TEMPORAL PROFILE OF GONADAL STEROIDS IN POPULATIONS OF ROOSTERS DIVERGENTLY SELECTED FOR MATING FREQUENCY

by

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INTRODUCTION

Although a great deal of information has been published regarding male sexual behavior, control of the intensity of the behavior being exhibited remains relatively vague. It has generally been accepted that basal endogenous levels of testosterone are not a primary factor regulating male libido. It is noteworthy that studies involving hormonal control of the intensity of the male sexual activity have focused primarily on testosterone; little regard has been given to other androgenic and estrogenic compounds. Since many gonadal hormones have been implicated in the control of sexual behavior, it would prove useful to assess circulating levels of these other steroids in addition to testosterone.

Several other factors should also be given consideration in order to further define male sexual behavior. A temporal hormonal study of animals differing in libido has yet to be performed. Research data exist suggesting that if gonadal hormonal secretion of cockerels is temporarily interrupted early in life, adult sexual behavior may be affected. Therefore, a maturational factor may be of significance. Additional data have implicated a gonadal substance other than testosterone which is capable of stimulating copulatory behavior in amphibians. The chemical composition of this substance has yet to be determined. Furthermore, it may be of importance to consider ratios of various hormonal compounds throughout development.

Hormonal studies involving male sexual behavior may be effectively executed utilizing genetic populations of animals differing in

mating behavior. Populations of chickens resulting from long-term bidirectional selection experiments for mating behavior have been developed at VPI&SU and are available for such endocrinological studies. Thus, if differences in basal steroid levels exist, or if differing patterns of steroid secretion are a factor regulating sexual behavior, then such hormonal differences should be evident in these genetic populations.

This study was conducted to analyze the hormonal states of roosters differing in sexual behavior. Through the efforts of this research, a profile of several testicular steroids was to be developed; their patterns of secretion were to be identified and the relationships between these hormones were to be assessed from hatch throughout sexual maturity.

LITERATURE REVIEW

Sex hormones, produced in male animals primarily by the testes, are capable of exerting profound effects upon sexual behavior. A wealth of information dealing with this area of research has been generated utilizing experimental animals from the amphibian, reptilian, avian and mammalian classes (review by Jones, 1974; Young, 1974; Adkins-Regan, 1981b; Bernon, 1981; and others). Due to the high degree of species specificity that exists in the animal kingdom, particularly between animal classes, the avian class will be given primary emphasis in this review of the literature.

It has been shown that castration and the concomitant decline in gonadal steroid production leads to a cessation in sexual displays for all animals studied (Adkins-Regan, 1981b). It was long believed that this declination in sexual behavior in male castrates was due to deprivation of the primary male sex hormone, testosterone, for it was found that exogeneous doses of this hormone successfully reinstated sexual behavior (Roussel, 1936; Shapiro, 1937; Stone, 1939; Beach, 1944; Beach and Holz-Tucker, 1949; Grunt and Young, 1952). This explanation, however, was later found to be grossly oversimplified.

It has been determined that in many cases, testosterone functions as a prohormone which is metabolized in neural or peripheral tissues to other steroids (McDonald et al., 1970). This conversion may follow one of two pathways. Testosterone, with the aid of the enzyme aromatase, may be converted (aromatized) to estradiol-17β. Under the influence of 5α or 5β-reductase, testosterone is reduced to 5α or 5β-

dihydrotestosterone, respectively. Dihydrotestosterone itself is nonaromatizable and cannot be converted to estradiol-17β (Ryan, 1963). Androstenedione, the precursor of testosterone, may be converted to estrone which is interchangeable with estradiol-17β (Naftolin et al., 1972).

Both aromatase and reductase activity have been identified in many members of the animal kingdom (Callard et al., 1978). More specifically, these biochemical substrates and subsequent conversion pathways have been identified in several avian species. Reductase activity has been observed in both juvenile (Balthazart and Hirschburg, 1979) and the maturing chicken (Massa and Sharp, 1981; Nakamura and Tanabe, 1974), the quail (Balthazart et al., 1979; Davies et al., 1980), the dove (Steimer and Hutchinson, 1980; 1981), the European starling (Massa et al., 1977), and the domestic duck (Willems, 1978). It is noteworthy that all of these researchers reported a predominance in 5β-reduction as compared to 5α-reduction in neural tissues. The aromatase pathway in the avian class has received little consideration but has been reported to exist in the juvenile chicken brain (Callard et al., 1978) and the dove brain (Hutchinson et al., 1981).

Quantification of Hormonal Levels

In addition to determining an animal's capacity for steroidal production, attempts have been made to quantitate the plasma and gonadal concentrations of steroid hormones in males of several avian species. Temporal studies have been performed utilizing the domestic chicken as an experimental model. Plasma testosterone was found

to increase throughout maturation, with the highest levels occurring in sexually mature birds (Mashaly and Glick, 1979; Tanabe et al., 1979). Gonadal testosterone concentrations were also observed to increase throughout maturity (Tanabe et al., 1979). Serum dihydrotestosterone levels in maturing roosters (12-24 weeks of age) were lower than testosterone levels and remained fairly constant throughout development with a slight increase occurring by the 24th week of age (Mashaly and Glick, 1979). Gonadal and plasma estradiol levels were reported to be very low or undetectable in the chicken from time of hatch throughout sexual development (Tanabe et al., 1979).

Plasma steroids in the quail have also been considered on a temporal basis (Ottinger and Brinkley, 1978; 1979). As in the chicken, testosterone levels were reported to increase with age. A critical review of their quantification procedures, however, reveals these values to represent testosterone plus 30% dihydrotestosterone. The importance of this study lies in the finding that increasing androgen levels were positively correlated with the onset of sex-related behavior.

Studies involving avian species have noted a defininte rhythm in circulating testosterone concentrations throughout the day. Schanbacher et al. (1974) observed hormonal fluctuations in domestic cocks. Fairly constant testosterone levels during the daylight hours were followed by higher levels throughout the dark period. A peak in the concentration occurred during early morning (0600 hr). Ottinger and Brinkley (1979) found similar results in Japanese quail. Highest androgen concentrations (testosterone + 30% dihydrotestosterone) occurred in the early morning (0800) hr) and evening (2000 hr). Levels were lowest during

late afternoon (1500 hr).

Hormonal Control of Overt Sexual Behavior

Many experiments have been performed to determine the effects of specific sex steroids on various sexual behaviors. These experiments normally have involved surgically castrated or functionally castrated (i.e., nonphotostimulated) animals and subsequent hormone therapy administered as injections or silastic implants. In all species studied, essentially total reinstatement of behavior was achieved with testosterone (chicken: McCollum et al., 1971; Jones, 1974; duck: Deviche, 1979; pigeon: Pietras and Wenzel, 1974; quail: Beach and Inman, 1965; Adkins, 1977; Cunningham et al., 1977; Adkins and Pniewski, 1978; Adkins et al., 1980; ring dove: Hutchinson, 1970; Martinez-Vargas, 1974; Cheng and Lehrmann, 1975; Adkins-Regan, 1981a). *Quail:* Japanese quail have been used extensively in studies involving hormonal control of sexual behavior. Many androgenic and estrogenic hormones have been tested in these experiments. Androstenedione has been found to be capable of stimulating copulation in castrated male quail (Adkins, 1977; Adkins et al., 1980). This hormone stimulated crowing and strutting as well. Copulation, but not crowing or strutting was stimulated by estradiol benzoate (Adkins and Adler, 1972; Balander et al., 1977; Adkins and Pniewski, 1978; Adkins et al., 1980). On the other hand, dihydrotestosterone has not been observed to stimulate copulation in this species (Adkins, 1977; Balander et al., 1977; Adkins and Pniewski, 1978; Adkins et al., 1980). Dihydrotestosterone does, however, effectively activate crowing and strutting (Adkins and

Pniewski, 1978; Adkins et al., 1980). It has also been shown that an aromatase inhibitor (1,4,6-androstatrien-3,17-dione) effectively inhibits testosterone-activated copulations but not estradiol benzoate-activated copulations (Adkins et al., 1980). Furthermore, Adkins and Nock (1976) found that CI-628, an antiestrogenic compound, was capable of inhibiting testosterone-induced copulations. These studies all suggest that copulation by quail involves androgen aromatization to estrogen, a conclusion derived by Adkins and Nock (1976) and Balander (1978).

Chicken: Experiments with domestic chickens have yielded similar findings. For complete understanding of these experiments, however, it is necessary to define mating behavior in the domestic chicken. The mating sequence in this species is rather complex (reviewed by Guhl and Fisher, 1969). Male courtship behavior, which is considered to contain an element of aggression, evokes one of several responses from the female. The hen may respond negatively to the male's sexual advances by exhibiting escape or avoidance behaviors, or she may respond positively by assuming a receptive crouch posture. This submissive gesture serves as a strong stimulus for the male to mount and tread; culmination of the sequence occurs with a completed mating. The importance of male aggressive behavior at the onset of the mating sequence has been illustrated by Balander (1977). He observed that males rendered nonaggressive through caponization did not mate with live hens. However, when these males were tested with a freshly sacrificed female serving as a model, copulation occurred. Presumably these capons were incapable of initiating the primary courtship

behaviors and, therefore, failed to copulate. With the use of the model, the necessity for aggression was removed thus enabling successful culmination of the behavioral sequence.

Recently an experiment was performed in our laboratories to ascertain the hormonal control of sexual behavior in the domestic cock. It is noteworthy that live hens were utilized in mating trial tests. In this experiment, it was observed that aside from testosterone, only a combination of 5α-dihydrotestosterone and estradiol benzoate was capable of stimulating the entire mating sequence. Furthermore, 5α-dihydrotestosterone-treated birds were observed only to court. Estradiol benzoate-treated birds displayed essentially no signs of sexual activity. Due to the usage of live females during the test situation, this lack of stimulation by estradiol benzoate was expected. These results suggest an androgenic control on courtship behaviors and an estrogenic control on copulatory behaviors thus lending support to the aromatization theory.

Ring dove: Hormonal control of reproductive behavior has also been studied in ring doves. These animals perform a series of sex-related behaviors including bow-cooing (courtship display), hop-charging (aggressive display), and wing-flipping and nest cooing (nest-soliciting displays). All of these behaviors are hormonally dependent and can be reinstated in the male castrate with testosterone therapy (Hutchinson, 1970; Martinez-Vargas, 1974; Cheng and Lehrman, 1975; Adkins-Regan, 1981a) or androstenedione treatment (Silver et al., 1979). Adkins-Regan (1981a) conducted further experiments to elucidate the hormonal control of male mating behavior in this species. She observed

that estradiol benzoate injections reinstated wing-flipping but very little copulatory behavior. It should be noted, however, that the estradiol benzoate-treated birds did not differ from control birds in copulatory behavior. The hormone 5α-dihydrotestosterone propionate, which cannot be aromatized, stimulated only bow-cooing and hop-charging. Dihydrotestosterone in its free form was found ineffective by both Adkins-Regan (1981a) and Saad and Silver (unpublished, as cited by Silver et al., 1979). It was suggested by Adkins-Regan (1981a; 1981b) that perhaps wing-flipping required aromatization of androgen to estrogen and that bow-cooing involved conversion of testosterone to dihydrotestosterone. A major contraindication to this theory exists; neither estradiol benzoate nor 5α-dihydrotestosterone propionate were as effective at stimulating their respective behaviors as the same dosage of testosterone propionate (Cheng and Lehrman, 1974; Adkins-Regan, 1981a). It remains to be known if stimulation of copulatory behavior requires the aromatization of testosterone to estradiol in the dove.

