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Gonadal Reaction in the Tree-Frog Larvae (*Hyla arborea japonica* Guenther) to the Androgen[1]

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(*With 1 Plate*)

Many contributions have been made on the sexual modification of anurans induced by steroid hormones. From these reports, as reviewed by Gallien (1955), it has been recognized that there is a certain variance in the type of reaction of the gonads to administered sex hormones according to taxonomic species. A group centering in Ranidae is characterized by their high sensitivity to androgens, which results in complete and permanent masculinization of genetic females (cf. Gallien 1955). This group shows also either temporary feminization or complete masculinization when treated with estrogens in various dosages. The other group, which includes lower anuran forms such as *Discoglossus*, *Bufo*, *Alytes* and *Xenopus*, on the other hand, shows a marked contrast with the former group in that the androgens scarcely interfere with sex differentiation of the female (Gallien 1950, 1956, Witschi & Allison 1950, Witschi 1951, Chang 1955).

So far as the writer is aware, Witschi (1931) is the first worker to perform experimental work on sex differentiation of the tree-frog. He states that Hylidae frogs belong to the first group in regard to gonadal reaction to sex hormones, showing similar type of the free-martin effect as in Ranidae frogs. However, experimental study on the result of administration of sex hormones has never been made on *Hyla*, although a number of works have been performed on *Rana*. In this paper the writer reports the effect of methyltestosterone on sex differentiation in *Hyla arborea japonica*.

Before going further the writer wishes to express his sincere appreciation to Professor Tohru Uchida for his invaluable criticism given throughout the course of the work. Acknowledgements are also due to the Teikoku Hormone Mfg. Co., Tokyo, whose donation of crystalline hormones enabled this work to be done.

Material and method

The species used is the tree-frog, *Hyla arborea japonica*, which is distributed widely in Japan. The preliminary work was done with the larvae soon after hatching. They were collected from a pond near Sapporo in 1955. The experiments were repeated twice in the following years, using larvae of a single spawn obtained naturally in the laboratory in each year. There were no essential differences between the results obtained in above

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three years. Methyltestosterone (Teikoku Hormone Mfg. Co., Tokyo) was dissolved previously in ethyl alcohol or in a mixture of ethyl alcohol and propylene glycol as solvents, which were proved non-effective in inducing any modifications of the gonadal development. The solution was then added to the aquarium water in desired dosages (0.01 to 2 mg per litre). In 1955 the administration was commenced on the seventh or eighth day after hatching, as presumed from body length of the collected larvae, and continued to the end of metamorphosis. At that time the animals were fixed with Bouin's or Zenker's fluid. In 1956 and 1957, the larvae just after hatching received the treatment over the period of 55 and 44 days respectively. The experimental and control tadpoles were reared under identical conditions in a room temperature ranging from 20° to 25°C, each aquarium water being changed every second day. Completion of metamorphosis followed about 60 days after hatching in these conditions. Mortalities in the experimental lots were 5.0 to 17.5% in 1957 and somewhat higher in the preceding two years.

For histological examinations the gonads and mesonephros were serially sectioned in 10μ thickness, and stained with Delafield's hematoxylin and eosin. In total 171 control and 320 experimental animals were examined in the course of the study.

Normal sex differentiation

In the 11-day old larvae, 14 mm in length, yolk platelets in the primordial germ cells are almost absorbed and mitotic division of the germ cells takes place actively. At the 18th day after hatching, 18 mm in total length, the indifferent gonads are formed, with the cortex consisting of unilayered germ cells and the medulla presented as about six rete cords in longitudinal section. Differentiation cf the testes is first observed in the tadpoles of 23 mm total length. The germ cells in the cortical region migrate into the bulky medulla, thus forming the juvenile testes. In the metamorphosed males the testes exhibit the primordial seminal tubules.

In the females, ovarian differentiation is noticed somewhat later. At the 30th day after hatching, 35 mm in total length, premeiotic oocyte-nests are found in the cortex. However, ovarian sacs are not observed at this stage. They put in an appearance in the animals at late metamorphic period. Their enlargement, as well as the time of appearance, is also markedly slow when compared with other anuran species. Even in the nearly metamorphosed females, the sacs are left rather small. Nevertheless, there is a rapid development of the cortex in which some oocytes enter into the auxocyte stage. In the just-metamorphosed frogs collected near a pond, the distention of the ovarian sacs in each gonomere is not so high as in *Rana temporaria*.

