Functional distinctions associated with the diversity of sex steroid hormone receptors ESR and AR

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***Abbreviated title*:** Functionalization of AR and ESR

***Keywords*:** androgen receptor, estrogen receptor, whole genome duplication, sex determination

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***Disclosure Statement*:** The authors have nothing to disclose.

Highlights:

Sex steroid hormones play fundamental roles in reproductive activities.

Sexually dimorphic development depends on sex steroid hormones.

The functions of both ESR and AR have diverged during vertebrate evolution.

In this review we provide a comprehensive analysis of the diversification of ESR and AR, and their functional associations.

We first briefly describe the evolutionary background of steroid hormone receptors (SRs) and then illustrate the roles established for sex steroid hormones and their receptors in sexually dimorphic development, and how this relates to their diversity in vertebrates.

Abstract

Sex steroid hormones including estrogens and androgens play fundamental roles in regulating reproductive activities and they act through estrogen and androgen receptors (ESR and AR). These steroid receptors have evolved from a common ancestor in association with several gene duplications. In most vertebrates, this has resulted in two ESR subtypes (ESR1 and ESR2) and one AR, whereas in teleost fish there are at least three ESRs (ESR1, ESR2a and ESR2b) and two ARs (ARα and ARβ) due to a lineage-specific whole genome duplication. Functional distinctions have been suggested among these receptors, but to date their roles have only been characterized in a limited number of species. Sexual differentiation and the development of reproductive organs are indispensable for all animal species and in vertebrates these events depend on the action of sex steroid hormones. Here we review the recent progress in understanding of the functions of the ESRs and ARs in the development and expression of sexually dimorphic characteristics associated with steroid hormone signaling in vertebrates, with representative fish, amphibians, reptiles, birds and mammals.

1. Introduction

Steroid hormones serve important functions in regulating a wide range of physiological processes including cell growth, differentiation, development, reproduction, and in overall homeostasis and health, throughout the life of vertebrates. Among the sex steroid hormones, estrogens and androgens play important roles in sexual differentiation and reproduction, particularly in the development and expression of male and female sexual characteristics. These effects are principally mediated by specific receptors, the estrogen and androgen receptors (ESRs and ARs), which belong to the nuclear receptor superfamily. As the main regulators of sex hormone signaling, ESR and AR have key roles in the molecular processes mediating reproductive development and behavioral patterns of organisms, and their diversity and evolution.

Most vertebrates have two ESR subtypes (ESR1 and ESR2) and one AR. ESRs share a certain degree of sequence similarity and bind the endogenous estrogen 17β-estradiol (E_2) with a high affinity. However, the two receptors exhibit clear differences in the tissue distribution and their target genes [1-4] and hence, functional diversification has been suggested among the ESR subtypes. To date, distinct roles of ESRs have been characterized in only a limited number of mammalian species, including in mouse and human. In the teleost lineage, the *esr2* gene has been further duplicated through a teleost-specific whole genome duplication (WGD) event, but for *esr1* only one gene remains. As such, most teleosts possess three ESR subtypes encoded by separate genes: *esr1*, *esr2a* and *esr2b*. [The published nomenclature for classification has been confusing, particularly with regards to nomenclature for ESR2 (formerly ERβ) subtypes. For example, the medaka ERβ1 (NM_001104702) is orthologous to ERβ2 in other fish species, including carp (AB334724) and zebrafish (AJ414567), whereas

medaka ERβ2 (NM_001128512) is orthologous to ERβ1 in carp (AB334723) and zebrafish (AJ414566). In human, the accepted nomenclature is "*ESR*" and this has subsequently also been adopted for other vertebrates in this review to avoid confusion]. The *ar* gene has also undergone duplication into $ar\alpha$ and $ar\beta$ in the teleost lineage, however, some fish species (*e.g.*, zebrafish and fathead minnow) have secondarily lost $ar\alpha$ [5]. The teleost-specific WGD event has led to the existence of more nuclear receptors in teleosts than in mammals (e.g., medaka has 69 nuclear receptors, whereas human and mouse have 48 and 49, respectively), with a difference also in functional diversity in fish compared with mammals. In this review, we provide a comprehensive analysis of the diversification of ESR and AR and their functional associations in a variety of vertebrate species, including fishes (teleosts such as medaka, stickleback, mosquitofish and zebrafish), amphibians (*Xenopus*), reptiles (alligator and turtle), birds (chicken, zebra finch and duck) and mammals (mouse and human). We first briefly describe the evolutionary background of steroid hormone receptors (SRs) and then illustrate the roles established for sex steroid hormones and their receptors in sexually dimorphic development, and how this relates to their diversity in vertebrates.

2. Evolutionary history of SR genes in vertebrates

Evolution of novel traits following genome duplication events has been considered to provide evolutionary innovations in the vertebrate lineage. Understanding the genetic mechanisms leading to functional diversity of SRs is one of the central challenges in comparative endocrinology and evolutionary biology. The SR family consists of ESR, AR, progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), and has been generated through a series of duplications of an ancestral

SR gene. Several gene duplication events, including two rounds of WGD occurring in the early vertebrate lineage, have lead to the current diversity of the SR family. The first duplication generated an *esr* and a 3-ketosteroid receptor from the ancestral SR [6]. The 3-ketosteroid receptor further duplicated into *a corticoid receptor* (*cr*) and a receptor for 3-ketosteroids (androgens, progestins). After the Cyclostome (jawless fish)-Gnathostome (jawed vertebrates) divergence, the *cr* and *3-ketosteroid receptor* each duplicated again, with the *cr* yielding the *gr* and the *mr*, and with the *3-ketosteroid receptor* leading to the creation of the *pr* and the *ar* [6]. As such, the four differently encoded genes, *mr*, *gr*, *pr* and *ar*, first appear in the common ancestor of gnathostome vertebrates [5, 7].

The evolution of *esr1* and *esr2* has been intensely studied (Fig. 1). Japanese lamprey (*Lethenteron japonicum*) (Cyclostomata; one of the earliest-branching lineages in vertebrates) has two distinct *esr* genes [8]. Some cartilaginous fish such as the elephant shark (*Callorhinchus milii*, Holocephali, a subclass of cartilaginous fish) also have two *esr* sequences similar to *esr1* and *esr2*. However, the catshark and whale shark (*Scyliorhinus torazame* and *Rhincodon typus*, Elasmobranchs, another subclass of cartilaginous fish) seem to have secondarily lost the *esr1* gene [9]. In Japanese lamprey, one Esr displays estrogen-dependent activation of gene transcription, whereas the other does not respond to E_2 [8], however, it remains controversial as to whether the two *esr* in lamprey are orthologs of vertebrate *esr1* and *esr2* or whether this duplication occurred after the split of cyclostomes from gnathostomes [8, 10]. Taken together, vertebrate *esrs* have emerged from an ancestral *esr* through a series of gene duplications. Duplication of the ancestral *esr* into *esr1* and *esr2* occurred early-diverging in the vertebrate lineage [6], however, additional ESR sequences from early diverging fish

species are required for establishing definitive phylogenetic relationships.

Teleosts experienced a teleost-specific WGD approximately 350 million years ago (MYA) [11, 12], which occurred after the split of the other ray-finned fish lineages (e.g. bichir, sturgeon, gar and bowfin) from the teleost lineage, and before the divergence of Osteoglossomorpha (e.g. arowana) and Elopomorpha (e.g. eel) [13, 14]. This teleost-specific WGD generated the additional copies of *gr* (*gr1* and *gr2*), *ar* (*ar*α and *ar*β) and *esr2* (*esr2a* and *esr2b*) compared with the gene repertoire in other jawed vertebrates [5, 15-17]. *Mr* and *pr* are also retained as single genes in teleosts [17]. To date, only a single *esr1* gene has been found from Silver arowana (*Osteoglossum bicirrhosum*) and Japanese eel (*Anguilla japonica*), suggesting that the *esr1* paralog has been lost in the early lineage of teleost fish species [10]. Two distinct paralogs of the *ar* gene, *ar*α and *ar*β, arose during the teleost-specific genome duplication and have been identified in a number of teleost fishes (Fig. 1) [5, 16]. In the history of the *ar* gene evolution, it is likely that the loss of the *ar*α gene occurred independently in Osteoglossiformes (e.g. arowana) [5], Cypriniformes (e.g. zebrafish and fathead minnow) [18, 19] and Siluriformes (e.g. catfish) [20]. Two *ar* genes have been identified in Salmoniformes [e.g. salmon and trout; and these diverged early in euteleost evolution [21], however, both are categorized into the *ar*β cluster [22]. Hence, the two *ar* genes in Salmoniformes arose by a lineage-specific gene duplication of *ar*β in the recent salmonid tetraploid event, estimated to have taken place 100-50 MYA [23], whereas, *ar*α gene might have been lost before this lineage-specific gene duplication.

