FUNCTIONAL SEX-REVERSAL IN GENETIC FEMALES OF XENOPUS LAEVIS, INDUCED BY IMPLANTED TESTES[1]

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IN his thesis, CHANG (1953) reports that testes implanted into female larvae of the frog Xenopus suppress ovarian development, and that initial cortical inhibition sometimes is followed by testicular differentiation of one or several gonomeres. These experiments have been taken up again with the aim of studying the morphologic effects induced by testicular implants, the sexual behavior of converts, their reproductive capacity, and their genetic constitution. Regarding the last point, it is already known that ordinary female larvae are heterozygous with respect to sex-determining genes. Proof of this has been obtained from breeding experiments (CHANG and WITSCHI 1955a, 1956; GALLIEN 1955, 1956). Recently reported cytologic evidence confirms that Xenopus belongs to the ZZ = male and ZW = female chromosomal type (WEILER and OHNO 1962).

Foremost remains the question whether the testicular nodes of sex-reversed females retain the ZW constitution, or assume a new one. The latter alternative could conceivably arise through migration of germ cells from the implant to the larval gonads or by chromosomal modifications. The observational material is not yet large enough to certify statistically that sex-reversal through testis implantation has occurred in CHANG's or in our own experiments. Obviously, unequivocal evidence can be obtained only by breeding tests, such as will be described in the following pages. By raising experimental animals to the age of maturity, mating behavior and fertility of sex-reversed animals may also be studied.

MATERIALS AND METHODS

Testis implantation was performed when normal larvae reached the late stage 25 (standard stages after WITSCHI 1962); that is, shortly before the beginning of sexual differentiation about three weeks after fertilization. A group of 31 hosts was obtained from the offspring of a normal male (ZZ) and a normal female (ZW). Donors were chosen from a similar but older set, two months after metamorphosis. The young males had a body length of 26 mm (range 24 to 27 mm). Their testes averaged 1.6 × 1.0 × 0.7 mm. Following immersion for a few minutes in amphibian Ringer's solution, one entire testis was inserted into the body cavity of larvae anesthetized in one percent ethyl urethane.

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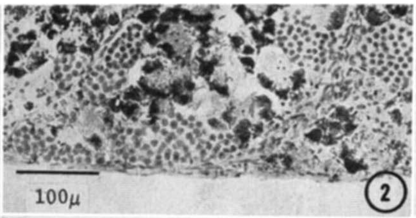

