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Intersex, Hermaphroditism, and Gonadal Plasticity in Vertebrates: Evolution of the Müllerian Duct and Amh/Amhr2 Signaling

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Abstract

In vertebrates, sex organs are generally specialized to perform a male or female reproductive role. Acquisition of the Müllerian duct, which gives rise to the oviduct, together with emergence of the Amh/Amhr2 system favored evolution of viviparity in jawed vertebrates. Species with high sex-specific reproductive adaptations have less potential to sex reverse, making intersex a nonfunctional condition. Teleosts, the only vertebrate group in which hermaphroditism evolved as a natural reproductive strategy, lost the Müllerian duct during evolution. They developed for gamete release complete independence from the urinary system, creating optimal anatomic and developmental preconditions for physiological sex change. The common and probably ancestral role of Amh is related to survival and proliferation of germ cells in early and adult gonads of both sexes rather than induction of Müllerian duct regression. The relationship between germ cell maintenance and sex differentiation is most evident in species in which Amh became the master male sex–determining gene.

INTRODUCTION

Hermaphroditism has always fascinated humans. In the myths of many cultures, the origin of man is traced back to a first hermaphrodite created by the gods, which is then bisected into a male and a female. In her classic paper, Ursula Mittwoch (1) explained that the description of the creation of Eve in the book of Genesis also leaves room for a hermaphrodite origin interpretation. She explains that the Old Testament was written in Hebrew, and that the word *zeal*, which is usually translated as "rib," can also mean "side," providing linguistic evidence for a bisected Adam.

In biology, hermaphroditism, the presence of both sexes in one individual, either simultaneously or consecutively, is quite frequent in plants and invertebrates; however, as an evolutionary reproductive strategy in vertebrates, it seems to be restricted entirely to fish. Hermaphroditism occurs in two forms: simultaneous and sequential hermaphroditism. The first occurs in species in which an adult individual possesses fully functional male and female reproductive functions. The sequential type is characterized by a switch from one sex to the opposite sex during adult life and comes in three primary forms: protogyny, in which an individual begins reproductive life as a female and later may switch to a male; protandry, in which an individual starts as a male and later may become female; and bidirectionality, in which an individual may switch back and forth between being functionally male and female (2).

The vertebrate gonad arises from a bipotential anlage, which later differentiates into testis or/and ovary. The molecular processes of gonadal sex determination are still not completely elucidated and can have high variation throughout phylogeny (reviewed in 3). The gonad is composed of two main lineages: somatic cells and germ cells. The somatic cells develop into two cell lineages: the supporting cells (granulosa cells in ovary or Sertoli cells in testis) and the steroidogenic cells (theca cells in ovary or Leydig cells in testis). The germ cells give rise to the gametes and are derived from the primordial germ cells (PGCs) (4, 5). The formation of the intraperitoneal genital ducts and external genitalia in male and female is normally characterized as secondary sex determination. Most vertebrates have distinct male or female duct systems, and their formation occurs during embryogenesis. In the male, the mesonephric (Wolffian) ducts and a group of mesonephric tubules connect with the testis to form a vas deferens and vasa efferentia (epididymis). In amniotes (reptiles, birds, and mammals), the Wolffian ducts regress in females, whereas in nonamniotes (fish and amphibians), they perform the excretory role in both sexes (6). Hence, the male gonad has a strictly necessary relationship with the excretory system to result in a functional adult male organ. In females, the oviducts differentiate from the Müllerian ducts, formed laterally to the mesonephric ducts. The Müllerian ducts can undergo regional specialization during development, which varies between species and with the mode of reproduction (7).

With the notable exception of the teleost fish (see below), the Müllerian ducts are present in the female of the majority of vertebrates. They exist as an anlage in both male and female fetuses, regressing in males of some vertebrates. In amniotes, the process of Müllerian duct regression in males is induced by anti-Müllerian hormone (AMH).[1] AMH, also known as Müllerian duct–inhibitory substance (MIS), is a member of the transforming growth factor-β superfamily (8) (**Supplemental Figure 1**). AMH binds preferentially to the AMH receptor type 2 (AMHR2), a paralog of bone morphogenetic protein receptor type 2 (BMPR2) (9). In male mammals, the process of Müllerian duct regression involves epithelial cell migration, epithelial-to-mesenchymal transformation, and apoptosis. Already at undifferentiated stages in mice, *Amhr2* is expressed throughout the length of the Müllerian duct in both sexes. After sex determination in the fetal testis, Amh is produced and induces Müllerian duct regression (10).

Supplemental Material >

In hermaphrodites, the sex-specific urogenital and reproductive system needs to be specialized for ovary and testis function in one individual to perform the reproductive roles of both sexes. We present evidence that hermaphroditism is related to a divergent evolution of the reproductive organ system, in particular the absence of the Müllerian duct in teleosts. However, the genes involved in regulation of Müllerian duct formation are present in fish, in which they acquired a major and even more prominent role in sex determination. Hence, a comparative view of the main bodies of evidence on the roles of Amh and its receptor in gonad development and formation of the male and female reproductive system may help us to better understand the evolutionary developmental biology of reproduction.

CYCLOSTOMATA[2]

The cyclostomes are represented by only two groups of living jawless fish: hagfish and lampreys (12). Regarding the structure of the reproductive system, cyclostomes do not have intraperitoneal genital ducts. The gametes are simply released into the body cavity. Even though Wolffian ducts are present, their role is restricted to excretion and not to reproduction (13).

In hagfish, Gorbman (14) correlated the gonadal differentiation with the body length of *Eptatretus stouti* and classified this species as being juvenile protogynous. He observed that the gonad differentiates into testis only in specimens longer than 28 cm, with the ovotestis representing an intermediate stage during the process of male gonadal differentiation (14). However, there is no evidence of any hagfish in which the transient hermaphroditic gonad morphology reflects a functional capability in reproduction (15). Thus, in males, there is a temporary preadult stage of ovotestis, which we define as juvenile intersex, because the mixed sex organs are not functional and the transition to male sex occurs before sexual maturation (**Figure 1**).

In contrast with hagfish, lampreys develop via a larval stage known as ammocoete. In a period between six months and two years of age, the gonad remains undifferentiated, followed by oogenesis that occurs in all larval lamprey gonads. In the female, after the metamorphosis, the oocytes enter maturation and the ovary develops further, whereas in the male the early oocytes undergo degeneration. From the few remaining residual germ cells, primary spermatogonia reenter mitotic division either shortly before or during metamorphosis in males (13). Therefore, like hagfish, lampreys have a relatively extended juvenile intersex stage before the gonadal sex differentiation toward male initiates. In the few cases in which adult male intersex was reported, it seems unlikely that those animals would be able to self-fertilize or spawn viable eggs. Hence, it was hypothesized that the presence of male intersex fish is a remnant larval or immature trait (16).

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[1] Gene and protein nomenclatures follow the guidelines for the corresponding organism mentioned in the context. This is based on *Trends in Genetics* Genetic Nomenclature Guide (11). If mammals in general are meant, the nomenclature for mouse is used.

[2] Correspondence of Latin and English (scientific and common) names of the main taxa is provided in the **Supplemental Appendix**.