3. Androgen-dependent secondary sex characteristics development in vertebrates

The development of vertebrate male reproductive organs and male secondary sexual traits is primarily regulated by androgens (Fig. 2). External genital organs have convergently evolved in vertebrates for efficient fertilization and reproduction. In mammals, the male external genitalia form a tubular urethra, as well as a well-developed prepuce and corporal body, and their development depends on androgens [24, 25]. Some fish species also have developed several types of copulatory organs for efficient sperm transport. In cartilaginous fishes, the midline pelvic fin is modified to form a tubular (glove-like) structure, termed the clasper in response to androgen [26]. In ovoviviparous fish such as Poecilidae (a group of Cyprinodontiformes), the development of a gonopodium (GP) through modification of the anal fin has generated a prominent male sexual characteristic [27-29]. The development of GP in ovoviviparous fish such as guppy, swordtail fish and mosquitofish enables internal fertilization. Oviparous fishes can also exhibit male-specific external structures associated with reproductive activities. For example, medaka (*Oryzias latipes*) exhibit a male-specific appendage structure, the elongation of fin rays and the formation of papillary processes in the anal fin [30, 31]. This enables mating males to embrace the posterior part of the female's body with the anal fin for efficient external fertilization [32].

Male secondary sexual characters also appear as an elongation of the fin ray, kidney hypertrophy, increase in skin thickness, and an appearance of breeding colors in some fishes [33]. Male stickleback (*Gasterosteus aculeatus*) produce spiggin in their kidneys in response to elevated circulating androgen levels and this glue protein is used during nest building. Sexually mature male stickleback also show a red coloration of their belly [34] and this prominent breeding color is attractive to females and

197 simultaneously serves as warning for competing males [35]. A recent study indicates
198 that androgen is a key factor in enhancing sensitivity to red light by regulating the
199 expression of the opsin gene [36]. Such visual sensitivity might be important for
200 territorial males to detect the presence of competitors [37, 38]. In mosquitofish, the
201 transition from anal fin to GP is induced by androgen treatment in both juvenile fry and
202 adult female [39, 40]. In medaka, castration causes regression of papillary processes,
203 whereas transplantation of a testis to an adult female or the administration of androgens
204 to females induces papillary processes formation [41, 42]. The androgen-dependent
205 development of the anal fin with the papillary process in medaka, the GP outgrowth in
206 mosquitofish, and the production of spiggin in stickleback have been used for the
207 detection of chemicals having androgen action [43-48].

208 In amphibians, the development of a nuptial pad and vocal organ called the larynx
209 are regulated by androgen [49, 50]. Adult male *Xenopus* form larger nuptial pads, which
210 are used for grasping females during amplexus. Gonadectomized females implanted
211 with a testosterone (T) pellet also form prominent nuptial pads [50]. The male larynx
212 undergoes a profound transformation involving rapid growth, fiber addition, and
213 conversion of fiber twitch type. Castration completely arrests fiber type conversion and
214 retards muscle growth and fiber addition, indicating the androgen-dependency of these
215 organs [49, 51].

216 Birds exhibit a diversified development of sex characteristics in appendicular and
217 reproductive organs, including comb, wattle, syrinx, urogenital tract and gonads [52-57].
218 In birds, androgens play a role in the developmental program of these hormone sensitive
219 tissues as well and therefore, AR expression in such tissues has been well analyzed [54,
220 58-61]. AR was exclusively detected in males in organs that display secondary sex

221 characteristics, such as Wolffian duct and peripheral cloacal regions that develop into
222 the prospective lymphobulbus [58]. By contrast, AR and ESR are both expressed in the
223 developmental syrinx [58]. T treatment does not induce the male syrinx in female birds
224 [62], while estrogen treatment feminizes the syrinx in zebra finch and duck [63, 64].
225 Thus, both hormones are involved in the sexual differentiation of vocal organ in birds,
226 although a sole treatment of androgen or estrogen is not sufficient to induce sex
227 reversed phenotypes [65].
228 Development of androgen-dependent secondary sexual characteristics in squamate
229 reptiles is also well documented. Castration inhibits and T stimulates rapid growth in
230 anole lizards, resulting in male-biased sexual size dimorphism [66]. T treatment
231 increases AR mRNA and protein expression in the copulatory organ (hemipenis) in
232 green anole [67].
233 The role of androgens in the development of sex characteristics has been studied
234 by pharmacological and genetic analyses. In mice, administration of the anti-androgen
235 flutamide, an AR antagonist [68-70] or the 5α-reductase inhibitors
236 4-methyl-4-aza-5-pregnan-3-one-20[s] carboxylate or finasteride [71, 72] interferes
237 with the development of male external genitalia, resulting in a hypospadias-like
238 phenotype. In human patients, hypospadias are a common malformation in which the
239 urethral meatus is located at the ventral side of the penis [73]. Target mutation in *Ar*
240 results in abnormalities in male sexual development including female-like external
241 genitalia formation and cryptorchidism in mice [74-76].
242 It has been known that the ligand selectivity of AR is different among species. In
243 mammals, T and 5α-dihydrotestosterone (5α-DHT) are considered to be effective
244 ligands for AR [77]. 11-Ketotestosterone (11KT) is known as a potent androgen in

teleost fishes [33]. Recent analyses, however, showed the presence of 11KT in early-branching actinopterygian fish (sturgeon) [78], urodele amphibian (*Necturus maculatus*) [79] and mammal (human) [80], suggesting a significant role of 11KT as an androgen in other vertebrates as well.

4. Molecular mechanisms of male sexual characteristics development; cross-talk between androgens and growth factors

Sexual differentiation is a remarkably complex process that depends on the orchestration of an intricate signaling network. Several effector genes that interact with androgen signaling have been identified [26, 39, 40, 52, 68, 81, 82]. Androgen-induced expression of *sonic hedgehog* (*shh*) is required for the formation of the GP in mosquitofish [40, 52], as well as the clasper function in cartilaginous fishes also [26]. During the androgen-induced transition from anal fin to GP, *shh* expression is closely associated with androgen-induced outgrowth of the anal fin, where *ar*s are expressed [40]. Flutamide treatment reduces cell proliferation in distal anal fin regions accompanied by reduced levels of the *shh* expression. These results suggest that androgen and hedgehog signaling are regulating cell proliferation and contributing to the development of new bone segments in the developing GP. It is clear that hedgehog signaling plays multiple roles on fin morphogenesis. The Shh is required for the anterior-posterior patterning of a developing fin [83], the growth and maintenance of the blastema, and patterning of the fin ray in adult fish, as illustrated following fin amputation [84, 85].

The androgen-dependent activation of hedgehog signaling is also necessary for male clasper development in cartilaginous fish [26]. By regulating *hand2*, androgens

control the male-specific pattern of *shh* in pelvic fins [26]. In mouse, *Shh* is expressed in the embryonic external genitalia (genital tubercle, GT) throughout the embryonic development and is indispensable for protrusion of the GT precursor during early embryogenesis [86, 87]. Shh signal facilitates the masculinization processes by modifying androgen-responsive gene expression [88]. Conditional mutation of *Shh* during sexual differentiation has been shown to lead to abnormal development of male external genitalia. *Indian hedgehog* (*Ihh*), another member of the hedgehog gene family, is also responsible for the development of male external genitalia [89]. These results indicate the close association between androgen and hedgehog signaling during the development of sexual characteristics in vertebrates. In sexually dimorphic organs, androgen signaling may re-activate hedgehog gene expression, which is necessary for both early morphogenesis and sexual development. The latter is associated with the androgen-induced heterochronic event.

Several growth factors also work as effectors in regulating reproductive organ formation in association with hormones. For example, the development of papillary processes is promoted by androgen-dependent increase of *bone morphogenic protein 7* (*bmp7*) and *lymphoid enhancer-binding factor-1* (*lef1*) expression. The Wnt/β-catenin signaling pathway has been identified as a masculine effector of androgen signaling in the development of both, papillary processes in medaka [81] and external genitalia in mouse [68]. The sexually dimorphic expression of several Wnt inhibitory genes, including *dickkopf 2* (*Dkk2*) and *secreted frizzled-related protein 1* (*Sfrp1*) have been identified in the developing external genitalia of mouse. These genes are more highly expressed in the female GT compared to males. In addition, loss-of-function and gain-of-function studies on *β-catenin* (*Ctnnb1*) mutants have shown impaired sexual

differentiation of the GTs, indicating that AR-dependent inhibition of Wnt inhibitory genes is necessary for masculinization of external genitalia [68].

5. Contribution of sex steroid hormone receptors to gonadal differentiation

Although the relative importance of sex steroid hormones in sex determination apparently seems to diminish in mammals, estrogens play a critical role in sex determination and particularly in ovarian development in most non-mammalian vertebrates. Sex is genetically determined in the medaka and administration of exogenous estrogens shortly after fertilization causes male to female sex-reversal, with the formation of a functional ovary and reproductive capabilities [90-92]. Likewise, exposure to estrogens throughout the larval period results in the formation of ovaries in males [93, 94]. In the chicken, sex reversal can be also induced experimentally, at least in part, by injecting eggs with estrogens, or by inhibiting estrogen production [95, 96].