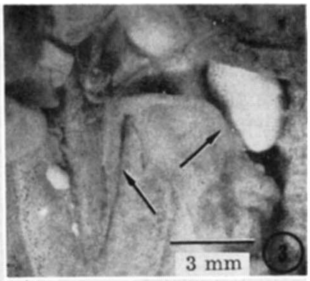

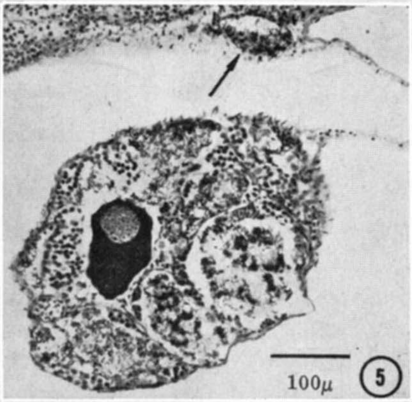

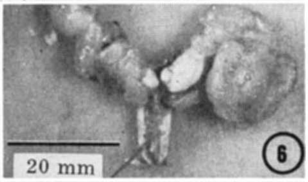

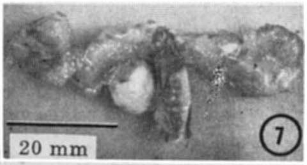

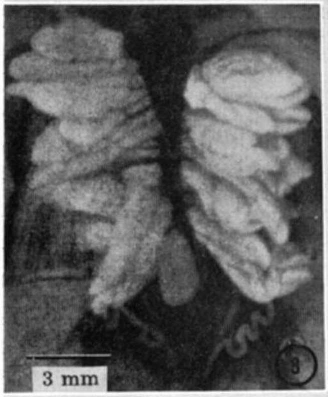

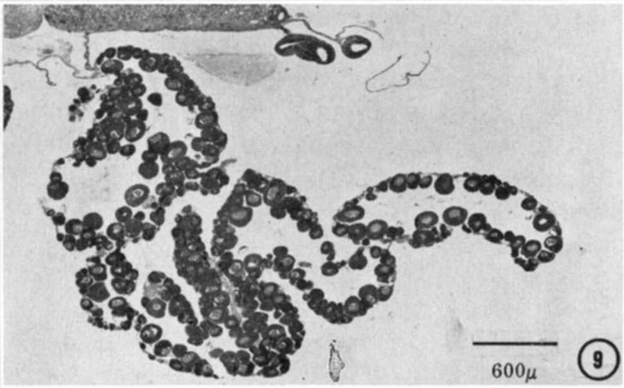

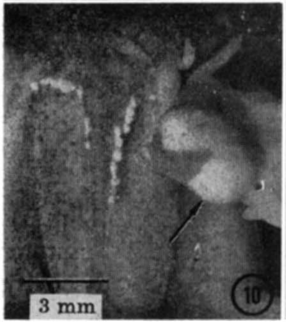

FIGURES 1–2.—Control male at 13 months. FIGURE 1.—Testes. FIGURE 2.—Section of a testis.

FIGURES 3–5.—Sex-reversed female (Case 13) at 13 months. FIGURE 3.—Arrows show respectively the large testis-graft and the sterile tissue, like a fat body, in the left gonadal region (right in the picture). Three testicular masses in the right side. FIGURE 4.—Cross section through

After metamorphosis, the hosts were numbered by a system of clipped toes. At the ages of 5 months and 13 months their gonads and testicular grafts were exposed, measured, and sketched during laparatomies. Sixteen animals were preserved immediately after the second laparotomy. At this time the graft-testis were removed from the remaining 15 animals to permit, from here on, independent development of the rudimentary host gonads. At the age of about three years also, five animals that had lost the male secondary sex characters were preserved. The other ten were retained for progeny tests. They were successively bred to sex-reversed males (ZZ) and to normal females (ZW). The sex of the offspring was determined at metamorphosis, or shortly after. There was no mortality throughout the experiment.

RESULTS

Morphology: Normally, the gonads of Xenopus have a distinctly segmental structure. Even preceding sexual differentiation they consist of chains of about 14 gonomeres (CHANG and WITSCHI 1956). Each bead-like swelling contains a cord of mesonephric derivation that enters through the hilus of the sex gland. Later the testes become compact (Figure 1) and gonomery persists only in the arrangement of rete canals and efferent ductules. A sequence of hollow segmental cords develop in the ovaries, so-called ovarial sacs, resulting in the lobed external appearance (Figures 8, 9, 12). At 13 months, spermatogenesis has advanced to maturity (Figure 2) but ovogenesis only to the stage of immature ovocytes, 0.15 mm in diameter (Figure 9).

In the experimental group, the repeated laparotomies reveal a direct relationship between health of the graft and inhibition of the host's gonads. At five months, the graft could not be located in four animals and at 13 months it had disappeared in 11. This implant degeneration is apparently due to an complete lack of, or at least an inadequate, blood supply. In the other cases, grafts were of varying size, from very small to that of unoperated donor controls.

The development of the host gonads is summarized in Table 1, which is based on the morphologic as well as the genetic studies. At five months, seven animals had distinctly recognizable testes (*T*) or ovaries (*Ov*). At 13 months the number had risen to 14. These, of course, are the hosts of poorly developed or entirely resorbed implants; but even of them, few if any had gonads that were equal to those of controls of same age. All other hosts had severely impaired sex glands

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middle part of the largest testicular node of Case 13. Arrow points at sterile tissue on the left side. FIGURE 5.—Same section as in FIGURE 4, highly magnified. Sperm in efferent ductules.

FIGURE 6.—Asymmetrical testes at 3 years.

