32 kDa bovine inhibin and quail testicular homogenates were assayed, each in quadruplicate, to generate FSH inhibition curves.

RIA of LH, ir-inhibin and testosterone

The LH concentrations were measured with a USDA-ARS RIA kit (Beltsville, MD, USA) for chicken LH. The antiserum used was anti-avian LH (HAC-CH27-01 RBP75). The hormone for iodination was chicken USDA-cLH-I-3. The results are expressed in terms of USDA-cLH-K-3. The intra- and interassay coefficients of variation were 5.2 and 11.2%, respectively. USDA-cLH-I-3 and USDA-cLH-K-3 were kindly provided by Dr J. A. Proudman (Biotechnology and Germplasm Laboratory, Animal and Natural Resources Institute, Beltsville, MD, USA) [24]. The antiserum against avian LH was kindly provided by the Biosignal Research Center (Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan) [25]. Plasma concentrations of ir-inhibin were measured as described previously [26]. The iodinated preparation used was 32 kDa bovine inhibin, and the antiserum used was rabbit antiserum against bovine inhibin (TNDH-1). The results are expressed in terms of 32 kDa bovine inhibin. The intra- and interassay coefficients of variation were 8.8 and 14.4%, respectively. The concentrations of testosterone were determined by a double-antibody RIA system with ^{125}I -labeled radioligands as described previously [27]. The antiserum against testosterone (GDN 250) was kindly provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and interassay coefficients of variation were 6.8 and 17.1%, respectively.

Immunohistochemistry

Serial sections of testes were incubated with 10% normal goat serum to reduce the background staining caused by the second antibody. The sections were then incubated with primary antibodies raised against chicken inhibin α subunit (1-25)-Nle-Tyr, cyclic inhibin β_A (81-113)- NH_2 (#305-24-D) and cyclic inhibin β_B (80-112)- NH_2 (#305-25-D) for 12 h at room temperature. The antibody against chicken inhibin α subunit was kindly provided by Dr. Pat Johnson (Animal Physiology, Cornell University, Ithaca, NY, USA). The antibodies for inhibin/activin (β_A and β_B) were kindly provided by Dr. W. Vale (Salk Institute for Biological Studies, La Jolla, CA, USA). The sections were then incubated with a second antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidin staining kit (Sigma). This was followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako) solution in 150 ml of 0.05 mol Tris-HCl 1-1 buffer (pH 7.6) and 30 μl H_2O_2 . Finally, the reacted sections were counterstained with hematoxylin solution (Merck, Tokyo, Japan). The control sections were treated with normal rabbit serum (Sigma) instead of the primary antisera. Specificity of antibodies against inhibin α , β_A and β_B subunits was not examined using neutralized antibodies instead of primary antibodies in the present study.

Fig. 1. Changes in body weight (A) of male quails from 1 to 7 weeks after hatching and changes in both the right and left testes weights (B) of male quails from 3 to 7 weeks after hatching (the testes weight at 1 and 2 weeks of age could not be measured since they were very light). Values are means $\pm$ SEM ($n=7-17$ for A and $n=5-10$ for B). Within the same panel, values with different superscripts are significantly different ($P<0.05$).

Statistical analysis

Mean values ($\pm$ SEM) were calculated and analyzed using one-way ANOVA. Duncan's multiplet range test was used for detection of significant differences using the SAS computer software package. A value of $P<0.05$ was considered to be statistically significant.

Results

Body and testes weight

The body and testicular weights of the male Japanese quail from one week of age to sexual maturity are shown in Fig. 1. Body weight increased gradually and reached a maximum at 7 weeks of age (Fig. 1A). On the other hand, testes weight did not show any significant change by 4 weeks of age, began to increase at 5 weeks of age and then increased significantly at 7 weeks of age (Fig. 1B).

Characterization of the inhibin RIA system

Displacement of tracer with partially purified bovine inhibin, serial dilution of serum obtained from adult female and male quail and homogenates of quail testes showed parallelism with the serial dilution of 32-kDa bovine inhibin standard. This result indicated

Fig. 2. Dose-response curves of bovine 32-KDa inhibin, the plasma of adult males and females and testicular homogenates of adult quails in the inhibin radioimmunoassay. Each value represents the mean of triplicate determinations.

that it was possible to measure the concentration of inhibin in the quail using the present RIA method. The concentrations of inhibin in the female quail were higher than in the male quail (Fig. 2).

