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UNIVERSITY OF LOUISVILLE

A STUDY OF SEX DIFFERENTIATION IN LEBISTES

RETICULATUS AS AFFECTED BY ESTRADIOL

STILBESTROL AND PREGNENINOLON

A Dissertation

Submitted to the Faculty

Of the Graduate School of the University of Louisville

In Partial Fulfillment of the

Requirements for the Degree

Of Master of Science

Department of Biology

By

Esther Coogle

Year

1943

NAME OF STUDENT: Esther Coogle

TITLE OF THESIS: A Study of Sex Differentiation in
Lobistes reticulatus as Affected by
Estradiol, Stilbestrol, and Pregne-
ninolon

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and guidance the author
is sincerely grateful.

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SECTION I

INTRODUCTION

SECTION I

INTRODUCTION

1. The Variety of Concepts of Sex Determination and Sex Differentiation in Animals

It is generally agreed that the sex of most animals is determined at fertilization by the sex chromosomes; and that sexual differentiation is effected, in most vertebrates at least, by internal secretions. Certain phenomena have been noted, however, that seem incompatible with this concept. Examples of such phenomena are:

1. Bipotentiality of germ cells
2. Hermaphroditism
3. Spontaneous sex reversals
4. Experimentally-induced sex inversions
5. Various sexual anomalies such as gynandromorphs, intersexes, supersexes, and freemartins

Before undertaking a study of sexual phenomena of any kind, it is well for the worker to consider critically various concepts of sex determination and sex differentiation that have been advanced in explanation of such phenomena. The following concepts are herewith presented more as points of attack than conflicting points of view now maintained by authorities. Nor is it to be expected that any one hypothesis among them may be found to explain fully all sexual phenomena, embodying the whole truth exclusive of the contributions of the other

hypotheses. We are reminded by Lillie (1939) of

. . . the possibilities of attainment of the functions of sexual differentiation by different mechanisms . . . Each species has something special to tell us, and contains a warning against the expectation of discovering some single principle of explanation.

Some of these explanations of the mechanisms underlying sexual phenomena, with an evaluation of each, are as follows:

Sex determination by sex chromosomes at fertilization. The combination of two X chromosomes or the X and Y chromosomes (or other variations of heterogamy) is assumed to insure the development of properly determined gonads and the subsequent appearance of secondary sexual characteristics. Difficulties are encountered, however, in the attempt to explain all sexual phenomena by this concept. That sex chromosomes may not be all-powerful is evidenced by the following observations: (Lillie)

. . . the primordial germ cells of a given embryo may under experimental conditions be caused to produce either ova or spermatozoa.¹ . . . The fact that complete sex inversion, i.e., the production of a functional male from a genetically-determined female, or vice versa, has been experimentally realized . . . is the definitive proof that the possibilities of either form of sex differentiation adhere in all developmental histories, whatever the original determination.

Sex determination by a balance of determiners located in both sex chromosomes and autosomes. Sex of an animal, according to the genic balance theory, is determined by the net effect of male-quality determiners and female-quality determiners. Variable degrees of

¹Often referred to as the theory of bipotentiality of germ cells.

maleness or femaleness may be produced through variations in the number, strength, or interactivity of these determiners. By experimentally altering the number and ratio of sex chromosomes and autosomes in Drosophila, workers have been able to breed intersexes, supermales, and superfemales (Bridges 1939). This concept, moreover, may help explain the experimental modification of sexual characteristics, as determiners for both sets of characters may be said to coexist in all cells of the organism. It has been found inadequate, however, to explain sex differentiation without other mechanisms, as indicated by the failure of castrates to develop secondary sex characters.¹

Sex determination by internal secretions, or hormones. It has been suggested that hormones produced by gonadal tissues may affect both the further development of the gonad itself, and the subsequent appearance of secondary sex characters. This concept does not attempt to dispense with the mechanism of sex determination by chromosomes, as a pre-determination of one sex or the other is assumed necessary to initiate the development of a male or a female gonad. This determination is, however, assumed to be easily overridden by a fortuitous excess or lack of sex hormones.

Various observations present evidence inconsistent with the above concept. In many animals, sex hormones are produced in tissues

¹An exception to this generalization occurs in insects, in which castration usually has no effect on secondary sex characters. In insects, therefore, both sex determination and sex differentiation probably are wholly under the control of the chromosomal constitution, which is presumably identical for both germinal and somatic tissues (Lillie 1939).

other than gonads. In certain animals, also, the hormonal make-up is not completely specific as to sex; i.e., female sex hormones are produced in the male animal, and male hormones in the female. When these heterotypic substances are present in unusual amounts, it is true, certain abnormalities may be manifested; it can be maintained, however, that in many animals the production of hormones of the opposed sex is a normal phenomenon.

Sex determination by inductors developed in the cortex and medulla of the embryonic gonad. It is held by some authorities that non-endocrine substances of a sex-determining nature are produced by the gonads early in their development (Willier 1939, Needham 1942). The substance produced by the cortex of the ambisexual gonad exerts a suppressing effect on the medulla, while the substance produced by the medulla exerts a corresponding effect on the cortex. The genic constitution of the tissues is assumed to be responsible for the relative strength or abundance of these substances. If cortexin is stronger the medulla is suppressed, the cortex develops, and the gonad becomes an ovary; if medullarin (medullin) is stronger the cortex is suppressed, the medulla develops, and the gonad becomes a testis. The gonad having once become a definitive ovary or testis, it becomes capable of the subsequent production of the hormonal secretions which are the effective agents of sex differentiation.

Though there is considerable evidence (mainly from the parabiosis experiments on amphibia by Witschi, 1927, 1931, 1937, and 1939) in support of this hypothesis, the exact role of such inductors

in sex determination remains to be clarified by further research.

Sex determination by factors extrinsic to the gonads. The idea of a causal relationship between environmental factors and sex is of frequent recurrence in discussions of sexual phenomena in animals. It is generally employed only in cases of sex reversal or delayed sex differentiation in which it is assumed that the appearance of secondary sex characters or even the determination of the gonads themselves wait upon environmental conditions.

In many animals having seasonal sexual activity (birds, certain mammals, etc.), the number of hours of daylight affect the hypophysis, causing it at a certain season to secrete gonadotropic substances which induce gonad activity and in many species the appearance of nuptial coloration. Temperature is known to be a deciding factor in controlling the seasonal reproductive cycles of certain fish, and in inducing the appearance of males rather than parthenogenetic females in the fall broods of certain species of aphids. Diet is a factor in the production of queen bees, as only larvae fed royal jelly become the fertile queens, while their less favored but identically constituted sisters become infertile workers.

Interesting though these exceptional cases may be in their application to the problems of sex determination, it is obvious that the great majority of species go on producing males and females in normal sex ratio regardless of variations in external conditions. Environment, then, can not be considered as significant in any general concept of sex determination in animals.

2. Selection of the Problem

There is considerable experimental evidence that the sex of animals may not be irreversibly determined at fertilization. By the use of such experimental techniques as parabiosis and hormone administration, a complete sex inversion has been induced in a few species, while many stages of intermediate sexuality have been produced in various experimental subjects.

The field of experimental sex modification in vertebrates offers no more promising and little-explored area than that of hormone administration to fishes. There is a great variety of sexual phenomena in fishes, including such diverse conditions as hermaphroditism, spontaneous sex reversal, ovoviviparity, seasonal sexual dimorphism, and many others, all of which offer the biologist rich material for experimentation. At present there is considerable confusion in the interpretation of these phenomena, both in their normal manifestations and in their experimental modifications. Witschi (1939) writes that it is simply impossible to harmonize the published experimental data. Berkowitz (1941) states after several series of experiments using sex hormones on fishes, that his results did not constitute clearly positive evidence for either theory of sex determination (the theory of sex hormones versus the theory of embryonic inductors). Some experimenters have administered sex hormones to fishes in the expectation of bringing about sex reversal, with more or less success.¹

¹By the use of heterotypic hormones, gonads containing both types of germinal tissue have been induced, and in a few cases, gonads consisting of tissues of the induced sex exclusively.

In selecting a field for experimentation by the writer it was decided to administer certain estrogenic substances to Lebistes reticulatus (Peters) (the common guppy), a small, live-bearing tropical fish. The conspicuous sexual dimorphism of this fish, and the prolonged period before sexual differentiation occurs, make this animal a suitable one for a study of the effects of hormonal agents on secondary sexual characteristics.

This study is undertaken, therefore, in the hope that some small contribution may be made to a solution of the problems of sex determination and sex differentiation in fishes. Where this investigation parallels those of other workers it may substantiate their findings; where it strikes out for itself it may add a fragment of evidence to the complex whole; and where it presents apparently contradictory evidence or inexplicable results it may open leads for future study.

SECTION II

REVIEW OF HORMONE EXPERIMENTATION

ON LEBISTES AND OTHER FISHES

SECTION II

REVIEW OF HORMONE EXPERIMENTATION

ON LEBISTES AND OTHER FISHES

The rather frequent occurrence of sex reversal in fishes, especially tropical fishes, has suggested to several investigators the project of using sex hormones and related substances in an attempt to induce this phenomenon in laboratory subjects. In most of the fishes in which reversals have been observed there is a marked sexual dimorphism. This variation in secondary sex characters between the male and the female has been used as an indication of the efficacy of various sex-affecting agents. A rather wide variety of effects has been noted from slight modifications of color and pattern through the complete assumption of all external characteristics of the opposite sex. The primary sex characters have also been affected in various ways; gonads have been induced that contain both male and female reproductive cells, and in a few instances a complete sex inversion has been brought about.

A digest of this experimentation is presented in Table I, p. 10. It will be seen that the results fall into certain categories; the administration of testosterone and pregnenolone to immature subjects inhibits female secondary sex characters and induces the precocious assumption of male secondary sex characters, while estradiol and stilbestrol hasten the development of female characters and inhibit the male characters.

There are, however, many inconsistencies in the results reported by various workers. Some investigators claim to have achieved reversal, while others report that the same agent falls short of this effect. These inconsistencies have largely been dispelled, however, by subsequent work along the same lines, the reversing effect of the agent having been found to vary with dosage and with the age of the subjects. Other inconsistencies in the effects of certain agents, as for example an androgenic effect of a substance normally exerting only an estrogenic effect in mammals, may presumably be due to overdosing. There seem to be few clear-cut results that will throw light on the fundamental problem of the mechanics of sex differentiation in fishes. Indeed, the ready response of the external sexual features of fishes to the agents, the ease with which the gonads are inhibited by a heterotypic agent or by an excess of a homotypic agent, and the evident compatibility of male and female characters exhibited concurrently by the experimental subject, throw the question of the normal methods of control of sex characters in fish into even greater confusion.

