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Changes in the activity of testosterone-metabolizing enzymes in the brain of male and female zebra finches during the post-hatching period

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The kinetic properties of the testosterone-metabolizing enzymes were studied in the hypothalamus of adult and young zebra finches of both sexes. Estradiol, 5 α -dihydrotestosterone, 5 β -dihydrotestosterone and 5 β -androstane-3 α ,17 β -diol were identified as metabolites of testosterone in males and females at different ages between 5 days post-hatch and adulthood. During maturation, the maximum velocity ($V_{\max}$) of the aromatase and 5 β -reductase decreased in males and females while the affinity of these enzymes for the substrate increased (decrease in K_m). These changes were more pronounced for 5 β -reductase than for aromatase. The affinity change of the 5 β -reductase occurred progressively during the post-hatching development between 5 and 30 days and is thus probably a true developmental process. In the case of the aromatase, the change in affinity occurred much later (after 30–40 days) and is thus probably related to the sexual maturation. These kinetic changes during development are directly related to the roles played by testosterone and its metabolites, in particular estradiol, in the differentiation and activation of reproductive behavior in the zebra finch. In particular, the dramatic decrease in 5 β -reductase activity during sexual maturation should correspond to a potentiation of testosterone action in the brain.

INTRODUCTION

For several years, the zebra finch (*Taeniopygia guttata castanotis*) has been used as a favorite model for studies in behavioral endocrinology and brain development. This, by and large, has been the consequence of the identification in the Seventies of the brain circuitry controlling the vocalizations in oscines²² followed by the discovery that these areas involved in song control are sexually differentiated²¹. These song nuclei are steroid sensitive: their total volume, the size of their neurons and/or their dendritic branching are modified by androgens in adulthood^{10–12,20}. It has been shown that the neonatal steroid environment is responsible, at least in part, for the sexual dimorphism in the brain. This is especially true in zebra finches in which adult treatment of

females fails to masculinize morphological aspects of the song control nuclei^{2,13,14}. Gurney and Konishi^{13,14} established that treatment of newly hatched female zebra finches with estradiol (E_2) masculinizes some nuclei in the song system and gives them the capacity to respond to testosterone (T) treatment in adulthood in the same way as observed in males. Interestingly, the same neonatal treatment with E_2 has permanent effects on the vocal behavior of the birds^{1,14,25,26}. From these observations, Gurney and Konishi assumed that sexual differentiation of singing behavior in zebra finches occurs by a masculinization of males under the influence of estrogens during the neonatal period. It was found later that plasma levels of E_2 are actually higher in males than females during the first 10 days after hatching¹⁹. During the first few days after hatching, substantial amounts of T, 5 α -di-

hydrotestosterone (5α -DHT) and androstenedione (A4) are also present in the plasma but the concentrations of these androgens are not sexually differentiated. These data are thus consistent with the idea that the increased plasma levels of E_2 are responsible for the masculinization of the song in males. However, it was demonstrated later²⁶ that estrogens must be present for more than 10 days in females in order to produce the full behavioral masculinization and that this is related to the degree of neuroanatomical masculinization²⁵. If the same hormonal stimuli are required in males, then the exact determination of the sexual differentiation of the song system remains unclear: peripheral E_2 levels could play a substantial role but it is possible that T is also acting through its central aromatization¹⁷.

Although an active aromatase has been identified in adults of several bird species (quail^{9,29,30}, ring dove³²) and is known to be present shortly after hatching in these species (quail¹⁸, dove¹⁸, chicken⁸), this enzyme has never been studied in the zebra finch. However, it was shown recently that a very active 5β -reductase is present in the zebra finch brain during the neonatal period⁵. This enzymatic system could potentially inactivate most of the T and in this way prevent it from having any organizing effect on the brain. To understand the hormonal bases of sexual differentiation, it is thus critical to evaluate both the plasma levels of steroids and their fate in the brain. This prompted us to carry out the present study.

In the adult zebra finch, it has also been demonstrated that some behavioral effects of T can be reproduced by treatment with the androgenic and/or estrogenic metabolites of the hormone¹⁵. Some behavioral components (high intensity aggressive displays, defense of the nest site) can be activated only by E_2 and by aromatizable androgens such as androstenedione. In these cases, concurrent treatment with the aromatase inhibitor, androstatrienedione (ATD) significantly suppresses the behavioral effects of androstenedione, suggesting that aromatization of this androgen is a critical step in its action. This is, however, surprising in view of the very high plasma levels of E_2 which were observed in adult males during that study¹⁶. This discrepancy was another incentive to study the actual aromatase activity in the zebra finch brain.

Because no study has described so far T metabolism in the brain of the zebra finch (with the exception of one paper devoted to the 5β -reductase pathway⁵), it was first necessary to characterize the enzymes responsible for the transformations of T. It was decided to carry out such a study using hypothalamic tissue because this is an area which has been shown in other species of birds and mammals to contain high levels of both aromatase and 5α -reductase activity. It is also implicated in the control of sexual behavior. In this paper, brain enzymes catalyzing T metabolism (aromatase, 5α - and 5β -reductase) were studied in the hypothalamus of male and female zebra finches at different ages between 5 and 85 days after hatching. Kinetic parameters (k_m , V_{max}) were obtained in each case because in a previous study⁵, we had found that the affinity of the 5β -reductase changes with age in this species so that results of one-point assays become impossible to interpret. It is critical to obtain evaluations of these parameters because, to some extent, they permit extrapolation of the results of in vitro assays in the physiological conditions of the living animal. An enzyme with higher affinity (lower k_m) will preferentially interact with the substrate (T in this case) by comparison with another enzyme which has a lower affinity. When the substrate is in limited concentrations, an enzyme with high affinity will still be biologically active while an enzyme with a lower affinity will produce almost no metabolites. On the other hand, the maximum velocity of the enzyme allows us to predict how much of the substrate could potentially be transformed in the living brain and therefore tell us something about the relative amounts of metabolites which can be produced in vivo provided enough substrate is available. These data should serve as a basis for the future investigations on the role of T metabolism in the control of both sexual and vocal behavior.

