Gene Duplication of Androgen Receptor As An Evolutionary Driving Force Underlying the Diversity of Sexual Characteristics in Teleost Fishes

Ryu, Tsukasa
Laboratory of Marine Biochemistry, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Okamoto, Keigo
Laboratory of Aquatic Molecular Developmental Biology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Ansai, Satoshi
Laboratory of Genome Editing Breeding, Graduate School of Agriculture, Kyoto University

Nakao, Miki
Laboratory of Marine Biochemistry, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

他

出版情報：Zoological Science. 41 (1), 2024-02-14. 日本動物学会
バージョン：

ZOOLOGICAL SCIENCE **41**: 68–76 (2024)

[REVIEW]

Gene Duplication of Androgen Receptor as an Evolutionary Driving Force Underlying the Diversity of Sexual Characteristics in Teleost Fishes

Tsukasa Ryu[1], Keigo Okamoto[2], Satoshi Ansai[3], Miki Nakao[1,4], Anu Kumar[5], Taisen Iguchi[6,7], and Yukiko Ogino[2,4]*

[1]*Laboratory of Marine Biochemistry, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 819-0395, Japan*
[2]*Laboratory of Aquatic Molecular Developmental Biology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 819-0395, Japan*
[3]*Laboratory of Genome Editing Breeding, Graduate School of Agriculture, Kyoto University, Kyoto 606-8507, Japan*
[4]*Center for Promotion of International Education and Research, Faculty of Agriculture, Kyushu University, Fukuoka 819-0395, Japan*
[5]*Commonwealth Scientific and Industrial Research Organization, CSIRO Environment, PMB2, Glen Osmond, 5064 South Australia, Australia*
[6]*Graduate School of Nanobioscience, Yokohama City University, Yokohama, Kanagawa 236-0027, Japan*
[7]*Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Ishikawa 927-0553, Japan*

Sexual dimorphism allows species to meet their fitness optima based on the physiological availability of each sex. Although intralocus sexual conflict appears to be a genetic constraint for the evolution of sex-specific traits, sex-linked genes and the regulation of sex steroid hormones contribute to resolving this conflict by allowing sex-specific developments. Androgens and their receptor, androgen receptor (Ar), regulate male-biased phenotypes. In teleost fish, *ar* ohnologs have emerged as a result of teleost-specific whole genome duplication (TSGD). Recent studies have highlighted the evolutionary differentiation of *ar* ohnologs responsible for the development of sexual characteristics, which sheds light on the need for comparative studies on androgen regulation among different species. In this review, we discuss the importance of *ar* signaling as a regulator of male-specific traits in teleost species because teleost species are suitable experimental models for comparative studies owing to their great diversity in male-biased morphological and physiological traits. To date, both in vivo and in vitro studies on teleost *ar* ohnologs have shown a substantial influence of *ar*s as a regulator of male-specific reproductive traits such as fin elongation, courtship behavior, and nuptial coloration. In addition to these sexual characteristics, *ar* substantially influences immunity, inducing a sex-biased immune response. This review aims to provide a comprehensive understanding of the current state of teleost *ar* studies and emphasizes the potential of teleost fishes, given their availability, to find molecular evidence about what gives rise to the spectacular diversity among fish species.

Key words: sexual characteristics, teleost, androgen, androgen receptor, whole genome duplication, gene duplication, immunity

INTRODUCTION

The evolution of sexual dimorphism can be driven in traits with different fitness optima between males and females (Darwin, 1871; Andersson, 1994; Arnegard et al., 2010). However, because males and females share much of their genome, the genetic correlation between the two sexes constrains the evolution of sexual dimorphism, which generates intra-locus sexual conflict (Cox and Calsbeek, 2009;

* Corresponding author. E-mail: ogino@agr.kyushu-u.ac.jp
doi:10.2108/zs230098

Van Doorn, 2009). In addition to sex-linked genes, male-biased gene expression under the control of androgens is a possible mechanism to resolve such sexual conflicts by allowing the expression of certain traits specifically in mature male vertebrates (Williams and Carroll, 2009; Kitano et al., 2020). Previous studies have demonstrated that androgens contribute to the expression of a wide range of male-specific traits, including morphology and behavior, in vertebrates (Hau, 2007; Mank, 2007). Among vertebrates, teleost fishes exhibit a variety of sexual characteristics, such as fin elongation (Zauner et al., 2003), nuptial coloration (Ansai et al., 2021), copulatory organs (Ogino et al., 2004), and reproductive behaviors such as nest building (Hoar, 1962), courtship (Borg and Mayer, 1995), and aggressive behavior (Yamashita et al., 2020), in response to androgens. The effects of androgens on each tissue are mediated by the androgen receptor (Ar), which belongs to the nuclear receptor superfamily (Mangelsdorf et al., 1995; Baker, 1997; Laudet, 1997; Thornton and Kelley, 1998; Escriva et al., 2000). The Ar plays key roles in controlling the molecular processes involved in the development and evolution of sex characteristics. Most vertebrates have one Ar gene, but two distinct paralogs of the Ar genes (*ara* and *arb*) have been identified in several teleosts (Ikeuchi et al., 1999; Sperry and Thomas, 1999; Ogino et al., 2004). Recent studies have shown that teleost species have undergone teleost-specific whole genome duplication (TSGD), resulting in the production of two Ar ohnologs: *ara* and *arb* (Douard et al., 2008; Ogino et al., 2009). Because gene duplication can reduce the negative selection pressure on duplicated copies and drive the establishment of lineage-specific traits with novel biological functions (Ohno, 1970), *ar* ohnologs may have contributed to the diversification of sexual characteristics in the teleost lineage. Recent in vitro functional analyses and knockout (KO) studies of *ar* ohnologs indicate that the functional differentiation of *ara* and *arb* is likely one of the key events that have caused the rapid diversification of sexual characteristics in teleost species (Ogino et al., 2016, 2023; Crowder et al., 2018; Tang et al., 2018; Yu et al., 2018; Alward et al., 2020). Teleosts account for nearly half of all extant vertebrate species (Ravi and Venkatesh, 2018), and the evolutionary acquisition of diverse sexual characteristics driven by sexual selection may have contributed to the radiation of teleosts.

In this review, we provide a comprehensive understanding of *ar* gene function in the context of the development of sexual characteristics in teleost fishes, by summarizing recent findings on the molecular evolution and functional diversification of *ar* genes. This review also emphasizes the involvement of Ar regulation in immunity of teleost fishes because the immune system is closely associated with androgen signals due to the expression of *ar*s in immune cells. The *ar* gene duplication may have contributed to wider variations in the immune response in teleost fish, which affect the survival and reproductive success of individuals according to environmental factors.

Evolutionary history of *ar* gene in vertebrates

Steroid hormone receptors are thought to have undergone molecular evolution through multiple gene duplications, including two whole genome duplication processes that occurred during early vertebrate evolution (Thornton, 2001). *ar* has not been identified in lamprey; however, genes showing ligand responsiveness almost identical to that of tetrapods are present in emerged cartilaginous fish (Ogino et al., 2009). Thus, it is thought that the *ar* gene emerged after the divergence of the jawless fish lineage, at least by the divergence of cartilaginous fishes through duplication of the gene encoding the progesterone receptor (Thornton, 2001; Ogino et al., 2018).