Sex determination in several species of reptiles involves temperature –a process called temperature-dependent sex determination (TSD) - where the incubation temperature of the egg, during a thermo-sensitive period (TSP) determines the sex of the offspring in, for example, all crocodilians studied, many turtles and some lizard species [97-99]. Gonadal differentiation in these species is also estrogen-sensitive. Administration of estrogens during the TSP induces male to female sex reversal even if eggs were incubated at a male-producing temperature. In general, expression of *cytochrome P450, family 19, subfamily a* (*cyp19a*; also named *aromatase*), which converts T to E_2, coincides with the later period of TSP in turtles and crocodilians [100-102] and thus, endogenous estrogen mediates terminal ovarian fate determination factor as a downstream signaling event in response to environmental temperature.

Expression pattern and distribution of *esrs* during the TSP have been studied extensively in the red-eared slider turtle (*Trachemys scripta*) and this has shown that *esr1* and *esr2* have distinct patterns of expression. *Esr1* mRNA expression peaks late during the TSP at both female- and male-producing temperatures (FPT and MPT), and at peak expression, gonadal *esr1* mRNA levels are 5-fold higher at FPT compared to MPT [103, 104]. By contrast, *esr2* expression increases after the TSP in the gonads that develop at FPT [103, 104]. It has been thus suggested that *esr1*, but not *esr2*, responds as an early target of estrogen-induced commitment to ovarian differentiation.

Functionalization of ESRs has been analyzed using selective ESR1 and ESR2 agonists in the American alligator (*Alligator mississippiensis*). Exposure of alligator eggs to the ESR1-selective agonist 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) induced ovarian differentiation at a MPT, whereas the ESR2-selective agonist 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY 200070), had no effect [105]. PPT-exposed embryos also show enlargement and advanced differentiation of the Müllerian duct, suggesting that ESR1 also plays a role in the development of the female reproductive tract [105]. In chicken, a sister group of crocodilians as Archosauria, PPT causes left-side ovotestis formation and retention of the Müllerian ducts in male embryos, whereas none of these effects are observed after exposure of embryos to the ESR2-selective agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) [106]. Taken together, these data suggest that ESR1 not only plays a central role in ovarian differentiation and the development of female reproductive organs, but also mediates induction of sex reversal in reptiles (and birds) after exposure to exogenous estrogen.

It is not clear whether natural testicular development in reptiles requires androgen

signaling. *In ovo* exposure of alligator or turtle embryos to the non-aromatizable androgen 5α-DHT or the anti-androgen flutamide have no effects on gonadal differentiation at both FPT and MPT, respectively [107, 108]. In contrast, at the pivotal temperature, that produces an approximately 1:1 sex ratio, exposure to androgens resulted in the male-biased hatchling production in turtles [109]. Androgens thus appear to play a more subtle role in gonadal fate determination. In the red-eared slider turtle, gonadal *ar* expression pattern is similar to *esr1*, which shows a spike late in the TSP [104]. By contrast, *ar* expression in the American alligator increases significantly over developmental time, but does not vary between MPT and FPT [110]. This implies different AR-mediating signaling pathways during gonadal differentiation between these two TSD species. Intriguingly, a spliced form of the AR, which lacks 7 amino acids within the ligand-binding domain, is expressed in the gonads of American alligator. This variant shows no response to androgens and perturbs intact AR transactivity as a dominant negative form [110].

6. Roles of ESR and AR in mammals as assessed via knockout studies

Since the establishment of the *Esr1* knockout (KO) mouse [111], the distinct roles of ESR subtypes have been extensively investigated. In mice, ESR1 plays an indispensable role in maintaining reproductive function. Although offspring were born without any gross effects on the gonad with normal reproductive organ morphogenesis, both female and male were infertile because of conditions including anovulation, hypoplastic reproductive organs, lack of any normal sexual behaviors, failure of response to estrogen in females, and abnormal water absorption in the epididymis in males [111-114]. The possible role of ESR2 in reproductive functions and fertility, on

the other hand, remains controversial. Several *Esr2* KO mouse lines have been established with phenotypic variation in terms of fertility, probably due to variation of residual ESR2 function [113, 115-118]. Taken together, *Esr* KO mice studies revealed that receptor subtypes exhibit distinct functions, which cannot be compensated by each other. One exception is maintenance of ovarian differentiation in mature animals where *Esr1* and *Esr2* double KO mice show transdifferentiation of ovarian somatic cells into testicular Sertoli cells, whereas this is not the case in single *Esr* KO mice [119].

In mammals, AR functional abnormalities cause a spectrum of disorders of androgen insensitivity syndrome (AIS) or testicular feminization mutation (Tfm) [77, 120, 121], showing that ARs are indispensable for male development. *Ar* KO male mice exhibit female-type external appearance and absence of seminal vesicles, vas deferens, epididymis and prostate, but retain a small inguinal testes with severely arrested spermatogenesis [75, 122], suggesting that although AR was not required for the formation of testis, it was essential for the development of male reproductive organs and spermatogenesis. AR-mediated androgen signaling also plays an important role in the female reproductive system. Female *Ar* KO mice show normal growth but are subfertile resulting in significantly fewer pups per litter compared to control mice. In the ovary of *Ar* KO mice, folliculogenesis is impaired with an increase in the number of atretic follicles [123].

7. Roles of ESR and AR in fish, as assessed via knockout studies

Above we illustrate the established fundamental roles of Esr and Ar in reproduction in mammals, as established through gene KOs. Such detailed information relating to the distinct roles of each subtype of Esr and Ar in non-mammalian

389 vertebrates is still limited. Recently the generation of *esr* KO zebrafish (*Danio rerio*)
390 and medaka by TALEN and CRISPR/Cas9 methods has been reported [124, 125].
391 Unexpectedly, KO of a single *esr* subtype alone showed normal reproductive
392 development and function in both female and male zebrafish [125]. By contrast, double
393 and triple KO (*esr2a*$^{-/-}$;*esr2b*$^{-/-}$ and *esr1*$^{-/-}$;*esr2a*$^{-/-}$;*esr2b*$^{-/-}$) develop all male phenotypes
394 and thus, Esr2a and Esr2b are, despite of the presence of functional redundancy among
395 Esr subtypes, essential for female development [125]. Zebrafish are juvenile
396 hermaphrodites, where all fish develop a so-called juvenile ovary and it followed by
397 sexual differentiation into testis or true ovary [126]. Some double and triple KO fish
398 appear to exhibit sex reversal and loss of Esr2s leads an arrest of folliculogenesis
399 resulting in female to male sex reversal, as intersexual gonadal phenotypes were often
400 observed after the window of natural sex differentiation stage [125]. In the zebrafish, all
401 *esr* subtypes are expressed in the mature ovary, and *esr2a* is most highly expressed
402 during folliculogenesis. *Esr2a* is also expressed in the oocytes and *esr2a* KO eggs
403 showed the unique phenotype of weakened chorion and early hatching [125]. It is thus
404 suggested that Esr2a is the most predominant Esr subtype contributing to ovarian
405 development in zebrafish.
406 The medaka exhibits XX-XY heterogamety with a distinct sex determination gene
407 called *DM-domain gene on the Y chromosome* (*dmy*) [127]. Hence, medaka is an
408 excellent model for studying sex determination and differentiation during early gonadal
409 development as genetic and intrinsic sexes can be identified. In our own studies we have
410 established *esr1* KO medaka and these did not show any significant defects in gonadal
411 development, sexual characteristics and reproductive activity [124] as in the case of
412 zebrafish. Intriguingly, *esr2a* KO female medaka show abnormal abdominal swelling

413 with ovarian expansion and are infertile (Fig. 3). Hence, even within the teleost lineage,
414 roles and functions of Esr are diverged. The development of *esr* KO zebrafish and
415 medaka provides important insights into receptor subfunctionalization between
416 mammals and fish and offers a powerful prospect for better understanding the distinct
417 roles of the different Esrs in vertebrates.

418 The hepatic *vitellogenin* (*vtg*) is a representative estrogen-responsive gene in
419 oviparous animals [128] and it has been shown that all three Esr subtypes are
420 functionally involved in E_2-induced *vtg* expression. Esr2a-mediated upregulation of
421 *esr1* induces enhanced *vtg* expression in primary hepatocytes of goldfish (*Carassius*
422 *auratus*) [129]. The need of both Esr1 and Esr2a for the induction of *vtg* has
423 furthermore been shown through morpholino (MO)-knockdown of each *esr* mRNA in
424 zebrafish embryos [130]. Estrogen stimulation significantly up-regulates *esr1*
425 expression in *in vivo* medaka study, while *esr2a* and *esr2b* expressions are unchanged,
426 indicating that *esr1* is the most highly expressed hepatic Esr subtype [124]. These
427 results suggest that estrogen stimulation primes and upregulates *Esr1* expression by
428 either Esr2 subtype and resulting in a continued *vtg* expression through augmented Esr1
429 in the liver. In fact, *vtg* expression is significantly lower in the liver of *esr1* KO medaka
430 than that of controls. However, the finding that *esr1* KO medaka show no significant
431 effects on reproductive activities suggests that Esr1 function could be partly
432 compensated for by one or both Esr2 subtypes.