MATERIALS AND METHODS

Animals

All birds used in these experiments were obtained from the breeding colony of zebra finches (*Taeniopygia guttata castanotis*) established at the Department of Ethology, University of Bielefeld (F.R.G.). Birds were killed by decapitation and brains were quickly dissected out of the skull and frozen on dry ice. This

procedure does not affect the T-metabolizing enzymes in the brain^{29,30} and thus samples can be saved in the time required to dissect several brains, in order to assay large number of homogenates within a same series.

All enzyme studies were done on the hypothalamus which was dissected out of the brain immediately before the experiment. The brain area which was dissected out extended in the rostrocaudal axis from the tractus septomesencephalicus to the level of the oculomotor nerves (posterior end of the tuberal hypothalamus). Its width and depth were about 1.5 mm. This dissection is identical to the one used previously⁵. Brain samples were then homogenized at a concentration of 20–40 mg/ml in ice-cold STMM buffer (0.25 M sucrose, 10 mM Tris HCl, pH 7.4 at 20 °C, 5 mM MgCl₂, 1 mM β -mercaptoethanol). Homogenates were immediately frozen in an acetone-dry ice bath (78 °C).

Radioenzyme assay

The radioenzyme assay for T-metabolizing enzymes has already been described³⁰. It was used here with only minor modifications. For the assay, 100 μ l of homogenates were thawed in an ice-water bath. Immediately after thawing, 100 μ l of homogenates were added to glass tubes containing radioactive tritiated testosterone (spec. act. 51–60 Ci/mmol from the Radiochemical Centre, Amersham). Tritiated testosterone had been added before to the tube in 50–100 μ l of methanol-toluene and carefully dried. According to the purpose of the experiment, different amounts of substrate were used in the range of 10 to 200 nM. At the start of the incubation, 100 μ l of STMM buffer containing 2 mg/ml of NADPH₂ (final concentration 1.2 mM = saturating levels; see ref. 30) was added to the individual tubes. Homogenates were then incubated at 41 °C for 5 or 15 min (or various durations if this was the purpose of the experiment). Reactions were stopped by freezing samples at 20 °C in some cases after cooling them in an ice water bath.

Radioactive steroids were extracted 3 times with diethyl ether after addition of 5 μ g of carrier steroids and about 2000 counts of [¹⁴C]E₂ (spec. act. 50 mCi/mmol from Amersham) and 2000 counts of [¹⁴C]5 α -DHT (spec. act. 50 mCi/mmol from Amersham) for calculation of recovery of estrogens and an-

drogens, respectively. Radioactive steroids were always repurified by thin layer chromatography (TLC) before use. After addition of 250 μ l of 1 M NaOH to the dry extracts, androgens were extracted 3 times with a mixture of toluene:cyclohexane (1:1, v/v). After neutralization of the aqueous phase by 1 M HCl, estrogens were extracted 3 times with diethyl ether. Both androgens and estrogens were separated by TLC on silica gel (estrogens, Macherey-Nagel no. 804023; androgens, Merck 5715) in a mixture of dichloromethane:ether (85:15, v/v). Androgens were revealed on the plates with Primulin (Sigma 7522) and estrogens with iodine vapors.

The different metabolites were eluted in 100 μ l of methanol and in 4 ml of Aqua-Luma and counted in a Beckman Liquid Scintillation Spectrometer using the preset ³H/¹⁴C windows. Counts were corrected for spillover, counting efficiency and tracer recovery. The amounts of metabolites produced in control tubes (blanks) containing buffer, NADPH₂ and tracer but no brain homogenate were subtracted from the final values. Triplicate determinations were performed in each case (blanks and actual determinations).

Four or sometimes 5 metabolites were quantified: E₂, 5 α -DHT, 5 β -dihydrotestosterone (5 β -DHT), 5 β -androstane-3 α ,17 β -diol (5 β -diol) and A4. The remaining substrate (radioactive T) was also measured. The identity of these 6 steroids was confirmed by recrystallizations to constant specific activity or constant isotopic ratio (see refs. 18, 30 for methods). This was done separately in adults and young of both sexes. Corresponding results are shown in Table I. Protein content of the homogenates was measured by the Coomassie blue reaction⁷.

The kinetic parameters of the enzymes (k_m and V_{max}) were evaluated by double reciprocal plots (Lineweaver–Burke plots) of the reaction speed (V) versus substrate concentration (S) using standard linear regression programs on two types of microcomputers. These provide us with the parameters of the regression line (slope $[a]$ and intercept $[b]$) as well as with their associated standard errors (Δa and Δb). The errors on the V_{max} ($1/b$) and k_m (b/a) were then calculated using a standard procedure for error estimation, namely: $|\Delta V_{max}| = 1/b^2 \times |\Delta b|$ and $|\Delta k_m| = 1/b \times |\Delta a| + |a/b^2| \times |\Delta b|$.

