Sex Determination and Primary Sex Differentiation in Amphibians: Genetic and Developmental Mechanisms

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ABSTRACT Most amphibians lack morphologically distinguishable sex chromosomes, but a number of experimental techniques have shown that amphibian sex determination is controlled genetically. The few studies suggesting that environment influences sex determination in amphibians have all been conducted at temperatures outside of the range normally experienced by the species under study, and these effects probably do not occur under natural conditions. No sex-determining genes have been described in amphibians, and sex differentiation can be altered by treatment with exogenous steroid hormones. The effects of sex steroids vary extensively between species, and a variety of steroids can alter the sex ratios of treated larvae. The role of endogenous sex steroids in gonadal differentiation has not been fully explored; thus the natural role of steroids in amphibian gonadal differentiation is unknown. Sex steroid receptors have not been examined in amphibian gonads, and the mechanism of steroid action on the gonad is unclear. In addition to steroids, the thyroid hormones may play a role in gonadal differentiation. Pituitary gonadotropins affect gonadal growth, but not differentiation or maturation of gonads.

In addition to the issue of resolving the mechanisms underlying hormone action in gonadal differentiation, other debates concerning interactions between the developing gonads and the invading germ cells, and even the origin of the medullary and cortical portions of the developing gonads, remain unresolved. Studies examining links between sex determination and gonadal differentiation are needed. In addition, examinations of variation in steroidal effects on gonadal development in a phylogenetic context are lacking. J. Exp. Zool. 281:373–399, 1998. © 1998 Wiley-Liss, Inc.

Amphibians are excellent models to study sex differentiation, for a number of reasons. Genetic sex-determining systems and sex chromosomes have evolved a number of times in this class, and gonadal differentiation (although under genetic control) is responsive to experimental manipulations. In addition, as laboratory animals, amphibians are ideal; because females produce up to 10,000 eggs per clutch in some species, large sample sizes can be obtained. Also, large numbers of individuals can be treated easily in experimental manipulations because larvae are typically aquatic and readily absorb hormones and other chemicals added to rearing water.

Despite their accessibility as study animals, relatively few recent studies have addressed sex determination and differentiation in amphibians.1 Also, there are no recent reviews that address both sex determination and sex differentiation in amphibians, although a few reviews addressed each topic separately: Dodd (’60) and Dournon et al. (’90) reviewed genetic and environmental mechanisms of sex determination (addressing amphibians and reptiles), and Schmid (’83) and Hillis and Green (’90) examined genetic mechanisms of sex determination with special reference to the evolution of sex chromosomes in amphibians. Gonadal differentiation in amphibians was reviewed by Gallien (’65), and at least four reviews (Burns, ’49, ’61; Adkins-Regan, ’81, ’87) addressed the effects of hormones on sex differentiation in vertebrates in general, with extensive discussions of amphibians.

1Although often used interchangeably, there is a fundamental difference between sex determination and sex differentiation. Here, “sex determination” will refer to mechanisms that direct sex (gonadal) differentiation, and “sex differentiation” will refer specifically to the development of testes or ovaries from the undifferentiated or bipotential gonad. In other words, sex determination refers to the switch (or the decision), whereas sex differentiation refers to the actual developmental process.

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ians. A single review addressed both sex determination and differentiation in amphibians (Witschi, '50), but this work was published prior to many primary studies on the topics. A second review (Adkins-Regan, '87) addressed both topics in nonmammalian vertebrates in general with reference to amphibians. Thus, with the exception of the recent reviews addressing sex chromosome evolution (Schmid, '83; Hillis and Green, '90), reviews on sex determination and sex differentiation (and, in particular, reviews addressing both sex determination and differentiation) are lacking. The goal of the current review is to synthesize work on sex determination and sex differentiation in amphibians. This simultaneous review of sex determination and differentiation will attempt to place the available information into an evolutionary framework and explore interactions between mechanisms of sex determination and sex differentiation.

SEX DETERMINATION

Genetic sex determination versus environmental sex determination

There are at least two mechanisms of sex determination recognized in vertebrates: genetic sex determination (GSD) and environmental sex determination (ESD). Genetic sex determination is a mechanism by which genes directly determine whether the gonads differentiate into testes or ovaries without external (environmental) influences. In ESD, the sex of individuals is determined by environmental factors: For example, in temperature-dependent sex determination (recognized in some fish, many turtles, some lizards, and all crocodilians), sex is determined by the incubation temperature of the eggs. Thus each individual has the full capacity to develop into males (testicular differentiation) or females (ovarian differentiation). In fact, sex differentiation is likely controlled by genetic mechanisms in animals with ESD and only the switch “sex determination” may be influenced by environmental factors.

Environmental sex determination

Several studies have suggested that some amphibian species display ESD (for review, see Dodd, '60; Gallien, '74; Dournon and Houillon, '84; Dournon et al., '90). In frogs, one species of Bufo (Piquet, '30) and four species of Rana (Witschi, '14; '29a; Piquet, '30; Yoshikura, '59b; Hsü et al., '71) were examined, in addition to three species of salamanders from two genera Pleurodeles (Dournon and Houillon, '84; Dournon et al., '84; Chardard et al., '95) and Hynobius (Uchida, '37a,b). These studies all showed that the temperature of the rearing water can alter the sex ratios of larvae. In all of these studies, however, effects were obtained by exposure to temperatures that are not normally experienced by the species under study. In fact, with one exception, these effects were produced only at high temperatures (27 to 36°C). In all of the studies on frogs, 100% males were produced at high temperatures, whereas either 100% females (Pleurodeles poireti; Dournon et al., '84) or 100% males (Hynobius retardatus: Uchida, '37b; Pleurodeles waltl: Dournon and Houillon, '84) were produced by high-temperature treatments in salamanders. A single study (Uchida, '37b) showed that low temperatures (10°C) can produce 100% females also.

When reared at temperatures within the ranges experienced naturally by the species under study, a 50:50 sex ratio was obtained in all of the studies cited above. For example, in Witschi’s ('29a) study on Rana sylvatica, larvae were reared at 32°C (which Witschi reported as a temperature near the lethal limit and a temperature that can be tolerated only for short periods). Rana sylvatica lives in northern North America where eggs are typically laid in early spring when water temperatures are as low as 4°C (personal observation; and Wright and Wright, '49; Herreid and Kinney, '66; Seale, '82; Waldman, '82). Larvae begin metamorphosing within 8 weeks, and water temperatures do not typically exceed 20°C during this time. When larvae were reared at 20°C in Witschi’s ('29a) study, or reared at a range of temperatures naturally experienced by this species (Hayes and Waldman, unpublished), the sex ratio was not significantly different from 50:50. Similarly, we (Hayes et al., unpublished) reared other species (Hyperolius viridiflavus, Bufo boreas, and Pyxicephalus adspersus) over a range of temperatures appropriate for each species and did not observe any effects on sex ratios.

The natural role of temperature in the sex determination in reptiles and fish is much more convincing, because effects have been observed at temperatures within ranges experienced by the species in the wild (fish: Conover and Heins, '87; turtles: Bull et al., '82), and effects have been shown in natural nests in turtles (Bull, '85; Rhodes and Lang, '95). Thus, given the lack of effects on sex ratio at temperatures appropriate for the species in the studies on amphibians, it is not likely that temperature is important in normal sex determination in amphibians. The reported environ-
mental influences are probably artifacts of the abnormally high temperatures at which the animals were reared. In addition, reptiles and fish displaying ESD typically lack sex chromosomes (morphologically distinguishable or otherwise), which is not the case in amphibians. Thus the available evidence for amphibians suggests that sex determination is controlled exclusively by genetics under natural conditions (see below).