433 Intriguingly, *Ar* is not primarily required for male sexual differentiation in the
434 zebrafish, as it is in mice, it is required for the development of secondary sexual
435 characteristics, and for proper organization of the testis in males and for oocyte
436 maturation in females [131]. The *ar* mutant male zebrafish fails to release sperm and

courtship behavior is significantly less [131]. To further understand functions of AR in fish, we are currently establishing *ar*α and *ar*β KO medaka.

7. Conclusion

Sex steroid hormone receptors are associated with the regulation of reproductive actions in vertebrates, and are most likely subject to directional selection. Cross-species comparative analyses from various vertebrates has revealed species differences in ESR sensitivity in response to endogenous estrogens, notably via the use of luciferase reporter gene assays [132]. For example, teleost Esr1s do not show much difference in responsiveness to E_2, whereas species differences are more pronounced in tetrapods [133, 134]. Amphibian Esrs appear to be less sensitive to E_2 generally [135, 136]. From vertebrates studies to date, the ESR1 in snakes - the Okinawa habu (*Protobothrops flavoviridis, Viperidae*) and Japanese four-striped rat snake (*Elaphe quadrivirgata, Colubridae*), have the highest estrogen sensitivity, followed by other reptilian and avian species [133, 137]. ESRs from high sensitive animals may respond more quickly and have a lower demand for the amount of hormone required to trigger hormone activity compared with low sensitive animals. However, the biological implications of such species differences in estrogen sensitivity have yet to be determined.

The presence of multiple SR subtypes, in particular in teleosts, may have significant bearing on the responsiveness and effects of steroid hormones. There are clearly different responses between receptor subtypes for the Esr in fish. As in the case for Esr1, inter-species differences in response to E_2 for both Esr2a and Esr2b are small. However, across the Esr subtypes Esr2a is generally the most sensitive to E_2 (i.e., Esr2a can be activated by the lowest concentration of E_2). An exception here is in the

zebrafish, where Esr2b is the most sensitive Esr subtype [138]. The transactivation property of teleost Arβ is similar with tetrapod and cartilaginous fish Ars, indicating that Arβ retains the original and common function throughout vertebrates. By contrast, teleost Arα shows a unique intracellular localization and significantly higher transactivating properties [5, 52, 139]. This has been observed for Arαs from spiny-rayed fishes (Acanthomorpha), but not for Japanese eel (Elopomorpha, an earlier branching lineage among teleosts), suggesting that *arα* has evolved after the divergence of the Elopomorpha lineage. The amino acids that are responsible for Arα specific hyper-transactivation and constitutive nuclear localization have been identified and are highly conserved in spiny-rayed fish Arα, but differ in Japanese eel [139]. Insertion of spiny-rayed fish type amino acids into Japanese eel Arα recapitulates the evolutionary novelty of euteleost Arα, indicating these substitutions generate a new functionality of Arα in the teleost genome after the divergence of the Elopomorpha lineage [139]. Such evolutionary novelty of protein function in *ar* genes might facilitate the emergence of divergent sex characteristics in teleost lineage.

Taken together, this review serves to illustrate that divergence of the sex steroid receptors, most notably for the estrogen receptor associates with functional complexity. Recent progress in genome editing approaches now allow for more practical capability to effectively target specific gene manipulations. Although adoption of these approaches has been reported in a few species only, application in future studies to genetically modify the estrogen and androgen receptors in animals throughout the vertebrate lineage is likely to enable the rapid advancement in our understanding of the evolution and functionalization of steroid hormone signaling.

8. Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (KAKENHI) [15K07138 (Y.O.), 15H04395 (Y.O.), 15H04396 (T.I., Y.O., S.M.), 26440173 (S.M.), 17H06432 (S.M.), 18H02474 (S.M.)] from the Japan Society for the Promotion of Science (JSPS): UK-Japan Research Collaboration Grants (T.K., N.T., A.L., C.R.T., S.M., T.I.) and grants (N.T., T.I., S.M.) from the Ministry of the Environment, Japan, and the Department for Environment, Food and Rural Affairs (Defra), UK: the NIBB Cooperative Research Program (Y.O., S.M) from National Institute for Basic Biology: The 2nd Women Researchers Promotion Program, Support for childbirth and childcare in Women Researchers Promotion Program and Support for Women Returning from Maternity and Parental Leave from Kyushu University (Y.O.).

References

[1] E.C. Chang, T.H. Charn, S.H. Park, W.G. Helferich, B. Komm, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Estrogen Receptors alpha and beta as determinants of gene expression: influence of ligand, dose, and chromatin binding, Mol Endocrinol. 22 (2008) 1032-1043.

[2] C. Williams, K. Edvardsson, S.A. Lewandowski, A. Strom, J.A. Gustafsson, A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells, Oncogene. 27 (2008) 1019-1032.

[3] P. Yi, M.D. Driscoll, J. Huang, S. Bhagat, R. Hilf, R.A. Bambara, M. Muyan, The effects of estrogen-responsive element- and ligand-induced structural changes on the recruitment of cofactors and transcriptional responses by ER alpha and ER beta, Mol Endocrinol. 16 (2002) 674-693.

[4] J.L. Bowers, V.V. Tyulmenkov, S.C. Jernigan, C.M. Klinge, Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta, Endocrinology. 141 (2000) 3657-3667.

[5] Y. Ogino, H. Katoh, S. Kuraku, G. Yamada, Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates, Endocrinology. 150 (2009) 5415-5427.

[6] J.W. Thornton, Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions, Proc Natl Acad Sci U S A. 98 (2001) 5671-5676.

[7] M.E. Baker, Y. Katsu, 30 YEARS OF THE MINERALOCORTICOID RECEPTOR: Evolution of the mineralocorticoid receptor: sequence, structure and function, J Endocrinol. 234 (2017) T1-T16.

521 [8] Y. Katsu, P.A. Cziko, C. Chandsawangbhuwana, J.W. Thornton, R. Sato, K. Oka, Y.
522 Takei, M.E. Baker, T. Iguchi, A second estrogen receptor from Japanese lamprey
523 (*Lethenteron japonicum*) does not have activities for estrogen binding and transcription,
524 Gen Comp Endocrinol. 236 (2016) 105-114.
525 [9] Y. Katsu, S. Kohno, H. Narita, H. Urushitani, K. Yamane, A. Hara, T.M. Clauss, M.T.
526 Walsh, S. Miyagawa, L.J. Guillette, Jr., T. Iguchi, Cloning and functional
527 characterization of Chondrichthyes, cloudy catshark, *Scyliorhinus torazame* and whale
528 shark, *Rhincodon typus* estrogen receptors, Gen Comp Endocrinol. 168 (2010) 496-504.
529 [10] S. Tohyama, S. Miyagawa, A. Lange, Y. Ogino, T. Mizutani, M. Ihara, H. Tanaka,
530 N. Tatarazako, T. Kobayashi, C.R. Tyler, T. Iguchi, Evolution of estrogen receptors in
531 ray-finned fish and their comparative responses to estrogenic substances, J Steroid
532 Biochem Mol Biol. 158 (2016) 189-197.
533 [11] F.G. Brunet, H. Roest Crollius, M. Paris, J.M. Aury, P. Gibert, O. Jaillon, V. Laudet,
534 M. Robinson-Rechavi, Gene loss and evolutionary rates following whole-genome
535 duplication in teleost fishes, Mol Biol Evol. 23 (2006) 1808-1816.
536 [12] O. Jaillon, J.M. Aury, F. Brunet, J.L. Petit, N. Stange-Thomann, E. Mauceli, L.
537 Bouneau, C. Fischer, C. Ozouf-Costaz, A. Bernot, S. Nicaud, D. Jaffe, S. Fisher, G.
538 Lutfalla, C. Dossat, B. Segurens, C. Dasilva, M. Salanoubat, M. Levy, N. Boudet, S.
539 Castellano, V. Anthouard, C. Jubin, V. Castelli, M. Katinka, B. Vacherie, C. Biemont, Z.
540 Skalli, L. Cattolico, J. Poulain, V. De Berardinis, C. Cruaud, S. Duprat, P. Brottier, J.P.
541 Coutanceau, J. Gouzy, G. Parra, G. Lardier, C. Chapple, K.J. McKernan, P. McEwan, S.
542 Bosak, M. Kellis, J.N. Volff, R. Guigo, M.C. Zody, J. Mesirov, K. Lindblad-Toh, B.
543 Birren, C. Nusbaum, D. Kahn, M. Robinson-Rechavi, V. Laudet, V. Schachter, F.
544 Quetier, W. Saurin, C. Scarpelli, P. Wincker, E.S. Lander, J. Weissenbach, H. Roest