Most tetrapod species have one copy of *AR*, whereas most teleost fish have two distinct *ar* ohnologs generated by TSGD (Fig. 1). Medaka *ara* and *arb* are mapped to chromosomes 10 and 14, respectively, with a conserved synteny relative to a single region located in the *AR* gene on human chromosome X, suggesting that teleost *ar* gene duplication occurred as a result of TSGD (Ogino et al., 2009). TSGD is an evolutionarily recent whole genome duplication that occurred approximately 350 Ma, which is after the split of the non-teleost actinopterygian lineage (namely, bichir, sturgeon, gar, and bowfin) from the teleost lineage, but before the divergence of Elopomorpha (i.e., Japanese eel) and Osteoglossomorpha (i.e., sliver arowana) (Hoegg et al., 2004; Jaillon et al., 2004; Pasquier et al., 2016). The fact that *ar* ohnologs are maintained in the genome approximately 350 million years after duplication suggests that these duplicated genes may have contributed to the evolution of the endocrine system and diverse reproductive functions in teleost fishes (Douard et al., 2008; Ogino et al., 2018). Indeed, the molecular evolutionary analysis of *ar* ohnologs in medaka and mosquitofish revealed that *ara* has a faster evolutionary rate than *arb*, with the accumulation of more novel substitutions in *ara* than in *arb* after the split of Elopomorpha (Douard et al., 2008; Ogino et al., 2009, 2018) (Fig. 1). The lineage-specific loss of *ara* likely occurred independently in Osteoglossiformes (Arowana), Cypriniformes (Zebrafish), Siluriformes (Catfish), and Salmoniformes (Rainbow trout) (Takeo and Yamashita, 1999; Hossain et al., 2008; Ogino et al., 2009; Huang et al., 2011).

Distinct molecular properties acquired in *ar* ohnologs in teleost fishes as assessed by in vitro functional analysis at the protein level

Ar is composed of three major functional domains: a hypervariable N-terminal domain (NTD), a central highly conserved DNA binding domain (DBD) consisting of two zinc finger motifs, and a COOH-terminal ligand binding domain (LBD) (Evans, 1988; Bertrand et al., 2004; Bridgham et al., 2010; Baker et al., 2015) (Fig. 2A). Testosterone (T) and 5α-dihydrotestosterone (DHT) are effective ligands for mammalian Ars (Quigley et al., 1995), whereas 11-ketotestosterone (11KT) is known as a potent androgen in teleost fishes (Borg, 1994). However, a recent analysis showed the presence of 11KT in urodele amphibians (Bolaffi et al., 1979) and humans (Imamichi et al., 2016), suggesting that 11KT is a functional androgen in other vertebrates and teleost fishes.

The molecular properties of *ar* ohnologs have been extensively studied in medaka and mosquitofish using in vitro reporter gene assays. Arb in these fishes exhibits a ligand-dependent transactivation capacity similar to that of AR in tetrapods, whereas Ara exhibits higher ligand-dependent transactivation capacity and constitutive nuclear localization in COS-7 cells (Ogino et al., 2009) (Fig. 2B). Based

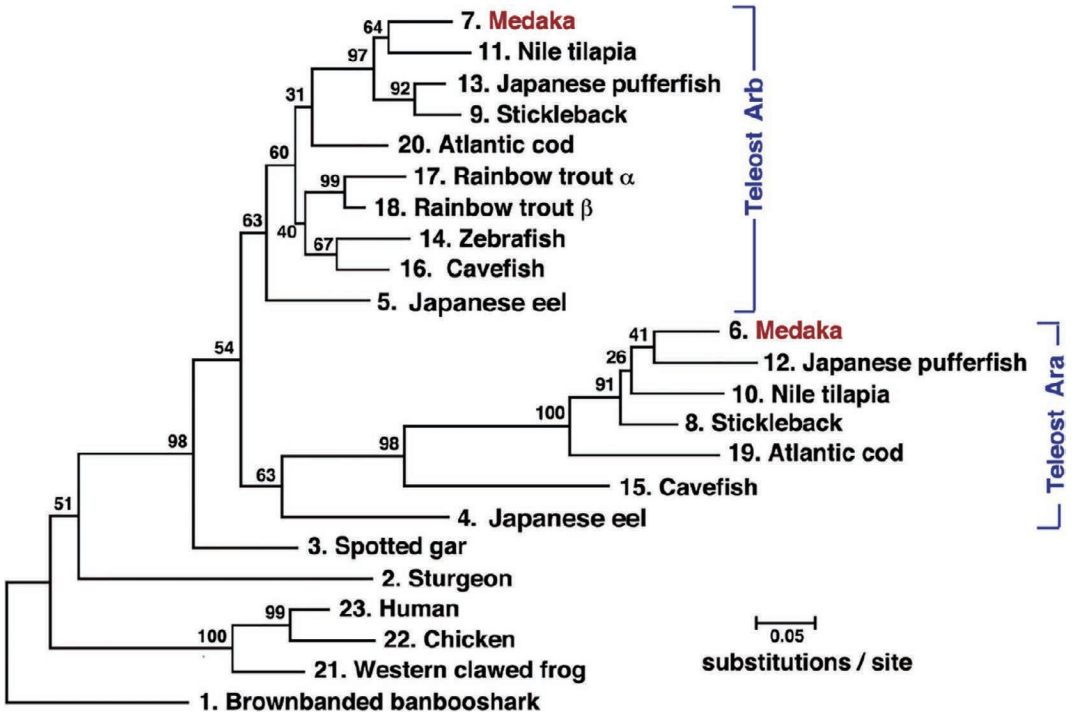

Fig. 1. Phylogenetic analysis of *Ar* genes. Molecular phylogenetic tree of vertebrate *ar* genes. This tree was constructed from the protein sequences of *ar* genes using the maximum-likelihood (ML) method combined with the JTT substitution model. The Brown-banded bamboo shark *ar* gene was used as an outgroup. The support values at the nodes represent bootstrap probabilities in the ML analysis. The GenBank and Ensembl accession numbers of the gene sequences used for this analysis are provided in Supplementary Table S1. Figures were modified from the previously published papers (Ogino et al., 2016, 2023) with permission for reuse (license number 5640600664159 from Oxford University Press).