TABLE I

DIGEST OF HORMONE EXPERIMENTATION ON LEBISTES AND RELATED FISHES

Experi- menter	Subject	Agent	Treatment	Effects
Berko- witz 1937	<u>Lebistes</u>	Estradi- ol	By mouth	Suppression of male secondary sex characters if treatment begun early
1941	<u>Lebistes</u>	Estradi- ol, stil- bestrol	By mouth By injec- tion	In young, inhibition of male and stimulation of female secondary sex characters, testis inhibited or changed to ovotestis in many (with moderate dosage only) In adolescents, mosaic of male and female secondary sex characters. In adult males, matured sperms, exhausted testes
1941	<u>Lebistes</u>	Gonado- tropins	By mouth	Precocious development of male secondary sex characters, pre- cocious development of testis
Regnier 1938	<u>Lebistes</u> females	"Male hormone"	By injec- tion	Heavy mortality, resorption of embryos, female secondary sex characters give place to male
	<u>Lebistes</u> males	"Female hormone"	By injec- tion	Inhibition of colors, check in spermatogenesis
	<u>Xiphopho- rus fe- males</u>	Testo- sterone	By injec- tion	Degeneration of ovary, stimula- tion of testis (but not complete development), male secondary sex characters
	<u>Xiphopho- rus juve- niles</u>	Testo- sterone	By injec- tion	Only those "genetically destined to become males" developed fully formed testes
Ever- sole 1939	<u>Lebistes</u>	Testo- sterone	By injec- tion	Gonopod develops in all, male pattern and pigment in some, no suppression of gravid spot
1941	<u>Lebistes</u>	Testo- sterone	By injec- tion	Male characters, except full colors, induced in all
	<u>Lebistes</u>	Pregne- ninolon	By mouth	Male colors and gonopod induced, puberty spot suppressed, ovaries of adult females degenerate

TABLE I (continued)

DIGEST OF HORMONE EXPERIMENTATION ON LEBISTES AND RELATED FISHES

Experi- menter	Subject	Agent	Treatment	Effects
Baldwin and Goldin 1939	<u>Xiphopho- rus vir- gin fe- males</u>	Testo- sterone	By injec- tion	Male secondary sex characters induced, ovaries regress, testicular tissue develops, with some phase of spermatogenesis
Baldwin and Min- Hsin-Li 1942	<u>Xiphopho- rus vir- gin fe- males</u>	Gonado- tropin	By injec- tion	Sex reversal in some but not all subjects
	<u>Xiphopho- rus males</u>	Gonado- tropin	In tank water	Acceleration of spermatogenesis, exhaustion of testis, increased vascularity and interstitial tissue formation
Grob- stein 1940	<u>Platypoe- cilus fe- males</u>	Testo- sterone	By injec- tion	A regenerating anal fin develops into a gonopod

SECTION III

THE EXPERIMENTAL AGENTS

SECTION III

THE EXPERIMENTAL AGENTS

Three substances were selected for administration to the experimental subjects. These agents were: (1)estradiol, a naturally-occurring estrogen; (2)stilbestrol (stilboestrol), a synthetic steroid having an estrogenic effect in mammals; and (3)pregneninolon (pregneninolone, pregninolon), a synthetic substance having a progestational effect in mammals.

1. Estradiol

Estradiol, or α -estradiol, is the primary hormone produced by the ovarian follicle of mammals, and is the most active estrogen known. In addition to its control of the uterine endometrium throughout the menstrual cycle, it helps maintain the normal size and function of the female reproductive organs. It has an inhibitory effect on the secretion of gonadotropic hormones by the pituitary gland. It also affects nasal and oral mucous membranes, metabolic rate, vasodilator actions, metabolism of calcium, and regulation of tissue fluids.

The form in which this agent was administered was Schering's Progynon-DH tablets derived from pregnancy urine.

2. Stilbestrol

Stilbestrol, or diethyl-stilbestrol, is a crystalline synthetic estrogen fully effective when administered orally. There are

occasional allergic or toxic reactions in patients undergoing stilbestrol therapy.

3. Pregneninolon

Pregneninolon, or anhydrohydroxy-progesterone (based on testosterone), is an orally effective progestin of synthetic derivation. It has been shown to have all the effects of the corpus luteum hormone in mammals and in man. It produces a secretory endometrium in the human female, inhibits uterine motility, and corrects endocrine imbalance in premenstrual tension. Its main clinical use is in cases of threatened abortion.

The form of pregneninolon used in this experimentation was Schering's Pranone.

SECTION IV

SUBJECTS USED IN THE EXPERIMENTATION

SECTION IV

SUBJECTS USED IN THE EXPERIMENTATION

The experimentation for this thesis was made upon Lebistes reticulatus Peters, the common guppy. This section will consider the following topics: (1) Description and Life History of L. reticulatus, (2) The Testis of L. reticulatus, (3) The Ovary of L. reticulatus, (4) Sex Inheritance in L. reticulatus, (5) L. reticulatus as the Subject of Biological Experimentation, and (6) Maintenance of L. reticulatus in the Laboratory.

1. Description and Life History of

Lebistes reticulatus

The guppy is a small, ovoviviparous, fresh water tropical fish native to the British West Indies, Venezuela, and parts of northern South America. The generic name "lebistes" or "pot" is descriptive of the pot-bellied shape of the female, while the specific name "reticulatus" refers to the net-like effect of the pigmented scale margins of the female. The earlier specific name "guppyi", honoring Mr. Lechmere Guppy, the discoverer of the fish, has become the accepted common name, though the names "rainbow fish", "peacock fish", and "millions fish" have been widely used.

Female guppies reach a length of two inches and are greenish-gray in color. Mature females have on each side above the anus a black spot where the pigmented peritoneal layer shows through the body wall

which is distended by developing ova or embryos. This spot is generally known as the "gravid spot", though the more recently applied term "puberty spot" (Essenberg 1923) seems preferable as the spot is evident on virgin as well as gravid females.

Male guppies reach a length of one inch and are more slender in outline than the females. It is the males that exhibit the great range of pattern and color which have helped make the guppy a favorite aquarium animal. There are usually one or more paired black spots on the body and fins, with bars or spots of red, blue, yellow, and green, and areas of iridescent purple. The fins of the male are broader and more varied in shape than those of the female. The caudal fin frequently has one or two pointed extensions or "swords" which may exhibit an opaque thickening and an intensified color. The dorsal fin may also have a pennant-like extension and may be brightly colored. The pectoral and ventral fins remain unmodified in shape and transparent in color, and are not used for purposes of display. When courting, the male assumes a position before the female, spreads the dorsal and caudal fins to the full extent, bends and vibrates the body, and makes quick forward thrusts of the gonopod. Males exhibit this mating behavior whenever near a female, and will attempt mating with females of other species.

Guppies are born in broods of 3 to 50, though under aquarium conditions, according to the writer's observation, the broods are usually not over 20. The young are about five-eighths of an inch in length and are indistinguishable sexually. At about seven weeks of age

the males begin to show the modification of the anal fin which adapts it as a gonopod or intromittent organ. The fin lengthens, becomes slender, and develops a groove for the passage of spermatophores. A membraneous hood develops near the tip of the gonopod, presumably for holding it in the genital pore of the female during transfer of spermatophores, though according to Innes (1942) its entrance has never actually been demonstrated. At about eight weeks of age the black spots develop and soon thereafter the male colors appear, although several more weeks may be required for the development of full color intensity. At about the age of three months the males are sexually mature, after which time they make only a very slow increase in length, though the fins continue to expand in width and the body to increase in depth, thus giving a more stocky appearance to older males.

At about the same time the males show anal fin modification the females begin to show the puberty spot. The abdomen enlarges greatly, giving the female an entirely different appearance from the male. Growth continues but there is no further change from juvenile characteristics as fins and body remain colorless.

Guppies are capable of breeding at about the age of three months, though early broods are usually small in number. One impregnation suffices for as many as five or six broods, produced at 28 day intervals under favorable conditions. According to Vaupel (1927) the sperm masses of the spermatophores

. . . are evidently broken up in their movement up the oviduct; the individual sperm are seen lying in the folds of the ovary, with the tip of the head tightly pressed against the

follicle cells separating them from the maturing ova. The spermatozoa evidently remain here for months, as one impregnation suffices for several broods.

Except for respiration and the removal of waste, the developing embryo is apparently independent of the mother, as no adaptations for nourishing the embryo are observable and as the quantity of yolk would seem adequate for the development of the young until birth. Prematurely born young are occasionally observed with a yolk sac comparable in every way with that of an oviparous fish.

As in other cold blooded vertebrates, growth and other processes depend greatly on temperature. Low temperatures may as much as double the time required for sexual maturity or gestation.

2. The Testis of L. reticulatus

The structure of the testis and the process of spermatogenesis in guppies are very different from those typical of vertebrate animals. Spermatogonia at the periphery of the testis by multiplication give rise to nests of spermatocytes which become invested with a sheath of connective tissue, thus forming cysts. As spermatogenesis proceeds in these cysts they are pushed nearer the center of the testis by younger cysts until they finally break into the centrally located sperm duct where they are stored until discharge. Except in the younger cysts, only a single stage of spermatogenesis is commonly found in any one cyst (Goodrich et. al., 1934). A mature cyst, when stained, is easily identified in a slide of the testis, as the dark line of sperm heads pressed against the Sertoli cells surrounding the cyst, and the delicate lines of the sperm tails extending toward the center of the cyst

are very conspicuous. Sertoli cells are no longer evident after the cyst has passed into the sperm duct, but the mass retains its shape through the intertwining of the sperm tails, thus forming a sperm ball or spermatophore. According to the writer's observation, these may frequently occur in a dumbbell shape, presumably through cohesion of two adjoining balls. Spermatophores may be forced from a mature male by gentle pressure against the sides of the abdomen.

The testis of the guppy is a bilobed structure produced by fusion of the two embryonic gonad structures. At the anterior end the two lobes remain distinct, the fusion becoming more intimate toward the posterior end where the two main sperm ducts from each lobe unite to form a single short duct. The main ducts of each lobe give rise to branch ducts, the entire duct system being lined with cuboidal epithelium.

3. The Ovary of L. reticulatus

The ovary of the guppy is paired at birth but begins to fuse into a single structure a few days after birth. A cavity lined with simple columnar epithelium runs the length of the ovary and continues as the oviduct. The young ovary is covered with a flattened layer of epithelial cells derived from the peritoneum, and contains oögonia derived from primordial germ cells plus a small amount of stroma. The epithelium lining the ovarian cavity (germinal epithelium) is assumed to produce oögonia from its own cells or from stroma cells that have become incorporated with it. As each oögonium develops it pushes outward from the cavity, carrying with it a few epithelial cells which help to form the follicle, but remaining attached by a neck of epithelial cells to the layer forming the cavity proper. An oögonium may form a nest of oöcytes, one or more of which may enlarge greatly, become invested with a yolk-secreting follicle, and thus become mature ova (Goodrich et. al., 1934).

In a freshly dissected ovary the mature ova are quite large, amber yellow in color, and full of oil droplets which readily escape upon puncture. Undeveloped ova are small, opaque white masses occurring in clumps at the base of the protruding large ova. In the stained and sectioned ovary the cavity is discernible only as folds of epithelium winding between the ova. The yolk is very difficult to section, cracking into strips and usually falling off the slide.

4. Sex Inheritance in L. reticulatus

Bridges (1939) in discussing the cytological and genetic basis of sex, makes frequent reference to Winge's work on the genetics of guppies (Winge 1922, 1923). His review presents a very useful outline and evaluation of this work, and is herewith summarized: (From Sex and Internal Secretion, pp 47-49).

The normal sex formula for *Lebistes* is XX = female and XY = male. Crossing experiments in various races show nine color patterns that are transmitted from father to son exclusively, never being exhibited nor transmitted by females. Thus on the basis of this discovery Winge announced a new type of sex-linked inheritance, that of factors exclusive to the Y-chromosome.