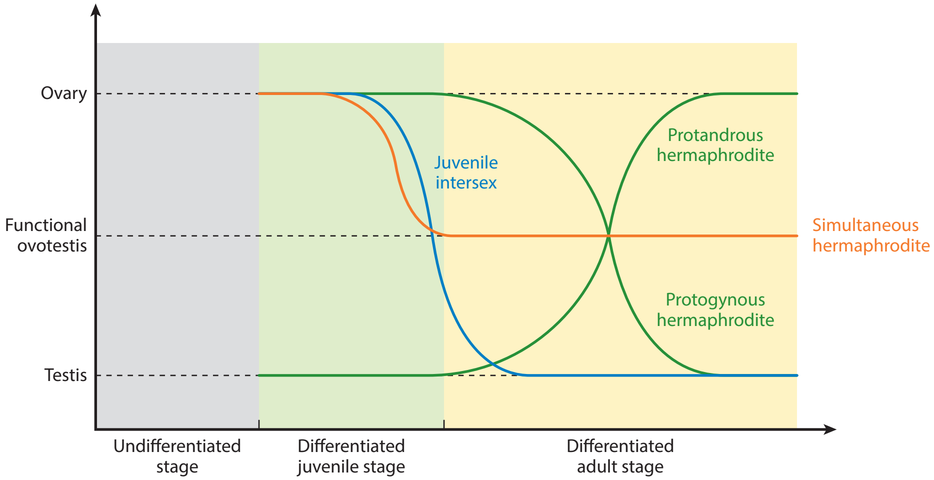

Figure 1

Diagram of gonadal sex change in juvenile intersex and hermaphroditism. In juvenile intersex, the sex change or ovotestis stage occurs during the juvenile stage. In hermaphrodites, the sex change or ovotestis formation takes place during the adult mature stage.

The jawless fish do not have a Müllerian duct or any other structure responsible for sperm or egg release. Consistently, we found no *amh* and *amhr2* genes in the whole-genome sequences available from the lampreys *Petromyzon marinus* and *Lethenteron camtschatium*.

CHONDRICHTHYES

Cartilaginous fish (Chondrichthyes) are the oldest living representatives of jawed vertebrates, comprising the elasmobranchs (sharks and rays) and the holocephalans (chimeras). This group presents a remarkable diversity of reproductive modes (17) and can be classified based on embryonic development being either external (oviparity) or internal (viviparity) (18).

In contrast to in all other vertebrates, in chondrichthyans the Müllerian ducts form through an anterior-to-posterior longitudinal splitting of the Wolffian duct (6, 19). The Müllerian duct later develops to form the oviduct, uterus, and uterine tubes in females. In males, the Müllerian ducts degenerate, but not completely, persisting in a rudimentary condition in adults (20). All chondrichthyan eggs deposited externally have leathery, structurally complex, and remarkably durable shells (21). The fertilized egg passes through the oviduct and is encapsulated by secretions produced by the oviductal gland (22). This gland is a specialized region of the anterior Müllerian duct–derived oviduct and is also important for the formation of the tertiary egg envelopes and for transferring the eggs to the uterus and storing sperm (23). Viviparity, in contrast, includes both lecithotrophic (yolk-sac viviparity) and a variety of matrotrophic modes of reproduction (histotrophy, oophagy, and placental) (17). In all cases of viviparity, specializations of the oviduct evolved to keep the egg or the embryo inside the female body, making maternal care possible (**Table 1**), with the Müllerian duct even becoming a "uterus" (17, 22). In conclusion, the production of the durable shell in the egg of oviparous chondrichthyans and the capacity to maintain the embryo inside the female in viviparous species are possible only owing to the presence of a Müllerian duct.

Table 1 Correlation between presence or absence of Müllerian ducts with modes of reproduction and deviation of gonochorism in extant vertebrates

Vertebrate clade		Mode of reproduction		Role of the Müllerian duct	Deviation from gonochorism
Cyclostomes	Hagfish	Oviparous		Absent; gametes released into the body cavity.	Juvenile intersex (all species)
	Lamprey	Oviparous		Absent; gametes released into the body cavity. Some species with seminiferous ampullae for sperm release.	Juvenile intersex in ammocoetes (all species)
Chondricthyes		Oviparous		Oviductal gland produces the egg jelly, forms the tertiary egg envelopes (durable shell), and stores sperm.	Natural intersex (rudimentary hermaphroditism) (*Apristurus longicephalus*)
		Viviparous	Yolk-sac viviparity	Retention of fertilized eggs within the uterus with no additional maternal nutritional input beyond the yolk.	Abnormal intersex
			Histotrophy	The uterus retains the developing embryo and produces mucus (limited histotrophy) or protein- and lipid-enriched histroph (lipid histotrophy) as a supplement to the egg.	Abnormal intersex
			Oophagy	Retention of the developing embryos and unfertilized eggs. The developing embryos ingest the unfertilized eggs or the small embryos to support further development.	Abnormal intersex
			Placental	Uteroplacental complex: umbilical cord; smooth, proximal portion of the placenta; distal, rugose portion of the placenta; egg envelope and uterine-attachment site.	Abnormal intersex
Actinopterygii	Cladistia	Oviparous		Male and female ducts appear to be homologous structures.	Not reported
	Chondrostei	Oviparous		Large funnel-shaped oviducts that lead directly into the caudal portion of the Wolffian duct. Well-developed and persisting Müllerian duct in adult males of *Acipenser*.	Abnormal intersex
	Holostei	Oviparous		In lepisosteids, the oviduct is continuous with the lumen of the ovary and extends from the ovary to the bladder. In *Amia*, the eggs are released into the peritoneal cavity and move into the oviduct.	Not reported
	Teleostei	Oviparous: cystovarian		Absent; male and female ducts (gonoducts) are homologous and responsible for gamete release.	Juvenile intersex; protandrous, protogynous, and simultaneous hermaphroditism

(*Continued*)

Table 1 *(Continued)*

Vertebrate clade			Mode of reproduction	Role of the Müllerian duct	Deviation from gonochorism
			Oviparous: gymnovarian	Absent; eggs are released into the body cavity. Transient gonoduct structures occur during embryonic development in females. Males have gonoducts (spermioducts), except in Anguillidae.	Protogynous and simultaneous hermaphroditism in Anguilliformes
			Viviparous and ovoviviparous	Absent; the gonoduct is modified for internal fertilization and sperm retention. Gestation occurs within the ovary (intraovarian).	Abnormal intersex
Sarcopterygii	Actinistia		Ovoviviparous	Compartmentalization of the oviduct for embryo retention during uterine gestation (embryoparity).	Not reported
	Dipnoi		Oviparous	Oviducts are long with multilayered secretory epithelium and end in the cloaca. Males retain the Müllerian duct.	Not reported
	Amphibians		Oviparous	Uterus secretes tertiary accessory envelopes onto the eggs. Ovisac formation for embryo retention in case of internal fertilization. Some cases of Müllerian duct retained in males.	Juvenile intersex in tadpoles (*Rana temporaria*)
	Reptiles		Viviparous	Fertilization; sperm storage and transportation; placental formation and maternal-fetal nutrient transfer.	Abnormal intersex
			Oviparous and ovoviviparous	Fertilization; sperm storage and transportation; egg transport; provision of water; shell membrane and eggshell production; expulsion and reabsorption of abortive eggs.	Abnormal intersex
	Birds		Oviparous		Gynandromorphy
	Mammals	Monotremes	Oviparous		Not reported
		Marsupials	Viviparous	Fertilization; sperm storage and transportation; shell membrane production; short-term placenta formation and maternal-fetal nutrient transfer.	Abnormal intersex
		Eutherians	Viviparous	Fertilization; sperm storage and transportation; placenta formation and maternal-fetal nutrient transfer. Vestigial structures in adult males as the appendix testis and prostatic utricle.	Abnormal intersex

In chondrichthyans, there are several reports of intersex individuals, in some cases described as hermaphrodites. However, this condition is relatively rare, and there is no evidence of reproductively functional ovotestis in such intersexual adults (24–26). The completely different internal reproductive structures in male and female adults, and the specializations of the oviduct in particular, may make it difficult, or even impossible, for a fully differentiated adult to change sex or fulfill both reproductive roles. Adult intersex chondrichthyan specimens possess different combinations of male and female sexual organs (gonads and ducts) and range from basic to complete intersexual (27). The only known example of a cartilaginous fish described as normal hermaphroditic is the oviparous species *Apristurus longicephalus*. In this species, 85% of the individuals develop the genital apparatus of one sex and the undeveloped genital apparatus of the opposite sex but develop the phenotype of the sex that corresponds to the fully developed genitals. The other 15% of the specimens have a gonochoristic phenotype, possessing only a male or female genital apparatus. The undeveloped oviduct in males of this species is not functional and cannot supply all the conditions for egg fertilization and maturation, restricting the animal to performing only male-specific reproductive features. Adult intersex females develop small claspers, but the oviduct and the ovaries are functional (28). Because the presence of intersex is the natural situation, but only one sex role is functional, this species was classified as rudimentary hermaphroditic (28). Nevertheless, the presence of undeveloped structures of the opposite sex displaces this species closer to intersex than to hermaphroditic, which we categorize as natural intersex.