TABLE I

Recrystallizations to constant specific activity (CSA) or constant isotopic ratio (CIR) of metabolites produced during the incubation of zebra finch hypothalamic homogenates with tritiated testosterone

In each case, 3 successive crystallizations were performed in methanol/water. Ratios of dpm to mg (CSA) or dpm-³H to dpm-¹⁴C (CIR) are given in the table.

Metabolite (method)	Number							
	Males				Females			
	Initial	1	2	3	Initial	1	2	3
E ₂ (CIR)								
Young	0.75	1.25	2.00	0.90	0.62	0.57	0.56	0.60
Adult	0.61	0.70	0.53	0.57	0.51	0.67	0.75	0.51
5 α -DHT (CIR)								
Young	6.36	3.62	3.12	3.15	5.28	3.40	2.63	2.80
Adult	5.65	4.10	3.60	3.20	3.27	2.58	2.68	2.43
5 β -DHT (CSA)								
Young	24,401	24,405	24,395	25,728	43,133	41,566	43,943	46,701
Adult	6,570	5,587	6,550	6,236	12,192	10,356	12,195	12,264
5 β -diol (CSA)								
Young	3,490	3,280	3,360	1,580	7,340	7,180	6,880	5,200
Adult	2,010	2,720	2,440	2,530	1,780	1,510	1,390	1,210
A4 (CSA)								
Young	542	552	453	577	330	266	279	330
Adult	430	421	391	443	665	609	713	—
T (CSA)								
Young	113,353	104,196	113,561	115,919	93,649	97,018	96,793	101,173
Adult	162,208	170,056	171,264	168,526	144,599	137,371	156,435	177,316

RESULTS

Experiment 1

Before starting any experiment on the sexual dimorphism or ontogenetical development of T-metabolizing enzymes, it was necessary to define incubation conditions in which the metabolites would accumulate linearly over time so that true enzymatic velocities would be measured.

The brains of 3 adult (2 males and 1 female) and of 8 young (4 males and 4 females, aged between 5 and 10 days post-hatch) animals were homogenized in STMM buffer at a concentration of 20 mg/ml.

Protein content in these homogenates was 2.44 and 1.72 mg/ml in adults and young, respectively. Aliquots of these homogenates (100 μ l) were added to tubes containing tritiated T and 100 μ l of STMM buffer containing NADPH₂ (2 mg/ml). Final concentration of radioactive T was 20 nM. Tubes were incubated at 41 °C for 5, 10, 15 or 30 min.

In these conditions, the most active enzymes were by decreasing order the 5 β -reductase (production of 5 β -DHT and the corresponding 5 β -diol), the 5 α -re-

ductase (production of 5 α -DHT), the 17 β -hydroxy-steroid dehydrogenase (17 β -HSDH, production of A4) and the aromatase (production of E₂). Enzymatic reactions were in each case more active in young than in adult birds. The accumulation of metabolites (and disappearance of T) were nearly linear between 5 and 30 min. However extrapolation of these lines almost never passed through the zero point especially in the data from young birds. This could have been the consequence of a systematic error or could represent a true phenomenon. The next experiment was designed to test these possibilities.

Experiment 2

The brains of 3 adult and 4 young male zebra finches were homogenized in STMM buffer as described above (20 mg fresh weight/ml; 2.75 and 1.95 mg protein/ml, respectively). One hundred μ l of these homogenates was incubated with radioactive T (final concentration 20 nM) and NADPH₂ (final concentration 1.2 mM). Durations of incubation this time were much shorter (1, 3, 5, 10 and 15 min).

Results of this experiment clearly showed that the

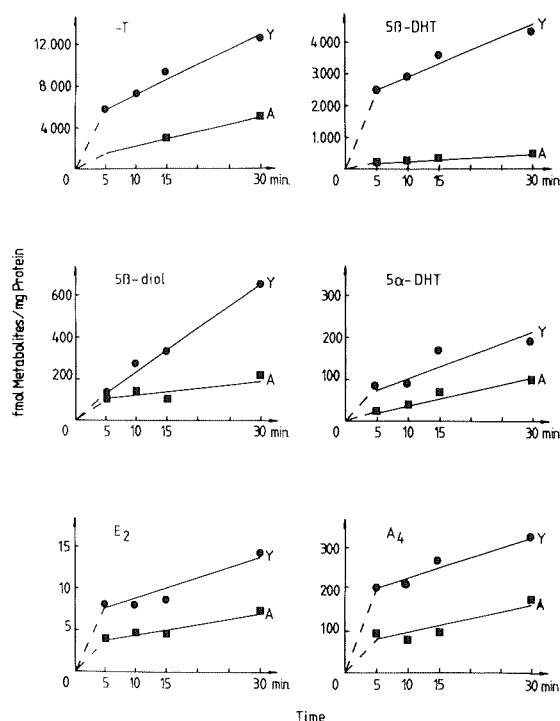


Fig. 1. Production of 5β-dihydrotestosterone (5β-DHT), 5β-androstane-3α,17β-diol (5β-diol), 5α-dihydrotestosterone (5α-DHT), estradiol (E₂) and androstenedione (A₄) and disappearance of testosterone (-T) during the incubation for different durations (5–30 min) of hypothalamic homogenates from young (●) and adult (■) zebra finches in the presence of tritiated testosterone (20 nM).

