SEXUAL DIFFERENTIATION IN THE TELEOST FISH XIPHOPHORUS HELLERII, AS MODIFIED BY EXPERIMENTAL TREATMENT

HENRY H. VALLOWE

Department of Zoology, Ohio University, Athens, Ohio

Attempts to alter the pattern of sexual differentiation in the Mexican swordtail fish, *Xiphoporus hellerii,* show a stability in sexual development usually not credited to this species. The swordtail has long been used as a classic example of lability of sexual differentiation and determination. The widespread reference to sex-reversal occurring in the species suggested a need for further investigation.

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Animals and Their Treatment

The fishes used were descendants of ten pairs from the Genetics Laboratory of the New York Zoological Society. They were reared under conditions similar to those described in earlier papers (Vallowe, 1952, 1953). The original ten pairs produced 618 males and 490 females in a period of approximately three years. The presence of a modified anal fin was the criterion used to establish maleness. Fish not possessing the gonopodium were anesthetized in Chlorobutanol and examined before a strong light. The characteristic amber color of the ova apparent in the ventral region of the posterior end of the body cavity was the criterion used to classify fish as females.

The sex ratios which have been established for this species show wide variations. Witchi (1939) considers *X. hellerii* to be a species without any hereditary sex determining mechanism although the presence of such mechanisms has been shown for other species in the genus. Geiser (1924) presents a comprehensive table showing the sex ratios reported by various authors. Harms (1926) gives a ratio of 24 males to 35 females. Bellamy (1922) reports 100 males to 66.7 females. Friess (1933) also reports a high proportion of males and considers temperature an important influence on the sex ratio. Breider (1935) made a study of the effects of light, nutrition, space and water and found that these had no certain influence on the sex ratio. In light of many conflicting reports, one cannot assume that a preponderance of one sex is due to sex-reversal unless all factors which may influence sexual differentiation are duly considered.

As a basis for interpreting the experimental results reported, a large series of fish were killed at various stages of development, beginning two weeks before birth

and continuing up to more than two years of age. The gonads of these fish were examined to determine the normal pattern of sexual development and differentiation. As early as three days after birth, the gonads could be recognized as potential testes or ovaries by the relative size, number and arrangement of the primordial germ cells. In the young male the germ cells were slightly smaller than in the female. In addition, there were fewer germ cells in each gonad primordium and they were concentrated at the periphery of the gland. In the young female the slightly larger cells were in greater numbers and were well distributed. The embryonic development of this species closely parallels the description given by Goodrich *et al.* (1934) and Dildine (1933, 1936) for *L. reticulatus,* that by Wolf (1931) for *X. maculatus,* and those by Geiser (1924) and Medlen (1950) for two species of *Gambusia.* Although sexual differentiation is conspicuous in these species shortly before birth, Essenberg (1923), Bailey (1933) and Regnier (1938) have shown it to be somewhat delayed in *X. hellerii.*

The differences which distinguish the early testes and ovaries are very subtle characteristics. The appearance of the early ovary and the indifferent gonad are so similar that Regnier (1938) considers that all the fish are born female and that the males later undergo a sex change. This introduces an unnecessary complication which is easily resolved if one assumes a longer period of indifferent development. Once differentiation begins, it progresses in an orderly sequence of events. Chavin and Gordon (1951) describe the differentiation of the testes in *X. maculatus* by using a series of six stages. With only slight modifications, these stages were used in the present study to classify the testes of *X. hellerii.* Gordon and Aronowitz (1951) make the observation that the histological structures of the testes of adult *X. maculatus* and *X. hellerii* are practically identical. The present study has shown that the development of the testis and the pattern of the differentiation of its structures are strikingly similar in the two species. Except for the time spent in the late stages of immaturity, the sequence of events is identical.

The process of sexual differentiation in *X. hellerii* as described by Essenberg (1923) and Van Oordt (1925) traces the origin of the definitive sperms to the epithelial cells of the testis tubules. Wolf (1931), Goodrich *et al.* (1934), and Chavin and Gordon (1951) were unable to find comparable stages of transformation in the species which they studied. The testis in *X. hellerii* is the acinus-type characteristic of the vivaparous Cyprinodonts. The arrangement and development of the structures within the testis indicate that the acini form from pre-existing acini at the periphery of the testis. As the acini are formed, they are pushed along the tubules toward the central ducts by the growth of new acini at the periphery. The peritoneal covering of the testis, the stroma cells, and the epithelium of the ducts and tubules gave no evidence of transforming into germ cells.