Interestingly, the presence of the Müllerian duct in cartilaginous fish coincides with the emergence of the *amh* gene in vertebrates. Unexpectedly, however, in all genomes available from Chondrichthyes to date, no *AMHR2* gene is annotated. Our own analysis uncovered that in the genome of the elephant shark (*Callorhinchus milii*), besides the bona fide Bmpr2-encoding gene, a sequence predicting a Bmpr2-like protein is present. The Bmpr2-like peptide sequence is not nested within the bona fide Bmpr2 at the expected position, but it is more closely attached to the Amhr2 clade (**Figure 2**). The genomic location harboring this gene in the elephant shark shows conserved synteny with the genomic region of *AMHR2* in other tetrapods (**Figure 3**). This result provides further evidence of the origin of *amhr2* as a paralog to *bmpr2* and suggests the presence of *amhr2* in cartilaginous fish. Thus, the appearance of AMH signaling parallels the emergence of the Müllerian duct. However, the persistence of the Müllerian duct in adult males indicates that the role of *AMH* in its regression appeared later in vertebrate evolution. Further functional analyses must be performed to confirm conserved functions of these genes.

OSTEICHTHYES

The group of bony fish is the largest class of vertebrates, with a high diversity of species, ecology, and reproductive strategies. Osteichthyes is divided into ray-finned (Actinopterygii) and lobe-finned fish (Sarcopterygii). Actinopterygii is represented mainly by the “modern” Teleostei and by ancient nonteleost groups, normally referred to as basal Actinopterygii (e.g., sturgeons, gars, and bowfins). The Sarcopterygii gave rise to all tetrapods and include at their base the coelacanths and the lungfish (12).

Regarding the reproductive system, the role of the Müllerian duct, in female, and the Wolffian duct, in male, as genital duct was maintained in the systematic group of Osteichthyes, except the teleosts (**Figure 4**). However, in contrast to in chondrichthyans and some amphibians, Müllerian ducts in Osteichthyes develop from the rostral mesonephric epithelium, extending caudally and parallel to the Wolffian ducts (10).

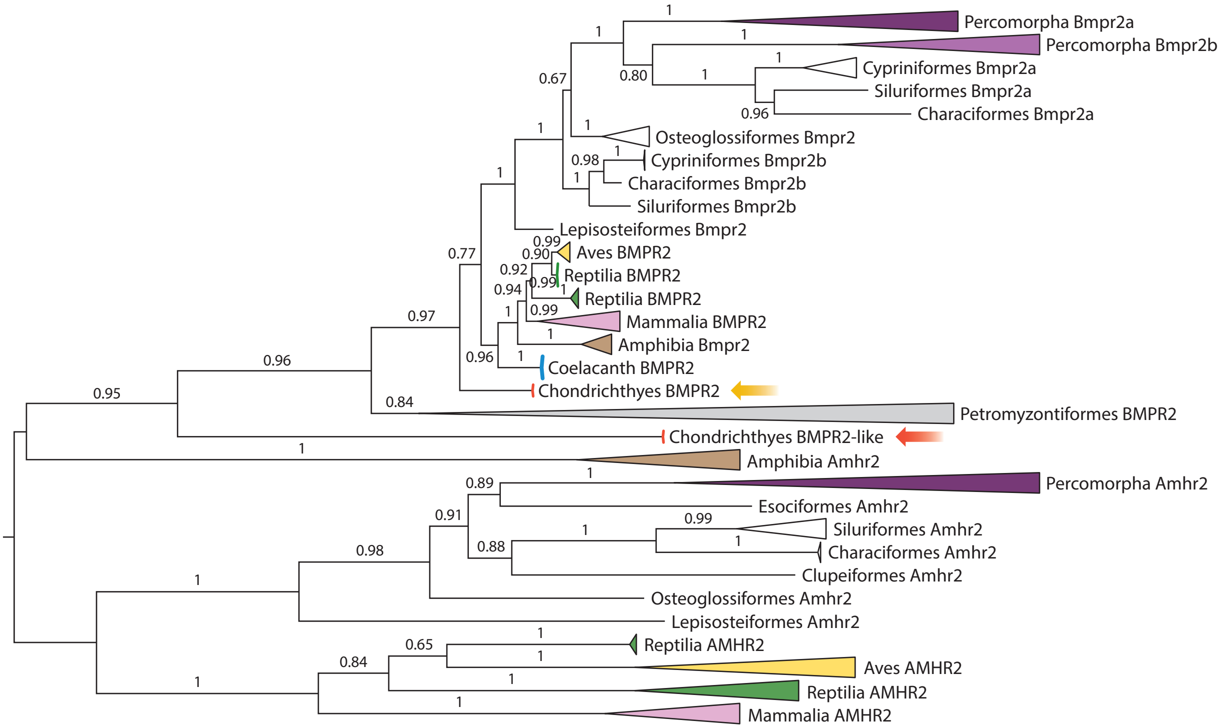

Figure 2

Phylogenetic analyses of Bmpr2 and Amhr2 amino acid sequences in vertebrates. Branch values represent the approximate likelihood ratio test (see **Supplemental Appendix**). The sequences demonstrate the common origin of Bmpr2 and Amhr2. The Chondrichthyes (*Callorhinchus milii*) Bmpr2-like sequence (*red arrow*) presents strong divergence from the Bmpr2 sequences in Chondrichthyes (*yellow arrow*) and other vertebrates. The sequences of both copies of the Bmpr2 genes in Osteoglossiformes are clustered together.

Supplemental Material >

Basal Actinopterygii

The basal Actinopterygii are represented by three groups: the Cladistia (bichirs), the Chondrostei (sturgeons and paddlefish), and the Holostei (bowfins and gars) (29). Information about urogenital duct formation in Cladistia and Holostei is scarce (30–32). Interestingly, in sturgeons, the Müllerian ducts in adult males do not regress. However, it is difficult to define a reasonable function of this duct in males, because its terminals end with blind tips in the wall or in the lumen of the caudal portion of the Wolffian duct (33). Intersex frequency in natural populations of sturgeon is usually low and is often related to water contamination with endocrine disruptors [e.g., dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs)]. In aquaculture, Rzepkowska (34) reported high frequency (more than 30%) of intersex caused by feminization in both Russian and Siberian sturgeons. In intersex of sterlet and shortnose sturgeons, the production of fertile gametes and successful cross- and self-fertilization have been demonstrated (35, 36). However, no data confirm cross- and self-fertilization in wild intersex sturgeon populations, which indicates an abnormal phenotype probably owing to aquaculture conditions.