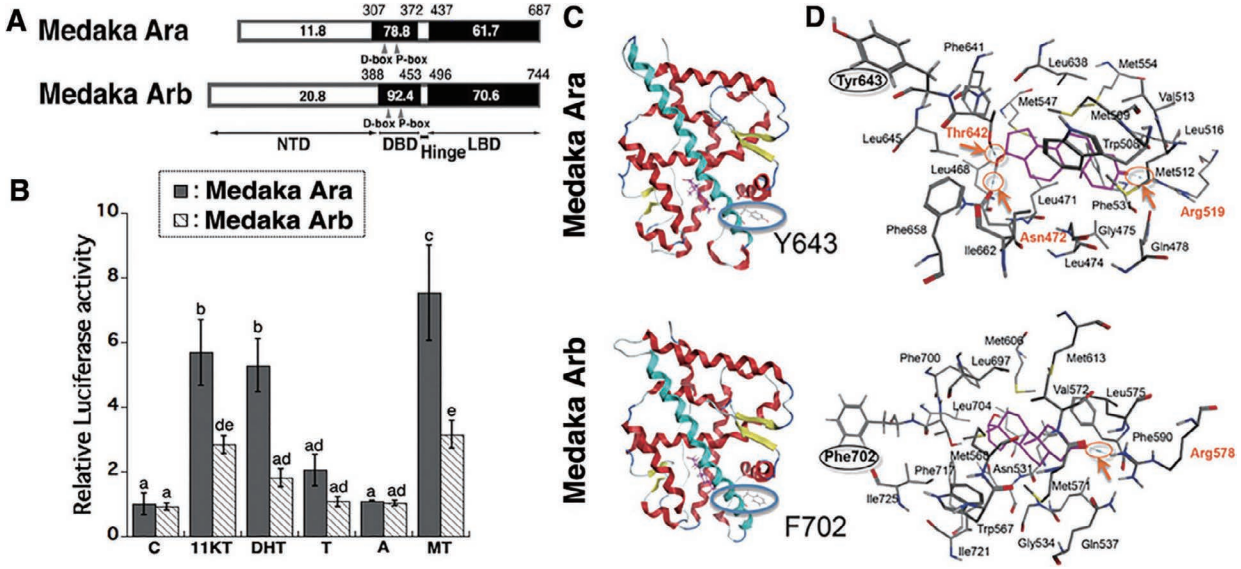

Fig. 2. Characterization of molecular property of medaka Ar ohnologs. **(A)** Structure of Ar ohnologs in medaka (GenBank accession numbers, Ara: AB252233 and Arb: AB252679). The numbers above each box refer to the amino acid positions in the putative DBD and LBD. The percentage of identity of the deduced amino acid sequences of each domain to the human AR (NM_000044.2) is shown in the boxes. **(B)** Ligand dependent transactivation properties of medaka Ar ohnologs in COS-7 cells. Transfected cells were treated with 10^{-9} M of various androgens, namely, 11KT, DHT, T, androstenedione (A), and 17α-methyl testosterone (MT), or vehicle only as a control (indicated as C). The relative transcriptional activity of Ar is shown as values normalized to pRL-induced activities. Vertical bars, Mean ± SD. Different letters indicate statistically significant differences in luciferase activity ($P < 0.05$) (ANOVA followed by the Tukey-Kramer test). **(C)** 3D ribbon diagrams of medaka Ara and Arb. The helices 10/11 are labeled in blue. The key amino acid substitutions that determine Ar ohnolog-specific transactivation properties, Ara Y643 and Arb F702 in helices10/11, are indicated. **(D)** Predicted modes obtained from the docking simulation analysis of 11KT for medaka Ara and Arb. Orange arrows indicate the predicted hydrogen bonds that stabilize the receptor-ligand complex. Figures were modified from Ogino et al. (2016) with permission to reuse; license number 5640600664159.

on site-directed mutagenesis and computational prediction of protein-ligand interactions between medaka Ar LBDs and 11KT, two key substitutions generating the new functionality of Ara have been identified (Ogino et al., 2016) (Fig. 2C, D). Substitution of the possible importin-binding site in the hinge region (medaka Ara K375 and Arb G456) contributes to the unique intracellular localization of medaka Ara in COS-7 cells. The substitution of helices 10/11 in the LBD (medaka Ara Y643, ArbF702) modulates the hydrogen bonds that stabilize the Ar LBD-11KT complex, leading to the generation of hyperactive Ara. These non-synonymous substitutions in medaka Ara are highly conserved in the LBD in teleost fishes, except the Japanese eel, which represents an earlier branching teleost group (Inoue et al., 2003; Li et al., 2008) expected to have Ar ohnologs derived from their respective ancestral genes before functional diversification. Both Japanese eel Ar ohnologs maintain ligand-dependent nuclear localization and show no significant differences in their transactivation responses (Ikeuchi et al., 1999; Ogino et al., 2016). The insertion of Ara-specific substitutions into Japanese eel Ar ohnologs recapitulates the evolutionary novelty of medaka Ara in COS-7 cells (Ogino et al., 2016), indicating that the substitution generating the new functionality of teleost Ara was fixed in the teleost genome after the divergence of the Elopomorpha lineage.

Functional diversity of *ar* ohnologs in sexual characteristics development assessed by genome engineering

In mammals, the role of AR in sexual dimorphism in morphology and reproductive behavior has been extensively investigated by AR KO (Yeh et al., 2002; Matsumoto et al., 2003; Juntti et al., 2010), the testicular feminization mutation (Tfm) rodents which lack functional AR, and disorders of androgen insensitivity syndrome (AIS) in humans (He et al., 1991; Griffin, 1992; Quigley et al., 1995; McPhaul, 1999). AR KO male mice exhibit a female-type external appearance with abnormal external genitalia, and the absence of sex accessory organs but retain small testes with cryptorchidism and severely arrested spermatogenesis (Yeh et al., 2002; Matsumoto et al., 2003; Zheng et al., 2015). These findings indicate the essential role of AR in the development of male reproductive organs and spermatogenesis. Such pleiotropic expression in various tissues may have constrained the molecular evolution of the *AR* gene in mammals. As described above, the fundamental roles of AR in reproduction have been illustrated in mammals. However, few empirical studies have investigated the molecular mechanisms underlying the evolutionary acquisition of diverse sexual characteristics in vertebrates. The acquisition of two duplicated copies of *ar* could have contributed to the diversification of masculine sexual characteristics found in teleosts, owing to the reduction in their pleiotropic constraints.

Recently, the generation of *ar* KO fishes from zebrafish (*Danio rerio*) (Yong et al., 2017; Tang et al., 2018; Yu et al., 2018), African cichlid (*Astatotilapia burtoni*) (Alward et al., 2020), and Japanese medaka *(Oryzias latipes*) (Ogino et al., 2023) was reported. In both zebrafish and African cichlids, *arb* KO showed smaller testis size. Zebrafish *arb* KO males are infertile during natural mating due to defective spermatogenesis, although a small number of mature sperm that can fertilize oocytes by in vitro fertilization are produced (Tang et al., 2018; Yu et al., 2018). Another possible reason for infertility is the reduced frequency of courtship-related behaviors, such as chases, zigzags, and tailnose, which are tactile behaviors related to spawning stimulation toward females and quivers (Yong et al., 2017). However, the zebrafish genome contains a single *ar* gene, possibly arising from the loss of a duplicate gene (Force et al., 1999), and genetic analysis of teleost fishes with both *ar* ohnologs is necessary to understand the contribution of *ar* gene duplication to the diversification of sexual characteristics.