Bioactivity of inhibin

The secretion of FSH from cultured rat pituitary cells was suppressed in a dose-dependent manner by testicular homogenates of quail as well as bovine 32 kDa inhibin (Fig. 3). This result indicated that the quail testis has inhibin bioactivity that is similar to bovine inhibin.

Plasma concentrations of LH, ir-inhibin and testosterone

The plasma concentrations of LH did not change until 4 weeks of age, increased significantly at 5 weeks of age and then remained at high levels until 7 weeks of age (Fig. 4A). The plasma concentrations of ir-inhibin maintained a steady increase until 7 weeks of age. There was a significant difference at 5 weeks of age compared with the values at 1 week of age (Fig. 4B). This was also true for the plasma testosterone concentrations at 6 and 7 weeks of age compared with the values at 1 week of age (Fig. 4C). The pituitary contents of LH gradually increased until 6 weeks of age and showed an abrupt increase at 7 weeks of age. The pituitary contents of LH were significantly high at 6 and 7 weeks of age compared with the values at 1 week of age (Fig. 5). A significant difference in the testicular contents of testosterone was observed from 5 to 7 weeks of age compared with 3 weeks of age. There is no data for the testicular contents of testosterone for 1 to 2 weeks because the testes were very small (Fig. 6).

Immunolocalization of inhibin/activin subunits

Immunolocalization of the inhibin α , β_A and β_B subunits in the Japanese quail testis is shown in Fig. 7. Immunoreactivity for the inhibin α and inhibin/activin (β_A and β_B) subunits was present in

Fig. 3. Bioactivity of quail testicular inhibin determined by the rat pituitary cell culture system. Each value represents the mean $\pm$ SEM of quadruplicate determinations. ■: Bovine inhibin standard. ▲: Quail testis homogenate. ▼: Male quail serum. ◆: Female quail serum.

Sertoli and Leydig cells from 1 to 7 weeks of age, respectively. Only the results for the inhibin α and inhibin/activin (β_A and β_B) subunits in the samples collected at 2, 4, 6 and 7 weeks of age are shown in the present study (Fig. 7Ai, Bi, Ci, Di; 7Aii, Bii, Cii, Dii; 7Aiii, Biii, Ciii, Diii). No immunostaining was detected in control sections in which normal rabbit serum was substituted in place of the primary antibody (Fig. 7Av, Bv, Cv, Dv).

Discussion

The present study demonstrated that developmental changes in plasma LH, ir-inhibin and testosterone occurred in the male quails from one week of age to sexual maturity, and plasma LH and ir-inhibin clearly increased after 5 weeks of age; the testosterone concentration increased significantly at 6 and 7 week of age. Correlation among the levels of circulating LH, ir-inhibin and testosterone in the male quails was also observed in this study, showing that testicular activity is accompanied by developmental changes in pituitary function. In addition, our immunohistochemical results indicated positive staining of inhibin α , β_A , β_B subunits in Sertoli and Leydig cells during different stages of development after hatching, suggesting that Sertoli and Leydig cells could secrete bioactive inhibins in the male quail from one week of age to sexual maturity and that inhibins may play an important role in the testicular development of Japanese quail. To our knowledge this is the first study showing the relationships among LH, inhibin and testosterone in the circulation in male Japanese quails during the developmental period. Our data on the variation in gonadal weight and LH level in the male quail support earlier data in the chicken [28–30] showing that the testis weight is low during the early stage and increases progressively at the time of sexual maturity. It is well known that pituitary gonadotropins promote testicular development

Fig. 4. Changes in the plasma concentrations of LH (A), immunoreactive (ir) inhibin (B) and testosterone (C) in male Japanese quails from 1 to 7 weeks after hatching. Values are mean $\pm$ SEM of (n=5–10). Within the same panel, values with different superscripts are significantly different ($P<0.05$).

and help insure maximum testicular growth; together, these gonadotropins orchestrate the production and development of spermatozoa [31, 32]. Our evidence here clearly demonstrated that testicular growth was proportional to the concentration of LH in the pituitary and circulation; when the LH level was low, the testicular growth was significantly depressed. It shows that clear relationships exist between testicular weight and the concentration of LH in the plasma. In this study, plasma ir-inhibin and testosterone increased during development and reached a peak at 7 weeks of age; these peaks are preceded by a rise in the plasma LH concentration. The increase in testosterone and ir-inhibin from Leydig cells is probably caused by LH. Feedback control of LH by testosterone is also well known [32, 33]. Risbridger *et al.* [34] demonstrated in

Fig. 5. Pituitary contents of LH in male Japanese quail from 1 to 7 weeks of age. Values are mean $\pm$ SEM ($n=5-10$). Within the same panel, values with different superscripts are significantly different ($P<0.05$).