T metabolism in young birds is a lot faster during the first 5 min of incubation than later on. Production of 5β-DHT and disappearance of T are shown as an example. The extremely high activity of the 5β-reductase in newly hatched zebra finches metabolized about 50% of the substrate (tritiated T) during the first 5 min of incubation. After that time the substrate thus became a rate-limiting factor for 5β-reduction but also for the other T-metabolizing enzymes. This explains the problems encountered in interpreting Expt. 1. For all subsequent experiments (except the first experiment on substrate effect which was studied concurrently with Expt. 1), a duration of 5 min was thus selected for incubation.

Experiment 3

The brains of 6 adult and 9 young male zebra finches were homogenized in STMM buffer at a concentration of 35 mg fresh weight/ml (protein concentrations: 2.7 and 2.3 mg/ml). One hundred μl of these

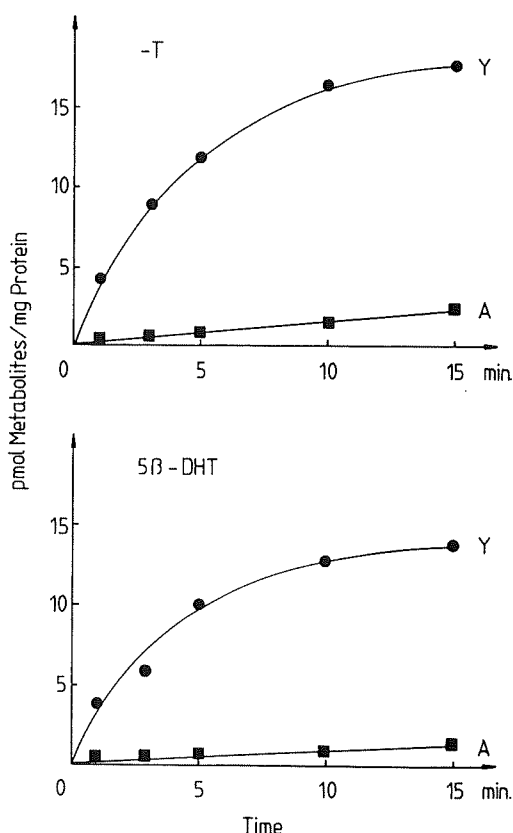


Fig. 2. Disappearance of testosterone (-T) and production of 5β-DHT during the incubation for short durations (1–15 min) of hypothalamic homogenates from young (●) and adult (■) zebra finches in the presence of tritiated testosterone (20 nM).

homogenates was incubated for 15 min at 41 °C with increasing concentrations of radioactive T in the range of 10 to 200 nM and NADPH₂ (1.2 mM).

Increasing amounts of the 4 major T metabolites were found with increasing concentrations of substrate. Some androstenedione was produced in the adult samples but not in the young at low concentrations of substrate indicating that too large a proportion of the substrate had been used (A₄ produced at the beginning of the incubation but then transformed back to T and then 5β-reduced, see above). This means that maximum velocities were probably underestimated in young birds. The kinetic parameters for the enzymes, estimated by double reciprocal plots (Lineweaver–Burke plots) are shown in Fig. 3.

The 4 plots were linear with correlation coefficients at least equal to 0.95. The maximum velocity of all reactions was higher in young than in adults.