Deal (1931) and Witschi (1931) reported many melanophores in the testes but none in the ovaries of *Hyla crucifer* and *H. regilla*. They regarded the presence of melanophores as the distinctive sex character. These testicular melanophores migrate from the dorsal renal capsule into the testis together with invading rete blastemata. In *Hyla arborea japonica*, the melanophores exist in association with the medullary tissue in the testes or with the rete cells preserved in the central portion of the juvenile ovaries. Thus the melanophores can be observed even in the ovaries of some metamorphosing females. Further-

more, the pigmentation is not so marked as to make the gonads dark externally. The melanophores are, in some cases, completely absent even in the testis. Accordingly, in this species it is impossible to determine the sex by the pigmentation of gonad.

Experimental results

As seen from the preceding description, the species used is of a sexually differentiated type. At late metamorphosis, both sexes are clearly distinguishable anatomically. Histologically, in the normal males, the testes were in the initial stage of tubule formation at this stage (Pl. III, Figs. 1, 2). The ovaries generally consisted of small auxocytes, premeiotic oocyte- and oogonia-nests (Pl. III, Figs. 3, 4). As already mentioned, the ovarian sacs made an appearance in some ovaries but there was no formation yet in others (Pl. III, Figs. 3, 5). Sometimes the medullary tissue was seen persisting in centroproximal part of the ovaries as a small but compact mass (Pl. III, Fig. 6).

In the animals treated with methyltestosterone, as shown in Tables 1 and 2, complete sex inversion was on no account caused in all the dosage levels used.

Table 1. Effect of methyltestosterone on gonadal development in
Hyla arborea japonica studied in 1955 and 1956

Group		Sex distribution*			Total
		♀ (PA)	♀ (P)	♂	
Methyltestosterone 200 μg/1	'56	1	14	15	
			(15)	(15)	(30)
400 μg/1	'56	8	1	14	
			(9)	(14)	(23)
500 μg/1	'55	2	5	9	
			(7)	(9)	(16)
600 μg/1	'56	1	14	19	
			(15)	(19)	(34)
1000 μg/1	'55	8	5	13	
		2	14	19	
			(29)	(32)	(61)
2000 μg/1	'56	6	8	9	
			(14)	(9)	(23)
Control	'55	21	16	34	
	'56	2	28	32	
			(67)	(66)	(133)

* Animals possessing ovaries with auxocytes are designated by ♀(PA), those with no auxocyte by ♀(P), and normal males by ♂.

Table 2. Sex distribution in the groups studied in 1957

Group	Sex distribution*		Total	Mortality (%)
	$♀_1\ ♀_2\ ♀_3$	$♂$		
Methyltestosterone 10 μg/1	9 7 1	19		
	(17)	(19)	(36)	5.0
100 μg/1	12 7 3	15		
	(22)	(15)	(37)	7.5
500 μg/1	8 7 1	17		
	(16)	(17)	(33)	17.5
1000 μg/1	14 4 0	18		
	(18)	(18)	(36)	10.0
Control	15 7 0	· 16		
	(22)	(16)	(38)	2.5

* Animals with well-developed ovaries are referred to as $♀_1$, those with ovaries retarded in development as $♀_2$, and those with ovarian medulla including a few germ cells as $♀_3$. $♂$ shows normal males.

More or less unevenness of gonadal development occurred in the experimental lots. But this was also the case in the controls. Moreover, no essential difference in the effect was detected among the treated groups of the different dosage levels.

In the treated males, there were no signs of accelerating the differentiation and development of the testis (Pl. III, Figs. 7, 8). Contrarily, in some males treated with 500 μg or more of methyltestosterone per litre of rearing water, the testes had reduced number of germ cells, showing rather arrested growth in respect to size.

In the treated females, the cortical germ cells differentiated as oocytes, sometimes attaining the auxocyte stage even under the influence of the hormone administered (Pl. III, Figs. 13, 16), as shown in Table 1.   When treated with intensive dosages of methyltestosterone, the ovaries were somewhat smaller in size than the normal ones. Histologically, however, cortical development in the affected ovaries was not so retarded as to reveal distinctly a suppressive influence of the treatment, though the germ cells were reduced in number.   In some influenced ovaries, underdevelopment of the premeiotic oocyte-nests was observed. But frequency of occurrence of such a type of ovary had no correlation to the elevation of the dosages (Table 2).   The ovarian sacs were defective in all the affected ovaries.   The region of the sacs appeared as if they were compressed by surrounding cortical elements in some parts (Pl. III, Fig. 14), or were segmentarily packed with rete cells in others (Pl. III, Figs. 9, 15).   Occasionally the medullary tissue was more bulky in the affected ovaries than that preserved in the normal

ones.   Nevertheless there appeared no clear relation between the state of medullary tissue and that of cortical differentiation.   Degenerative changes of the cortical elements were rare both in the affected and in the control ovaries.