Eight other primary patterns of male coloration were found to be transmitted by the X-chromosome and thus passed by males to their daughters to be passed on in turn by them to half of their sons. All of these genes were thought at one time or another during the course of Winge's breeding experiments to have crossed over and become part of the Y-chromosome.

Two races of L. reticulatus having masculinized females yielded on repeated crossings a new strain in which both males and females were XX. Since the X-borne characters were now inherited equally from both sexes, the X-chromosome was assumed to have ceased to be a sex differential and to have become an autosome, while sex differentiation was supposed to have been taken over by a chromosome that was formerly an autosome, through the accumulation in it of male-tendency genes.

Likewise, by concentrating female-tendency genes in an autosome, Winge succeeded in producing XY females and YY males. As a result of these findings Winge concludes that numerous female-tendency genes and male-tendency genes exist in both autosomes and sex-chromosomes alike, and that sex is the outcome of the specific balance between these genes, among which there is no valid distinction as to "primary" sex genes versus "modifiers".

Winge's assumptions as to the scattering of sex genes among the autosomes and the experimental shifting of sex determination from sex-chromosomes to autosomes are questioned by Goldschmidt (1937), who maintains that Winge's data can be explained by the application of the M-F balance theory.¹

It seems certain however, in spite of the unsettled status of theories of sex determination in L. reticulatus, that sex in this species is genetically determined, but by a set of genetic factors of a nearly equal balance.

Other workers have studied the genetics of Lebistes. Vaupel (1929) has tentatively identified a pair of small similar-appearing chromosomes as the sex chromosomes. It has been established that the diploid number of chromosomes is 46 and the haploid number 23.

¹Strong and weak female determining factors (F and f) in the X- and Y-chromosomes respectively, with male factor(s) M(m) in autosomes, cytoplasm of egg, or Y-chromosome, or in a pair of accessory sex chromosomes. (Present writer's formulation). M and F vary in strength in various races, or may be differently located in sex chromosomes according to the type of heterogamy.

5. Lebistes as the Subject of Biological Experimentation

For several reasons guppies have proved popular as experimental subjects. They are hardy, require little attention, breed at short intervals, and produce large numbers of young. They endure handling better than do most tropical fish, exhibiting only a temporary excitement at being transferred from tank to tank or being held in a net for examination. The writer has often kept specimens in a petri dish of water for several minutes while examining them under a microscope or while applying medicines to discourage fungus growth; the animals endure such treatment well, behaving in an entirely normal manner shortly after their return to the tank. They are not greatly upset by changes of water and can go without feeding for two days or more, though this practice is likely to encourage their cannibalistic tendencies.

The sexual dimorphism of guppies makes them suitable material for experimentation with sex hormones or similar substances, and their peculiar type of sex-linked inheritance offer a challenging field for genetic experimentation. Many fish breeders and amateur aquarists have also taken an interest in developing color strains of guppies.

6. Maintenance of L. reticulatus

in the Laboratory

In common with all tropical aquarium fishes, guppies require a warm temperature, well-aerated water free from injurious chemicals, and a varied diet. In this series of experiments the water was ordinarily maintained by thermostat-controlled heaters within a range of 75- 78° F. These limits were exceeded in summer in very hot weather, and in winter

when the heating apparatus proved inadequate to cope with very low temperatures. In spite of occasional drops of water temperature into the low sixties, the subjects survived without apparent injury.

Aeration was provided at all times by an air pump. Water used in the tanks was Louisville tap water allowed to stand 24 hours or more before use, in order to release harmful gases. The tanks were of one gallon capacity, and usually contained a strand of Cabomba or Anacharis which served as refuge for the fish rather than as oxygenators, as a lack of direct sunlight kept them from flourishing. The use of sand in tanks was abandoned, as it proved difficult to keep clean. Food and sediment were not allowed to accumulate and thus foul the water; tanks were siphoned out and the water filtered and returned or replaced by fresh, about once per week. The animals were maintained on a diet of prepared fish food occasionally supplemented with lettuce, shredded liver or beef, and minced fresh earthworm.

SECTION V

ADMINISTRATION OF THE AGENTS

SECTION V

ADMINISTRATION OF THE AGENTS

Two topics relating to administration of the experimental agents will be discussed in this section: (1) Considerations Regarding Dosage and (2) Experimental Procedures.

1. Considerations Regarding Dosage

At the beginning of this experimentation several questions regarding the administration of hormonal substances to fishes presented themselves. Most of the preceding investigators had injected an oily solution of the male or female hormone into the body cavity of the fish. It was hoped by the present investigator that this tedious procedure would be unnecessary, and that a water soluble form of the agent might be procured, a form which would be absorbed through the gills of the fish. and possibly to some extent also through other body membranes, in sufficient amounts to be effective. This expectation was realized through the selection of each agent in a form suitable for oral administration in clinical practice, such tablets proving to be freely water soluble and to retain effectiveness in solution for several days. The prolonged effectiveness of one of the agents (pregneninolon) was tested by keeping a set of juveniles¹ in water taken from a tank in which pregneninolon tablets had been administered

¹For the purposes of this experimentation a juvenile may be defined as a sexually undifferentiated individual.

regularly twice per week to a set of experimental subjects. The agent had been in the water not less than four days at the time of removal. This old water proved to be fully as effective as tablets freshly administered. There was one animal in the old pregnenolone water that seemed to be responding in an unusual way to the agent,¹ whereupon the experiment was repeated with another set of juvenile animals, with results in all ways identical with those produced by feeding the tablets. It was then assumed that the anomaly had had some cause other than that of a variation in concentration or method of administration of the agent.

This experiment also helped clear up the question of a possibly augmented effect of the agent in fish that ate it as well as breathed it. No differences in effect were observed between subjects that merely absorbed pregnenolone through the gills and those that in addition swallowed some of the crushed tablets as food. Another agent, estradiol, also proved acceptable as food, but is thought likewise to have been fully effective in solution.

It was noted, also, that in all tanks the dosage produced a complete or maximum effect. It was at first anticipated that limited solubility of the agent might maintain it at a certain level of concentration in the water, a level which might prove less effective than a feeding method of administration. If there was any limit in solubility of the agents, however, it must have been above that of any threshold

¹For an account of this specimen see footnote to Table VIII, p.43

or minimum effect on the subjects, as all the observed effects were produced promptly and fully in all specimens regardless of dosage or method of administration. See Table II, p. 27, for dosage.

It was observed, however, that all tablets left an undissolved residue in the water. This substance was assumed to be an insoluble filler rather than any undissolved portion of the agent, as the addition of more water to the tank had no effect in further dissolving the residue.

Being largely without data from other experimenters as to the administration of water soluble hormone preparations, the present investigator selected tablets of the smallest dosage prepared for medicinal purposes, and except in the case of one pregneninolon experiment, for which the regular 5 mg. tablet was divided into fifths, gave as regular dosage one tablet twice per week in all tanks of experimental subjects. The tanks each held approximately one gallon.

Another consideration was that of a possible toxic effect of the agents, a condition which might call for new experiments with a modified dosage. Though there was a depressing effect on the growth of most subjects receiving treatment (see p. 67) for a suggested explanation) and a slight depression of activity, in most cases there was little indication that the administered substances were threatening the life of the subjects and thus the success of the experimentation. Exceptions to this statement are the cases of the stilbestrol-juveniles, the pregneninolon-gravid-females, and the estradiol-gravid-females. These gravid individuals (Tables III and VII, pp. 34,43) endured treat-

TABLE II
ADMINISTRATION OF AGENTS

Agent	Tablet Size	p.p.m	Subjects Treated
Stilbestrol	.1 mg.	.025	Juveniles
Estradiol	.1 mg.	.025	Juveniles Adolescent males ¹ Gravid females
Pregneni- nolon	5.0 mg.	1.250	Adolescent females Gravid females
Pregneni- nolon	1.0 mg.	.250	Juveniles
Pregneni- nolon water	. . .	. . .	Juveniles

¹For the purposes of this experimentation an adolescent may be defined as an individual undergoing differentiation of secondary sexual characters.

ment very poorly, appearing languid and becoming diseased from fungus attacks early in treatment (pp. 37, 50). It is thought, however, that interference with gestation rather than other systemic effects may have caused their death.

Stilbestrol-treated juveniles, though suffering a marked suppression of growth, seemed otherwise almost normal in condition until the close of the fourth month of treatment, at which time they succumbed in short order (p. 38).

Most of the subjects undergoing pregnenolone treatment suffered less suppression of activity than subjects of other experiments. Their keen appetites, attenuated forms, and overactive behavior when startled, suggest that the agent may have exerted a stimulating effect on metabolism.

In general, it was found upon dissection of subjects at the close of the experimentation that all body organs except gonads were healthy in appearance and quite comparable to those of the controls, though fatty tissues were less abundant. It may be appropriate to state here that any harmful effect of the agents was assumed to be exerted through an interference with the endocrine balance of the animal rather than through any direct effect on such vital functions as respiration, assimilation, or others.

2. Experimental Procedures

Before the actual experimental procedures were inaugurated it was considered essential to establish optimum laboratory conditions for the subjects, as activity which necessitated a careful study of such

factors as aeration, temperature control, feeding, water changing, and disease control. The effects of these factors were observed while the stock of experimental animals was being built up; efforts meanwhile were made to ascertain the best possible conditions for their maintenance. As stated earlier (section IV), guppies are easy to culture; it was found throughout the experiments that few losses and ill effects could be attributed to unsuitable conditions. Only once was there a threat to the successful maintenance of the subjects; this consisted of an outbreak of the dreaded mouth fungus in the estradiol-juvenile tank. One specimen succumbed, but the others recovered after receiving salt treatment. Salt was used freely also in all other tanks as a preventive, and no further outbreaks of the disease occurred.

A puzzling feature in many of the experimental tanks was a gradual decrease in the number of animals, with no dead bodies as evidence. It was at first thought that specimens might have been lost by leaping from the tanks, but careful search disclosed only an occasional suicide. (Losses from leaping were less than 1% of all animals kept on hand). The probable explanation is that once an animal succumbs (from whatever cause) it becomes a food object to its companions and is consumed, leaving no trace. On one occasion a non-experimental adult was found dead in the water; examination disclosed that the intestine and abdominal wall had ruptured, presumably from the intake of too much dried food. Such accidents may have taken some toll of the experimental

animals, but are thought to have been a negligible factor in mortality.¹

All of the reported experiments except those using old pregnenolone water were begun Feb. 1, 1943, at which time there were available 3 broods of juveniles of about 2 weeks of age, one brood of adolescents just reaching sexual differentiation, and a number of adults. For each of the experiments using juveniles a single brood was selected, six individuals being reserved as controls. For the experiments using adolescent males and females, individuals of the adolescent brood were sex segregated, three individuals of each sex being reserved as controls.

For the experiments using old pregnenolone water, begun about April 1 and June 1, the young of two broods were combined for each experiment. The age of these young appears in Table VI, p. 41.

It was thought that the use of animals of a single brood would insure the greatest possible uniformity in the subjects of a single experiment. This was probably an unnecessary precaution, as guppies of the same age appear to respond in the same way to any factor, the only observed variation among broods being in the time required to reach sexual differentiation. This variation may possibly be accounted for on the basis of difference in strains; inbreeding may possibly account for slow development.

