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FINAL REPORT

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Project Titles:

Overall Project Title: The Role of Population - Dependent Factors in
Altering Adrenal Steroidogenesis and Reproduction
in the Cottontail Rabbit in Ohio

Subproject Titles:

- I. The Role of Quantitative Differences in Adrenal Steroid Secretion
in Cottontails from Different Physiographic Regions
- II. The Role of Population-Dependent Factors in Altering Adrenal Physiology
Relative to Reproduction

Introduction

Previous studies demonstrated that cottontail rabbits reproduce at different rates in Ohio's various physiographic regions (Stevens, 1962). This study was designed to demonstrate whether population-dependent factors may be the cause of such variations and whether these causes are reflected in the metabolic capacity of adrenal glands in female rabbits. A total of 64 female cottontails were collected during their first pregnancy in order to determine reproductive performance. Thirty-one animals were taken from a region of high productivity (Westcentral Region) and 33 were collected from a region of lower productivity (Southeast Region). The adrenal glands were removed from all animals and the metabolic activity (as reflected in steroidogenic biosynthesis in vitro) compared with the reproductive rates as individuals and as a group.

Methods

Reproductive Performance - The reproductive performance of each pregnant female collected was evaluated in the following manner:

- 1) All pregnant uteri were dissected and the number of implantation sites noted.
- 2) The number of viable embryos present were counted.
- 3) Both ovaries from pregnant females were fixed, serially sectioned and stained with hematoxylin and eosin. Slides were examined and the number of corpora lutea counted.

These data provided an evaluation of 1) ovulation rates, 2) preimplantation loss and 3) post implantation loss.

Adrenal Gland Steroid Biogenesis - The biosynthetic capacity of adrenal glands from each rabbit were evaluated as follows: The adrenals were allowed to come to 4° C., stripped of all fat and other debris; they were minced to a fine brei (0.2 - 0.6 cm.) in preweighed 24 dram vials. The vial containing the tissue was again weighed. To the vial was added the incubation media. All incubations were carried out with a Krebs-Ringer bicarbonate buffer at pH 7.4 and the following concentrations of cofactors: adenosine triphosphate, 4.0mM; nicotinamide-adenine dinucleotide (NAD), 0.4 mM; nicotinamide-adenine dinucleotidephosphate (NADP), 0.1 mM; reduced nicotinamide-adenine dinucleotide phosphate (NADPH), 0.01 mM; glucose-6-phosphate, 0.5 mM; sodium fumerate, 70mM; D-glucose-6-phosphate: NADP oxidoreductase 0.5 units, and glucose concentration of 200 mg.%. These values represent final concentration. The incubations were carried out at 37°C in an atmosphere at 95% oxygen-5% carbon dioxide. Radiochemical purity of the labeled steroid was established in several chromatographic systems prior to its use in the incubation. Each vial contained a little more than 2.0 uc of 7 - H³ - pregnenolone or 1.0 uc of 4-C¹⁴ pregnene - 3,20 dione (C¹⁴ - progesterone). The incubations were ended by the addition of 10 ml. of dichloromethane, shaken and the organic solvent removed. Each was re-extracted twice more and centrifuged to break any emulsion that may have formed. All solvents were freshly redistilled to assure purity, and all chemicals were reagent grade. The enzymes and cofactors were purchased from Sigma Chemical Company and were the best grade obtainable.

The combined organic extracts were dried with sodium sulfate (anhydrous) and dried down in centrifuge tubes; to each dried residue was added 1.0 ml. of 25% benzene in hexane. The tube was warmed to 45°C and an area of activated silica gel "G" equivalent to 50 cm

was added to the tube and slurried. An additional 5.0 ml. of the solvent was added, mixed and centrifuged. The solvent was evaporated in another tube after transfer. Randomly, aliquots were removed for counting. At no time did the 25% benzene in hexane solution contain greater than 1% of the radioactive counts. The silica gel was eluted three times with dichloromethane. The elutions were combined and dried to a residue. This residue was then chromatographed.

The defatted extraction residue was chromatographed on washed papers in the toluene/propylene glycol (T/PG) system for 72 hours; one milliliter of seven different steroid standards was dried down with the residue to serve as ultraviolet markers. The chromatographic separation of these seven were well established previously. The run-off beakers of the T/PG were rechromatographed in the same system for 24 hours; the run-offs from the 24 hour papers were placed in ligroene/propylene glycol (L/PG) for 24 hours and the run-off in the hexane/propylene glycol system for a single length run. The papers were dried and scanned for radioactivity in the Packard 4 pi radiochromatogram scanner. The radioactive areas were compared with the UV absorbing areas of the added inert carrier compounds. The area where the UV and radioactivity corresponded well were eluted and rechromatographed to resolve any overlapping compounds and to give the best possible resolution. Areas of radioactivity not resolving completely were eluted and rechromatographed in appropriate systems to effect the desired separation.