The Amh sequence in basal actinopterygians was annotated in spotted gar and sturgeon, and the phylogenetic analyses cluster both groups in their expected basal evolutionary positions (**Figure 5**). Interestingly, Amh sequences of cartilagenous fish show stronger similarity with the sarcopterygians, instead of being in the expected more basal position relative to bony fish. In

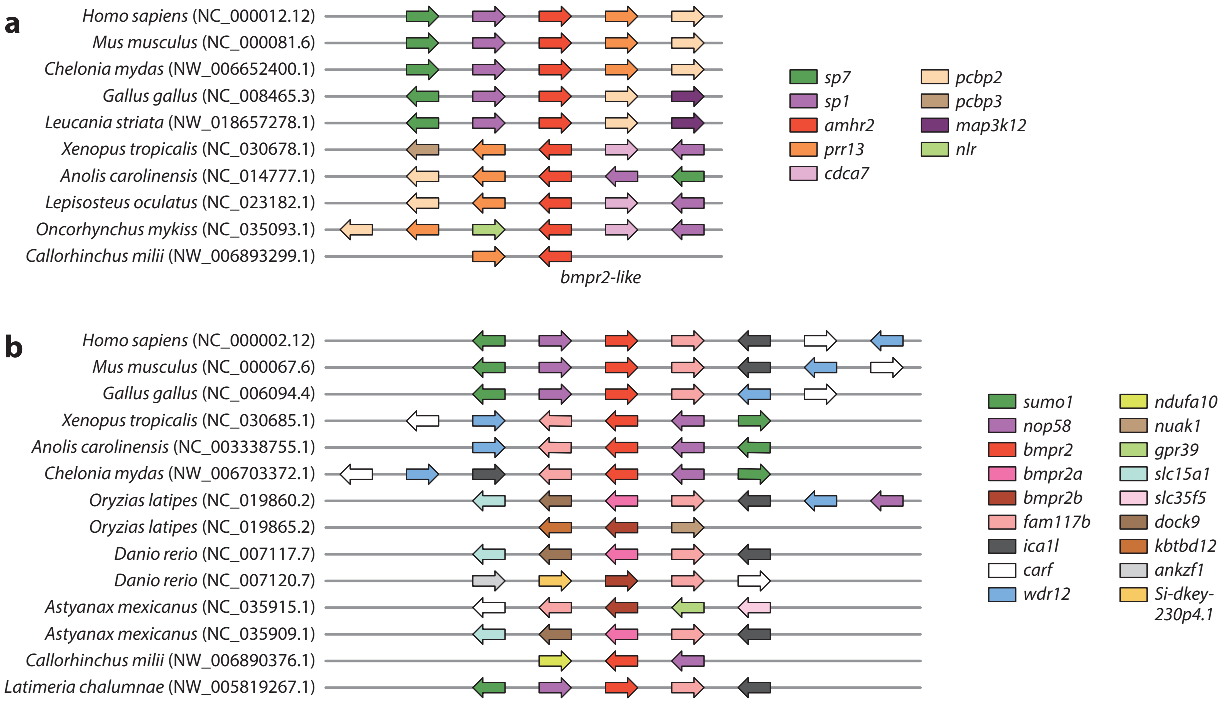

Figure 3

Genomic organization and gene synteny comparisons across vertebrates for (*a*) Amhr2 and (*b*) Bmpr2 genes. The position of *bmpr2-like* in the genome of *Callorhinchus milii* indicates that the annotated gene has conserved synteny to the Amhr2 of other vertebrates. The syntenic regions of *amhr2* and *bmpr2* genes were analyzed based on alignment of the target gene and genomic annotation according to the GenBank database (see **Supplemental Appendix**).

Supplemental Material >

elephant shark, the *amh* gene consists of five exons, and this structure was maintained in sarcopterygians. In Actinopterygii, *amh* contains seven exons, except in trout, which have six exons. These two additional exons are caused by insertions of intronic sequences into exon 1 and 5 of mammals and birds (37). The lineage specification of *amh* in Actinopterygii could indicate a role in divergence in this group. Expression analyses of *amh* in gonads of sturgeons presented higher transcription levels in the testis than in the ovary, but no sexually differential expression level in juveniles (38, 39). Additionally, in sterlets (*Acipenser ruthenus*), high expression of *amh* in early spermatogenesis indicates an important role in early testicular differentiation (40).

Teleostei

Compared with other vertebrates, teleosts have important differences in genital duct formation. In all other jawed vertebrates, the egg is released into the coelomic cavity and collected through a funnel into the oviduct, derived from the Müllerian duct. Teleosts have evolved gonoducts that are not derived from the Müllerian ducts, and their formation can vary by species. In general, male and female gonoducts originate from the dorsal peritoneum, the suspensory mesentery of the gonad, the posterior peritoneum of the gonad, the somatic mass posterior to the gonad, or some combination thereof (41).

Male and female gonoduct formation can also vary and in some species may be reduced or absent in one or both sexes. In most females, the gonoducts are connected to the ovarian cavity, so the

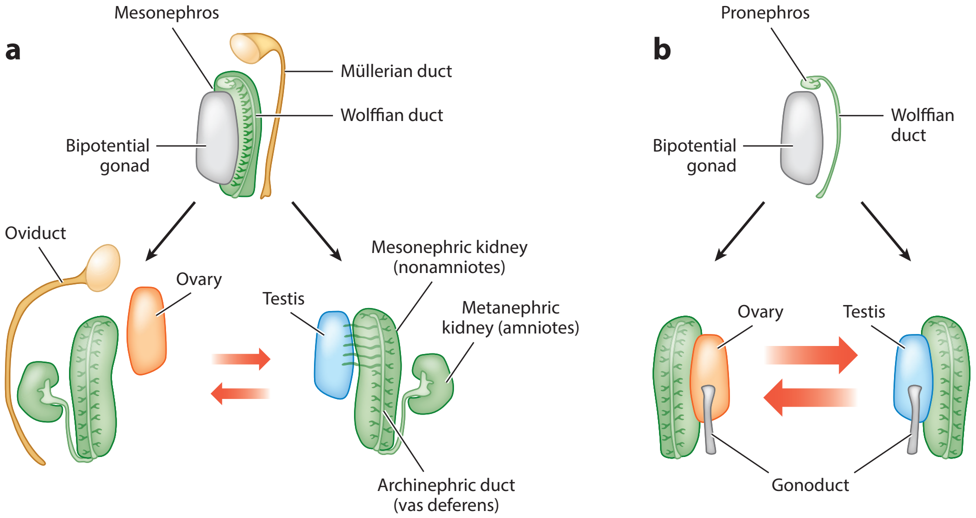

Figure 4

Urogenital system development in jawed vertebrates. (*a*) In nonteleosts, the Müllerian duct is reduced or regresses completely in males, whereas in females it develops into the oviduct. The testis cords and the spermatic duct originate from the mesonephros, showing a straight dependency of the male gonad with the excretory system. Some rare cases of artificially induced sex change in amphibians indicate a mild potential to develop hermaphroditism in a nonteleost group (*red arrows*). (*b*) In teleosts, the Müllerian duct is absent, and the gonoduct has the same origin in both sexes. There is complete independence of the excretory and reproductive systems. The smaller sex-specific specializations of the urogenital system in teleosts, in comparison with the other jawed vertebrates, increase the potential to sex change in adult stages and develop hermaphroditism (*red arrows*).

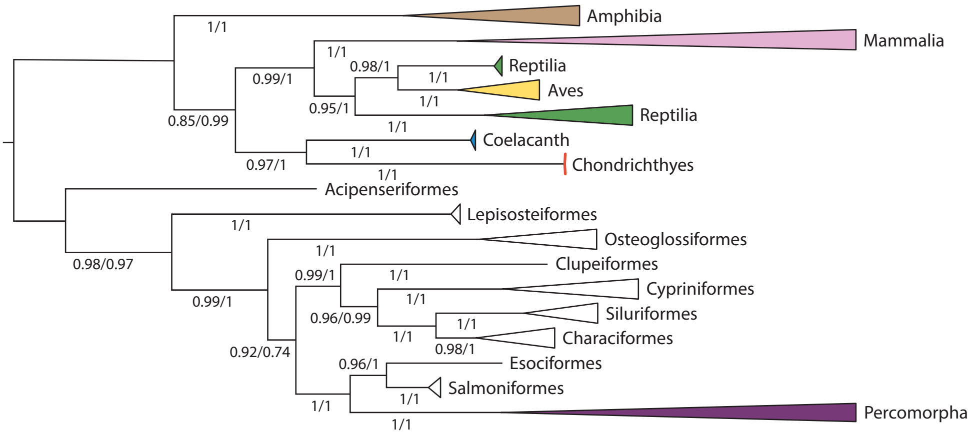

Figure 5

Phylogenetic analyses of Amh amino acid sequences in vertebrates. The branches of the tree were collapsed according to the taxa. First and second branch values represent the approximate likelihood ratio test and the posterior probability from the PhyML and BEAST programs (see **Supplemental Appendix**).