545 Crollius, Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early
546 vertebrate proto-karyotype, Nature. 431 (2004) 946-957.
547 [13] S. Hoegg, H. Brinkmann, J.S. Taylor, A. Meyer, Phylogenetic timing of the
548 fish-specific genome duplication correlates with the diversification of teleost fish, J Mol
549 Evol. 59 (2004) 190-203.
550 [14] C.H. Chiu, K. Dewar, G.P. Wagner, K. Takahashi, F. Ruddle, C. Ledje, P. Bartsch,
551 J.L. Scemama, E. Stellwag, C. Fried, S.J. Prohaska, P.F. Stadler, C.T. Amemiya, Bichir
552 HoxA cluster sequence reveals surprising trends in ray-finned fish genomic evolution,
553 Genome Res. 14 (2004) 11-17.
554 [15] G.N. Eick, J.W. Thornton, Evolution of steroid receptors from an estrogen-sensitive
555 ancestral receptor, Mol Cell Endocrinol. 334 (2011) 31-38.
556 [16] V. Douard, F. Brunet, B. Boussau, I. Ahrens-Fath, V. Vlaeminck-Guillem, B.
557 Haendler, V. Laudet, Y. Guiguen, The fate of the duplicated androgen receptor in fishes:
558 a late neofunctionalization event?, BMC Evol Biol. 8 (2008) 336.
559 [17] A.K. Greenwood, P.C. Butler, R.B. White, U. DeMarco, D. Pearce, R.D. Fernald,
560 Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns,
561 and transcriptional activities, Endocrinology. 144 (2003) 4226-4236.
562 [18] M.S. Hossain, A. Larsson, N. Scherbak, P.E. Olsson, L. Orban, Zebrafish androgen
563 receptor: isolation, molecular, and biochemical characterization, Biol Reprod. 78 (2008)
564 361-369.
565 [19] V.S. Wilson, M.C. Cardon, J. Thornton, J.J. Korte, G.T. Ankley, J. Welch, L.E. Gray,
566 Jr., P.C. Hartig, Cloning and *in vitro* expression and characterization of the androgen
567 receptor and isolation of estrogen receptor alpha from the fathead Minnow (*Pimephales*
568 *promelas*), Environ Sci Technol. 38 (2004) 6314-6321.

[20] B.F. Huang, Y.L. Sun, F.R. Wu, Z.H. Liu, Z.J. Wang, L.F. Luo, Y.G. Zhang, D.S. Wang, Isolation, sequence analysis, and characterization of androgen receptor in Southern catfish, *Silurus meridionalis*, Fish Physiol Biochem. 37 (2011) 593-601.

[21] T.J. Near, R.I. Eytan, A. Dornburg, K.L. Kuhn, J.A. Moore, M.P. Davis, P.C. Wainwright, M. Friedman, W.L. Smith, Resolution of ray-finned fish phylogeny and timing of diversification, Proc Natl Acad Sci U S A. 109 (2012) 13698-13703.

[22] J. Takeo, S. Yamashita, Two distinct isoforms of cDNA encoding rainbow trout androgen receptors, J Biol Chem. 274 (1999) 5674-5680.

[23] F.W. Allendorf, G.H. Thorgaard, Tetraploidy and evolution of salmonid fishes, in: B.J. Turner (Ed.) Evolutionary Genetics of Fish, Plenum Press, New York, 1984, pp. 1-53.

[24] G. Yamada, Y. Satoh, L.S. Baskin, G.R. Cunha, Cellular and molecular mechanisms of development of the external genitalia, Differentiation. 71 (2003) 445-460.

[25] R. Murakami, T. Mizuno, Proximal-distal sequence of development of the skeletal tissues in the penis of rat and the inductive effect of epithelium, J Embryol Exp Morphol. 92 (1986) 133-143.

[26] K.L. O'Shaughnessy, R.D. Dahn, M.J. Cohn, Molecular development of chondrichthyan claspers and the evolution of copulatory organs, Nat Commun. 6 (2015) 6698.

[27] E. Rosa-Molinar, A.C. Burke, Starting from fins: parallelism in the evolution of limbs and genitalia: the fin-to-genitalia transition, Evol Dev. 4 (2002) 124-126.

[28] F.W. Allendorf, G.H. Thorgaard, Tetraploidy and the evolution of Salmonid fishes, in: J.B. Turner (Ed.) Evolutionary genetics of fishes, Plenum Publishing Corp., New York, 1984, pp. 1-53.

593 [29] A. Kuntz, Notes on the habits, morphology of the reproductive organs, and
594 embryology of the viviparous fish *Gambusia affinis*, Bull US Bur Fish. 33 (1914)
595 177-190.
596 [30] H. Uwa, The synthesis of collagen during the development of anal-fin processes in
597 ethisterone-treated females of *Oryzias latipes*, Dev Growth Differ. 13 (1971) 119-124.
598 [31] T.B. Oka, On the processes on the fin-rays of the male of *Oryzias latipes* and other
599 sex characters of this fish, J Fac Sci Imp Univ Tokyo Sec. 2 (1931) 209-218.
600 [32] M. Yamamoto, N. Egami, Fine structure of the surface of the anal fin and the
601 processes on its fin rays of male *Oryzias latipes*, Copeia. 1 (1974) 262-265.
602 [33] B. Borg, Androgens in teleost fishes, Comp Biochem Physiol. 109C (1994)
603 209-245.
604 [34] C. Darwin, The descent of man, and selection in relation to sex, John Murray,
605 London, 1871.
606 [35] R. Künzler, T. Bakker, C, M., Female preferences for single and combined traits in
607 computer animated stickleback males, Behav Ecol. 12 (2001) 681-685.
608 [36] Y.T. Shao, F.Y. Wang, W.C. Fu, H.Y. Yan, K. Anraku, I.S. Chen, B. Borg,
609 Androgens increase lws opsin expression and red sensitivity in male three-spined
610 sticklebacks, PLoS One. 9 (2014) e100330.
611 [37] C.R. Largiader, V. Fries, T.C. Bakker, Genetic analysis of sneaking and
612 egg-thievery in a natural population of the three-spined stickleback (*Gasterosteus*
613 *aculeatus L.*), Heredity (Edinb). 86 (2001) 459-468.
614 [38] J. Van den Assem, Territory in the Three-Spined Stickleback *Gasterosteus*
615 *aculeatus L.*: An Experimental Study in Intra-Specific Competition, Brill, Leiden, 1967.
616 [39] E.K. Brockmeier, Y. Ogino, T. Iguchi, D.S. Barber, N.D. Denslow, Effects of

17beta-trenbolone on Eastern and Western mosquitofish (*Gambusia holbrooki* and *G. affinis*) anal fin growth and gene expression patterns, Aquat Toxicol. 128-129 (2013) 163-170.

[40] Y. Ogino, H. Katoh, G. Yamada, Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates, FEBS letters. 575 (2004) 119-126.

[41] Y.K. Okada, H. Yamashita, Experimental investigation of the manifestation of secondary sexual characters in fish, using the medaka *Oryzias latipes* (Temminck, Schlegel) as material, J Fac Sci Imp Univ Tokyo Sec. 6 (1944) 383-437.

[42] T.O. Hishida, N. Kawamoto, Androgenic and male inducing effects of 11-ketotestosterone on a teleost the medaka *Oryzias latipes*, J Exp Zool. 173 (1970) 279-284.

[43] G. Toft, T.M. Edwards, E. Baatrup, L.J. Guillette, Jr., Disturbed sexual characteristics in male mosquitofish (*Gambusia holbrooki*) from a lake contaminated with endocrine disruptors, Environ Health Perspect. 111 (2003) 695-701.

[44] A. Sebillot, P. Damdimopoulou, Y. Ogino, P. Spirhanzlova, S. Miyagawa, D. Du Pasquier, N. Mouatassim, T. Iguchi, G.F. Lemkine, B.A. Demeneix, A.J. Tindall, Rapid fluorescent detection of (anti)androgens with spiggin-gfp medaka, Environ Sci Technol. 48 (2014) 10919-10928.

[45] L.G. Parks, C.S. Lambright, E.F. Orlando, L.J. Guillette, Jr., G.T. Ankley, L.E. Gray, Jr., Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity, Toxicol Sci. 62 (2001) 257-267.

