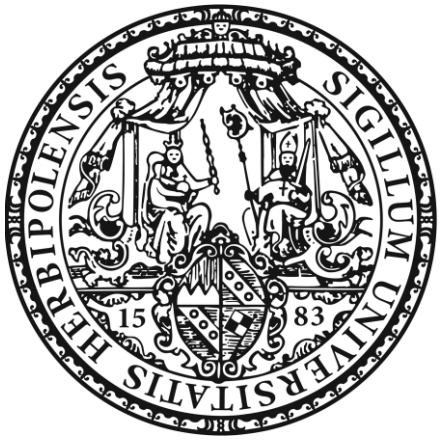


# Sex determination and meiosis in medaka:

# The role of retinoic acid

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
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**Mateus Contar Adolfi**

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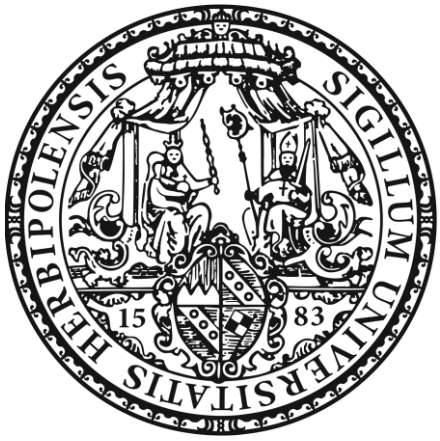


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Contar Adolfi, Mateus
Hauptstraße, 16
97218 Gerbrunn
Tel. +49 (0)931 31-89171
E-Mail: mateus.adolfi@biozentrum.uni-wuerzburg.de

**Erklärungen nach §4 Abs. 3 Satz 3, 5, 8 der Promotionsordnung der Fakultät für Biologie**

**Affidavit**

I hereby declare that my thesis entitled: „**Sex determination and meiosis in medaka: The role of retinoic acid.**" is the result of my own work.

I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore I verify that the thesis has not been submitted as part of another examination process neither in identical nor in similar form.

**Eidesstattliche Erklärung**

Hiermit erkläre ich an Eides statt, die Dissertation: „**Sex determination and meiosis in medaka: The role of retinoic acid.**", eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 16. Juni 2016

_____
(Mateus Contar Adolfi)

Nothing in biology makes sense except
in the light of evolution
(*Theodosius Dobzhansky*)

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## Summary

Sex determination (SD) is a complex and diverse developmental process that leads to the decision whether the bipotential gonad anlage will become a testis or an ovary. This mechanism is regulated by gene cascades, networks and/or chromosomal systems, and can be influenced by fluctuations of extrinsic factors like temperature, exposure to hormones and pollution. Within vertebrates, the group of fish show the widest variety of sex determination mechanism. This whole diversity of processes and mechanisms converges to the formation of two different gametes, the eggs and the sperm, the first bigger and static, and the second smaller and motile. Meiosis is crucial for the formation of both types of gametes, and the timing of meiosis entry is one of the first recognizable differences between male and female in vertebrates. The germ cells go into meiosis first in female than in male, and in mammals, this event has been shown to be regulated by retinoic acid (RA). This small polar molecule induces in the germ cells the expression of the pre-meiotic marker *Stra8* (stimulated by retinoic acid gene 8), which is necessary for meiosis initiation. Interestingly, genome analyzes have shown that the majority of fish (including medaka) lack the *stra8* gene, adding a question mark to the role of RA in meiosis induction in this group. Since a role of RA in entry of meiosis and sexual development of fish is still far from being understood, I investigated in medaka (*Oryzias latipes*) a possible signaling function of RA during the SD period in embryos and in reproductively active gonads of adults. I generated a transgenic medaka line that reports responsiveness to RA *in vivo*. With this tool, I compared RA responsiveness with the expression of the main gene involved in the synthesis of RA. My results show that there is a de-correlation between the action of RA with its source. In adults, expression of the RA metabolizing enzymes show sexually dimorphic RA levels, with *aldh1a2* levels being higher in testis, and *cyp26a1* stronger in female gonad. In ovary, the responsiveness is restricted to the early meiotic oocytes. In testis, RA is acting directly in the pre-meiotic cells, but also in Sertoli and Leydig cells. Treatment experiments on testis organ culture showed that RA pathway activation leads to a decrease in meiosis markers expression levels. During the development, RA responsiveness in the germ cells was observed in both sexes much earlier than the first female meiosis entry. Treatments with RA-synthesis inhibitor show a decrease in meiosis markers expression levels only after the sex differentiation period in female. Expression analyzes of embryos treated with exogenous RA showed induction of *dmrt1a* at the gonad levels and an increase of *amh* levels. Both genes are not only involved in male formation, but also in the regulation of germ cell proliferation and differentiation. RA is important in meiosis induction and gametogenesis in adult medaka. However, there is no evidence for a similar role of RA in initiating the first meiosis in female germ cells at the SD stage. Moreover, contrary to common expectation, RA seems to induce sex related genes that are involved indirectly in meiosis inhibition. In this thesis, I showed for the first time that RA can be involved in both induction and inhibition of meiosis entry, depending on the sex and the developmental stage in a *stra8*-independent model organism.

# Zusammenfassung

Geschlechtsbestimmung ist ein komplexer und vielfältiger Entwicklungsprozess, der zu der Entscheidung führt, ob sich aus der bipotenten Gonadenanlage Hoden oder Ovarien entwickeln. Dieser Mechanismus ist durch Genkaskaden, Netzwerke und/oder chromosomale Systeme reguliert, kann aber auch durch Fluktuation äußerer Faktoren wie beispielsweise Temperatur, durch Hormonexposition oder durch Umweltverschmutzung beeinflusst werden. Innerhalb der Wirbeltiere zeigen Fische die größte Vielfalt in Bezug auf die Mechanismen der Geschlechtsbestimmung. Die unterschiedlichen Mechanismen der Geschlechtsbestimmung konvergieren in der Entstehung von der beiden unterschiedlichen Geschlechtszellen, der Eizelle und des Spermiums. Die Eizelle ist groß und statisch, das Spermium hingegen ist kleiner und beweglich. Die entscheidende Rolle für die Entstehung der Geschlechtzellen spielt die Meiose. Der Zeitpunkt, an dem zum ersten Mal in der Entwicklung die Meiose einsetzt, ist der erste erkennbare Unterschied in der Gonadenentwicklung zwischen männlichen und weiblichen Wirbeltieren. Die Meiose der Keimzellen beginnt bei Weibchen früher als bei Männchen. Bei Säugetieren reguliert Retinsäure (RA) diesen Prozess. Dieses kleine polare Molekül induziert die Expression des Prä-Meiose-Markers *Stra8* (stimulated by retinoic acid gene 8) in den Keimzellen, welcher für den Eintritt in die Meiose essentiell ist. Interessanterweise haben Genomanalyzen gezeigt, dass das *stra8* Gen in Medaka sowie in den meisten anderen Fischarten nicht vorhanden ist. Dies stellt eine vergleichbare Rolle von RA für die Induktion der Meiose wie bei Säugetieren in diesen Fischen in Frage. Da die Rolle von RA für den Eintritt in die Meiose sowie für die Geschlechtsentwicklung in Fischen bisher nur unzureichend untersucht und verstanden ist, habe ich bei Medaka (*Oryzias latipes*) eine mögliche Funktion von RA für die Geschlechtsdetermination in Embryonen sowie in Gonaden geschlechtsreifer Tiere untersucht. Ich habe im Rahmen dieser Arbet eine transgene Medakalinie generiert, die *in vivo* eine RA induzierte Genexpression durch ein GFP Reportergen anzeigt. Mit Hilfe dieser Linie wurde die transkriptionsreulierende Aktivität von RAmit der Expression der wichtigsten Gene, die in die RA Synthese involviert sind, verglichen. Meine Ergebnisse zeigen eine Diskrepanz zwischen dem Wirkungs- und Syntheseort von RA. Die RA metabolisierenden Enzyme zeigten eine geschlechtsdimorphe Expression in adulten Medakas, mit einer höheren *aldh1a2* Expression im Hoden sowie einer stärkeren *cyp26a1* Expression in weiblichen Gonaden. Im Ovar sind lediglich frühe meiotische Eizellen RA-sensitiv. Im Hoden wirkt RA direkt in prä-meiotische Zellen, aber auch in Sertoli und Leydig Zellen. Stimulations-Experimente an Hoden Organkulturen ergaben, dass eine Aktivierung des RA Signalwegs zu einer Abnahme des Expressionslevels von Meiose-Markern führt. Während der Embryonalentwicklung konnte in den Keimzellen beider Geschlechter eine transkriptions-induziernende Aktivität von RA bereits zu einem Zeitpunkt beobachtet werden, der deutlich vor dem ersten Meiose Eintritt in Weibchen liegt. Behandlungen mit einem RA-Synthese Inhibitor zeigten lediglich nach der Geschlechtsdifferenzierung in Weibchen eine verminderte Expression der Meiose-Marker. Expressionsanalysen von Embryonen, die mit exogener RA behandelt wurden, ergaben eine Induktion von *dmrt1a* in den Gonaden und ein Anstieg von *amh*. Beide Gene sind sowohl in die männliche Geschlechtsentwicklung involviert, als auch in die Regulation der Keimzellproliferation und –differenzierung. Zusammen ergaben meine Untersuchungen, dass RA für die Induktion der Meiose und der Gametogenese in adulten Medakas wichtig ist. Allerdings gibt es keinen Hinweis für eine ähnliche Rolle

von RA bei der Initiierung der ersten Meiose in weiblichen Keimzellen während der Geschlechtsdetermination. Im Gegensatz zur bisher beschriebenen Situation, scheint darüber hinaus RA die Expression geschlechtsspezifischer Gene zu induzieren, die indirekt in die Inhibition der Meiose involviert sind. In der vorliegenden Arbeit konnte in einem stra8-unabhängigen Modelorganismus das erste Mal gezeigt werden, dass RA – abhängig vom Geschlecht und vom Stadium der Entwicklung - sowohl in die Induktion als auch in die Inhibition des Meiose-Eintritts involviert ist.

