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Development of Ovaries and Sex Change in Fish: Bringing Potential into Action

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Abstract
Background: Encompassing about half of the 60,000 species of vertebrates, fish display the greatest diversity of sex determination mechanisms among metazoans. As such that phylum offers a unique playground to study the impressive variety of gonadal morphogenetic strategies, ranging from gonochorism, with either genetic or environmental sex determination, to unisexuality, with either simultaneous or consecutive hermaphroditism. ***Summary:*** From the two main types of gonads, the ovaries embrace the important role to produce the larger and non-motile gametes, which is the basis for the development of a future organism. The production of the egg cells is complex and involves the formation of follicular cells, which are necessary for the maturation of the oocytes and the production of feminine hormones. In this vein, our review focuses on the development of ovaries in fish with special emphasis on the germ cells, including those that transition from one sex to the other as part of their life cycle and those that are capable of transitioning to the opposite sex depending on environmental cues. ***Key messages:*** Clearly, establishing an individual as either a female or a male is not accomplished by the sole development of two types of gonads. In most cases, that dichotomy, be it final or transient, is accompanied by coordinated transformations across the entire organism, leading to changes in the physiological sex as a whole. These coordinated transformations require both molecular and neuroendocrine networks, but also anatomical and behavioural adjustments. Remarkably, fish managed to tame the ins and outs of sex reversal mechanisms to take the most advantages of changing sex as adaptive strategies in some situations.

Introduction

Generating sexually dimorphic adult individuals with respective reproductive organs is probably one of the most pervasive features of animal development, morphology, physiology, and behaviour. Intriguingly, the apparently conserved initial ontogenetic process that triggers a bipotential gonad anlage to develop into either a testis or an ovary, relies on astonishingly plastic, complex,

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tightly regulated, and rarely conserved stochastic regulatory networks [Bachtrog et al., 2014; Herpin and Schartl, 2015; Pan et al., 2021]. Strikingly, even among very closely related species that do not display any noticeable morphological, histological or cell biological differences, sex determination schemes and gonadal morphogenetic mechanisms can be substantially different [DeFalco and Capel, 2009; Bachtrog et al., 2014; Myosho et al., 2015]. While organismic biologists want to decipher the "ultimate" causes leading to transitions of sex determination mechanisms or systems between lineages, molecular biologists focus on the so-called "proximate causes" of these diverse mechanisms in order to understand how they set up and interact to bring about such an amazing plasticity of the respective genetic networks.

Encompassing about half of the 60,000 species of vertebrates, fish display the greatest diversity of sex determination mechanisms among metazoans [Bachtrog et al., 2014; Pan et al., 2016]. As such, fish offer a unique playground to study the impressive variety of gonadal morphogenetic strategies, ranging from gonochorism, with either genetic or environmental sex determination (GSD and ESD, respectively) to unisexuality, with either simultaneous or consecutive hermaphroditism [Devlin and Nagahama, 2002; Nagahama et al., 2021]. Changing sex, either as a response to external environmental stimuli or inherently as part of the life cycle, demonstrates that sexual identity of the gonadal soma is a non-static state, resulting from the dynamic equilibrium reached by the antagonistic male and female gene regulatory networks [Brennan and Capel, 2004; DeFalco and Capel, 2009]. Clearly, establishing an individual as either a female or a male is not accomplished by the sole development of two types of gonads. In most cases, that dichotomy, be it final or transient, is accompanied by coordinated transformations across the entire organism, resulting in changes in the physiological sex as a whole. These coordinated transformations require both molecular and neuroendocrine networks, but also anatomical and behavioral adjustments. Remarkably, fish managed to tame the ins and outs of sex reversal mechanisms to take the most advantages of changing sex as adaptive strategies in some situations. Despite this great sex plasticity, the ancestral feature of producing two types of gametes, sperm in males and eggs in females, is still present. Hence, any morphogenic strategy that varies from gonochorism in fish is derived from that ancestral feature, and the process of changing sex is the (necessary?) transition from the production of one type of reproductive cell to the other.

From the two main types of gonads, the ovaries embraced the important role to produce the larger and non-motile gamete, which is the basis for the development of a future organism [Kimble and Page, 2007]. The production of the egg cells is complex and involves the formation of follicular cells, which are necessary for the maturation of the oocytes and the production of feminine hormones [Guiguen et al., 2010]. In this vein, our review will focus on the development of ovaries in fish with special emphasis on the germ cells (see Box 1), including species that change sex as part of their life cycle and those that are capable of transitioning to the opposite sex depending on environmental cues.

Box 1. From primordial germ cells to germ cells

The notion of "germ cells" embraces all cells belonging to the germline at all stages of development. Nevertheless, being subjected to an active developmental process, their differentiation states, fates or behaviours delineate different phases of an overall progressive commitment. Hence, segregated from a subset of cells of the somatic lineage, sometimes referred as presumptive primordial germ cells (pPGCs), the first morphologically distinguishable germ cells are referred as primordial germ cells (PGCs). Distinctive nuclear morphology, specific gene expression or presence of a unique electron-dense cytoplasmic structure (better known as "germ plasm" in certain organisms), but also an active migration toward the somatic part of the primordial forming gonad are the typical hallmarks of the newly differentiated PGCs. Once embedded into the primordial (and undifferentiated) gonad, PGCs will alternate between quiescence and mitotic phases. Concomitantly with the male or female commitment of the somatic part of the primordial gonad, these PGCs will further differentiate into gonocytes that are characterized by their intimate relationship together with the somatic supporting cells of the gonad. These gonocytes will then resume either type I (that is characteristic of stem type germ cells) or type II divisions (see Fig. 2). Alternatively, after meiotic entry, gonocytes might also give rise to either oogonia, oocytes, and eggs or spermatogonia, spermatocytes, and sperm in female or male gonads, respectively. Interestingly, data suggesting that XX and XY "PGCs" possess different characters even before gonadal primordium formation and the onset of sex determination by gonadal somatic cells (in fish [Nishimura et al., 2014] and mammals [Jameson et al., 2012]) might point out another "phase" of their commitment occurring in between PGCs and germ cell states.