**Genetic sex determination**

In genetic sex determination, the direction of sex differentiation is controlled genetically, such that one sex has a gene (or more likely a set of genes) that the other lacks. The sex-determining genes likely control only the “decision,” and are not likely involved in sex differentiation, but rather initiate a sequence of events that results in gonadal differentiation. Normally, the different sexes develop because individuals are directed through different developmental pathways as a result of the activation of the sex-determining gene(s). This last point is important, because all individuals may have the capacity to develop into either sex. This capacity is evident from the ability to experimentally sex-reverse amphibians: Depending on the species, genetic males can be experimentally induced to develop ovaries and vice versa with steroid hormone treatments.

The presence of sex chromosomes is the clearest indication that a species possesses GSD. In these cases, the sex-determining genes are on a chromosome that has evolved such that it is morphologically distinguishable from its homologue upon cytological examination (the X and Y in mammals, for example). The evolution of sex chromosomes often involves the loss of genetic material: For example, in mammals, the Y chromosome is smaller than the X as a result of limited chromatin exchange between the X and Y chromosome and subsequent loss of genes from the Y chromosome (see J. Graves, this issue).

Sex chromosomes can be distinguished morphologically by a number of techniques. Examining preparations of metaphase chromosomes from males and females for chromosomes in which the homologues differ in size between the sexes is the simplest technique. Some sex chromosomes differ in the centromeric index as a result of pericentric inversions, which can be observed cytologically. In addition, as chromosomes lose genetic material (such as in the mammalian Y chromosome), they accumulate repeats, resulting in heterochromatism. Heterochromatism can be detected by a variety of techniques that produce different banding patterns on the chromosomes. Finally, some accumulated repeats result in differences in replication time between chromosomes. A technique using the incorporation of bromodesoxyuridine can detect differences in replication time to identify otherwise homomorphic sex chromosomes (Schmid, ’83).

More than 1500 amphibian species have been examined cytologically using the techniques described above. As few as two species of frogs (*Pyxicephalus adspersus*: Schmid, ’80; Schmid and Bachman, ’81; *Gastrotheca riobambae*: Schmid, ’83) and 11 salamanders (all Plethodontidae: Mancino, ’65; Kezer and MacGregor, ’71; León and Kezer, ’78; Sessions, ’84; Nardi et al., ’86) have sex chromosomes that differ in size. Sex chromosomes are distinguishable in at least another 30 species of salamanders and frogs by examining banding patterns or differences in replication time. So, less than 4% of the amphibians examined cytologically possess morphologically distinguishable sex chromosomes (no data are available for caecelians).

Despite the absence of morphological distinguishable sex chromosomes in the majority of amphibians, sex is probably under genetic control in all amphibians. The inability to distinguish sex chromosomes in amphibians may indicate only that the sex chromosomes have not evolved to where they are morphologically distinguishable: Although there may be chromosomes possessing sex-specific genes, the differentiated regions are small and not distinguishable cytologically. Hillis and Greene (’90) suggested that the lack of differentiation in amphibian sex chromosomes indicates that a much smaller region may be involved in sex determination in amphibian sex chromosomes relative to other vertebrates with morphologically distinct sex chromosomes.

Furthermore, other experimental techniques that do not require morphological identification of sex chromosomes have been used to identify the sex-determining systems in amphibians. In most cases, experimental manipulations were used to obtain information regarding heterogamy. These studies involved back-crossing experimentally “sex-reversed” individuals to normal individuals, and using the resulting sex ratio to infer the genetic constitution (male heterogamy: XXXY versus female heterogamy: ZZZW). For example, 100% females were produced when larvae of the African clawed frog (*Xenopus laevis*) were exposed to exogenous estrogens (Witschi and Allison, ’50). When raised to sexual maturity, approximately 50% of these females produced 100% male offspring when
bred to normal males. Thus 50% of the estrogen-treated animals were assumed to be sex-reversed males (ZZ females) that produced only male (ZZ) offspring, when crossed back to normal (ZZ) males (Chang and Witschi, '55b; Hayes, '98; Figure 1). These types of studies have been used to show female heterogamety in other species (Ambystoma spp.; Humphrey, '45; '48). In similar studies with Rana spp., androgen treatment produced 100% males (Kawamura and Yokota, '59; Richards and Nace, '78). Approximately 50% of these males produced 100% female offspring when crossed back to normal females, showing that the species under study were male heterogametic (XX/XY). Other studies used breeding experiments to track sex-linked (but not sex-determining) genes in frogs (Wright and Richards, '83; Sumida and Nishioka, '94; Rudolf and Dournon, '96).

Expression of the HY antigen (see Bennet et al., '75; Wachtel et al., '75; Zaborski et al, '88) has been used to identify the heterogametic sex in frogs (Engle and Schmid, '81) also. In this study, species in which the heterogametic sex was identifiable by independent methods were used to test the use of HY antigen as a tool for identifying the heterogametic sex. HY antigen was always expressed in the heterogametic sex in this and subsequent studies, suggesting that the test was valid (Wachtel et al., '75; Zaborski, '79). Later studies showed that the HY antigen was not the testis inducer, although it was sex-linked (McLaren et al., '84, '88; Goldberg, '88). Furthermore, in nonmammalian vertebrates, the gene for HY antigen is present in both sexes (not sex-linked) and can be induced by hormone treatment. For example, in birds, only females (ZW) normally express HY antigen. When males were treated with estrogens, however, they expressed HY antigen (Müller et al., '79, '80; Zaborski et al., '81; Ebensperger et al., '88). Given the presence (but not necessarily expression) of the HY antigen gene in both sexes in lower vertebrates, its use as a tool to identify the heterogametic sex in amphibians is limited, and results must be confirmed by other methods. Nevertheless, experimental techniques, such as the ones described above, identified the sex-determining systems in another 17 species of frogs (in addition to those identified by cytological techniques) and two more salamanders.

One other method for assessing the sex-determining mechanism in amphibians has provided confusing results. Several workers showed that amphibian eggs can be induced to divide without fertilization by pricking the eggs with a glass needle (Parmenter, '25; Kawamura, '39, '49; Moriwaki, '54, '57, '59, '60a,b). Pricking induced chromosome doubling in some eggs (diploidy) and presumably normal development in these "parthenogenetic" embryos. All of these studies were conducted in Rana spp., which are male heterogametic (XX/XY: Schmid, '83; Hillis and Green, '90), so all parthenogenetically produced animals should be females (the eggs contain only an X chromosome). In several studies, however, males resulted from pricked

Fig. 1. Experimental techniques used by Mikamo and Witschi ('64) to determine the genetic sex-determining system of the African clawed frog (Xenopus laevis). Normal males and females were bred to produce larvae. The resulting larvae were treated with estrogen and produced 100% females. Fifty percent of the treated larvae were genetic females, and the remaining 50% were genetic males, “sex-reversed” by the hormone treatment so that they developed ovaries. When bred back to normal males, the estrogen-treated females produced a normal sex ratio of 50% male/50% female offspring. The sex-reversed males, however, produced only male offspring when bred back to normal males, indicating that males are homogametic (ZZ) in this species.
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(unfertilized) eggs. For example, Parmenter ('25) reported 18 male and three female diploid animals produced by pricking in *Rana pipiens*; Kawamura ('49) reported 32 normal females, seven females with degenerating ovaries, three males with rudimentary testes, and two normal males in *Rana nigromaculata* and *R. japonica*; and Moriwaki ('54) reported seven males and nine females in *R. japonica*. One interpretation is that the sexes were misidentified, but when a parthenogenetically produced male was used to fertilized a normal female (Moriwaki, '54), 100% females (72 animals) were produced, compared to 56% females (out of 86 animals) in controls. These data suggest that the parthenogenetically produced male was indeed a homogametic (XX) individual. It is difficult to reconcile these data with other evidence for male heterogamety in this genus, and no recent studies have followed up on these studies.