¹Breder and Coates (1932) report that guppies kill and eat excess individuals in overcrowded tanks, reducing the population to a stable number (proportional to the size of the tank) in which two females survive for every male.

All experiments were continued until July 1, 1943, with the following exceptions: one animal each from most of the experimental tanks was taken for dissection and study of the gonads in April, and on May 1 approximately 50% of the survivors in each hormone experiment were withdrawn from the treatment tanks and placed in untreated water.

At the conclusion of the experiments a histological study was made of the gonads of most of the surviving experimental animals and withdrawals. Not all animals were used in every case, but enough specimens were sacrificed to insure whenever possible a fair sampling of males and females from each set.

It may be remarked here that the external appearance of the treated animals never gave any indication of the true sex. Usually upon dissection, however, the gonad was easily identifiable as an ovary or a testis; only in cases of extreme suppression was the genetic sex still in doubt. A study of the microscope slides of these suppressed gonads (fixed in Bouin's and stained in iron hematoxylin) served in most cases to clear up the difficulty.

SECTION VI

RESULTS OF THE EXPERIMENTATION

SECTION VI

RESULTS OF THE EXPERIMENTATION

Tables III through X present observations upon the experimental animals during treatment, the effects of withdrawal from treatment, and data on freshly dissected and on sectioned and stained gonads. Plates I through VI are photographs of these sets of experimental animals at the close of treatment, before any were sacrificed for dissection (except for a few specimens taken midway in the experimentation, as mentioned in the preceding section). Plates VII through XVII are photomicrographs of certain gonads described in the tables.

The experimental groups have been named according to the conditions of the experiment rather than with code numbers or other symbols. For example, an "Estradiol-juvenile-withdrawal, apparent female" is an animal subjected to estradiol treatment while still sexually undifferentiated, later withdrawn from hormone treatment, and appearing more feminine than masculine at the time of killing for histological examination. Where more than one animal from any experimental group was studied histologically, the specimens were assigned letters A, B, C, etc., with the photomicrographs of the gonads of these animals bearing the same key letters.

A discussion of the effects of treatment is presented below under appropriate headings.

1. Observations on Estradiol-Treated Subjects

Juveniles. Estradiol-treated young guppies showed no immediate marked response to the agent, though a comparison with the controls after a week or two of treatment showed a retardation of growth in the treated subjects. As treatment progressed, the subjects were observed to assume a somewhat more rounded outline than normal in juveniles (a characteristic of maturing females), though the more diagnostic puberty spot never appeared at any time even though treatment was prolonged far past the time at which the females among these juveniles would normally have reached sexual differentiation (Plate I, Fig. 1). The actions of the treated animals were somewhat sluggish, but otherwise they seemed normal in behavior. Near the close of the experimentation the anal fin assumed the narrow shape characteristic of the male gonopod; in no case, however, did it complete its development and become identical with that of a normal male (Table III, p. 34).

It may be remarked here that in all sets of experimental juveniles the responses of the subjects were in all observed respects the same without regard to genetic sex.

Estradiol-withdrawn-juveniles exhibited an immediate acceleration of growth and activity upon withdrawal from treatment, and soon assumed the fully rounded abdomen and dark puberty spot of the female (Plate I, Fig. 2). There were only three of these withdrawal specimens; all proved on dissection to have fully developed and apparently normal ovaries (Plate X, Fig. 1).

TABLE III
EFFECTS¹ OF ESTRADIOL OBSERVED DURING TREATMENT

Age, Number, and Sexual Condition of Subjects	Systemic Effects	Effects on Secondary Sex Characters	Mortality
Seventeen undifferentiated juveniles, age 16 days	Growth greatly inhibited, color pale, gills very pink	Feminized body outlines, no puberty spot, no male color. After 4 months of treatment a gonopod develops	About 50%
Eight adolescent males, age 4 months	Slight inhibition of growth	None	Negligible
Three adult gravid females	Depressed, weakened, susceptible to disease	None	100%

¹The word "effect" here and elsewhere in this paper is used only to avoid such circumlocutions as "modifications observed after treatment", or "phenomena associated with the use of the agent". Unless otherwise indicated by the context, "effect" is not intended to imply a direct causal relationship of the agent.

The gonads of the estradiol-juveniles showed a range of effects (Table IV, p. 36). The ovary of one specimen, though small and apparently lacking a well developed epithelium, was essentially normal (Plate IX, Fig. 2). The ovary of another specimen sacrificed earlier in treatment showed great suppression (Plate IX, Fig. 1). The testis of one of the males was greatly suppressed in size, contained but a few spermatogonia, and had poorly developed ducts. The testes of two other males clearly showed a feminizing effect of the agent. One of these testes contained a large ovum (Plate VIII, Fig. 2), while the other had many germ cells (some spermatogonia, some oögonia), with numerous oöcytes and a few unmistakable ova with follicle cells (Plate VIII, Fig. 1. See the Appendix, also, for a discussion of this case).

Adolescent Males. Males just past sexual differentiation at the beginning of treatment with estradiol exhibited no effects of treatment on secondary sexual characters and little on activity. There was a slight depression, as was the case of most animals treated with these agents. The withdrawn specimens became somewhat more active and exhibited a slight intensification of the male colors.

Upon dissection the testis of one of the estradiol-adolescent males proved to be heavily loaded with spermatophores, many being discharged when the sperm duct was cut. A photomicrograph of this gonad (Plate XI, Fig. 1) shows it to contain a few young cysts and many later cysts and spermatophores, some abnormal. There is some evidence that hypertrophy of the duct system may have occurred earlier, as there are

TABLE IV
EFFECTS OF ESTRADIOL ON GONADS

Experimental Subjects	Gonad Size and Condition at Time of Killing	Effect of Experimental Agent on Gonad Tissues
Estradiol-juveniles, apparent females	A. Very small testis	Marked suppression of germinal elements, only a few spermatogonia, ducts evident but not well developed
	B. Testis bilobed, small	Easily identifiable as genetic testis. Has a few spermatogonia, a cyst of spermatocytes, with many oögonia, oöcytes, and ova, some with definite follicle cells
	C. Small, apparently normal ovary	Ovary normal except for poorly organized epithelium
	D. Greatly reduced gonad	A genetic testis having numerous ducts with degenerated epithelium, a few large cysts of spermatogonia, and one large degenerated ovum
	E. Small ovary	Suppressed but not degenerate ovary (sacrificed early in treatment). No yolk, no well developed follicle cells
Estradiol-juvenile-withdrawal, apparent female	Large and apparently normal ovary	Ovary normal in every way
Estradiol-adolescent-genetic male	Large bilobed white testis which discharged many spermatophores	Evidence of abnormal stimulation. Probably earlier hypertrophy of duct system, now consisting mainly of basement membrane and masses of debris. Young cysts near periphery, some late and mature cysts throughout. Mature cysts small, abnormal, easily broken, sperms poorly formed, no spermatogonia
Estradiol-adolescent-withdrawal, genetic male	Large white testis	Abnormal testis. There are a few irregular cysts, much connective tissue, and evidently a regenerating epithelium. Numerous spermatogonia, some beginning to form cysts

large areas of a homogenous debris adjacent to the remains of the duct epithelium, which at this stage is represented mainly by a basement membrane and an occasional strip of degenerated epithelium. No spermatogonia could be identified.

An estradiol-withdrawal adolescent male had a large testis with a few cysts and large areas of connective tissue (Plate XI, Fig. 2). The sperm ducts were centrally located and lined with a single layer of epithelium (probably in the process of regenerating after treatment). There were numerous spermatogonia, some beginning to form cysts.

Adult Gravid Females. Estradiol exerted a depressing effect on the activity of adult gravid females and apparently caused susceptibility to a fungus disease, as all specimens died within two months of the beginning of treatment. One of the females gave birth to eight apparently normal young three weeks after treatment began; in the other females the embryos were apparently resorbed, as no further births occurred. Probably the physiological upset of such large masses of degenerating tissue and the increased susceptibility to disease were the immediate cause of death in these specimens, as the agent was not observed to exert actually toxic effects in these or other experimental animals. At any rate, there were no survivors after two months. Dissection of a dead specimen showed no developing embryos.

2. Observations on Stilbestrol-Treated Subjects

Juveniles. Only one set of experimental animals was treated with stilbestrol. There was suppression of growth, a slightly femini-

zing effect on body proportions, and late in treatment a slight modification of the anal fin (Table V). Upon withdrawal from treatment there was an increase in growth and activity, and the assumption of a more feminine outline and a definite puberty spot in all individuals. Certain of these specimens later assumed male outlines and markings; two of them proved on examination to be genetic males, and a third (still living) has lost all feminine characteristics and seems to be a normal male.

One unexpected development was noted in a withdrawal animal which proved upon subsequent examination to be a female. There was a regeneration of fin rays and membranes beyond the margins of the narrowed anal fin assumed by this specimen under treatment. It is generally reported in the literature of similar experiments that once the shape of the anal fin is set in the gonopod pattern it is incapable of further modification; perhaps in this case the slight degree of initial modification had not destroyed all growth capacities in the fin.

It so happened that withdrawals were made at a critical period in the treatment of these juveniles with stilbestrol. All withdrawn specimens lived and flourished, while those remaining under treatment soon succumbed. No clue as to the lethal action of the agent was observed. Of the three agents used in this experimentation, however, stilbestrol is the only one reported to produce occasional allergic or toxic reactions in patients. It is quite likely that the fish would have survived longer with less massive dosage.

One of the experimental animals had been killed for examination

TABLE V
EFFECTS OF STILBESTROL OBSERVED DURING TREATMENT

Age, Number, and Sexual Condition of Subjects	Systemic Effects	Effects on Secondary Sexual Characters	Mortality
Nine sexually undifferentiated juveniles, 13 days old	Marked inhibition of growth, pale and pinkish color, very pink gills. Activity sluggish near end of experimentation	Feminized body outlines but no puberty spot. No male colors. After 4 months a gonopod develops	100% of those remaining under treatment

after two months of treatment and before any withdrawals had been made. This animal's gonad was apparently a testis with only a few spermatogonia in poorly formed cysts at the periphery, and with degenerated sperm ducts making up most of the organ (Table VI; Plate X, Fig. 2).

Among the withdrawal animals sacrificed for examination, however, the males (two individuals) had testes apparently normal in size and development (Plate XII). All stages of spermatogenesis were represented in the testes of both animals. Hardly any stroma was evident, and ducts were rare and slightly developed. The unusual size and number of blood vessels in these organs is an indication of the rapidity with which regeneration must have been taking place. One female withdrawal had a large ovary with five well developed ova, the organ proving on histological examination to be essentially normal except for an increased vascularity. As no female specimen had been sacrificed during treatment, it is impossible to state what the effects of stilbestrol on the ovary might be, or how much regeneration may have taken place in the ovary of this withdrawn specimen.

3. Observations on Pregneninolon-Treated Subjects

Eversole (1941) observed a marked androgenic effect of pregneninolon on L. reticulatus. (A comparison of his results with those of the present investigator may be seen in Table XII, p. 58). Though strictly speaking pregneninolon is not an estrogen, the similarity of its effects to those of the corpus luteum in mammals might arouse an expectation of some kind of feminizing effect on laboratory subjects.