Ovarian Development in Gonochoristic Species: The Medaka Model

With respect to mechanisms of gonadal sexual dimorphism, fishes from the *Oryzias* genus are certainly the best studied gonochoristic fish species. Both XY and ZW systems are found within this group displaying undifferenti-

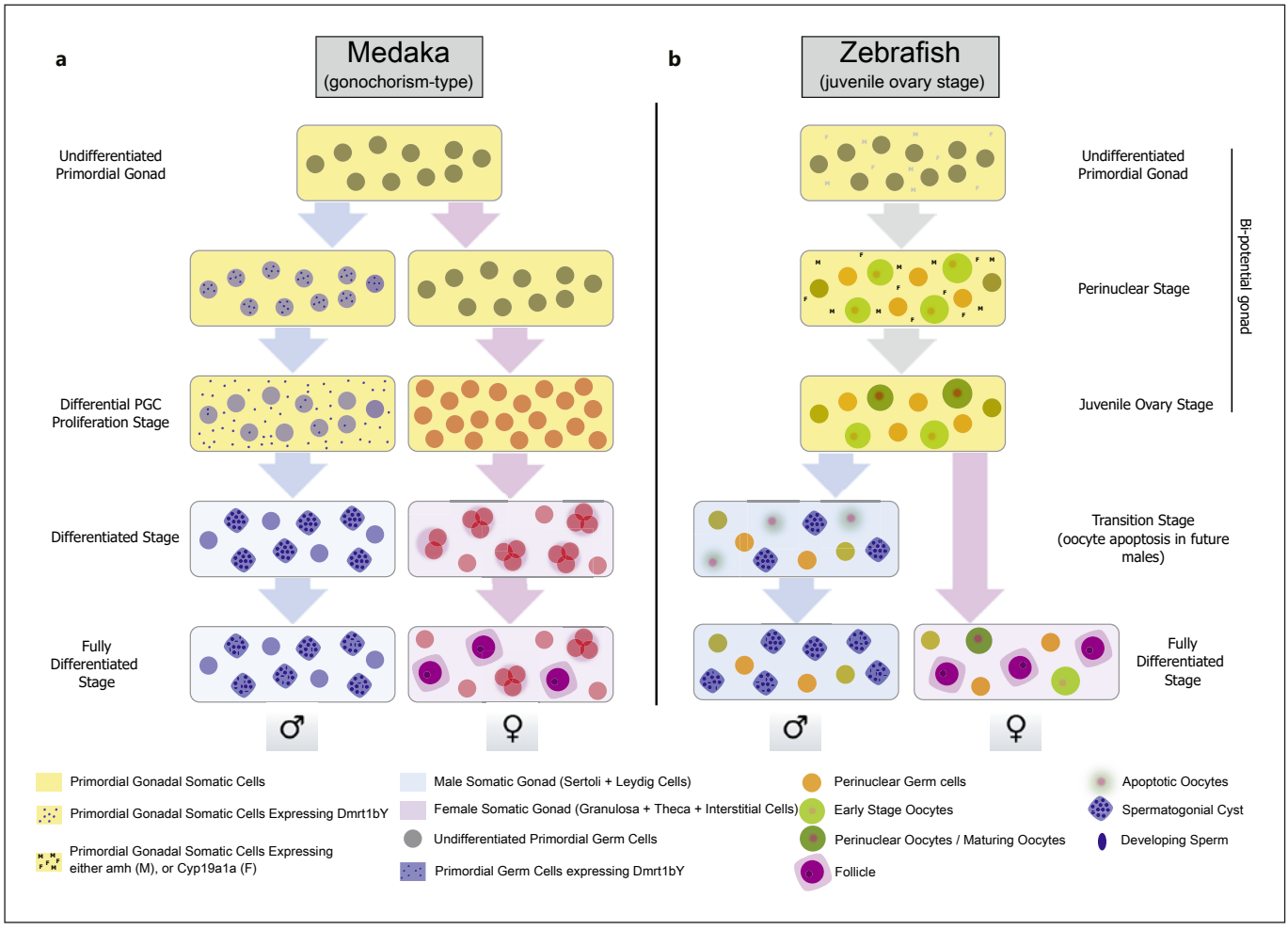

Fig. 1. Comparative overview of the main stages of early gonadal commitments and morphogenesis in medaka (*Oryzias latipes*) (**a**) and zebrafish (*Danio rerio*) (**b**).

ated (homomorphic) sex chromosomes [Takehana et al., 2007a, b]. Despite having different genetic sex determinants (Dmrt1bY [also known as "dmy"], *Oryzias latipes* [Matsuda et al., 2002; Nanda et al., 2002] and *Oryzias curvinotus* [Kondo et al., 2003]; GsdfY, *Oryzias luzonensis* [Myosho et al., 2012]; and Sox3Y, *Oryzias dancena* [Takehana et al., 2014]) and likely different plastic genetic regulatory networks underlying gonadal ontogeny amongst these closely related *Oryzias* species, their adult gonads are however very similar in morphology, cellular organization, and physiology.

In contrast to the complexity of gonadal development in mammals and birds (see other reviews in this issue), the histological structures involved in gonadal formation are relatively simple in medaka fish (Fig. 1a). Indeed, both female and male medaka gonads are initially established by the coordinated development of two distinct cell lineages: (1) the germ cells, and (2) the somatic gonadal mesoderm surrounding the germ cells. Primordial germ cells (PGCs) are specified with the inheritance of cytoplasmic determinants, including specific RNAs and proteins (the so-called "germ plasm"), on cellularization (cf. preformation in contrast to the epigenesis reported in mammals for example; see Extavour and Akam [2003] for review and Box 1). Then PGCs remain closely associated with endodermal tissues and then migrate via the dorsal gut mesentery to the region of presumptive gonad [Nakamura et al., 2006; Herpin et al., 2008]. On the other hand, the embryological origin of the somatic cells of the gonadal anlage is distinct from that of PGCs [Nakamura et al., 2006]. The epithelium of the genital ridge mainly develops the external somatic layer of the developing gonads.