As shown in Table 2, a few of the affected ovaries showed an irregular gonado-genesis which was suggestive of intersexual phase (Pl. III, Figs. 10, 11).   For the most part, the gonads were small in size and somewhat retarded in cortical differ-entiation.   In a certain section of these gonads, the rete mass took the centro-proximal part, in which a few germ cells were surrounded by the rete cells.   some-times such a feature was observed in the cranial region of the gonad, and the usual ovarian structure was maintained in the caudal region (Pl. III, Figs. 11, 12).   In general, the feature was found only in a certain gonomere.   There is cephalo-caudal gradient in the normal gonadal differentiation in this species.   But the above structure was found in a part of the gonad without any regularity in po-sition.

The fat bodies were well developed in the experimentals as well as in the controls, and examination of the mesonephros indicated no notable difference between the treated and the control animals.

To exclude any doubt of invalidation of methyltestosterone by an un-known factor, some of the 20 day-old tadpoles received injections of testosterone propionate (Enarmon, Teikoku Hormone Mfg. Co., Tokyo) of total $120\mu g$ per animal.   At the 18th day after the first injection the larvae were sacrificed, and the histological examination of the animals gave almost the same results as those already described.   Rearing the larvae of the present species in alcoholic solution of testosterone propionate ($500\ \mu g/1$) also gave the same results after 40 days of treatment.

Discussion

It is obvious from the facts mentioned already that, at least under the present experimental conditions, methyltestosterone failed to bring about com-plete sex inversion in *Hyla arborea japonica*.   It has been well recognized that the result of a given hormone upon gonadal differentiation may vary depending on dosage, time and duration of treatment, and taxonomic species.   In the present species, no apparent difference in the result was produced among the dosages used. All the present treatment was started before the sex differentiation of gonad had taken place, and continued over sufficient period to cause the sex reversal in Ranidae frog tadpoles.

In *Rana temporaria*, methyltestosterone causes complete masculinization of genetic females in a very weak dosage of $8\mu g/1$ (Takahashi unpubl.).   In *Rana sylvatica*, complete masculinization is attained by $2\mu g/1$ of testosterone propionate (Mintz & Witschi 1946). The five-days treatment of *R. sylvatica* with 50 $\mu g/1$ of testosterone propionate is enough to induce complete masculinization if the treatment is performed during the period of sex differentiation (Witschi 1948).   Compared with the facts above quoted, *Hyla arborea*

japonica seems to be not necessarily similar to Ranidae frogs in the type of response of gonad to androgen from without.

In 1931, Witschi performed heterosexual parabiosis in *Hyla regilla*. The result was the same as that obtained in parabiotic twins of *Rana sylvatica* and of *R. aurora*, the testicular medulla of the male partner suppressing the ovarian cortex of the female co-twin and thus inducing the sex reversal. In the present study, the degree of the testicular development is almost equal to that of the normal animal. The ovaries, however, are a little undersized when affected by intensive dose of methyltestosterone. Even in these ovaries, the process of oogenesis is not hindered and the auxocyte formation occurs in the usual manner. Reduction in the number of germ cells may be one of the signs of cortical inhibition, but it is still of slight degree.

While the cortical maturation proceeds almost normally, the affected ovaries were lacking in the ovarian sacs. Sometimes, the region is occupied instead by medullary cells. Maintenance of such medullary tissue does not, however, lead the ovary to intersexual phase. Exceptionally, a feature like intersexual one was observed in some sections of the affected ovaries. As already mentioned, even in the normal case, the medullary tissue of the indifferent gonad remains instead of the ovarian sacs till the larvae reach to the late metamorphosis. The treatment may cause the medullary tissue to be held longer in the affected ovaries than in the normal ones. In other words, the treatment may hinder the differentiation of medullary tissue as lining layer of the ovarian sacs. Thus it seems probable that a few germ cells, which are in close contact with the medulla in the underdeveloped ovary, became enclosed with the rete cells and in consequence the gonad presents the intersexual feature.