Supplemental Material >

eggs are not released into the coelom. Unlike in other vertebrates, in male teleosts the gonoducts that transfer the sperm from the testis are completely independent from the kidney–urinary system (42). The epithelium of the testicular ducts provides the spermatozoa with nutrition (43).

Teleosts present different types of reproductive modes, with oviparity as the dominant mechanism. Females of viviparous and ovoviviparous species have specializations of the gonoduct for internal fertilization and sperm retention. Unlike in other vertebrates, embryo development in viviparous teleosts occurs within the ovary (intraovarian gestation), probably owing to the lack of a Müllerian duct–derived oviduct (44). Viviparity is present in only approximately 2% of teleost species (45). Interestingly, neither intersex nor hermaphroditism has been found in viviparous species under natural conditions, which could be related to the gonoduct modifications needed to accomplish this reproductive mode, making development of fertile hermaphrodites or sex-changed animals less likely.

The oviparous teleosts are the only group of vertebrates in which the potential to change sex in adult life is used as a reproductive strategy in natural populations. Individuals of some species are able to perform both male and female reproductive functions simultaneously or sequentially and are therefore considered true hermaphrodites (**Figure 1**). Hermaphroditism evolved several times in teleosts and has been documented in approximately 6% of all extant teleost species (46). Nevertheless, it is clearly a derived feature relative to gonochorism. In zebrafish (47) and black tetra (48), the gonad of all individuals starts development as ovary. Later, some individuals complete ovarian differentiation, whereas in others oocytes enter apoptosis and testicular development takes place. Generally, these species are described as juvenile hermaphroditic. However, similar to cyclostomes, they cannot perform the reproductive role of both sexes via a sex change after sexual maturation. Hence, we also classify these species as juvenile intersex (**Figure 1**).

The structure of the hermaphroditic gonad varies according to the taxonomic group and the type of hermaphroditism. Protogynous hermaphroditism is the most common type, particularly in tropical reef fish (49). In some protogynous species, the gonoduct structure of the so-called secondary males arises secondarily along the periphery of the gonad and surrounding the persisting ovarian lumen. In contrast, in primary males, which are not hermaphrodites, the gonoduct is centrally located, with no trace of a previous ovarian duct (50). In some protandrous groups, the two heterosexual regions are separated by connective tissue and have separate gonad cavities, whereas in other groups there is no connective tissue between the testicular and ovarian tissues, with a common gonad cavity (50, 51). In both cases, each sexual region develops separate ducts to release the gametes (52). During the process of sex change, in species with clear separation of male and female parts, the gonoduct of the opposite sex develops, whereas the other regresses together with the corresponding gonad tissue (50). In some simultaneous hermaphroditic species, the testicular and ovarian parts have completely separate ducts (53). However, the simultaneous hermaphrodite mangrove killifish (*Kryptolebias marmoratus*) is the single documented case of self-fertilization in vertebrates; the gonadal lumina begin at the upper central part of each lobe, continue posteriorly, and unite in the common genital sinus (54). Hence, reproductive plasticity in teleosts is not restricted to the gonad, and the gonoducts can also change during the transition to the other sex.

Hermaphroditism arose independently several times within teleosts, indicating that species in this group have all the prerequisites to perform this role. The molecular mechanisms behind sex determination and sex change in teleosts, like in all vertebrates, are only incompletely elucidated and show variation throughout phylogeny (55). In hermaphrodites, the sex phenotype is a consequence of the interaction between the genes that determine ovary and testis development and the environment, although the environmental triggers vary according to the reproductive strategy of the species. Several theoretical models have been forwarded to explain the evolutionary advantage to changing sex in adult animals or to being a simultaneous hermaphrodite, by combining body size, critical age, fecundity, and social and behavioral factors (2, 50). Nevertheless, the current explanations for fitness increase owing to sex change do not address why Teleostei is the only vertebrate group presenting this feature.

The role of Amh in teleosts got particular attention because of the intriguing fact that teleosts lack Müllerian ducts. During embryonic development, the undifferentiated gonads of both sexes have *amh* expression at relatively low and equal levels. Later, *amh* expression increases during male gonad development and remains high during and after puberty in adults. In medaka (*Oryzias latipes*), expression of *amh* starts in the somatic gonad upon the arrival of migrating PGCs to the somatic gonad precursor in both sexes (56). Interestingly, the medaka *amhr2* loss-of-function mutant *hotei* leads to a hyperproliferation of mitotically active germ cells and male-to-female sex reversal in 50% of the XY animals (57, 58). In *Takifugu* species, two alleles of the *amhr2* gene were identified, in which one allele has a missense SNP (*amhr2-SNP*) and is linked to primary sex determination. Studies in two pejerrey species (*Odontesthes*) identified a second copy of *amh*, named *amhY*, which is expressed exclusively in males prior to gonad sex differentiation and acts as the male sex–determining gene (59, 60). In Nile tilapia, *amh* is also present on the sex chromosome, together with a tandem duplication of the *amhy* named *amhΔ-y*. Functional analyses showed that *amhy*, not *amhΔ-y*, is essential for male sex determination in Nile tilapia (61). In all studies, the *amh*/*amhr2* system in teleosts is involved in regulation of germ cell proliferation and differentiation and can play a crucial role in sex determination.

Accumulated knowledge has shown that Amh also exerts a role in adult teleost gonads, in particular, at early stages of germ cell development in both males and females (37). Most of the experimental data regarding Amh function in the testis come from zebrafish (62–65) and medaka (57, 58). It is important to emphasize that zebrafish lack *amhr2*, but AMH still has an important physiological function, though putative target cells of Amh are unknown (37). In zebrafish and Japanese eel testis, Amh is characterized as a "spermatogenesis-preventing substance" (62, 66). Interestingly, recent studies showed that Amh counteracts gonadotropin-induced effects on Leydig cell steroidogenesis (62) and Sertoli cell stimulatory growth factor production (i.e., insulin-like growth factor 3) (63, 64). However, inhibitory signals, such as *inha* (inhibin-a), are upregulated by Amh (64). Taken together, these data clearly demonstrate that Amh inhibits germ cell development through modulation of growth factor production and suppression of Leydig cell function (**Figure 6**). In consequence, these mechanisms lead to a condition in which spermatogonia are maintained in an undifferentiated state.

Similar to the medaka mutant *hotei* (58), *amh* loss of function in zebrafish showed phenotypic characteristics that reinforced the above-mentioned functions of Amh (65). Both mutants exhibited uncontrolled proliferation, impaired germ cell differentiation, and hyperplastic gonads, with a higher number of proliferating germ cells in both sexes (65). In females, the ovary is composed mostly of perinucleolar oocytes, which are arrested at the early vitellogenesis stage (57, 65). Altogether, the phenotype of both mutants suggests that Amh presumably has a role in regulating germ cell proliferation and differentiation in both sexes (57, 65).