TABLE VI
EFFECTS OF STILBESTROL ON GONADS

Experimental Subjects	Gonad Size and Condition at Time of Killing	Effects of Experimental Agent on Gonad Tissues
Stilbestrol-juvenile, apparent female	Very small gonad	Apparently a testis with a few degenerate cysts near periphery. Degenerated epithelial tubes with wide lumens. If spermatogonia are present they are abnormal. This animal sacrificed early in treatment
Stilbestrol-juvenile-withdrawals, apparent females	A. Testis	Apparently normal testis, all stages of spermatogenesis, no hypertrophy of ducts
	B. Testis, one lobe blood-shot	Testis normal except for increased vascularity, little stroma, few ducts. Att stages of spermatogenesis
	C. Large ovary, 5 well-developed ova	Ovary normal except for increased vascularity

In order to test its action through a wide range of subjects, several experiments were performed. Juveniles, adolescent females, and gravid females were treated with tablets crumbled into the tank water (Table VII). Two additional sets of juveniles were treated with water withdrawn from tanks receiving pregneninolon treatment (Table VIII, p. 44).

Juveniles. In the case of juveniles treated with fresh tablets, growth was greatly inhibited and male secondary sex characters were assumed in all individuals males and females alike. These characters were induced at a much earlier age than that of their normal appearance in males. The beginning of gonopod modification appeared a few days after the start of treatment; soon thereafter male colors, and finally male behavior, were observed. Though growth was greatly inhibited, activity seemed only slightly affected. Juveniles treated with old pregneninolon water exhibited the same effects as those treated with the fresh hormone tablets, except that these effects were perhaps not quite so marked (Table VIII).

Withdrawals of juveniles from fresh pregneninolon treatment were made (Table X, p. 49). For the entire period of withdrawal (two months) the subjects remained apparently male, though one proved upon subsequent examination to be female. There was the characteristic spurt in growth upon withdrawal.

Both males and females suffered gonad suppression from pregneninolon treatment (Table IX, p. 46; Plate XIII; Plate XIV). There was marked stimulation of duct epithelium in the testes of withdrawn sub-

TABLE VII
EFFECTS OF PREGNENINOLON OBSERVED DURING TREATMENT

Age, Number, and Sexual Condition of Subjects	Systemic Effects	Effects on Secondary Sexual Characters	Mortality
Twelve sexually undifferentiated juveniles 11 days old	Marked inhibition of growth, little effect on activity	Gonopod appears within a few days, male colors appear long before normal time and in all subjects regardless of genetic sex	Less than 50%
Twelve adolescent females 4 months old	Growth slowed, appetite increased, body slender	Gonopod appears after few days, black spot after 14 days, colors (paler than normal) then appear, puberty spot disappears after 6 weeks, male behavior appears, caudal and dorsal fins become attenuated, long, and ragged	About 25%
Three adult gravid females	Weakening, causing susceptibility to disease, sores on body	Puberty spot disappears and black spots appear, anal fin modified	100%

TABLE VIII

EFFECTS OF PREGNENINOLON WATER AGED 4 DAYS OR MORE, OBSERVED DURING TREATMENT

Age, Number, and Sexual Condition of Subjects	Systemic Effects	Effects on Secondary Sexual Characters	Mortality
Five sexually undifferentiated juveniles aged 2 to 4 weeks	Inhibition of growth	Gonopod and male colors appear in few days, body typically male in outline with exception of one individual ¹	Negligible
Seventeen sexually undifferentiated juveniles aged 1 to 3 weeks	Inhibition of growth	Gonopod and male colors appear, body outline typically male in all subjects	Negligible

¹One of these specimens presented a very curious anomaly presumably in response to the agent. This individual, a few days after treatment began, showed a marked distension of the abdomen (Plate V, Fig. 1.) The outline was similar to that of a gravid female, except that the distension was two or three times as great. The internal pressure was so strong that the ribs could be distinguished through the stretched skin. That this was an abnormal condition was evident from the discrepancy in size of the abdomen and other body parts. It was conjectured that the distension might be caused by an ovary stimulated to precocious development by the agent. At the termination of the experiments this specimen was examined under strong light with a binocular microscope, at which time the clear yellow color of ripe ova was discernible through the thinned body wall at the puberty spot. This specimen was not sacrificed for histological examination, but was reserved for possible breeding. It was noted that after withdrawal from treatment the ovary regressed slowly in size.

No similar anomaly of development occurred in the duplicate experiment. No explanation is offered as to the cause of this unusual development.

jects (Plate XV, Fig. 1), and also in those undergoing treatment for a short time only (Plate XIV).

Adolescent Females. Probably the most interesting set of effects observed were those of pregneninolon on adolescent females (Table VII, p. 43). After only a week of treatment masculinization of the anal fin was noted. In two weeks several of the individuals had developed either black spots or colored margins on the caudal fin. After six weeks of treatment the puberty spot had disappeared and a male type of behavior had appeared, the individuals occasionally showing fight or tensing and vibrating the body in the courting display.

Other behavior effects were noted. There was a timidity and nervousness at the slightest disturbance, a behavior noted in normal guppies only as a result of very rough treatment. These experimental animals seemed also to have an abnormally eager appetite.

Though all subjects treated with pregneninolon showed definite masculinizing effects, the masculinization was never so complete as to cause these animals to be easily mistaken for genetic males. The colors never became as intense, nor the dorsal and caudal fins as large, as in control males. These subjects did in certain ways, however, exhibit exaggerated effects of the agent. The body became excessively slender, and there was a great lengthening and narrowing of the caudal and dorsal fins, with the development of iridescent colors along the margins (Plate VI, Fig. 1). As the narrowing of these fins is not normal in either males or females, it is assumed that the agent may have exerted a local eroding action on the tissues of the fin margins. It had previously

TABLE IX
EFFECTS OF PREGNENINOLON ON GONADS

Experimental Subjects	Gonad Size and Condition at Time of Killing	Effects of Experimental Agent on Gonad Tissues
Pregneninolon-juveniles, apparent males	A. Suppressed gonad, opaque white mass at anterior end	Very degenerate gonad, presumably an ovary. One large ovum at anterior end, and one or more large collapsed structures probably degenerated ova
	B. Small and degenerate gonad	Suppressed or degenerate gonad, presumably a testis, no germinal elements distinguishable, impossible to identify definitely
Pregneninolon-juvenile-withdrawals, apparent males	A. Ovary with one large ovum	Apparently normal ovary, one large and many small ova
	B. Very degenerate gonad	Very degenerate, much connective tissue, no germinal elements identifiable. Probably a testis
	C. Very small testis	Testis very small, with hypertrophied duct system, no germinal elements except possibly a few peripheral spermatogonia
	D. Small testis	Testis with hypertrophied duct system, no germinal elements identifiable, much stroma at anterior end
Old-pregneninolon-water-juveniles, 5 month treatment, apparent males	A. Small gonad	Presumably a testis, no germinal elements distinguishable, much loose connective tissue, very spongy
	B. Small, whitish mass	Ovary with ova in various stages, none mature, many degenerate. Whole organ very vascular, no ovarian cavity discernible, much degenerate epithelium
	C. Very small testis	Testis with much duct system, much connective tissue, no germinal tissue identifiable

TABLE IX (continued)

EFFECTS OF PREGNENINOLON ON GONADS

Experimental Subjects	Gonad Size and Condition at Time of Killing	Effects of Experimental Agent on Gonad Tissues
Pregneninolon-adolescent-genetic females	A. Distinct ovary, oviduct, and genital pore	Ovary of fair size, with a few large ova and many oocytes. Epithelium hypertrophied. Genital pore consists of epithelium, connective tissue, and striated muscle fibers
	B. Small ovary	Ovary small, with many ova, many oocytes, and much epithelium
	C. Small ovary	Ovary small, compact, with many ova and a large lateral ovarian cavity with a proliferated epithelium. (This subject examined after short treatment)
Pregneninolon-adolescent-female-withdrawal	Definite ovary	Ovary medium-sized, with one very large ovum and many smaller ova. Yolk abundant, follicle cells normal, few oocytes. Heavily vascularized, no distinct ovarian cavity

been observed that in a fish of another genus (Mollenisia) a disease known as fin rot had caused an intensification of pigment at the eroding margins of the fins. It was thought for a time that the narrowing and pigmentation of the caudal and dorsal fins of the guppies under treatment with pregnenolone was an indication of this disease, but as no morbid effects were observable upon microscopic examination, and as the condition did not produce systemic effects as in the case of true fin rot, it was concluded that in the case of these treated females there was a particular sensitiveness of fin margins to the agent. A similar though less marked effect was noted in treated juveniles.

These pregnenolone-treated adolescent females were so greatly affected by treatment, in both physical features and behavior, that their survival until the close of the experiments seemed unlikely. The mortality, however, was only about 25%, most of the losses occurring early in treatment. At the time of withdrawal care was taken to select one of the most severely affected specimens as a withdrawal subject; this specimen as well as those less affected made a rapid recovery from the effects of pregnenolone treatment (Table X). The body became more rounded, the colors faded, and the fins almost completely regained their typical feminine transparency and rounded outline (Plate VI, Fig. 2). At the time of writing four of the original set of these female guppies remain: two withdrawals that have had three months of treatment and three of withdrawal, and two others that have had five months of treatment and one of withdrawal. (At time of writing, August 1, all hormone feedings have been discontinued). It may prove interesting to breed these females and watch for possible offspring.

TABLE X

EFFECTS OF WITHDRAWAL FROM HORMONE TREATMENT

Experimental Treatment	Condition at Time of Withdrawal	Effects of Withdrawal on Appearance and Behavior
Stilbestrol-juveniles	Stunted, weakened, no secondary sexual characters except gonopod	Improved growth, nutrition, and activity, development of puberty spot (some prove on histological examination to be males)
Estradiol-juveniles	Stunted, have faint puberty spot, gonopod, no color	Improved growth and nutrition, all develop puberty spot and typical female appearance
Estradiol-adolescent-males	Depressed activity	Improvement in general activity, intensification of male coloring
Pregneninol-juveniles	Stunted, typical male colors though somewhat pale	Improved growth, continued lengthening of caudal and dorsal fins, otherwise seem typically male though some prove later to be female
Pregneninol-adolescent-females	Attenuated body, long ragged caudal and dorsal fins, male coloration (pale), male behavior	Marked improvement in nutrition, growth, and activity, and an immediate disappearance of color with resumption of normal fin contours. One individual develops puberty spot

Histological examination of the ovary of one of the females after only two months of treatment showed it to be small and compact, with many ova and no follicle cells nor indications of yolk deposition (Table IX, p. 46, pregneninolon-adolescent-female, specimen C; also Plate XVI, Fig. 1). The ovaries of other specimens that had undergone the whole five months of treatment were still of about the same size, but contained fewer ova and more epithelium (Plate XVI, Fig. 2; Plate XVII). An interesting anomaly was the hypertrophy of the genital pore of these long-term specimens; a slide made of this tumorous mass showed it to consist of irregular masses of epithelium, connective tissue, and striated muscle.