FIGURE 7.—Unilateral sterility at 3 years. The right testis is about twice normal testicular size at the same age.

FIGURES 8–9.—Control female at 13 months. FIGURE 8.—Ovaries. FIGURE 9.—Section of an ovary.

FIGURES 10–11.—Case with inhibited ovaries, fixed at 13 months. FIGURE 10.—Arrow points at testicular graft with about double normal testicular size. FIGURE 11.—Cross section of gonads of same case as FIGURE 10. In the small ovaries, the ovogenesis is advanced as in control females. Spermatogenesis in graft is normal at 13 months. Arrow indicates transition area of oviduct differentiation.

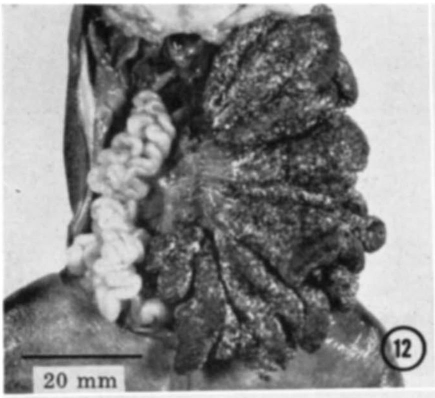
with three types of manifestation: retardation, fatty degeneration, and nodular testicular development. The first type is represented by the left gonad of Figure 10. Instead of having become an ovary (Figure 8) or a testis (Figure 1) it is still an undifferentiated slender fold with 12 recognizable gonomeres. Fatty degeneration may destroy only a few gonomeres (Figure 10, right) or an entire gonad (Figure 3, left side). The cross section through the case of Figure 10 shows (Figure 11) besides the large graft testis, a small left gonad that is an ovary with a few ovocytes of almost normal size. The lower end of the right gonad is entirely sterile, a mere peritoneal fold filled with a few fat cells. Of major interest is the third type of impairment, the combination of small testicular nodes with otherwise severe regression. Figure 3 shows a characteristic case. Both gonads are practically destroyed with the exception of the nodule on the right side which probably includes elements of two gonomeres. Cross sections (Figures 4, 5) reveal that spermatogenesis has progressed locally a far as in controls (Figure 2); but it is lagging in the major part which has the structure of an ovotestis, including even an ovocyte of normal size for this age. The bisexual condition of this gonad makes it almost certain that the animal was of female genic sex and is undergoing sex-reversal. Since it was preserved at an early age, no breeding test is available to verify this surmise.

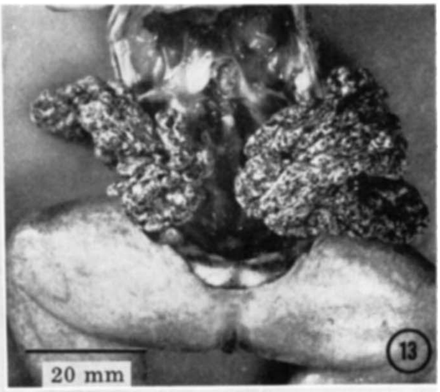
The relatively large number of cases with testicular nodules (designated with *t* in Table 1) suggests that some are genic males, others genic females. Indeed, of ten that later were submitted to breeding tests, two had the female and eight the male constitution. The two adult cases depicted in Figures 6 and 7 are genetically ascertained males. By the age of 13 months their testes had been reduced to only a few gonomeres; one had completely disappeared (left side of case Figure 7). However, following removal of the implant, existing remnants enlarged, some almost to normal size (Figure 6, left; Figure 7, right).

