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Plasma levels of luteinizing hormone and of five steroids in photostimulated, castrated and testosterone-treated male and female Japanese quail (Coturnix coturnix japonica)

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Abstract

Sensitive radioimmunassay techniques were developed and valiated to permit the assay in small volumes of quail plasma of five steroids including testosterone, 5 α -dihydrotestosterone, androstenedione, estradiol and progesterone. The characteristics of the assays are presented. They are then used in a study of plasma steroid levels in male and female Japanese quail following photostimulation, gonadectomy and testosterone therapy. The levels of all steroids are affected by the experimental treatments and some by the sex of the birds. Levels of androgens, estradiol and progesterone after gonadectomy and testosterone therapy are similar in males and females. The differential sensitivity of males and females to the activating effects of testosterone on cloacal gland growth and sexual behavior thus cannot be explained by a differential catabolism of the implanted hormone and must be researched at the level of the target organ for steroid action.

Introduction

The technique of gonadectomy and hormone replacement therapy is frequently used in endocrine studies and especially in studies on the hormonal controls of behavior. In these studies, it is frequently assumed that a gonadectomized animal is essentially hormone-free and that treatment with a given steroid (e.g. testosterone) in the form of daily injections or silastic implants restores the endocrine condition which was present before gonadectomy. This simplistic view however ignores a number of facts such as the possible adrenal contribution to the steroid environment and the fact that other hormones such as those produced by the pituitary gland are affected by gonadectomy and not necessarily restored to the level seen in intact animals following subsequent treatment with testosterone. These observations are of variable importance according to the specific aim of the experiment under progress but in any case, it is always important to know precisely what is the hormonal condition of the animals which are studied.

For the last 3-4 years, we have been engaged in a long-term project whose general aim is to uncover the molecular mechanisms underlying the activation by testosterone of copulatory behaviour in quail. Although many studies have been published in which plasma levels of hormones had been measured in quail (e.g. Doi, Takai, Nakamura and Tanabe, 1980; Balthazart, Schumacher and Ottinger, 1983; Ottinger and Brinkley, 1979), no systematic description of the levels of gonadal steroids in male and female quail following gonadectomy and testosterone replacement was available.

We recently developed and validated specific radioimmunoassays to measure testosterone (T), 5 α -dihydrotestosterone (5 α -DHT), androstenedione (A4), estradiol (E2) and progesterone (P) in quail. The present article describes with some detail these assay procedures and their application to the study of these steroids in the plasma of male and female quail following gonadectomy and testosterone treatment. The relationships of these hormone levels with plasma levels of luteinizing hormone (LH), with the area of the cloacal gland (an androgen-dependent structure; Sachs, 1967), with the cloacal diameter (which is estrogen-dependent; Noble, 1972; 1973) and with the copulatory behaviour is also examined.

Materials & Methods

BIRDS AND TREATMENTS : This experiment was carried out on 43 male and female Japanese quail (Coturnix coturnix japonica) bought from a local dealer at the age of 3 weeks. Until that time they had been raised in continous light. From their arrival in the laboratory till the end of the experiment, they were exposed to a photoperiod of 16 hours light and 8 hours dark with food and water always available ad libitum.

At the age of 4 weeks, all birds were gonadectomized or sham-operated under total anesthesia (Hypnodil, Janssens Pharmaceutica, Belgium; 15 mg/kg body weight; see Schumacher and Balthazart, 1984 for detail of methods).

Two weeks later, they were weighed, their cloacal gland area was measured with a calliper (greatest length multiplied by greatest width in mm 2 is referred to here as cloacal gland area) and they were randomly separated into 6 experimental groups defined as follows:

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-Control males (n=10) : Sham-gonadectomized birds (SHAM).
-Castrated males (n=7): gonadectomized males with no hormonal treatment (CX).
-Castrated and testosterone treated males (n=4): castrated birds which received 60 mm (3 X 20) silastic implants (Dow Corning silastic tubing 602-265; see Schumacher and Balthazart, 1984 for detail of methods) filled with crystalline testosterone (T males).
-Control females (n=9): sham-gonadectomized females (SHAM).
-Ovariectomized females (n=7): gonadectomized females receiving no hormonal treatment (OVEX).
-Ovariectomized and testosterone-treated females (n=6): Ovex females receiving 3 x 20 mm of silastic implants (see males T) filled with testosterone (T females).

The birds which were not treated with testosterone received 3 empty silastic implants as control.

The day when the birds received the silastic implants is considered in the following as day 0 and birds were then approximately 6 weeks old. From that time on, all birds were kept in isolation to prevent aggressive interactions.