It is slightly more difficult to distinguish the newly differentiated ovary from the indifferent gonad because the arrangement of the cells remains about the same. However, the relative size and number of the cells characterize the gonad as an immature ovary. Early in the differentiation process the primordial germ cells are found scattered throughout the stroma of the ovary. When the fusion of the paired ovaries is completed, the germ cells come to lie within the wall of the cavity formed between them or in the stroma layer which supports this wall. In the mature female oogonia are found developing within the wall of the cavity, hence the name germinal epithelium. The larger and more advanced cells are pushed

outward toward the peritoneal covering. Essenberg (1923) feels that all the primordial germ cells disintegrate and do not take part in the formation of the germinal epithelium. Although there is great difficulty in determining the origin of the cells within the germinal epithelium, no disintegrating primordial cells were encountered in the early stages of gonad fusion and cavity formation. The primordial cells appeared to remain in normal active condition and were arranged in small groups surrounded by stroma cells. In the germinal epithelium it is possible to see all stages of transition in the shape of the nuclei from elongate oval, typical of the epithelial cells, to the spherical nuclei of oogonia. A comparable situation exists in *L. reticulatus* according to Goodrich *et al.* (1934) and leads them to the conclusion that epithelial cells as well as primordial germ cells may give rise to the definitive ova. Wolf (1931) arrived at a similar conclusion. The origin of the sex cells appears to be different in the two sexes of these species. While the primordial germ cells are the only source of definitive sperms, the peritoneal cells forming the wall of the ovarian cavity may contribute to the formation of definitive ova.

The following series of experiments reports the effects of estrogenic and androgenic hormones on gonad development. The results of the treatments described are interpreted in light of their effect on the normal sexual differentiation.

Experiments and Results

Immature fish

Young sexually immature fish (49 to 55 days old) were given nine weekly injections of 0.01 cc. of sesame oil containing 0.25 mg. of testosterone propionate into the body cavity. During this period of time there was a conspicuous thickening and elongation of the anal fin rays, a growth and pigmentation of the sword-like extension of the caudal fin and an intensification of the lateral line and dorsal fin coloration. In short, the young fish appeared to be miniatures of the sexually mature adult males. The histological picture presented by the gonads of these fish was one of radical change in the immature ovaries and one of general stimulus in the young testes.

The ovaries of the testosterone-injected immature females lacked any sign of ova. There were follicles present but these were filled with a loose collection of cells. This indicates that ova had been present previously, but that resorption had taken place. In other follicles primary spermatocytes, spermatids and spermatophores were observed. The ovarian cavity was obscured and much stroma filled the gonad. The brood-mate control females showed ovaries in which fats and oils were being deposited in the maturing ova.

The testes of immature males treated with this androgen showed an acceleration in spermatogenesis. The tubules and sperm ducts were filled with all stages of spermatogenesis including well formed spermatophores. The epithelium lining the sperm ducts was greatly hypertrophied but otherwise the testes appeared very similar to those of mature males. Brood-mate control males showed testes in less advanced stages of development.

Another group of immature fish at comparable ages was given nine weekly injections of 0.01 cc. sesame oil containing 0.00083 mg. of estradiol benzoate. At the end of the treatment period these fish were deep-bodied, showed only faint

indications of coloration and, in general, appeared to be miniatures of the adult females. The histological picture presented by these fish was the reverse of the situation found in those injected with testosterone. The ovaries of the estradiol-treated females showed a general stimulus while the testes of the treated males showed radical changes.

The testes of the estrogen-treated males showed a modification from the bipartite gonad to a fused structure in which the two lobes were no longer distinct. The peritoneal covering of the gonad had thickened, the germinal elements were no longer concentrated at the periphery of the gonad, the sperm ducts were obscured, and the blood vessels were enlarged. Some acini contained what appeared to be oogonia. Other acini contained disintegrating cells which resembled primary spermatocytes. The testes were larger than those of the brood-mate controls. In some of the gonads the sperm ducts appeared only as spaces with no organized epithelium. Two new dorso-lateral cavities had formed and were lined with a well organized epithelium. Oocytes were common in the testes showing this degree of modification.