[46] E.F. Orlando, W.P. Davis, L.J. Guillette, Jr., Aromatase activity in the ovary and

641 brain of the eastern mosquitofish (*Gambusia holbrooki*) exposed to paper mill effluent,
642 Environ Health Perspect. 110 Suppl 3 (2002) 429-433.
643 [47] J. Batty, R. Lim, Morphological and reproductive characteristics of male
644 mosquitofish (*Gambusia affinis holbrooki*) inhabiting sewage-contaminated waters in
645 New South Wales, Australia, Archives of environmental contamination and toxicology.
646 36 (1999) 301-307.
647 [48] W.M. Howell, D.A. Black, S.A. Bortone, Abnormal expression of secondary sex
648 characters in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for
649 environmentally induced masculinization, Copeia. 4 (1980) 676-681.
650 [49] D. Sassoon, D.B. Kelley, The sexually dimorphic larynx of *Xenopus laevis*:
651 development and androgen regulation, Am J Anat. 177 (1986) 457-472.
652 [50] D.B. Kelley, D.W. Pfaff, Hormone effects on male sex behavior in adult South
653 African clawed frogs, *Xenopus laevis*, Horm Behav. 7 (1976) 159-182.
654 [51] M.L. Tobias, M.L. Marin, D.B. Kelley, The roles of sex, innervation, and androgen
655 in laryngeal muscle of *Xenopus laevis*, J Neurosci. 13 (1993) 324-333.
656 [52] Y. Ogino, S. Miyagawa, H. Katoh, G.S. Prins, T. Iguchi, G. Yamada, Essential
657 functions of androgen signaling emerged through the developmental analysis of
658 vertebrate sex characteristics, Evol Dev. 13 (2011) 315-325.
659 [53] J.A. Mayer, C.M. Chuong, R. Widelitz, Rooster feathering, androgenic alopecia,
660 and hormone-dependent tumor growth: what is in common?, Differentiation. 72 (2004)
661 474-488.
662 [54] J.M. Gasc, W.E. Stumpf, Sexual differentiation of the urogenital tract in the
663 chicken embryo: autoradiographic localization of sex-steroid target cells during
664 development, J Embryol Exp Morphol. 63 (1981) 207-223.

665 [55] A.P. Arnold, The effects of castration and androgen replacement on song, courtship,
666 and aggression in zebra finches (*Poephila guttata*), J Exp Zool. 191 (1975) 309-326.
667 [56] A.L. Romanoff, The Avian Embryo. Structural and Functional Development.,
668 Macmillan, New York, 1960.
669 [57] E. Wolff, Endocrine function of the gonad in developing vertebrates, in: A.
670 Gorbman (Ed.) Comparative Endocrinology, Wiley, NewYork, 1959, pp. 569-573.
671 [58] H. Katoh, Y. Ogino, G. Yamada, Cloning and expression analysis of androgen
672 receptor gene in chicken embryogenesis, FEBS letters. 580 (2006) 1607-1615.
673 [59] B.A. Shanbhag, P.J. Sharp, Immunocytochemical localization of androgen receptor
674 in the comb, uropygial gland, testis, and epididymis in the domestic chicken, Gen Comp
675 Endocrinol. 101 (1996) 76-82.
676 [60] Y. Tanabe, T. Nakamura, K. Fujioka, O. Doi, Production and secretion of sex
677 steroid hormones by the testes, the ovary, and the adrenal glands of embryonic and
678 young chickens (*Gallus domesticus*), Gen Comp Endocrinol. 39 (1979) 26-33.
679 [61] F.J. Zeller, The effects of testosterone and dihydrotestosterone on the comb, testis,
680 and pituitary gland of the male fowl, J Reprod Fertil. 25 (1971) 125-127.
681 [62] J. Wade, L. Buhlman, Lateralization and effects of adult androgen in a sexually
682 dimorphic neuromuscular system controlling song in zebra finches, J Comp Neurol. 426
683 (2000) 154-164.
684 [63] J. Wade, L. Buhlman, D. Swender, Post-hatching hormonal modulation of a
685 sexually dimorphic neuromuscular system controlling song in zebra finches, Brain Res.
686 929 (2002) 191-201.
687 [64] T. Noumura, E. Matsumoto, M. Takahashi, On the development of sexual
688 dimorphism in the duck syrinx and estradiol binding, in: B. Lofts, W.N. Holmes (Eds.)

689 Current Trends in Comparative Endocrinology, Hong Kong University Press, Hong
690 Kong, 1985, pp. 601-602.
691 [65] S.L. Veney, J. Wade, Post-hatching syrinx development in the zebra finch: an
692 analysis of androgen receptor, aromatase, estrogen receptor alpha and estrogen receptor
693 beta mRNAs, J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 191 (2005)
694 97-104.
695 [66] R.M. Cox, D.S. Stenquist, R. Calsbeek, Testosterone, growth and the evolution of
696 sexual size dimorphism, J Evol Biol. 22 (2009) 1586-1598.
697 [67] H.N. Kerver, J. Wade, Seasonal and sexual dimorphisms in expression of androgen
698 receptor and its coactivators in brain and peripheral copulatory tissues of the green
699 anole, Gen Comp Endocrinol. 193 (2013) 56-67.
700 [68] S. Miyagawa, Y. Satoh, R. Haraguchi, K. Suzuki, T. Iguchi, M.M. Taketo, N.
701 Nakagata, T. Matsumoto, K. Takeyama, S. Kato, G. Yamada, Genetic interactions of the
702 androgen and Wnt/beta-catenin pathways for the masculinization of external genitalia,
703 Mol Endocrinol. 23 (2009) 871-880.
704 [69] K. Suzuki, Y. Ogino, R. Murakami, Y. Satoh, D. Bachiller, G. Yamada, Embryonic
705 development of mouse external genitalia: insights into a unique mode of organogenesis,
706 Evol Dev. 4 (2002) 133-141.
707 [70] D.W. Silversides, C.A. Price, G.M. Cooke, Effects of short-term exposure to
708 hydroxyflutamide *in utero* on the development of the reproductive tract in male mice,
709 Can J Physiol Pharmacol. 73 (1995) 1582-1588.
710 [71] R.L. Clark, J.M. Antonello, S.J. Grossman, L.D. Wise, C. Anderson, W.J. Bagdon,
711 S. Prahalada, J.S. MacDonald, R.T. Robertson, External genitalia abnormalities in male
712 rats exposed in utero to finasteride, a 5 alpha-reductase inhibitor, Teratology. 42 (1990)

91-100.

[72] J. Imperato-McGinley, Z. Binienda, A. Arthur, D.T. Mininberg, E.D. Vaughan, Jr., F.W. Quimby, The development of a male pseudohermaphroditic rat using an inhibitor of the enzyme 5 alpha-reductase, Endocrinology. 116 (1985) 807-812.

[73] L.S. Baskin, A. Erol, P. Jegatheesan, Y. Li, W. Liu, G.R. Cunha, Urethral seam formation and hypospadias, Cell Tissue Res. 305 (2001) 379-387.

[74] T. Sato, T. Matsumoto, T. Yamada, T. Watanabe, H. Kawano, S. Kato, Late onset of obesity in male androgen receptor-deficient (AR KO) mice, Biochem Biophys Res Commun. 300 (2003) 167-171.

[75] S. Yeh, M.Y. Tsai, Q. Xu, X.M. Mu, H. Lardy, K.E. Huang, H. Lin, S.D. Yeh, S. Altuwaijri, X. Zhou, L. Xing, B.F. Boyce, M.C. Hung, S. Zhang, L. Gan, C. Chang, Generation and characterization of androgen receptor knockout (ARKO) mice: an *in vivo* model for the study of androgen functions in selective tissues, Proc Natl Acad Sci U S A. 99 (2002) 13498-13503.

[76] R. Murakami, A histological study of the development of the penis of wild-type and androgen-insensitive mice, J Anat. 153 (1987) 223-231.

[77] C.A. Quigley, A. De Bellis, K.B. Marschke, M.K. el-Awady, E.M. Wilson, F.S. French, Androgen receptor defects: historical, clinical, and molecular perspectives, Endocr Rev. 16 (1995) 271-321.

[78] P.M. Lokman, B. Harris, M. Kusakabe, D.E. Kime, R.W. Schulz, S. Adachi, G. Young, 11-Oxygenated androgens in female teleosts: prevalence, abundance, and life history implications, Gen Comp Endocrinol. 129 (2002) 1-12.

[79] J.L. Bolaffi, V. Lance, I.P. Callard, J.M. Walsh, D.R. Idler, Identification of 11-ketotestosterone, 11 beta-hydroxytestosterone, and testosterone in plasma of

737 Necturus maculosus (Rafinesque), Gen Comp Endocrinol. 38 (1979) 127-131.
738 [80] Y. Imamichi, K.I. Yuhki, M. Orisaka, T. Kitano, K. Mukai, F. Ushikubi, T.
739 Taniguchi, A. Umezawa, K. Miyamoto, T. Yazawa, 11-Ketotestosterone Is a Major
740 Androgen Produced in Human Gonads, J Clin Endocrinol Metab. 101 (2016)
741 3582-3591.
742 [81] Y. Ogino, I. Hirakawa, K. Inohaya, E. Sumiya, S. Miyagawa, N. Denslow, G.
743 Yamada, N. Tatarazako, T. Iguchi, Bmp7 and Lef1 are the downstream effectors of
744 androgen signaling in androgen-induced sex characteristics development in medaka,
745 Endocrinology. 155 (2014) 449-462.
746 [82] Y. Pu, L. Huang, L. Birch, G.S. Prins, Androgen regulation of prostate
747 morphoregulatory gene expression: Fgf10-dependent and -independent pathways,
748 Endocrinology. 148 (2007) 1697-1706.
749 [83] C.J. Neumann, H. Grandel, W. Gaffield, S. Schulte-Merker, C. Nusslein-Volhard,
750 Transient establishment of anteroposterior polarity in the zebrafish pectoral fin bud in
751 the absence of sonic hedgehog activity, Development. 126 (1999) 4817-4826.
752 [84] E. Quint, A. Smith, F. Avaron, L. Laforest, J. Miles, W. Gaffield, M.A. Akimenko,
753 Bone patterning is altered in the regenerating zebrafish caudal fin after ectopic
754 expression of sonic hedgehog and bmp2b or exposure to cyclopamine, Proc Natl Acad
755 Sci U S A. 99 (2002) 8713-8718.
756 [85] L. Laforest, C.W. Brown, G. Poleo, J. Geraudie, M. Tada, M. Ekker, M.A.
757 Akimenko, Involvement of the sonic hedgehog, patched 1 and bmp2 genes in patterning
758 of the zebrafish dermal fin rays, Development. 125 (1998) 4175-4184.
759 [86] S. Miyagawa, A. Moon, R. Haraguchi, C. Inoue, M. Harada, C. Nakahara, K.
760 Suzuki, D. Matsumaru, T. Kaneko, I. Matsuo, L. Yang, M.M. Taketo, T. Iguchi, S.M.