# Chapter 1

## General Introduction:

## Sex Determination and Meiosis

# 1. General Introduction

## 1.1 Sex Determination

### 1.1.1 A Brief History

The question about the meaning of sex in animals is a long time discussion, and it is still a big topic in the society and in the academic world. Nowadays, this theme gained a lot of focus since the concept of sexual orientation and gender identity started to be used interchangeably with the biological gonadal sex determination definition [1, 2].

Aristotle (384–322 BCE), interestingly, already contemplated the understanding of the diversity of reproduction strategies and the presence or absence of two separate sexes [3]:

> *"Now some animals come into being from the union of male and female, i.e. all those kinds of animal which possess the two sexes. This is not the case with all of them, (...). Male and female differ in their essence by each having a separate ability or faculty, and anatomically by certain parts; essentially the male is that which is able to generate in another,(...) the female is that which is able to generate in itself and out of which comes into being the offspring previously existing in the parent".*

In the same way, Aristotle hypothesized that the heat of the male partner determines the sex of the animals during the intercourse. The more heated the passion, the greater the probability of male offspring [3]. The view that women were but poorly developed men, was for over thousand years accepted and spread throughout different intellectuals, which, at that time, was restricted to religious authorities [4].

Charles Darwin in 1871 called attention to the importance of different sexes and their role in evolution. Even though the exact mechanism of sex determination in the 19$^{th}$ century was not understood, the presence of two different sexes, the secondary sexual characters and their respective habits of live act as strong selective factor in the nature[5]:

> *"Besides the primary and secondary sexual differences, such as the foregoing, the males and females of some animals differ in structures related to different habits of life, and not at all, or only indirectly, to the reproductive functions".*

Hence, to understand the mechanism of formation of the two different sexes is, in last instance, to understand one important mechanism that drives evolution.

The environmental view of sex determination was the main hypothesis for a long time, and just after rediscovery of Mendel's work and the characterization of the sex chromosome a new view was add to the field. In 1905 the correlation between sex chromosomes in insect of the female sex with XX and the male sex with XY or XO was established [6, 7].Today, we know that in different species sex determination can be influenced by different factors like environmental and internal genetic mechanism [8].

### 1.1.2 Gonad Development

Sexual reproduction is fundamental in maintaining the variation and allows the surviving of a species, and this process involves the formation of gametes, that in some species is restricted to the male or female gonad. [4]. In vertebrates and invertebrates, the gonads differentiate in ovary and testis during ontogenesis. In mammals, the precursor of the adrenal glands and gonads develop from a common primordium. In birds and some other vertebrates, there is an important mesonephric contribution to the stromal cells of the gonads [9-12]. Different from other vertebrates, the development of the urogenital system of teleost has a complete separation of the urinary and gonadal tubule systems [13]. The undifferentiated gonad is formed by two components of somatic cells: the cortex and medulla. The first has its origin in the peritoneum and the second is derived from the mesonephric blastema. In most vertebrates, during ovary formation, the cortex develops and the medulla degenerates, on the other hand, in testis formation, the medulla develops further and the cortex regresses. However, differently from other vertebrates, the medullar tissue is not found in the gonad of teleost [8]. In medaka fish, the lateral plate mesoderm moves to the prospective gonadal region at the dorsal region of the hindgut, where a single genital primordium is formed. From this primordial tissue, the organogenesis of the gonad is then initiated, resulting in the formation of bilateral split gonads [14].

The gonad is composed not only of somatic cells, but also of germ cells, which give rise to the gametes. In mouse and other mammals, primordial germ cells (PGC) are induced from pluripotent epiblast cells before and during gastrulation by

extraembryonic cell-derived signals [15-17]. In many invertebrates and non-mammalian vertebrates, the determination of the germ line starts just after the first embryonic cleavages, and is independent from the somatic gonadal tissue [18]. All gametes originate from the PGCs, and their differentiation depends on the presence of a cytoplasmic component called germ plasm [4]. The germ plasm forms *nuages* characterized by polar granules, mitochondria and an mRNA concentrate [19]. The PGCs have to migrate from the position where they were specified toward the region of the future gonad. The PGCs migrate due to chemoattractive proteins such SDF1 (stromal cell-derived factor 1) and its receptor CXCR4 [20-22]. SDF1 is produced in the somatic cells of the presumptive gonad, and the CXCR4 is located on the germ cells. Two copies of the *sdf1* gene are found in teleost, and, in medaka it was shown that SDF1a and SDF1b have complementary roles in the fine-tuning of PGC migration [23].

### 1.1.3 Molecular Control of Gonadal Sex Determination and Differentiation

The molecular process of sex determination is still not completely elucidated, and can have a big variation throughout phylogeny [24]. In addition, the gonad development can be influenced by fluctuations of extrinsic factors like temperature, exposure to hormones and pollution [25]. Treatment with hormones can induces gonadal sex reversal, not only in the undifferentiated gonad, but also in animals that already completed the gonadal development [26]. Based on experiments using a diverse number of hormones to induce gonad development or sex reversal, it was suggested that sex hormones could act like endogenous inductors during sexual differentiation [27-29]. In therian mammals (Marsupials and Placentalia), it is known that the process of sex determination involves the regulatory action of a gene linked to the Y chromosome, called *SRY* [30, 31]. This gene starts the development of testis instead of ovary from the gonad primordium, being the only gene necessary for development of the testis [32, 33]. Transgenic XX mice that carry the *Sry* gene sex reverse to sterile males [34].

However, the presence of a master sex-determination gene is not observed in all species, and the chromosomal system is diverse between taxonomic groups [35]. There are some downstream genes products, which lead to gonadal differentiation and which

kept the same functions during evolution. One strong actor in this scene is a gene family discovered in insects (*doublesex*) and nematodes (*mab-3*) that encode proteins with a *zinc-finger*-like motif, called DM domain, with DNA binding properties [36-38]. The DSX gene was the first gene from the DM family that has its structure described. The gene *doublesex* (DSX) occupies a key position in the hierarchy of the regulatory network in sexual differentiation in *Drosophila*, been the unique required gene in somatic differentiation in both sexes; all the other genes are required only in female. Both sexes of *Drosophila* transcribe DSX, however, depending of the sex, the mRNA is alternatively spliced and thus can encode sex specific proteins; the $DSX_m$ male specific and the $DSX_f$ female specific [36].

The *dmrt1* gene (doublesex/mab-3 related transcription factor-1), that contains the DM domain, seems to be an important gene that has a conserved structure and role in gonad determination and differentiation in males, and is found in vertebrates and invertebrates [38-44]. In amphibians, it was shown that *dmrt1* is expressed exclusively in gonads of males, and testosterone treatments of females of *Rana rugosa* induces sex reversal and *dmrt1* expression [42]. In *Xenopus laevis*, which has a ZZ/ZW chromosomal system, the DM-W gene was isolated (located on the W chromosome) in females [45]. This gene is a paralogue of the *dmrt1* gene, generating a truncate protein without the trans-activation domain, where DM-W is dominant negative and direct the ovary formation in female, and the *dmrt1* to testis in male [46].

In snakes, in general, the female is heterogametic with the ZZ/ZW system. Most turtles and all crocodilians have no sex chromosomes, but there are some species of turtles where sex chromosomes were found [47]. In turtles with TSD, the expression of *dmrt1* in the genital ridge of the embryos is higher in those that were incubated at low temperature, leading to male formation, and lower in the embryos incubated at higher temperatures, which leads to female formation [48]. In birds, like in snakes, the chromosomal system is the ZZ/ZW and the best candidate gene that leads to sex differentiation is *dmrt1*, which is located in the Z. This gene is expressed in the embryos of both sexes, but in higher levels in males (ZZ) than in female (ZW) during the critical period of gonad differentiation. Hence, it was inferred that male development in birds is dose-dependent of *dmrt1* [49]. It was also seen that genes linked to the W chromosome control the expression of a hypermethylated region of the Z chromosome

next to *dmrt1*, indicating that this mechanism acts to repress the expression of genes adjacent to *dmrt1* in ZW females, leading to ovary formation [50].

In mammals, the *Dmrt1* gene is found on the short arm of the chromosome 9, and is expressed in germ cells and in Sertoli cells. It acts downstream of the *Sry* gene [51]. Knocking out *Dmrt1* in mice leads to severe defect in the testis, mainly in the Sertoli cells and loss of germ cells. Hence, *Dmrt1* is necessary for the maintenance and differentiation of the germ cells and the soma of male gonad in mammals [52]. Recent studies showed that transgenic overexpression of *Dmrt1* mice causes female-to-male sex reversal, revealing that *Dmrt1* alone can also have a role as primary testis-determining trigger [53].

In fish, highest diversity of genetic sex determination systems can be observed, from heterogametic males or females, with or without autosomal influence, to complex molecular sex systems without a major sex chromosome, or with a diverse number of chromosomes that harbor several loci participating in the process of sex determination [54]. In medaka (*Oryzias latipes*), the animal model species of this thesis, the chromosomal sex determination system is male heterogametic (XX/XY), with homomorphic sex chromosomes [55]. At the sex determination locus (SDL) of the Y chromosome of medaka, a gene was isolated that codes for a protein that contains the DM domain. This Y specific gene was called *dmrt1bY* or *dmy*. It is a duplication of the autosomal *dmrt1* (*dmrt1a*) [56, 57]. The role of this gene has been widely studied, and shown to be involved not only in the determination and differentiation to male, but it is also involved the mitosis arrest of the PGCs specifically in G2 [58, 59]. Mutants for *dmrt1bY* show sex reversal in the absence of the gene product [60]. Recently, the master sex-determining genes have been identified in several other teleost, namely *dmrt1bY* (*dmy*) in *Oryzias curvinotus* (the same gene as in *O. latipes*) [61], *gsdfY* in *Oryzias luzonensis* [62], $sox3^{Y}$ in *Oryzias dancena* [63], *gsdf* in *Anaplopoma fimbria*, *amhy* in *Odonthestes hatchery* [64], *amhr2* in *Takifugu rubripes* [65], and *sdY* in *Oncorhynchus mykiss* [66] and several other salmonids [67].