Pigeon: Concerning the domestic pigeon, Pietras and Wenzel (1974) observed that testosterone propionate, androstenedione and androsterone were all effective at restoring courtship behavior; androstenedione, however, was the most effective in this respect. Dihydrotestosterone (5α) was found to be entirely ineffective, and estradiol benzoate activated only chasing bahavior. These researchers suggest that conversion of androgen to estrogen is unnecessary for the activation of courtship behaviors in the pigeon.

Duck: The majority of experiments dealing with the domestic duck have involved induction of adult behavior in juveniles (see Balthazart et al., 1980) and will not be discussed. Testosterone propionate treatment, however, has been observed to restore social behavior in the castrated drake (Deviche, 1979).

Assessment of Factors Controlling Intensity of Male Sexual Behavior

Chicken: Androgen influence upon the exhibition of sexual behavior has been studied by several researchers utilizing lines of chickens produced as a result of bidirectional selection for mating activity (Siegel, 1965). Benoff et al. (1978a) and Bernon (1981) have determined baseline testosterone levels in intact males from these lines. Benoff et al. (1978a) reported higher testosterone levels in birds from the high mating line as compared to the low mating line. The within line variation was, however, quite large. Conversely, Bernon (1981) did not find a correlation between mating frequency and testosterone levels. Bernon also observed a high degree of sample variation.

McCollum et al. (1971), Jones (1974), and Van Krey et al. (1977) injected birds from these genetic lines with varying dosages of testosterone cypionate. Although mating behavior was restored, in none of these studies did the sexual behavior increase above the baseline level exhibited by intact males from the respective lines. Two theories of control were developed in explanation. McCollum et al. (1971) suggested the possibility of two genetically controlled systems for mating behavior, one neural and the other endocrine. The neural

system was viewed as being primary to the behavior, and the endocrine system became effective only when threshold levels were reached in the neural system. It was suggested that through selection for increased mating behavior, the neural threshold, and consequently the sensitivity of the target tissue to the hormone, was being affected. Alternatively, Van Krey et al. (1977) hypothesized an inhibitory mating center (IMC) and a stimulatory mating center (SMC). The IMC was thought to be independent of, and unaffected by, sex steroids. Androgens proposedly had a permissive role in the control of sexual behavior merely allowing mating activity to be expressed. Through genetic selection, activity of the androgen independent IMC was affected.

Benoff et al. (1978b) measured uptake of tritiated testosterone by hypothalami of birds from the high and low mating lines and the randombred control line from which these lines originated. No differences were found suggesting the existence of relative rates of assimilation and metabolism and a comparable number of available binding sites for testosterone between the lines. Although the results were not significant, it was noted that hypothalamic tissue of the high mating line reached a maximum concentration thirty minutes before the low mating line and the randombred control line. Several factors were considered as a possible explanation: differences in receptor site affinity, membrane permeability of neurons, or permeability of the blood-brain barrier.

Balander (1977) performed hormone implanation experiments utilizing cockerels from high and low mating lines. A model hen was used

during mating trials. Testosterone propionate placed into the praeopticus paraventricularis magnocellularis (PPM) and praeopticus medialis (POM) nuclei of low mating line birds had no behavioral effect. Implanted birds from the high mating line were stimulated to copulate, but the level of activity exhibited was never equal to that of intact counterparts. Balander (1977) presented two theories in explanation. The first of these suggests that the two genetic lines differ in the level of aromatase enzymes present. Lower levels in the low mating line would lead to decreased levels of available estrogen necessary for stimulation of copulatory behavior. The second theory suggests the possible existence of additional androgen sensitive nuclei responsible for sexual stimulation.

Quail: Experiments have also been performed utilizing lines of Japanese quail resulting from bidirectional selection for mating frequency (Sefton and Siegel, 1975). Capons of these lines were stimulated to mate by testosterone cypionate injections, but the behavior exhibited by each line was never equal to that of intact male counterparts; also, intact males from the control and low mating lines which received testosterone cypionate injections never reached the intensity of behavior displayed by males of the high mating line (Cunningham et al., 1977). These findings are in agreement with similar experiments performed with domestic fowl (McCollum et al., 1971; Jones, 1974; Van Krey et al., 1977); the same theories of control were, therefore, suggested to operate in this species.

Balander et al. (1977) injected male quail from these genetic lines with various sex steroids and evaluated the sexual behavior

exhibited during tests with a female model. Sexual behavior was stimulated in high mating line males by testosterone propionate and estradiol benzoate. Dihydrotestosterone (5α) was ineffective in this respect. Concerning the low mating line males, only those receiving estradiol benzoate treatment were stimulated to copulate. These findings lend support to the aromatization theory proposed by Adkins (1977). Balander (1978) noted the significance of estradiol benzoate stimulation in the low mating line. He concluded that selection for frequency of mating activity had affected the capacity for aromatization.

Mammals: Similar experiments involving the mammalian class are in agreement with studies performed with birds. Regardless of the dosage of androgen administered to orchidectomized deer mice (Dalterio et al., 1979), guinea pigs (Grunt and Young, 1952; Riss and Young, 1954), mice (Champlin et al., 1963) and rats (Larsson, 1966; Damassa et al., 1977), the levels of sexual activity exhibited cannot be raised above precastrate levels. Also, the introduction of supraphysiological levels of testosterone into middle-aged rats showing characteristic declines in both circulating testosterone levels and sexual arousal was incapable of increasing the behavior above the performance of intact middle-aged males (Gray et al., 1981).

Of the studies cited, with the exception of Benoff et al. (1981a), all suggested that some factor(s) in addition to circulating androgens is/are responsible for the degree of sexual behavior exhibited. Several possible theories have already been presented for consideration (McCollum et al., 1971; Van Krey et al., 1977; Balander, 1977; Balander,

1978; Benoff et al., 1978b).

A third theory of control, although not directly related to the avian class, is worthy of mention. It has been shown through experiments with the frog that injections of purified sex steroids are incapable of inducing sexual behavior in castrates (Palka and Gorbman, 1973). Crude testicular extracts, however, have been found quite effective in this respect suggesting that some factor of the testes aside from the variables considered was required for induction of male copulatory behavior (Palka and Gorbman, 1973).

A fourth theory, as discussed by Leshner (1979) and Harding (1981), concentrated on the hormonal changes that occur in an individual during social interactions and the role these endocrinological changes play in directing an animal's short-term behavioral responses to social stimuli. Leshner (1979) presented evidence for a "baseline hormonal state" which refers to the hormonal state of an animal prior to exposure to a behavior eliciting stimulus. In addition, he presented a second situation termed the "feedback effects on behavior" which pertains to behaviors evoked as a result of "acute hormonal responses" to environmental stimuli or behavioral actions. The majority of the experiments cited here, excluding those involving brain manipulation, have dealt with either the measurement of hormones during the baseline state, or the induction of unnatural baseline hormonal states in experimental subjects. If these experiments had been conducted with consideration given to the effect of hormonal changes on short-term behavioral responses and feedback effects on behavior, perhaps different results would have been obtained. This situation has been

discussed in detail in the papers of Leshner (1979) and Harding (1981), and examples were presented in support of this concept. The following is a discussion of experiments dealing specifically with mating behavior and their relation to the aforementioned theory.

Hormonal Responses to Environmental Stimuli and Behavioral Actions

Physical condition and behavior of the female have been associated with changes in male hormonal condition. Exposure to a conspecific female has been positively correlated with increased testosterone levels above the baseline level for many mammalian species (bull: Weathersbee and Lodge, 1976; guinea pig: Harding and Feder, 1976; hamster: Macrides et al., 1974; rat: Kamel et al., 1975; rhesus monkey: Bernstein et al., 1974). Similar incidences have been reported concerning members of the avian class. Male domestic pigeons paired with a female showed tendencies toward higher testosterone levels when compared to males housed alone (Haase et al., 1976). O'Connell et al. (1981a) concluded from experiments with ring doves that the female's gonadal condition influenced the male's hormonal state. In addition, the female's behavior (i.e., courting, non-courting, incubating) also had an effect on the circulating hormone levels in the male (Feder et al., 1977; O'Connell et al., 1981a; 1981b).

Increases in androgen levels above the baseline state have also been associated with copulatory activity. Androgen levels in males of several mammalian species (bull: Katongole et al., 1971; boar: Liptrap and Raeside, 1978; guinea pig: Harding and Feder, 1976; man: Fox et al., 1972; rabbit: Saginor and Horton, 1968; Haltmeyer

and Eik-Nes, 1969; ram: Moore et al., 1978; rat: Purvis and Haynes, 1974; Kamel et al., 1975) are known to increase following a sexual encounter. Increased androgen levels are also characteristic of periods of intense breeding activity in the rough-skinned newt (Speckler and Moore, 1980) and of the breeding season of the rhesus monkey (Berstein et al., 1974).

More specifically, Batty (1978a) compared testosterone levels of sexually active and inactive mice. She observed that mice exhibiting copulatory behavior had higher testosterone levels following a sexual encounter than did inactive mice. Damassa et al. (1977) observed similar increases in testosterone in male rats. Furthermore, both of these studies reported peaks in androgen concentration to occur at the onset of sexual behavior thus leading to the conclusion that hormonal mediation of the initiation of sexual behavior was more important than the execution of the behavior (Batty, 1978a).

Several studies have linked hormonal condition following environmental stimuli or behavioral actions with the degree of sexual behavior exhibited. Harding and Feder (1976) reported testosterone levels of high activity male guinea pigs to be higher than low activity males following a sexual encounter. Also, testosterone levels in high activity males were higher upon exposure to an estrous female. Studies with mice are in partial agreement (Batty, 1978a). Male mice exhibiting high mount frequency and short mount latency had low basal testosterone levels, but showed rapid increases upon exposure to a receptive female. Conversely, males with a low mount frequency and a low mount latency had high basal testosterone levels with no increase occurring

upon exposure to an estrous female.

Studies involving pituitary and hypothalamic secretions have also been enlightening. Circulating levels of luteinizing hormone (LH) have been observed to increase in male rodents during the initiation stages of mating behavior (Kamel et al., 1977; Coquelin and Bronson, 1979) and also upon exposure to the odor of an estrous female (Kamel et al., 1977). Luteinizing hormone releasing hormone (LHRH) has been given consideration in the control of male sexual behavior. Moss et al. (1975) found this releasing hormone capable of facilitating sexual behavior in castrated rats receiving simultaneous hormone therapy.

A hormonal scheme for the control of male sexual behavior has been proposed by Kamel et al. (1977) utilizing the results of these studies. According to this model, exposure to a female causes increases in LHRH levels which in turn leads to increased LH and testosterone secretion. Furthermore, LHRH was suggested as possibly being responsible for the execution of copulatory behaviors.

MATERIALS AND METHODS

Stock and Management

Group 1: Chicks were obtained from pedigreed matings among each of two lines of chickens divergently selected over 21 generations for cumulative number of completed matings (Siegel, 1965; 1972). A total of 46 high mating line (HML) and 34 low mating line (LML) chicks were utilized.

On the day of hatch, the birds were exsanguinated by cardiac puncture with a heparinized syringes; the blood samples were stored temporarily on ice. Following collection of samples, the subjects were sexed by laparotomy. Samples obtained from males were centrifuged at 4850 X g for 10 minutes; the plasma fraction was recovered and stored at -20°C.