The retention of the two *ar* ohnologs with distinct protein properties may have contributed to reproductive diversification in the teleost lineage. African cichlid and Japanese medaka are excellent model species for investigating the role of *ar* ohnologs in the development of sexual characteristics because they show social and reproductive behaviors and morphological traits such as coloration and fin elongation in the laboratory as in nature (Egami and Ishii, 1956; Yamamoto and Egami, 1974; Yokoi et al., 2015; Alward et al., 2020; Yamashita et al., 2020). Recent *ar* KO studies in African cichlid and Japanese medaka revealed that both *ara* KO males and *arb* KO males are fertile, but their frequency of reproductive success and fertilization rate has decreased because the two *ar* ohnologs have diverged in their functions for male sexual characteristics (Alward et al., 2020; Ogino et al., 2023). The African cichlid *ara* codes for reproductive and aggressive behaviors and *arb* for dominant bright coloration; either *ar* is sufficient for attacking males (Alward et al., 2020). Consistent with this, the Japanese medaka *arb* predominantly regulates external sexual characteristics such as fin morphology, pigmentation pattern, and sexual motivation (Fig. 3). The *arb* KO male medaka has shorter anal fin rays without any papillary processes which develop as branched bone nodules derived from the anal fin rays, as in females (Fig. 3). These masculine fin morphologies enable males to rub and prevent females from escaping during wrapping for spawning (Ono and Uematsu, 1957). However, in contrast to cichlids, medaka *ara* regulates not only reproductive behavior but also external sexual characteristics, such as tooth enlargement, which can be used in intra-male competition, suggesting that the functional contribution of *ara* varies among species (Ogino et al., 2023).

Recent technological advances in improving the resolution of androgen research have included the establishment of two knock-in medaka strains, *ara*$^{\text{FLAG-2A-mClover3}}$ (Ara-KI) and *arb*$^{\text{FLAG-2A-mClover3}}$ (Arb-KI), which express two distinct proteins, an epitope (3xFLAG)-tagged Ar and a green fluorescent protein (mClover3), from endogenous *ar* loci (Ogino et al., 2023). These strains enable the visualization of the expression patterns of Ara and Arb with green fluorescence and their intracellular localization by immunohistochemistry using an anti-FLAG antibody. For example, prominent green fluorescence and nuclear localization of Arb have been observed in the developing papillary processes of the anal fin. This is likely why *arb* KO males lack papillary processes (Ogino et al., 2023). Such differences in the expression patterns and the molecular property of Ar ohnologs indicate that they have distinct *cis*-reguatory sequences and substitution in protein coding sequences. In fact, the *cis*-regulatory sequences that are conserved in the 5′ upstream regions of medaka, stickleback, and fugu *ara* genes are not conserved

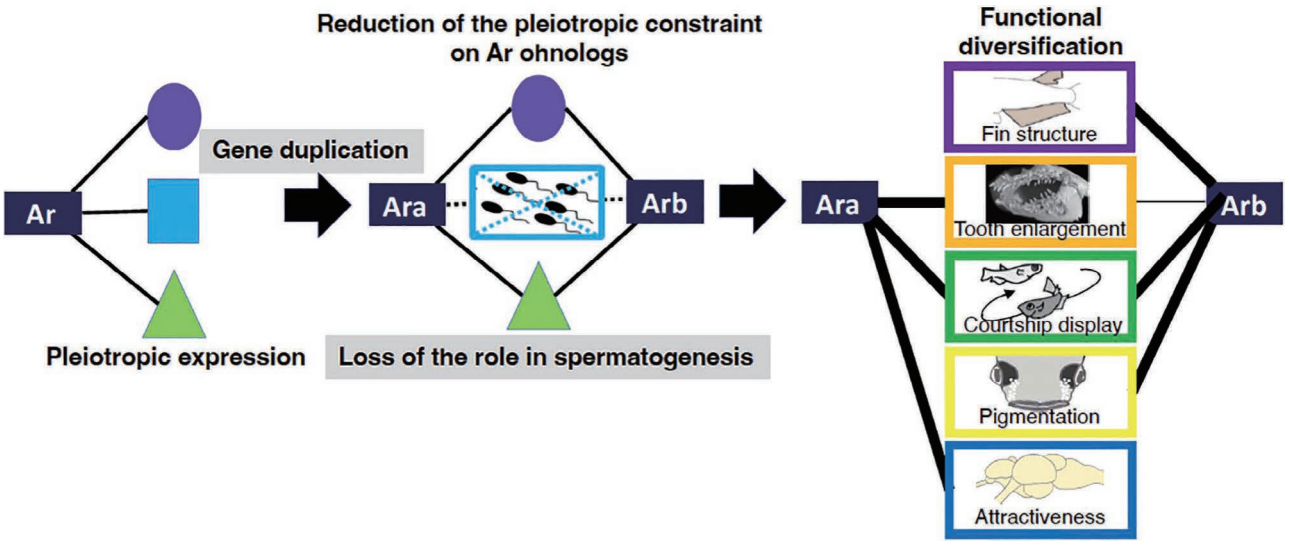

Fig. 3. Evolutionary scenario for the acquisition of male-specific sexual characteristics through *ar* gene duplication. It is thought that the increase in the copy number of the *ar* gene due to TSGD and the relaxation of evolutionary constraints due to the loss of its role in spermatogenesis serve as driving forces for the evolution of sexual characteristics in Japanese medaka.

in those of medaka *arb* gene and zebrafish *ar* gene (Ogino et al., 2023). An Ara-specific substitution has been identified in the the nuclear localization signal (NLS) that contacts the NLS binding proteins such as the importin α proteins (Ogino et al., 2016). These findings indicate that differences in the regulation of both transcription and subcellular localization of Ars may result in the regulation of different target genes and determine functional differences in Ar ohnologs. Chromatin immunoprecipitation sequence analysis of target genes for each Ar ohnolog using these epitope-tagged Ar-KI medaka lines will provide a more detailed molecular mechanism of the functional differences of Ar ohnologs in vivo. Further analyses targeting the evolutionary acquisition of Ar-target genes as well as the changes in *ar* gene function will enable us to understand the mechanisms of the evolutionary diversification of sexual characteristics driven by *ar* gene duplication.

The evolutionarily conserved and diverged functions of *ar* genes affecting sexual characteristics among vertebrates

In Japanese medaka, not only single *ar* KOs (*ara* KO and *arb* KO) but also the double KO (*ar* DKO) has been established (Ogino et al., 2023), which enables the evolutionary comparison of *ar* function among vertebrates. Japanese medaka *ar* DKO males show a female-like appearance with shorter anal fin rays without any papillary processes or lack of tooth elongation. The *ar* DKO males could not breed successfully with females under natural mating, with mostly abolished courtship displays, whereas the single KO males displayed courtships, indicating the functional redundancy of *ar* ohnologs for courtship displays (Ogino et al., 2023). In rodents, *AR* KO resulted in the ablation of male-typical sexual and aggressive behaviors (Sato et al., 2004). These findings indicate that androgens play an essential role in male sexual behavior in vertebrates.