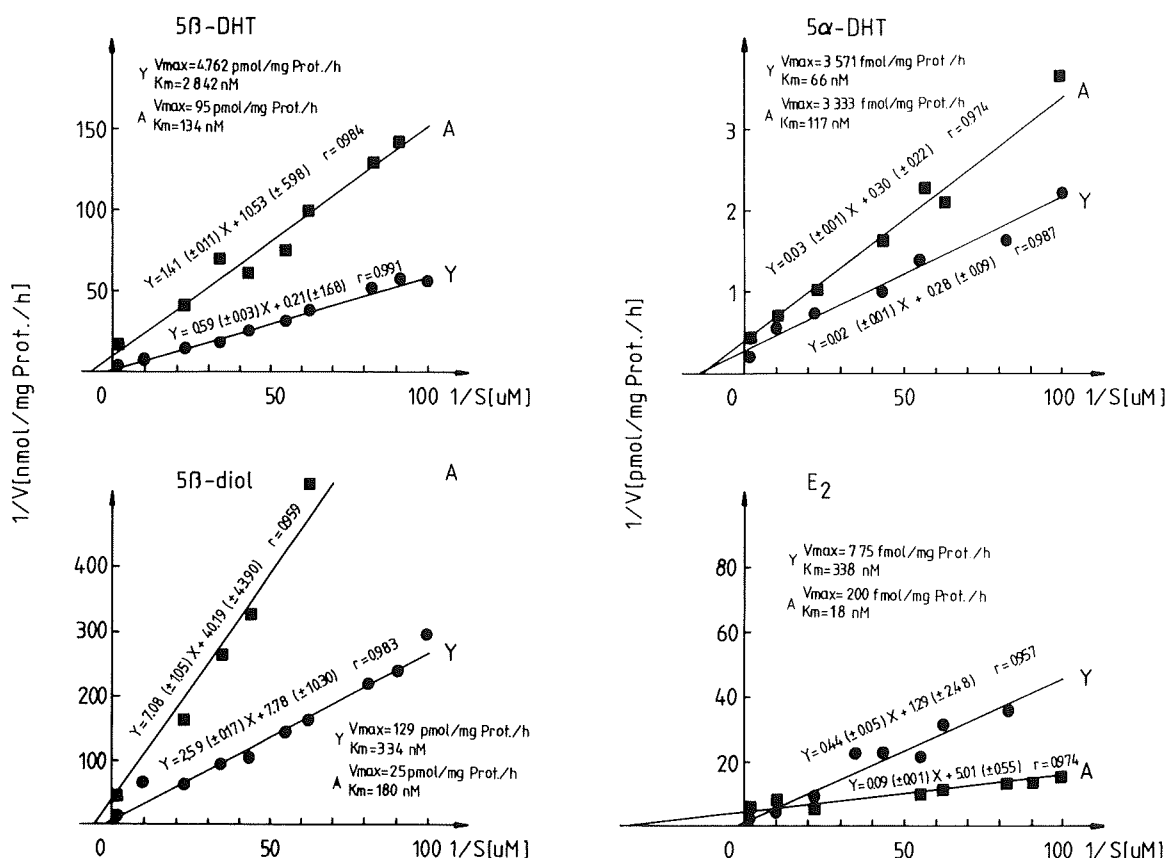


Fig. 3. Effects of substrate concentration on the productions of 5β-DHT, 5β-diol, 5α-DHT and E₂ by hypothalamic homogenates from adult (■) and young (●) zebra finches. Incubation time was 15 min and substrate concentrations were in the range 10 to 200 nM. The double reciprocal plots (1/V versus 1/S) are shown. Please note that different units were used for the right and left graphs to reflect very different velocities of the 4 enzymes.

This difference was especially large in the case of 5β-reduction. The k_m of the enzymes involved in 5β-reduction (production of 5β-DHT and 5β-diol) was much higher in young than adults (lower affinity in young). This difference was even more pronounced for the aromatase. The k_m and V_{max} of 5α-reductase were not markedly affected by age.

This experiment revealed major differences in enzyme characteristics (k_m , V_{max}) according to the age of the birds. The standard errors on the slope and intercept of the regression lines usually did not overlap for the adult and young data (see Fig. 3 for details). This suggested that the observed differences were real. However when the errors were calculated on the actual kinetic parameters (V_{max} and k_m), they were found to overlap extensively due essentially to their mathematical properties (errors are calculated on the inverse of the regression parameters which

enormously magnifies differences observed for small numbers). The differences which are pointed out here were, however, reproduced in Expt. 4 and it can thus be safely said that they represent real properties of the enzymes. In several cases, maturation seemed to be associated with decreased V_{max} and at the same time increased affinity of the enzymes for their substrate. In the last experiment we investigated at what age these changes occur and whether they are different in the two sexes. This also enabled us to confirm these changes of kinetic parameters in more adequate conditions (shorter incubation time).

Experiment 4

Brains from birds of both sexes with ages ranging between 5 and 85 days were used. The animals were divided into 12 groups according to their age and sex. Twelve brain homogenates were then prepared in

STMM buffer (concentration 20 mg fresh weight/ml) starting from the following numbers of birds: 3 males and 3 females aged 5–10 days (protein content of the homogenates: 1.19 and 1.28 mg protein/ml, respectively), 4 males and 3 females aged 10–15 days (1.59 and 2.21 mg protein/ml), 3 males and 2 females aged 20–25 days (1.92 and 2.01 mg protein/ml), 3 males and 3 females aged 30–35 days (2.13 and 2.10 mg protein/ml), 3 males and 2 females aged 40–45 days (1.98 and 1.70 mg protein/ml) and 4 males and 3 females aged 80–85 days (2.66 and 2.27 mg protein/ml). Sexual maturation occurs between 70 and 80 days in zebra finches³¹. These 12 homogenates were incubated for 5 min at 41 °C with increasing concentrations of radioactive T in the range of 20–160 nM in the presence of NADPH₂ (1.2 mM). The maximum velocity of the enzymes and their affinity constant were evaluated for the different ages and sexes by double reciprocal plots. All these plots were linear. From the standard errors on the slope and intercept of these linear regressions, the errors on $V_{\max}$ and k_m were calculated (see Table II). Correlation coefficients calculated on the production of 5 β -DHT, 5 β -diol and E₂ were always higher than 0.92 (usually above 0.98) with only one exception in the

case of aromatase (0.86 in 40–45-day females). For some unknown reason, the correlations were always lower for 5 α -DHT and were in the range of 0.84 to 0.96. Some examples of these plots are given in Figs. 4 and 5. A summary of all kinetic parameters and their associated errors is presented in Table II.