The modern understanding of the balance between male and female pathways during gonad sex determination and differentiation suggests that, once one of the sex-specific networks is active, the gene-regulatory network of the opposite sex is repressed (67). At the same time, when genetic fate fails to differentiate the gonad into testis or ovary, the molecular pathway of the opposite sex becomes activated and leads to sex change. In medaka, XY mutants for *dmrt1a* (an important testis-development gene) are not able to develop testis and later transdifferentiate the gonad into ovary (68). Hermaphroditic fish, however, are able to start the development of one sex at the same time or even after the sex-specific molecular pathway of the opposite sex becomes active. Hermaphroditic teleosts, therefore, must have developed mechanisms by which both sexes can

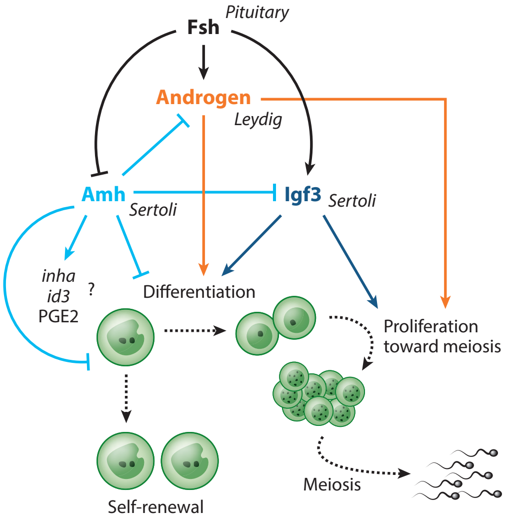

Figure 6

Role of Amh in adult fish testis. Amh inhibits the stimulatory effects of gonadotropins in spermatogenesis, in both Leydig and Sertoli cells. In Leydig cells, Amh inhibits the gonadotropin-induced functions of Leydig cells to secrete androgens. Androgens stimulate spermatogonial proliferation and entry toward meiosis. In Sertoli cells, Amh counteracts the effects of gonadotropin-induced stimulatory growth factors of spermatogenesis (e.g., insulin-like growth factor 3, Igf3). Amh also increases Sertoli cell production of inhibitory growth factors, such as inhibin-a (*inha*), transcription factor inhibitor of DNA binding 3 (*id3*), and prostaglandin E2 (PGE$_2$) signaling. Taken together, these mechanisms lead to an inhibition of germ cell proliferation and differentiation, maintaining spermatogonia in an undifferentiated state. Arrows indicate stimulation, whereas a perpendicular bar at the end of a line represents inhibition. Green cells represent different spermatogonial generations. Abbreviations: Amh, anti-Müllerian hormone; Fsh, follicle-stimulating hormone.

develop and, in some cases, coexist in the same gonad. It was hypothesized that residual stem cells originating from the PGCs remain quiescent during the first sex phase and subsequently become activated to form the gonadal structure of the opposite sex. A study with intersexes in medaka suggests that the testis contains undifferentiated gonial stages throughout life (69), and these gonia can be induced to differentiate into female germ cells by female hormone treatments (70). As discussed above, Amh plays a strong role in maintaining germ cell quiescence. Studies in black porgy, a protandrous species, revealed that early germ cell proliferation was arrested at the area containing type A spermatogonia in the oocyte-bordering region, with *amh* strongly expressed in the Sertoli cells in this region (71, 72). In the protogynous orange-spotted grouper, Amh is mainly localized in the Sertoli cells surrounding type A spermatogonia, especially during reversible sex change (73). Hence, Amh is likely important for maintaining the undifferentiated germ cells and thereby protecting the testicular tissue from repression by the female pathway during sex change.

In summary, teleosts have unique anatomical and genetic features, which created the optimal precondition to respond to genetic and environmental cues, making it possible to functionally change sex or even perform both male and female reproductive roles at the same time (**Figure 4*b***).

Sarcopterygii

The extant sarcopterygians (lobe-finned fish) include all tetrapods (amphibians, birds, reptiles, and mammals) and the groups of coelacanths (Actinistia) and lungfish (Dipnoi). Fertilization in coelacanths is internal. After fertilization, the embryo is formed and develops to an advanced state prior to its release from the female reproductive tract, a pattern of oviparous reproduction called embryoparity (6). The embryos develop to term in compartmentalized regions of the oviduct. In Dipnoi, the males have a Wolffian duct as a functional genital duct but retain the Müllerian ducts, at least to some extent, though they are extremely reduced in some species. However, like in sturgeon, no indication of a functional role of the Müllerian duct in Dipnoi males was suggested (74).

In amphibians, the general configuration of the urogenital morphology is basically similar among all groups. The oviduct secretes a tertiary accessory envelope for the eggs. In cases of internal fertilization, a widened region in the oviduct, also known as the ovisac, differentiates for embryo retention (6). Traces of Müllerian duct retention in male amphibians are represented by connective tissue strips beside the kidneys or, as in gymnophiones, by connective tissue strips anteriorly followed by glandular tissue posteriorly. The glandular tissue of the male Müllerian duct and its opening into the cloaca suggest a possible exocrine function of this duct related to internal fertilization and terrestriality (74).

The presence of hermaphroditism and the capacity to sex reverse after gonad maturation are hard to evaluate in wild amphibian populations. Sex reversal has been observed in several species in the laboratory. In captivity, some protogynous individuals of *Hyperolius viridiflavus* with reduced fertilization rates were observed under low–male density conditions (75), and some protandrous individuals of *Triturus alpestris* were found under conditions of food deprivation (76). A well-known example of artificial sex change is found in *Bufo bufo*, in which chirurgical removal of the testes of sexually mature males is followed by the development of the Bidder's organ into functional ovaries (77). In this situation, the retention of the Müllerian duct in adult males may allow the testis-to-ovary, i.e., male-to-female, sex change. The only case described as juvenile hermaphroditic (here, juvenile intersex) in natural populations of amphibians was observed in *Rana temporaria*. In this species, all tadpoles are female, and during metamorphosis, half of them transiently develop ovotestis and finally become males (78).

During the Paleozoic era, the female oviduct of a group of tetrapods passed through crucial evolutionary changes. Shifting of egg fertilization from outside to inside the female oviduct enabled evolution of the amniotic egg. The oviduct of this vertebrate group, the amniotes, produces and deposits components necessary for adaptation to terrestrial development (e.g., eggshell and albumen) (79). Modifications of oviduct (Müllerian duct) function therefore were essential for the radiation of vertebrates in conquering dry land.

In reptiles, different modes of reproduction are found, e.g., viviparity, oviparity, and ovoviviparity, and the oviduct performs a variety of reproductive functions depending on the reproductive strategy (**Table 1**). The reproductive functions of the oviduct in all oviparous reptiles include fertilization, sperm storage, egg transport, embryo maintenance, egg shell deposition, and delivery. In viviparous species, the oviduct contributes to the formation of a placenta-like structure (79). There are no reports of hermaphroditism or functional sex change in adults. Intersexes are rare among hatchlings in laboratory and field studies, except in some temperature-shift experiments, and those are sterile (80).

In birds, the structure of the oviduct is basically similar to in crocodiles. The oviduct develops regional specialization for the production of the shell membrane and calcium deposition (81). Birds employ a ZZ/ZW system, in which the female is the heterogametic sex (ZW), and despite the genetic background, ZZ male embryos can be sex reversed to female by estrogen treatments during the critical period of gonadal sex determination but not thereafter (82, 83). Intersex individuals are common in triploid chickens (84), and the presence of ovotestis is observed in infertile domestic hens (85). However, birds have an unusual characteristic, which they share with invertebrates, namely, the development of gynandromorphs. In this case, not only the two types of gonads but also genitalia, plumage, and other sexual dimorphisms are divided bilaterally into a male phenotype on one side and a female phenotype on the other. Despite the similarities with hermaphrodites, gynandromorphic individuals are not able to change sex or to perform the role of both sexes. The gynandromorphy cases suggest that the chromosomal constitution of cells in birds influences their perception of the hormone environment (86).

In mammals, like in most vertebrates, Müllerian duct specializations differ according to reproductive mode (**Table 1**). Monotremes (platypus and echidnas) are ovoviviparous, and like those of reptiles and birds, their oviduct is responsible for sperm storage, fertilization, egg transport, and shell membrane and eggshell production. In therians (marsupials and eutherians), the reproductive parts are in general similar (87). Marsupials produce immature offspring and develop a marsupium or pouch. Eutherians capitalized on the allantoic placenta, a prolongation of intrauterine life, and birth of somewhat more mature offspring. The Müllerian duct also gives rise to the vagina, which remains paired in monotremes and marsupials but is a single fused tube in eutherians (87). In males of many mammals, rudiments of the Müllerian ducts form vestigial adult structures, such as the appendix testis and prostatic utricle (6).