Ovaries of the estradiol-injected females showed ova in the stage of oil deposition. There was a slight increase in the amount of stroma present but the blood vessels and follicular epithelium appeared normal. The germinal epithelium appeared very active and abundant oogenesis was observed.

Mature fish

The histological picture presented by the testes of sexually mature males after ten weekly injections of 0.02 cc. sesame oil containing 0.00166 mg. estradiol benzoate was one of general suppression and destruction. The most conspicuous effects obtained were those of an enlargement of the sperm ducts and the destruction of the spermatogenic elements. In normal mature males the sperm ducts were paired, centrally located tubes which contained spermatophores suspended in a lightly staining, non-granular fluid. In estrogen-treated males the ducts were greatly enlarged and in some cases occupied most of the testes when viewed in cross-section. They seemed to be distended with fluid and contained spermatophores in various stages of disintegration. Quantities of free spermatozoa were observed in the lumen of the ducts; this condition is not found in normal mature males. However, free spermatozoa are found in the ducts of senile males which have passed the reproductive age and are no longer capable of fertilization.

The effect on the germinal elements was most drastic in the intermediate stages of spermatogenesis. While spermatozoa and spermatogonia were still abundant, primary and secondary spermatocytes and spermatids were usually reduced in number or entirely absent. The acini which, by their position, should have contained the intermediate stages of spermatogenesis, had hypertrophied walls in which the cell outlines were conspicuous. These acini were smaller than normal and were either filled with disintegrating germ cells or were completely empty. The reduction of the acini was accompanied by a slight increase in the amount of stroma tissue and the appearance of a fibrous tissue network. The blood vessels were numerous and enlarged. The testes appeared less bipartite than the normal condition, but none were found in which fusion was as complete as that found in the ovary.

The histological picture presented by the ovaries of females given ten weekly injections of 0.02 cc. sesame oil containing 0.5 mg. testosterone propionate was also one of general suppression and destruction. None of the ovaries showed any signs of spermatogenesis even when injections were continued for as long as eighteen weeks. A conspicuous activity was noted in the germinal epithelium but there was no indication that the primary germ cells being proliferated could be spermatogonia. The cells were in groups of as many as ten, but their arrangement, size, and staining properties were strikingly similar to the oogonia found in normal ovarian development. The larger ova that were present in the gonad were in various stages of disintegration. Only a few of the ovaries examined showed the presence of mature ova; in most cases, there remained only large, empty follicles which were in various stages of collapse. There seemed to be little effect of the hormone treatment upon the smaller ova; they were still firm, were surrounded by a well organized follicle, and were in their normal arrangement in the ovary. There was very little increase in the amount of stroma in the ovary and only isolated areas in which a fibrous network was formed. On the other hand, the blood vessels had increased in size and were found throughout the ovarian tissue. In gross appearance, the ovaries of the androgen-treated females resembled those of immature normal females.

In the empty follicles and ovarian cavity of many of the virgin ovaries the presence of a secretion was observed. This secretion appeared as a mass of crumpled membranes which is normally found only in the non-virgin ovary. The frequency of its appearance indicated that this is a typical response to androgenic injections and it may be indicative of an expulsion of mature ova from their follicles. However, no mature eggs were found in the ovarian cavities or oviducts. If the eggs were completely evacuated from the body, they were never found in the aquaria.

The ovaries of the gravid females did not differ from the ovaries of virgin females given the same treatment. In two cases where the females were killed three weeks after the initial injection of testosterone propionate, embryos in early stages of development still remained in the follicles. The characteristic membrane-like secretions were observed in the follicles of some of the gravid females.

Discussion

The results obtained from the study of the normal sexual differentiation of *X. hellerii,* and from the attempts to alter the pattern of this differentiation by experimental treatment, point to a stability in sexual development and differentiation which has usually not been credited to this species. Much of the experimental and descriptive work dealing with Xiphophorin fishes has emphasized the ease with which the secondary sexual characteristics of these species can be made to respond to hormonal treatment. The effects of many hormone substances on the primary sex organs have also been described but the results are not always in agreement. However, in all cases of sex-reversal reported for adult *X. hellerii* the change has always been from female to male, whether such reversals were naturally occurring phenomena or whether they were induced by hormone treatments. During the course of this investigation no case of natural sex-reversal was observed in the laboratory population; further, the sex ratio obtained for this

population indicates that unobserved sex-reversals could not have occurred in large numbers. The adult sex ratio and the sex distribution of the fishes used in the study of the normal development of the gonads did not indicate that a shift in the ratio occurred between the juvenile and mature populations. Essenberg (1923) postulated that a sex-reversal of 50 per cent of the immature females would explain the shift he observed in the sex ratio (a change from a ratio among immature fish of three females to one male to the adult condition of one female to three males).