A study of the ovary of one of the withdrawals indicates that there was rapid recovery from the effects of treatment. The organ was much larger than the suppressed ovaries of treated animals, was heavily vascularized, and had many small ova and several larger yolk-containing ova (Plate XVII, Fig. 2). The follicle cells were very conspicuous, in some sections appearing in heavily massed rows.

Adult Gravid Females. Adult gravid females treated with pregneninolon showed a regression of the puberty spot and a faint indication of male coloring. It was assumed that the embryos were resorbed, as there were no births. After about two months of treatment the last of these specimens succumbed. Autopsy showed a few external lesions or congested areas and great swelling and congestion of viscera. Dissection of the ovary showed it to be an encapsulated structure surrounded by a fatty substance in which were numerous small, ovoid, firm, unde-

veloped ova. The center of the ovary was occupied by a clear, amber, rounded mass containing one or more degenerate or abnormal embryos.

An interesting conjecture is the possible effect of a much lighter dosage of pregneninolon. In sub-lethal concentrations the treated females might live to bear young which might show at birth some effect of the agent, or at the time of sexual differentiation an altered sex ratio, as a result of prenatal treatment.

SECTION VII

DISCUSSION

SECTION VII

DISCUSSION

This section will be divided into the following subsections:

(1) Critique, (2) Comparison of Results with Those of Other Investigators, (3) Interpretation of Results, and (4) Inferences Relating to Action of the Agents.

1. Critique

Certain questions as to the validity of experimental procedures may occur to readers. Certain doubts arose also in the mind of the experimenter, some of which are presented herewith.

It was anticipated that difficulties might be encountered in ascertaining the genetic sex of experimental subjects having a gonad modified into an ovotestis. The external appearance of such an animal never provided any clues to the genetic sex, but the gonad upon gross dissection usually was easily identifiable as a testis or an ovary through its general appearance. Any testis except a very degenerate one has a bilobed structure and a whitish or transparent appearance quite different from the irregular bumpy appearance of an ovary. Mature ova have a distinctive clear yellow color easily distinguishable through the thin ovarian membrane. Even a suppressed ovary contains small opaque white lumps which prove on examination to be undeveloped ova. In no case did an ovary ever undergo suppression to the extent evidenced by an occasional testis. It usually happened in the case of

gonads so greatly suppressed as to be mere threads of tissue, that examination after sectioning showed a few cysts indicative of some stage of spermatogenesis, thus identifying the gonad as a testis.

The small number of subjects treated in each experiment, and the even smaller number surviving at the end of the treatment, might throw some doubts upon the validity of the interpretations. It is true that a larger number of survivors would have permitted a better study of the variety of effects in different individuals. On the whole, however, the effects of treatment were so marked and so constant that the examination of a single specimen usually sufficed to show the general response of the animal to the agent. When the number of survivors permitted, however, enough animals were sacrificed and examined to provide a selection including both males and females.

A criticism more fundamental to the validity of these experiments might reasonably be made. It is fairly obvious that the experiments were allowed to run over too long a time, and that the agents were administered in too great concentrations. If it had been feasible to run duplicate experiments with varying concentrations of hormone, or if sampling of effects by histological examination could have been made at frequent intervals, the dosage and periods of treatment might have been better adjusted. In this way the effects of treatment might have more closely approximated the physiological action of naturally-present hormones (assuming a close relationship of such secretions and the experimental agents), and the experiments might have been terminated at the time of maximum effect of the agents.

In certain experiments with a high mortality, the question of a possible selective lethal effect of the agent on either males or females arose. Though this point has little bearing on the actual results of the experimentation, it is interesting enough in its own right to be worth considering.

According to most authorities, the normal sex ratio in L. reticulatus is 1:1. Breder and Coates (1932), however, report a ratio of one male to two females. It has been the present writer's observation, also, that in the limited number of broods reared to sexual differentiation, females preponderate over males. At any rate, among the eight survivors of the estradiol-treated juveniles, there were five females to three males; among the six survivors of the stilbestrol-treated juveniles there were two females to four males (including one female and one male still living), and among the seven survivors of the pregnenolone-treated juveniles there were two females to five males. Little significance can be attached to these ratios because of the small number of animals involved, but it is fairly evident that no such factor as a wholesale destructive effect on either males or females was operating in these experiments. The one-hundred-per cent mortality suffered by gravid females subjected to estradiol and pregnenolone has already been discussed (Section V) as probably due mainly to interference with gestation.

It may be stated here that all reasonable precautions were taken to insure accuracy of dosage and to prevent any contamination of tank water with accidentally-introduced hormones. Considerable perturbation

was caused the experimenter by the observation that certain untreated females were showing masculinization effects, i.e., the appearance of faint gray spots, or of colored bars on the caudal fin, and a narrowing of the anal fin. If these unfeminine characteristics were to be interpreted as a result of careless handling of the crushed hormone tablets, or of the accidental transfer of hormone-containing water from tank to tank in a siphon tube, the results of much of the experimentation might be invalidated. These atypical female specimens were subsequently removed to a part of the laboratory distant from the treatment tanks and were observed for possible regression of the masculine traits. (It had been well established that withdrawal specimens uniformly lose all color indications, and even in some cases regain the feminine outline of the anal fin after initial modification toward the male contour). These females under observation for effects of possible accidental contamination with the agents did not, however, exhibit any regression but continued to develop normally into fine large specimens still showing a slightly narrowed anal fin and faint traces of color. It was therefore concluded that no such contamination had occurred.

A possible explanation may appear through a consideration of Winge's experiments with masculinized females (p. 20). His subjects consisted of females presumed to carry crossed-over traits from the Y-chromosome, and thus to have a genotypic basis for their masculinity. It is possible that such variation from the more standard phenotype is common, and that the animals discussed in the preceding paragraph may be examples of it.

2. Comparison of Results with Those of Other Investigators

The work of Berkowitz (1937, 1941) and Eversole (1941) has already been presented in brief form in Table I, p. 10. It may be useful here to give a detailed account of their results with an item by item comparison with those of the present investigator (Tables XI and XII).

It will be noted that there are several items in which the results of the two series of experimentations are not in perfect agreement. It is thought that the massive dosage of the present series produced unfavorable systemic effects and slight androgenic effects, none of which were reported by Berkowitz. This same variation in treatment is assumed to account for the smaller percentage of reversals in the more recent investigation.

In general, the experiments of the writer were allowed to run a longer period before the sacrifice of subjects for examination; reversals may have occurred in many specimens early in the treatment, especially in specimens that finally showed degenerate gonads.

It will be seen, however, that there is a general agreement in results, indicating that even when the conditions of the treatment vary considerably the effects of the agents are profound and consistent.

In the case of pregneninolon also, as with the two estrogens discussed above, there is substantial agreement of the writer's results with those of previously reported experiments along the same line.

TABLE XI
COMPARISON OF RESULTS WITH THOSE OF BERKOWITZ

Administration of Estrogens by Berkowitz	Present Results Compared with Those of Berkowitz
Juveniles fed estrogens failed to develop male characters, did develop gravid spot, larger size, and general female appearance	Juveniles fed estrogens remained small and feminine in appearance, finally developing a modified anal fin
Males showed 90% reversals, compact testes with many yolk-containing ova, large spermatogonial cells in cysts, and hypertrophy of stroma and ducts	Lower ratio of reversals, no yolk in ova
Females unaffected except for less yolk deposition	Same effects, except for inhibition of growth and slight modification of anal fin
Prolonged treatment results in inhibited testes with very little germinal tissue	Same effects, except that a few reversed testes retained germinal tissue
Testes of estrogen-injected mature males showed marked inhibition and occasional egg cells	Estrogen-fed adolescent males showed marked stimulation of spermatogenesis, inhibition of early stages, and no reversal
Ovaries of estrogen-injected females were normal except that they lacked large, yolk-filled eggs	Estrogen-fed mature females suffered lethal effects. Embryos were resorbed
Stilbestrol-injected females had normal gonads	No adults treated with stilbestrol. Juveniles fed stilbestrol suffered suppression of gonads

TABLE XII

COMPARISON OF RESULTS WITH THOSE OF EVERSOLE

Administration of Pregneninolon by Eversole	Present Results Compared with Those of Eversole
All fish developed gonopod regardless of age, sex, and dosage	Same results
Most fish showed masculine colors (yellow, orange, red) within 15 to 20 days after treatment	Colors noted, but thought not to be as intense as in control males
Immature males were more responsive than females in these color changes, but in most cases the sexes were indistinguishable	No differences noted
Gonads had to be studied histologically before sex could be definitely established	Same observation
Puberty spot suppressed in all juvenile females	Same results
Inhibitory effect on somatic growth of all immature subjects	Same results
Male mating behavior induced in immature subjects and in mature females	Same results
No indication of sex reversal in immature females. Ovogenesis and yolk deposition inhibited	Same results

No additional experiments of similar nature have come to the attention of the writer, except a recently-reported work of Mlle. Regnier, which because of war conditions is as yet unavailable. In her earlier report of experiments with Xiphorhynchus helleri, however, she includes a brief account of the injection of "female hormone" into adolescent males of Lebistes reticulatus, in which she stated that male colors, and all except the first stages of spermatogenesis, were inhibited.

3. Interpretation of Results

In this, as in all biological research, observations and the accumulation of data are of little utility in themselves; they become useful only as they help clarify underlying principles or exemplify mechanisms of physiological phenomena. The data of this and similar investigations, if they are to be of much value, must be utilized in some general principle of sex determination or sex differentiation in fishes. Unfortunately we seem not to be dealing with any one simple mechanism; there are many factors involved, factors whose interactions are as yet largely unexplored by experimentation. A partial list of such factors might include endocrine activity of the gonads; inductor substances in the gonads; the effects of gonad secretions on the hypophysis and vice versa; the genetic constitution of tissues as effecting their responses to morphogenetic influences; the possible chemical antagonism of the experimental agents and the subject's own secretions; and many others.

The present investigation offers little new in the way of evidence or interpretation. It is hoped, however, that its data may be of value in the sense that all careful observations are useful in adding to the weight of accumulating evidence that may sometime prove more significant. In the effort to interpret the results of this experimentation, the writer has found certain of the concepts stated in Section I to be more useful than others, and has found certain theories of sex determination in L. reticulatus to be more in accord with present results than are others. The remainder of this section will deal with the application of the present findings to these theories.

Sex-Linked and One-Sided Sexual Inheritance in

L. reticulatus

In view of the generally-accepted status of Winge's theory of one-sided sexual and sex-linked inheritance in L. reticulatus, any further supporting data may seem superfluous. At the time of his latest writing on the subject, however, (1934) no experimental administration of sex hormones to Lebistes reticulatus had been reported.

In the present series of experiments, the observation that females treated with the strongly androgenic steroid, pregneninolon, exhibit a modified range of male colors and certain of the male spots, suggests that the agent brings into action certain color genes carried in the X-chromosome and not normally exhibited by the female, though handed down by her to her sons. Likewise, gonopod formation, the masculine shape of dorsal and caudal fins, and the male type of behavior, may be represented by determiners in the X-chromosome, since they are

stimulated to phenotypic expression by administered androgenic substances. Other colors and patterns normal to males of the same strains are never exhibited by these treated females; we may assume that these factors are carried only by the Y-chromosome and are thus never genotypically present in the female.