Unexpectedly, *ar* DKO can produce functional sperms. The frequency of sperm movement, average speed of sperm, and in vitro fertilization rate were not significantly different from those of wild type males; moreover, total sperm number is higher in *ar* DKO than wild-type in Japanese medaka (Ogino et al., 2023). In contrast, *AR* KO mice exhibit spermatogenesis arrest at the pachytene spermatocyte stage (Yeh et al., 2002), and *ar* KO zebrafish exhibit a decreased number of mature sperms (Tang et al., 2018; Yu et al., 2018). Japanese eel spermatogenesis is stimulated by 11KT in cultured testes (Miura et al., 1991). Furthermore, previous studies in African cichlid showed that the *ar* DKO possess small testes (Alward et al., 2020; Hoadley et al., 2022) although it has not been investigated whether spermatogenesis proceeds normally. Given these situations, the essentiality of *ar* ohnologs in spermatogenesis has been lost at least in the lineage leading to medaka. The loss of their function in spermatogenesis and gene duplication likely resolved the pleiotropic function derived from their ancestral gene, which reduced the evolutionary constraints on *ar* ohnologs and accelerated the acquisition of exaggerated male-specific traits in medaka (Fig. 3). The medaka species are widely distributed in southern and southeast Asia and they have acquired diverse male-specific sexual characteristics such as fin outgrowth, pigmentation, and reproductive behavior (Ono and Uematsu, 1957; Yamamoto and Egami, 1974; Mokodongan and Yamahira, 2015; Nishiike et al., 2021).

Androgen-mediated immunomodulation in teleost fishes

It is widely known that vertebrates show sex differences in several aspects of immunity (Roved et al., 2017; Yang et al., 2022). For instance, the susceptibility of the immune system to sex steroid hormones is largely responsible for sex differences in fish immunity (Chavez-Pozo et al., 2018). Among teleost fishes, the expression of *ar* in the major immune organs, namely the head kidney, liver, and spleen, has been reported in salmonids, gilthead seabream, and zebrafish (Slater et al., 1995; Hossain et al., 2008; Aguila et

al., 2013). Teleost immune relevant leukocytes, namely acidophilic granulocytes and macrophages, have also been reported to express *ar* (Sanchez-Hernandez et al., 2014). These observations indicate that androgen signaling exhibits diverse effects on fish immunity.

Studies in various teleost species identified both positive and negative effects of androgen on immunity. In gilthead seabream, enhanced phagocytic activity and accumulation of immune-related cytokines, peroxidases, and complement activity have been reported in response to T and 11KT (Chaves-Pozo et al., 2003; Cuesta et al., 2007; Aguila et al., 2013; Castillo-Briceno et al., 2013). These features represent enhanced innate immunity against diverse pathogens, some of which are possibly influenced by androgen signaling. In contrast, studies on other fish species have depicted androgens as immunosuppressors, as shown below. Leukocytes in salmonid species express *ar* and are sensitive to T and its derivatives, but respond negatively in that leukocyte populations decrease in response to direct exposure to physiological levels of T (Slater et al., 1995; Slater and Schreck, 1997). In addition, the intraperitoneal injection of androgen derivatives including 11KT to common carp suppresses phagocytosis and ROS activities of macrophages (Watanuki et al., 2002). Rainbow trout B lymphocytes, which are key mediators of humoral adaptive immunity, were also affected by T or 11KT treatment and showed reduced secretion of IgM against lipopolysaccharides in the liver and spleen (Hou et al., 1999).

The negative effects of androgen on the immune system are in agreement with the immunocompetence handicap hypothesis (ICHH), as stated by Folstad and Karter (1992). The ICHH states the existence of a trade-off between the development of secondary sexual characteristics and immunity due to the immunosuppressive effect of androgens. In birds, individuals with more developed secondary sexual traits have a higher parasite load (Folstad and Karter, 1992). Similar examples have been observed in teleost fishes, such as arctic charr, stickleback, and cyprinids (Folstad and Karter, 1992; Liljedal et al., 1999; Kurtz et al., 2007; Rohlenova and Simkova, 2010).

However, some studies have questioned the ICHH. For example, the following results are inconsistent with this hypothesis. In the tench (*Tinka tinka* L.), T administration repressed the immune respons, whereas the immune response stimulated by β-glucan did not affect plasma T levels (Vainikka et al., 2005). Also, tested immunological parameters had only weak or no correlation with T levels to support ICHH in humans, bluegill, or common carp (Saha et al., 2003, 2004; Loggie et al., 2018; Nowak et al., 2018). In the bluegill, 11KT implantation reduced the expression of immune-related genes (Partridge et al., 2014). However, the stimulation of immune response with vaccination composed of formalin-inactivated *Vibrio anguillarum* and *V. ordalii* did not decrease the circulating 11KT level (Loggie et al., 2018). These results imply that additional androgens could down-regulate immune-related gene expressions, while the immune response induced by infection does not influence circulating androgen levels.

These reports imply that there is no absolute enhancement or suppression of immunity solely due to the action of androgens. However, immunity encompasses a wide range of self-defense systems, and the above-discussed studies varied in the depiction of immune-related features, as well as the species. To gain comprehensive insight into the influence of androgens and their receptors on immune response, it is imperative to systematically dissect and classify the

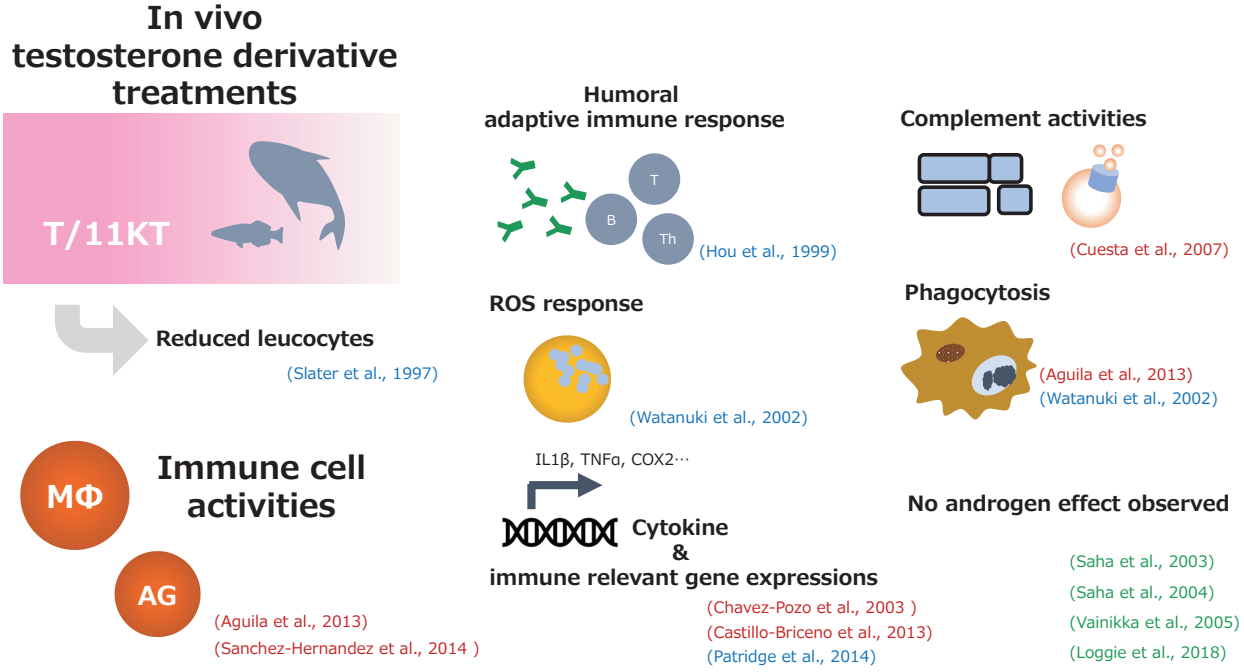

Fig. 4. Androgen-mediated immune modulation. Schematic diagram of androgen-mediated immune modulation in teleost fish. In both in vivo and in vitro studies, the red letters represent enhanced modulation, whereas the blue letters represent suppressive modulation. The lists of references list previous studies.