**Evolution of sex chromosomes**

Using cytological data and data from published studies such as the ones described above, Hillis and Greene ('90) examined the evolution of sex-determining mechanisms in amphibians. Unlike many other major vertebrate groups (all mammals are male heterogametic, all birds are female heterogametic, etc.), sex chromosomes have evolved several times in amphibians. The ancestral state appears to be female heterogamety, and although male heterogamety has evolved several times (Figure 2), a reversion to female heterogamety in a male heterogametic lineage has occurred only once (*Chiropterotriton dimidatus* in the bolitoglossin plethodontids).

Schmid ('83) pointed out that the ancestral Z chromosome in amphibians does not share a common ancestor with the Z chromosome of snakes and birds. Similarly, it is not likely that the sex chromosomes are related between amphibian families: The X and Y chromosomes in derived Ranids are not evolved from the Z or W chromosomes of the more basal *Pyxicephalus adspersus* and *Tomopterna* spp. For example, the nucleolus organizing region (NOR) is on the Z chromosome in *Leiopelma* (Green, '88), on the X chromosome in *H. femoralis* (Anderson, '91) and *Gastrotheca* (Schmid et al., '86, '88, '90), on the Z chromosome in *Buergeria buergeri* (Ohta, '86; Schmid et al., '93). As a result, males have two nucleoli per nucleus and females only one. Males express twice as much ribosomal RNA as females, further suggesting that there is no dosage compensation.

**SEX (GONADAL) DIFFERENTIATION**

**Developmental mechanisms**

Once sex determination mechanisms are activated, primary sex differentiation ensues. The majority of amphibians are gonochoristic, and gonadal differentiation follows a similar pattern in frogs and salamanders (little is known about caecilians). The gonads consist of the somatic gonad and germ cells.
The somatic gonadal tissues in males and females likely have different origins, and the primordial germ cells arise from yet a third origin.

The gonads originate from an outpocket of cells on the ventral surface of the kidney. Initially, there are no histologically observable differences between males and females. The undifferentiated (or bipotential) gonad is a solid structure with an intact cortex and medulla. In females, the cortex develops and grows, whereas the medulla regresses, leaving a hole (ovarian vesicle) which is observable upon histological analysis. The cells in the cortex become large follicles, and oocytes can be observed during fairly early stages of differentiation. Male development is characterized by medullary development and cortical regression. The gonads in males have smaller densely packed cells (Figure 3).

**Origin of the somatic gonadal tissue**

There is still debate regarding the origin of the cells that form the gonadal ridge (which becomes
the undifferentiated gonad) and whether different tissues contribute to the cortex and medulla. In Witschi's studies on *Rana sylvatica* (Witschi, '27) and *R. sylvatica, R. arora,* and *Hyla regilla* (Witschi, '31), he reported that the cortex (the primordium of the ovary) is derived from the coelomic epithelium and that the medulla (the testicular primordium) originated from the mesonephric blastema, with no contributions from the coelomic epithelium. Several later studies and reviews agreed with Witschi’s findings (Witschi, '67; Mittwoch, '73; Deuchar, '75), but Vannini ('41, '42) suggested that the medulla was derived from the interrenal blastema and not the mesonephric blastema in *Rana agilis.* Studies in *Bufo bufo* and *Bufo viridis* (Vannini and Busetto, '45), in *Bombina pachypus* (Vannini, '47), and in two salamanders (*Triton* spp.; Papi, '47, '49) produced findings similar to Vannini ('41, '42). Later studies in *Rana esculenta* (Sabbadin, '51a; Vannini and Sabbadin, '54) and *Rana dalmatina* (Sabbadin, '51b) suggested that the interrenal blastema contributed cells to the cortex (which is derived primarily from cells from the coelomic epithelium) and to the medulla of the developing gonads. In these early stages of gonadal development, the cortex and medulla are reportedly not distinct and migrations from the interrenal blastema to the cortex do not cease after the two layers separate. Vannini and Sabbadin ('54) suggested that the differences in their findings and those findings of Witschi were, in part, due to what they interpreted as Witschi's unclear understandings of the origins of the mesonephric and interrenal blastema. The
significance of the difference between Witschi's and Vannini's model is that the gonadal primordia and the interrenal primordia (both steroid-producing tissues) share a common origin in Vannini's model, whereas the gonads share a common origin with the kidney primordia (non-steroid-producing) in Witschi's model.

Recent studies examining the origins of the gonadal ridge created more confusion: Studies in *Xenopus laevis* and *R. pipiens* (Merchant-Larios and Villalpando, '78, '81) suggested that the mesonephric blastema may not contribute to the gonadal medulla, but further suggested that the coelomic epithelium was the origin of the gonadal medulla, giving the cortex and medulla a common origin. Lopez ('89) suggested that there was no medulla in *Bombina orientalis* initially, and that the germ cell–filled cortex was invaded by the medulla, which originated from cells of the mesonephric blastema. Furthermore, Tanimura and Iwasawa ('87, '89) suggested that the entire gonad was formed from the coelomic epithelium in *Rhacophorus arboresus* and that the medulla is formed by cells segregating within the primordial gonad. Furthermore, at least two studies suggested that cells migrate from the cortex into the medulla during male development (Vannini and Sabbadin, '54; Amanuma, '63). Thus an examination of several studies reveals conflicting results. Although species variation is possible, more comparative ultrastructural studies may resolve this problem.

**Origin of the primordial germ cells**

Like the origins of the cortex and the medulla, the origin of the primordial germ cells in amphibians has been heavily debated. The early history of these debates was documented by Witschi ('29c): The origin of the primordial germ cells was first described by Goette (1875), who suggested that they arose from yolk-laden embryonic cells. Allen ('07) was the first to report that the primordial germ cells had an endodermal origin. Other authors described secondary germ cells, and some claimed that these secondary germ cells had a mesodermal origin (Bouin, '01; Dustin, '07). Humphrey ('25) even reported that germ cells in salamanders were derived from mesoderm, whereas germ cells in frogs were endoderm-derived. Humphrey suggested also that germ cells did not migrate or move independently, but rather shifted their position throughout development as a result of “growth of shifting of related parts” (Humphrey, '25; page 1). Other authors had previously made similar claims that germ cells were mesoderm-derived in salamanders (Hall, '04; Dustin, '07; Allen, '11; Schapitz, '12; Beccari, '22). Kuschakewitsch ('10) reported secondary germ cells that arose from a variety of tissues; and Gatenby ('16) claimed that oogonia arose de novo in the ovary. King ('08), Witschi ('14), Beccari ('24, '26), Bounoure ('25) did not find any evidence for secondary germ cell differentiation, and it is not likely that such occurs, based on recent studies cited below.

Most workers currently agree that the germ cells originate in the endoderm from the dorsal mesentary of the gut. The formation and migration of the primordial germ cells have been well studied in *Xenopus laevis* (Blackler, '58; Al-Mukhtar and Webb, '71; Kerr and Dixon, '74; Ijiri and Egami, '75; Ikenishi and Kotani, '75; Whittington and Dixon, '75; Wylie and Heasman, '76; Kamimura et al., '76; Wylie et al., '76; Züst and Dixon, '77; Yamaguchi and Iwasawa, '81), including light microscopic and ultrastructural electron microscopic studies. These studies found that germ cells originated solely from primordial germ cells that differentiated and migrated as early as the gastrula stage. Thus no secondary or spontaneous differentiation of germ cells in the gonads is likely.