It is interesting that the effect has, in a certain respect, resemblance to the case in Bidder's organ of the toad. Upon administrating 0.1 mg/1 of testosterone to *Bufo viridis* larvae, Vegni Talluri and Padoa (1953) found that the medullary tissue in Bidder's organ becomes compact and fills in the rudimentary ovarian cavity without bringing about inversion in the ovarian nature of the organ. But the development of the organ is generally inhibited by treatment with androgens, as is revealed in reduction in size of the organ (Vegni Talluri & Padoa 1953, Chang 1955, Takahashi 1956). In *Hyla arborea japonica*, at least under the conditions of the present study, the cortical development appears to be not likely to suffer serious influence by the administration of methyltestosterone. Consequently, it seems probable that compensatory potency of the medulla which gives rise to masculinization cannot be released effectively.

Cases in which male hormone has no masculinizing effect on gonad have been found in *Alytes obstetricans* by Witschi and Allison (1950) and Witschi (1951), in *Discoglossus pictus* and *Xenopus laevis* by Gallien (1950, 1956), and in *Bufo americanus* by Chang (1955). In these species, however, medullary suppression is more or less noted. The type of reaction of the gonad to androgen in the present species differs also from that in the above species, because the medullary development is not intensely influenced by the treatment.

In conclusion, while androgen treatment cannot cause complete masculiniza-

tion, larval *Hyla arborea japonica* has a potentiality to invert its sex toward masculinization. In this respect, the reaction to androgen in this species can be said to be *Rana* type. But to the writer, it is of great interest that the persistency of the essential nature of the ovary is rather suggestive of the lower anuran type.

Summary

The larvae of the tree-frog, *Hyla arborea japonica* were reared in water solution of methyltestosterone ranging in dosage from 10 to 2000 μg per litre cf the rearing water. The treatment failed to cause either complete masculinization or feminization in all the dosage levels used. The testes differentiated almost normally. The ovarian cortex developed without suffering serious suppression by the treatment, though the ovaries were somewhat reduced in size. The affected ovaries were lacking in the ovarian sacs, and instead the medullary tissue was present in the region. Maintenance of the medulla was, however, not so intensive as to lead the gonad to an intersexual phase. It seems to be appropriate to consider that the treatment could not induce intensive cortical inhibition sufficient to cause masculinization of the gonad.

Literature

Chang, C.Y. 1955. Hormonal influences on sex differentiation in the toad, *Bufo americanus*. Anat. Rec. 123 : 467–485.

Deal, R.E. 1931. The development of sex characters in the tree frog. Anat. Rec. 48 : 27–45.

Gallien, L. 1950. Étude de quelques facteurs intervenant dans l'action des hormones sexuelles au cours de la différenciation du sexe chez les amphibiens anoures. Bull. Biol. 84 : 341–375.

———— 1955. The action of sex hormones on the development of sex in amphibia. Mem. Soc. Endocr. 4 : 188–204.

———— 1956. Inversion expérimentale du sexe chez un anoure inférieur, *Xenopus laevis* Daudin. Bull. Biol. 90 : 163–184.

Mintz, B. & E. Witschi 1946. Determination of the threshold dose of testosterone propionate inducing testicular development in genetically female anurans. Anat. Rec. 96 : 526–527.

Takahashi, H. 1956. The effects of some sex hormones on the development of Bidder's organ in *Bufo vulgaris formosus* Boulenger. Jour. Fac. Sci. Hokkaido Univ., Ser. VI, Zool. 12 : 297–308.

Vegni Talluri, M. & E. Padoa 1953. L'organo del Bidder nei girini di *Bufo viridis* trattati con testosterone. Monit. Zool. Ital. 61 : 186–197.

Witschi, E. 1931. Studies on sex differentiation and sex determination in amphibians. V. Range of cortex-medulla antagonism in parabiotic twins of Ranidae and Hylidae. Jour. Exp. Zool. 58 : 113–145.

———— 1948. Geschlechtsbestimmung durch Chemikalien. Julius Klaus Archiv. 23 : 525–538,

———— 1951. Hormonal modification of sex differentiation in the obstetric toad, *Alytes obstetricans*. Anat. Rec. 109 : 389–390.

Witschi, E. & J. Allison 1950. Responses of *Xenopus* and *Alytes* to the administration of some steroid hormones. Anat. Rec. 108 : 589–590.

Explanation of Plate III

All figures show cross sections of gonad. ×240.

Fig. 1. A testis of control animal at late metamorphosis.

Fig. 2. A testis of control animal at end of metamorphosis.

Figs. 3, 5–6. Ovaries of control animals at late metamorphosis. In Fig. 3 small ovarian cavity is seen, whereas in Fig. 6 rete cell mass exists instead in the region.

Fig. 4. A ovary with auxocytes of control animal at end of metamorphosis.

Fig. 7. A testis of animal treated with 10 μg/1 of methyltestosterone.