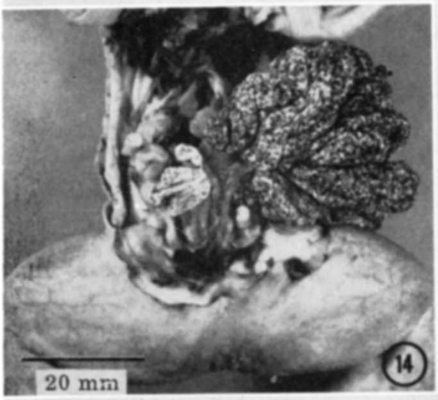
The ovaries of the females suffer similar reductions in the number of gonomeres as the testes of the males. All five animals which, at 13 months, had severely inhibited gonads but no medullary nodules, developed ovaries after removal of the implant-testis. Nevertheless, at three years a comparison with control ovaries (Figure 12) reveals various types of reduction. Often simply the number of gonomeres is diminished (Figure 13). In other cases, some parts of the ovaries show a delay in growth and maturing of the ovocytes (Figure 15, white lobes), or an entire ovary may persist at an immature condition (Figure 14, small right ovary). Ovaries may even be suppressed completely as in the case of Figure 16, where the left ovary is missing.

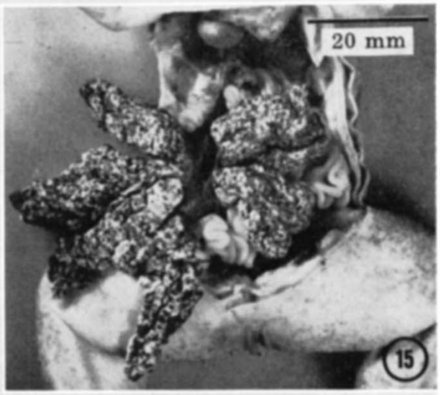
In conclusion, it appears that implantation of a testis into Xenopus larvae at the stage preceding sexual differentiation inhibits testicular as well as ovarian development. The occurrence of sex-reversal cannot be demonstrated convincingly by direct observation alone, but needs to be supported by genetic proof gained from breeding tests (see below).

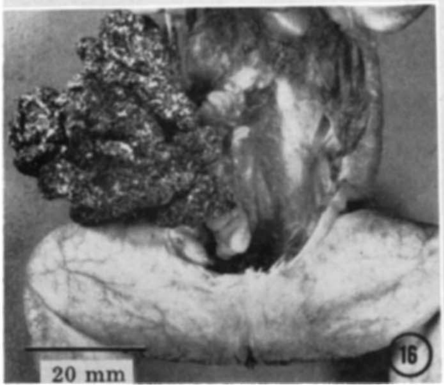
Sex behavior and reproductive capacity: Adult females of Xenopus are usually larger than males of same age; they are recognizable, when in breeding condition, by a cloacal protrusion serving as an ovipositor.











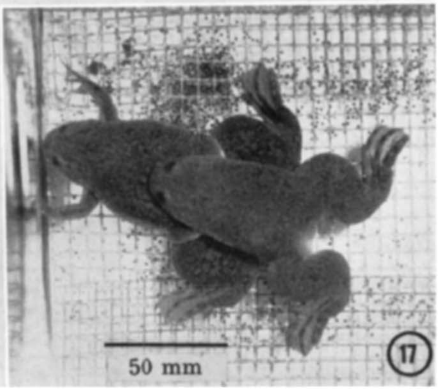


FIGURE 12.—Ovary and oviduct of normal female aged 3 years. Left ovary removed to show the oviduct.

FIGURES 13–16.—Variable conditions of ovaries and oviducts in animals that have recovered from an arrested condition, after removal of the testicular grafts at 13 months. FIGURE 13.—Case without oviduct. FIGURE 14.—Case which has oviduct on both sides, but the lower parts are not developed. FIGURE 15.—Case with almost perfect oviduct development on both sides. FIGURE 16.—Shows unilateral sterility and missing of the left oviduct. The right oviduct lacks the lower part.

FIGURE 17.—Mating of sex-reversed male with sex-reversed female. Genetically, the mother frog is male (ZZ) and the father (Case 12) is female (ZW).