Blackler ('62) provided additional evidence against secondary formation of germ cells: Germ cells were reciprocally transferred between two subspecies of *Xenopus laevis* (one that produced light brown eggs and one that produced dark brown eggs). In some crosses, the eggs produced by adult females were solely of the donor type: If any secondary production from a source other than the grafted primordial germ cells occurred, then all individuals would have produced eggs of mixed types.

Although all of the studies cited above agree in regards to the origin of primordial germ cells, very little comparative work has been conducted. Two recent studies (Tanimura and Iwasawa, '87; Nomura and Iwasawa, '92) examined germ cell kinetics in a toad (*Bufo japonicus formosus*) and a salamander (*Onychodactylus japonicus*), respectively, but no studies have examined the ultrastructure and migration of primordial germ cells to the extent of the studies in *Xenopus laevis*.

**Germ cell–somatic gonadal tissue interactions**

Several studies examined the potential role of the germ cells in gonadal differentiation, with mixed results. Witschi ('29c) and Cheng ('32) suggested that the histological changes associated with masculine and feminine differentiation occurred before the germ cells migrated into the go-
nad, but Dantchakov ('32, '33) proposed that the germ cells secreted a substance that induced gonadal differentiation.

The most convincing experimental evidence suggesting that the gonads differentiate independent of germ cells was presented by Humphrey ('38), who conducted reciprocal unilateral transplants of the intermediate segment and adjacent mesoderm in *Rana sylvatica* during early tail bud stages (prior to migration of the primordial germ cells). Thus the mesonephros and gonad on the operated side differentiated from the donor's mesoderm, whereas the primordial germ cells that migrated into the developing gonad were from the host's endoderm (because no endoderm was transplanted). In cases where the sex of the donor and host differed, the gonad on the operated side followed the sex of the donor, as did the germ cells. For example, when male mesoderm was transplanted to a female embryo, an ovary developed on the unoperated side, whereas a testes developed on the operated side. The primordial germ cells that entered this testes developed into sperm, showing that the developing testes induced the invading germ cells to develop into sperm rather than oocytes. Similar studies were conducted in salamanders (Humphrey, '27), and showed that gonads developed in the absence of primordial germ cells, but only testicular development was described in detail. Humphrey's studies showed, however, that gonads develop in the absence of germ cells and that germ cells do not stimulate differentiation of the gonads.

Later ultrastructural studies suggested that germ cells may even differentiate independent of the gonads (Kalt, '73). Primordial germ cells were more numerous (Ijiri and Egami, '75), and the germ cell division rate was faster in females than in males (Züst and Dixon, '77), even before the germ cells migrated into the gonads. These data suggested that germ cells may sexually differentiate independent of the gonads, although Humphrey's findings ('27, '38) suggested that the gonads can redirect germ cell differentiation.

Despite evidence that germ cells are not required for gonadal differentiation, more recent studies suggested, on the contrary, that germ cells were inductive and directed differentiation of the gonads. Shirane pressed eggs of *Rana japonica* and *Rana brevipoda* ('82) and *Rana nigromaculata* ('84) against quartz and exposed the vegetal pole to UV irradiation to destroy primordial germ cells before they differentiated and migrated to the gonadal ridge. In the earlier ('82) study, germ cell-free embryos were produced, and although the gonads were sometimes smaller in size, fertile males and females were produced. Shirane ('82) reported a skewed ratio at sexual maturity in *Rana brevipoda*, but the sample size (33 total: 8 females/25 males) was low, and mortality between metamorphosis and the stage of examination was greater than 30%. The sex ratio determined before metamorphosis (at metamorphosis) was normal (54% female), and although the author claimed that the skewed sex ratio at sexual maturity was due to sex reversal (female to male), it is equally likely that the change was due to differential mortality. In Shirane's later study ('84), he reported female-to-male sex reversal (but again sample sizes were low). He hypothesized that UV irradiation reduced the number of germ cells that migrated into the gonadal ridge. He claimed that ovarian differentiation failed and that medullary cells surrounded the remaining germ cells and induced testicular development (female-male sex reversal). With this evidence, he concluded that germ cells were necessary for ovarian differentiation.

Shirane ('87) further tested the hypothesis that germ cells affected gonadal differentiation by using the peanut lectin (PNA), which binds to the surface of migrating primordial germ cells. Shirane ('87) suggested that the observation that the primordial germ cells lost their affinity for PNA coincident with gonadal differentiation indicated that the germ cells induced gonadal differentiation. He further suggested that the primordial germ cells secreted substances that induced gene expression in the developing gonad, resulting in differentiation (Shirane, '87). To test these hypotheses, Shirane injected embryos with PNA or N-acetylgalactosamine (galNA; which is recognized by PNA). He proposed that the sex-inducing activity of the migrating primordial germ cells would be decreased by treatment with lectins (which would presumably occupy the binding sites of the lectins and prevent binding and activation of the secreted substances in the gonadal ridge) and that treatment with galNA would enhance the activity. Shirane ('87) suggested that PNA inhibited female development in *Rana japonica* and inhibited both male and female development in *R. nigromaculata* based on the results of this study. Furthermore, treatment with galNA reportedly inhibited male development in both species.

There are several problems with Shirane's ('87) study and the interpretation of results, however. The sample sizes were low (30 or fewer in the experimental groups discussed) and not adequate
for assessing effects on the sex ratio. In addition, Shirane examined gonadal development only in early developmental stages (TK V; Taylor and Kollros, '46) and admits that “the results at TK V were sometimes ambiguous” and “perhaps discrepancies in these results may be caused by the fact that the decision about gonadal sex was histologically more difficult at TK V” (Shirane, '87; page 500). Attempts to reanalyze data at TK X failed because treatments with galNA were lethal by this stage, and the experiment was discontinued. Furthermore, the author discussed the effects of his treatment on male and female genes, when there is no evidence that any observed histological effects resulted from changes in gene expression. Shirane's ('82, '86, '87) studies are thus inconclusive, and earlier studies (discussed previously) provided stronger evidence for the lack of a role for primordial germ cells in gonadal differentiation.

**Cortical-medullary interactions**

Witschi ('27, '31, '34, '57) proposed that gonadal differentiation was the result of antagonistic interactions between the developing cortex and medulla (Figure 4). He proposed that the cortex of genetic females produced a compound (cortexin) that induced regression of the medulla (or alternatively accelerated cortical growth) and/or that a substance from the medulla (medullarin) induced cortical regression (or medullary growth) in males. Furthermore, Witschi proposed that these compounds did not behave like typical hormones, but traveled in tissue rather than being secreted into the blood (Witschi, '27).

Several studies showed that sex reversals occurred when males and females of the same species were surgically connected as parabiotic twins, which Witschi viewed as evidence supporting his theory: Witschi and McCurdy ('29) showed that ovaries transformed into testes when female larvae were paired with male larvae in *Taricha torosa* (*Triturus torosus*) with only minor effects on the testes by the female. Burns's ('30) data supported these findings and showed that male *Ambystoma tigrinum* induced sex reversal when parabiosed with females. In frogs, similar findings were reported: Parabiosis between male and female *Xenopus laevis* resulted in sex reversal of females (degeneration of the cortex and oocytes, and hypertrophy of the medulla; Chang, '53). Similarly, in parabiotic twins of *Rana sylvatica* (Witschi, '29c), *Rana aurora*, and *Hyla regilla* (Witschi, '31) females initially developed ovaries when parabiosed with males, but subsequently developed testes. Witschi viewed the above data as evidence for his inductor substances (Witschi, '31), but these data suggested that the putative inductors traveled in the blood shared by the parabiotic twins (not only through tissues as suggested by Witschi ['27]).