Fig. 8. A testis of treated animal of 100 μg/1 group.

Figs. 9–12. Ovaries of treated animals of 100 μg/1 group. Figs. 10 and 11 showing the proximal rete mass in which a few germ cells are enclosed. Fig. 12 showing a section of caudal part of the same gonad as shown in Fig. 11.

Fig. 13. A ovary of treated animal of 400μg/1 group.

Figs. 14, 15. Ovaries of treated animals of 600μg/1 group.

Fig. 16. A ovary of treated animal of 1000 μg/1 group, revealing the medullary tissue persisting in the proximal region.
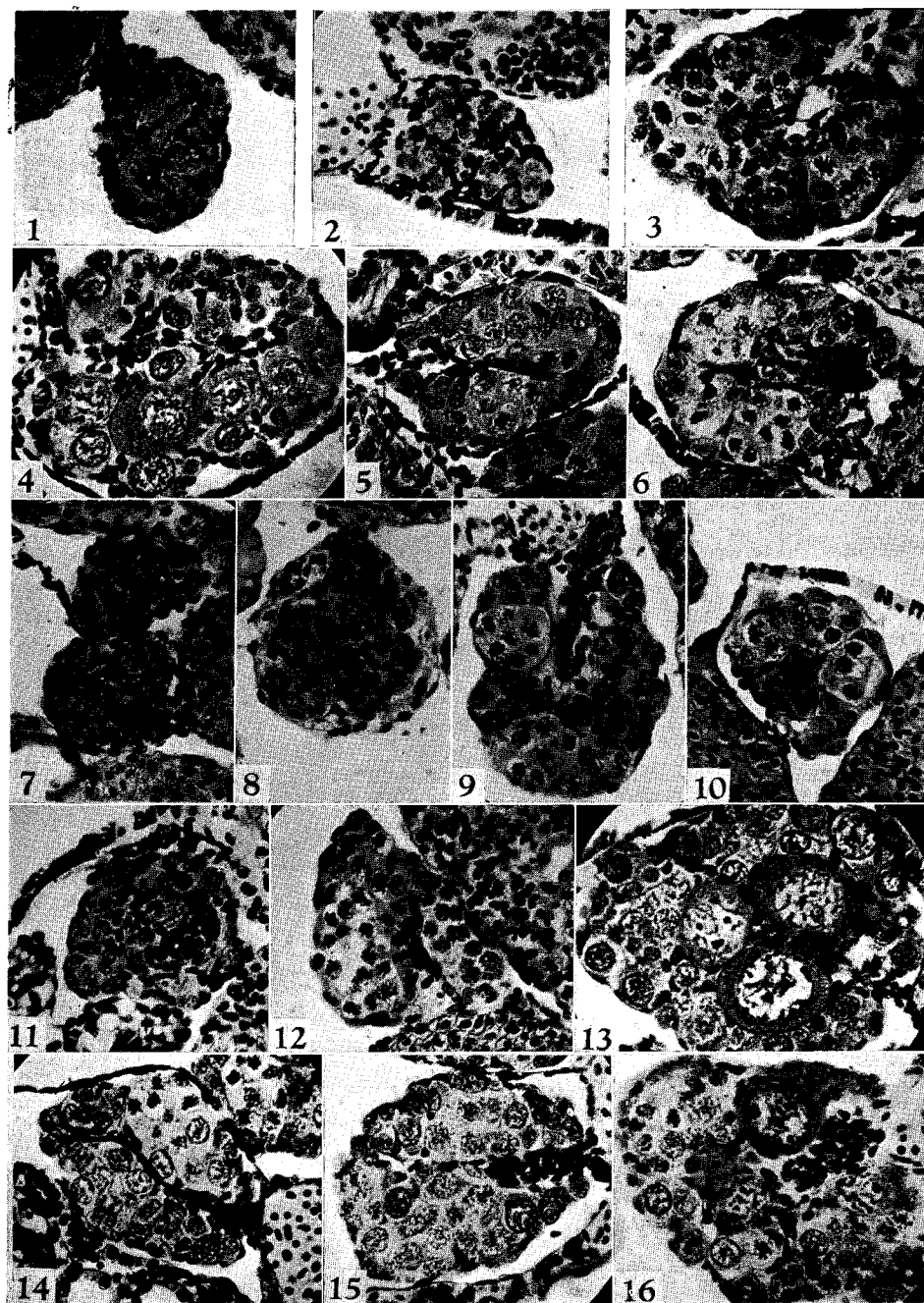
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