Regarding intersex and hermaphrodites in mammals, several cases of intersex in marsupials were reported with different combinations of male and female sexual organs, varying from basic to complete intersexual reproductive traits (88). In eutherians, androgen administration in pregnant rats induced varying degrees of masculinization and intersex, depending on the dosage and the period of pregnancy (89). Nevertheless, XX mice can be fully sex reversed to phenotypic male if the gonad is induced genetically to differentiate as a testis (90, 91). In both marsupials and placental mammals, there is no natural hermaphroditism and the intersexes are not fertile, owing to the incomplete capacity of the genital ducts to change sex.

As discussed for teleosts, the role of AMH was long correlated only to the regression of the Müllerian duct (92) and was also suggested to be involved in the control of germ cell maturation (93). However, the role of this molecule, besides in regression of the Müllerian duct, only got attention more recently. In teleosts and sarcopterygians, especially birds and mammals, the role of AMH/AMHR2 in the gonads is better understood but still not fully comprehended.

In lungfish and coelacanths, *amh* is highly expressed in differentiated testis. *amhr2* is expressed in testis of *Latimeria*, whereas in lungfish its expression is found only in female gonads. The female expression bias of *amhr2* in lungfish was also observed in some tetrapods, whereas in *Latimeria*, *amhr2* is expressed only in the gonad, similar to in teleost fish (94).

In amphibians, spatiotemporal gene expression and protein localization revealed *amh* to be sex biased toward testis development, especially in differentiating Sertoli cells. However, Amh localization in somatic and germ cells of undifferentiated gonads and expression in females in some species were reported (95). In *Xenopus tropicalis*, *amhr2* expression was already detected in the undifferentiated gonad, presenting higher expression in females only at later developmental stages (96). Interestingly, in phylogenetic analyses of both Amh and Amhr2, the amphibians present higher divergence from other sarcopterygians (**Figure 2** and **5**). This diversity was proposed to correlate with the lack of regression of the Müllerian duct in some adult males (97). However, the divergence of the Amh structure of amphibians from the other basal sarcopterygians indicates a specific gene or pathway modification in this group.

In many reptile species, temperature plays an important role in sex determination; RNAseq data on turtles showed *amh*, together with other known male-related genes, to exhibit strong thermoresponsiveness (98). Together with the expression analyses in alligators, *amh* and *amhr2* were revealed to be the putatively critical temperature sex–determining elements for male gonadogenesis in reptiles (99–101).

In birds, similar to in other vertebrates, *AMH* is already active in the undifferentiated gonad of both sexes and is upregulated in developing testis, preceding the sex-determination period (102). Suppression of *AMH* expression by RNA interference in chicken leads to a reduction in size of the mesonephros and gonads in both sexes owing to a decrease in cell proliferation. Germ cell number was also reduced in knockdown males (103). Interestingly, overexpression of *AMH* caused the gonads of both sexes to develop small and undeveloped structures at embryonic and adult stages (104). *AMHR2* is expressed in the gonads and Müllerian ducts of both sexes and is strongly expressed in Sertoli cells and testis cords of males after gonadal sex differentiation (105). Similar to in teleosts, *AMH* appears to play a crucial role in germ cell survival and regulation of germ cell proliferation, as well as in maintaining the gonad in an undifferentiated state.

In platypus, *AMH* is located on the Y_5 chromosome, and transcriptome sequencing analyses suggested this gene, named *AMHY*, as a promising candidate sex-determining gene (106). This observation extends the organismal range of the potential of *AMH* to become a sex-determination gene. In the marsupial tammar wallaby, *Amh* is equally expressed in the developing gonads of both males and females, being upregulated mainly in Sertoli cells during testicular differentiation and exhibiting significantly lower expression in ovaries. Interestingly, sexual dimorphism of *Amh* expression precedes the peak of the male sex-determination gene *SRY* (107). *AMH* messenger RNA was also observed in the female marsupial brushtail possum, being limited in granulosa cells once the follicles had formed in ovary (108). In both marsupial species, *AMH* expression is decreased or almost absent in adult gonads.

In eutherians, *AMHR2* is expressed in the Müllerian duct of both sexes during embryonic stages. Therefore, the developing testis is predicted to produce the molecule, which leads to the regression of the Müllerian duct in males. Indeed, *AMH* is one of the earliest Sertoli cell–specific genes expressed in the male gonad (109). In postnatal testis, AMH secretion is maintained at high levels until puberty, when Sertoli cell maturation is characterized by decreasing AMH activity (110, 111). During puberty, downregulation of AMH is correlated with the onset of germ cell meiosis and the increase of testosterone in the serum (112, 113). In females, *AMH* is expressed in granulosa cells (homologous to Sertoli cells) of postnatal ovary, remaining low throughout reproductive life and becoming undetectable after menopause (114). Interestingly, the expression of *AMH* is high in small follicles, decreasing gradually in larger follicles. In adult males and females, AMH expression is high in actively dividing Sertoli and granulosa cells, respectively. AMH production is stronger in Sertoli and granulosa cells surrounding the meiotically arrested germ cells (115–117). Hence, the levels of AMH correlate with the amount of meiotic germ cells. In summary, functional analyses, in all vertebrate species studied so far, indicate a conserved and important role of AMH in regulating germ cell differentiation.

BEYOND THE MÜLLERIAN DUCT

Explanations for the evolutionary success of the amniotes and their adaptations are manifold. The transition from water to land required modifications of several important systems, such as locomotion, feeding, reproduction, water evaporation control, gas exchange, and sensory systems. As mentioned above, the oviduct modifications could have been essential for the transition from water to land, but not much is known about the key innovations that led to the success of teleosts. Classical explanations correlate this success to changes in jaw mechanisms, reproductive biology, and teleost-specific whole-genome duplication (118). In a recent analysis of fossil records and living animals, early teleosts did not show enhanced phenotypic evolution relative to their sister group, the holosteans (bowfins and gars) (119). Nevertheless, teleost diversity extends beyond increasing fossilizable morphological complexity. This group presents marvelous reproductive strategies, enabling them to occupy several ecological niches and presenting unique modifications in the urogenital system, gonadogenesis, and sex determination. Here, we have reviewed the main modifications of the Müllerian duct and Amh/Amhr2 pathway, but obviously, other anatomic and molecular changes followed vertebrate evolution.

In vertebrates, the undifferentiated gonad is formed by two components of somatic cells: the cortex and medulla. The first has its origin in the peritoneum, and the second is derived from the mesonephric blastema. In most vertebrates, during ovary formation the cortex develops and the medulla degenerates, whereas in testis formation, the medulla develops further and the cortex regresses. However, in contrast to in other vertebrates, in teleosts the medullar tissue is not found in the gonad, and from this primordial tissue common to male and female, gonad organogenesis is then initiated, resulting in the formation of bilateral split gonads (120). This common embryologic origin of testis and ovary in teleosts could have also facilitated the gonad to pass through molecular and morphological alterations during sex change (2). Nevertheless, we demonstrated that other vertebrates also present gonad plasticity independent from single or separate origin.

Müllerian duct acquisition must be seen together with the dependency of the testis from the Wolffian duct and the excretory system. Different branches of vertebrate groups evolved independent adaptations to avoid the mixture of sperm and urine. In amniotes, the strategy was the development of metanephric kidneys (121). However, important testicular structures are still derived from the mesonephric system in all nonteleost vertebrates. Sperm release and all reproductive function are completely independent from the Wolffian duct in teleosts (20). But the loss of the Müllerian duct in this group should have consequences in egg formation. The oviduct, derived from the Müllerian duct, is responsible for the production of the tertiary accessory envelope of the egg in most vertebrates, especially all oviparous species (6). For instance, it is necessary for the formation of the egg capsule in chondrichthyans (22), the egg jelly coat in amphibians (122), and the eggshell in amniotes (79). Teleosts produce eggs without a tertiary envelope, and all modifications come from the chorion (primary and secondary accessories). The teleost chorion is thick and impenetrable, with the exception of the entry portal for the sperm, an opening or hole called the micropyle (6). Egg–sperm recognition in teleosts is performed by contact of the sperm with the micropyle, and therefore, the sperm lacks acrosome. The acquisition of the micropyle and loss of the acrosome was a gradual evolutionary shift, with the presence of multiple micropyles in the eggs of Chondrostei and Holostei, the latter of which also lack acrosomes (see in 123). Hence, throughout actinopterygian evolution, a gradual independence from the Müllerian duct is observed for some reproductive functions, leading to a complete loss of this structure in teleosts (**Figure 7**).