Previously was said that environmental factors such temperature can determine sex. However, experiments show that temperature can also influence the sex in species that already developed a firm genetic sex determination system [25, 68]. In medaka, incubation of the embryos at high temperature during specific development stages can

lead to a female-to-male sex reversal, by upregulating the autosomal *dmrt1a* gene [69, 70]. This indicates that the *dmrt1bY* in medaka can be both a strong factor for sex determination and weak at the same time, because it can promptly be overtaken by alternative environmental and genetic factors [69]. Medaka with a knockout for *dmrt1a* present male-to-female sex reversal later in life, indicating an important role of this gene in testis maintenance. In other fish species, *dmrt1* was shown to be important for gametogenesis regulation [44].

*DMRT1* is an extremely important conserved downstream gene involved in sex determination, but is not the only one. By comparing the different species from a large variety of taxa, a great number of other genes downstream to the master sex gene was found. Those genes can act as transcription factors (*SOX9*, *DMRT1*) or signaling pathways (TGF-β/Amh, Wnt4/β-catenin, Hedgehog), with a known or presumed role in gonadogenesis or gonadal differentiation. The presence of conserved downstream genes and a high diversity of master sex-determination genes permeate the central paradigm of sex determination in the last 20 years, that "master change, slaves remain". However, recent comparative studies on the downstream regulatory network brought new insight into how sex development is regulated in different organism, and how new sex determiners have evolved. As result, we observe a change of paradigm, where also the downstream regulators show a great degree of plasticity, like the master sex determining genes. However, still several downstream transcription factors have a conserved role in gonad differentiation: *DMRT1* for male and *FOXL2* for female differentiation [71].

The FOXL2 (forkhead box L2) transcription factor has an important role in ovary development and maintenance, being mainly expressed in somatic cells of the female gonad [72]. The *foxl2* expression in ovary is positively correlated with the expression of *aromatase* (*cyp19*), suggesting that *foxl2* in involved in regulation of estrogen synthesis via direct upregulation of *aromatase* (more information in Appendix 1) [73].

## 1.2 Meiosis and Retinoic Acid

Despite this huge diversity of upstream and downstream factors and networks involved in the process of sex determination, some phenomena still appear to be highly

conversed throughout evolution. A common feature found in most studied vertebrates from mammals to fish is an early morphological difference between male and female that becomes apparent on the germ cell level. In both sexes, the number of PGCs is the same until late embryogenesis. Then PGCs start to proliferate first in female embryos and enter meiosis afterwards. In male embryos, the PGCs stay in a quiescent status and only later PGCs resume proliferation and become spermatogonia [74-77].

The control of meiosis entry has been intensively studied and becomes reasonably well understood in mammals. Studies indicate that exposure to retinoic acid (RA) makes mouse fetal germ cells entering meiosis in female and initiating oogenesis, while the somatic cells of the testis produce the degrading enzyme (CYP26) that destroys RA and delays entry into meiosis in males [78, 79]. However, the information if and how RA is playing a role in this first decision of germ cell proliferation and meiosis entry in other species, especially in fish, are scarce.

### 1.2.1 Retinoic Acid, Signaling and Degradation

Vitamin A is known to be essential for embryonic development and the proper function of many different cells, tissues and organs in many species [80, 81]. Retinoic acid (RA) is a small molecule polar active metabolite of vitamin A that easily diffuses through tissues. This molecule is a classical morphogen. Generally, it is produced at the posterior end of the early vertebrate embryo during gastrulation, and is therefore expected to form posterior-anterior gradient [82-87].

Figure 4 offers an overview of vitamin A metabolism, signaling and binding. In mammals the large majority of dietary vitamin A is transported through maternal circulation as retinol bound to retinol-binding protein 4 (RBP4), and is uptake into the cells mainly by the interaction of the transmembrane receptor STRA6 (stimulated by retinoic acid 6). In oviparous species, the retinoid sources comprise carotenoid stores in the yolk (reviewed in Refs. [88, 89]). Inside the cell, retinol is bound by CRBPs (cellular retinol-binding proteins) and after can be stored as retinyl ester or oxidized to RA via a two-step enzymatic process. The first chemical reaction is the reversible conversion of retinol to retinaldehyde, with the dehydrogenation reaction catalyzed by the retinol dehydrogenases (RDHs), mainly RDH10. The cleavage of beta-carotene by beta-carotene oxygenase (BCO) enzyme(s) directly generates retinaldehyde.

Retinaldehyde dehydrogenases (RALDHs or ALDHs) then generate retinoic acid (RA). Once the synthesis is completed, the cellular RA binding proteins (CRABPs) direct the function of RA within the cell. In general, the RA bound to CRABP2 is directed toward the RA receptors (RARs), and RA bound to CRABP1 is targeted to degradation. RA exists as at least four different isomers: *all-trans*-RA (ATRA), 9-*cis*-RA, 13-*cis*-RA, and 9,13-*dicis*-RA. Of these, ATRA acts by binding to RARs regulating gene transcription [90, 91]. The retinoic acid receptors (RARs) are members of the steroid binding superfamily of transcription factors that are bound to their DNA responsive elements (retinoic acid responsive elements, RAREs for RARs) in the absence of their ligand. The RARs, through heteromeric binding with a retinoid X receptor (RXR) on RAREs, control the expression of RA-responsive genes that possess RAREs in their promotor region [82, 85, 92, 93].

Finally, RA signaling is terminated or, in some cases, prevented by the action of the RA-degrading enzymes, cytochrome P450 family 26 (CYP26) [86, 94-97]. The CYP26 family consists of three genes: CYP26A1, CYP26B1 and CYP26C1. Both CYP26A1 and CYP26B1 are hydroxylases that form 4-OH-RA, 18-OH-RA and 4-oxo-RA from ATRA [98, 99], whereas CYP26C1 appears to prefer 9-*cis*-RA as subtract [100]. Information about of a possible role of the many oxidative metabolites that can be formed by P450s are scarce. However, it was shown that metabolites like 4-OH-RA, 18-OH-RA and 4-oxo-RA can have a pharmacological activity and bind to RARs [101]. The spatio-temporal distribution of RA in the embryo is determined by the balance of the RA synthesis and degradation, generating a gradient along the embryo, been important for the anterior-posterior axis formation [84, 102].

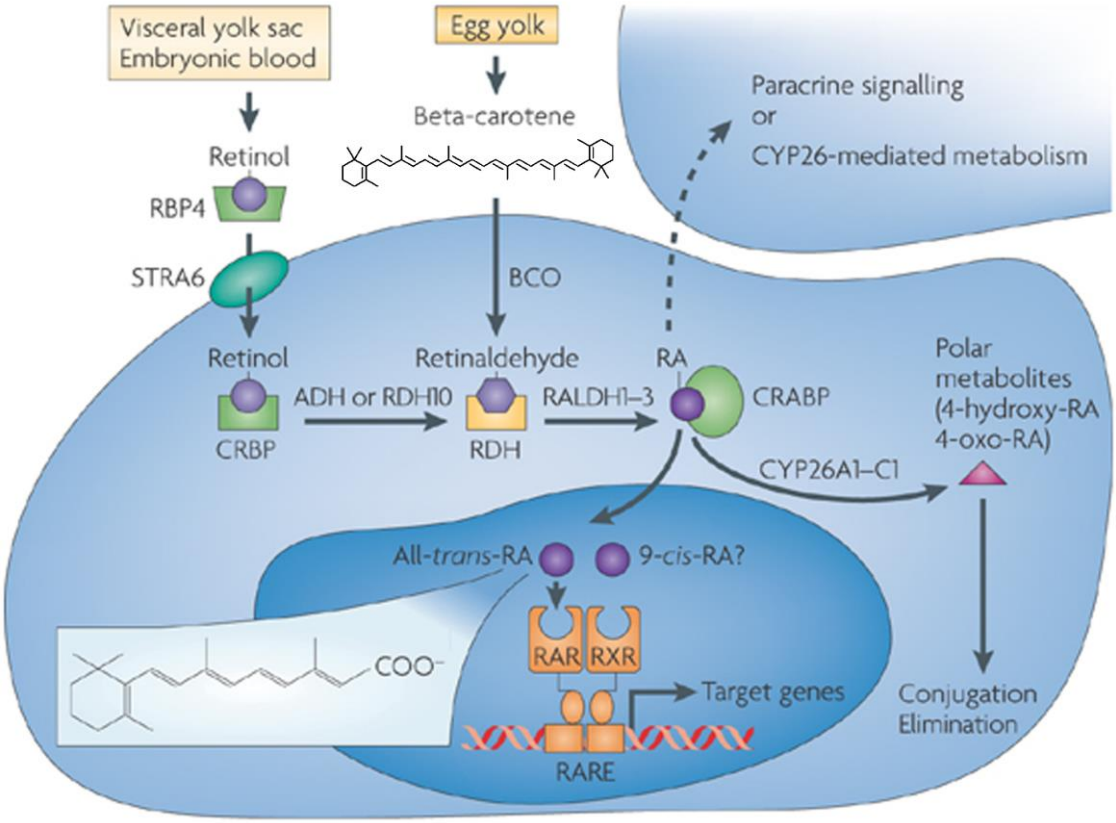


**Figure 1 Regulation of retinoid signaling in embryonic cells.** Retinoid sources comprise circulating maternal retinol in placental species and carotenoid stores in the yolk of oviparous species. Retinol is taken up by embryonic retinol binding protein 4 (RBP4), transferred intracellularly by the receptor protein stimulated by retinoic acid 6 (STRA6), and transformed into retinaldehyde, mainly by retinol dehydrogenase 10 (RDH10). Cleavage of beta-carotene by beta-carotene oxygenase (BCO) enzyme(s) directly generates retinaldehyde. Retinaldehyde dehydrogenases (RALDHs) then generate retinoic acid (RA), which acts within the nucleus as a ligand for nuclear receptors (heterodimers of RA receptors (RARs) and retinoid X receptors (RXRs) to regulate transcriptional activity of target genes. Binding proteins for retinol (cellular retinol binding proteins; CRBPs) and retinoic acid (cellular retinoic acid binding proteins; CRABPs) are involved in this pathway. All-trans-RA (formula depicted in figure) is the major *in vivo* RAR ligand, whereas the 9cis-RA stereoisomer acts *in vitro* as a RXR ligand, although its *in vivo* presence is debated. In cells expressing cytochrome P450 26 (CYP26) enzymes, RA is transformed into more polar compounds (4-hydroxy-RA and 4-oxo-RA), which are subject to further metabolism and elimination. Not only can RA act in an intracrine manner in cells that synthesize it, but also there is evidence for non cell-autonomous (paracrine) effects on neighboring cells or tissue layers (dashed arrow). ADH, alcohol dehydrogenase; RARE, RA-response element. Adapted from Ref. [88].