TABLE 1

Summary record of gonad condition of the 31 hosts at the times of two laparotomies (5 months and 13 months) and at preservation (ages 13 months to 4 years)

Sex class	5 months	13 months	Preservation	Breeding type
Males (17)	6 T	6 T	6 T	
	9 t	3 T	3 T	ZZ(2)
		6 t	6 T	ZZ(4)
	2 i	2 t	2 T	ZZ(2)
Females (11)	1 Ov	1 Ov	1 Ov	
	6 ov	4 Ov	4 Ov	
		1 ov	1 ov	
		1 i	1 Ov	
	4 i	4 i	4 Ov	
Converts (3)	3 i	3 t	1 ovotestis	
			2 breeding males	ZW(2)

All gonads are more or less irregular. Genic type of ten animals submitted to breeding tests:—i: sexually undifferentiated gonad rudiments. t: at least one testis; small size. ov: at least one ovary; small size. T: at least one testis of about normal size. Ov: at least one ovary of about normal size.

Adult males have black nuptial pads that spread over the inside of fingers and arms. They become detectable under the dissecting microscope at about ten months (body length 40 mm). At this stage, they appear as small horny spines on the second and third fingers. Simultaneously clusters of ripe sperm occur in the seminal tubules. With advancing age the area of spines gradually spreads over all fingers and much of the arms. The spines become longer and brown with black tips. In control males, the patches become directly visible at the age of about 18 months. Females also show some rudimentary development of spines, though even at the adult age no pads are detectable by the naked eye.

All experimental animals with established testis grafts show a distinctly precocious development of the patches. At 13 months they become slightly dark and have spread over the insides of four fingers. Following removal of the graft further development stops, to be resumed only at the age of about two years by those that carried testicular nodes. The two animals which later proved to be genetic females developed entirely like true males, maintaining nuptial pads, assuming the masculine body-shape and size, and lacking the protruding ovipositor. Attaining sexual maturity, they behaved like normal males, particularly toward females that were injected with hypophyseal preparations. They embrace and inseminate in the usual manner, without having received themselves any stimulating treatment. Between January 1962 and February 1963, one of these sex-reversed animals (Case 6) has been mated successfully six times, the other (Case 12) seven times, each producing offspring with females of various genetic types (Table 2). The fertility rate was not lower than that of normal males in parallel breedings, averaging about 75 percent.

The pair shown in Figure 17 is a copula of a sex-reversed male (ZW) and a sex-reversed female (ZZ). Already over 1000 eggs have been shed and fertilized.

Breeding tests: The results of the progeny tests are presented in Table 3. In every instance, only a restricted number of larvae was raised to sexual maturity (50 to 150). Since relatively simple sex ratios could be expected, there was no need to maintain entire progenies. Moreover, some larvae were used for other experiments about which we hope to report in coming years.

Eight of the ten cases, all apparent males, prove to be genic males (Cases 3, 9, 16, 20, 21, 24, 25, 31 of Table 3). They produce all-male offspring with sex-converted males (ZZ), and a 1:1 sex ratio with normal females (ZW). Evidently they are of the ZZ constitution, i.e. true genic males. Experiments with these animals were discontinued after the first tests had given unmistakable results.

TABLE 2

Matings of cases 6 and 12
(sex-chromosome constitution of mates)

	January 1962	March 1962	June 1962	November 1962	February 1963	Total
Case 6	(ZZ)	. . . . .	. . . . .	(ZZ)*	(ZZ)*	(3ZZ)
	(ZW)	. . . . .	(ZW)	(ZW)*	. . . . .	(3ZW)
Case 12	(ZZ)	. . . . .	. . . . .	(ZZ)	(ZZ)*	(3ZZ)
	(ZW)	(ZW)	(ZW)	(ZW)	. . . . .	(4ZW)

* Sex of the offspring not yet established, and not entered in Table 3.