diverse facets of the immune system. To the best of our knowledge, most studies on the effects of androgens on teleost immunity have focused on the innate immune system (Fig. 4). The *ar* ohnologs with distinct molecular properties could have contributed to the diversification of the immune system in teleost fishes. Further studies on the effect of androgens on teleost adaptive immunity, considering functional differences in *ar* ohnologs, are highly anticipated.

CONCLUSION

The diversity of the teleost fishes accounts for the majority of the diversity of currently existing vertebrates. TSGD was the key event that formed the foundation of teleost radiation. In addition to sex-linked genes, evidences showed that the Ar is responsible for sexual dimorphism by regulating the susceptibility to androgens. The emergence of duplicated genes with overlapping roles that allow organisms to be phenotypically stable may relax selection pressures and enable innovation. Thus, duplicated *ara* and *arb* ohnologs may have had a significant effect on the emergence of variations in reproductive traits. One representative example is the medaka *ar* ohnologs, wherein the *arb* is likely to have been biased to maintain ancestral function under the stronger pressure of sexual selection compared to *ara* which has functionally diverged in each species. Interestingly, the *ar* ohnologs of medaka lost their essentiality in spermatogenesis derived from their ancestral gene, which can be thought to resolve pleiotropic constraints on genes. The functional diversification of *ar* ohnologs could have been promoted by the loosened constraint on *ar* ohnologs by gene duplication and following the loss of the ancestral gene function.

To elucidate the whole function of Ar, further studies on other teleost species and non-teleost ray-finned fishes are required for comparison; studies should give deeper attention to the functional differences of *ar* ohnologs. Fortunately, most teleost fish lineages based on their phylogeny are present to date. The availability of wide lineages and recent advancements in genome information render teleost fish suitable models for genetic comparisons. Since genetic analysis methods such as KO and knock-in studies are available for many teleost fishes, cutting-edge molecular biology techniques could be applied to these models. Hence, teleost fish are ideal models for comparative studies to understand the molecular mechanisms underlying the evolutionary diversification of sexual traits. Studies using epitope-tagged Ar-KI medaka lines indicate that differences in the regulation of both the transcription and intracellular localization of Ars can be possible determinants of the functional differences of Ar ohnologs. Further studies to elucidate the gene regulatory mechanisms of *ar*s with higher resolution, and chromatin immunoprecipitation sequencing analysis of their target genes for Ar ohnologs using Ar-KI lines will enable us to understand the genetic changes underlying the evolutionary novelty of sexual traits regulated by androgen signaling. The expression of Ar ohnologs in immune-related cells and the positive and/or negative effects of androgens on the immune system suggest that androgen signaling can cause sex differences in immune responses and possibly affect individual survival. In conclusion, from external phenotypes such as fin elongation to the internal phenotype exemplified by immune responses, variations in reproductive and survival strategies imply that Ar contributes to the evolutionary radiation of teleost fishes.

ACKNOWLEDGMENTS

We thank Kohei Ohta (Kyushu University) for discussions. This study was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and the Japan Society for the Promotion of Science (JSPS, grant numbers JP15K07138, JP19K06741, JP20H04928, and JP16H06280 to YO, and JP19H03049 and JP22H00386 to KO). This work was also supported by the Astellas Foundation for Research on Metabolic Disorders, The Naito Foundation, NIBB Collaborative Research Program (17-317, 18-324, 19-309, 20-309, 21-203, 22-309), the 2nd Women Researchers Promotion Program, AY2016 Support for Childbirth and Childcare in Women Researchers Promotion Program, Support for Women Returning from Maternity, Parental Leave Program and SENTAN-Q program at Kyushu University to YO, and grants from the Ministry of the Environment, Japan to TI.

COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Funding acquisition, YO and TI; Writing—original draft, TR and YO; Writing— review and editing, TR, KO, SA, MN, AK, TI, and YO.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online. (URL: https://doi.org/10.2108/zs230098)

Supplementary Table S1. Accession numbers of the sequences used for the phylogenetic analysis.

REFERENCES

Aguila S, Castillo-Briceno P, Sanchez M, Cabas I, Garcia-Alcazar A, Meseguer J, et al. (2013) Specific and non-overlapping functions of testosterone and 11-ketotestosterone in the regulation of professional phagocyte responses in the teleost fish gilthead seabream. Mol Immunol 53: 218–226

Alward BA, Laud VA, Skalnik CJ, York RA, Juntti SA, Fernald RD (2020) Modular genetic control of social status in a cichlid fish. Proc Natl Acad Sci U S A 117: 28167–28174

Andersson M (1994) Sexual Selection. Princeton University Press, Princeton, NJ

Ansai S, Mochida K, Fujimoto S, Mokodongan DF, Sumarto BKA, Masengi KWA, et al. (2021) Genome editing reveals fitness effects of a gene for sexual dichromatism in Sulawesian fishes. Nat Commun 12: 1350

Arnegard ME, McIntyre PB, Harmon LJ, Zelditch ML, Crampton WG, Davis JK, et al. (2010) Sexual signal evolution outpaces ecological divergence during electric fish species radiation. Am Nat 176: 335–356

Baker ME (1997) Steroid receptor phylogeny and vertebrate origins. Mol Cell Endocrinol 135: 101–107

Baker ME, Nelson DR, Studer RA (2015) Origin of the response to adrenal and sex steroids: Roles of promiscuity and co-evolution of enzymes and steroid receptors. J Steroid Biochem Mol Biol 151: 12–24

Bertrand S, Brunet FG, Escriva H, Parmentier G, Laudet V, Robinson-Rechavi M (2004) Evolutionary genomics of nuclear receptors: from twenty-five ancestral genes to derived endocrine systems. Mol Biol Evol 21: 1923–1937

Bolaffi JL, Lance V, Callard IP, Walsh JM, Idler DR (1979) Identification of 11-ketotestosterone, 11 beta-hydroxytestosterone, and testosterone in plasma of *Necturus maculosus* (Rafinesque). Gen Comp Endocrinol 38: 127–131