On days 18 and 19, all birds, were tested for sexual behavior but putting them with a sexually mature female in a test arena for 5 min. The frequencies and latencies of the following behaviour patterns were recorded: neck grab (NG), mount attempt (MA) and cloacal contact movements (CCM; see Adkins and Adler, 1972 or Hutchison, 1978 for description of the behavior, Schumacher and Balthazart, 1984 for detail on the test procedure). Only the total frequency of NG and CCM during the two tests will be presented here; other measures lead to similar conclusions.

Body weight was measured on day 19 and cloacal gland area and cloacal diameter (an estrogen-dependent variable; see Noble, 1972, 1973) on day 18 after the behavioral tests.

On day 20, between 9.00 a.m. and 6.00 p.m. one blood sample (2 ml) was collected from the wing vein of each bird using heparinized syringes, Samples were then centrifuged and plasma stored at -20° C until assayed for hormone concentrations.

On days 21-22 all birds were sacrificed and completeness of gonadectomy as well as presence of the silastic implants were controlled. Birds with gonadal remnant or without implants were removed from the analysis and the number presented above reflects those birds which were retained. All sham males had at that time developed testes and all females but one had mature ovaries (largest follicle larger than 15 mm in diameter; egg laying had been recorded for at least one week).

HORMONE RADIOIMMUNOASSAYS : All samples were assayed for luteinizing hormone (LH) and for five steroid hormones including testosterone (T), 5 α -dihydrotestosterone (5 α -DHT), androstene-3, 17 dione (A4), estradiol (E2) and progesterone (P).

The LH assay has been described in detail by Follett, Scanes and Cunningham (1972), validated for quail plasma and used extensively during the last decade.

Steroids were assayed following extraction and purification/separation on celite columns by procedure that we have just set up and validated in our laboratory and which are inspired from those described in Abraham, Manlimos and Garza (1977) and Wingfield and Farner (1975). Parts of these validations have been previously published (Delville, Sulon and Balthazart, 1985; 1986).

Two separate aliquots of plasma (300 µl each) were used to assay P on one hand and T, 5 α -DHT, A4 and E2 on the other hand.

In a first step, steroids were extracted and separated from lipids on Extrelut microcolumns (Merck 11738) by diethylether.

P was then purified in one aliquot on celite columns as described by Abraham, Manlinos and Garza (1977, system II; celite:ethylene glycol columns were used and elution was obtained with a mixture of iso-octane:ethyl acetate, 95:5).

The other aliquot was deposited on a celite:ethylene glycol:propylene glycol column (Abraham, Manlimos and Garza, 1977, system I) fitted with a glycol water "trap" (see Wingfield and Farner, 1975)and the steroids were eluted with mixtures of iso-octane:ethyl acetate in the following order: A4 (99:1), 5 α -DHT (95:1), T (85:15) and E2 (50:50). Recoveries through the extraction procedures were checked in each sample for all steroids by adding known amounts (1000-2000 dpm) of tritiated steroids in the plasma and counting aliquots of the fractions eluted from the columns (see table 1).

The fractions containing the steroids were then dried and steroids were quantified by specific radioimmunoassay procedures validated for quail plasma (see Delville, Sulon and Balthazart, 1985, 1986; and table 1). For each assay, the specificity of the antibody combined with the purification on celite ensures that only one steroid is included in the measure. All assays have been shown to be parallel for quail plasma in the range of sample volume which is used (usually 20-80 µl) and precision has been tested by adding different amounts of cold steroids to plasma pools from young individuals or from castrates or to plasma which had been charcoaled to remove endogenous steroids. The correlation between amounts added and measured was always good and the slope of the regression line close to one (see table 1).

TABLE - 1

Characteristics of the radioimmunoassays used to measure steroids in the quail plasma. Mean recovery indicated is for the extraction and celite purification procedure. Titer for the antibody is initial dilution added to the assay tubes. Final dilution is the same divided by 5 (100 µl antibody is added to 400 µl containing sample and tracer). Cross-reactions are all supplied by the sellers of antibody except for the cross-reaction with 5 β -DHT that we have measured.