Other studies examined interactions between ovaries and testes by transplanting testes into females and ovaries into males, for comparisons with same-sex reciprocal transplants as controls. Such studies in salamanders (*Ambystoma maculatum* and *A. tigrinum*: Humphrey, '29; '31a; *Cynops* (*Triturus*) pyrrhogaster: Ichikawa, '37; *A. mexi-
canum: Humphrey, '42) showed that transplanted donor testes developed normally, but caused sex reversal (transformation of ovaries into testes) in female hosts and transformation of donor ovaries when placed in males (provided that the donor and host were of similar age and developmental stage). Humphrey ('31b) later showed that when the grafted testes were removed from the female hosts, the sex-reversed females (neomales) were sterile (Humphrey, '42). Furthermore, the rudimentary ovary underwent reversal again (after testes removal) and produced functioning ovaries, showing that the effects were not permanent.

Humphrey ('35) showed that heterosexual parabiosis between two different species produced different results: Testes donated from A. maculatum were transformed into ovaries by the female A. jeffersonium. Also, A. tigrinum female hosts transformed testes grafted from A. maculatum into ovaries (Humphrey, '35). In these cases, the effects of the ovary on the testes were believed to be due to the intrinsic faster development rate of the female host species.

In frogs, similar examinations revealed significant variation between studies. In *Rana temporaria* (Witschi, '27), testes implanted into sexually undifferentiated tadpoles had no effect on developing ovaries. These studies (Witschi, '27) were conducted in a race described as protogynous, so this peculiar sex differentiation in this species may have affected results. Witschi ('27) viewed these data as evidence that did not support his inductor theory of sex differentiation, as he predicted that the testes should interfere with cortical development. A number of studies in *Xenopus laevis*, however, yielded positive results, but created some confusion: When implanted with testes, genetic females developed testes (Chang, '53; Mikamo and Witschi, '63, '64). In addition, Chang ('53) found that testis grafts induced sex reversal (development of testes from ovaries in females) only when the grafts were made during the larval phase. Also, testes from only immature postmetamorphic males induced complete sex reversal: The testes of adult sexually mature males lost their inductive capabilities.

The ability of implanted testes from immature males (but not adult males) in *Xenopus laevis* is especially interesting, in light of experiments examining the effects of crystalline sex steroids on gonadal development in amphibians. Testosterone was incapable of inducing male development in *Xenopus laevis* larvae; early studies suggested that testosterone produced 100% males, but proved incorrect. The gonads were not affected by androgen treatment, although androgens induced secondary sex characters such as thumb pad development (Chang and Witschi, '55a). Thus sex steroids were likely candidates for Witschi's cortexin and medullarin, except that testosterone had no effect on gonads, whereas whole testes were effective. In fact, if androgens were the inductive substances, then the testes of adult males should be more potent, but Chang's ('53) studies showed that only immature testes were capable of inducing testicular development in females.

Furthermore, estrogens were 100% effective at inducing complete sex reversal of genetic males, but in parabiosis experiments, the ovary was affected by the male (or the testis graft) and not the other way around. Even more confusing, ovarian extracts were capable of inducing sex inversion in adult male *Triturus pyrrhogaster*, whereas estrogen treatment could not produce this effect (Hanaoka, '61). Chang and Witschi ('55a) turned away from the idea that steroids were the putative inductors, speculating that the cortexin and medullarin were proteins, and perhaps that the cortex even produced antibodies (antimedullarin) against the medulla and vice versa. In the same report (Chang and Witschi, '55a), the authors also reexamined Witschi's earlier thesis (Witschi, '27) that the inductor substances traveled through tissues rather than through blood, and suggested that the putative inducers were blood-bound.

**Role of steroid hormones**

Padoa ('36) was the first study to examine the effects of exogenous steroids on gonadal differentiation in amphibians. In Padoa's ('36) study on *Rana esculenta*, exogenous estrogen treatment resulted in 100% male offspring. Later studies showed that a variety of steroids “sex-reverse” frog and salamander larvae when added to the water or injected into larvae (Gallien, '59), although Swingle ('22) suggested that the true transformations do not occur. Paradoxically, a number of chemically unrelated steroids have similar effects: For example, progestins, corticoids, androgens, and estrogens can all induce testicular development producing 100% male progeny (Table 1). Equally puzzling, a single steroid can have a variety of effects, depending on the species: Estradiol can produce 100% females, have no effect at all, or produce 100% males (see Table 1). A single steroid can have drastically different effects even in the same species, depending on dose. Moreover, in ranids estradiol induces 100% females at low
TABLE 1. Steroidal effects on primary sex differentiation in anurans

<table>
<thead>
<tr>
<th>Steroid 1</th>
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<th>Species 3</th>
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(continued)
**TABLE 1. Steroidal effects on primary sex differentiation in anurans (continued)**

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**Estrogens**

| Estrone | 3.7 | Bufo vulgaris | MF | Takahashi ('56) |
| Estrone | U1 | Rana esculenta | MIF | Padoa ('38) |
| Estrone | U1 | Rana esculenta | M | Padoa ('38) |
| Estrone | U1 | Rana esculenta | M | Padoa ('36) |
| Estradiol | 3.7 | Bufo vulgaris | MF | Takahashi ('56) |
| Estradiol | .07–.14 | Buergeria buergeriana | MIF | Ohta ('87) |
| Estradiol | .46–1.85 | Buergeria buergeriana | M | Ohta ('87) |
| Estradiol | ? | Alytes obstetricians | F | Witschi and Allison ('50) |
| Estradiol | .37–3.7 | Bufo americanus | IF | Chang ('55) |
| Estradiol | 1.0 | Bufo boreas | MF | Hayes et al., unpubl. |
| Estradiol | 0.037–0.37 | Hyperolius argus | IF | Hayes et al., unpubl. |
| Estradiol | 0.37 | Hyperolius viridiflavus | IF | Hayes et al., unpubl. |
| Estradiol | .18 | Hyperolius viridiflavus | MIF | Richards ('82) |
| Estradiol | 0.37 | Ptychoeuphalus adspersus | MF | Hayes and Licht, unpubl. |
| Estradiol | ? | Rana agilis | F | Vannini ('46) |
| Estradiol | Inj | Rana clamitans | MF | Foote and Witschi ('39), Mintz et al. ('45) |
| Estradiol | Inj | Rana catesbeiana | MF | Puckett ('39, '40) |
| Estradiol | U1 | Rana esculenta | M | Padoa ('38) |
| Estradiol | .1–.21 | Rana esculenta | F | Padoa ('42) |
| Estradiol | .42 | Rana esculenta | MIF | Padoa ('42) |
| Estradiol | .92–3.7 | Rana esculenta | M | Padoa ('42) |
| Estradiol | ? | Rana esculenta | M | Padoa ('36) |
| Estradiol | 3.68 | Rana japonica | MF | Yoshikura ('62) |
| Estradiol | .07–18 | Rana pipiens | F | Richards and Nace ('78) |
| Estradiol | 3.68 | Rana pipiens | M | Richards and Nace ('78) |
| Estradiol | .03–.92 | Rana sylvatica | F | Witschi ('48) |
| Estradiol | .7 | Rana sylvatica | M | Dale ('62) |
| Estradiol | 3.7–4.6 | Rana sylvatica | IF | Witschi ('48) |
| Estradiol | ? | Rana temporaria | M | Gallien ('40, '41) |
| Estradiol | .07–.37 | Rana spp. | F | Chang and Witschi ('55c) |
| Estradiol | 1.85 | Rana spp. | MIF | Chang and Witschi ('55c) |
| Estradiol | 3.7–18.5 | Rana spp. | M | Chang and Witschi ('55c) |
| Estradiol | .04 | Xenopus laevis | MF | Chang and Witschi ('55a,b) |
| Estradiol | .18–36 | Xenopus laevis | F | Chang and Witschi ('55a,b) |
| Estradiol | .185–2.2 | Xenopus laevis | F | Gallien ('53) |
| Estradiol | .185–2.2 | Xenopus laevis | F | Gallien ('57) |
| EB | ? | Xenopus laevis | IF | Gallien ('54) |
| EB | .13–5.2 | Discoglossis picta | IF | Gallien ('50) |
| EB | Inj (150) | Pelobates cultripes | F | Gallien and Collenot ('58) |
| EB | Inj (150) | Pelodytes punctatus | IF | Gallien and Collenot ('58) |
| EB | .21 | Rana esculenta | M | Padoa ('42) |
| EB | .11–.66 | Rana sylvatica | MF | Witschi ('48) |
| EB | 1.6 | Rana sylvatica | F | Witschi ('48) |
| EB | .13 | Xenopus laevis | F | Gallien ('56) |
| EB | .001–1.6 | Xenopus laevis | F | Gallien ('55, '62) |
| EBB | Inj | Rana clamitans | MF | Mintz et al. ('45) |
| EDP | Inj | Rana clamitans | MF | Mintz et al. ('45) |
Mechanisms of steroid action