There seem to be no strongly feminine characters induced in male fishes by estrogenic hormone treatment. This is partly accounted for by the tendency of adult females to retain juvenile color and proportions except for the distension of the abdomen and the appearance of the puberty spot, both of which are mechanical features rather than sex-linked color patterns such as males exhibit. Consequently the only feminizing effect noticeable in male guppies treated with estrogens is the retention of juvenile characteristics.

The use of hormonal agents in connection with breeding experiments should be of value in a further study of the genetics of guppies.

The Genic Balance Theory. It is considered by some authorities that there is an almost even balance between male and female determiners in L. reticulatus (Goldschmidt 1937). It has been stated also that a primarily neutral soma probably occurs in fishes (Witachi 1939). The ease with which genotypically-present sexual characters may be induced by hormonal treatment, as well as the actual sex reversals in this species, both induced and spontaneous, lend some support to this view of the genetic constitution of guppies.

There is nothing in this series of experiments to point to one theory of sex determination in preference to another, however. Cer-

tainly the genetic determination of these subjects is not so easily overthrown by treatment as to imply that guppies are developmental gonochorists as are the related fish Xiphophorus helleri. It is worthy of note that all guppies withdrawn from hormone treatment make a rapid recovery from any sex reversing effects of treatment. These observations may imply that the sex determination, though perhaps close to an even balance of male and female determiners, is still definite enough to provide a stable sex ratio under normal conditions.

Winge, who has probably studied the genetics of Lebistes reticulatus more than has any other authority, claims to have shifted sex determiners from sex chromosomes to autosomes in certain strains of this species (Winge 1922, 1923). He states that in so doing he has established new strains of this animal heterozygous for the female rather than for the male. This hypothesis is discredited by Goldschmidt (1937) who believes that the application of the F-M balance theory to Winge's experiments will adequately explain his results without the need for any new theories. The assumption that especially strong male factors or especially weak female factors had been introduced by crossing various strains could account for the production of an XX male all of whose offspring were female, without assuming that the determiners for the male characters had been transferred from a sex chromosome to an autosome. It is possible that sex hormone treatments might be of some value in future experiments directed toward a definite solution of this problem.

The Concept of Hypophyseal Involvement. The observation that treated juveniles of the experiments sustained a general suppression of growth and of germinal development suggests that the hormones of the anterior pituitary may have been suppressed. It is well known that removal of the hypophysis before sexual maturity has a castration effect in vertebrate animals. It is probable that at least two factors complicate the action on the gonad of any administered sex-affecting agent: (1) the suppressing effect of the administered substance on the hypophysis and a consequent lack of gonad-stimulating secretions, and (2) an intensifying or neutralizing effect (or other possible interference) of the administered agent with the normal gonad secretions.

Not a great amount of experimentation with pituitary substances has been done on fish. Berkowitz (1941) reports that gonadotropins stimulate an immature testis to develop in a way normal for later stages, but that there is no effect on ovaries. In adult males the gonad regressed in size and developed hypertrophy of the stroma. Baldwin and Min-Hsin Li (1942) report that gonadotropins administered to adult males of Xiphophorus helleri hasten spermatogenesis and bring about an exhaustion effect simulating a condition of sterility.

In an effort to determine the effects of sex hormones on gametogenesis, Bullough (1942) injected estrone into female minnows and testosterone propionate into males. He concluded that homotypic sex hormones have a direct stimulating effect on gametogenesis in both males and females, but that some other substance (presumably gonadotropin from the

pituitary) is necessary for the further development of oögonia into mature ova. He found also that a certain proportion of primary germ cells and spermatogonia were unaffected by the male hormone, remaining dormant. In conclusion he states "the primary effect of the gonadotropic secretions of the anterior pituitary gland is to cause the secretion by the gonads of a sex hormone which in turn acts on the gonads and induces the nuclear divisions associated with gametogenesis." He also states that injected heterotypic sex hormones have a disintegrating effect on gonads of both male and female minnows.

These views are hardly in accord with those of Moore and Price (1932) who explain suppression of gonads in hormone-treated rats as caused by a lack of pituitary gonadotropins which has been brought about by an excess of sex hormones. It is well known that gonad and pituitary secretions in many animals regulate reproductive cycles through their alternating mutual suppressing action. Perhaps cases of marked gonad suppression in treated animals may be explained by presuming a more serious interference with the delicate balance of sex and gonadotropic hormones than would be likely to occur under normal physiological conditions.

In the present series of experiments, the suppression of both spermatogenesis and epithelium development in the juvenile testis by estradiol may best be explained by assuming a lack of gonadotropic secretions from the hypophysis. It is not necessary in this case to assume any directly suppressing nor degenerating effect of the administered agent, as it is certain that gonads do not normally develop to maturity

without hypophyseal secretions. The development of ova in certain of the juvenile testes may be accounted for either by a direct stimulating effect of the administered female hormone, or by the removal of some check to their development through the suppression of male elements in the testis. The latter view seems the more plausible, as, in this experimentation at least, ova could be induced to appear only if males were subjected to treatment at a very early age, implying that bipotentiality of the young gonad persists until such a time as it is suppressed by the development of one sex tissue predominantly, and that in the absence of this suppression the growth potentialities of the heterotypic sex tissues may be expressed. In some fishes (i.e., Xiphophorus), in many amphibians, and in occasional anomalous individuals of many vertebrate animals, the potentiality for sex reversal may be retained by the mature gonad.

The observed stimulation of spermatogenesis in adolescent males by estradiol treatment, as well as the stimulation of epithelial tissue, seem to be specific effects of the agent. The absence of new cyst formation from spermatogonia in a resting condition is assumed to be caused by a lack of gonadotropic hormones. After a few weeks of treatment the testis of an adolescent male shows a proliferated or finally degenerated epithelium, a preponderance of the later stages of spermatogenesis, and usually a few apparently unaffected resting spermatogonia. If such a distinction is valid, the heterotypic administered agent might be considered a foreign substance of morphogenetic action, and the gonadotropic hormones simply normal growth-regulating substances.

The ovaries of estradiol-treated juveniles showed suppression of germinal epithelium and failure of ova to develop yolk. This observation is not in agreement with Bullough's results in Phoxinus laevis, in which he found that estrone stimulated the production of oogonia. A difference in developmental processes between the oviparous minnow and the ovoviviparous guppy may explain this inconsistency, or a difference in effect between estrone and estradiol. At any rate, the present experimenter is in accord with Bullough's observation that it is a lack of gonadotropic secretions which is responsible for the failure of ova to develop yolk. In estrogen-treated female Lobistes the follicle cells of the arrested ova are always poorly developed while those in withdrawn specimens are quite conspicuous.

The effects of stilbestrol need not be discussed here, as except for the lack of sex-reversing action they are the same as in the estradiol-treated subjects.

The effects of pregnenolone on the gonads of juvenile guppies parallel those of estradiol and stilbestrol in certain ways but diverge widely in others. The main similarity of action is in the suppression of germinal elements and epithelium in subjects treated for the full term of the experiments. In subjects treated one month only, there was stimulation of spermatogenesis and suppression of oogenesis, with stimulation of epithelium in both types of gonad. In male subjects withdrawn after long treatment, a great hypertrophy of the duct system was noted; in nearly all gonads of pregnenolone-treated subjects numerous enlarged blood vessels were observed.

In view of the definite androgenic effect of pregneninolon on secondary sex characters, the correspondence between its internal effects and those of the two estrogens seems surprising. This suppression of germinal elements in the gonads may be only another indication, however, of an indirect action involving the pituitary, in which case the suppression would be the same whether the administered agent were an estrogen or an androgen. The stimulating effect on the epithelium may, however, be specific to the agent itself. It is not necessary to seek any parallel for this effect in the animal's own physiological secretions. We may admit that certain steroid substances may have a morphogenetic action on animal tissues without implying that these agents are identical with, or even closely related to, the specific secretions of the animal's own body.

Suppression of anterior pituitary secretions may also be responsible for the inhibition of growth observed in all treated juveniles in these experiments.

The Concept of Cortical-Medullary Inductors. The terms "sex reversal" and "sex inversion" as applied to the gonads of experimental animals is not to be considered as the complete metamorphosis of a gonad of one sex into one of the other. It is rather to be taken as the development of germinal tissue of the opposed sex, with or without the retention of germinal tissues of the genetic sex. These tissues need not have reached the stage of mature germ cells to permit a diagnosis of reversal; if they can be identified as oögonia or spermatogonia (or later developmental stages) a condition of reversal is assumed.

The cases of reversal observed in this experimentation were testes that had developed one or more ova. In one case the one ovum caught in sectioning was a large, somewhat degenerated mass located laterally in the testis. In another case the gonad was a rather well-developed testis with definite cysts (though no mature sperms), and well-developed ducts with apparently normal epithelium. Ova of various sizes, all immature, were found, and what appeared to be many intermediate stages between spermatogonia and young ova. If these observations are correct, it would seem that the germ cells themselves are capable of reversal. See the Appendix for a more complete consideration of this case.

It is becoming more generally accepted that the germ cells of vertebrates are bipotential; though the primordial germ cells by their zygotic determination have a chromosomal makeup characteristic of either sperm or egg cells, this chromosome content has no effect in determining which type of germ cell they shall become (p. 2). In other words, the germ cells of a genetic male animal may become egg cells, if the genetic determination of the individual is overridden in such a way as to change the testis into an ovary.¹

¹A parallel condition may exist in plants. Hartmann has shown that both male and female strains of the brown alga, Ectocarpus siliculosus, of various degrees of sexuality, when subjected to extractives of the tissues of the opposed sex, "changed sex" and developed normally functioning reproductive structures of the induced sex. By a reinterpretation of the sexual phenomena of sea weeds, fungi, and various other plants as well as animals, he developed the theory of "relative sexuality", which implies varying degrees of maleness or femaleness in many organisms, with a capacity to deviate from the normal sex type in either direction according to the sex valence of the mating individuals. He designates valence as each individual's inherited mixture of male and female potencies.

Witschi, in discussing experimental sex transformations, remarks,

Sex reversal in vertebrates is possible as long as an ambisexuality of organs is maintained. In other words, it depends on the presence in one sex of the rudiments of sex organs of the other sex, or of cells and tissues of primordial character which may differentiate either in the male or the female direction. As a rule the technique of experimental sex inversion consists in the partial or complete elimination of the dominant inductor system, either the cortex of the ovary or the medulla of the testis. The development of the suppressed sex, if it follows, is due to a spontaneous compensatory growth of whatever rudiments of gonadic structures might be left, rather than to stimulation by some experimentally introduced factor.

Authorities are not in general agreement with Witschi's theory of cortical-medullary inductors. It has generally been assumed that the substances responsible for normal sex development in the gonads (gonadal determination) are in the nature of sex hormones, if not identical with them as demonstrable in the later stages of development of the animal. The point of disagreement seems to be the nature of the determining substances rather than their mode of action. Willier (1939) says, "Evidence has accumulated which in general indicates that the early differentiation of the gonad rudiment is independent of hormone action". Witschi, Needham (1942) and others maintain that chemical substances of the nature of inductors are produced in the bisexual stage of the gonad; these substances are not carried by the blood, are only slowly diffusible, and act through only a limited area.