Fig. 6. Testicular contents of testosterone in male Japanese quail from 3 to 7 weeks after hatching. Values are mean $\pm$ SEM ($n=5-10$). Within the same panel, values with different superscripts are significantly different ($P<0.05$).

Fig. 7. Immunohistochemical localization of the inhibin/activin subunits in the Japanese quail testis. The positive results of the inhibin α and inhibin/activin (β_A and β_B) subunits for the samples collected at 2, 4, 6 and 7 weeks of age are shown in the present study (Fig. 7Ai, Bi, Ci, Di, 7Aii, Bii, Cii, Dii; 7Aiii, Biii, Ciii, Diii). No immunostaining was detected in control sections in which normal rabbit serum was substituted in place of the primary antibody (Fig. 7Av, Bv, Cv, Dv). The scale bars represent 100 and 50 μm , respectively.

rats that LH stimulates secretion of ir-inhibin in Leydig cells. Previous studies have indicated that inhibins and activins have roles to play in the development and function of the avian gonads during post-hatch prepubertal life [1]. Developmental changes in inhibins have been observed in female Japanese quails, and these observations have shown that plasma ir-inhibins are significantly increased

during sexual maturation [35]. In other aves, such as chickens [18, 19] and ducks [14, 20], there are similar developmental changes in the circulating ir-inhibin concentrations in accordance with the testicular activity. Taken together, our experimental results suggest that developmental changes in testicular weight may be involved by an increased circulating LH, ir-inhibin and testosterone concen-

tration in male Japanese quail. Identification of the sites of expression and production of inhibin subunit messenger RNA and protein is critical to understanding the biology of inhibins [12]. Inhibin and activin are structurally related dimeric glycoproteins, and it is now generally accepted that the main sites of production of inhibin reside within the gonads, primarily the Sertoli cells in the testis and the granulosa cells in the ovary [36, 37]. Differential localization of inhibin subunits was demonstrated in the testis of the quail from one week of age to sexual maturity in the present study. Positive immunostaining for the inhibin α , β_A and β_B subunits was detected in Sertoli and Leydig cells from 1 to 7 weeks of age. In mammals, many studies have reported that inhibin subunits may play some important roles in spermatogonial development [38, 39], Sertoli cell proliferation [40, 41] and steroid biosynthesis [42, 43]. In bull calves [44], testicular inhibin is involved in controlling FSH secretion during early prepubertal development and may act as a regulator of Sertoli cell differentiation and population. In ducks [20], inhibin/activin subunits are present in Sertoli and Leydig cells during embryonic and newly hatched development, suggesting that monomers of these proteins play an important autocrine or paracrine role during early stages of embryo development. In the chicken [1, 18, 20], expression of the mRNA subunits in the testis during post-hatch prepubertal development suggests that inhibins and activins are being produced but may principally be involved in autocrine/paracrine functions within the developing gonads. Thus, our present immunohistochemical results also support the suggestion that inhibin/activin subunits from Sertoli and Leydig cells act as a paracrine or autocrine factor involved in testicular development in the Japanese quail.