All the differences in enzyme velocities and affinities which had been discussed in Expt. 3 were reproduced in this last experiment. In both sexes the $V_{\max}$ of the 5 β -reductase progressively decreased with age while the affinity was progressively increasing. The change in $V_{\max}$ was especially striking (from about 4000 down to about 200 pmol/mg protein/h depending on the sex of the birds; see Table II for details). There was some decrease with age in the maximal production of 5 β -diol but this is probably only related to the decreased availability of the substrate (5 β -DHT) for this reaction. The k_m of the 3 α -hydroxysteroid dehydrogenase (which reduces 5 β -DHT into 5 β -diol) did not systematically change with age. This experiment also confirmed the decrease of aromatase activity ($V_{\max}$) with age and the corresponding increase of the enzyme affinity for the substrate. Contrary to what had been observed for the 5 β -reductase, these changes did not take place progressively

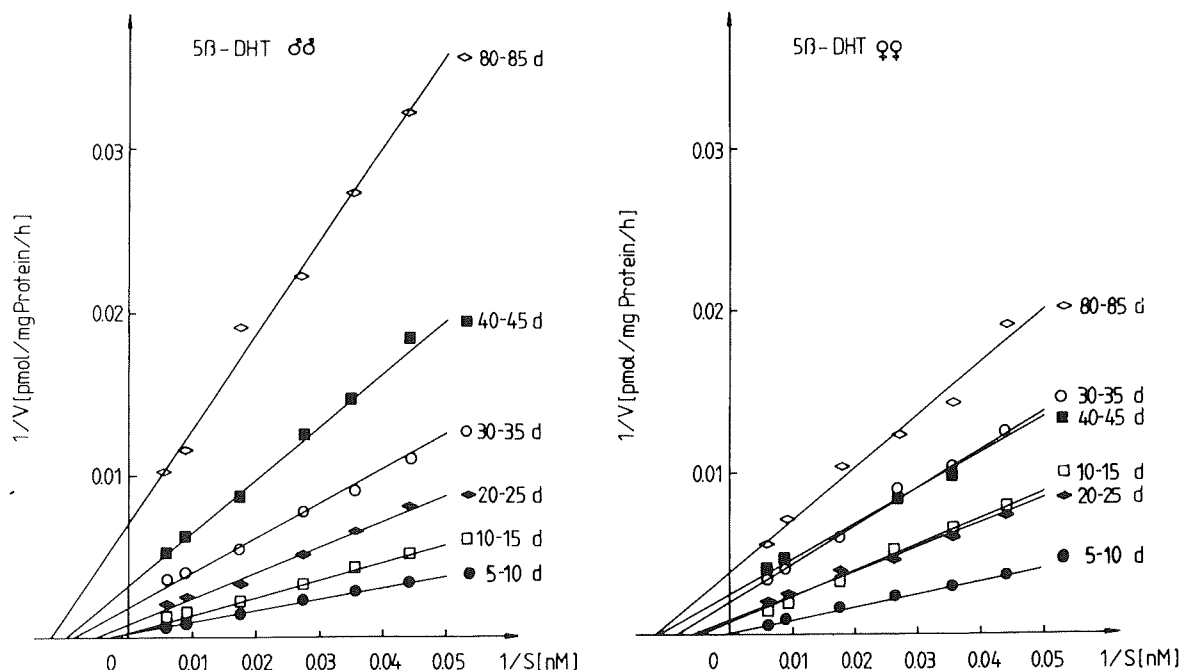


Fig. 4. Double reciprocal plots of the production of 5 β -DHT versus substrate concentration in male and female zebra finch hypothalamus of different ages between 5 and 85 days (d).

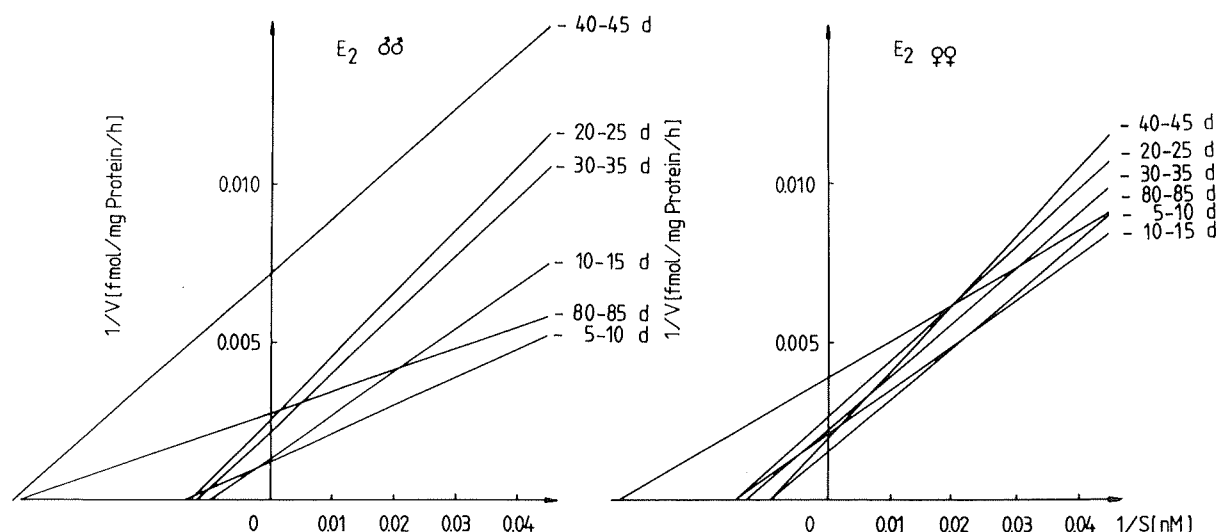


Fig. 5. Double reciprocal plots of the production of E_2 versus substrate concentration in male and female zebra finch hypothalamus of different ages between 5 and 85 days (d). The actual experimental points have been omitted to preserve clarity of the drawing.

with age (compare Figs. 4 and 5) but occurred abruptly when birds initiated their sexual maturation (at 40 days of age or later). No systematic change of V_{max} and k_m for the 5α -reductase could be detected: some decrease of these parameters seemed to occur with age but for the technical reason mentioned above (lower correlation on the linear regression),

this suggestion must be considered cautiously.