A definite development of cortical and medullary tissues of the gonad may be demonstrated in the guppy during embryonic development (Dildine 1936). Though there is no external indication of sex until the age of 6 or 7 weeks, the gonads are sexually distinguishable shortly before birth. It is unlikely, therefore, that the experimental

agents, administered no earlier than the second week after birth, could have had any effect in the primary sex determination of the gonads.

In considering cases of reversal, however, we are probably dealing with a modification of normal developmental processes rather than with the specific morphogenetic action of an administered agent. It is more reasonable to assume that the agent acts through modification of an already existing mechanism than by any specific action of its own, particularly in view of the frequent reports of inhibitory action of administered hormones. But whether hormones or inductors, the normal regulating substances may be assumed to be suppressed in some way by the administered agent, probably through suppression of the gonadotropic hormones of the pituitary. The chain of reactions might be as follows: estrogenic substances administered to a young male guppy might decrease the production of gonadotropic hormones by the pituitary, thus causing atrophy of the testis. This condition might result in a lack of male hormone in the subject, which might then permit the continued production of inductor substances as in embryonic development, either allowing an equal chance for development of male and female components of the gonad, or through some as yet unexplained mechanism exerting a greater suppressing effect on male tissues thus allowing a great degree of reversal.

It is difficult to make any general statement as to the bearing of this investigation on the inductor versus the sex hormone theories of gonad determination. It would seem, however, that there may be a shade more evidence in support of the inductor theory. If hormones

were the natural agents of sex determination in the gonads, it is reasonable to assume that more numerous and more complete reversals of sex might have occurred, since the administered agent might have been considered as supplementing and prolonging the action of early-produced gonadal substances of the same nature. The following case of a hermaphrodite of L. reticulatus, reported by Blacher (1926), may also indicate the presence of inductors in the gonad.

The observed animal, a genetic female, had a combination of male and female secondary sex characters. On dissection,

. . . a fully developed ovary containing almost completely formed embryos was discovered. . . . All along the ovary, gradually tapering, was found a well-developed testicle. Contiguous with the testicular tissue lies a mass containing disintegrated ovarian tissue. In the testicular portion the various stages of spermatogenesis can be found. To all appearance normal and mature sperm cells gathered in cysts (spermatophores) are just as abundant and regular as can be observed in normal testes.

Whatever anomaly of heredity or development may be accountable for this hermaphroditic gonad (for which no explanation is offered here), it would appear that the presence of testicular tissue exerts a suppressing effect on ovarian tissue. It is assumed that the testicular tissue must have developed subsequently to the maturity of the ovary; the large size and female proportions of the specimen indicate that there were probably no testicular secretions to interfere with growth and body proportions until the full size characteristic of females was achieved. The presumption may be made that testicular tissue, appearing after the ovary was fully developed, by means of a diffusing inductor substance exerted a suppressing effect on the strip of ovarian

tissue with which it was in contact. This hypothesis is quite in accord with the theory of cortical-medullary inductors; had the suppressing factor been of the nature of a sex hormone, it would have been distributed by the blood stream and would have exerted an effect throughout the entire ovary. Such male sex hormones were also undoubtedly produced by the testis, as the specimen exhibited male coloration and a modified anal fin.

The writer is in general agreement with Berkowitz (1941) however, in regards to any decisive evidence in support of either the inductor theory or the hormone theory of sex determination in the gonad. Berkowitz writes,

Our . . . results suggest that egg cells develop when spermatogenic elements are suppressed, but only if the suppressing agent is not in too high concentration. . . These results do not constitute clearly positive evidence for either of the two alternative theories mentioned above.

The concepts of sexual mechanisms that have been found most useful in explaining the observed effects of the present experiments are as follows:

- (a) The XX-XY type of sex determination with male characters in both X and Y, and with certain sex-linked characters in Y only
- (b) Sex determination of the gonad by inductors
- (c) Bipotentiality of germ cells
- (d) Stimulation of secondary sex characters by hormones presumably secreted by the gonads
- (e) Activation of mating behavior by sex hormones.

4. Inferences Relating to Action of the Agents

1. The responses of secondary sexual characters to experimentally-administered sex hormones or other related substances indicate that these characters may be normally activated in fish by their own endocrine substances.

2. The fact that in general no organs nor tissues except those of the reproductive system and those features concerned with sex differentiation seem to be affected by the experimental agents implies that the agents are closely related to sex hormones of the subjects.

3. The fact that secondary sex characters are susceptible of modification by the agents while at the same time the normal source of such activating substances (the gonad) may be suppressed into functionlessness, implies that the mechanisms of sexual differentiation in fishes are hormonal.

4. The fact that the agents in general exert a suppressing effect on both male and female gonads implies a suppression of gonadotropic hormones of the pituitary.

5. The fact that spermatogenesis is abnormally stimulated by estrogenic agents, without replenishment by development of new spermatogonial cysts, implies that the process of spermatogenesis is regulated by substances similar to the agents. The fact that early spermatogonia may remain latent under treatment of the agents implies that the early stages of spermatogenesis may be under a different control from that of the later stages.

6. The fact that certain of the administered agents bring about inhibition of spermatogenesis and at the same time allow the development of one or more ova in the testis implies that it is the presence of male elements in the testis, or some substance produced by them, that normally inhibit the development of female tissue in a young gonad.

7. The fact that a male fish can be induced to assume certain of the female secondary sexual characters, and that females can be induced to assume certain of the male characters, implies that the potentialities for these characters exist in the somatic cells of the individual, possibly in the form of determiners of sexual characteristics, in a more or less balanced or sexually bipotential condition, capable of responding in a male or female direction as stimulated by a preponderance of male or female hormone.

SECTION VIII

SUMMARY

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SUMMARY

1. Estradiol was found to exert a suppressing effect on growth of juvenile guppies, a suppressing effect on both male and female secondary sex characters with a slightly androgenic later effect on females, and a sex-reversing effect on the gonads of a proportion of young males. On adolescent males it had a stimulating and finally exhausting effect on the testis.

2. Stilbestrol had the same effect on juveniles as did estradiol, except that among the limited number of animals examined histologically there was no indication of sex-reversing tendencies.

3. Stilbestrol (and presumably also estradiol) brought about hypertrophy of the duct system of the testis, frequently with the development of stratified epithelium in place of simple cuboidal epithelium.

4. Pregneninolon had a suppressing effect on growth but none on activity. Its effect on secondary sexual characters was markedly androgenic in both juveniles and adolescent females, and slightly so in adult females. The ovaries of treated animals were suppressed but not otherwise abnormal; the testes were greatly suppressed or degenerate, with hypertrophy of ducts.

SECTION IX

APPENDIX

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APPENDIX

The case of a certain ovotestis (Plate VIII, Fig. 1) deserves special consideration as a gonad in which there seem to be various stages of transition from male germ cells to female.

From its bilobed structure the organ is undoubtedly a genetic testis. The frequent occurrence of cysted male germ cells of a single stage of development is noted, this being characteristic of the male gonad. These developing germ cells are not always identifiable as male or female; one large cyst has every appearance of primary spermatocytes; other cysts contain a number of quite similar cells and in addition contain one or more greatly enlarged cells having every appearance of oögonia. A progressive increase in size of these oögonia can be traced in various groups, up to ova having definite follicle cells.

Though follicles are evident around the larger ova, it is not clear how they could have been formed. There is a definite investment of stroma cells around each cyst of germ cells, and it is possible that the stroma may have given rise to follicle cells. On the other hand there are a number of sperm ducts with a dense cuboidal epithelium and containing sometimes additional cells, sometimes debris, in the lumen. Certain cysts have peripheral cells very similar to these epithelial cells. It is not too improbable that a portion of a sperm duct may have

constricted to form a cyst, and that one or more cells of this epithelium may have developed into oöcytes while others became follicle cells.

There is evidence of degeneration in some of the larger ova. In no ova are yolk granules apparent.

Except for early spermatogonia, there seem to be more germ cells developing in the female direction than in the male.

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PLATES

EXPLANATION OF LABELED FIGURES

bl. v.	---	blood vessel
con. t.	---	connective tissue
ch. gr.	---	chromatin granules
du.	---	duct
deb.	---	debris
dg. ep.	---	degenerated epithelium
dg. ov.	---	degenerated ova
ep.	---	epithelium
fol.	---	follicle
nu.	---	nucleus
ooc.	---	oocyte
ov.	---	ovum
ov. cav.	--	ovarian cavity
pr. ep.	---	proliferated epithelium
Ser.	---	Sertoli cells
spcy.	---	spermatocytes
spgo.	---	spermatogonia
sphr.	---	spermatophore
sptd.	---	spermatids
yo.	---	yolk

PLATE I

Fig. 1. Estradiol-juveniles, apparent females.

Fig. 2. Estradiol-juveniles-withdrawals, apparent females.

PLATE II

Fig. 1. Estradiol-adolescent, genetic male.

Fig. 2. Estradiol-withdrawal-adolescent, genetic male.

PLATE III

Fig. 1. Stilbestrol-juvenile.

Fig. 2. Stilbestrol-juveniles-withdrawals.

PLATE IV

Fig. 1. Pregneninolon-juveniles.

Fig. 2. Pregneninolon-juveniles-with-drawals.

PLATE V

Fig. 1. Old-pregneninolon-water-juveniles, Set 1.

Fig. 2. Old-pregneninolon-water-juveniles, Set 2.

PLATE VI

Fig. 1. Pregneninolon-adolescent-females.

Fig. 2. Pregneninolon-adolescent-females-with-drawals.

PLATE VII

Fig. 1. Testis of normal male, x 140, all stages of spermatogenesis.

Fig. 2. Ovary of normal female, x 140.

PLATE VIII

Fig. 1. Ovotestis, x 140, Estradiol-juvenile, specimen B.

Fig. 2. Ovotestis, x 140, Estradiol-juvenile, specimen D.

PLATE IX

Fig. 1. Ovary, x 140, Estradiol-juvenile, specimen E. Short treatment.

Fig. 2. Ovary, x 140, Estradiol-juvenile, specimen C.

PLATE X

Testis, x 120, Stilbestrol-juvenile, short treatment.

PLATE XI

Fig. 1. Testis, x 140, Estradiol-adolescent-male.

Fig. 2. Testis, x. 90, Estradiol-adolescent-male-withdrawal.

PLATE XII

Fig. 1. Testis, x 140, Stilbestrol-juvenile-withdrawal, specimen A.

Fig. 2. Testis, x 140, Stilbestrol-juvenile-withdrawal, specimen B.

PLATE XIII

Fig. 1. Ovary, x 140, Pregneninolon-juvenile.

Fig. 2. Ovary, x 116, Pregneninolon-water-juvenile.

PLATE XIV

Fig. 1. Testis, x 140, Pregneninolon-water-juvenile.

Fig. 2. Testis, x 140, Pregneninolon-water-juvenile.

PLATE XV

Testis, pregnenolone-juvenile-withdrawal, x 140.

PLATE XVI

Fig. 1. Ovary, x 140, Pregneninolon-adolescent-female, short treatment, specimen C.

Fig. 2. Ovary, x 140, Pregneninolon-adolescent-female, specimen A.

PLATE XVII

Fig. 1. Ovary, x 25, Pregneninolon-adolescent-female, specimen B.

Fig. 2. Ovary, x 140, Pregneninolon-adolescent-female-withdrawal.