761 Evans, G. Yamada, Dosage-dependent hedgehog signals integrated with
762 Wnt/beta-catenin signaling regulate external genitalia formation as an appendicular
763 program, Development. 136 (2009) 3969-3978.
764 [87] R. Haraguchi, R. Mo, C. Hui, J. Motoyama, S. Makino, T. Shiroishi, W. Gaffield, G.
765 Yamada, Unique functions of Sonic hedgehog signaling during external genitalia
766 development, Development. 128 (2001) 4241-4250.
767 [88] S. Miyagawa, D. Matsumaru, A. Murashima, A. Omori, Y. Satoh, R. Haraguchi, J.
768 Motoyama, T. Iguchi, N. Nakagata, C.C. Hui, G. Yamada, The role of sonic
769 hedgehog-Gli2 pathway in the masculinization of external genitalia, Endocrinology. 152
770 (2011) 2894-2903.
771 [89] Z. Zheng, B.A. Armfield, M.J. Cohn, Timing of androgen receptor disruption and
772 estrogen exposure underlies a spectrum of congenital penile anomalies, Proc Natl Acad
773 Sci U S A. 112 (2015) E7194-7203.
774 [90] H. Kobayashi, T. Iwamatsu, Sex reversal in the medaka *Oryzias latipes* by brief
775 exposure of early embryos to estradiol-17beta, Zoolog Sci. 22 (2005) 1163-1167.
776 [91] T. Iwamatsu, H. Kobayashi, S. Hamaguchi, R. Sagegami, T. Shuo, Estradiol-17beta
777 content in developing eggs and induced sex reversal of the medaka (*Oryzias latipes*), J
778 Exp Zool A Comp Exp Biol. 303 (2005) 161-167.
779 [92] T. Yamamoto, Hormonic factors affecting gonadal sex differentiation in fish, Gen
780 Comp Endocrinol. Suppl 1 (1962) 341-345.
781 [93] I. Villalpando, H. Merchant-Larios, Determination of the sensitive stages for
782 gonadal sex-reversal in *Xenopus laevis* tadpoles, Int J Dev Biol. 34 (1990) 281-285.
783 [94] C.Y. Chang, E. Witschi, Genic control and hormonal reversal of sex differentiation
784 in Xenopus, Proc Soc Exp Biol Med. 93 (1956) 140-144.

785 [95] A. Elbrecht, R.G. Smith, Aromatase enzyme activity and sex determination in
786 chickens, Science. 255 (1992) 467-470.
787 [96] D. Scheib, Effects and role of estrogens in avian gonadal differentiation,
788 Differentiation. 23 Suppl (1983) S87-92.
789 [97] J.J. Bull, Sex determination in reptiles, Q Rev Biol. 55 (1980) 3-21.
790 [98] M. Charnier, Action of temperature on the sex ratio in the *Agama agama*
791 (Agamidae, Lacertilia) embryo, C R Seances Soc Biol Fil. 160 (1966) 620-622.
792 [99] T. Rhen, A. Schroeder, Molecular mechanisms of sex determination in reptiles,
793 Sexual development : genetics, molecular biology, evolution, endocrinology,
794 embryology, and pathology of sex determination and differentiation. 4 (2010) 16-28.
795 [100] R. Yatsu, S. Miyagawa, S. Kohno, B.B. Parrott, K. Yamaguchi, Y. Ogino, H.
796 Miyakawa, R.H. Lowers, S. Shigenobu, L.J. Guillette, Jr., T. Iguchi, RNA-seq analysis
797 of the gonadal transcriptome during *Alligator mississippiensis* temperature-dependent
798 sex determination and differentiation, BMC Genomics. 17 (2016) 77.
799 [101] C. Murdock, T. Wibbels, Cloning and expression of aromatase in a turtle with
800 temperature-dependent sex determination, Gen Comp Endocrinol. 130 (2003) 109-119.
801 [102] W.N. Gabriel, B. Blumberg, S. Sutton, A.R. Place, V.A. Lance, Alligator
802 aromatase cDNA sequence and its expression in embryos at male and female incubation
803 temperatures, J Exp Zool. 290 (2001) 439-448.
804 [103] M. Ramsey, D. Crews, Steroid signaling and temperature-dependent sex
805 determination-Reviewing the evidence for early action of estrogen during ovarian
806 determination in turtles, Semin Cell Dev Biol. 20 (2009) 283-292.
807 [104] M. Ramsey, D. Crews, Steroid signaling system responds differently to
808 temperature and hormone manipulation in the red-eared slider turtle (*Trachemys scripta*

809 *elegans*), a reptile with temperature-dependent sex determination, Sexual development :
810 genetics, molecular biology, evolution, endocrinology, embryology, and pathology of
811 sex determination and differentiation. 1 (2007) 181-196.
812 [105] S. Kohno, M.C. Bernhard, Y. Katsu, J. Zhu, T.A. Bryan, B.M. Doheny, T. Iguchi,
813 L.J. Guillette, Jr., Estrogen receptor 1 (ESR1; ERalpha), not ESR2 (ERbeta), modulates
814 estrogen-induced sex reversal in the American alligator, a species with
815 temperature-dependent sex determination, Endocrinology. 156 (2015) 1887-1899.
816 [106] A. Mattsson, J.A. Olsson, B. Brunstrom, Activation of estrogen receptor alpha
817 disrupts differentiation of the reproductive organs in chicken embryos, Gen Comp
818 Endocrinol. 172 (2011) 251-259.
819 [107] V.A. Lance, M.H. Bogart, Disruption of ovarian development in alligator embryos
820 treated with an aromatase inhibitor, Gen Comp Endocrinol. 86 (1992) 59-71.
821 [108] D. Crews, T. Wibbels, W.H. Gutzke, Action of sex steroid hormones on
822 temperature-induced sex determination in the snapping turtle (*Chelydra serpentina*),
823 Gen Comp Endocrinol. 76 (1989) 159-166.
824 [109] T. Wibbels, J.J. Bull, D. Crews, Steroid hormone-induced male sex determination
825 in an amniotic vertebrate, J Exp Zool. 262 (1992) 454-457.
826 [110] S. Miyagawa, R. Yatsu, S. Kohno, B.M. Doheny, Y. Ogino, H. Ishibashi, Y. Katsu,
827 Y. Ohta, L.J. Guillette, Jr., T. Iguchi, Identification and Characterization of the
828 Androgen Receptor From the American Alligator, *Alligator mississippiensis*,
829 Endocrinology. 156 (2015) 2795-2806.
830 [111] D.B. Lubahn, J.S. Moyer, T.S. Golding, J.F. Couse, K.S. Korach, O. Smithies,
831 Alteration of reproductive function but not prenatal sexual development after insertional
832 disruption of the mouse estrogen receptor gene, Proc Natl Acad Sci U S A. 90 (1993)

833 11162-11166.
834 [112] S.C. Hewitt, G.E. Kissling, K.E. Fieselman, F.L. Jayes, K.E. Gerrish, K.S. Korach,
835 Biological and biochemical consequences of global deletion of exon 3 from the ER
836 alpha gene, FASEB J. 24 (2010) 4660-4667.
837 [113] S. Dupont, A. Krust, A. Gansmuller, A. Dierich, P. Chambon, M. Mark, Effect of
838 single and compound knockouts of estrogen receptors alpha (ERalpha) and beta
839 (ERbeta) on mouse reproductive phenotypes, Development. 127 (2000) 4277-4291.
840 [114] R.A. Hess, D. Bunick, K.H. Lee, J. Bahr, J.A. Taylor, K.S. Korach, D.B. Lubahn,
841 A role for oestrogens in the male reproductive system, Nature. 390 (1997) 509-512.
842 [115] A.K. Binder, K.F. Rodriguez, K.J. Hamilton, P.S. Stockton, C.E. Reed, K.S.
843 Korach, The absence of ER-beta results in altered gene expression in ovarian granulosa
844 cells isolated from in vivo preovulatory follicles, Endocrinology. 154 (2013) 2174-2187.
845 [116] M.C. Antal, B. Petit-Demouliere, H. Meziane, P. Chambon, A. Krust, Estrogen
846 dependent activation function of ERbeta is essential for the sexual behavior of mouse
847 females, Proc Natl Acad Sci U S A. 109 (2012) 19822-19827.
848 [117] M.C. Antal, A. Krust, P. Chambon, M. Mark, Sterility and absence of
849 histopathological defects in nonreproductive organs of a mouse ERbeta-null mutant,
850 Proc Natl Acad Sci U S A. 105 (2008) 2433-2438.
851 [118] J.H. Krege, J.B. Hodgin, J.F. Couse, E. Enmark, M. Warner, J.F. Mahler, M. Sar,
852 K.S. Korach, J.A. Gustafsson, O. Smithies, Generation and reproductive phenotypes of
853 mice lacking estrogen receptor beta, Proc Natl Acad Sci U S A. 95 (1998) 15677-15682.
854 [119] J.F. Couse, S.C. Hewitt, D.O. Bunch, M. Sar, V.R. Walker, B.J. Davis, K.S.
855 Korach, Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha
856 and beta, Science. 286 (1999) 2328-2331.