Group 2: Thirty-six HML and 37 LML chicks were generated as in the manner described above using the same parental stock. Upon hatching, each bird was wingbanded, vaccinated against Marek's disease and vent-sexed. The males were raised in unisexual flocks in litter-floor pens and received food and water ad libitum. A 16 hour light cycle (0600 to 2200 hr) was maintained for the duration of this experiment.

Between 0800 and 0930 hr when the birds were 56, 112, 168 days of age, 3.0 ml blood samples were obtained by cardiac puncture with heparinized syringes. The samples were stored temporarily on ice and then centrifuged at 4850 X g for 10 minutes. The plasma fraction was recovered and stored at -20°C.

On day 182 of development, individual body weights were obtained to the nearest 10 grams. Also on day 182 of development, packed cell

volumes (PCV) were determined for each bird in duplicate. Blood was collected into heparinized microhematocrit tubes from the brachial vein, spun for 3 minutes in a Drummond centrifuge at 6130 X g and the values determined utilizing a Drummond hematocrit reader.

Steroid Analysis

The procedure employed for hormone analysis allows for the extraction, purification and quantification of four steroidogenic compounds (androstenedione, dihydrotestosterone, testosterone and estradiol-17β) from a single plasma sample. A detailed outline of this procedure is presented in appendix A and will be only briefly related in this text.

Chemical Reagents: A list of all reagents used can be found in appendix A. All of the organic solvents, with the exception of spectroanalyzed grade chemicals, were distilled before use and redistilled if not used within three days.

Extraction: Approximately 1200 cpm of each of the tritiated hormones (1,2-^{3}H(N)-androst-4-ene-3,16-dione; 1,2,4,5,6,7-^{3}H(N)-dihydrotestosterone; 1,2-^{3}H(N)-testosterone; and 2,4,6,7-^{3}H(N)-estradiol) were added to screw top extraction tubes. One ml plasma samples were then added to each tube and allowed to equilibrate in a 45°C water bath for 15 minutes. The samples were extracted three times with 5.0 ml of iso-octane to liberate the androgens. The three supernates collected for each sample were combined and evaporated to dryness. Extraction with iso-octane allowed for differential removal of the androgens without extraction of fat or estradiol. High fat content in the extract is undesirable when performing chromatographic separation with celite. The samples were next extracted three times with 5.0 ml of anhydrous diethyl ether.

The three supernates collected for each sample were combined and evaporated to dryness. Both the androgen and the estradiol extracts were stored at 4°C until chromatography was performed.

Column Chromatography: Celite columns (Abraham, 1977) (68 mm packing height; .6 cm radius) were used for the separation of the androgens. The dried residue was transferred to the column in 1.0 ml of iso-octane. The elution procedure which was adapted from Saksena et al. (1977) was as follows: 3.5 ml iso-octane (androstenedione), .5 ml iso-octane (discard), 3.5 ml iso-octane (dihydrotestosterone), 1.5 ml iso-octane (discard), 4.0 ml iso-octane:benzene (7.3, v/v) (testosterone).

The estrogenic fraction was chromatographed on Sephadex (LH20) mini-columns (42 mm packing height; 1.3 cm radius) to fractionate estrone and estradiol. In addition, this step served to separate the fat from the estradiol fraction. The dried residue was transferred to the column in .3 ml of elution solvent (benzene:methanol; 1:1, v/v). The elution procedure was as follows: 1.2 ml elution solvent (discard), .5 ml elution solvent (discard-contains fat and some estrone), 2.0 ml elution solvent (discard, contains estrone), .5 ml elution solvent (discard), 3.5 ml elution solvent (estradiol-17β).

Sample Preparation: For androstenedione, dihydrotestosterone and testosterone, the dried extracts were redissolved in 2.0 ml methylene chloride:methanol (9:1, v/v). Known aliquots were then pipetted into disposable culture tubes for assay.

The sample preparation method used for estradiol was an extension of the procedure employed by Carruthers and Hafs (1980). To each tube

was added .6 ml of phosphate buffered saline with merthiolate and .1% gelatin added (PBS-mer-ga), and the samples allowed to equilibrate first in a 45°C water bath for 15 minutes and then overnight at 4°C. Known aliquots were removed and placed in disposable culture tubes for assay.

For all four hormones assayed, a .2 ml aliquot was transferred to a mini-vial and 5.0 ml scintillation cocktail added for determination of procedural losses. Average values for percentage of recovery for each hormone appear in Table 1.

Assays

Androstenedione: This assay was adapted from that of Abraham et al. (1975) and used rabbit antiserum[1] prepared against androstenedione-3-oxime-human serum albumin as reported by Abraham (1974). Percent cross reactivities of this antibody with other hormones appear in Table 2. One hundred µl of antibody (1:2000) dilution was added to each sample and to the standard curve tubes (0, .025, .05, .10, .25, .50, and 1.0 ng androstenedione), vortexed and incubated 15 minutes at room temperature. Following incubation, 5.0 X 10^4 dpm of 1,2,6,7-^{3}H(N)-androst-4-ene-3,17-dione were added to each tube, vortexed and incubated 14 to 16 hours at 4°C.

Following the incubation period, 1.0 ml of dextran coated charcoal was added to each sample, vortexed and incubated in an ice bath for 10 minutes. The tubes were then centrifuged (1732 X g, 4°C, 10 minutes).

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[1]Androstenedione antibody (batch #OP/09) was obtained from Optimox, Inc., Palos Verdes, CA.

Table 1. Percent recoveries, antibody sensitivities and between and within assay coefficients of variation for various steroidogenic radioimmunoassay procedures

Hormone	Percent recovery	Antibody sensitivity	Between assay coefficient of variation (%)	Within assay coefficient of variation (%)
Androstenedione	69	.025-1 ng	6.60	4.04
5α-dihydrotestosterone	50	.005-1 ng	8.33	13.61
Testosterone	56	.005-1 ng	5.70	11.41
Estradiol-17β	49	1-100 pg	54.54	45.97

Table 2. Cross reaction of various steroids with androstenedione (AE) antibody (#OP/09) (Optimox, Inc., Palos Verdes, CA).

Steroid	Cross-reactivity[1] (%)
Etiocholanolone	10.00
Androsterone	5.00
Dehydroepiandrosterone	2.00
Testosterone	0.10
Cholesterol	< .05
5α-Dihydrotestosterone	0.01
Pregnenolone	<0.01
17-Hydroxypregnenolone	<0.01
Progesterone	<0.01
17-Hydroxyprogesterone	<0.01
Desoxycorticosterone	<0.01
Corticosterone	<0.01
Aldosterone	<0.01
Cortisol	<0.01
Androst-5-ene-3β,17β-diol	<0.01
5α-Androstane-3α,17β-diol	<0.01
5α-Androstane-3β,17β-diol	<0.01
Estradiol-17β	<0.01
Estrone	<0.01
Estriol	<0.01

[1] Cross-reactivity is defined as:

$$\frac{\text{quantity of steroid required to displace 50\% of bound } H^3\text{-steroid}}{\text{quantity of AE required to displace 50\% of bound } H^3\text{-AE}} \times 100$$

After centrifugation, .5 ml supernate was removed to a mini-vial and 5.0 ml assay scintillation cocktail added. The vials were counted on a Searle Beta Scintillation Counter (Model Delta 300).

Dihydrotestosterone: This assay was adapted from the procedure used by Abraham et al. (1975) and utilized rabbit antiserum[2] prepared against testosterone-3-oxime-human serum albumin. Percent cross reactivities of this antibody with other hormones appear in Table 3. One hundred µl of antibody was added to each sample and to the standard curve tubes (0, .005, .01, .02, .03, .05, .08, .10, .25, .50, and 1.0 ng 5α-dihydrotestosterone). Following addition of antibody, approximately 2.0 X 10^4 dpm of 1,2,4,5,6,7-H^3(N)-dihydrotestosterone were added to each tube, vortexed and incubated 14 to 16 hours at 4°C.

Following the incubation period, .4 ml of dextran coated charcoal was added to each sample, vortexed and incubated in an ice bath for 10 to 15 minutes. The tubes were then centrifuged (1018 X g, 4°C, 15 minutes). After centrifugation, .4 ml supernate was removed to a mini-vial and 5.0 ml assay scintillation cocktail added. The vials were counted on a Searle Beta Scintillation Counter (Model Delta 300).

Testosterone: This assay was adapted from the procedures of Smith and Hafs (1973) and Kattesh (1979) using rabbit anti-serum[3] prepared against testosterone-3-oxime human serum albumin. Percent cross reactivities

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[2] 5α-dihydrotestosterone antibody (batch #OP-08A) was obtained from Optimox, Inc., Palos Verdes, CA.

[3] Testosterone antibody (MSU #74) was donated by Dr. J. Ireland, Michigan State University, East Lansing, MI.

Table 3. Cross reaction of various steroids with 5α-dihydrotestosterone (5α-DHT) antibody (#OP/08A) (Optimox, Inc., Palos Verdes, CA).

Steroid	Cross-reactivity[1] (%)
Testosterone	90.00
Androst-5-ene-17β-diol	80.00
5β-Dihydrotestosterone	30.00
Androstenedione	4.00
Androsterone	4.00
Etiocholanolone	0.50
Dehydroepiandrosterone	0.06
Cholesterol	<0.01
Pregnenolone	<0.01
17-Hydroxypregnenolone	<0.01
Progesterone	<0.01
17-Hydroxyprogesterone	<0.01
20α-Hydroxypregn-4-ene-3-one	<0.01
Desoxycorticosterone	<0.01
Corticosterone	<0.01
Aldosterone	<0.01
Cortisol	<0.01
11-Desoxycortisol	<0.01
Estrone	<0.01
Estradiol-17β	<0.01

[1]Cross-reactivity is defined as:

$$\frac{\text{quantity of steroid required to displace 50\% of bound } H^3\text{-steroid}}{\text{quantity of 5α-DHT required to displace 50\% of bound } H^3\text{-5α-DHT}} \times 100$$

of this antibody with other hormones appear in Table 4. Two hundred µl of antibody (1:50,000 dilution) was added to each sample and to the standard curve tubes (0, .005, .01, .03, .05, .10, .25, .50, 1.0 ng testosterone), vortexed and incubated 15 minutes at room temperature. Following incubation, 5.9 X 10^4 dpm of 1,2-^{3}H(N)-testosterone were added to each tube, vortexed and incubated 14 to 16 hours at 4°C.

Following the incubation period, 1.0 ml of dextran coated charcoal was added to each sample, vortexed and incubated in an ice bath for 10 minutes. The tubes were then centrifuged (1732 X g, 4°C, 10 minutes). After centrifugation, .5 ml supernate was removed to a mini-vial and 5.0 ml assay scintillation cocktail added. The vials were counted on a Searle Beta Scintillation Counter (Model Delta 300).

Estradiol-17β: This assay was developed by Anderson (1982) and utilizes a double antibody system and iodinated estrogen. The estradiol antibody[4] used was generated in rabbits against estrone-17-oxime-bovine serum albumin according to the procedure of Erlanger et al. (1958). Percent cross reactivities of this antibody with other hormones appear in Table 5. Two hundred µl of antibody (1:60.5 dilution) was added to each assay tube and to the standard curve tubes (0, 1, 3, 5, 10, 20, 50, and 100 pg estradiol-17β). Next, ~10,000 cpm of iodinated estrogen were added to each tube. Finally, .2 ml of second antibody which was generated in sheep against rabbit gamma globulin was added to the tubes. The dilution of the second antibody used was selected according to the normal rabbit serum used and was determined by assessing

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[4]Estradiol antibody (E17-94; batch #427) was obtained from Endocrine Sciences, Tarzana, CA.