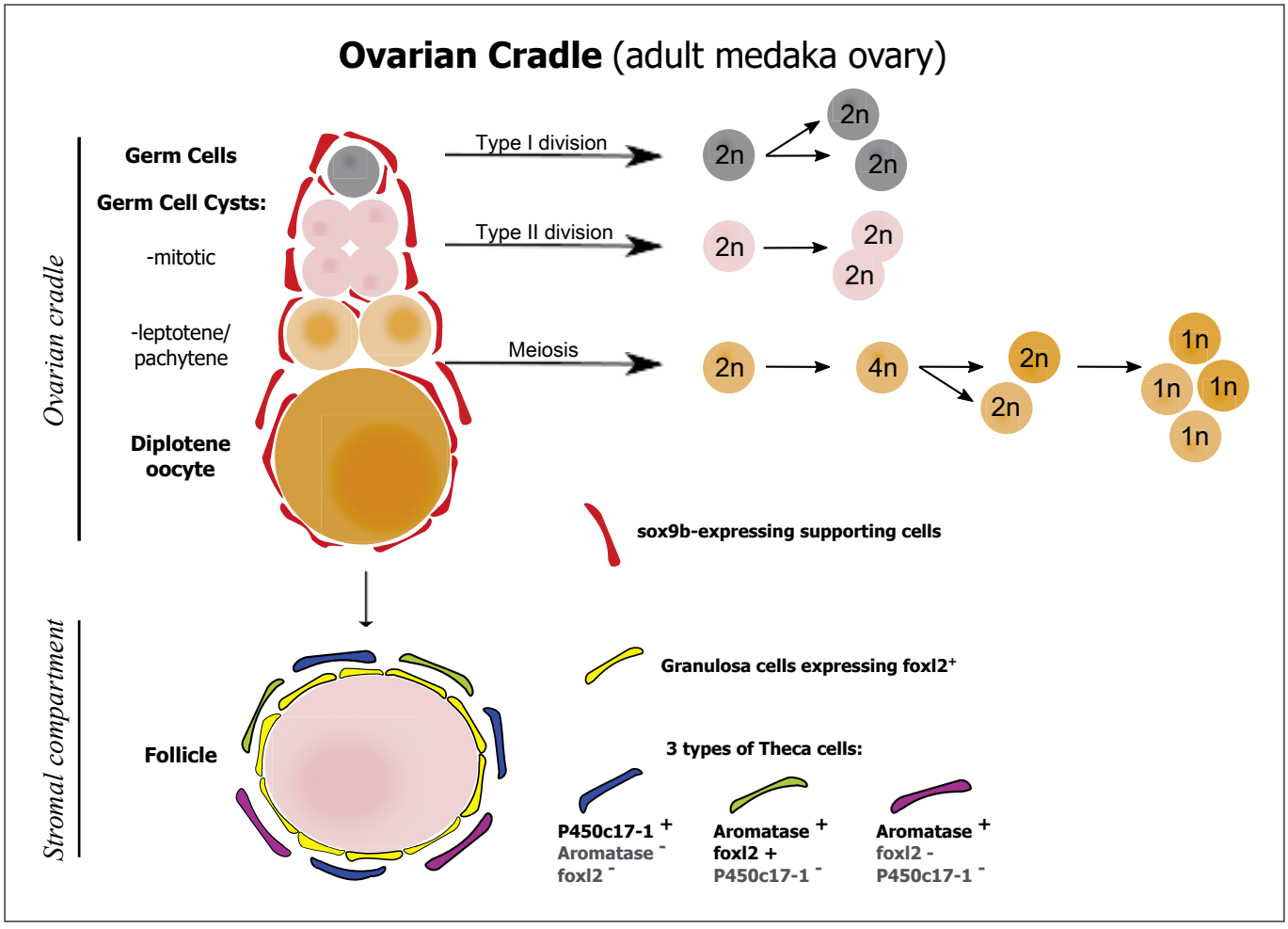

Fig. 2. Early medaka oogenesis occurring within the "germinal cradle." In medaka ovary, oogenesis occurs within a functional unit called the germinal cradle [Nakamura et al., 2010] composed of intermingled *sox9b*-expressing supporting cells and germ cells. First, germ cells or oogonia initially undergo type I proliferation. Further on, cystic type of germ cells adopt a type II proliferation scheme and eventually subsequently perform meiosis. These diplotene oocytes then exit the cradle and form follicles. In follicles of medaka, like in mammals, granulosa cells express both *foxl2* and aromatase. The strict co-expression of *Foxl2* and *Aromatase* (*Cyp19*) in the mammalian ovary led to the further demonstration that Foxl2 is involved in the regulation of estrogen synthesis via direct transcriptional upregulation of ovarian-type Aromatase [for review, see Pannetier et al., 2006]. Surprisingly, in medaka aromatase-only positive theca cells that remained foxl2-negative are found within the thecal layer [Nakamura et al., 2009; Herpin et al., 2013]. In that perspective, it is interesting to note that birds also have multiple populations of theca cells some of which are also steroidogenic [Nitta et al., 1991]. In contrast to the main consensus, the discordance of spatial expression patterns of Foxl2 and ovarian-type aromatase (Cyp19a1) calls into question an exclusive transcriptional regulation of cyp19a1 by foxl2 in the ovary of medaka. In type I division (stem-cell like division), germ cells divide completely, generating two daughter cells. In type II division (gametogenesis-committed cystic division), germ cells remain connected via intercellular bridges [Saito et al., 2007].

In addition, similar to mammals, mesenchymal cells probably also contribute to the formation of some somatic cells. Interestingly, the undifferentiated gonads of most vertebrates present two portions of the tissue, one more external, called cortex, and another named medulla. It is widely accepted that most of the ovarian somatic cells originate from the cortex, while the testis has its origin from the medulla with important mesonephric contribution. However, different from mammals, fish present only germinal epithelium with no clear separation between medullary and cortical tissues in the gonad [Devlin and Nagahama, 2002] (Fig. 1).

The epithelial layer appears to be the origin of most somatic cells in both presumptive male and female go-