### 1.2.2 Entry into Meiosis

Since 1925 it is known that vitamin A is required for production of fertile sperm in mammals [103]. The role of RA in triggering meiosis has been mainly described in mammals; however it seems that this mechanism is also conserved in other vertebrate species, like amphibians and birds [104, 105].

In mammals, like in other vertebrates, the entry into meiosis in male and female germ cells occurs at different stages of development. In the mouse, the bipotential germ cells continue to divide mitotically for 2-3 days after entry into the genital ridge in both sexes. Then germ cells cease mitosis and enter prophase of the first meiotic division in the developing ovary at about 12.5-13.5 days post-coitum (dpc). In the developing testis the germ cells do not enter meiosis during fetal life but instead arrest in the $G_0/G_1$ phase of the cell cycle also about 12.5-13.5 dpc and postpone meiosis until birth. It was shown that the initial sex differentiation of a germ cell is determined not by its intrinsic chromosomal constitution, but by its cellular environment. It was hypothesized, that the developing testicular tissue produces a meiosis-inhibiting factor. Studies indicate that exposure to RA makes mouse fetal germ cells entering meiosis in female and initiates oogenesis, while testis produce the CYP26 enzyme that degrades RA and thereby delays entry into meiosis in male. It was shown that *Cyp26b1* transcripts are present in mouse in developing gonads of both sexes, but then is upregulated in testis and downregulated in ovary [78]. Hence, after 11.5 dpc, *Cyp26b1* is not anymore expressed in female gonads, but very high in male gonads, particularly at the critical time of germ cell meiosis entry [79]. This suggested that CYP26B1 acts as a meiosis inhibitor destroying RA coming from the mesonephros to which the gonads are attached [106, 107]. Treatments with RA (or RAR agonists) in organ culture of early gonad induces expression of the pre-meiotic marker Stra8 (stimulated by retinoic acid gene 8), followed by the expression of the early meiotic markers Sycp3 (synaptonemical complex protein 3) and Dmc1 (dosage suppressor of mck1 homolog) [74, 78, 107, 108]. However, in *Cyp26b1* knockout (KO) mice XY embryos form an apparently normal testis, but RA levels are raised, *Sycp3* and *Stra8* are highly upregulated, and XY germ cells enter and progress through meiosis [78, 109].

Analysis of *Stra8* KO mice demonstrated a key role of STRA8 as a gatekeeper for meiosis initiation. In XX *Stra8* KO mice there is no evidence that germ cells have

entered meiosis [110]. It seems that, in male mouse, postnatal entry in meiosis requires RA and *Stra8* [111]. The evidence that both RA and STRA8 are involved in initiating meiosis in both sexes suggests that the molecular control of pre-meiosis S-phase entry is conserved and gender-neutral [74].

On the other hand, in non-tetrapod vertebrates the information about the control of meiosis entry and if this event is related to RA function remains uncertain. Analyzes of different genomes of fish suggested that RA could regulate meiotic initiation via *stra8*-independent pathway, since no *stra8* homologue is present in the genomes of majority of the analyzed species. However, recently *stra8* was identified in some fish species and specially characterized in the catfish *Silurus meridionalis* [112]. In this species, RA triggers the onset of meiosis via *stra8*-dependent pathway, as it does in tetrapod [113]. Not much is known from other the majority of fish species that lack *stra8*. In Tilapia treatments with an inhibitor of RA synthesis or disruption of Aldh1a2 leads to a delay in entry of meiosis [114]. A recent study in zebrafish tried to correlate the role of RA to meiosis by analyzing the expression of the metabolic enzymes during gonad development and in the adult, but no functional information was provided to confirm this function [115].

In medaka, information on a possible role of RA in meiosis entry of the PGCs is lacking. It has been shown that during late embryogenesis the number of PGCs is approximately the same in both XY and XX embryos, The PGCs start to proliferate in female embryos and enter meiosis around the hatching stage. In male medaka, only at around 15 to 20 dph (days post hatching) the PGCs resume proliferation and become spermatogonia [77]. *In vitro* and *in vivo* studies showed that *dmrt1bY* expression inhibits PGC proliferation at the sex determination stage by a G2 arrest of the germ cell cycle [59]. Further experiments including depletion of PGCs revealed that the number of PGCs could also determine the sex phenotype. For instance, medaka lacking germ cells develop as males [116]. Overproduction of germ cells induces female development as demonstrated by a medaka mutant, named *hotei,* which has an excess of germ cells and shows male-to-female sex reversal [117]. The *hotei* phenotypes is caused by a mutation of the Amh (Anti-Müllerian hormone) type II receptor (*amhrII*) [118]. Characterization of this mutant showed that the Amh signaling acts in supporting cells to regulate the proliferation of the mitotically active germ cells but does not trigger

quiescent germ cells to proliferate in the developing gonad [117]. Of note, the master sex determination region on the Y chromosome of the tiger pufferfish, *Takifugu rubripes* (fugu), contains a more active version of the *amhrII* gene than the X chromosome version, and that is responsible for the sex determination to male in this species[65]. Those results demonstrate that the *amh*/*amhrII* system, which is related to germ cell proliferation and differentiation, can play a crucial role in sex determination.

## 2. Medaka, *Oryzias latipes,* as animal model

In this thesis, the animal model used is the teleost fish medaka (*Oryzias latipes*). Medaka belongs to the order Beloniformes (needlefishes), family Adrianichthyidae and subfamily Oryziinae. The medakas, also known as ricefishes, are small size fish (maximum length about 9 cm) mainly found in freshwater and brackish water [119].The model species the Japanese medaka, *Oryzias latipes*, has several advantageous features as an experimental animal. As it is an oviparous fish, embryonic development occurs externally and embryos are completely transparent throughout most of their embryonic development [120]. The genome size of medaka is only about 800 million base pairs (Mbp) compare to that of zebrafish, which is 1700 Mbp. The small genome size is advantageous for isolating entire genomic regions from a relatively small genomic DNA library, such as a fosmid library [121]. With respect to its sex determination system, medaka has been shown to be a better suited model in comparison with other fish species. The sex determination gene (*dmrt1bY*) has been identified and well characterized [56, 57]. Therefore, it is possible to genotype molecularly males and females even during the embryonic development, before the gonad is differentiated. For retinoic acid (RA) studies, analyses of the functional evolution of Aldh1a family members showed that teleost lost some genes, and in medaka only the Aldh1a2 gene is still presented, making medaka an easier organism to describe the synthesis of RA [122].

## 3. Aims and Outline of the Thesis

The process of meiosis entry in the early embryo is still not elucidated, and the function of RA in this process seems to vary in different species, especially in higher teleost. However, the timing of meiosis entry, first in female and later in male, is conserved in vertebrates, and the master sex determination genes and other sex related

genes are somehow involved in the controlling of PGC proliferation and/or meiosis (see above). Therefore, to characterize the relation between the process of sex determination/differentiation and meiosis entry, and a possible role of retinoic acid in this process, I decided to use the medaka fish, *Oryzias latipes*, as model species. Since medaka is one of the teleost species where *stra8* is absent [123], my studies were also intended to help to understand meiosis regulation in a Stra8-independent manner.

In **Chapter 2**, a descriptive study of the synthesis and the responsiveness of RA was carried out to characterize a possible role of RA on meiosis entry in medaka. Furthermore, exogenous treatments with RA inducing pathway and RA inhibition chemicals were performed in embryos and adult fish. Hence, we could analyze the role of RA not only in the first meiosis but also in the adult fish. **Chapter 3**, focuses in the changes of the sex related genes to the exogenous treatments performed in **Chapter 2**. Mutants were developed for the RA degrading enzyme genes *cyp26a1* and *cyp26b1*, and described the knockout situation. **Chapter 3** also investigate a possible induction of meiosis *in vitro*, using medaka spermatogonia stem cells (SG3). Finally, **Chapter 4** summarizes, discusses and integrates the main results obtained from this thesis.

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# Chapter 2

## Retinoic Acid and Meiosis Induction in Adult versus Embryonic Gonads of Medaka

Mateus C. Adolfi[1], Amaury Herpin[1,2], Martina Regensburger[1], Jacopo Sacquegno[1], Joshua S. Waxman[3] and Manfred Schartl[1,4]

1 -University of Wuerzburg, Physiological Chemistry, Biocenter, Am Hubland, D-97074 Wuerzburg, Germany.
2 - INRA, UR1037, Fish Physiology and Genomics, Rennes F-35000, France.
3 - The Heart Institute, Molecular Cardiovascular Biology and Developmental Biology Divisions, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA.
4- Comprehensive Cancer Center Mainfranken, University Clinic Würzburg, Josef Schneider Straße 6, 97074 Wuerzburg, Germany and Texas Institute for Advanced Study and Department of Biology, Texas A&M University, College Station, Texas 77843, USA.