The changing levels of each inhibin/activin subunit with age probably indicates changing functional importance during development of the gonads [1]. The pattern of expression of inhibins varies through different stages of development for the male [11–13]. In the present study, positive staining for inhibin α , β_A and β_B subunits was found in Sertoli and Leydig cells for all ages of development after hatching; testes weights began to increase at 5 weeks of age, and this was followed by a significant increase at 7 weeks of age; the plasma concentrations of ir-inhibin maintained a steady increase until 7 weeks of age. These results indicate that the inhibin/activin subunits may play differentially regulated roles during development of the gonads, and enlarged gonads have the ability to produce large amounts of inhibins [45]. The cellular localization of inhibin subunits in the testes varies among species and during testicular development. In the rat testis, the mRNA of the α -, β_A -, and β_B -subunits has been demonstrated at different ages before puberty [46, 47], and Sertoli cells express different subunits and amounts at different stages of testicular development. In Shiba goats [11], the inhibin α and inhibin/activin (β_A and β_B) subunits are expressed in Leydig cells, but not in Sertoli cells, during fetal/neonatal life and are present in the Sertoli and Leydig cells of the adult testes, suggesting that the changing immunostaining for each subunit with age is associated with testicular function. A previous report showed that the inhibin α and β_A -subunits are expressed differentially at different stages of embryonic development in the male and female chick gonads [48]. Thus, our present immunohistochemical results support published views [1] that the changing

levels of mRNA may reflect the available contribution of each subunit to form a dimeric protein of functional relevance at any one time during the development of the gonads. In the present study, the plasma FSH, inhibin A and inhibin B concentrations could not be determined because there are no measuring methods currently available for the male Japanese quail. Future studies are needed to investigate the relationships among FSH, inhibin A and inhibin B in the male Japanese quail. In summary, our current results provide new endocrine physiological evidence for the Japanese quail and demonstrated that the plasma ir-inhibin and testosterone levels rose with testicular development. Furthermore, Sertoli cells and Leydig cells are the major source of inhibin secretion from one week of age to sexual maturity in the male Japanese quail.

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References

1. Onagbesan, OM, Safi M, Decuypere E, Bruggeman V. Developmental changes in inhibin alpha and inhibin/activin beta and betaB mRNA levels in the gonads during post-hatch prepubertal development of male and female chickens. *Mol Reprod Dev* 2004; 68: 319–326.
2. Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* 1986; 321: 776–779.
3. Vale W, Rivier C, Hsueh A. Chemical and biological characterization of the inhibin family of protein hormones. *Recent Prog Horm Res* 1988; 44: 1–34.
4. Tanaka Y, Taniyama H, Tsunoda N, Shinbo H, Nagamine N, Nambo Y, Nagata S, Watanabe G, Herath CB, Groome NP, Taya K. The testis as a major source of circulating inhibins in the male equine fetus during the second half of gestation. *J Androl* 2002; 23: 229–236.
5. Lin T, Calkins H, Morris PL, Vale WW, Bardin CW. Regulation of Leydig cell function in primary culture by inhibin and activin. *Endocrinology* 1989; 125: 2134–2150.
6. Chen CC. Editorial: Inhibin and activin as paracrine/autocrine factors. *Endocrinology* 1993; 132: 4–5.
7. Weng Q, Medan MS, Okano T, Tsubota T, Xu Meiyu, Watanabe G, Taya K. Changes in serum inhibin levels and immunolocalization of inhibin/Activin subunits during the breeding season in the wild male Japanese black bears (*Ursus thibetanus japonicus*). *Endocrine* 2006; 29: 345–350.
8. Weng Q, Medan MS, Xu Meiyu, Tsubota T, Watanabe G, Taya K. Seasonal changes in immunolocalization of inhibin/activin subunits and testicular activity in wild male raccoon dogs (*Nyctereutes procyonoides*). *J Reprod Dev* 2006; 52: 503–510.
9. Spencer SJ, Rabinovici J, Jaffe B. Human recombinant activin-A inhibits proliferation of human fetal adrenal cells *in vitro*. *J Clin Endocrinol Metab* 1990; 71: 1678–1680.
10. Albano RM, Arkell R, Beddington RSP, Smith JC. Expression of inhibin subunits and follistatin during postimplantation mouse development: decidual expression of activin and expression of follistatin in primitive streak, somites, and hindbrain. *Development* 1994; 120: 803–813.
11. Weng Q, Medan MS, Ren G, Watanabe G, Arai K, Taya K. Immunolocalization of inhibin/activin subunits in the Shiba goat fetal, neonatal, and adult testes. *J Reprod*