TABLE 3

Results of progeny tests with ten implanted animals that developed abnormal testes
(compare Table 1)

	ZZ mothers					ZW mothers				
	Offspring									
		Male		Female			Male		Female	
Testis implanted fathers	Total frogs	No.	Percent	No.	Percent	Total frogs	No.	Percent	No.	Percent
Case 3	69	69	100	0	0	99	53	53.5	46	46.5
Case 6	84	40	47.6	44	52.4	56	15	26.8	41	73.2
Case 6	. .	. .	. . .	. .	. . .	117	32	27.4	85	72.6
Case 9	70	70	100	0	0	96	51	53.1	45	46.9
Case 12	65	37	56.9	28	43.1	85	21	24.7	64	75.3
Case 12	. .	. .	. . .	. .	. . .	129	32	24.8	97	75.2
Case 12	. .	. .	. . .	. .	. . .	100	26	26.0	74	74.0
Case 12	59	30	50.8	29	49.2	75	18	24.0	57	76.0
Case 16	62	62	100	0	0	100	52	52.0	48	48.0
Case 20	98	98	100	0	0	126	58	46.0	68	54.0
Case 21	63	63	100	0	0	98	51	52.0	47	48.0
Case 24	68	68	100	0	0	60	32	53.3	28	46.7
Case 25	52	52	100	0	0	95	46	48.4	49	51.6
Case 31	57	57	100	0	0	84	39	46.4	45	53.6

The two remaining cases (in addition to the one that was preserved; Figure 3–5) obviously are of female constitution, hence sex-converts (Cases 6 and 12 of Table 3). They were bred repeatedly, always producing a balanced male and female offspring with ZZ mates (estrogenized males), and a ratio of *1 male: 3 females* with ZW mates (normal females). It must be concluded that they are of ZW constitution, i.e. genic females. Consequently, the 1:3 ratio of their offspring groups is expected to consist really of the genic proportion of 1 ZZ:2 ZW:1 WW. The viability of the entire offspring generation is so good that further breeding tests should easily lead to the identification of the WW individuals. It can also be pointed out that in both breeding combinations the W sperm of the sex-converted female prove physiologically equal to the Z sperm. Control males, of course, produce only Z sperm.

A summary of all breeding results from the ten functional males (Table 4) provides clear evidence of the complete viability of all gametes of sex converts, of genic male as well as of genic female constitution.

DISCUSSION

The observation that a well implanted testis graft impairs gonad development of male as well as of female hosts so far defies efforts to find an adequate explanation. Certainly it is not a consequence of mechanical pressure; implants into head cavities exert the same kind of inhibition as those in the body cavity (Chang 1953). Nor is it brought about through release of steroid hormones by the implant; for in Xenopus, androgens do not interfere with the normal development of ovaries and testes (Witschi 1950; Chang and Witschi 1955a, 1956; Gallien 1962). It seems rather that this is another instance of controlled growth of an organ. Organisms establish size limits possibly by the production of restricted amounts of organ-specific growth substances (Witschi and McCurdy 1943). Such substances must be different from gonadotropins, since growth and differentiation of amphibian gonads up to quite advanced stages progress normally in the absence of the hypophysis (Chang and Witschi 1955b). The same prin-

TABLE 4

Classified summary of breeding tests (from Table 2)

	ZZ mothers (estrogen converts)					ZW mothers (controls)				
	Offspring									
Testis implanted fathers	Total frogs	Male		Female		Total frogs	Male		Female	
		No.	Percent	No.	Percent		No.	Percent	No.	Percent
ZW (2 converts)	208	107	51.4 ±2.71	101	48.6 ±2.71	562	144	25.6 ±0.53	418	74.4 ±0.53
ZZ (8 genetic males)	539	539	100	0	0	758	382	50.4 ±1.07	376	49.6 ±1.07

ciple of controlled organ size is likewise manifest in the compensatory growth of the rudiments of host gonads which immediately follows removal of the testicular grafts.

Since at the age of five months genetically male hosts carrying healthy testis implants still may have indifferent gonads—while testes and ovaries normally are recognizable by external examination at two months—it is not permissible to classify animals with inhibited gonads as sex-reversed females, even if at the age of 13 months, some nodules may have developed (compare Table 1). Of the two cases of such appearance, six months old, which CHANG (1953) considered as converts, only one (with some cortical development) may truly have been of female genic constitution. The great prevalence of "heterosexual" combinations in his material almost certainly derives in part from a mistaken assumption that strongly inhibited gonads always are stunted ovaries.