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## Abstract

Sex determination (SD) is a complex developmental process regulated by cascades or networks of genes, which establish the cellular fate of the undifferentiated gonadal soma and of the primordial germ cells (PGC). Although the molecular mechanisms involved in SD are highly diverse in vertebrates, there are a few conserved features. In all vertebrates studied so far one of the first recognizable differences between male and female embryos is the onset of meiosis. In mammals, retinoic acid (RA) has been shown to be regulate this initiating event. Since a role of RA in entry of meiosis and sexual development of fish is still far from being understood, we investigated in medaka a possible meiotic function of RA during (i) the SD period in embryos and (ii) in reproductively active gonads of adults. We generated a transgenic medaka line that reports responsiveness to RA *in vivo*. We observed different expression domains for the RA-synthesizing (*aldh1a2*) and the RA-degrading gene (*cyp26a1*). Expression of the *aldh1a2* gene in the early gonad did not reflect action of RA in those same cells, showing that production of RA is not necessarily connected to transcriptional control activity. Interestingly, we find RA mediated transcriptional activation in germ cells of medaka embryos of both sexes but unexpectedly much earlier than the cognate SD stage, which is around hatching. No activity of RA was seen in germ cells at the SD stage when meiosis commences in female hatchlings. In adults, there is a clear sex specific expression of the *aldh1a2* gene in males, and of the *cyp26a1* gene in females, indicating an important sex-bias of RA metabolism in the gonads. In ovaries, the responsiveness to RA in early oocytes, especially in oocytes in meiosis arrest. In the testis, retinoic acid is acting directly in the pre-meiotic cells, but also in the Sertoli cells and Leydig cells. Our results show that RA plays an important role in meiosis induction and gametogenesis in medaka in adults like in mammals. However, contrary to common expectations, we find no evidence for a similar role of RA in initiating the first meiosis in female germ cells at the sex determination stage. We correlated for the first time the source of RA metabolism and the cell-specific responsiveness in the gonads of embryo and adult fish.

## 1. Introduction

In vertebrates, the decision whether the bipotential gonad anlage will become testis or ovary is a critical stage in embryonic development. This complex sex determination process includes fate determination and cell differentiation within two fundamentally different programs, the female and the male. These intricate processes are regulated and fine-tuned by cascades or networks of genes. In the past 20 years the paradigm that ruled the sex determination field was that, the genetic machinery controlling gonad development is broadly conserved. Results indicated that the downstream components tend to converge upon the regulation of common effectors, while the 'master sex-determining genes', at the top of the genetic hierarchies, show an impressive diversity in different organisms [1, 2]. However, more recent data comparing fish and mammals indicated also discrepancies in the gene expression patterns and in the interactions of the downstream gonadal regulatory network, which mighty reflect a plasticity of this regulatory network to various degrees during vertebrate evolution [3, 4]. In this respect, the development of the gonad is different from all other major vertebrate organs, where generally a high conservation of molecular mechanisms from fish up to humans has been noted [5, 6]. Despite the modest degree of conservation on the molecular level, there are however some commonalities in sexual development of vertebrates.

The undifferentiated gonad of all vertebrates is composed of somatic cells and germ cells, the latter giving rise to the gametes. All gametes originate from primordial germ cells (PGCs), which migrate into the developing gonad [7-9]. Germ cells are confronted there to take two major decisions; one is the sexual identity of the cell to differentiate as sperm or egg. The other decision is to remain in mitotic division cycles, or to entry into meiosis. The timing of the mitosis/meiosis decision and features of meiosis itself are often sex-specific-triggered, suggesting a close relationship between the mitosis/meiosis and sperm/egg decisions [10]. A common feature found in most vertebrates from fish to mammals is an early morphological difference between male and female that becomes apparent on the germ cell level. For instance in medaka, in both XY and XX embryos, the number of PGCs is the same until late embryogenesis. Then PGCs start to proliferate in female embryos and enter meiosis around the hatching stage. In males, it is only at around 15 to 20 days post hatching (dph) the PGCs proliferate again and become spermatogonia [11]. This is a similar situation as in

mammals where PGCs in females resume mitosis and enter prophase of the first meiotic division much earlier than in males [12, 13].

In mammals is well known that the critical molecule in the control of meiosis entry is retinoic acid (RA) [14-17]. RA is a polar derivative of vitamin A that easily diffuses through tissues and is a classical diffusing morphogen [18-22]. The balance of the RA-metabolizing enzymes also determines the spatio-temporal distribution of RA [23]. These are the RA synthesizing enzyme Aldh1a, a member of the retinaldehyde dehydrogenase family (also known as raldh and which can be present in one to several isozymes depending on the species), and the RA-degrading enzymes from the Cyp26/ P450-cytochrome family [24-27]. It acts by binding to nuclear retinoic receptors (RARs), which through binding to retinoic acid response elements (RAREs) control the expression of RA-responsive genes [28, 29].

Initial sex differentiation of a mammalian germ cell is not determined by its intrinsic chromosomal constitution, but rather by its cellular environment [30, 31]. This also includes the first entry into meiosis. It was hypothesized, that the developing testicular tissue produces a meiosis-inhibiting factor, since the germ cells go through meiosis first in female while testicular germ cells do not enter meiosis during fetal life [13, 32, 33]. Studies indicated that exposure to RA makes mouse fetal germ cells entering meiosis in female and initiate oogenesis, while the somatic cells of the testis produce the Cyp26 enzyme that degrades RA and delays entry into meiosis in male [34, 35]. Expression studies during mouse gonadogenesis indicated that CYP26B1 acts as a meiosis inhibitor degrading RA coming from the mesonephros to which the gonads are attached [14, 36]. RA acts to initiate meiosis by inducing expression of the meiosis marker *Stra8* (*stimulated by retinoic acid gene 8*), followed by the expression of the early meiotic markers *Sycp3* (*synaptonemical complex protein 3*) and *Dmc1* (*dosage suppressor of mck1 homolog*) [14, 16, 34, 37].

Although such a role of RA in triggering meiosis was mainly described in mammals, evidence show that this mechanism is likely to be conserved in amphibians and birds [38, 39]. However, information about such a function in fish is lacking. in zebrafish Recent work based on expression profiles of the RA metabolism enzymes during gonad development and in the adult indicated a possible role of RA for meiosis, but no functional information is available so far to confirm this function [40].

Disruption of the *ald1a2* in Tilapia leads to a delay in meiosis entry [41]. Analyses of the evolution of *aldh1a* gene family members revealed that teleost lost some paralogs, and in medaka only the *aldh1a2* gene is still presented [42]. This information together with the fact that no sequence of *stra8* was found so far in most teleost genomes including medaka, poses the question whether RA has indeed a similar role in meiosis and gonadogenesis in fish as in mammals.

To get a better understanding of the role of RA in meiosis induction in fish we used the model species medaka, where the primary processes of sex determination are well studied and reasonably understood. We generated transgenic lines to analyze the expression pattern of the RA synthesizing gene and the transcriptional regulation activity of RA in the gonad during sexual development. Further, we used these tools to analyze the role of RA in the adult gonad of the fish. Results show that the expression pattern of the RA biosynthesis gene is similar to zebrafish and Tilapia. Studying the timing of production of RA and its activity in the germ cells during development and in the adult gonad of medaka, we show that RA has an important role in meiosis at later stages of gonad development rather than in initial sex determination in the embryo.

## 2. Materials and methods

### 2.1 Animals

Medaka (*Oryzias latipes*) fish belongs to the class Actinopterygii, order Beloniformes and the family Adrianichthyidae. It is tiny fresh water species, which is widely used as a laboratory organism for many biological questions and in biomedical research [43].

All experiments were performed with Carbio strain of medaka. The animals were kept under standard photoperiod cycle of 14 hr/10 hr light/dark at 26°C (±1°C). Eggs were collected 1-2 hr after starting the light cycle and raised at 26°C in Danieau's medium (17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)2$, 1.5 mM Hepes, pH 7.2). The stages of development were identified according to Iwamatsu (2004) [44].

## 2.2 Production of 12XRARE reporter transgenic fish and imaging analyses

To make a transgenic RA reporter, we used the 12XRARE reporter vector (12XRARE-ef1a:gfp), which has a concatenation of 12 direct repeat 5 (DR5) retinoic acid response element (RARE) sites. As described previously, the RARE sites were placed upstream of an elongation factor-1 alpha (ef1a) minimal promoter in a vector containing *egfp* flanked by adeno-associated viral inverted terminal repeat elements and I-SceI sites [45]. The plasmid was injected at a concentration of 100 ng/µl together with the *I-SceI* meganuclease enzyme (0.5 unit/µL in 0,5 X *I-SceI* buffer), through the chorion into the cytoplasm of the one-cell stage embryo [46]. Embryos were kept at 28.5°C until hatching. GFP- positive $G_0$ fish were mated to each other and the offspring were again sorted for fluorescence.

We generated double transgenic reporter lines by crossing 12XRARE-ef1::gfp with Vasa::H2B::mCherry and sox9b::mCherry [3] fish to identify the germ cells and the somatic cells, respectively. The Vasa::H2B::mCherry medaka transgenic line was kindly supplied by Dr. Alexander Froschauer from the Zoology and Developmental Biology, Technical University of Dresden.

For imaging embryos, hatchlings or tissues were mounted with 1-2% low melting temperature agarose. Confocal pictures and image stacks were acquired using a Nikon C1 (eclipse Ti) confocal laser scanning microscope and the NIS elements AR software.

## 2.3 *In vivo* all-trans retinoic acid, Citral and Diethylaminobenzaldehyde (DEAB) treatments

All-*trans*-retinoic acid (ATRA; Sigma-Aldrich) and DEAB (Sigma-Aldrich) were diluted in DMSO as 1 mM and 10 mM stocks, respectively, and stored at -20°C in the dark. Serial dilutions for RA (100nM, 200nM, 500nM and 1000nM) and DEAB (5 µM, 10 µM, 25 µM and 50 µM) were made in Danieau's solution just prior to use. Medaka embryos were collected and raised in Danieau's medium. From the dome stage (stage 14) onwards, the embryos were kept in the dark for 24 hours (stage 22). The concentration range is based on reports of similar treatments in other fish [21, 47].