A possible explanation of naturally occurring sex-reversal in adult fish, in which virgin females become males, lies in improper initial classification. In this study only those fish which possessed amber ova that could be observed through the body wall when viewed against a strong light source were designated as females. This procedure reduced to a minimum the possibility of erroneous sex classification. The classification of immature males as females may account for a few, but certainly not all, of the cases of hormone-induced sex-reversal reported by other investigators.

Another factor which may play a significant role in the discrepancies of the results obtained from experiments with *X. hellerii* lies with the variations in the fish used. The very important reviews by Gordon (1931, 1937) concerning the history of Xiphophorin species as aquarium fishes give substantial evidence to indicate that the *X. hellerii* available from commercial hatcheries have possibly been produced by hybridization with *X. maculatus*. For the aquarist the hybrid fish has many traits that are desirable. The hybrid is usually more highly colored, more robust and larger than either parent species, and more prolific in the production of large broods of young. These factors would account for its selection and propagation by commercial hatcheries. Gordon and Rosen (1951) suggest that the hybrid possesses an imbalanced chromosomal arrangement that may have endocrinological significance in that this imbalance may initiate abnormal or nonfunctional gonads in the hybrid. Although hybrids may usually be identified by color or color patterns, some are practically identical with the wild-type swordtail. The few cases of natural sex-reversal that have come to my attention have always been in hybrid fishes or in fish with unknown origins. A strong possibility exists that the sex-reversals reported by Essenberg (1926) occurred in hybrid stocks. (He gives the origin of the stock as the Crescent Fish Farm, a commercial fish hatchery.) Further application of this possibility suggests that the work of other investigators may also be based on commercially available hybrid strains rather than on pure species stocks.

The results of the experimental treatments given to the pure species used in this study were found to be in close accord with the results obtained by many investigators using the closely related species which are not known for their tendencies to produce sex-inversions. The response to the techniques employed indicated that these fish were in no way aberrant but were in close agreement with the other members of the viviparous Cyprinodont group.

Although the immature fish developed the germ cells of the opposite sex under hormone treatment, the sexually mature fish did not when subjected to the same hormone preparations. Since the gonads are homologous structures, it is possible that they retain a few common properties until differentiation becomes so advanced that these must be sacrificed. Evidence from the treatment of maturing males

with estradiol benzoate shows that the ability of the testis to produce ova is retained from a short time after gonopodium elongation. However, this response is lost after the later stage of male development, *i.e.,* gonopodium differentiation, is initiated. In the mature male the effects of estrogenic treatment were a destruction and suppression of existing elements. Unlike the results obtained in immature and maturing males, there was no stimulus to develop female characteristics.

Immature females produced spermatozoa within their ovaries under the influence of testosterone propionate. This response, however, was not found in the ovaries of mature females given twice the amount of the same hormone preparation.

Summary

1. The normal sexual development and differentiation of *Xiphophorus hellerii* have been observed in the light of the adult sex ratio and the differentiation of the gonads from birth to maturity. The sexual differentiation has been shown to follow a definite pattern in both sexes. The gonads of both sexes are homologous and indifferent at birth, but within a few days testes and ovaries can be distinguished by the relative size, number and arrangement of the primordial germ cells. No atypical gonads were discovered in the course of this study which would indicate the possibility of sex-reversal.

2. Injections of testosterone propionate induced spermatogenesis in the ovaries, of immature females but had no comparable effect in mature specimens.

3. Injections of estradiol benzoate induced oogenesis in the testes of immature males but had no comparable effect in mature specimens.

4. Improper initial sex classification and hybrid origin of the fish used are suggested as possible explanations for some of the discrepancies between the results obtained in this study and those reported in earlier investigations.

5. The results of the study of the normal sexual development and the attempts to alter the differentiation pattern by experimental means indicate a stable sexual development and differentiation for this species.