Mechanism of steroid action on the gonads are not known. No steroid receptors have been identified in amphibian gonads. Furthermore, gonads respond to steroids at times when other tissues lack sensitivity to steroids. For example, estrogens induce ovarian development in *Xenopus laevis* larvae during stages when this same hormone is unable to induce vitellogenesis in the liver, oviductal growth, or cloacal labial growth (Hayes, ’97b). In the case of the liver, estradiol is not capable of inducing vitellogenin because the liver lacks estrogen receptors until metamorphosis (Rabeo et al., ’94), and this mechanism likely explains why other tissues do not respond. Similarly, testosterone induced testicular differentiation in *Hyperolius argus* and *Hyperolius viridiflavus* during stages in which this hormone is unable to induce secondary sex characters such as gular pouch development (Hayes, ’97a). So, either sex steroids do not act through conventional steroid receptors in their actions on the gonads, or gonadal expression of steroid receptors is regulated differently when compared with other tissues.

Tadpoles metabolize steroids administered into the water or injected into the body cavity (Dale, ’62; Rao et al., ’68, ’69; Hayes and Licht, ’93, ’95), and this metabolism may play a role in the varied effects of steroids on sex differentiation. Species that are unaffected by steroids may deactivate these hormones faster by metabolizing them to inactive compounds, or alternatively, species that are affected may activate the hormones by metabolizing them to more potent compounds. Data from one study (Hayes et al., submitted) suggested that the metabolites of estradiol produced by *Xenopus laevis* larvae are active compounds capable of inducing ovarian differentiation. Rao et al. (’68) also claimed that female tadpoles metabolized steroids to active compounds, whereas male tadpoles converted them to inactive compounds.
Incomplete reporting and inconsistent designs between published studies create a problem when interpreting data on the effects of steroids in different species. For example, with a few exceptions, none of the studies used to estimate the effects of steroids in amphibians (see Figure 2) reported the total amount of hormone added to the water, the number of tadpoles per treatment tank, the developmental stages during which the tadpoles were treated, the total amount of time over which animals were treated, or the temperature maintained during treatments (Hayes and Licht, '95). Variation in effects between species may be an artifact of different experimental designs and treatment regimes: The total amount of hormone and number of tadpoles are important in determining dose (in addition to reporting hormone concentrations). A given amount of hormone likely decreases in effectiveness as more tadpoles are added to a tank (50 tadpoles sequester and use more hormone than 10 tadpoles). Also, the developmental stage during the treatment is important: Estrogens induce ovarian differentiation in Xenopus laevis only during specific stages of development (Chang and Witschi, '55a; Gallien and Foulgoc, '60; Witschi, '71; Iwasawa et al., '79; Villalpando and Merchant-Larios, '90; Hayes et al., '97b). Thus some studies may fail to show effects of steroids on gonadal development because the treatments were administered during incorrect stages. In addition, these critical periods likely vary between species. Likewise, the total amount of time that animals are exposed and the temperature during exposure likely affect the potency of steroids. In addition, all of these factors (concentration, total mass of steroid, developmental stage, temperature, and duration of exposure) alter steroid metabolic rates (which also vary between species; Hayes and Licht, '93, '95). Thus, if steroid metabolism by tadpoles represents activation or deactivation of the steroids (and steroid metabolism likely affects potency), then the interaction between tadpoles and treatment conditions and effects on steroid metabolism provide another mechanism by which artifacts may be produced when comparing effects between species from reports in the literature. These problems may be solved by careful studies of multiple species under similar controlled conditions in parallel studies.

Endogenous steroids

It is not likely that steroid hormones play a role in sex determination, although they may be involved in normal gonadal differentiation in amphibians. In order for males and females to produce different sex steroids, they have to express different steroidogenic enzymes. If males and females express different functional enzymes, then they are already differentiated (even if this differentiation cannot be detected ultrastructurally). In fact, the steroids may be responsible for inducing this detectable (structural and ultrastructural) differentiation, but the hormones themselves must be the product of prior differentiation.

In addition to the lack of understanding of the mechanisms of exogenous steroid action on gonadal differentiation, little is known about the role of endogenous steroids in normal gonadal differentiation. Amphibian larvae possess steroidogenic enzymes in their gonads, and steroid metabolism and production have been measured, but only after gonadal differentiation had occurred (Hsü et al., '77, '78, '79, '85) or when the sexes were not identifiable (Kang et al., '95). Also, several studies have shown that amphibian larvae metabolize exogenous steroids (Rana sylvatica and Rana pipiens: Dale, '62; Pleurodeles waltl: Ozon, '63; Xenopus laevis: Rao et al., '68, '69; nine species, representing eight families: Hayes and Licht, '93, '95), and one study even suggested that male and female larvae metabolize estrogens differently (Rao et al., '68). Although steroid metabolism was not localized to the gonads, these data showed that larvae are capable of metabolizing and possibly producing sex steroids. In salamanders, Chardard et al. ('95) showed that Pleurodeles waltl had active aromatase in its gonads that increased in females during larval development. Rearing female larvae at high temperatures (which sex-reverses female P. waltl) caused a decrease in aromatase activity, suggesting that estrogens may be involved in sex differentiation of females in this species (Chardard et al., '95).