To investigate an effect on meiosis entry, we made long term treatments from stage 29 until 10 days after hatching (dah) of Citral (1 μM; Sigma-Aldrich), and the medium was changed every 2 days. Specimens were collected at 1 dah, 5 dah, 10 dah, 20 dah, 30 dah, 40 dah and 50 dah and genotyped for sex by PCR amplification of the Y-linked male determining gene *dmrt1bY* using genomic DNA as template. For genomic DNA extraction, a piece of tissue was incubated for 1 hour at 95°C in 100 μL of a basic buffer solution (25 mM NaOH, 0,2 mM EDTA, pH=12) with gentle shaking. The solution was cooled down on ice, 100 μL of neutralization solution (40mM Tris-HCl pH=5.0) added and vortexed. 2 μL of the total volume was used in a 25μL PCR reaction. The PCR products were resolved in 1% agarose gels.

## 2.4 Testis organ culture

For experiments on meiosis induction and inhibition in adult testis, we performed *ex vivo* organ cultures as developed for zebrafish testis [48]. Medaka gonads during the development start as two separated tissues and later on fuse together into one [49]. The gonads of adult male were dissected, and the fused testes were separated in two parts; one was used as experimental control and the other for treatments. Considering that the cell cycle time of spermatogonia in fish is approximately 30 hours [50] we used an incubation period of 3 days.

Triplicates of testis samples were incubated either with *all-trans*-retinoic acid (ATRA, 1 μM), DEAB (2,5 μM), Citral (1 mM), CYP26 inhibitor Talarazole (1 μM) and RARα agonist AM580 (10 nM), in presence or absent of Talarazole. After incubation the testes were used for gene expression studies.

## 2.5 Real time quantitative RT-PCR

Tissues of adult males and females and whole embryos of different developmental stages from the Carbio strain were collected. Total RNA was extracted from 3 pools of adult fish tissues (n=4) and whole embryos (n=20) using the TRIZOL reagent (Invitrogen) according to the supplier's recommendation. After DNase treatment, reverse transcription was done with 2 μg total RNA using RevertAid First Strand Synthesis kit (Fermentas) and random primers. Real-time quantitative PCR was carried out with SYBR Green reagent and amplifications were detected with a *Mastercycler® ep realplex* (Eppendorf). All results are averages of at least three

independent RT reactions from three independent RNA preparations. Transcript levels of the target genes were normalized against the medaka elongation factor-1 alpha (*ef1a*) gene. The ΔCt values presented as means ± standard error of the mean (SEM), were analyzed by one-way ANOVA, Tukey's and Student's t-test. A significance level of $P < 0.05$ was used for all tests.

## 2.6 Whole mount in situ hybridization

To generate riboprobes, cDNA fragments corresponding to the 3' untranslated region (UTR) and the open reading frame (ORF) of *aldh1a2* and *cyp26a1* were PCR amplified from cDNA of adult medaka testis and ovary respectively (for primers sequences see Table 1). The amplified fragments were subcloned and used as template for *in vitro* transcription. Whole-mount RNA in situ hybridization was performed as described [51]. Stained embryos were dissected and mounted in glycerol for photography. Staining time was individually adjusted for each probe to get the best signal and does not reflect the endogenous transcript expression level. For a more sensitive fluorescence *in situ* hybridization detection, we used the HNPP Fluorescent Detection Set (Roche) according to the manufacturer's instructions.

| ***Primer*** | **Sequence** | **Purpose** |
|---|---|---|
| Aldh1a2_RT_F | 5'-GCCTTCTCTTTGGGCTCAGT-3' | qRT-PCR |
| Aldh1a2_RT_R | 5'-GGAAATGCGGTGTAGTGGTC-3' | qRT-PCR |
| Cyp26a1_RT_F | 5'-AGAGATGAAGCGGCTGATGT-3' | qRT-PCR |
| Cyp26a1_RT_R | 5'-TCTTCTTGCTGCTGTCGATG-3' | qRT-PCR |
| Cyp26b1_RT_F | 5'-ATCCGAGCACGAGACATCTT-3' | qRT-PCR |
| Cyp26b1_RT_R | 5'-GGATGTTGGAGGAGTTGCAT-3' | qRT-PCR |
| Cyp26c1_RT_F | 5'-CAGAGGGGCTTGACTTTGAC-3' | qRT-PCR |
| Cyp26c1_RT_R | 5'-CAGCCTTTGGGAATCTGGTA-3' | qRT-PCR |
| Dmc1-RT-F | 5'-GTTTCCAGACGGCCTTTGAG-3' | qRT-PCR |
| Dmc1-RT-R | 5'-CGATGTCTCTCAGCCTGTCT-3' | qRT-PCR |
| Ef1a-RT-F | 5'-GCCCCTGGACACAGAGACTTCATCA-3' | qRT-PCR |
| Ef1a-RT-R | 5'-AAGGGGGCTCGGTGGAGTCCAT-3' | qRT-PCR |
| Aldh1a2_Probe_F | 5'-CAGAGTGGAATCCAGGAAGG-3' | *in situ* hybridization probe |
| Aldh1a2_Probe_R | 5'-GGAAATGCGGTGTAGTGGTC-3' | *in situ* hybridization probe |
| Cyp26a1_Probe_F | 5'- CTTTCAGCTCAATGGCTTCC -3' | *in situ* hybridization probe |
| Cyp26a1_Probe_R | 5'-CAAACGTCATGAGCAGCCTA-3' | *in situ* hybridization probe |

**Table 1** –Sequence of primers used in the present study

*In situ* hybridization on sections was carried out on 5 µm-thick paraffin sections. After paraffin removal the sections were rehydrated and treated with proteinase K (10

μg/mL; Sigma-Aldrich) at room temperature for 10 min. Hybridization with 400 ng riboprobe per milliliter was carried out in hybridization solution (5x standard sodium citrate, 50 % deionized formamide, 50 μg/ml heparin, 0,1 % Tween-20, 50 mg/ml yeast tRNA) at 70 °C overnight. The slides were then washed in 5x and 0,5x standard sodium citrate in 20% formamide at 60 °C, each 3 times for 20 min, and blocking was performed with 1 % Blocking Reagent (Roche, Basel) at room temperature for 1 h. Subsequently, the slides were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche) diluted 1:2000 in blocking solution at 4 °C overnight. Color development was conducted by incubating the sections in Nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Roche) or with the HNPP Fluorescent Detection Set (Roche) in the dark. After air-drying, the slides were mounted in glycerol for photography and micrographs were taken with a ZEISS Axiophot microscope.

### 2.7 Light microscopy

Larvae and gonads from adult fish were dissected and fixed in 4% paraformaldehyde solution for 24 h at 4°C, subsequently dehydrated, embedded in paraffin, and then serially sectioned at 5 μm thickness. The sections were counterstained with hematoxylin & eosin (HE).

## 3. Results

### 3.1 Synthesis of RA and RA activity during embryonic gonad development.

Transcripts levels of *aldh1a2* of different embryonic stages were determined from pooled embryo extracts. Levels are undetectable during early stages but become upregulated around early blastula stage (stage 10) and steadily increase. After stage 33, the levels remain relatively constant until hatching (stage 39) (Figure 1 A).

To identify the source of RA in the gonadal region of the embryo we analyzed expression of *aldh1a2* by *in situ* hybridization (Figure 1 B-G). During early somitogenesis, the gene is strongly expressed in the first four pairs of somites (Figure 1 B). Later, at stage 26, the RNA is still present in the paraxial mesoderm and additionally strongly expressed in the brain, eyes and fin bud (Figure 1 C). From stage 32 until hatching, the expression of *aldh1a2* was detected in cells surrounding the gonad

and in the intestine (Figure 1 D-G).