Steroid	Mean Recovery	Antibody	Titer	Cross-Reactions	Precision
A4	79 + 12%	Anti-androstenedione- 19-CME-BSA from RSL (California)	1/56,000	Dihydroepiandrosterone: 4% DHEA-S: 4% Estrone: 2 %	Y=0.90 X + 55.3 (r=0.995) for added amounts between 125 and 1000 pg/ml (pool=66 + 3 pg/ml, n=2)
5α-DHT	85 + 9%	Anti-15b-CME-5a-DHT:BSA from RSL (California)	1/21,000	T: 23% 5α -A-3,17-dione:17% A4 : 2% all others < 1%	Y= 1.10 X + 15.2 (r=0.948) for added amounts between 125 and 1000 pg/ml (pool=44 + 25 pg/ml, n=7)
T	73 + 6 %	Anti-T-19-CME-BSA from RSL (California)	1/56,000	5α -DHT: 19% 5 β -DHT: 16% 5 α -A-3α, 17 β -diol: 3% all others < 1%	Y= 1.25 X + 95.5 (r=0.995) for added amounts between 312 and 2500 pg/ml (pool = 36 + 11 pg/ml,n=3)
E2	99 + 15 %	Anti-E2-6-CMO-BSA from RSL (California)	1/200,000	Estrone : 15% Estriol : 1%	Y= 1.31 X -37 (r=0.971) for added amounts between 125 and 500 pg/ml (pool = -21 + 3 pg/ml,n=2)
P	70 + 6%	Anti-P-hemisuccinate-BSA from Biodata-Serono	1/8,000	11α -OH-P: 87 % 11β -OH-P: 16 % all others: < 1%	Y= 0.89 X + 32 (r=0.997) for added amounts between 250 and 1000 pg/ml (pool = 48 pg/ml, n=1)

RSL = Radioassay Systems Laboratories, Inc (California, USA), CME: carboxymethylmecapto, BSA: bovine serum albumin, CMO : carboxymethyloxime, A: androstane (in the name of androgens), DHEA-S : dihydroepiandrosterone- sulfate.

The tracer used in the radioimmunoassays was labelled with iodine 125 except in the case of the 5α -DHT assay where tritium was used.

STATISTICS : Behavioral data (frequencies) were analyzed by non-parametrical tests (Kruskal-Wallis analysis of variance on the 6 groups followed by Mann-Whitney tests to compare groups two by two). Median and range are used as parameters for central tendency and dispersion, respectively.

All other data were first analyzed by a two-way analysis of variance with sex and treatment as factors. The different groups were then compared with the Newman Keuls for multiple comparisons. Differences are considered significant only for bilateral probabilities inferior to 5%.

Results & Discussion

At the start of the experiment, the ANOVA showed that females were heavier than males (197.8 versus 184.1 g) although this difference was not large enough to generate a significant difference between any pair of groups (all Newman Keuls tests not significant). Treatment (gonadectomy versus sham operation) had no effect on body weight. At the end of the experiment, body weight was statistically similar in all groups (no significant effect of sex nor treatment) although females still tended to be slightly heavier (230.6 versus 214.2, $P < 0.10$ in the ANOVA).

SHAM males had fairly large cloacal glands (CGA=212 mm^2) at the start of the experiment while this structure was not developed by the other groups. The ANOVA on these data thus revealed an extremely significant ($P < 0.001$) effect of sex and treatment and an extremely significant interaction between the two.

The mean cloacal gland area and cloacal diameter in the different groups at the end of the experiment is presented in figure 1 together with a summary of the behavioral data.

These results confirm and extend observations already made previously (e.g. Balthazart, Schumacher and Ottinger, 1983). Cloacal gland area is sensitive to androgen so that it regress in castrated animals and is restored to the level seen in intact birds with treatement by testosterone. The same is true for sexual behavior in males as exemplified here by the neck grab and cloacal contact movement frequencies. Especially interesting is the sexual dimorphism in responsiveness to testosterone treatment. Not only is cloacal gland area smaller and sexual behavior less frequent in intact females than in intact males but also these sexual differences are maintained when birds are gonadectomized and submitted to the same hormonal treatment (see figure 1 for detail of statistics).

Cloacal diameter was significantly affected by the treatments (decrease after gonadectomy and increase after T treatment) but this effect was not different in males and females.

The hormonal conditions associated with these experimental situations are summarized in figure 2 which presents the mean plasma levels of hormones measured in the 6 groups of birds.