Table 4. Cross reaction of various steroids with testosterone (T) antibody (MSU #74) (Kattesh, 1979).

Steroid	Cross-reactivity[1] (%)
5α-androsten-17-ol-3-one	53.1
Estradiol-17β	17.5
Progesterone	17.5
Estrone	10.0
Dehydroepiandrosterone sulfate	9.7
Androstenedione	6.5
5Δ-androsten-3β,17β-diol	5.7
5,16-androstadien-3β-ol	4.6
Dehydroepiandrosterone	2.7
Corticosterone	1.6
16Δ5α-androsten-3-one	1.2
16Δ5α-androsten-3α-ol	1.1
16Δ5α-androsten-3β-ol	1.0
Cortisol	.8

[1]Cross-reactivity is defined as:

$$\frac{\text{quantity of steroid required to displace 50\% of bound } H^3\text{-steroid}}{\text{quantity of T required to displace 50\% of bound } H^3\text{-T}} \times 100$$

Table 5. Cross reaction of various steroids with estradiol (E_2) antibody (E17-94; batch #427) (Endocrine Sciences, Tarzana, CA).

Steroid	Cross-reactivity[1] (%)
Estrone	130.0
Estriol	3.0
1,3,5(10)estratrien-3,16α-diol,17-one	3.0
1,3,5(10)estratrien-3,16β,17α-triol	2.5
17-ethynyl-3,17β-estradiol	2.0
1,3,5(10)estratrien-2,3-diol-17-one	1.0
Dihydrotestosterone	0.2
19-nor progesterone	<0.1
Progesterone	<0.1
17-OH-progesterone	<0.1
Testosterone	<0.1
11-deoxycortisol	<0.1
Cortisol	<0.1
Cortisone	<0.1
Androstenedione	<0.1
Pregnanediol	<0.1
Etiocholanolone	<0.1
1,3,5(10)estratrien-3,16α,17β-triol,3-methyl ether	<0.1

[1] Cross-reactivity is defined as:

$$\frac{\text{quantity of steroid required to displace 50\% of bound } H^3\text{-steroid}}{\text{quantity of } E_2 \text{ required to displace 50\% of bound } H^3\text{-}E_2} \times 100$$

the dilution which yielded optimum precipitation of bound hormone. Following addition of all solutions, the tubes were vortexed and allowed to incubate at room temperature overnight. Tests for nonspecific binding and for effects due to the addition of tritiated estradiol (added for recovery determinations) were ascertained.

Following incubation, 1.0 ml of PBS-mer-ga was added, and the tubes were centrifuged for 30 minutes at 1203 X g. The supernate was decanted, the tubes wiped dry of any residual moisture and the precipitate counted on a Beckman Gamma 4000 Counter.

Calculations

Assay concentration values for all hormones were assessed utilizing a log transformation of a standard curve. The final hormone concentrations (expressed in either ng/ml or pg/ml) were adjusted using dilution factors and percent recoveries.

In addition to these adjustments, the final estradiol concentrations also took into account the assay concentration of tritiated estradiol added for recovery purposes.

Precision of Assay

Validation tests were performed on each assay procedure to assess the reliability of the methods in our laboratories utilizing chicken plasma. The first test involved the quantification of hormone present in increasing levels of plasma (i.e., .5, 1.0, 1.5, and 2.0 ml). Secondly, known amounts of unlabelled hormone were added to 1.0 ml plasma samples and the concentrations of hormone determined. The results of both of these tests are represented in Tables 6 and 7.

Table 6. Accuracy of measuring various steroids in chicken plasma[1]

Hormone	Plasma extracted (ml)	Amount of hormone measured[2] (ng/ml)	n
Androstenedione	.5	.44± .02	2
	1.0	.37± .01	2
	1.5	.54± .03	2
	2.0	.57± .09	2
Dihydrotestosterone	.5	.36± .06	3
	1.0	.34± .06	5
	1.5	.31± .04	2
	2.0	.36± .10	2
Testosterone	.5	1.90± .15	2
	1.0	1.80± .15	6
	1.5	2.20± .01	2
	2.0	1.80± .21	2
Estradiol-17β	.5	135.19± 9.71	3
	1.0	133.99± 2.50	2
	1.5	139.96± 7.57	3
	2.0	150.47±16.77	2

[1]Androstenedione, dihydrotestosterone and testosterone tests were performed utilizing adult rooster plasma; estradiol-17β tests were performed on adult laying hen plasma.

[2]Mean ± standard error

Table 7. Accuracy of measuring increasing amounts of steroid hormones added to 1.0 ml of rooster plasma

Hormone	Hormone added	Added hormone recovered[1]	n
Androstenedione	.05 (ng)	.05± .01	3
	.10	.12± .04	3
	.50	.42± .18	3
	1.00	.83± .10	3
	5.00	4.48± .19	3
Dihydrotestosterone	.05 (ng)	.03± .01	3
	.10	.08± .02	3
	.50	.52± .07	3
	1.00	.96± .06	3
	5.00	4.77± 1.11	3
Testosterone	.05 (ng)	.04± .00	3
	.10	.09± .02	3
	.50	.46± .15	3
	1.00	.74± .14	3
	5.00	4.60± .55	3
Estradiol-17β	10 (pg)	13.54± 6.01	3
	25	24.20± 8.17	3
	50	45.63±14.17	3
	100	94.90±26.64	3
	250	242.28±93.94	3

[1]Mean ± standard error

The lowest sensitivity was also determined for each assay. The results are presented in Table 1. Within assay and between assay coefficients of variation were obtained and the results appear in Table 1.

Statistical Analyses

Mean levels for each hormone were compared between lines at each age using analysis of variance. Analyses were made using the statistical model:

$$(1) \qquad Y_{ij} = \mu + L_i + e_{ij}$$

where i = 1,2 lines (L); j = 1,2...n individuals per line and Y_{ij} = hormone level.

The age effect within a given hormone for each line was determined by analysis of variance. The statistical model used was:

$$(2) \qquad Y_{ij} = \mu + A_i + e_{ij}$$

where i = 1,2,3,4 ages (A); j = 1,2...n individuals per age and Y_{ij} = hormone level. Significant age differences were then examined utilizing Duncan's multiple range test.

Sire family contribution to within line variations for each hormone at each age were assessed using a nested analysis of variance. The model utilized for this analysis was:

$$(3) \qquad Y_{ijk} = \mu + L_i + S(L)_{ij} + e_{ijk}$$

where i = 1,2 lines (L); j = 1,2...n sires within a line (S(L)); k = 1,2...n progeny per sire and Y_{ijk} = hormone level.

Pearson product moment correlation analysis of mean hormone values was performed for each age.

The mean values obtained for packed cell volume and body weight for each line were compared utilizing analyses of variance per equation (1).

RESULTS

Absolute Hormone Levels and Temporal Relationships

Mean plasma concentrations of androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgen (Total A), and estradiol-17β (E_2) are presented by lines in Table 8. No significant differences were found between the lines for any hormone at any age up through sexual maturity. The coefficients of variation for these values were quite high (Table 9), ranging from 23.9% to 188.1%, indicating a great deal of within line variability.

Mean plasma levels of AE were low in both of the selected lines on Day 1 (.091 and .068 ng/ml for the HML and LML, respectively). The levels increased considerably by Day 56 (.498 and .409 ng/ml for the HML and LML, respectively) and were unchanged at Day 112 (.483 and .461 ng/ml for HML and LML, respectively). Further increases occurred by Day 168 (.967 ng/ml for the HML and .908 ng/ml for the LML). Values for days 56 and 112 were not significantly different from one another, but were different ($P \leq .01$) from Day 1 and Day 168. Days 1 and 168 were significantly different ($P \leq .01$) from one another.

Mean plasma levels of DHT remained low through Day 112 with values ranging from .058 to .082 ng/ml for the HML and from .063 to .094 ng/ml for the LML. Values for these ages were not significantly different. By Day 168, mean plasma levels of DHT increased to .159 ng/ml in the HML and .146 ng/ml in the LML. These values were significantly different ($P \leq .01$ for HML and $P \leq .05$ for LML) from all other ages.

Table 8. Plasma concentration of androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgen (Total A) and estradiol-17β (E_2) in White Rock, high and low mating line roosters at various ages.

Hormone	Age (days)	High mating line Plasma Conc. (ng/ml)[1]	n	Low mating line Plasma Conc. (ng/ml)[1]	n
AE	1	.091 ± .017^{a**}	16	.068 ± .008^{a**}	17
	56	.498 ± .089^{b}	20	.409 ± .074^{b}	20
	112	.483 ± .044^{b}	20	.461 ± .060^{b}	20
	168	.967 ± .129^{c}	20	.908 ± .076^{c}	20
DHT	1	.072 ± .009^{a**}	9	.094 ± .094^{a*}	17
	56	.082 ± .014^{a}	16	.063 ± .010^{a}	10
	112	.058 ± .010^{a}	13	.074 ± .008^{a}	12
	168	.159 ± .012^{b}	15	.146 ± .017^{b}	15
T	1	.160 ± .084^{a**}	13	.084 ± .013^{a**}	16
	56	.455 ± .097^{ab}	20	.584 ± .131^{b}	20
	112	.578 ± .085^{b}	20	.518 ± .075^{b}	20
	168	1.119 ± .138^{c}	20	.980 ± .142^{c}	20
Total A	1	.159 ± .019^{a**}	4	.231 ± .027^{a**}	11
	56	1.157 ± .212^{a}	16	1.164 ± .236^{b}	10
	112	1.176 ± .162^{a}	13	1.023 ± .174^{b}	12
	168	2.107 ± .325^{b}	15	1.927 ± .268^{c}	15
E_2	1	.030 ± .009^{a}	6	.037 ± .007^{a}	9
	56	.055 ± .013^{a}	8	.096 ± .044^{a}	8
	112	.086 ± .047^{a}	7	.034 ± .023^{a}	4
	168	.081 ± .026^{a}	14	.134 ± .044^{a}	14

[1]Mean ± standard error

Differences between lines within an age were not significant ($P \geq .05$).

Means within a line and a hormone with different superscripts are significant (*$P \leq .05$;**$P \leq .01$).

Table 9. Coefficients of variation for androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgen (Total A) and estradiol-17β (E_2) values for high and low mating line White Rock roosters at various ages.

Hormone	Age (days)	High mating line C.V.(%)	n	Low mating line C.V.(%)	n
AE	1	76.9	16	48.5	17
	56	80.0	20	80.4	20
	112	40.8	20	57.9	20
	168	59.6	20	37.4	20
DHT	1	38.9	9	116.0	17
	56	69.5	16	49.2	10
	112	67.2	13	39.2	12
	168	29.6	15	46.6	15
T	1	188.1	13	64.3	16
	56	95.4	20	100.3	20
	112	65.6	20	65.1	20
	168	55.2	20	64.8	20
Total A	1	23.9	4	38.5	11
	56	73.2	16	64.3	10
	112	49.6	13	58.9	12
	168	59.8	15	53.9	15
E_2	1	77.9	6	59.2	9
	56	66.5	8	128.9	8
	112	145.1	7	134.1	4
	168	120.1	14	122.6	14

Plasma T concentrations followed the same pattern as that exhibited by AE. On Day 1, the T values were low (.160 ng/ml for the HML and .084 ng/ml for the LML). Concentrations were similar between Days 56 and 112 being .455 and .578 ng/ml for Days 56 and 112, respectively in the HML, and .584 and .518 ng/ml for Days 56 and 112, respectively in the LML. As with AE, plasma T concentrations increased with the advent of sexual maturity. Day 168 concentrations were 1.119 and .980 ng/ml for the HML and LML, respectively. Significant differences ($P \leq .01$) between the ages in levels of T were similar to that observed with AE, the only difference being that for the HML, the level at Day 56 was not found to differ significantly from Day 1.