Further examination of Table 1 also reveals that of seven genic females showing highly inhibited gonads at the time of the first laparotomy, four developed ovaries and two became functional males. At the age of 13 months the latter still had mere thread-like gonads with a few nodules. Evidently the sexual character of the compensatory hypertrophy, which follows removal of the graft, depends on the relative size and condition of cortical and medullary rudiments. A study of indifferent gonads of controls shows a considerable variation in size and shape of medullary cords. Such initial differences certainly play some role in the type of reaction of each gonomere to the experimental procedures. Unfortunately, the available material is still too small to make a detailed analysis of other factors. However, the work of CHANG (1953) already furnishes clear evidence that the effectiveness of grafted testes changes with the age of the donors. So far, testes of animals two months past metamorphosis were the most effective inhibitors. Equally important are changes in sensitivity and responsiveness of the host gonads. Implants introduced before the start of sexual differentiation (stages 26–27) seem to induce greatest reactions. Similar quantitative time variations for gonadal antagonisms have been exposed also in transplantation experiments with adult salamanders (*Ambystoma tigrinum*). During the first year following metamorphosis testis grafts had little influence on host ovaries. However, in the course of the second year there occurred a period of rapid reduction of the ovarian cortex and formation of medullary, testicular nodes (WITSCHI 1939). Likewise the analysis of the development of parabiotic pairs of salamanders leads to the assumption of quantitative time variations in the antagonistic potencies of ovaries and testes (WITSCHI 1936). It can be expected that with continued experimentation it will become possible to increase the frequency of masculinization of Xenopus females by the implantation method. It should then be feasible to analyze also the mechanics and chemistry of gonadal antagonism.

The genetic analysis of the sex-reversed females of Xenopus leads to the same results that were obtained by HUMPHREY (1945) in Ambystoma. Breeding converts with normal females proves that (1) In animals with the female (ZW) constitution spermatogenesis can run a normal course. (2) Sperm with the W sex chromosome, which are not normally produced in these species, are viable and capable of fertilizing. (3) Eggs with WW constitution and complete lack of Z chromosomes develop normally and differentiate as females. These results are of particular interest since in Xenopus sex-reversed males have furnished corresponding proof that ovogenesis can occur and may progress normally in animals of male (ZZ) constitution, i.e. in the absence of the W chromosome. Final proof that such reversals do not in any sense involve or impair the genetic factors is furnished by the results here reported of breeding sex-reversed females with sex-reversed males. Their offspring is of normal viability and consists again of equal numbers of males and females, even though, quite naturally, the males have now the constitution of the mother and the females have that of the father. The apparently sharp restriction of genic influence on the decision of the alternative of testicular or ovarian differentiation is the more surprising as, according to WEILER and OHNO (1962) the W chromosome is larger than the Z and all other chromosomes of this species.

The availability now of ZZ males, ZZ females, WW females, and probably soon also WW males, makes it possible to produce at will all-male (ZZ) and all-female (ZW) offspring. This will greatly help all further experimental work on sex differentiation, cytogenetics and sex endocrinology with Xenopus. Steroid determinations at early larvae stages, for which DALE (1962) has developed some new methods, should now furnish more significant results. They may even contribute toward the elucidation of the nature of the inductor substances of sexual differentiation.

SUMMARY

Testicular grafts placed in the body cavity of larvae of the frog Xenopus greatly inhibit the development of male and female host gonads. Removal of the graft after one year is followed by a rapid recovery of the remaining rudiments of the host gonads. Recovery is incomplete, since usually a varying number of gonomeres have been destroyed completely. In the recovery of gonads of genetically female animals, medullary elements may gain the lead, with subsequent testicular development. Breeding tests prove that in such cases complete spermatogenesis occurs, though the female genic constitution (ZW) is maintained. Breeding tests further show that sperm carrying a W chromosome are as fully functional as those with the Z chromosome. Breeding sex-reversed females with sex-reversed males furnishes unequivocal evidence that the genic and chromosomal elements do not become impaired or otherwise changed by the process of experimentally induced postgenetic sex inversion.

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