nads. The PGCs, on the other hand, migrate into the germinal ridge, in which gametogenesis takes place, concomitant with the migration and differentiation of somatic cells. Shortly before hatching (stages 38/39) [Iwamatsu, 2004], at the peak of expression of *dmrt1bY/dmy* (the male-specific master sex-determining gene in *O. latipes*) in the male gonad primordium, the germ cells in the female gonad first actively proliferate and then undergo meiosis, a process not observed in male gonads [Kobayashi et al., 2004] (Fig. 1a). In detail, two patterns of PGC divisions (type I and type II) [Saito et al., 2007] have been described (Fig. 2): (1) Type I PGCs self-renew in a typical linear division scheme that is characteristic of stem cells. All daughter cells are identical to one another, dispatched all over the primordial gonad, and surrounded by somatic supporting cells [Saito et al., 2007]; (2) Type II germ cells divide synchronously and successively, and remain interconnected by intercellular bridges that they form clusters due to incomplete cytokinesis (Fig. 2) [Saito et al., 2007]. Afterward, type II proliferating PGCs will eventually enter meiosis. In females, type II germ cell divisions are observed as early as 2 days after the primordial gonad is formed (around hatching stage), while males will continue with type I PGC proliferation until around 10–15 days post-hatching [Satoh and Egami, 1972]. Interestingly, prior to any somatic expression of *dmrt1bY/dmy* and far before any somatic gonadal dimorphism, germ cell-specific expressions of *dmrt1bY/dmy* and *sdgc* genes are reported as early as stage 33 in XY individuals [Nishimura et al., 2014]. This sexually different expression is induced in a germ cell-autonomous manner, since XX and XY germ cells exhibit different mitotic activities in vitro [Nishimura et al., 2014]. It is until 10 days later that the first somatic gonadal dimorphisms are apparent with the formation of the acinus (the seminiferous tubule precursor) and the follicles in gonads of males and females, respectively (Fig. 1a). Together, these data suggest that XX and XY germ cells possess different characters even before gonadal primordium formation/commitment and the onset of sex determination by gonadal somatic cells [Nishimura et al., 2014]. Interestingly, during the entire process of gonadal differentiation, *sox9b* expression is reported for both XX and XY supporting cells of the medaka bipotential primordial gonads, suggesting a common origin between Sertoli and granulosa cells [Nakamura et al., 2012a]. In XY individuals, *sox9b*-positive cells additionally express *dmrt1* as an indication of their further differentiation into Sertoli cells in the testes. In XX individuals, ovarian cords within the germinal epithelia are characterized as gonadal primordia, which in turn develop into ovarian structures [Nakamura et al., 2010]. These cords, composed of somatic *sox9b*-expressing cells and mitotic *nanos2*-expressing oogonia, continually give rise to germ cells and form a stem cell niche within the ovary referred to as germinal cradle [Nakamura et al., 2010] (Fig. 2). These cradles containing germline stem cells contribute to the production of fertile eggs and are reminiscent of the germanium of the *Drosophila* ovary, therefore indicating that these fundamental processes governing oogenesis are likely to be conserved across animal species. In addition, *sox9b* is already expressed in the somatic cells of the early gonads of males and females prior to sex determination, surrounding the PGCs [Nishimura and Tanaka, 2014].

Interestingly, the loss of the autosomal *dmrt1a* gene in XY medaka (*O. latipes*) leads to reprogramming of the sexual fate of the gonad. These individuals, although first developing testes according to their genotype (XY with *dmrt1bY/dmy*), soon fail to maintain their (male) gonadal fate due to the absence of the autosomal *dmrt1a* gene. Sertoli cells trans-differentiate to granulosa cells with upregulated level of Foxl2 expression, and eventually, a fully functional ovary develops instead of a testis which was first initiated [Masuyama et al., 2012].

After the onset and even completion of gonadal differentiation, a subset of medaka germ cells, the germline stem cells remain undifferentiated, and are located inside the germinal cradle (Fig. 2). Indeed, intensive research has revealed that sexual fate decision of the germ cells is governed by a germ cell autonomous switch gene, *foxl3* [Nishimura et al., 2015]. The expression of *foxl3* can be detected as early as 5 days post fertilization (dpf) in both male and female embryonic gonads. While *foxl3* expression halts in male gonads after hatching, it persists in female gonads. The cell-autonomous manner of regulation of *foxl3* on the sexual fate of germ cells was evident when mutant females of *foxl3* developed an ovary but produced fertile sperm, indicating that *foxl3* represses the initiation of spermatogenesis in a germ cell-autonomous manner [Nishimura et al., 2015]. Surprisingly, even though *foxl3* mutant females continue to produce sperm in the ovary, they switch to produce oocytes in sperm-filled mature ovary after 1–2 months after hatching [Nishimura et al., 2015]. This later switch of germ cell sexual fate suggests that oogenesis could take place in the absence of *foxl3* that represses spermatogenesis, and emphasizes again the malleability of (fish) sexual fate over the course of life in an individual. Recent studies in Nile tilapia demonstrated that the sexual fate of the germ cells is determined by the antagonistic interaction between *foxl3* (oogonia) and

dmrt1 (spermatogonia), and the response to environmental factors relies on the presence of those genes [Dai et al., 2021].

Taken together, these results suggest that even in species with a strict mono-factorial GSD system, the gonadal fate can still remain highly malleable. The signal from the sex determination gene for either repression or activation of male or female sex determination networks can be (easily) overridden by factors other than the original sex determination gene, including different genes, metabolic status or temperature, stress, hormones, and pollutants, being mainly mediated through either lipid or cortisol levels [for review, see Sakae and Tanaka, 2021].

Ovarian Development without Branded Master Sex-Determining Gene: The Zebrafish Model

In contrast to medaka, in which sex determination relies on a (relatively) straightforward XX/XY monofactorial genetic system, laboratory strains of zebrafish cope with a weak and complex polyfactorial genetic system involving four different chromosomes (*D. rerio* chromosomes 3, 4, 5, and 16) that is also highly susceptible to environmental modulators (hypoxia [Shang et al., 2006], high density [Lawrence et al., 2008], temperature [Abozaid et al., 2012; Ribas et al., 2017], altered thermocycles [Villamizar et al., 2012], poor nutrition [Liew and Orbán, 2014] or even gamma rays [Walker and Streisinger, 1983]). A consequence, likely resulting from the absence of any strong sex determiner, is that all embryonic gonads initially develop as presumptive ovaries [Takahashi, 1977]. For that reason, embryonic development of gonad in zebrafish is referenced as a case of protogynous juvenile hermaphroditism (also named juvenile intersex) [Uchida et al., 2002] (Fig. 1b). Interestingly, discrepancies in the duration of this juvenile hermaphroditism stage have been reported, ranging from 5 up to 11 weeks after fertilization [Takahashi, 1977]. Such a variation highlights the labile mechanism and factors that influence and modulate the initial gonadal development in zebrafish.

Similarly to medaka, zebrafish PGCs migrate to the site of the future gonadal primordium by the first day of embryonic development after they are specified with the inheritance of germ plasm components during the first cleavages post fertilization [for review, see Herpin et al., 2008 and Aalto et al., 2021]. At this stage, germ cells are positioned in close association with the somatic cells adjacent to somite 5, flanking the midline [Weidinger et al., 2002], and remain mitotically quiescent until 8–10 dpf [Leerberg et al., 2017]. Interestingly, starting from 8 dpf, somatic cells of the presumptive primordial gonad specifically and distinctly express either female (cyp19a1a) or male (amh) markers, in an apparent mosaic of male-type and female-type cells [Leerberg et al., 2017]. This early "molecular" male-type and female-type cell commitment, before any cell fate differentiation, is a hallmark of the singular bi-differentiated and bi-potential primordial (although on its whole undifferentiated) gonad of zebrafish (Fig. 1b).