Total plasma androgen (AE + DHT + T) concentrations in the HML, did not differ significantly through 112 days of age. Values ranged from .159 ng/ml on Day 1 to 1.176 ng/ml on Day 112. The level rose significantly ($P \leq .01$) to 2.107 ng/ml by Day 168.

In the LML, plasma androgen was low at Day 1 (.231 ng/ml), increased by Day 56 (1.64 ng/ml), and remained unchanged on Day 112 (1.023 ng/ml). It peaked on Day 168 (1.927 ng/ml). Values for Days 56 and 112 were not different from one another, but were significantly different ($P \leq .01$) from Day 1 and Day 168. Values at Day 1 and Day 168 also differed significantly ($P \leq .01$) from one another.

Estradiol concentration remained stable throughout development. Levels ranged from .030 to .086 ng/ml in the HML and .034 to .134 ng/ml in the LML. None of the values differed significantly.

Correlations Between Hormones

On Day 1, no positive correlations were observed in the HML. In

the LML, Total A was found to be positively correlated ($P \leq .05$) with DHT (Table 10).

On Day 56 in the HML, both AE and T were positively correlated ($P \leq .01$) with Total A and were also positively correlated ($P \leq .01$) with each other. In the LML, T was found to be positively correlated ($P \leq .01$) with Total A (Table 11).

On Day 112, all androgenic hormones were positively correlated ($P \leq .01$) with one another in the HML. In the LML, AE and T were found to be positively correlated ($P \leq .05$) with one another (Table 12).

On Day 168, in both lines, AE and T were positively correlated ($P \leq .01$) with Total A and also with each other. Also, DHT was found to be positively correlated ($P \leq .05$) with T in the HML (Table 13).

None of the correlations between E_2 and the androgens were significant in either line.

Sire Family Contribution to Within Line Variation

The percentage of variation attributable to sire was determined (Table 14). Values ranged from zero to 17.43% for AE, zero to 55.15% for DHT, zero to 14.02% for T, zero to 50.56% for Total A and zero to 68.16% for E_2.

Packed Cell Volume and Body Weight

Mean values for packed cell volume (PCV) and body weights are represented in Table 15. Significant differences ($P \leq .01$) were found between the lines for both characteristics; the HML exhibited a greater mean PCV and a lesser mean body weight as compared to the LML.

Table 10. Correlation between plasma androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgens (Total A) and estradiol-17β (E_2) in White Rock, high mating line (HML) and low mating line (LML) roosters at 1 day of age.

		HML				
		AE	DHT	T	Total A	E_2
LML	AE		.12 n=6	-.22 n=11	.79 n=4	.45 n=5
	DHT	-.06 n=14		.41 n=6	.54 n=4	.76 n=4
	T	-.03 n=14	-.23 n=13		-.18 n=4	.80 n=4
	Total A	-.04 n=11	.72* n=11	.41 n=11		.00 n=1
	E_2	.69 n=6	.50 n=8	-.51 n=5	-1.0 n=2	

*$P \leq .05$

Table 11. Correlation between plasma androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgens (Total A) and estradiol-17β (E_2) in White Rock, high mating line (HML) and low mating line (LML) roosters at 56 days of age.

		HML				
		AE	DHT	T	Total A	E_2
LML	AE		-.18 n=16	.86** n=20	.96** n=16	.00 n=8
	DHT	.03 n=10		-.16 n=16	-.11 n=16	-.92 n=4
	T	-.09 n=20	.03 n=10		.96** n=16	.03 n=8
	Total A	.12 n=10	.07 n=10	.97** n=10		-.16 n=4
	E_2	-.08 n=8	.35 n=4	-.26 n=8	-.48 n=4	

**$P \leq .01$

Table 12. Correlation between plasma androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgens (Total A) and estradiol-17β (E_2) in White Rock, high mating line (HML) and low mating line (LML) roosters at 112 days of age.

		HML				
		AE	DHT	T	Total A	E_2
LML	AE		.71** n=13	.82** n=20	.91** n=13	.57 n=7
	DHT	.01 n=12		.79** n=13	.82** n=13	-.11 n=6
	T	.46* n=20	.40 n=12		.98** n=13	.21 n=6
	Total A	.89** n=12	.27 n=12	.90** n=12		.34 n=6
	E_2	-.89 n=4	.00 n=1	-.53 n=4	.00 n=1	

*$P \leq .05$
**$P \leq .01$

Table 13. Correlation between plasma androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgens (Total A) and estradiol-17β (E_2) in White Rock, high mating line (HML) and low mating line (LML) roosters at 168 days of age.

		HML				
		AE	DHT	T	Total A	E_2
LML	AE		.22 n=15	.70** n=20	.95** n=14	-.32 n=14
	DHT	-.19 n=15		.56* n=15	.44 n=15	.23 n=9
	T	.74** n=20	.08 n=15		.97** n=15	-.36 n=14
	Total A	.88** n=15	.05 n=15	.97** n=15		-.31 n=9
	E_2	.08 n=14	.43 n=11	.14 n=14	.00 n=11	

*P<.05
**P<.01

Table 14. Sire family contribution to within line variations in plasma levels of androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgen (Total A) and estradiol-17β (E_2) in high and low mating line White Rock roosters at various ages.

Hormone	Age (days)	High mating line Sire family % variation	n	Low mating line Sire family % variation	n
AE	1	0	16	0	17
	56	14.57	20	0	20
	112	17.43	20	9.04	20
	168	0	20	0	20
DHT	1	0	9	0	17
	56	.05	16	0	10
	112	4.38	13	22.05	12
	168	37.48	15	55.95	15
T	1	3.72	13	0	16
	56	14.02	20	0	20
	112	13.31	20	0	20
	168	0	20	0	20
Total A	1	-	4	50.56	11
	56	8.72	16	0	10
	112	5.59	13	0	12
	168	0	15	0	15
E_2	1	0	6	0	9
	56	0	8	68.16	8
	112	0	7	0	4
	168	0	14	40.68	14

Table 15. Means and standard errors of packed cell volumes and body weights for high and low mating line White Rock roosters at 182 days of age.

Line	Packed cell Volume (%)	Body Weight (kg)
High Mating Line (n)	45.09 ± .61[a] (20)	2.31 ± .04[a] (20)
Low Mating Line (n)	41.82 ± 1.00[b] (20)	2.56 ± .04[b] (19)

Means with different superscripts within a column are different ($P \leq .01$).

DISCUSSION

Comparison of Absolute Hormone Levels to Those in the Literature

The high coefficients of variation in hormone levels observed within the mating lines is in agreement with the works of Benoff et al. (1978a) and Bernon (1981) who both reported wide variations in plasma T levels in the selected mating lines of chickens. A similar situation has been observed in genetic strains of rodents (Bartke et al., 1973; Batty, 1978b). High coefficients of variation can also occur within individual animals. This was demonstrated by Katongole et al. (1974) and Bartke and Dalterio (1975) with rams and mice, respectively. Therefore, because a high degree of variation was expected, relatively large sample sizes from the selected genetic populations were used in the current experiments.

Of the hormones considered during this study, circulating T levels have received the most attention from avian endocrinologists. To aid in placing the current data into perspective, Mashaly and Glick (1979) found serum T levels in New Hampshire roosters to be .221 $\pm$ .083 ng/ml on day 112 and 1.936 $\pm$ .262 ng/ml on day 168 of age. Tanabe et al. (1979) reported T levels for newly hatched White Leghorn roosters to be .293 $\pm$ .048 ng/ml. Plasma testosterone levels for earlier generations of the HML and LML have also been determined. Benoff et al. (1978a) found levels for HML and LML males 315 days old to be 7.3 $\pm$ 1.0 ng/ml and 4.5 $\pm$ 0.6 ng/ml, respectively. Bernon (1981) reported levels of 2.5 $\pm$ 0.7 ng/ml and 2.5 $\pm$ 1.1 ng/ml for the HML and LML, respectively at 238 days of age.

The values reported by Mashaly and Glick (1979) are comparable to those obtained as a result of the current research. The remaining investigators cited, however, have reported values of a greater magnitude. Nevertheless, it is important to note that the time of day when samples are collected can influence the results obtained. A definite rhythm in hormonal concentration has been observed in the domestic fowl (Schanbacher, et al., 1974) and the Japanese quail (Ottinger and Brinkley, 1979) with higher T levels occurring during the early morning and late evening. The time of day blood samples were collected by these earlier researchers (Benoff et al., 1978a; Tanabe et al., 1979; Bernon, 1981) is unknown. Furthermore, Benoff et al. (1978a) and Bernon (1981) collected samples from older animals. For these reasons, comparison of absolute values is risky.

Serum DHT levels were measured in New Hampshire roosters by Mashaly and Glick (1979). The values obtained for Day 112 (.059 $\pm$ .013 ng/ml) agreed closely with our values, but the Day 168 values (.075 $\pm$ .013 ng/ml) were somewhat lower than those resulting from the current research. Although the cause for this discrepancy is unknown, the effects of hormonal fluctuation and breed differences may be of importance.

Androstenedione and consequently Total A have not been reported previously in the literature for the rooster.

Concerning E_2 levels, it should be noted that the values reported here may not be representative of the levels present in the birds. Although preliminary validation procedures indicated the assay method to be acceptable (Tables 6 and 7), the within and between assay

coefficients of variation (Table 1) were of a questionable magnitude. Irregardless, the work of Tanabe et al. (1979) is presented for comparison. They reported E_2 levels in White Leghorn roosters from hatch through adulthood as being less than 7.5 pg/ml, values considerably less than those obtained as a result of this study. Again, while sampling time may be of importance, assay procedural differences may be the primary reason for the discrepancy. It is unlikely that E_2 values for these lines would be as low as suggested by the data of Tanabe et al. (1979). The values obtained as a result of the current research are consistent with values obtained during validation procedures utilizing a pooled plasma sample obtained from these genetic lines. The latter value was found to be 227 pg/ml.

Temporal Relationships

Temporal hormone profiles were relatively consistent between the lines with greater levels of hormone being present in mature animals. Estradiol is the exception to this pattern as E_2 levels were not found to change significantly with age.

The apparent differences observed in temporal patterns of Total A between the mating lines may be misleading. This variation might be attributed to the small sample size present in the HML for Day 1; the patterns of androgen concentration were actually similar between the lines.

Correlations Between Hormones

One of the primary reasons for performing correlation analyses was to assess the interrelationship of E_2 with the androgens. No significant results were obtained in this respect, however. Nevertheless,

it is possible that low sample sizes and high variability between assay values for E_2 limited the sensitivity of the statistical analysis.

Striking differences were found between the lines in other respects. A higher incidence of significant correlations were discovered in the HML. It is suggested by these correlation analyses that the hormonal profile of the HML is relatively more orderly and interrelated while the LML has a less uniform hormonal state. Although evidence is currently undefined, and no outside data exists to offer support to this concept, it is interesting to speculate that an orderly versus an aberrant hormonal condition may in some way be affecting the degree of sexual behavior exhibited.