Despite the paucity of studies examining endogenous hormone production, a few studies have addressed the role of endogenous hormones in amphibian gonadal differentiation by treating amphibian larvae with reagents that block endogenous steroid production. Zaccanti et al. ('94) treated larval Bufo bufo and Rana dalmatina with androsten-3 one 17 β-carbossilic acid, which inhibits 5 α reductase (prevents the production of 5α reduced androgens). This study showed accelerated ovarian development with larger and more numerous oocytes in the Bidder’s organ of Bufo bufo and in the ovary of Rana dalmatina. Although sex reversal did not occur, these data suggested that endogenous androgens might be
involved in gonadal differentiation. Hsü et al. (‘79, ’81a) also showed that blocking estrogen synthesis resulted in transformation of ovaries into testes and that blocking both androgen and estrogen synthesis with 17 β-ureide (Hsü et al., ’81b) resulted in sterile gonads. Similarly Yu et al. (’93) showed that endogenous steroids may be involved in sex differentiation in Rana catesbeiana. Implanting tadpoles with capsules containing 4-OH-androstenedione (an aromatase inhibitor) resulted in testicular development. The authors suggested that the sex reversal was due to a buildup of androgen that acted on the gonad to induce sex reversal. These studies all suggest that aromatase (and low androgens) may be required for ovarian differentiation. An earlier study suggested that endogenous steroids may not be involved in normal sex differentiation, however. Treatments with an anti-estrogen (tamoxifen) or an anti-androgen (cyproterone acetate) were unable to block the effect of exogenous estrogen or testosterone treatment, respectively. Also, neither anti-steroid affected normal sex differentiation (Rastogi and Chieffi, ’75).

One difficulty in understanding the potential role of steroids in normal gonadal differentiation is that steroid production must be analyzed prior to sex differentiation to discern whether sex steroids organize the gonad or whether differences in sex steroid production are the result of organization (sex differentiation) that has occurred already. This task is difficult, because identification of sexes in most species is based on gonadal morphology, since only a few species have easily distinguishable sex chromosomes. Although steroid levels were measured in eggs of two species (Rana sylvatica and Xenopus laevis; Hayes, unpublished), the effects of steroids during these early stages are not known to influence sex differentiation. Also, hormones found in eggs are of maternal origin, because the steroidogenic tissue has not developed in early embryos at these stages.

**Other hormones**

**Thyroid hormones**

Thyroid hormones are best known for their role in amphibian metamorphosis, but have been implicated in gonadal development in amphibians. Their role in gonadal differentiation is debated, and some workers claim that gonadal differentiation is one of the few aspects of development that is not under the control of thyroid hormones (Hayes, ’97b; for review).

The order and coordination of developmental events are relatively consistent in amphibians: Forelimbs emerge before the tail reabsorsbs (in frogs), the lungs mature before the gills reabsorb (in frogs and metamorphosing salamanders), etc. The timing of gonadal development varies significantly, however. For example, Xenopus laevis have differentiated gonads (based on histological analysis) by NF (Nieuwkoop and Faber, ’56) Stage 52, whereas Hyperolius viridiflavus are differentiated as early as NF Stage 48 (even by morphological examination). Other species, such as Bufo boreas, are not sexually differentiated until after metamorphosis (Gosner ’60 Stage 48 = NF Stage 66), and gonads are not distinguishable by histological examination even 1 week after metamorphosis (Hayes, ’97b). Even within a species, there may be variation in the timing of gonadal differentiation (Iwasawa, ’69; Chang and Hsu, ’87), especially in the sexually undifferentiated races of Rana temporaria (Witschi, ’30) and Rana catesbeiana (Puckett, ’40). In addition, Ohira (’87) reported that Buergeria buergeri from Hiroshima had differentiated gonads by Gosner Stage 40, but our experience raising the same species from Yamanashi under similar conditions showed that gonads were not differentiated even at metamorphosis (Hayes and Wong, unpublished). One interpretation is that this variation is an indication that thyroid hormones do not affect gonadal differentiation. On the other hand, thyroid hormones may regulate gonadal differentiation, but different species may show different sensitivities: some respond to very low doses of thyroid hormones and differentiate early in development, whereas others require high titers of thyroid hormone and differentiate at metamorphic climax, etc. Also, thyroid hormones may regulate gonadal differentiation in some species, but not others. This possibility is certainly realized in other tissues, such as development of the hind limbs, which are thyroid hormone–dependent in Xenopus laevis but not in Bufo boreas (Hayes, ’95, ’97a).

Several studies suggested that thyroid hormones regulate gonadal development. In recent studies, we (Hayes, ’97b) showed that treatment with thiourea (a goitrogen) resulted in 100% females in Xenopus laevis (a species in which estradiol induced ovarian differentiation but testosterone had no effect) and 100% males in Hyperolius viridiflavus (an unrelated species in which estradiol had no effect but testosterone induced testicular development). In fact, the ste-
Pituitary hormones

Several studies addressed the role of pituitary hormones and the pituitary gonadotrop(h)ins in sex differentiation. Gonads developed normally in both males and females when larvae were hypophysectomized early in development prior to gonadal differentiation in *Rana japonica* (Yoshikura, ’60, ’61, ’62b,c; Yü et al., ’72; Rengel et al., ’93). On the contrary, several studies suggested that thyroid hormones are not involved in gonadal differentiation (Allen, ’18; Yoshikura, ’60, ’61, ’62b,c; Yü et al., ’72; Rengel et al., ’93). Thus a definite role for thyroid hormones and their mechanism of action remains to be shown.

Undifferentiated races

So called undifferentiated races have been reported in a number of ranid species, including *Rana temporaria* (Pflüger, 1882; Ponse, ’30; Witschi, ’30), *R. catesbeiana* (Pucket, ’40), and *R. esculenta*, R. ornativentris (Iwasawa, ’69) and in a single bufonid (*Bufo bufo*: Ponse, ’49; Foote, ’64) and in *Rhacophorus arboreus* (Iwasawa, ’69a,b). Similar reports were made in salamanders (*Hynobius retardatus*: Uchida, ’37b; and *Ambystoma maculatum*: Witschi, ’33b). In *R. catesbeiana* (Pucket, ’40), the animals were not truly undifferentiated, but ovarian differentiation was observed in larval stages. The testes remained a “protestis” (as described by Swingle, ’26), however, until metamorphosis, when they developed into recognizable testes. Development in undifferentiated races of *R. temporaria* is quite distinct, however. In some populations, all of the animals metamorphose with ovaries. In the following 6 to 9 months after metamorphosis, some of the individuals reportedly transform into males going through an intersexual stage (Witschi, ’21, ’30). Similar reports were made for *Rhacophorus schlegeli* (Amanuma, ’63a,b).

Sequential hermaphroditism and spontaneous sex reversal

Although Witschi (’29b, page 236) stated, “Some of the most common anurans are rudimentary hermaphrodites which eventually may ripen fertile eggs and sperms either in consecutive years, as in the toad *Bufo vulgaris*, or simultaneously, as in the frog *Rana temporaria*” and even claimed that artificial self-fertilization was possible in some of these rudimentary hermaphrodites, he does not cite any reference where data and observations were reported. Crew (’21) also reported a number of incidences of abnormal sex reversals and intersexual animals, but not much information was provided for review, and the report is simply a categorical listing of cases.

At least two published studies since Witschi (’29b) and Crew (’21) addressed the possibility of sequential hermaphroditism (natural sex reversal without any hormone treatments, etc.). Grafe and Linsenmair (’89) suggested that seven out of 24 captive female *Hyperolius viridiflavus ommatostictus* (which reportedly laid at least one clutch of eggs) began calling, developed gular pouches, and apparently fertilized eggs. No histological evidence or further examinations were published in this case, however. In another study
(Collenot et al., '94), the salamander (Pleurodeles waltl) reportedly underwent spontaneous sex reversal in captivity. The authors reported that some animals (all apparently of the ZZ type) developed into females, and intersexes. This study used a supposed sex-linked peptidase in which one type is supposedly linked to the W chromosome (Ferrier et al., '80; Dournon et al., '88) to identify genetic types of individuals. Animals lacking the proposed W-linked peptidase were assumed to be ZZ, and any animals possessing an ovary but lacking the W-linked form of the enzyme were assumed to be sex-reversed. An alternative explanation is that the peptidase may not be as tightly linked as proposed and thus may not be a reliable sex marker. Their results may be explained by exchange between the Z and W sex chromosomes. Evidence for such exchange is found in the X and Y chromosome in mammals, for example (Kent et al., '88).