During the bipotential period (13–14 dpf) that follows, perinucleolar oocytes are distinguishable in all gonads regardless of the future gonadal fate of the animals, indicating that most of the germ cells follow a female-type developmental program (Fig. 1b) [Takahashi, 1977]. Then, germ cells and somatic cells of the primordial gonad proliferate until around 20–25 dpf, when the first signs of sexual differentiation become apparent [Tzung et al., 2015]. At this "transition" stage, oocytes in the gonads of the future males undergo apoptosis, switching these gonads from an ovary-type to a complete testis-type development (Fig. 1b). On the other hand, oocytes present in gonads of the future females progress with the regular development [Uchida et al., 2002]. That clearing/transitioning phase of oocyte apoptosis in the gonads that will develop as testes continues until around 30 dpf [Uchida et al., 2002] when either male or female gonads are fully differentiated. Sexual maturity is reached by 60 dpf [Takahashi, 1977].

Of note, the timeline of gonadal differentiation and development in zebrafish is highly variable, depending on individuals, fish strains, and rearing conditions [Presslauer et al., 2014]. Furthermore, several reports [Luzio et al., 2015, 2021] also point out that some males (up to 20% of the future males) develop testes directly without going through a juvenile ovarian structure. Interestingly these individuals, at as early as 20 dpf, display a reduction of their number of PGCs compared to the other fish [Luzio et al., 2021]. These observations, although inherent to the genetic background and rearing conditions of the individuals, nevertheless call attention to the critical role of PGCs into promoting female gonadal development.

Of the Importance of Germ Cells during Gonadal Development of Gonochoric Fish Species

In mammals, pluripotent cells from the epiblast are transformed by diverse signals from surrounding tissues and give rise to PGCs (epigenesis; see other review in this

issue). In contrast, PGCs in fish are specified by maternally derived molecules, better known as germ plasm (preformation) [for review, see Johnson et al., 2011]. Having a unique control over their basic cell functions (including transcriptional and translational controls, RNA and protein stability or responses to differentiation signals) [Seydoux and Strome, 1999; Wylie, 2000; Starz-Gaiano and Lehmann, 2001], the germ plasm also specifies these cells in a cell-autonomous way. As such, their active role(s) for primary sex determination, and later for gonadal morphogenesis is particularly important in fish, especially during ovarian development [Slanchev et al., 2005; Kurokawa et al., 2007; Siegfried and Nüsslein-Volhard, 2008].

A common feature among most studied vertebrates from mammals to fish is that the first recognizable sex difference is at the germ cell level. PGCs start to proliferate as gonocytes in female embryos and enter meiosis, while in male embryos they stay quiescent [Saito et al., 2007; Bowles and Koopman, 2010]. The stage when this happens is usually seen as the critical sex determination stage [Feng et al., 2014; Adolfi et al., 2016]. Consequently, several studies focused on the possible role of germ cells (or gonocytes) in sex determination [Tanaka et al., 2008; Ribas et al., 2017; Adolfi et al., 2019a]; and in fish, the complete ablation of germ cells induced maleness in many species, including medaka [Kurokawa et al., 2007] and zebrafish [Slanchev et al., 2005], but with a few exceptions [Fujimoto et al., 2010; Goto et al., 2012]. For example, using germ cell-deficient medaka models, Kurokawa et al. [2007] showed that germ cells are required for the formation of ovary and the maintenance of theca and granulosa cells. More precisely, to determine a critical gametogenetic stage(s) for that observed feminization, Nishimura et al. [2018], using medaka mutants in which gametogenesis was blocked at specific stages, provided evidence that the feminizing effect of germ cells seems to be underlain by a mechanism that is distinct from the mechanisms of commitment to gametogenesis, entering meiosis and sexual fate determination [Nishimura et al., 2018]. This underlined that germ cells in medaka are predisposed to develop the ability to feminize the gonad independently of the somatic sex or the developmental stage of oogenesis [Nishimura et al., 2018]. Similarly, in zebrafish, different studies have reported the importance of germ cells and oocytes for feminizing the gonads [Slanchev et al., 2005; Siegfried and Nüsslein-Volhard, 2008]. Moreover, other studies in zebrafish [Tzung et al., 2015; Ribas et al., 2017] and medaka [Adolfi et al., 2019a] have shown that a threshold number of PGCs is required for the stability of the ovarian fate. These findings demonstrated that a small number of germ cells, independent from the sex chromosome constitution of the individual, are enough to maintain the sexual development program, supporting the idea that germ cell signals could influence the sex of the somatic gonad.

The relationship between proliferation of germ cells and sex determination is not only morphologically apparent, but also trackable at the molecular level via a crosstalk between germ cells and somatic cells [Tanaka et al., 2008; Saito and Tanaka, 2009]. It is then not a surprise that some factors described as male-biased genes, such as *gsdf* and *amh*, have been shown to be related also to germ cell proliferation and differentiation (zebrafish [Lin et al., 2017]; medaka [Nakamura et al., 2012b; Zhang et al., 2016]). One preeminent example for this relationship is that the medaka master sex-determining gene *dmrt1bY/dmy* was functionally proved to be an inducer of mitosis arrest of PGCs [Paul-Prasanth et al., 2006; Herpin et al., 2007].

Hence, while the fine signaling and crosstalk between germ and the somatic cells of the primordial gonad remain to be elucidated, these observations suggest that during the sex-determining period the regulation of germ cell proliferation within the primordial gonad is key to influence whether that gonad will adopt a testis or an ovarian fate. It is then tempting to speculate that any genetic (amh signaling, gsdf, dmrt1 for instance) [for review, see Herpin and Schartl, 2015; Pan et al., 2016, 2021] or environmental factors [Kossack and Draper, 2019] that could influence the number of germ cells at the sex-determining period would have the potential to become a master sex-determining trigger in fish [Adolfi et al., 2021].