Sire Family Contribution to Within Line Variation

A greater percentage of variation in AE and T was sire related in the HML. With respect to DHT, sire family variation was greater among the LML. Sire family variation for Total A in the LML was high at Day 1, but a value for the HML for comparative purposes was lacking. The LML showed a great deal of sire related variability for E_2; this result is questionable, however, due to the uncertainty of the E_2 assay.

Packed Cell Volume and Body Weight

The highly significant line difference in packed cell volume and the values themselves are in agreement with previous studies. McCollum (1969) and Benoff (1977) reported higher values for the HML as compared to the LML. The difference between lines has

been attributed to the erythropoetic properties of androgen by Benoff (1977) who found higher T levels in the HML. The correlation between the two factors, however, was not significant. The difference in packed cell volume obtained as a result of the current research is obviously not due to variations in T levels, for no significant differences were found between the lines in this respect. A variety of factors including age, exercise, excitement, time of day, temperature and altitude can affect erythrocyte numbers. Therefore, the differences between the lines must be left open to speculation at present.

The significant weight differences observed between the mating lines are in accordance with other reports. Both Benoff (1977) and Bernon (1981) observed greater body weights among the LML as compared to the HML.

Baseline Hormone Levels

A great deal of effort has been exerted by researchers attempting to link hormonal control with the degree of sexuality exhibited by an individual. For obvious reasons, the gonadal hormones, particularly basal levels, have received much attention. With the exception of one study (Benoff et al., 1978a), baseline hormone levels, whether natural or induced, have not been implicated in the control of the degree of sexual behavior. It has been shown repeatedly that sexual activity of mammals cannot be raised above precastrate levels, regardless of the dosage of androgen administered (Grunt and Young, 1952; Riss and Young, 1954; Champlin et al., 1963; Larsson, 1966; Damassa et al., 1977; Dalterio et al., 1979; and Gray et al., 1981). Similar results have been obtained utilizing genetically selected mating lines

of quail (Cunningham et al., 1977) and chickens (McCollum et al., 1971; Van Krey et al., 1977). Furthermore, two researchers have assessed basal T levels in roosters of these genetically selected lines. Benoff et al. (1978a) reported a positive correlation between circulating T titers and the number of completed matings, but these results were not confirmed by Bernon (1981). This latter study found no difference in T levels between roosters of these genetic mating lines. Regardless of this contraindication, the remainder of the existing literature supports the findings of the present study in that no significant differences were found between the lines in circulating T levels.

Androstenedione, DHT and E_2 have not been considered with respect to regulation of intensity of sexual behavior. Therefore, baseline levels of these hormones in animals differing in sexual behavior were previously unknown. It was found through the efforts of this research that the levels of AE, DHT and E_2 did not differ between the mating lines.

It is noteworthy concerning quantification of DHT, that primarily the 5α form was measured. This factor is of importance considering the evidence that has been presented concerning T metabolism in avian species. Several studies have reported a predominance of T conversion to the 5β reduced form in brain tissue of several avian species (Massa and Sharp, 1981; Massa et al., 1977; Balthazart and Hirschburg, 1979; Balthazart et al., 1979; Nakamura and Tanabe, 1974; Steimer and Hutchinson, 1980; 1981). The primary peripheral conversion pathway is unknown, thereby making it difficult to ascertain whether

or not this factor is of importance when assaying circulating levels of DHT.

With the assumption that basal hormone levels are not responsible for regulation of the degree of sexual behavior, earlier researchers proposed alternative theories to explain the line differences in sexual behavior; these were discussed previously in the review of the literature. One additional theory which has yet to be given consideration takes into account the interrelationship of environmental stimuli, behavioral actions and hormonal fluctuations (Leshner, 1979; Harding, 1981). This phenomenon has been observed in a variety of animal species (see literature review for references).

It has been shown that high activity males possess greater T levels following a sexual encounter than do low activity males. These high activity males also display increased T levels upon exposure to an estrous female (Harding and Feder, 1976). In addition, it has been illustrated that vigorously mating males have lower basal hormone levels than less active males, but show rapid increases in T levels upon exposure to a receptive female (Batty, 1978a; Damassa et al., 1977). Also noteworthy is the fact that behaviors leading up to the sexual behavior, rather than copulation itself, are correlated with increasing T levels.

Although the studies cited indicate that increases in baseline T levels are important for increased sexual activity, it may be premature to make such a generalization. The reaction rate of gonadal hormones in this type of situation is unknown and, therefore, should be determined experimentally.

In conjunction with studies linking mating behavior with gonadal

hormones, similar studies have gone a step further to link mating behavior with pituitary and hypothalamic secretions. In rodents, it has been determined that luteinizing hormone (LH) levels rise during the initiation stages of behavior (Kamel et al., 1977; Coquelin and Bronson, 1979). Furthermore, LH releasing hormone (LHRH) given in addition to gonadal hormones is capable of facilitating male sexual behavior (Moss et al., 1975).

When these concepts are coupled with the finding of Balander (1977) that LML roosters exhibit increased copulatory behavior when tested with restrained females, an attractive hypothesis evolves. Perhaps genetic selection is affecting the endocrine system at the level of the hormonal responsiveness to behavioral and environmental stimuli. Without this initial surge of hormone, preliminary sexual behaviors are not displayed, thus precluding copulatory behavior.

SUMMARY AND CONCLUSIONS

The primary concern of this research project was to assess the endocrine relationships of several gonadal steroids in genetic populations of roosters divergently selected for mating activity. A procedure combining column chromatography and radioimmunoassay procedures was developed to enable quantification of circulating levels of androstenedione, dihydrotestosterone, testosterone, total androgens (androstenedione + dihydrotestosterone + testosterone) and estradiol-17β in roosters of high and low mating lines at various ages.

No consistent differences between lines or sire families within a line were found in basal hormone levels from hatch up through sexual maturity. These results indicated that baseline levels of hormones are not the primary factor regulating male libido, nor are temporal hormonal concentrations responsible for subsequent differences in mating activity. An alternative hypothesis was, therefore, presented in explanation of differences in sexual behavior between the mating lines. This method of control gives consideration to momentary hormone changes occurring in response to environmental stimuli or behavioral actions. Plausible hormonal constituents which could be implicated in this mechanism include gonadal as well as hypothalamic and pituitary secretions.

Correlation analyses between the hormonal traits yielded interesting results. A higher incidence of significant correlations were observed in the high mating line. This observation suggests that individuals of the high mating line possess a more orderly, interrelated

hormonal state throughout development. A uniform versus an aberrant hormonal state was, therefore, considered as a possible explanation for differences in libido.

Finally, packed cell volume and body weight were observed to differ significantly between the mating lines with the high mating line exhibiting a greater mean packed cell volume and a lesser mean body weight. These variations were expected and concur with former studies. The higher mean packed cell volume among the high mating line, however, could not be attributed to the erythropoetic capacity of androgen for the lines were not found to differ in this respect. Many factors have been observed to effect PCV and, therefore, the differences between the lines must be left open to speculation at present.

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APPENDIX A

Procedure for extraction and quantification of androstenedione, dihydrotestosterone, testosterone and estradiol-17β

PROCEDURE FOR EXTRACTION AND QUANTIFICATION OF ANDROSTENEDIONE, DIHYDROTESTOSTERONE, TESTOSTERONE AND ESTRADIOL-17β

I. Chemical Reagents

A. All organic solvents except spectro-analyzed chemicals were freshly distilled before use (shelf life is three days).

1. Anhydrous diethyl ether (Fisher Scientific)
2. Benzene (Eastman Kodak)
3. Ethanol (Mallinckrodt)
4. Ethylene glycol (Mallinckrodt)
5. Methanol (Fisher Scientific)
6. Methylene chloride (Axton-Cross)
7. 2,2,4-trimethyl pentane (iso-octane) (Fisher Scientific and Eastman Kodak)
8. Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) (Fisher Scientific)
9. Sodium phosphate dibasic ($Na_2HPO_4 \cdot 12H_2O$) (Fisher Scientific)
10. Sodium phosphate dibasic, anhydrous (Na_2HPO_4) (Sigma)
11. Sodium azide (Fisher Scientific)
12. Sodium chloride (Fisher Scientific)
13. Norit A Charcoal (Sigma)
14. Dextran (T70 or T80) (Sigma)
15. Preblend 2a70 (scintillation grade) (Research Products International)
16. Triton X-100 (Research Products International)
17. Ethylmercurithiosalicyclic acid sodium salt (Merthiolate sodium) (Fisher Scientific)
18. Ethylene dinitrilo acetic acid disodium salt (EDTA) (Sigma)

II. Extraction of samples

A. General

1. Rinse extraction tubes (16 X 125 mm screw top test tubes) with methylene chloride:methanol (9:1, v/v). Invert to dry.

2. Rinse a set of collecting tubes as described above for androgens (18 X 155 mm test tubes) and a set for estrogens (15 X 85 mm disposable culture tubes).

3. Add 10 µl (~1200 cpm) of the following steroids (New England Nuclear) to each extraction tube:

   1,2-^{3}H(N)-androst-4-ene-3,17-dione
   1,2,4,5,6,7-^{3}H(N)-dihydrotestosterone
   1,2-^{3}H(N)-testosterone
   2,4,6,7-^{3}H(N)-estradiol

4. Prepare three total count (TCT) vials for each of the steroids listed above to determine procedural losses. Air dry.

5. Add plasma samples (1.0 ml) to extraction tubes. Incubate in 45°C water bath for 15 minutes to allow for equilibration.

B. Androgens

1. Add 5.0 ml iso-octane to each plasma sample.

   a) Shake for 1 minute.

   b) Freeze at -75°C for 7 minutes.

   c) Decant iso-octane into collecting tube.

   d) Thaw plasma samples and repeat extraction procedure two additional times.

   e) Combine all supernates and evaporate to dryness in a 45°C water bath under air. This extract contains androstenedione, dihydrotestosterone and testosterone.

   f) Store at 4°C until column separation is performed.

C. Estrogens

1. All ether extractions must be performed under the hood.

2. Add 5.0 ml fresh anhydrous diethyl ether to each plasma sample previously extracted for androgens.

   a) Shake for 1 minute.

   b) Freeze at -75°C for 7 minutes.

c) Decant ether into a collecting tube.

d) Thaw plasma samples and repeat extraction procedures two additional times.

e) Combine all supernates and evaporate to dryness in a 45°C water bath under air.

f) After drying, rinse each tube with 1.0 ml of ether to concentrate the hormone extract in the bottom of the tube. Evaporate to dryness as above.

g) Store dried extract at 4°C until column chromatography is performed.

III. Column chromatography of extract

A. Androgens

1. Celite column preparation

a) Heat celite analytical filter aid (C-211, by Fisher Scientific) in oven at 538°C for 12 hours to burn off impurities. Keep celite dry and free of contaminants until used.

b) Mix celite with ethylene glycol in a ratio of 2:1 (.88 g to with .44 ml for each column).

c) Place a glass bead in the bottom of a 5.0 ml pyrex disposable serological pipette (Scientific Products).

d) Pack small quantities of the celite mixture into pipette approximately .5 ml at a time using a plastic rod. Pack to 3.0 ml mark (68 mm packing height).

e) Flush column with 15.0 ml iso-octane before use.

2. Addition of sample to column

a) Add 1.0 ml phosphate buffered saline with .1% gelatin (PBS-ga) (appendix B) to sample and vortex 30 sec.

b) Add 1.0 ml iso-octane to tube, vortex 30 sec and freeze at -75°C for 7 minutes.

c) Transfer supernate to top of column using a Pasteur pipette.