Borg B (1994) Androgens in teleost fishes. Comp Biochem Physiol

109C: 219–245
Borg B, Mayer I (1995) Androgens and behaviour in the three-spined stickleback. Behaviour 132: 1025–1035
Bridgham JT, Eick GN, Larroux C, Deshpande K, Harms MJ, Gauthier ME, et al. (2010) Protein evolution by molecular tinkering: diversification of the nuclear receptor superfamily from a ligand-dependent ancestor. PLoS Biol 8: e1000497
Castillo-Briceno P, Aguila-Martinez S, Liarte S, Garcia Alcazar A, Meseguer J, Mulero V, et al. (2013) In situ forming microparticle implants for delivery of sex steroids in fish: Modulation of the immune response of gilthead seabream by testosterone. Steroids 78: 26–33
Chaves-Pozo E, Pelegrin P, Mulero V, Meseguer J, Garcia Ayala A (2003) A role for acidophilic granulocytes in the testis of the gilthead seabream (*Sparus aurata* L., Teleostei). J Endocrinol 179: 165–174
Chaves-Pozo E, Garcia-Ayala A, Cabas I (2018) Effects of sex steroids on fish leukocytes. Biology (Basel) 7: 9
Cox RM, Calsbeek R (2009) Sexually antagonistic selection, sexual dimorphism, and the resolution of intralocus sexual conflict. Am Nat 173: 176–187
Crowder CM, Lassiter CS, Gorelick DA (2018) Nuclear androgen receptor regulates testes organization and oocyte maturation in zebrafish. Endocrinology 159: 980–993
Cuesta A, Vargas-Chacoff L, Garcia-Lopez A, Arjona FJ, Martinez-Rodriguez G, Meseguer J, et al. (2007) Effect of sex-steroid hormones, testosterone and estradiol, on humoral immune parameters of gilthead seabream. Fish Shellfish Immunol 23: 693–700
Darwin C (1871) The Descent of Man, and Selection in Relation to Sex. J Murray, London, UK
Douard V, Brunet F, Boussau B, Ahrens I, Vlaeminck-Guillem V, Haendler B, et al. (2008) The fate of the duplicated androgen receptor in fishes: a late neofunctionalization event? BMC Evol Biol 8: 336
Egami N, Ishii S (1956) Sexual differences in the shape of some bones in the fish, *Oryzias latipes*. J Fac Sci, Tokyo Univ IV: 563–571
Escriva H, Delaunay F, Laudet V (2000) Ligand binding and nuclear receptor evolution. Bioessays 22: 717–727
Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240: 889–895
Folstad I, Karter AJ (1992) Parasites, bright males, and the immunocompetence handicap. Am Nat 139: 603–622
Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J (1999) Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151: 1531–1545
Griffin JE (1992) Androgen resistance--the clinical and molecular spectrum. N Engl J Med 326: 611–618
Hau M (2007) Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. Bioessays 29: 133–144
He WW, Kumar MV, Tindall DJ (1991) A frame-shift mutation in the androgen receptor gene causes complete androgen insensitivity in the testicular-feminized mouse. Nucleic Acids Res 19: 2373–2378
Hoadley AP, Fernald RD, Alward BA (2022) Control of testes mass by androgen receptor paralogs in a cichlid. J Comp Physiol B 192: 107–114
Hoar WS (1962) Hormones and the reproductive behaviour of the male three-spined stickleback (*Gasterosteus aculeatus*). Anim Behav 10: 247–266
Hoegg S, Brinkmann H, Taylor JS, Meyer A (2004) Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. J Mol Evol 59: 190–203
Hossain MS, Larsson A, Scherbak N, Olsson PE, Orban L (2008) Zebrafish androgen receptor: isolation, molecular, and biochemical characterization. Biol Reprod 78: 361–369
Hou Y, Suzuki Y, Aida K (1999) Effects of steroids on the antibody producing activity of lymphocytes in rainbow trout. Fisheries Science 65: 850–855
Huang BF, Sun YL, Wu FR, Liu ZH, Wang ZJ, Luo LF, et al. (2011) Isolation, sequence analysis, and characterization of androgen receptor in Southern catfish, *Silurus meridionalis*. Fish Physiol Biochem 37: 593–601
Ikeuchi T, Todo T, Kobayashi T, Nagahama Y (1999) cDNA cloning of a novel androgen receptor subtype. J Biol Chem 274: 25205–25209
Imamichi Y, Yuhki KI, Orisaka M, Kitano T, Mukai K, Ushikubi F, et al. (2016) 11-Ketotestosterone is a major androgen produced in human gonads. J Clin Endocrinol Metab 101: 3582–3591
Inoue JG, Miya M, Tsukamoto K, Nishida M (2003) Basal actinopterygian relationships: a mitogenomic perspective on the phylogeny of the “ancient fish”. Mol Phylogenet Evol 26: 110–120
Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, et al. (2004) Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate protokaryotype. Nature 431: 946–957
Juntti SA, Tollkuhn J, Wu MV, Fraser EJ, Soderborg T, Tan S, et al. (2010) The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors. Neuron 66: 260–272
Kurtz J, Kalbe M, Langefors A, Mayer I, Milinski M, Hasselquist D (2007) An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. Am Nat 170: 509–519
Kitano J, Kakioka R, Ishikawa A, Toyoda A, Kusakabe M (2020) Differences in the contributions of sex linkage and androgen regulation to sex-biased gene expression in juvenile and adult sticklebacks. J Evol Biol 33: 1129–1138
Laudet V (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. J Mol Endocrinol 19: 207–226
Li C, Lu G, Orti G (2008) Optimal data partitioning and a test case for ray-finned fishes (Actinopterygii) based on ten nuclear loci. Syst Biol 57: 519–539
Liljedal S, Folstad I, Skarstein F (1999) Secondary sex traits, parasites, immunity and ejaculate quality in the Arctic charr. Proc R Soc B 266: 1893–1898
Loggie JW, Garner SR, Partridge CG, Dixon B, Knapp R, Neff BD (2018) A test of the effects of androgens on immunity: No relationship between 11-ketotestosterone and immune performance in bluegill (*Lepomis macrochirus*). Gen Comp Endocrinol 261: 1–8
Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. (1995) The nuclear receptor superfamily: the second decade. Cell 83: 835–839
Mank JE (2007) The evolution of sexually selected traits and antagonistic androgen expression in actinopterygiian fishes. Am Nat 169: 142–149
Matsumoto T, Takeyama K, Sato T, Kato S (2003) Androgen receptor functions from reverse genetic models. J Steroid Biochem Mol Biol 85: 95–99
McPhaul MJ (1999) Molecular defects of the androgen receptor. J Steroid Biochem Mol Biol 69: 315–322
Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991) Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc Natl Acad Sci U S A 88: 5774–5778
Mokodongan DF, Yamahira K (2015) Origin and intra-island diversification of Sulawesi endemic Adrianichthyidae. Mol Phylogenet Evol 93: 150–160
Nishiike Y, Miyazoe D, Togawa R, Yokoyama K, Nakasone K,