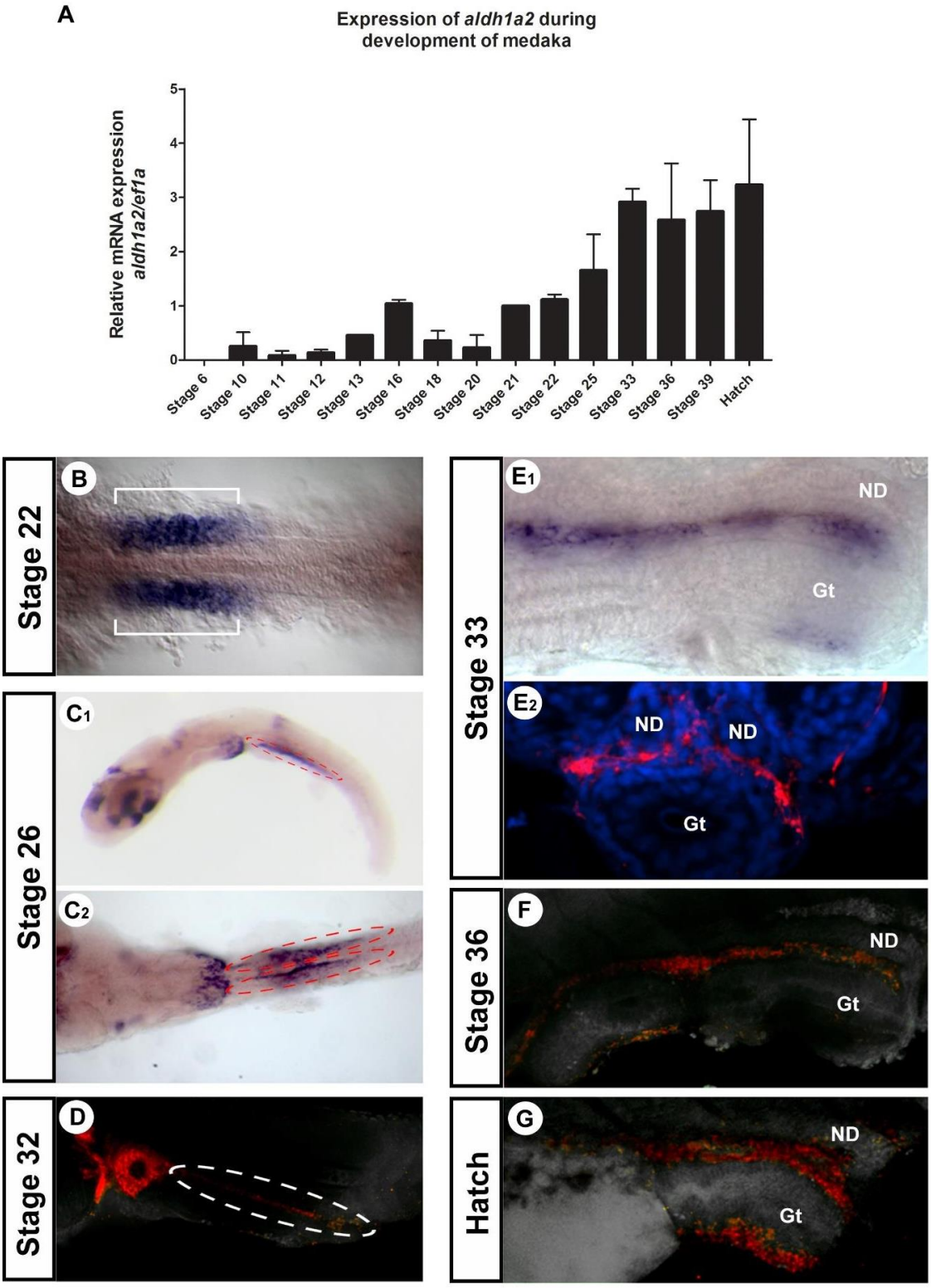


**Figure 1. Expression pattern of *aldh1a2* during medaka development.** *aldh1a2* (A) mRNA expression in embryos of medaka in different stages of development. Values are expressed as arbitrary units of mRNA normalized against the expression levels of ef1a amplified from the same template, relative to the expression observed in the stage 21. *In situ* hybridization of *aldh1a2* (B-G) in different stages of development. Gt, gut; ND, nephric duct.

In order to find out if the regions that metabolize RA are also responding to RA, we analyzed responsiveness to RA during embryonic development by means of a transgenic reporter line where 12 copies of the Retinoic Acid Responsive Element (12X RARE) drive eGFP expression. Ubiquitous GFP signals were already observed at early stages of the embryos and could be observed throughout all stages of development (Supplementary Figure 1). When the production of RA was compared to the responsiveness to his molecule, we did not observe any correlation between site of synthesis and transcription regulating activity of RA. For instance, in the eye at stage 26, the expression pattern of *aldh1a2* in the retina is clearly restricted to a ventral and a dorsal domain (Supplementary Figure 2 A). In the 12XRARE reporter fish, both domains are present; however, the dorsal part of the retina shows only barely detectable activity (Supplementary Figure 2 B). At stage 22, the first four somites present expression of *aldh1a2* (Supplementary Figure 2 C); however, the neural tube, where *aldh1a2* is not expressed, also showed responsiveness to RA (Supplementary Figure 2 D). These results indicate that the RA responsive cells are not restricted to the region where RA is produced and that production of RA is not necessarily connected to transcriptional control activity, in agreement with a non-cell autonomous action of this diffusible signal molecule (more information in Appendix 2).

At the level of the gonads, we were able to see GFP positive cells. At the 35 somite stage (stage 30), we identified responsiveness for RA in germ cells of both sexes (Figure 2 A-C). Thereafter, no signal was observed in germ cells or in somatic cells until one day after hatching, when the germ cells in females already have entered into meiosis. We observed clear expression of *aldh1a2* in the region of the gonads in both male and female embryos at stage 33 (Figure 2 F and G). Despite expression of *aldh1a2* in the gonads of males and females, the GFP signal indicating RA responsiveness was clearly restricted to the nephric ducts (Figure 2 D, E, H and I). In particular, during the sex determination stages (stage 26 to 36) when meiosis starts in female germ cells, no responsiveness was seen (more information in Appendix 3).

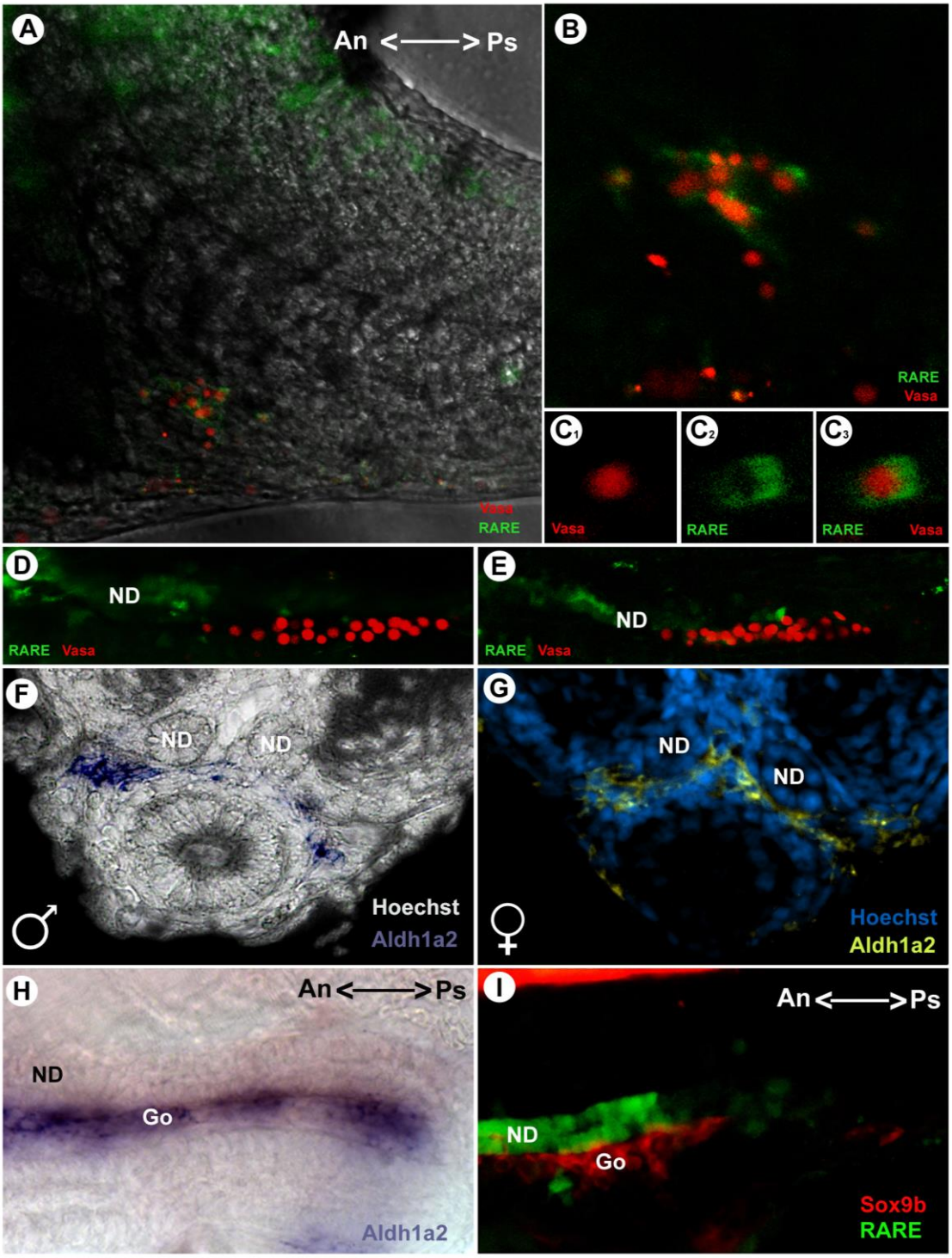


**Figure 2. Responsiveness to RA in the gonad during medaka development and correlation with *aldh1a2* expression at stage 33.** Responsiveness to retinoic acid (Green) in embryonal stages 30 (A-C) 35 (D) and 37 (E) of double transgenic embryos (12XRARE-ef1::gfp::Vasa::H2B::mCherry). Germ cells, vasa positive (Red), at the gonad level. Responsiveness in the germ cells is apparent at stage 30 and switches to the nephric duct at later stages. *In situ* hybridization for *alh1a2* transcripts show expression in the gonadal region in male (F) and female (G) in transversal section, and lateral view (H). Lateral view of double transgenic embryos (12XRARE-ef1::gfp (Green):: sox9b::mCherry (Red)) (I) showing no co-localization at this stage. An, anterior region of the embryo; Go, gonadal region; ND, nephric duct; Ps, posterior region of the embryo.

## 3.2 Tissue specific expression of RA metabolism genes in adult medaka.

The transcript levels of RA metabolism enzyme genes (*aldh1a2*, *cyp26a1*, *cyp26b1*, *cyp26c1*) were analyzed for differential expression between male and female medaka (Figure 3 A-D). The *aldh1a2* gene showed high expression levels in the kidney in both sexes. The organ that showed pronounced differential *aldh1a2* expression between the sexes was the gonad, with testis presenting significantly higher expression than ovary. On the contrary, expression levels of *cyp26a1* are extremely high in the ovary, compared to all the other organs in both sexes. The *cyp26b1* and *cyp26c1* genes showed high expression in the gills, but also in brain for *cyp26b1* and in eyes for *cyp26c1*. No significant differences were observed between testis and ovary for both genes. This indicates that *cyp26a1* has a more critical role for sexual development amongst the RA degrading enzymes. Thus, the *cyp26b1* and *cyp26c1* genes were not followed up further.