Rudimentary hermaphroditism in Bufonids

The natural condition in Bufonids is the most compelling case for hermaphroditism in amphibians (although not functional). In all bufonids, both males and females possess Bidder's organs. Bidder's organs are a pair of rudimentary ovaries that develops at the anterior portion of the gonad in both males and females. Witschi ('33a) provided an excellent review of the early descriptions of the Bidder's organ: The Bidder's organ was first described as fat (Roesel, 1758) and then as the primordium of the testes (Rathke, 1825). Ironically, Bidder (1846), for whom the organ was named, argued that the Bidder's organ was an accessory structure involved in male gametogenesis. Later, Von Wittich (1853) suggested that the Bidder's organ was a rudimentary ovary developing in both males and females, and further histological data from several workers supported Von Wittich's claim (see references in Witschi, '33a). Even more convincing, Harms ('21, '23) and Guyénot and Ponse ('23a,b,c, '27) showed that the Bidder's organ developed into a fully functional ovary that can produce fertilizable eggs after the testes were surgically removed.

Since its discovery, a number of papers have described the development of the Bidder's organ (Witschi, '33a; Horié, '38; Vannini, '56; Zaccanti et al., '71; Tanimura and Iwasawa, '86), and more recent studies have examined its contribution to steroid hormones (Colombo and Colombo-Belvedere, '80; Ghosh et al., '84; Pancak-Roessler and Norris, '91). The Bidder's organs always develop at the cephalic end of the gonad and may differentiate at the same time as the gonads. Meiotic activity in the Bidder's organ is greater in females than in males (Tanimura and Iwasawa, '86), and these data, combined with the observation that removal of the testes results in the transformation of the Bidder's organ into fully functional ovaries (references above), suggested that the testicular secretions may antagonize the development and function of this rudimentary ovary in males.

Despite the presence of the Bidder's organ, however, bufonids are not truly hermaphroditic (neither simultaneous or sequential): Under normal conditions, the Bidder's organs do not function as ovaries in males. Although Witschi suggested that sequential hermaphroditism occurred in Bufo vulgaris, the primary description of this observation and details of studies are not cited and the literature is not available. Thus the function (if any) and the mechanisms underlying development of this structure remain unclear. The realization that the Bidder's organ is a rudimentary ovary stimulated much discussion about the ancestral state of amphibians, however. Several authors proposed that the rudimentary ovary in bufonids is evidence of a hermaphroditic ancestry of the bufonids, if not all amphibians (Witschi, '33a). Interestingly, the cephalic portion of the gonad in Xenopus laevis can be induced to develop an ovarian-like structure (with male characteristics developing caudally) if male larvae are treated with estrogens for a short time near the critical period (Chang and Witschi, '55a; Hayes et al., unpublished). The fate of such intersex animals is unknown since none have been followed to metamorphosis and adulthood. These data suggest that the cephalic portion of the gonad is more sensitive to sex steroids and transforms before the caudal end. Alternatively, gonads normally differentiate posterior to anterior, so that short-term hormone treatments transform the cephalic end, which remains bipotential, but are unable to transform the caudal end, which may differentiate earlier. Whatever the mechanism, such studies may provide interesting models for examining mechanisms underlying Bidder's organ development in bufonids.

Parthenogenesis

Natural parthenogenesis has been reported in Ambystomatid salamanders and is well studied. The Jeffersonian salamander complex consists of two diploid species (Ambystoma jeffersonium and Ambystoma laterale), which reproduce normally. There are at least two triploid species (Ambystoma
platineum and Ambystoma tremblayi) whose ranges overlap with the ranges of the diploid species (Clanton, '34; Minton, '54; Uzzell, '64; MacGregor and Uzzell, '64). The two triploid species primarily consist of females. These triploids depend on diploid males because sperm is required to stimulate development of the eggs of the parthenogens, although genetic material from the males is not incorporated (Licht, '89).

Uzzell ('64) showed that triploid females consistently produced only female (and triploid) offspring, and suggested that the triploid species resulted from hybridization between the two diploid species (Uzzell, '63). Later electrophoretic studies supported this hypothesis (Uzzell and Goldblatt, '67; Downs, '78; Bogart et al., '85; Lowcock and Bogart, '89; Kraus, '89; Lowcock, '89) and showed that some island species may have arisen from hybridizations between Ambystoma laterale and Ambystoma texanum as well. Uzzell and Goldblatt ('67) went further to hypothesize that a tetraploid hybrid, producing diploid gametes, may have served as an intermediate that, when back-crossed to one of the diploid parental species (producing haploid gametes), produced triploid offspring.

Triploid salamanders have been produced in the laboratory also. Ambystoma tigrinum and Ambystoma mexicanum eggs can be induced to develop as triploids by cold treatment and by pricking with a needle. These animals undergo normal ovarian development during early stages, but oocytes degenerate later in development. This degeneration may be related to the ploidy of the animals and not some physiological deficiency, since grafted ovaries from normal diploid females develop normally (Humphrey and Frankhauser, '46).

Studies in natural triploid Notophthalmus (Triturus) viridescens (Salamandridae) showed that, unlike Ambystomatids, both male and female triploids developed (Frankhauser, '40). In addition, not only did males develop normally, but females suffered a loss of germ cells, and only rudimentary ovaries developed. However, this study (Frankhauser, '40) was based on only four animals (one male and three females) that were captured from the wild and raised in the laboratory. One possible explanation for the potential differences in the effects of triploidy between the Ambystoma spp. and Notophthalmus may be related to the different sex-determining systems (Ambystoma are ZZ/ZW whereas Notophthalmus spp. are XXXY). Studies on these animals may contribute to our understanding of the relationship between mechanisms of sex determination and sex differentiation.

The existence of parthenogenesis has not been reported in frogs, although natural polyploids exist in frogs (Wasserman, '70; Bogart and Tandy, '76; Bogart, '80). Frogs can be induced to develop polyploidy (Parmenter, '33; Rugh and Marsland, '43; Dasgupta, '62), and a single study showed that both males and females resulted from triploidy induced in Rana pipsiens by cold shock, but by metamorphosis all females were sex-reversed and developed testes (Humphrey et al., '50).

CONCLUSIONS

All amphibians probably display genetic sex determination, but external factors can influence sex (gonadal) differentiation. The external factors include temperature, physical manipulations, and exogenous hormones. Only endogenous hormones may naturally affect gonadal differentiation, however.

A lack of work in the field over the last two decades has left many questions concerning sex differentiation unanswered. Mechanisms regulating germ cell differentiation are unclear. The contributions of coelomic epithelium and kidney and interrenal primordia to the cortex and medulla of the gonads remain undetermined. The role of endogenous steroids and thyroid hormones and their mechanisms of action on the development of the gonad are also unclear. Other future studies should address the relationship between mechanisms of GSD (female versus male heterogamety) and mechanisms of sex differentiation and how GSD may be related to the susceptibility to sex reversal by some steroids but not by others (known to be active in other species).

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