- Dev* 2005; 51: 521–526.
12. O'Connor AE, De Kretser DM. Inhibins in normal male physiology. *Semin Reprod Med* 2004; 22: 177–185.
 13. Kaneko H, Matsuzaki M, Noguchi J, Kikuchi K, Ohnuma K, Ozawa M. Changes in circulating and testicular levels of inhibin A and B during postnatal development in bulls. *J Reprod Dev* 2006; 52: 741–749.
 14. Yang P, Medan MS, Watanabe G, Taya K. Developmental changes of plasma inhibin, gonadotropins, steroid hormones, and thyroid hormones in male and female Shao ducks. *Gen Comp Endocrinol* 2005; 143: 161–167.
 15. Johnson PA, Brooks C, Wang SY, Chen CC. Plasma concentrations of immunoreactive inhibin and gonadotropins following removal of ovarian follicles in the domestic hen. *Biol Reprod* 1993; 49: 1026–1031.
 16. Vanmontfort D, Berghman LR, Rombauts L, Verhoeven G, Decuypere E. Changes of immunoreactive inhibin, FSH, LH, and progesterone in plasma after short-term food deprivation and during the ovulatory cycle of the domestic hen. *Gen Comp Endocrinol* 1994; 95: 117–124.
 17. Vanmontfort D, Room G, Bruggeman V, Rombauts L, Berghman LR, Verhoeven G, Decuypere E. Ovarian and extragonadal sources of immunoreactive inhibin in the chicken; effects of dexamethasone. *Gen Comp Endocrinol* 1997; 105: 333–343.
 18. Lovell TM, Knight PG, Groome NP, Gladwell RT. Measurement of dimeric inhibins and effects of active immunization against inhibin α -subunits on plasma hormones and testis morphology in the developing cockerel. *Biol Reprod* 2000; 63: 213–221.
 19. Davis AJ, Johnson PA. Expression pattern of messenger ribonucleic acid for follistatin and the inhibin/activin subunits during follicular and testicular development in *gallus domesticus*. *Biol Reprod* 1998; 59: 271–277.
 20. Yang P, Medan MS, Arai KY, Jin WZ, Watanabe G, Taya K. Secretion of inhibin and testicular expression of inhibin subunits in male duck embryos and newly hatched ducks. *Endocrine* 2005; 28: 171–179.
 21. Inooka S, Takahashi S, Takahashi H, Mizuma Y. Immunological traits in generations 7 to 12 of two lines of Japanese quail selected for high or low antibody response to Newcastle disease virus. *Poult Sci* 1984; 63: 1298–1302.
 22. Takahashi S, Inooka S, Mizuma Y. Selective breeding for high and low antibody responses to inactivated Newcastle disease virus in Japanese quail. *Poult Sci* 1984; 63: 595–599.
 23. Taya K, Sasamoto S. Selective release of FSH in lactating rats during the period of follicular atresia induced by the administration of antiserum to LH-releasing hormone. *J Endocrinol* 1988; 118: 455–464.
 24. Krishnan KA, Proudman JA, Bahr JM. Purification and partial characterization of isoforms of luteinizing hormone from the chicken pituitary gland. *Comp Biochem Physiol Biochem Mol Biol* 1994; 108: 253–264.
 25. Hattori M, Wakabayashi K. Isoelectric focusing and gel filtration studies on the heterogeneity of avian pituitary luteinizing hormone. *Gen Comp Endocrinol* 1979; 39: 215–221.
 26. Hamada T, Watanabe G, Kokuho T, Taya K, Sasamoto S, Hasegawa Y, Miyamoto K, Igarashi M. Radioimmunoassay of inhibin in various mammals. *J Endocrinol* 1989; 122: 697–704.
 27. Taya K, Watanabe G, Sasamoto S. Radioimmunoassay for progesterone, testosterone and estradiol-17 β ¹²⁵I-iodohistamine radioligands. *J Anim Reprod* 1985; 31: 186–197.
 28. Sharp PJ. A comparison of variations in plasma luteinizing hormone concentrations in male and female domestic chickens (*Gallus domesticus*) from hatch to sexual maturity. *J Endocrinol* 1975; 67: 211–223.
 29. Tanabe Y, Nakamura T, Fujioka K, Doi O. Production and secretion of sex steroid hormones by the testis, the ovary, and the adrenal glands of embryonic and young chickens (*Gallus domesticus*). *Gen Comp Endocrinol* 1979; 39: 26–33.