Miyata M, et al. (2021) Estrogen receptor 2b is the major determinant of sex-typical mating behavior and sexual preference in medaka. Curr Biol 31: 1699–1710 e1696
Nowak J, Pawlowski B, Borkowska B, Augustyniak D, Drulis-Kawa Z (2018) No evidence for the immunocompetence handicap hypothesis in male humans. Sci Rep 8: 7392
Ogino Y, Katoh H, Yamada G (2004) Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates. FEBS Lett 575: 119–126
Ogino Y, Katoh H, Kuraku S, Yamada G (2009) Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates. Endocrinology 150: 5415–5427
Ogino Y, Kuraku S, Ishibashi H, Miyakawa H, Sumiya E, Miyagawa S, et al. (2016) Neofunctionalization of androgen receptor by gain-of-function mutations in teleost fish lineage. Mol Biol Evol 33: 228–244
Ogino Y, Tohyama S, Kohno S, Toyota K, Yamada G, Yatsu R, et al. (2018) Functional distinctions associated with the diversity of sex steroid hormone receptors ESR and AR. J Steroid Biochem Mol Biol 184: 38–46
Ogino Y, Ansai S, Watanabe E, Yasugi M, Katayama Y, Sakamoto H, et al. (2023) Evolutionary differentiation of androgen receptor is responsible for sexual characteristic development in a teleost fish. Nat Commun 14: 1428
Ohno S (1970) Evolution of Gene Duplication. Springer Verlag, New York NY, USA
Ono Y, Uematsu T (1957) Mating ethogram in *Oryzias latipes*. J Fac Sci Hokkaido U Ser VI Zool 13: 197–202
Partridge C, Rodgers CMC, Knapp R, Neff BD (2015) Androgen effects on immune gene expression during parental care in bluegill sunfish (*Lepomis macrochirus*). Can J Zool 93: 9–13
Pasquier J, Cabau C, Nguyen T, Jouanno E, Severac D, Braasch I, et al. (2016) Gene evolution and gene expression after whole genome duplication in fish: the PhyloFish database. BMC Genomics 17: 368
Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS (1995) Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16: 271–321
Ravi V, Venkatesh B (2018) The divergent genomes of teleosts. Annu Rev Anim Biosci 6: 47–68
Rohlenova K, Simkova A (2010) Are the immunocompetence and the presence of metazoan parasites in cyprinid fish affected by reproductive efforts of cyprinid fish? J Biomed Biotechnol 2010: 418382
Roved J, Westerdahl H, Hasselquist D (2017) Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. Horm Behav 88: 95–105
Saha NR, Usami T, Suzuki Y (2003) A double staining flow cytometric assay for the detection of steroid induced apoptotic leucocytes in common carp (*Cyprinus carpio*). Dev Comp Immunol 27: 351–363
Saha NR, Usami T, Suzuki Y (2004) In vitro effects of steroid hormones on IgM-secreting cells and IgM secretion in common carp (*Cyprinus carpio*). Fish Shellfish Immunol 17: 149–158
Sanchez-Hernandez M, Arizcun M, Garcia-Alcazar A, Sarropoulou E, Mulero V, Garcia-Ayala A (2014) Fish granulocytes express a constitutively active androgen receptor variant. Dev Comp Immunol 45: 115–122
Sato T, Matsumoto T, Kawano H, Watanabe T, Uematsu Y, Sekine K, et al. (2004) Brain masculinization requires androgen receptor function. Proc Natl Acad Sci U S A 101: 1673–1678
Slater CH, Schreck CB (1997) Physiological levels of testosterone kill salmonid leukocytes in vitro. Gen Comp Endocrinol 106: 113–119
Slater CH, Fitzpatrick MS, Schreck CB (1995) Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. Gen Comp Endocrinol 100: 218–225
Sperry TS, Thomas P (1999) Characterization of two nuclear androgen receptors in Atlantic croaker: comparison of their biochemical properties and binding specificities. Endocrinology 140: 1602–1611
Takeo J, Yamashita S (1999) Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. J Biol Chem 274: 5674–5680
Tang H, Chen Y, Wang L, Yin Y, Li G, Guo Y, et al. (2018) Fertility impairment with defective spermatogenesis and steroidogenesis in male zebrafish lacking androgen receptor. Biol Reprod 98: 227–238
Thornton JW (2001) 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: 5671–5676
Thornton JW, Kelley DB (1998) Evolution of the androgen receptor: structure-function implications. Bioessays 20: 860–869
Vainikka A, Jokinen EI, Kortet R, Paukku S, Pirhonen J, Rantala MJ, et al. (2005) Effects of testosterone and beta-glucan on immune functions in tench. J Fish Biol 66: 348–361
Van Doorn G (2009) Intralocus sexual conflict. Ann N Y Acad Sci 1168: 52–72
Watanuki H, Yamaguchi T, Sakai M (2002) Suppression in function of phagocytic cells in common carp *Cyprinus carpio* L. injected with estradiol, progesterone or 11-ketotestosterone. Comp Biochem Physiol C 132: 407–413
Williams TM, Carroll SB (2009) Genetic and molecular insights into the development and evolution of sexual dimorphism. Nat Rev Genet 10: 797–804
Yamamoto M, Egami N (1974) Fine structure of the surface of the anal fin and the processes on its fin rays of male *Oryzias latipes*. Copeia 1974: 262–265
Yamashita J, Takeuchi A, Hosono K, Fleming T, Nagahama Y, Okubo K (2020) Male-predominant galanin mediates androgen-dependent aggressive chases in medaka. eLife 9: e59470
Yang C, Jin J, Yang Y, Sun H, Wu L, Shen M, et al. (2022) Androgen receptor-mediated CD8(+) T cell stemness programs drive sex differences in antitumor immunity. Immunity 55: 1747
Yeh S, Tsai MY, Xu Q, Mu XM, Lardy H, Huang KE, et al. (2002) 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: 13498–13503
Yokoi S, Okuyama T, Kamei Y, Naruse K, Taniguchi Y, Ansai S, et al. (2015) An essential role of the arginine vasotocin system in mate-guarding behaviors in triadic relationships of medaka fish (*Oryzias latipes*). PLoS Genet 11: e1005009
Yong L, Thet Z, Zhu Y (2017) Genetic editing of the androgen receptor contributes to impaired male courtship behavior in zebrafish. J Exp Biol 220: 3017–3021
Yu G, Zhang D, Liu W, Wang J, Liu X, Zhou C, et al. (2018) Zebrafish androgen receptor is required for spermatogenesis and maintenance of ovarian function. Oncotarget 9: 24320–24334
Zauner H, Begemann G, Mari-Beffa M, Meyer A (2003) Differential regulation of msx genes in the development of the gonopodium, an intromittent organ, and of the “sword”, a sexually selected trait of swordtail fishes (*Xiphophorus*). Evol Dev 5: 466–477
Zheng Z, Armfield BA, Cohn MJ (2015) Timing of androgen receptor disruption and estrogen exposure underlies a spectrum of congenital penile anomalies. Proc Natl Acad Sci U S A 112: E7194–7203

(Received October 6, 2023 / Accepted January 15, 2024 /
Published online February 14, 2024)