Vertebrates present a high diversity of reproductive modes, and each reproductive strategy requires specific modifications of the urogenital system (**Table 1**). However, the correlation between viviparity and the absence of juvenile intersex and hermaphroditism is obvious. The most likely explanation is that viviparous species developed strong and irreversible sex-specific modifications, like external genitalia for internal fertilization and oviduct specialization for gestation. Hence, the potential of a species to sex reverse depends on how different the reproductive structures are between males and females in the same species. Teleosts, except for viviparous species, show the lowest internal anatomic differences between sexes and the highest potential to perform functional sex reversal in adults. Amniotes are dependent on internal fertilization and eggshell production for survival in the terrestrial environment, and the maintenance of internal sex dimorphism is essential in this group, thus reducing drastically the preconditions to develop functional hermaphrodites (**Figure 8**).

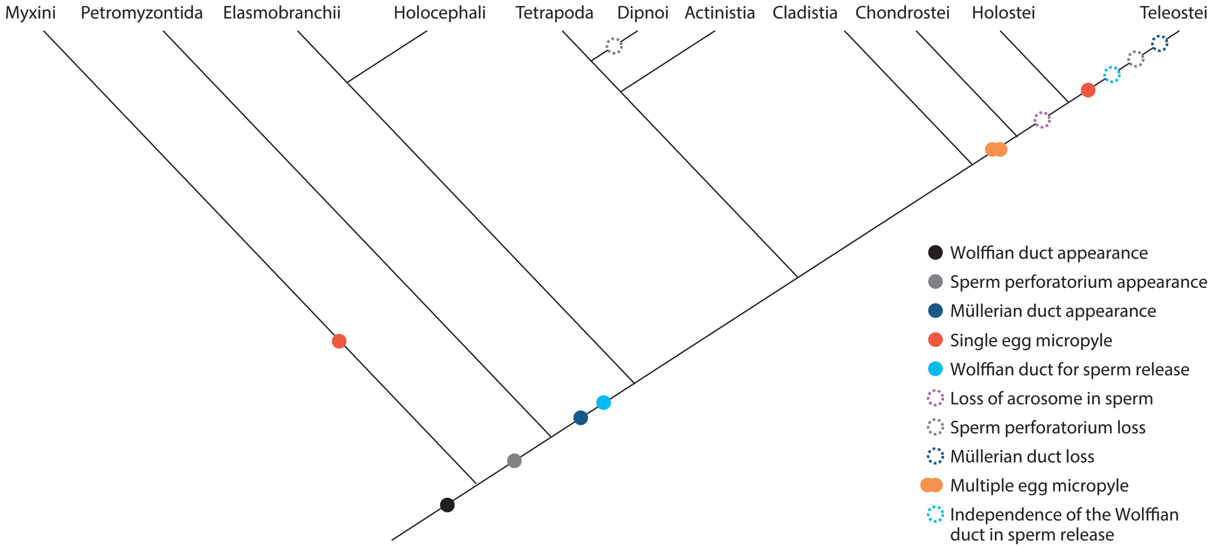

Figure 7

Urogenital system and gamete modifications in vertebrates. Gradual modifications of the urogenital system in vertebrates led to the complete loss of the Müllerian duct and independence of the Wolffian duct in sperm release in Teleostei. Consequences of the urogenital modifications are also reflected in the lack of acrosome in sperm and egg micropyle development. Scheme modified after 123.

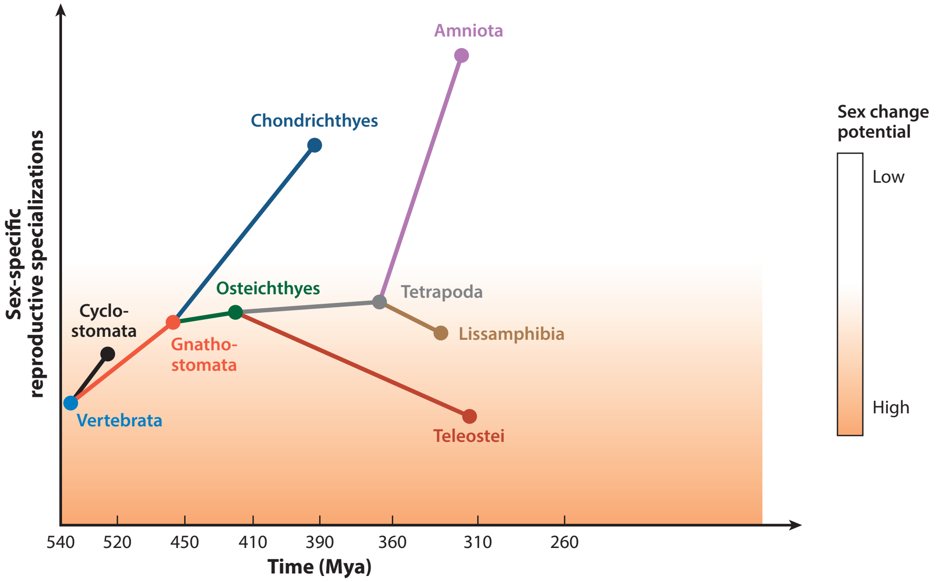

Figure 8

Hypothetical diagram correlating the potential for sex change (*orange scale*) with the general sex-specific reproductive specializations (*y*-axis) of all living vertebrate groups through time (*x*-axis). Chondrichthyes and Amniota developed independent sex-specific modifications, which reduced their potential to change sex at adult stages (e.g., viviparity, eggshell, external genitalia). Teleostei is the group with the highest potential for sex change, creating the optimal conditions to develop hermaphroditism. The nodes indicate the origin of each vertebrate group in millions of years.

CONCLUSIONS

Different molecular mechanisms of sex determination, and a huge variety of reproductive modes, evolved independently in vertebrates. Gonadal plasticity allowed vertebrates to respond to environmental changes and to maximize sexual breeding. Juvenile intersex seems to be the ancestral condition in vertebrates. The acquisition of the Müllerian duct, which develops in a specialized oviduct, together with evolution of the Amh/Amhr2 system favored the conditions to evolve viviparity in jawed vertebrates. However, species with high sex-specific reproductive adaptations (e.g., external genitalia, eggshell production, internal gestation) have less potential to reverse sex once established, making intersex an abnormal and infertile condition. Teleosts lost the Müllerian duct and developed complete independence from the urinary system for gamete release, whereas males and females share the same genital duct in most species.

Amh has long been correlated with regression of the Müllerian duct and male reproductive functions. New functional analysis of this molecule in different groups of vertebrates shows that this old concept needs to be reinterpreted. The common and probable ancestral role of Amh in jawed vertebrates is related to the survival and proliferation of germ cells in the bipotential gonad. This role is also observed in adult gonads of both sexes, where Amh is implicated in the fine-tuning of gametogenesis. The relationship between germ cell maintenance and sex differentiation is observed in several species, and Amh has an essential role in this process, creating a strong potential in regulating the sex identity of the gonad. This potential is more evident in platypus and some teleost species in which Amh turned out to be the main sex-determining gene. In hermaphrodites, Amh seems to be important in the maintenance of both male and female gonadal tissues in the same organ.

Considering the natural strategy to change sex after reaching sexual maturity, teleosts are the only group with true hermaphroditism. Teleosts are unique in developing specific reproductive strategies derived from the conserved sexual plasticity of the gonad and overcoming the reciprocal repression of the male and female sex-determination and -differentiation pathways. This special reproductive feature of teleosts is observed via anatomic changes (e.g., loss of the Müllerian duct) and molecular adaptations (e.g., Amh/Amhr2 evolution, whole-genome duplication), which increase the potential to sex change in adult life.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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