 30. Vanmontfort D, Berghman LR, Rombauts L, Verhoeven G, Decuypere E. Developmental changes in immunoreactive inhibin and FSH in plasma of chickens from hatch to sexual maturity. *Br Poult Sci* 1995; 36: 779–790.
 31. Kerr JB, Maddocks S, Sharp RM. Testosterone and FSH have independent, synergistic and stage-dependent effect upon spermatogenesis in the rat testis. *Cell Tissue Res* 1992; 268: 179–189.
 32. Kirby JD, Froman DP. Reproduction in male birds. In: Whittow GC (ed.), *Sturkie's Avian Physiology*. New York: Academic Press, 2000: 597–612.
 33. Maung SL, Follet BK. The endocrine control by luteinizing hormone of testosterone secretion from the testis of the Japanese quail. *Gen Comp Endocrinol* 1978; 36: 79–85.
 34. Risbridger GP, Clement J, Robertson DM, Drummond AE, Muir J, Burger HG, de Kretser DM. Immunoreactive and bioactive inhibin and inhibin α -subunit expression in rat Leydig cell cultures. *Mol Cell Endocrinol* 1989; 66: 119–122.
 35. Sedqyar M, Weng Q, Watanabe G, Kandiel MM, Takahashi S, Suzuki AK, Taneda S, Taya K. Secretion of inhibin in female Japanese quails (*Coturnix japonica*) from hatch to sexual maturity. *J Reprod Dev* 2008; 54: 52–57.
 36. De Kretser DM, Robertson DM. The isolation and physiology of inhibin and related proteins. *Biol Reprod* 1989; 40: 33–47.
 37. De Kretser DM, Hedger MP, Loveland KL, Phillips DJ. Inhibins, activins and follistatin in reproduction. *Hum Reprod Update* 2002; 8: 529–541.
 38. Van Dissel-Emiliani FM, Grootenhuys AJ, de Jong FH, de Rooij DG. Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. *Endocrinology* 1989; 125: 1899–1903.
 39. Mather JP, Attie KM, Woodruff TK, Rice GC, Phillips DM. Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. *Endocrinology* 1990; 127: 3206–3214.
 40. Lin T, Calkins JK, Morris PL, Vale W, Bardin CW. Regulation of Leydig cell function in primary culture by inhibin and activin. *Endocrinology* 1989; 125: 2134–2140.
 41. Boitani C, Stefanini M, Fragale A, Morena AR. Activin stimulates Sertoli cell proliferation in a defined period of rat testis development. *Endocrinology* 1995; 136: 5438–5444.
 42. Hsueh AJ, Dahl KD, Vaughan J, Tucker E, Rivier J, Bardin CW, Vale W. Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc Natl Acad Sci* 1987; 84: 5082–5086.
 43. Mauduit C, Chauvin MA, de Peretti E, Morera AM, Benahmed M. Effect of activin A on dehydroepiandrosterone and testosterone secretion by primary immature porcine Leydig cells. *Biol Reprod* 1991; 45: 101–109.
 44. Kaneko H, Noguchi J, Kikuchi K, Akagi S, Shimada A, Taya K, Watanabe G, Hasegawa Y. Production and endocrine role of inhibin during the early development of bull calves. *Biol Reprod* 2001; 65: 209–215.
 45. Tanaka Y, Taniyama H, Tsunoda N, Shinbo H, Nagamine N, Nambo Y, Nagata S, Watanabe G, Herath CB, Groome NP, Taya K. The testis as a major source of circulating inhibins in the male equine fetus during the second half of gestation. *J Androl* 2002; 23: 229–236.
 46. Kaipia A, Toppari J, Huhtaniemi I, Paranko J. Sex differences in the action of activin A on cell proliferation of differentiating rat gonad. *Endocrinology* 1994; 134: 2165–2170.
 47. Madjic G, McNeilly AS, Sharpe RM, Evans LW, Groome NP. Testicular expression of inhibin and activin subunits and follistatin in the rat and human foetus and neonate and during postnatal development in the rat. *Endocrinology* 1997; 138: 2136–2147.
 48. Safi M, Onagbesan OM, Volckaert G, Vanmontfort D, Bruggeman V, Decuypere E. Developmental expression of activin/inhibin α - and β _A-subunit genes in the gonads of male and female chick embryos. *Gen Comp Endocrinol* 2001; 122: 304–311.