857 [120] M.J. McPhaul, Molecular defects of the androgen receptor, J Steroid Biochem
858 Mol Biol. 69 (1999) 315-322.
859 [121] J.E. Griffin, Androgen resistance--the clinical and molecular spectrum, N Engl J
860 Med. 326 (1992) 611-618.
861 [122] T. Matsumoto, K. Takeyama, T. Sato, S. Kato, Androgen receptor functions from
862 reverse genetic models, J Steroid Biochem Mol Biol. 85 (2003) 95-99.
863 [123] T. Matsumoto, H. Shiina, H. Kawano, T. Sato, S. Kato, Androgen receptor
864 functions in male and female physiology, J Steroid Biochem Mol Biol. 109 (2008)
865 236-241.
866 [124] S. Tohyama, Y. Ogino, A. Lange, T. Myosho, T. Kobayashi, Y. Hirano, G. Yamada,
867 T. Sato, N. Tatarazako, C.R. Tyler, T. Iguchi, S. Miyagawa, Establishment of estrogen
868 receptor 1 (ESR1)-knockout medaka: ESR1 is dispensable for sexual development and
869 reproduction in medaka, *Oryzias latipes*, Dev Growth Differ. 59 (2017) 552-561.
870 [125] H. Lu, Y. Cui, L. Jiang, W. Ge, Functional Analysis of Nuclear Estrogen
871 Receptors in Zebrafish Reproduction by Genome Editing Approach, Endocrinology. 158
872 (2017) 2292-2308.
873 [126] D. Uchida, M. Yamashita, T. Kitano, T. Iguchi, Oocyte apoptosis during the
874 transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish,
875 J Exp Biol. 205 (2002) 711-718.
876 [127] M. Matsuda, Y. Nagahama, A. Shinomiya, T. Sato, C. Matsuda, T. Kobayashi, C.E.
877 Morrey, N. Shibata, S. Asakawa, N. Shimizu, H. Hori, S. Hamaguchi, M. Sakaizumi,
878 DMY is a Y-specific DM-domain gene required for male development in the medaka
879 fish, Nature. 417 (2002) 559-563.
880 [128] J.P. Sumpter, S. Jobling, Vitellogenesis as a biomarker for estrogenic

881 contamination of the aquatic environment, Environ Health Perspect. 103 Suppl 7 (1995)
882 173-178.
883 [129] E.R. Nelson, H.R. Habibi, Functional significance of nuclear estrogen receptor
884 subtypes in the liver of goldfish, Endocrinology. 151 (2010) 1668-1676.
885 [130] L.B. Griffin, K.E. January, K.W. Ho, K.A. Cotter, G.V. Callard,
886 Morpholino-mediated knockdown of ERα, ERβa, and ERβb mRNAs in zebrafish
887 (*Danio rerio*) embryos reveals differential regulation of estrogen-inducible genes,
888 Endocrinology. 154 (2013) 4158-4169.
889 [131] C.M. Crowder, C.S. Lassiter, D.A. Gorelick, Nuclear androgen receptor regulates
890 testes organization and oocyte maturation in zebrafish, bioRxiv. (2017) bioRxiv 058552.
891 [132] S. Kohno, Y. Katsu, T. Iguchi, L.J. Guillette, Jr., Novel approaches for the study of
892 vertebrate steroid hormone receptors, Integrative and comparative biology. 48 (2008)
893 527-534.
894 [133] R. Yatsu, Y. Katsu, S. Kohno, T. Mizutani, Y. Ogino, Y. Ohta, J. Myburgh, J.H.
895 van Wyk, L.J. Guillette, Jr., S. Miyagawa, T. Iguchi, Characterization of evolutionary
896 trend in squamate estrogen receptor sensitivity, Gen Comp Endocrinol. 238 (2016)
897 88-95.
898 [134] S. Miyagawa, A. Lange, I. Hirakawa, S. Tohyama, Y. Ogino, T. Mizutani, Y.
899 Kagami, T. Kusano, M. Ihara, H. Tanaka, N. Tatarazako, Y. Ohta, Y. Katsu, C.R. Tyler, T.
900 Iguchi, Differing species responsiveness of estrogenic contaminants in fish is conferred
901 by the ligand binding domain of the estrogen receptor, Environ Sci Technol. 48 (2014)
902 5254-5263.
903 [135] Y. Katsu, E. Taniguchi, H. Urushitani, S. Miyagawa, M. Takase, K. Kubokawa, O.
904 Tooi, T. Oka, N. Santo, J. Myburgh, A. Matsuno, T. Iguchi, Molecular cloning and

905 characterization of ligand- and species-specificity of amphibian estrogen receptors, Gen
906 Comp Endocrinol. 168 (2010) 220-230.
907 [136] Y. Katsu, S. Kohno, T. Oka, N. Mitsui, O. Tooi, N. Santo, H. Urushitani, Y.
908 Fukumoto, K. Kuwabara, K. Ashikaga, S. Minami, S. Kato, Y. Ohta, L.J. Guillette, Jr., T.
909 Iguchi, Molecular cloning of estrogen receptor alpha (ERalpha; ESR1) of the Japanese
910 giant salamander, *Andrias japonicus*, Mol Cell Endocrinol. 257-258 (2006) 84-94.
911 [137] Y. Katsu, K. Matsubara, S. Kohno, Y. Matsuda, M. Toriba, K. Oka, L.J. Guillette,
912 Jr., Y. Ohta, T. Iguchi, Molecular cloning, characterization, and chromosome mapping
913 of reptilian estrogen receptors, Endocrinology. 151 (2010) 5710-5720.
914 [138] S. Tohyama, S. Miyagawa, A. Lange, Y. Ogino, T. Mizutani, N. Tatarazako, Y.
915 Katsu, M. Ihara, H. Tanaka, H. Ishibashi, T. Kobayashi, C.R. Tyler, T. Iguchi,
916 Understanding the molecular basis for differences in responses of fish estrogen receptor
917 subtypes to environmental estrogens, Environ Sci Technol. 49 (2015) 7439-7447.
918 [139] Y. Ogino, S. Kuraku, H. Ishibashi, H. Miyakawa, E. Sumiya, S. Miyagawa, H.
919 Matsubara, G. Yamada, M.E. Baker, T. Iguchi, Neofunctionalization of androgen
920 receptor by gain-of-function mutations in teleost fish lineage, Mol Biol Evol. 33 (2016)
921 228-244.
922
923

Figure legends

Fig. 1

Composite phylogeny of vertebrates with the hypothesized scenario of ESR and AR evolution. The evolutionary tree illustrates that Chondrichthyes (shark), the earliest branching group of living jawed vertebrates, possess ESR1, ESR2 and AR. The teleost-specific whole genome duplication (WGD) gave rise to two different teleost ARs (ARα and ARβ) and ESRs (ESR2a and ESR2b). Figure modified from Ogino *et al*., 2016, Tohyama *et al*., 2016.

Fig. 2

Androgen-dependent development of sex characteristics. (A) Male mosquitofish and bone staining of gonopodium (GP). The distal portion of the GP is composed of the 3rd, 4th, and 5th fin rays and the distal tip is equipped with spines, serrae, an elbow, and hooks. (B) Male medaka and bone staining of papillary processes that develop as an outgrowing bone nodule from the anal fin rays. (C) Mouse external genitalia in male. The development of copulatory organs is one of the representative models to investigate androgen-dependent organogenesis. (D) A schematic diagram of the possible signaling cross-talk between androgen and growth factor signaling for development of secondary sex characteristics. Figure modified from Ogino et al. 2004.

Fig. 3

Esr2a KO female medaka exhibit abnormal abdominal swelling and are infertile. (A) Wild-type female, (B) *esr2a* KO female.