3. Chromatographic separation

a) Elution solvents

(1) Solution A. . . . . .iso-octane

(2) Solution B. . . . . .iso-octane:benzene (7:3, v/v)

b) Elution procedure

1.0 ml sample	Discard
3.5 ml solution A	ANDROSTENEDIONE
.5 ml solution A	Discard
3.5 ml solution A	DIHYDROTESTOSTERONE
1.5 ml solution A	Discard
4.0 ml solution B	TESTOSTERONE

c) Columns are run under a positive air pressure adjusted such that a flow rate of 10 drops/minute is achieved.

d) Fractions are collected into separate disposable culture tubes (15 X 85 mm).

e) Columns must be rinsed with two bed volumnes of iso-octane between samples.

f) Evaporate elution fraction to dryness under air in 45°C water bath and store at 4°C until assayed.

g) When not in use, columns should be filled with iso-octane and stoppered on both ends.

B. Estrogens

1. Sephadex column preparation

a) Soak Sephadex LH-20 (Sigma Chemical Corp.) overnight in a solution of spectroanalyzed benzene:methanol (9:1, v/v).

b) Place a filter disc at bottom of 3.0 ml glass tuberculin syringe. Add 1.0 ml solvent.

c) Using a pasteur pipette transfer the LH-20 mixture to the syringe allowing for drainage of solvent. Continue this procedure until sephadex level has reached 2.6 ml mark on syringe (42 mm packing height).

d) Place filter disc on top of sephadex and wash column with 8.0 ml elution solvent (benzene:methanol; 9:1, v/v.

2. Dried collection fraction is transferred to column with a Pasteur pipette by rinsing once with .3 ml of elution solvent.

3. Chromatographic separation

a) Elution procedure

.3 ml Sample	Discard
1.2 ml Elution Solvent	Discard
.5 ml Elution Solvent	Discard (contains fat)
2.0 ml Elution Solvent	Discard (estrone)
.5 ml Elution Solvent	Discard
3.5 ml Elution Solvent	ESTRADIOL-17β

b) Columns are free flowing.

c) Fractions are collected into separate disposable culture tubes (10 X 75 mm).

d) Columns must be rinsed with three bed volumes of elution solvent between samples.

e) Evaporate collection fraction to dryness under air in 45°C water bath and store at 4°C until assayed.

f) When not in use, columns should be filled with elution solvent and stoppered at both ends.

IV. Sample preparation and assay procedure

A. Androgens

1. Sample preparation

a) Redissolve samples with 2.0 ml methylene chloride: methanol (9:1, v/v), vortex, and pipette a known amount for assay into each of two culture tubes (10 X 75 mm) and .2 ml into a mini-vial for determination of procedural losses.

b) Allow recovery vials to air dry, and add 5.0 ml recovery scintillation cocktail (appendix B) to each of the TCT vials prepared in step II.A.4. and to three background (BKG) vials.

c) Dry down culture tubes with air in 45°C water bath and store at 4°C until assay is performed.

2. Assay procedure

a) Androstenedione

(1) Prepare one set of standards (Sigma Chemical Co.) made from an ethanol solution containing 1 ng/ml for each set of 38 assay tubes: 0, .025, .05, .10, .25, .50, 1.0 ng. Place one blank tube at the beginning (0-0 tube) of the assay and one at the end of the assay tubes. Evaporate in 45°C water bath under air.

(2) Prepare a 1:1000 dilution of androstenedione antibody made up with PBS-ga. The antibody was obtained from Optimox, Inc. batch #OP.09. Add .1 ml to each tube, vortex and incubate at room temperature for 15 minutes.

(3) Following incubation, add .1 ml of a PBS-ga solution containing 1,2,6,7-^{3}H(N)-androst-4-ene-3,17-dione (~5.0 X 10^{4} dpm) to each tube and vortex. Incubate 14 to 16 hours at 4°C.

b) Dihydrotestosterone

(1) Follow the above procedure using the following levels for the standard curve: 9, .005, .01, .02, .03, .05, .08, .10, .25, .50, 1.0 ng.

(2) Prepare a 1:2500 dilution of dihydrotestosterone antibody made up with PBS-ga. The antibody was obtained from Optimox, Inc., batch #OP/08A. Add .1 ml to each tube. Vortexing is unnecessary.

(3) Following addition of antibody, add .1 ml of PBS-ga solution containing 1,2,4,5,6,7-^{3}H(N)-dihydrotestosterone (~2.0 X 10^{4} dpm) to each tube and vortex. Incubate for 14 to 16 hours at 4°C.

c) Testosterone

(1) Follow above procedure using the following levels for the standard curve: 0, .005, .01, .03, .05, .10, .25, .50, 1.0 ng.

(2) Prepare a 1:50,000 dilution of testosterong antibody made up with PBS-ga. The antibody was obtained from Dr. James Ireland, Michigan State University, batch MSU #74. Add .2 ml to each tube, vortex and incubate at room temperature for 15 minutes.

(3) Following incubation, add .1 ml of a PBS-ga solution containing 1,2-^{3}H(N)-testosterone (~5.0 X 10^{4}) to each tube and vortex. Incubate 14 to 16 hours at 4°C.

3. Assay

a) Androstenedione and testosterone

(1) Add 1.0 ml deionized water to first blank tube and 1.0 ml cold dextran coated charcoal solution (appendix B) to other tubes and vortex.

(2) Incubate 10 minutes at 4°C and centrifuge at 3000 RPM (1732 X g) for 10 minutes at 4°C.

(3) Pipette .5 ml supernate into mini-vial and add 5.0 ml assay cocktail (appendix B).

(4) Vials are stored at 4°C for 24 hours and then counted on a Searle Beta Liquid Scintillation Counter.

b) Dihydrotestosterone

(1) Add .4 ml deionized water to first blank tube and .4 ml cold dextran coated charcoal solution (appendix B) to other tubes and vortex.

(2) Incubate for 10 to 15 minutes at 4°C and centrifuge at 2300 RPM (1018 X g) for 15 minutes at 4°C.

(3) Pipette .4 ml supernate into mini-vial and add 5.0 ml assay cocktail.

(4) Vials are stored at 4°C for 24 hours and then counted on a Searle Beta Liquid Scintillation Counter.

B. Estrogens

1. Sample preparation

a) Add .6 ml PBS with merthiolate containing .1% gelatin (PBS-mer-ga) (appendix B) to each sample and incubate 15 minutes in 45°C water bath, vortex briefly and store overnight at 4°C.

b) Following incubation, vortex shortly and transfer known aliquots into disposable culture tubes (10 X

75 mm) for assay and .1 ml into a mini-vial for determination of procedural losses. Eppendorf pipettes and disposable tips are used for removal of aliquots. Add PBS-mer-ga to culture tubes to bring volume up to .2 ml if necessary.

c) Add 5.0 ml recovery scintillation cocktail to each recovery vial and to the TCT vials prepared in step II. A.4.and to three BKG vials. Also add .1 ml PBS-mer-ga to the TCT and BKG vials. Vials are then counted in a liquid scintillation counter.

d) Store culture tubes at 4°C until assayed.

2. Preparation of standard curves

a) Prepare 4 curves using a PBS-mer-ga solution containing 1 pg/ml: 0, 1, 3, 5, 10, 20, 50, 100 pg.

b) Prepare the following additional tubes for assay:

	Solutions added			
Tubes	Buffer	First Antibody	Second Antibody	Iodinated Estrogen
No first antibody	+	-	+	+
No second antibody	+	+	-	+
TCT	-	-	-	+
BKG	+	+	+	-
H^3 (contains ~1200 cpm 2,4,6,7-^{3}H(N)-estradiol)	+	+	+	+
Nonspecific binding (contains 10 ng estradiol)	+	+	+	+

Add .2 ml PBS-mer-ga to each tube (except TCT tubes) to equal volume of unknowns. Incubate 15 minutes in 45°C water bath and vortex.

3. Preparation of estradiol antibody

a) Estradiol antibody (#E17-94) was obtained from Endocrine Sciences, batch #427.

b) Prepare antibody by adding 3.0 ml of 1:400 normal rabbit serum with EDTA in PBS (NRS-EDTA-PBS) (appendix B) to 25 µl stock estradiol antibody (1:20) dilution.

c) Add .2 ml antibody to all assay tubes except No First Antibody and TCT tubes.

4. Preparation of iodinated estrogen

a) The iodinated estrogen was obtained from New England Nuclear.

b) Add 10,000 cpm (~13,888 dpm/100 µl) contained in .1 ml of PBS-mer-ga of iodinated estrogen to all assay tubes except BKG tubes.

5. Preparation of second antibody

a) The antibody was generated in sheep against rabbit gamma globulin (ARGG) and was diluted in PBS-mer-ga. The concentration of second antibody used was determined according to the dilution that yielded the optimum precipitation of bound hormone.

b) Add .2 ml to all assay tubes excluding the TCT and No Second Antibody Tubes. Vortex and allow to incubate overnight at room temperature.

6. Assay

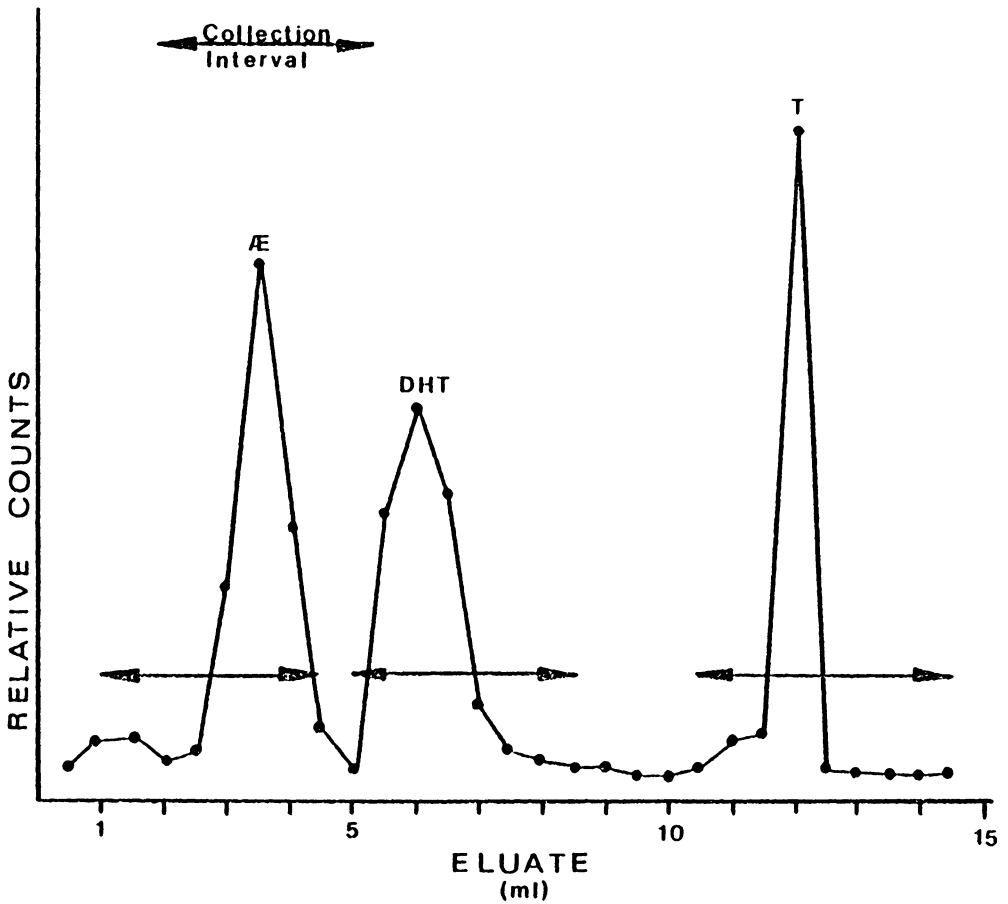
a) Add 1.0 ml cold PBS-mer-ga to all tubes excluding TCT tubes.

b) Centrifuge for 30 minutes at 2500 RPM (1203 X g).