DISCUSSION

In this paper, we have described and validated a radioenzyme assay which can be used to study T-metabolizing enzymes in the brain of zebra finches of

TABLE II

Estimation of the maximum velocity (V_{max}) and the affinity constant (k_m) of T-metabolizing enzymes in the brain of male and female zebra finches at different ages between 5 and 85 days post-hatch

Kinetic parameters were estimated by linear regression on double reciprocal plot and their errors were derived by the formula described in the Methods. V_{max} is expressed as pmol/mg protein/h except in the case of E_2 where it is in fmol/mg protein/h, and k_m is expressed in nM concentrations.

Age post-hatch (days)	E_2		5α -DHT		5β -DHT		5β -diol	
	V_{max}	k_m	V_{max}	k_m	V_{max}	k_m	V_{max}	k_m
Males								
5-10	833 $\pm$ 215	75 $\pm$ 29	15 $\pm$ 16	128 $\pm$ 180	3982 $\pm$ 1048	281 $\pm$ 84	171 $\pm$ 34	176 $\pm$ 41
10-15	784 $\pm$ 244	109 $\pm$ 45	12 $\pm$ 4	73 $\pm$ 23	3571 $\pm$ 1699	388 $\pm$ 202	81 $\pm$ 37	180 $\pm$ 103
20-25	394 $\pm$ 139	79 $\pm$ 42	21 $\pm$ 9	264 $\pm$ 138	1176 $\pm$ 374	184 $\pm$ 70	63 $\pm$ 17	170 $\pm$ 56
30-35	481 $\pm$ 69	92 $\pm$ 18	6 $\pm$ 3	56 $\pm$ 48	571 $\pm$ 101	124 $\pm$ 29	53 $\pm$ 12	93 $\pm$ 20
40-45	138 $\pm$ 10	24 $\pm$ 4	11 $\pm$ 20	284 $\pm$ 566	302 $\pm$ 23	98 $\pm$ 10	36 $\pm$ 15	135 $\pm$ 69
80-85	369 $\pm$ 16	29 $\pm$ 3	5 $\pm$ 2	69 $\pm$ 53	137 $\pm$ 16	79 $\pm$ 13	33 $\pm$ 28	159 $\pm$ 138
Females								
5-10	495 $\pm$ 95	64 $\pm$ 21	17 $\pm$ 12	131 $\pm$ 129	4673 $\pm$ 1712	368 $\pm$ 148	156 $\pm$ 69	289 $\pm$ 129
10-15	368 $\pm$ 38	66 $\pm$ 7	10 $\pm$ 6	174 $\pm$ 143	1124 $\pm$ 112	175 $\pm$ 21	48 $\pm$ 19	140 $\pm$ 56
20-25	633 $\pm$ 184	106 $\pm$ 44	12 $\pm$ 9	128 $\pm$ 122	1042 $\pm$ 253	156 $\pm$ 47	130 $\pm$ 37	191 $\pm$ 74
30-35	444 $\pm$ 146	76 $\pm$ 36	10 $\pm$ 8	143 $\pm$ 138	474 $\pm$ 65	111 $\pm$ 20	58 $\pm$ 9	132 $\pm$ 21
40-45	493 $\pm$ 723	105 $\pm$ 199	16 $\pm$ 7	274 $\pm$ 139	394 $\pm$ 27	81 $\pm$ 21	74 $\pm$ 30	243 $\pm$ 117
80-85	252 $\pm$ 14	29 $\pm$ 4	7 $\pm$ 2	92 $\pm$ 34	251 $\pm$ 45	87 $\pm$ 9	60 $\pm$ 15	120 $\pm$ 38

different ages from hatching to sexual maturity. Essentially, the same technique as previously used in Japanese quail was used with the exception that a much shorter incubation time had to be selected. This is due to the presence of an extremely active 5β -reductase activity in zebra finches and especially in the newly hatched birds. This high reductase activity very quickly decreases the substrate concentration in the incubation tubes so that reactions deviate from linearity. It is only during the first 5 min that reasonable approximations of the true enzyme velocities can be obtained. The identity of the T metabolites formed in zebra finches could be confirmed in both sexes and at different ages by successive recrystallizations to constant specific activity or constant isotopic ratio.

The k_m of the 5α -reductase and of the aromatase evaluated here in adult zebra finches are in the same order of magnitude as those observed earlier in quail³⁰. The apparent k_m for the 5β -reductase is substantially lower here (about 80 nM) in adult zebra finches than in quails (about 500 nM). However as mentioned earlier, the kinetic parameters of 5β -reductase were measured here at relatively low substrate concentrations far away from saturating levels so that the results suffer from some imprecision. In an earlier study in which we had measured the k_m of 5β -reductase in the presence of saturating concentrations of radioactive T, a value of 588 nM had been obtained⁵. It thus appears extremely likely that all 3 enzymes have very similar affinities in both species and the observed discrepancy only reflects a procedural artifact.

By contrast, important species differences are observed in the maximum velocities of the enzymes. The 5α - and 5β -reductase are more active in adult zebra finches (about 3 and 150 pmol/mg protein/h, respectively) than in quails (about 1.5 and 20 pmol/mg protein/h) while the aromatase demonstrates a higher activity in the latter species (2.4 and 0.3 pmol/mg protein/h in quail and zebra finch, respectively). Even the relative activities of these enzymes are different in these two species: in zebra finches the 5α -reductase is much more active than the aromatase while in quail the reverse relationship is observed.