Interestingly, even in GSD species with a stable genetic sex-determining gene, as medaka and pejerrey, environmental cues or feeding conditions that affect fat metabolism [Sakae et al., 2020] are still able to reverse the sex and modulate germ cell number [Hattori et al., 2007; Yamamoto et al., 2014]. Several studies showed that changes in the rearing conditions of eggs at the sex-determining window could be stress factors, increasing the cortisol levels and leading to female-to-male sex reversal [Sato et al., 2005; Selim et al., 2009]. Experiments in medaka fish showed that cortisol treatments induced expression of male-related genes, as *amh* and *dmrt1a* (the autosomal version of *dmrt1bY/dmy*) in XX animals, leading to masculinization and reducing the germ cell proliferation [Adolfi et al., 2019a]. Similar effects were also observed in embryos treated with steroid hormones, which are known

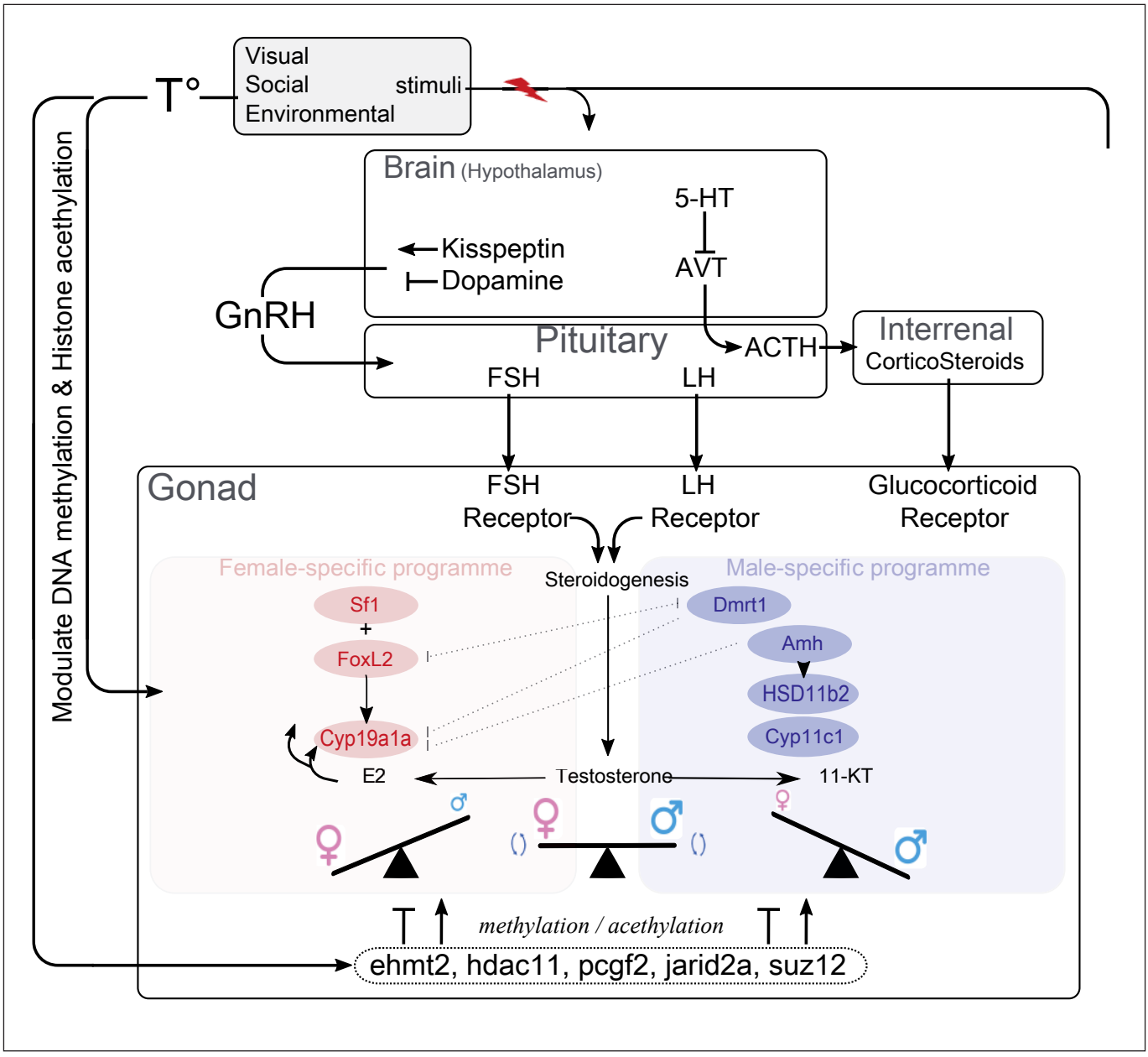

Fig. 3. Overview of the known stimuli and pathways operating through the hypothalamus-pituitary-interrenal axis during sex change in fish. The sex determination mechanism is a fine balance between the antagonistic male (blue) and female (pink) pathways. At the same time, the sex-specific networks are also connected to the production of the main steroid hormones in male (11-KT) and female (E2), which are important for the differentiation of the gonads. Testosterone is the precursor of both 11-KT, converted by hsd11b2 and cyp11c1, and E2, converted by aromatase (cyp19a1a). The expression of *cyp19a1a* is regulated directly by the female-related transcription factor foxl2, while hsd11b2 and cyp11c1 can be regulated by male-related genes as *dmrt1* and *amh*. Gonadotropins (FSH and LH) are produced in the pituitary and can influence the regulation of steroid hormones. Visual, social, and environmental cues lead to the alterations in the hypothalamus, leading to the regulation of the gonadotropins and cortisol, which in turn regulate the synthesis of E2 and 11-KT during sex change. In addition, epigenetic factors lead to DNA methylation and histone modifications, regulating the expression of genes related to sex determination and steroidogenesis. ACTH, adrenocorticotropic hormone; AVT, arginine vasotocin; E2, 17β-estradiol; GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; 5-HT, serotonin; 11-KT, 11-ketotestosterone. Scheme adapted from Capel [2017].

to regulate the expression of important sex-related genes [Baroiller and D'Cotta, 2016].

Specifying sexual fate will ultimately trigger the production of sex hormones (androgens vs. estradiol in males and females, respectively) in order to instruct the rest of the body and organs to adopt the sex-appropriate physiology and behavior. In some cases, obviously, external signals can modulate the reproductive functions and, in fish, environmental, social or visual cues, through the neuroendocrine path, are able to deconstruct and reshape the entire gonad and the rest of the body, including behavioral, anatomical, and hormonal characteristics in order to make the most out of changing sex in an adaptive perspective.

Ovary Development in Sex-Changing Fish

In nature, several species and taxonomic groups developed the capacity of changing sex, substituting the sexual characteristics of one sex into the opposite. Several hypotheses were put forward to explain sex change as an adaptive strategy, relating to factors such as body size, critical age, fecundity, as well as social and behavioral aspects [Gemmell et al., 2019]. However, the transition of the functional gonad of one sex to another involves changes not only in the morphological gonad, but also in sexual and social behavior of an individual, which combines molecular, hormonal, and environmental factors (e.g., stress, social and population changes).