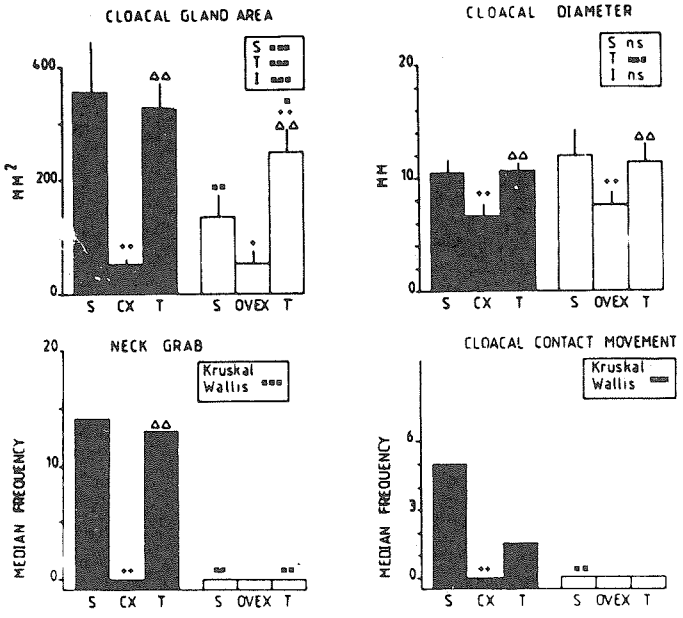

Fig. 1: Mean cloacal gland area and cloacal diameter in the six groups of birds at the end of the experiment and sexual behavior (median frequencies) measured during the two behavioral tests.

Behavioral data were analyzed by the Kruskal-Wallis analysis of variance (see results next to the title for each graph) followed by Mann-Whitney tests to compare groups two by two (results above the columns). The results of the ANOVA performed on morphological data are shown in the insets next to the titles (S = sex, T= treatments and I= interaction) and the comparisons by Newman Keuls tests of the groups two by two are shown above the columns.

The following symbols have been used *:P< 0.05, **: P < 0.01 and ***: P < 0.001 (ANOVA or comparison with the corresponding male group, e.g. SHAM female with SHAM male), + : P < 0.05, ++: P < 0.01 (comparison with the SHAM group of the same sex) and A: P.0.05 , AA: P < 0.01 (comparison with the gonadectomized group of the same sex).

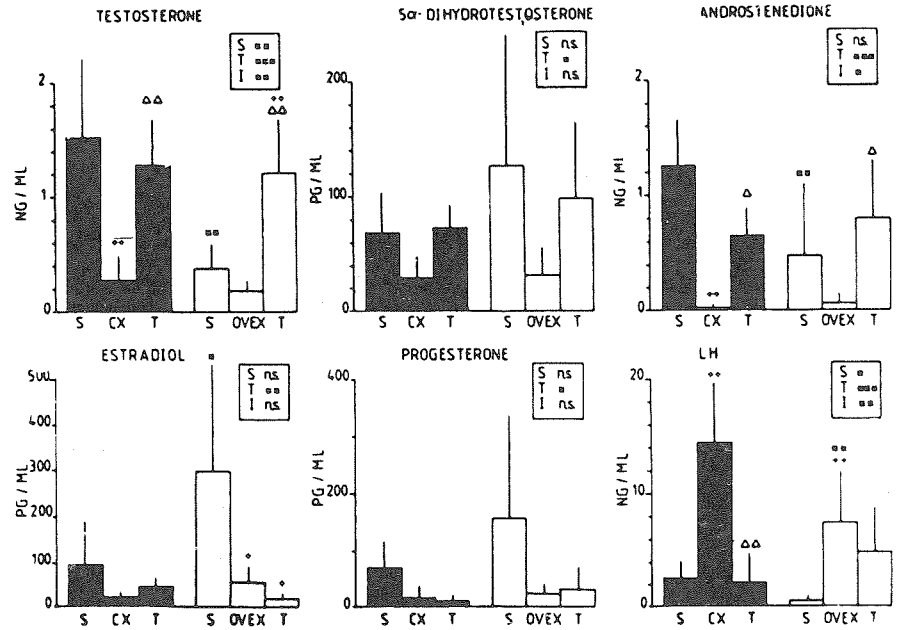

Fig. 2: Plasma levels of five steroids and of luteinizing hormone (LH) in the six groups of birds at the end of the experiment. See figure 1 for the explanation of the statistical results.

The ANOVA revealed significant differences associated with the sex of the birds for each hormone except 5 α -DHT and P. Each hormone was however significantly affected by the experimental treatments. Interactions between the two factors were observed in many cases (see figure for detail of statistics)

Newman Keuls tests revealed additional features of these results. Intact males had more T, A4, and LH and less E2 in their plasma than females but no difference was observed for 5 α-DHT or P. All these sexual differences were abolished by gonadectomy except for LH which remained higher in CX males than in OVEX females. No significant difference could be observed in the hormone levels of males and females following treatment with testosterone. The testosterone treatment which was applied here (60 mm silastic implants) elevated plasma levels of T but also of A4 to levels seen in intact males. The same effect was observed in the two sexes.