The upper half of the 72 hour (T/PG) paper was rerun in TE 91/50 which gave three distinct areas (BF, F and E). The areas of radioactivity on the 24 hour T/PG was placed on thin layer chromatography system (chloroform; methanol: water, 141;9:0.7 parts); this resolved compound S and corticosterone; the areas of interest on the L/PG was separated

on a TLC system of ethyl acetate: benzene, 50:50. This gave four distinct areas: desoxycorticosterone (DOC), testosterone-epitestosterone, 17-hydroxyprogesterone-pregnenolone and androstenedione. The progesterone area was eluted from the H/PG paper and rerun in H/70 system. Unknown areas were repeatedly rechromatographed with various standards, run on a side limb, to provide evidence for their identification.

Thirty-three pairs of adrenal gland incubations have been resolved; the resolved radiolabeled compounds were eluted, and an aliquot was taken for quantitation in the Packard Tri-Carb Spectrometer. The remaining amount was dried down for further work. The results of these counts, following calculation for conversion to dpm/gm of tissue, were further used to express the results as per cent substrate conversion of labeled steroid.

Results

The reproductive performance of the limited number of cottontails collected in this study reflected similar productivity rates as was found in earlier studies. Ovulation rates were higher in females collected in the Westcentral Region than in the Southeast Regions which resulted in a larger number of viable embryos in the uterus of rabbits of the first group. No difference in pre- or post-implantation loss was observed. The reproductive data are shown in Tables I and II.

As seen in Table III, the biosynthetic steroids produced from C^{14} progesterone precursor in adrenal glands of the two regions were quite similar (W-Westcentral Region; S - Southeast Region). There was, however, one outstanding exception. An unidentified steroid, or steroid complex (H_x) was produced in much greater quantity in adrenals from the W-region. Since this steroid (S) represented the only major difference between regions, extensive and considerable effort was directed towards identifying this substance (S).

The unknown Hx, following quantitation separately, was combined and chromatographed in various paper and thin layer systems. It has a polarity that lies between desoxycorticosterone and progesterone. The following compounds were run with the unknown Hx in the various systems, but failed to demonstrate a mobility similar to the unknown compound:

16-hydroxyprogesterone, 6-oxo-progesterone, 11 β -hydroxyprogesterone, 11-oxo-progesterone, 6 β -hydroxyprogesterone, Δ^4 -pregnene-20 α -ol, 3-one, Δ^4 -pregnene-20 β -ol, 3-one, Δ^4 -pregnene - 3 α -ol, 20-one, Δ^4 -pregnene - 3 β -ol, 20-one. None of these compounds co-chromatographed with the unknown. It would appear from the various mobilities, that the unknown Hx may have a structure similar to pregnane-3,6,20-trione. This standard was not available for comparative evaluation.

The results of the continued further analysis of the original twenty incubations has selectively identified and quantitated the unknown Hx compound from the W and S regions. It can be seen that this compound is greater than four (4) fold higher in the West when compared to the South region. This four fold increase and it's significance or lack of significance can not be evaluated at this time. As pointed out above, the identification remains a problem.

In the second set of incubations, the concentration of 18-hydroxycorticosterone was determined. Again, the average values of the two groups were very similar (Table IV). Surprisingly, no unknown Hx was encountered despite a comparable chromatographic separation. The two different incubations are compared, the first being with labeled progesterone and the second with H³-pregnenolone, it is quite apparent that the corticoids produced during the incubations, are similar within groups and between groups. However, the total percentage conversion per gram tissue is different. This may be due to length of storage of the original glands or that pregnenolone is a more direct pathway, that is, hydroxylations at 11,17 and 21 occur prior to 3 β -hydroxysteroid dehydrogenase activity.

An apparent and most interesting feature of the latter incubation data is the twofold increase of testosterone and androstenedione in the Southern group. These differences are significant since these hormones are potent inhibitors of pituitary secretion and could be responsible for pituitary changes that would reflect in the population data.

Discussion

The results of these studies are significant but not conclusive. Both the incubations with C^{14} - progesterone and H^3 - pregnenolone indicated a difference between regions in the steroidogenic profile of the adrenal glands from respective animals. Although the sample size was too small to show individual productivity - adrenal function relationships, comparisons as a group were possible.