Most knowledge on the regulation of the sex determination network in vertebrates is based on gonochoric species, as described above. At the sex determination period, the gonads follow the molecular pathway toward ovaries or testes. The main ovary regulators are the steroidogenic enzyme aromatase (encoded by the *cyp19a1a* gene) and the transcription factor Foxl2. Aromatase is responsible for the conversion of androgen into estrogen, the main female hormone, which in turn regulates *foxl2* expression [Bertho et al., 2016]. Foxl2 acts in opposition to Dmrt1, being responsible for the differentiation of the somatic cells into granulosa cells in females instead of Sertoli cells in males. Studies showed that Dmrt1 and Foxl2 can block each other and thus repress the genetic pathway of the opposite sex, and this mutual repression persists until adulthood and thus is crucial for the phenotypic sex and gonad maintenance [Li et al., 2013; Lindeman et al., 2015] (Fig. 3).

This outstanding gonad plasticity and mutual repression between the male and female regulatory networks are generally observed in vertebrates, but teleosts, being able to change sex, seem to be the only group that turned this potential into action. Sex change in fish may occur naturally as observed in sequential hermaphrodites, and it is classically divided into: (1) protandry, in which an individual may change from a mature male into female later in life; (2) protogyny, the most common type of hermaphroditism, in which an individual starts as a female and later may become male; and (3) bidirectionality, in which an individual may switch back and forth between male and female [Chan and Yeung, 1983; Avise and Mank, 2009]. Recently, it was proposed that anatomic and developmental changes in the urogenital system of teleosts, such as the loss of the müllerian duct and the independency of the urinary ducts for sperm release, create the optimal conditions to develop hermaphroditism [Adolfi et al., 2019b]. In addition, those changes also explain why hermaphroditism appeared several times and is scattered in the evolutionary tree of teleosts [Adolfi et al., 2019b]. Hermaphroditism is a derived state from gonochorism with only about 6% presenting sex change as a mode of reproduction. Hence, those species and groups needed to overcome and/or adapt from the molecular mutual inhibition between the male and the female sex pathways.

During sex change, the gonads pass through radical morphological changes, which vary according to the taxonomic groups and types of hermaphroditism. In wrasse, a protogynous species, the process of sex change starts by oocyte degeneration and follicle atresia, followed by testicular tissue arising at the periphery of the ovarian lamella, having no clear physical separation between testis and ovary parts [Nakamura et al., 1989]. Some protandrous fish also present no specific compartments for the female or the male tissues, but in porgies, which include both protandrous and protogynous species, connective tissues are present separating the two heterosexual regions ("ovotestis") [De Mitcheson and Liu, 2008]. In the protandrous black porgy (*Acanthopagrus schlegelii*), until the second spawning season, the ovotestis gonads are dominated by testicular tissue with a small ovary portion containing mainly oogonia and primary oocytes. Before the third breeding season, the testicular tissue reduces in some individuals, and the ovarian part starts to be more prominent in the ovotestis [Wu et al., 2010a].

In those protogynous species with no connective tissue separating the male and female regions, the origin of the germ and somatic cells of the future testis remains unclear. In swamp eel, it was demonstrated that the early testis tissues are first observed in the proximities of the

germinal epithelium [Lo Nostro et al., 2003]. The epithelium located at the periphery of the ovarian lamella seems to be the source of Sertoli cells, since the male-related gene *gsdf* (gonadal somatic-derived factor) is detected in the somatic cells surrounding the gonial cells prior to sex change [Shibata et al., 2010]. Similarly, the simultaneous hermaphrodite mangrove killifish (*Kryptolebias marmoratus*) first develops ovary, and the cells surrounding undifferentiated gonial cells express Amh (anti-müllerian hormone) in the early gonad, and these cells will later develop into the testicular part [Qu et al., 2020]. Interestingly, *Kryptolebias* are the only documented cases of self-fertilization in vertebrates [Costa, 2011]. As discussed in gonochoric medaka, the germinal epithelium expresses the same genes observed in the early gonads prior to sex determination (e.g., *sox9b*) [Nishimura et al., 2016], indicating that these tissues may be a constant source of bipotential germ and somatic cells (Fig. 2). In addition, studies with intersexes in medaka showed that adult testis also present bipotential germ cells (gonia), which can differentiate into female germ cells after treatments with feminizing hormones [Balch et al., 2004]. However, the capacity of the already differentiated somatic and germ cells to reprogram during sex change still needs further investigation.

In gonochoric and hermaphroditic species, the molecular male and female networks are also connected to the synthesis and regulation of hormones, which are important in testis and ovary development and differentiation. In teleosts, 11-ketotestosterone (11-KT) is the main androgen for testis development, while 17β-estradiol (E2) is the principal estrogen responsible for regulating ovary functions, produced by aromatase (*cyp19a1a*). The 11-KT is converted from testosterone via 11β-hydroxylase (*cyp11c1*) and 11β-hydroxysteroid dehydrogenase (*hsd11b2*) [Casas and Saborido-Rey, 2021]. During sex change, the balance of sex steroid levels in blood changes strongly from one sex to the opposite, but the exact trigger of this variation is still unknown and can be group- or species-specific [Godwin, 2010] (Fig. 3). Exogenous treatments with sex steroids can induce masculinization and feminization in fish, especially at the sex-determining window. However, upon the withdrawing of the hormone treatments, the sex change is not sustained in some species, indicating that exogenous treatments are not able to switch completely the molecular program of the gonads [Wu et al., 2015].

Some hermaphroditic species use social cues as triggers for sex change, such as the protogynous wrasses. In these species, alterations in the brain driven by neuroendocrine pathways lead to behavioral changes prior to gonad sex transition [Lamm et al., 2015]. In such cases, the hypothalamus-pituitary-gonad (HPG) and the hypothalamus-pituitary-interrenal (HPI) axes regulate the sex change by controlling the balance between androgens and estrogens (Fig. 3). The gonadotropins are the principal pituitary hormones represented by the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), which regulate the steroidogenesis via receptor-mediated stimulation of ovarian follicle cells or Leydig cells in testis. The expression of FSH and LH, and their corresponding receptors, varies during sex change, and the gonadotropins are mainly regulated by the gonadotropin-releasing hormone (GnRH) from the hypothalamus [Schulz et al., 2010]. Nevertheless, the patterns of expression and regulation of GnRH, FSH, and LH during sex change are not consistent and seem to vary among different species and types of hermaphroditism [An et al., 2009, 2010; Kobayashi et al., 2010]. The behavior modulation and the regulation of the HPG axis during sex change also involve several neuropeptides. Arginine vasotocin (AVT) is a neurotransmitter described to stimulate behavioral sex change, such as courtship and aggression, in protogynous wrasses [Semsar et al., 2001; Godwin and Thompson, 2012]. However, other neurotransmitters such as dopamine (DA) and serotonin (5-HT) act as inhibitory factors of sex change [Larson et al., 2003a, b]. The neuropeptide kisspeptin is well-known to stimulate the release of GnRH and mediates the transition of social status [Shi et al., 2010]. However, studies of the expression patterns of kisspeptin and its receptors during sex change are still insufficient (Fig. 3).