The data presented above confirm a number of known facts about the reactions of male and female quail to gonadectomy and replacement therapy with testosterone. They show namely that in males castration produces a complete involution of the cloacal gland and suppresses copulatory behaviour. These effects can however be completely reversed following two weeks of treatment with 60 mm of silastic implants filled with T (see Balthazart, Schumacher and Ottinger, 1983; Schumacher and Balthazart,1984; Balthazart, Schumacher and Malacarne, 1985). The present study shows under which precise hormonal conditions this restoration to intact level is observed. Plasma T levels in testosterone-treated males is nearly identical to that seen in intact males as already shown previously (Schumacher and Balthazart, 1986). LH levels are also at the same levels in these testosterone-treated male as in intact males showing that the replacement therapy represents physiological levels of steroid exerting a natural level of feedback at the hypothalamic and pituitary levels.

More interesting is the fact that A4 levels were similar in testosterone-treated and intact males despite the fact that they were almost equal to zero in castrated animals. The suppression in castrates shows that this steroid has a gonadal origin but the presence of high levels in testosterone-treated males suggest that it can be formed in the periphery from the oxidation of testosterone and there is no need to postulate that the testis itself is directly secreting A4. It is indeed well established that the 17β-hydroxy-steroid-dehydrogenase which catalizes the transformation of T into A4 is present in several tissues of quail (namely brain, pituitary gland and cloacal gland; see Balthazart, Schumacher and Ottinger, 1983) as shown by *in vitro* radioenzymoassays. The present data show that this enzymatic activity is actually significant *in vivo* and is sufficient to restore quantitatively the plasma levels of A4 to the level seen in intact males when sufficient amounts of substrate (T) are provided.

The same is probably true for the enzyme producing 5 α -DHT from T (the 5 α -reductase) although data are less clear in this case. Plasma levels of 5 α -DHT were significantly affected (ANOVA) by the treatment in this experiment (reduction following castration and increase after testosterone therapy) but the magnitude of the effects was too small and variance too large so that none of the separate comparisons between pairs of groups was significant. The changes in mean levels of hormones (see figure 2) however clearly suggest that the 5 α -reductase which has been identified by *in vitro* assays in quail (Balthazart, Schumacher and Ottinger, 1983; Delville, Hendrick, Sulon and Balthazart,1984) is active *in vivo* and contributes to the plasma levels of 5 α -DHT.

The data also show that it is by far an oversimplification to consider androgens as the " male " hormones and estrogens and progestagens as the " female " hormones. All steroids are present in both sexes and only differ by their concentration (except for P). E2 and P measures were associated in intact females with a very large variance. This is probably related to the fact that blood samples were collected during an extended period of the day which very probably covered most of the egg laying cycle. As these hormones vary very significantly during the ovulatory cycle and show clear preovulatory peaks (Doi, Takai, Nakamura and Tanabe, 1980; Delville, Sulon and Balthazart, 1986), the large variance is likely to result from this temporal effect rather than from genuine individual differences of assay errors. Had females been all sampled at a preovulatory peaks, we would probably have observed higher levels of E2 and P associated with lower variance.

It is finally very interesting to relate the differential sensitivity to testosterone treatment of males and females to the plasma levels of hormones which are actually present in birds submitted to these treatment. It has been known for some time that testosterone injections or implants activate copulatory behavior in male but not in female quail and that a same testosterone treatment results in larger cloacal glands in males than females (Adkins and Adler, 1972; Adkins, 1975; Balthazart, Schumacher and Ottinger, 1983). It was however quite possible that the peripheral catabolism of the injected or implanted hormone was different in the two sexes so that a same hormonal treatment resulted in different circulating levels of hormone and thus in a differential activation of testosterone-dependent characteristics. We had shown previously that this interpretation could not be retained as far as plasma levels of T were concerned: the circulating concentration of this hormone is indeed similar in males and females following implantation of 60 mm silastic capsules filled with the crystalline steroid (Balthazart, Schumacher and Ottinger, 1983; Schumacher and Balthazart, 1986). The present study shows in addition that the metabolites of T which are formed following implantation of this steroid (5 α -DHT, A4) also are present in similar concentrations in males and females. Other hormones such as E2 which could exert some anti-androgenic effects (see Balthazart, Schumacher and Malacarne, 1985) are also equally concentrated in the plasma of males and females following castration and testosterone and testosterone therapy. All available data thus show that in such birds, the hormonal milieu is quite similar in males and females and the differential response to testosterone treatment results thus very probably from a differential sensitivity of the target structures to the steroid action. Experiments are currently in progress in our laboratory to identify at the biochemical level the mechanisms underlying this differential sensitivity to steroid action.

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