The presence and absence of the unknown Hx in the two incubation sets is of interest. In the progesterone experiments usually better than 50% of the labeled precursor remained unconverted following the incubation. Due to the larger amount of precursor present, the dihydrocompound (allopregnandione) was formed in significant amounts and enough was present at the end of the incubation to permit identification. When there are larger amounts of progesterone available, and the dihydro-derivative is formed, this flattening of the rings due to this delta-4-reduction makes the molecule more accessible to further hydroxylation or metabolic transformation resulting in the unknown Hx encountered only in the progesterone incubations. This may account for the failure to locate and identify not only the dihydrocompound, but likewise the unknown Hx, when biotransformations are rapid from a rather limited supply of progesterone. Only 6.72 and 13.75 percent conversion per gram was found when using pregnenolone as the labeled substrate.

Several pertinent observations have been made that apply to the overall ecological problem in this study. It has become quite clear that the reproductive difference between the two study regions is distinct and consistent. Secondly, the reproductive difference is certainly environmentally induced. Rabbits from both regions confined to the same area for equal periods of time had no significant difference in reproductive rate. This study has suggested that adrenal gland function, as indicated by in vitro incubations, is different in the two physiographic regions of Ohio. This difference may be indicative of hyper- or hypo-function of this organ in the lower producing region. The identity and biological activity of compounds in the Hx of glands incubated with C^{14} - progesterone is important in this regard. Should this compound (s) be reproductive inhibitory, a hyper-function can be assumed. The second incubations suggest testosterone as the agent reducing productivity in Southeastern Ohio cottontails. Certainly a different approach to manipulating environmental factors in wildlife management would be taken if stress (hyper-function) was responsible for lower reproduction than if reduced metabolic activity (hypo-function) was involved. The basic information obtained in this study concerning the population physiology of an important game species as the cottontail, is of itself significant.

Table 1

Reproductive Performance of Cottontails
Collected in the Westcentral Region

<u>Rabbit No.</u>	<u>No. of Corpora Lutea</u>	<u>Implantation Sites</u>	<u>Living Embryos</u>
W8	7	7	-
W11	7	-	-
W9	6	5	5
W20	7	7	6
W26	5	5	3
W21	7	5	4
W23	6	5	5
W16	4	4	4
W27	9	8	8
W10	6	4	4
W12	7	5	5
W15	7	6	6
W14	5	5	5
W13	-	6	3
W17	5	5	5
W19	5	4	4
W18	-	5	5
W22	6	6	6
W5	4	4	4
W7	8	-	-
W1	-	-	-
W6	5	5	5
W4	5	5	5
W3	3	-	-
W28	5	5	5
W29	7	7	6
W30	6	6	6
W31	6	6	5
W32	5	-	-
W33	4	3	3
W34	5	5	5
AVERAGE	5.79	5.31	4.88

Table II

Reproductive Performance of Cottontails
Collected in the Southeastern Region

<u>Rabbit No.</u>	<u>No. of Corpora Lutea</u>	<u>Implantation Sites</u>	<u>Living Embryos</u>
S18	4	4	4
S3	5	5	5
S5	4	4	4
S1	7	5	4
S4	5	5	5
S2	5	5	5
S6	3	3	3
S8	6	4	4
S12	4	4	2
S14	-	5	4
S7	5	5	5
S9	4	4	3
S11	4	2	2
S17	5	4	4
S15	4	4	4
S16	5	5	5
S24	4	3	3
S19	6	4	4
S27	4	4	3
S28	4	4	4
S21	5	3	3
S20	4	4	4
S22	5	5	5
S23	4	2	2
S26	5	6	6
S25	4	4	4
S28	4	-	-
S29	3	3	2
S30	4	4	4
S31	5	4	4
S32	5	5	5
S33	4	4	3
AVERAGE	4.52	4.10	3.52

Table III

Per cent Substrate Conversion of 4-C¹⁴ - Progesterone to
Various Compounds Isolated from the Incubations ^(a)