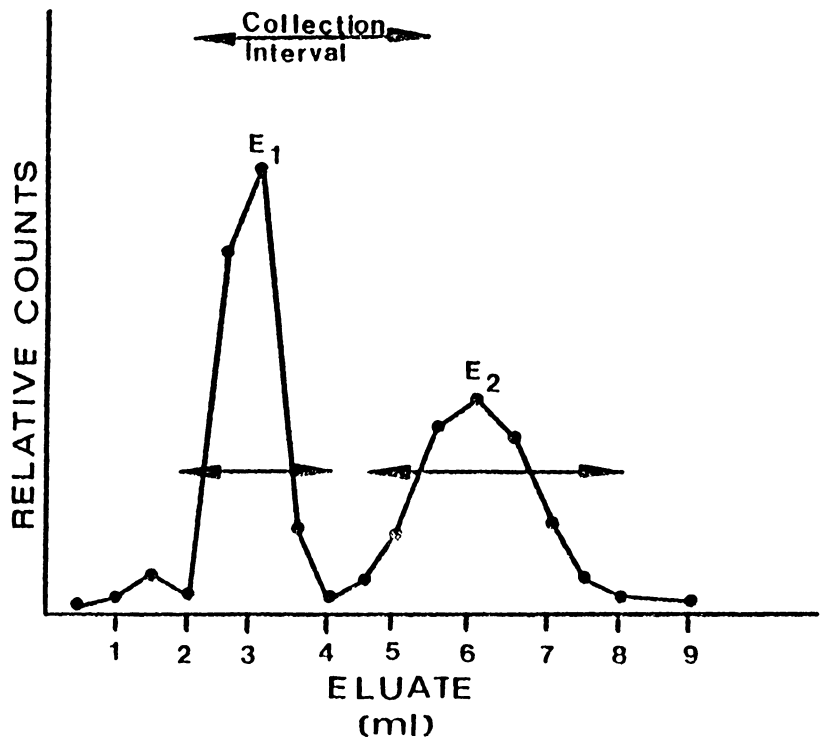
c) Decant supernate and wipe out tubes (except TCT tubes) with a disposable tissue.

d) Tubes are then counted utilizing a Beckman Gamma 4000 Counter.

Appendix Figure 1. Elution profile for androstenedione (AE), dihydrotestosterone (DHT) and testosterone (T)



Appendix Figure 2. Elution profile for estrone (E_1) and estradiol (E_2)



APPENDIX B

Protocol solutions

PHOSPHATE BUFFERED SALINE .1% GELATIN SOLUTION (PBS-ga)

Solution A (0.2M):

$NaH_2PO_4 \cdot H_2O$	27.6 g
Distilled H_2O	1000 ml

Solution B (0.2M):

$NaHPO_4 \cdot 12\ H_2O$	71.64 g
[or Na_2HPO_4 (anhydrous)]	28.4 g
Distilled H_2O	1000 ml

Phosphate buffer (PBS-a)

Sodium azide	1 g
Sodium chloride	9 g
Solution A	195 ml
Solution B	305 ml
Distilled H_2O	500 ml

Adjust pH to 7.0. The buffer may be kept at room temperature for up to one month.

Phosphate buffer with .1% gelatin (PBS-ga)

Gelatin (unflavored)	100 mg
PBS-a	100 ml

Mix on heated stir plate until gelatin goes into solution. Adjust pH to 7.0. Store at 4°C.

SCINTILLATION COCKTAILS

1. Recovery cocktail

Preblend 2a70	12 g
Toluene (purified grade). .	3000 ml

2. Assay cocktail

Recovery cocktail	1600 ml
Triton X-100.	400 ml

Note: Modifications in the preparation of the above have occurred since completion of laboratory work for this thesis.

DEXTRAN COATED CHARCOAL SOLUTIONS

1. Androstenedione and testosterone

Charcoal (Norit A).	.25 g
Dextran (T70 or T80). . . .	.025 g
PBS-ga.	100 ml

2. Dihydrotestosterone

Charcoal (Norit A).	.313 g
Dextran (T70 or T80). . . .	.031 g
PBS-ga.	100 ml

PHOSPHATE BUFFERED SALINE WITH MERTHIOLATE AND .1% GELATIN (PBS-mer-ga)

Phosphate buffer with merthiolate (PBS-mer) (.15M)

Sodium chloride	28.6 g
Ethylmercurithiosalicyclic acid sodium salt	.35 g
.5M Na_2PO_4	48.0 ml
.5M NaH_2PO_4	24.0 ml
Distilled H_2O	to 3540 ml

Adjust pH to 7.0

Phosphate buffer with merthiolate and .1% gelatin (PBS-mer-ga)

Gelatin (unflavored)	100 ml
PBS-mer	100 ml

Mix on heated stir plate until gelatin goes into solution. Adjust pH to 7.0. Store at 4°C.

PHOSPHATE BUFFERED SALINE WITH EDTA
(.05M EDTA-PBS)

[ethylenedinitrilo]tetraacetic acid, disodium salt	18.6 g
PBS-mer	to 1000 l

Warm to dissolve. Adjust pH to 7.0 with NaOH.

NORMAL RABBIT SERUM IN EDTA-PBS
(NRS-EDTA-PBS)

Normal rabbit serum.	150 µl
.05M EDTA-PBS.	60 ml

APPENDIX C

Analysis of variance tables

Appendix Table 1. Analysis of variance for plasma levels of androstenedione in high and low mating line roosters at various ages

Source of variation	df	Sum of squares	F-value
Day 1			
Line	1	.0043	1.49
Error	31	.0896	
Total	32	.0939	
Day 56			
Line	1	.0786	.59
Error	38	5.0927	
Total	39	5.1714	
Day 112			
Line	1	.0047	.08
Error	38	2.0919	
Total	39	2.0965	
Day 168			
Line	1	.0343	.15
Error	38	8.4938	
Total	39	8.5281	

F-values indicate no significant differences.

Appendix Table 2. Analysis of variance for plasma levels of 5α-dihydrotestosterone in high and low mating line roosters at various ages

Source of variation	df	Sum of squares	F-value
Day 1			
Line	1	.0028	.34
Error	24	.1959	
Total	25	.1987	
Day 56			
Line	1	.0022	.94
Error	24	.0573	
Total	25	.0595	
Day 112			
Line	1	.0015	1.29
Error	23	.0273	
Total	24	.0289	
Day 168			
Line	1	.0012	.35
Error	28	.0955	
Total	29	.0967	

F-values indicate no significant differences.

Appendix Table 3. Analysis of variance for plasma levels of testosterone in high and low mating line roosters at various ages

Source of variation	df	Sum of squares	F-value
Day 1			
Line	1	.0412	.98
Error	27	1.1321	
Total	28	1.1732	
Day 56			
Line	1	.1666	.63
Error	38	10.0949	
Total	39	10.2615	
Day 112			
Line	1	.0363	.28
Error	38	4.8866	
Total	39	4.9229	
Day 168			
Line	1	.1937	.49
Error	38	14.9110	
Total	39	15.1047	

F-values indicate no significant differences.

Appendix Table 4. Analysis of variance for plasma levels of total androgen in high and low mating line roosters at various ages

Source of variation	df	Sum of squares	F-value
Day 1			
Line	1	.0152	2.37
Error	13	.0835	
Total	14	.0987	
Day 56			
Line	1	.0003	0.00
Error	24	15.8409	
Total	25	15.8412	
Day 112			
Line	1	.1467	.42
Error	23	8.0516	
Total	24	8.1983	
Day 168			
Line	1	.2422	.18
Error	28	37.3174	
Total	29	37.5596	

F-values indicate no significant differences.

Appendix Table 5. Analysis of variance for plasma levels of estradiol-17β in high and low mating line roosters at various ages

Source of variation	df	Sum of squares	F-value
Day 1			
Line	1	.1705	.35
Error	13	6.3870	
Total	14	6.5575	
Day 56			
Line	1	6.6484	.80
Error	14	116.9657	
Total	15	123.6141	
Day 112			
Line	1	7.0055	.63
Error	9	100.2388	
Total	10	107.2443	
Day 168			
Line	1	19.8596	1.08
Error	26	476.3176	
Total	27	496.1772	

F-values indicate no significant differences.

Appendix Table 6. Analysis of variance for various plasma steroids in high mating line White Rock roosters

Source of variation	df	Sum of squares	F-value
Androstenedione			
Age	3	6.9676	16.48**
Error	72	10.1485	
Total	75	17.1161	
Dihydrotestosterone			
Age	3	.0858	13.43**
Error	49	.1043	
Total	52	.1901	
Testosterone			
Age	3	8.3122	13.06**
Error	69	14.6411	
Total	72	22.9533	
Total Androgen			
Age	3	15.2216	6.02**
Error	44	37.0850	
Total	47	52.3066	
Estradiol-17β			
Age	3	14.8276	.67
Error	31	230.1352	
Total	34	244.9628	

**$P \leq .01$

Appendix Table 7. Analysis of variance for various plasma steroids in low mating line White Rock roosters

Source of variation	df	Sum of squares	F-value
Androstenedione			
Age	3	6.6614	28.84**
Error	73	5.6195	
Total	76	12.2809	
Dihydrotestosterone			
Age	3	.05413	3.32*
Error	50	.2718	
Total	53	.3259	
Testosterone			
Age	3	7.1980	10.54**
Error	72	16.3835	
Total	75	23.5814	
Total Androgen			
Age	3	18.5411	11.23**
Error	44	24.2084	
Total	47	42.7496	
Estradiol-17β			
Age	3	66.0971	1.45
Error	31	469.7739	
Total	34	535.8710	

**$P \leq .01$

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TEMPORAL PROFILE OF GONADAL STEROIDS IN POPULATIONS OF ROOSTERS DIVERGENTLY SELECTED FOR MATING FREQUENCY

by

Toni Linn De Santo

(ABSTRACT)

This research was conducted to establish a temporal hormone profile in genetically selected populations of roosters differing in sexual activity. Plasma levels of androstenedione (AE), dihydrotestosterone (DHT), testosterone (T), total androgens (Total A) and estradiol-17β (E_2) were measured at 1, 56, 112 and 168 days of age by radioimmunoassay.

No significant differences in mean hormone values were found between the lines at any age. Temporal hormonal patterns for each hormone were also similar for the lines. The levels of AE, T and Total A were observed to increase from Day 1 to 56, stabilize at Day 112, and rise again prior to Day 168. Dihydrotestosterone levels were relatively low throughout development but did show increases by Day 168. Estradiol-17β concentration did not change significantly with age.

The percentage of variation attributable to sire family was determined. No consistent differences between sire families within a line were observed.

Within line correlation analyses were calculated between each of the hormonal traits. A higher incidence of significant correlations

was observed among the high mating line suggesting these animals to have a more uniform hormonal state throughout development as opposed to the low mating line.

Packed cell volume and body weight measurements were obtained at the termination of the experiment. Significant differences were found between the mating lines for both characteristics; the high mating line exhibited a greater mean packed cell volume and a lesser mean body weight.