LITERATURE CITED

Bailey, Ralph J., 1933. The ovarian cycle in the viviparous teleost, *Xiphophorus helleri. Biol. Bull.,* **64**: 206–225.

Bellamy, A. W., 1922. Breeding experiments with the viviparous teleosts *Xiphophorus helleri* and *Platypoecilus maculatus* (Günth.). *Anat. Rec.,* **23**: suppl. 98–99.

Breider, Hans, 1935. Über Aussenfaktoren, die das Geschlechtsverhältnis bei *X. helleri* Heckel kontrollieren sollen. *Zeitschr. wiss. Zool.,* **146**: 383–416.

Chavin, Walter, and Myron Gordon, 1951. Sex determination in *Platypoecilus maculatus.* I. Differentiation of the gonads in members of all-male broods. *Zoologica,* **36**: 135–146.

Dildine, Glenn C., 1933. Germ cell origin and gonad differentiation in the viviparous top minnow, *Lebistes reticulatus. Anat. Rec.,* **57**: suppl. 88.

Dildine, Glenn C., 1936. Studies in teleostean reproduction. I. Embryonic hermaphroditism in *Lebistes reticulatus. J. Morph.,* **60**: 261–277.

Essenberg, Jacob M., 1923. Sex-differentiation in the viviparous teleost, *Xiphophorus helleri* Heckel. *Biol. Bull.,* **45**: 46–96.

Essenberg, Jacob M., 1926. Complete sex-reversal in the viviparous teleost, *Xiphophorus helleri. Biol. Bull.,* **51**: 98–111.

Friess, Else, 1933. Untersuchungen über die Geschlechtsumkehr bei *Xiphophorus helleri* Heckel. *Arch. f. Entw.,* **129**: 255–355.

Geiser, S. W., 1924. The sex ratio in *Gambusia holbrooki. Biol. Bull.,* **47**: 175–212.

GOODRICH, H. B., J. E. DEE, C. M. FLYNN AND ROWENA N. MERCER, 1934. Germ cells and sex differentiation in *Lebistes reticulatus*. *Biol. Bull.*, **67**: 83–96.

GORDON, MYRON, 1931. Hereditary bases of melanosis in hybrid fishes. *Amer. J. Cancer*, **15**: 1495–1523.

GORDON, MYRON, 1937. Heritable color variations in the Mexican swordtail fish. *J. Hered.*, **28**: 222–230.

GORDON, MYRON, AND OLGA ARONOWITZ, 1951. Sex determination in *Platypoecilus maculatus*. II. History of a male platyfish that sired all-female broods. *Zoologica*, **36**: 147–153.

GORDON, MYRON, AND DONN E. ROSEN, 1951. Genetics of species differences in the morphology of the male genitalia of xiphophorin fishes. *Bull. Amer. Mus. Nat. Hist.*, **95**: 409–464.

HARMS, J. W., 1926. Beobachtungen ueber Geschlechtsumwandlungen reif der Tiere und deren F_1 Generation. *Zool. Anz.*, **67**: 67–79.

MEDLEN, AMMON B., 1950. Sperm formation in *Gambusia affinis*. *Texas Jour. Sci.*, **2**: 395–399.

REGNIER, M. T., 1938. Contribution a l'étude de la sexualité des cyprinodont vivipares (*Xiphophorus helleri, Lebistes reticulatus*). *Bull. Biol.*, **72**: 385–493.

VALLOWE, HENRY H., 1952. Boiled egg yolk an essential ingredient. *Aquarium*, **21**: 350.

VALLOWE, HENRY H., 1953. Some physiological aspects of reproduction in *Xiphophorus maculatus*. *Biol. Bull.*, **104**: 240–249.

VAN OORDT, G. J., 1925. The relation between the development of the secondary sex characters and the structure of the testis in the teleost *Xiphophorus helleri*. *J. Exp. Biol.*, **3**: 43–59.

WITCHI, EMIL, 1939. Modification of the development of sex in lower vertebrates and in mammals. *In:* Sex and internal secretions, ed. by Edgar Allen. Baltimore. Williams and Wilkens.

WOLF, L. E., 1931. The history of the germ cells in the viviparous teleost *Platypoecilus maculatus*. *J. Morph. and Physiol.*, **52**: 115–153.