Incubation	Compound or groups of compounds (b)							
	BF	F	E	S	B Complex	DOC	H _x	Allop
W-4	0.83	0.46	0.31	0.79	12.83	1.60	7.60	3.94
W-5	0.81	0.22	0.18	0.69	9.71	1.55	0.62	3.05
W-6	0.64	0.32	0.26	0.59	10.41	0.82	-	3.06
W-8	1.35	0.38	0.31	0.24	12.48	1.43	1.20	3.84
W-11	0.80	0.38	0.37	0.58	9.73	1.95	4.35	2.67
W-23	0.63	0.25	0.30	0.60	8.07	1.65	0.71	2.72
W-1	0.59	0.19	0.13	1.28	9.05	1.29	1.04	3.96
W-7	0.77	0.34	0.27	0.85	14.85	2.60	3.36	5.07
W-2	0.48	0.06	0.07	1.45	7.72	1.47	7.15	3.74
W-18	1.50	0.51	0.39	1.08	13.64	2.70	1.89	3.66
Average	0.84	0.31	0.26	0.82	10.85	1.71	3.10	3.57
S-20	0.77	0.34	0.18	0.84	8.02	0.75	0.32	1.67
S-19	0.81	0.27	0.13	2.32	8.04	1.70	0.27	1.83
S-4	0.66	0.23	0.19	0.69	6.76	1.10	0.43	1.68
S-16	1.05	0.28	0.32	1.69	9.03	1.81	0.52	2.71
S-2	0.56	0.22	0.13	1.16	7.28	1.71	0.74	2.11
S-6	1.06	0.33	0.39	0.96	9.76	1.83	0.75	2.07
S-5	1.54	0.40	0.58	1.48	10.58	1.65	1.84	3.25
S-21	1.82	0.74	0.34	2.09	14.32	3.08	0.58	5.53
S-22	0.73	0.22	0.25	1.19	5.94	0.67	0.93	1.63
S-25	0.40	0.16	0.15	2.09	4.87	0.96	0.75	1.15
Average	0.94	0.32	0.27	1.45	8.46	1.53	0.71	2.36

a) Calculated from conversion expressed as dpm/gm tissue.

b) Where BF represents more polar than hydrocortisone complex;
F is hydrocortisone; E is cortisone; S is Reichstein's compounds; B
complex is for a large group of compounds (see text); DOC is 11-
desoxycorticosterone; H_x is an unidentified compound; and Allop is
for allopregnanedione.

Table IV

Per cent Substrate Conversion of $7\alpha\text{-H}^3$ -Pregnenolone to
Various Compounds Isolated from the Incubations (a)

Compound or groups of compounds (b)											
Incubation	BF	F	E	S	18OH-B	B	DOC	T ₄ - Epit	17-OH- Prog.	A-4- DHEA	Prog.
W-26 28	11.17	5.85	7.32	0.08	1.64	0.11	8.31	4.29	2.93	2.36	-
W-27 29	5.18	3.36	2.36	0.29	0.82	0.25	1.74	1.48	.83	.69	3.79
W-28 30	5.50	3.32	2.43	0.79	1.21	1.24	3.02	3.40	1.21	.78	5.64
W-29 31	3.95	1.84	1.69	1.07	1.11	1.28	2.26	1.59	1.23	1.08	8.58
W-30 32	9.42	5.84	5.10	1.32	2.16	2.33	3.77	2.44	1.29	1.44	5.19
W-31 33	14.72	9.16	9.25	2.41	3.86	3.47	5.42	.41	.62	1.72	11.94
W-32 34	9.85	6.48	7.31	1.48	1.81	2.98	5.10	2.10	1.35	1.75	5.18
Average	8.54	5.12	5.06	1.06	2.10	1.66	4.23	2.24	1.35	1.40	6.72
S-33 28	10.33	6.39	6.13	2.07	2.46	4.47	8.80	5.98	2.55	2.10	15.68
S-34 29	10.03	9.92	7.46	1.65	3.14	2.53	7.12	4.89	2.17	2.21	15.58
S-35 30	8.33	7.13	4.79	1.58	2.64	1.91	4.41	3.27	1.93	1.93	13.97
S-36 31	12.56	5.59	8.42	1.95	3.54	2.69	6.09	3.25	1.32	1.62	6.55
S-37 32	8.30	9.01	2.61	1.27	2.43	1.83	5.63	6.23	2.77	2.09	16.97
Average	9.91	7.60	5.88	1.70	2.84	2.68	6.41	4.72	2.14	1.99	13.75

a) Calculated from conversion expressed as dpm/gm tissue.

b) Where BF represents more polar than hydrocortisone complex;

F is hydrocortisone; E is cortisone; S is Reichstein's compound S;

18-OH-B is 18-hydroxycorticosterone; DOC is 11-desoxycorticosterone;

T₄-Epit is the testosterone-epitestosterone complex; 17OH-Prog is

17-hydroxyprogesterone; A₄-DHEA is the androstenedione-dehydroepiandrosterone complex; and Prog is progesterone.