One of the most striking features of this study is the very low level of aromatase activity which was detected in the zebra finch brain. We tested the reality

of this finding during one experiment in which hypothalami of both quail and zebra finch were studied simultaneously. When tested in identical conditions the enzyme activity was 5 times lower in zebra finch than in quail (data not presented here). This low activity of the aromatase in zebra finch could possibly be explained by the relatively lower plasma levels of testosterone found in this species by comparison with quail (in adult males: around 300 pg/ml usually in zebra finches with occasional maximum at 1 ng/ml, between 1 and 3 ng/ml in quails^{6,23,24,27,28}). It is well established that in quails and in ring doves, testosterone increases the activity of the aromatase in the hypothalamus^{29,33} and preliminary evidence suggest that the same applies to zebra finches. Future experiments should establish whether it is possible to increase aromatase activity of zebra finches to levels comparable to those seen in quail by treating the birds with high levels of exogenous testosterone.

The low activity of the aromatase, however, fits in well with the supposed actions of E_2 and the available data on peripheral levels of this hormone. In the very young birds, plasma levels of E_2 are high in the male and this hormonal stimulus is thought to be responsible for the masculinization of reproductive behavior and of brain structures implicated in its control^{14,19,26}. By comparing the period with elevated plasma E_2 and the sensitive period for masculinization it could be speculated that peripheral hormones are perhaps not providing the full stimuli needed for sexual differentiation. From the present study it seems likely that central aromatization of T in the hypothalamus does not substantially contribute to this process. The presence of extremely high levels of 5β -reductase activity which is likely to inactivate most of the endogenous testosterone makes the contribution of central aromatase even less probable (see ref. 5 for further discussion). Enzyme activities in the song control nuclei should, however, be quantified in order to validate this conclusion.

In adult finches, Harding and collaborators¹⁶ showed that E_2 as well as androstenedione (A4) activate some aspects of reproductive behavior and that the effects of A4 can be blocked by concurrent administration of the aromatase inhibitor, androstatrienedione (ATD). Considering the very low levels of aromatase found in the hypothalamus of adult finches in the present study, it would be tempting to

speculate that either these behavioral effects of A4 take place in another brain region (e.g. limbic structures) or that A4 aromatization occurs in the periphery and then the resulting E_2 reaches the hypothalamus to activate sexual behavior. This, of course, does not exclude that the very low levels of aromatase found in the hypothalamus play a significant role in behavioral activation and only additional studies using brain implants of E_2 , A4 and ATD will give a clear answer to this question.

No important sexual differences in enzyme activities nor in enzyme affinities were detected in the present study. It must, however, be remembered that only large brain areas were studied in pools of animals. It is therefore quite possible that sexual differences would be observed if more limited brain regions were studied in a large number of individuals.

With the exception of 5α -reductase, all enzymatic activities were markedly affected by the age of the birds. It has been shown previously in quail that during the ontogenetic development the 5β -reductase activity decreases while the aromatase increases^{3,4,18}. In this species, however, the maximum velocity of the enzymes changed with age but their affinity was not modified at least during post-hatching life (embryonic data on k_m are not available). By contrast, in the zebra finch both V_{max} and k_m of the aromatase and of the 5β -reductase decrease during the first two months after hatching. It must be remembered that the zebra finch is an altricial species while the quail is precocial so that, at hatching, the maturation of their brain is quite different. Further studies should characterize the k_m of these enzymes in embryonic quail in order to evaluate whether this discrepancy is a true species difference or only reflect differences of maturation at the time of hatching.

Very interesting is also the fact that aromatase activity (V_{max}) increases very much with age in the quail¹⁸ while it remains stable or even decreases in the zebra finch. Once again, this could be related to the presence of high peripheral levels of E_2 which reduces the need for central aromatase and/or possibly to the absence of stimulation of the enzyme activity by testosterone (see above).

The enzyme kinetics observed here in adult and young birds which demonstrate differences in apparent k_m and V_{max} are complex to interpret. They could reveal the presence of non-competitive inhibitors of the enzymes or alternatively suggest that during maturation there is a change in the type of enzyme catalyzing the reaction. In any case, these ontogenetical changes in enzyme properties make it impossible to compare directly results of one point assays made on brain from different ages. An increased production of metabolites can indeed result from a change in enzyme concentration or in enzyme apparent affinity.

Finally it is interesting to observe that the developmental changes in the properties (k_m and V_{max}) of the 5β -reductase and of the aromatase were not parallel in time. The largest decrease of k_m and V_{max} for the 5β -reductase occurred between 5 and 20 days of age while the k_m of the aromatase remained stable until about 40 days and then dropped rapidly. The decrease of V_{max} for this enzyme was however more progressive. This difference between the enzymes suggests that the mechanisms which control their ontogenetical differentiation are quite different. The major change in aromatase activity occurred at a time when birds begin their sexual maturation as evidenced by molting into adult plumage and change of beak color from black to red³¹. It is thus likely that this enzymatic change is controlled by the hormonal events associated with puberty. Other causal factors must, however, be invoked to explain the maturation of the 5β -reductase system which occurs much earlier in life. Castration and steroid replacement therapies are now in progress in order to investigate the hormonal control of the activity of these enzymes.

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