Like in gonochoric species, stress can play important roles in influencing sex in hermaphrodites [Yamaguchi et al., 2010; Fernandino et al., 2013; Goikoetxea et al., 2017]. The reaction to stress is regulated by the HPI neuroendocrine system through the actions of corticotropin-releasing hormone (CRH) and glucocorticoid steroids [Castañeda Cortés et al., 2019]. The main corticosteroid related to stress is cortisol, and studies with hermaphrodites demonstrate that cortisol levels vary during the process of sex change. In protogynous species and in bidirectional hermaphrodites, cortisol promotes female-to-male sex change [Solomon-Lane et al., 2013; Nozu et al., 2015]. However, the exact influence of stress in the sex determination pathway is unclear. The current hypothesis, based on gonochoristic fish and in bluehead wrasse, is that cortisol reduces the *cyp19a1* expression and induces male-related genes as *amh*, regulating the germ cell differentiation and testis formation [Goikoetxea et al., 2017] (Fig. 3).

In addition, Amh is expressed in the Sertoli cells surrounding undifferentiated spermatogonia in the oocyte-bordering region of black porgy, and it was proposed that Amh inhibits the spermatogonia proliferation and female growth [Wu et al., 2010b, 2015].

While the male and female sex determination and steroidogenic pathways are strongly connected, it is now clear that the whole mechanism of sex determination and sex change is a balance and fine tuning between those two opposite pathways [Gemmell et al., 2019]. It is well-accepted that the genes responsible for testis developments (e.g., *dmrt1*, *amh,* and *gsdf*) and for ovary formation (e.g., *foxl2* and *cyp19a1a*) regulate the production of androgens and estrogens, respectively [Pfennig et al., 2015; Bertho et al., 2016]. Several of these genes and regulatory factors were identified as possible initiators of natural sex change in sequential hermaphrodites. In protogynous species, as expected, expression of female-related genes (e.g., *dax1*, *figla*, *gdf9*) declines progressively, while male-related genes (e.g., *gsdf, sox9*, *amh, cyp11c1*, *dmrt1*) increase their levels. Nevertheless, the expression of *foxl2* and *dmrt1* presented their peak of mRNA levels at mid-late stages of sex change [Liu et al., 2017]. Those results suggest that *dmrt1* and *foxl2* are not responsible for initiating sex change but are necessary for maintaining the sex-specific program. Cause or consequence, the question remains.

The aromatase gene, *cyp19a1a*, seems to be the best candidate in regulating sex change, since its expression is strongly downregulated in early protogynous, and it is consistent with the reduction in E2 production at this stage [Li et al., 2006]. In addition, *foxl2* expression is not tightly coupled with aromatase, since its expression level only decreases at the terminal phase during sex transition [Zhang et al., 2017] (see also Fig. 2). Aromatase inhibitor treatments were able to promote sex change in protogynous species, while in protandrous species the transition was blocked [Lee et al., 2002; Horiguchi et al., 2018; Breton et al., 2019]. However, the upstream regulator(s) of aromatase is/are still unknown. Promoter analyses of teleost *cyp19a1a* demonstrate that this gene may be regulated by numerous factors, as steroidogenic factor 1 (Sf1), Sox9, wt1, GATA-binding proteins, cAMP, glucocorticoids, estrogens, progesterone, and androgens [Gardner et al., 2005; Guiguen et al., 2010]. Hence, the current hypothesis is that visual, social, and environmental *stimuli* lead to the induction or repression of *cyp19a1a*, which in turn leads to regulation of the main sex-related genes during the process of sex change [Gemmell et al., 2019].

Recently, epigenetic modulators, such as DNA methylation, histone modifications, and non-coding RNAs, have been demonstrated to play an important role in sex determination, not only in gonochoric species, but also in hermaphrodites [Capel, 2017; Best et al., 2018]. Studies in protogynous and protandrous species demonstrated that *cyp19a1a* methylation led to reduced expression of this gene. Similarly, protandrous species showed progressive methylation of *dmrt1*, while demethylation of this gene was observed in protogynous fish [Domingos et al., 2018; Todd et al., 2019]. Interestingly, genes related to the regulation of the methylation machinery, such as *dnmt1* and *dnmt3*, vary their expression during sex change. Moreover, some hermaphrodite species showed that histone modifications are involved in maintaining the differentiated gonad [Zhang et al., 2013; Tsakogiannis et al., 2018].

Conclusion

The gonads are unique organs, not only due to their special role in producing gametes, but also to their outstanding capacity of changing sex or even produce testis and ovary in the same organ. Here, we reviewed the intricated molecular and developmental process of ovary formation, and the role of the germ cells in sex determination of fish. At this point should become clearer that it is impossible, biologically and conceptually, to regard the male and female sexes independent from each other. Both male and female pathways are entangled, and the genes related to the formation of one sex may have an influence on the opposite one. Hence, for a general comprehension of ovary development and sex change, it is necessary to understand and integrate the molecular network and the process of sex determination as a whole. In addition, the capacity of the gonad to respond to environmental cues, and their connection with the endocrine system, are in last instance acting on the gonads through the sex determination genetic network.

Together with the specific changes in the reproductive system, the remarkable plasticity in ovarian development in fish and the flexibility in germ cell fate throughout the life of an individual, eventually facilitated the adaptation of variable forms of sex change among teleosts. The central task of reverting the sexual fate of the germ cells is also accompanied with the orchestrated transition throughout the entire body, permitting this group to adapt to different niches and expand its diversity of reproductive modes, and being the most diverse group of vertebrates.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Mateus Contar Adolfi, Qiaowei Pan, and Amaury Herpin performed the primary review of the theoretical research and led the writing of the review. Alexandra Depincé and Ming Wen provided substantial written and intellectual contributions. The final manuscript has been revised, seen and approved by all authors (Mateus Contar Adolfi, Alexandra Depincé, Ming Wen, Qiaowei Pan, and Amaury Herpin).

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