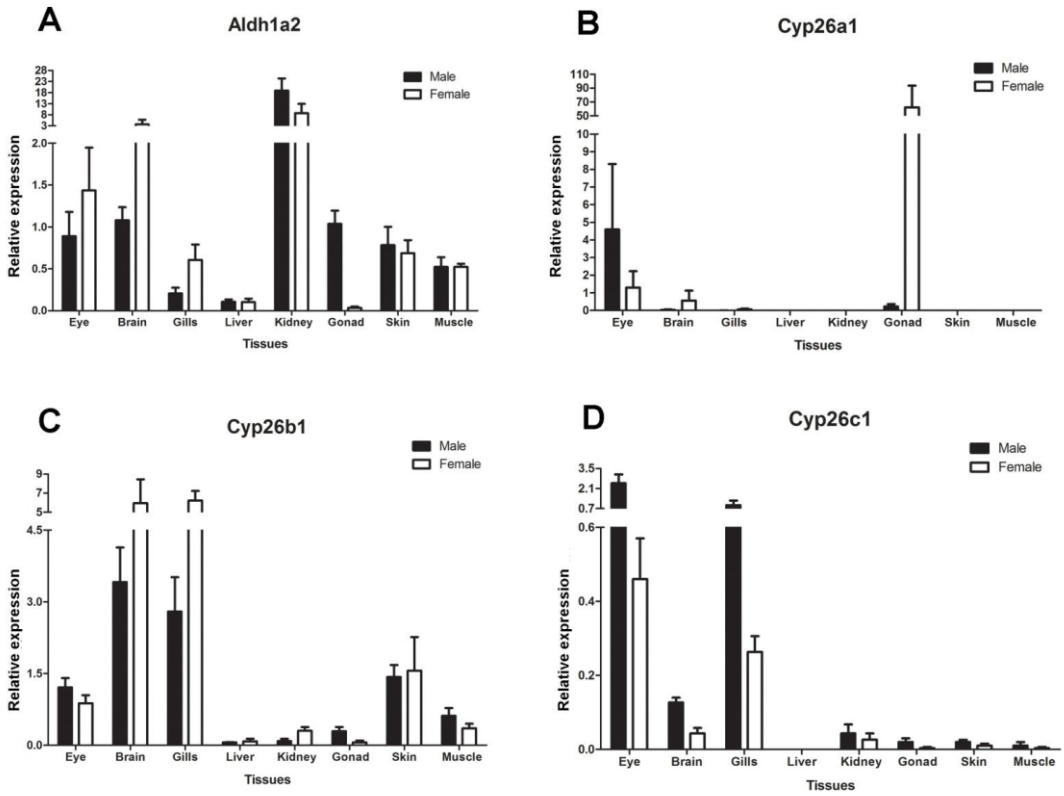


**Figure 3. Expression of retinoic acid biosynthesis and degradation enzyme genes in adult medaka**
Expression levels of *aldh1a2* (A), *cyp26a1*, (B), *cyp26b1* (C), *cyp26c1* (D) in organs of adult male and female medaka determined by qRT-PCR. Values are expressed as arbitrary units of mRNA levels normalized against the expression levels of ef1a amplified from the same template and relative to the average expression observed in eyes of males and females.

## 3.3 Transcript localization of *aldh1a2* and *cyp26a1* and RA responsiveness in adult gonads.

To investigate a possible role of RA in male and female gonads of medaka, we attempted to identify the cells that express the RA biosynthesis enzyme gene and the sites of responsiveness to RA in ovary and testis of adult fish.

RNA *in situ* hybridization for *aldh1a2* in adult ovary showed no localization in any steroidogenic cells or germ cells (Figure 4A). *cyp26a1* expression was exclusively seen in the early oocytes and was most prominent in pre-vitellogenic oocytes (Figure 4 B). Analysis of 12XRARE adult females showed that the germ cells are responsive to RA, however only the early oocytes. The signal in early oocytes was detectable already in the pre-vitellogenic stage, increased gradually during vitellogenesis, and vanished completely in post-vitellogenic oocytes (Figure 4 C).

In adult testis, *aldh1a2* was specifically expressed in Leydig cells (Figure 4 D). For *cyp26a1*, no signal was detectable. In the 12XRARE transgenic line, strong reporter expression was observed in the somatic cells in whole-mount preparations (Figure 4 E). In transversal sections, responsiveness to RA was also seen in germ cells at the tip of the germinal epithelium lobe (Figure 4 F). Leydig cells that were positive for *aldh1a2* also presented responsiveness to retinoic acid (Figure 4 G). At the germ cell level, only the early spermatogonia were GFP positive, and only those Sertoli cells close to GFP positive spermatogonia showed RARE reporter expression. (Figure 4 H).

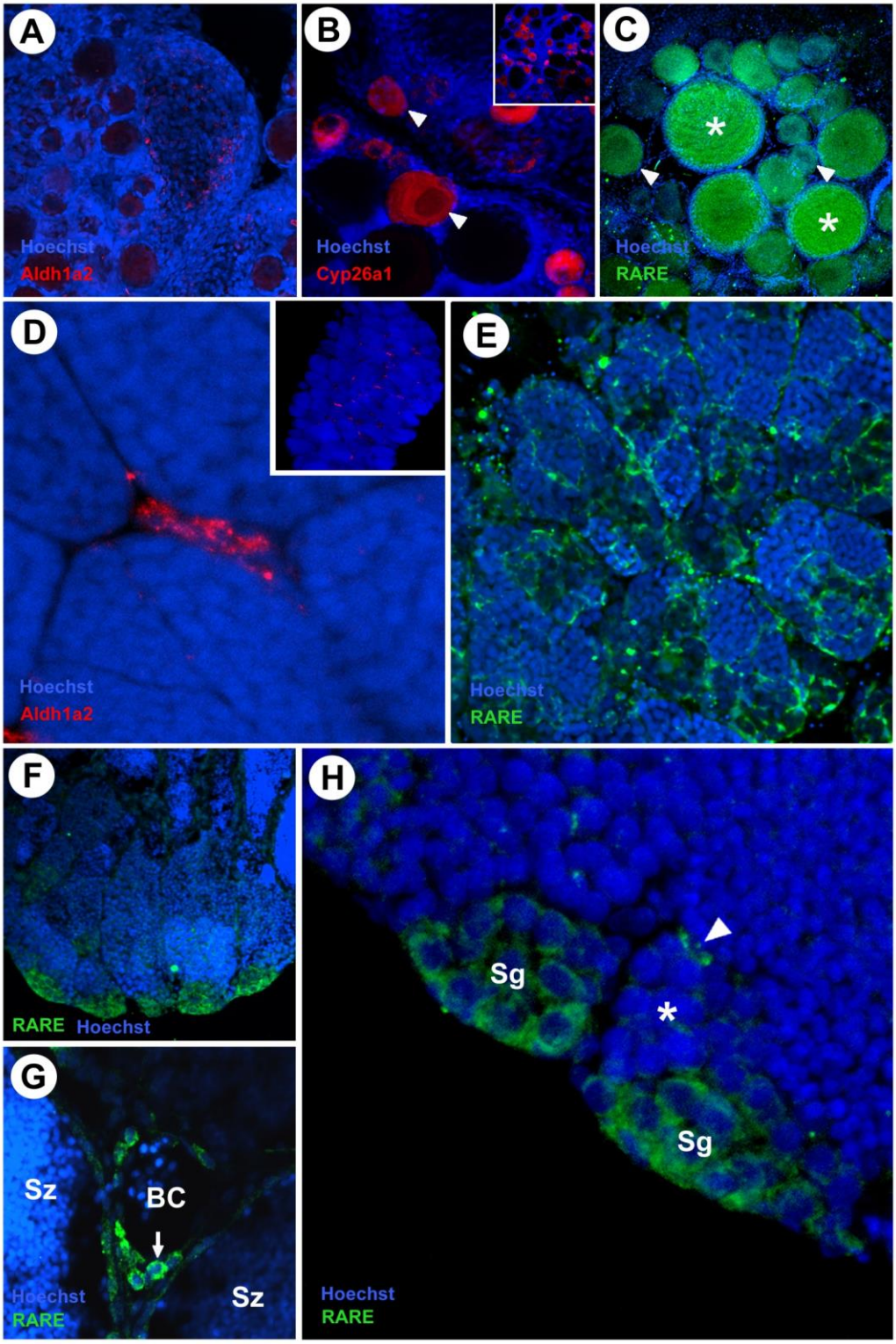


**Figure 4. Fluorescent *in situ* hybridization of *aldh1a2, cyp26a1* expression and RA responsiveness in adult gonads of medaka.** (A) *aldh1a2* expression in the parenchyma cells. (B) *cyp26a1* expression restricted to early oocytes; strong signal in the prophase I-arrested oocytes (arrowhead). (C) Responsiveness to RA present in early oocytes. Note the strong signal in the vitellogenic oocytes (asterisks) (D) *aldh1a2* expression in the Leydig cells. (E) Responsiveness to RA present in Sertoli and Leydig cells; no germ cells appear to be responsive to RA. (F) Responsiveness to retinoic acid at the tip of the germinal epithelium lobe. (G) Leydig cells (arrow) close to blood vessels are positive for RARE driven GFP. (H) Strong GFP signal in Sertoli cells (arrowhead) and in spermatogonia closer to the tip of the germinal epithelium lobe; Small group of spermatogonia with weak expression (asterisks) more distant from the lobe. BC, blood cells; Sg, spermatogonia; Sz, spermatozoa.

## 3.4 Effects of modulation of RA signaling in medaka embryos and adult testis.

For a functional evaluation of a possible role of RA for meiosis induction, we performed drugs treatments on embryos and adult testis to modulate endogenous RA levels.

Since it we did not know how the RA metabolic enzymes genes would respond to exogenously applied agonists or antagonists, medaka embryos were treated 24 hours (from stage 15 to stage 23) with various concentrations of ATRA and DEAB, and the expression levels of all RA metabolism genes were quantified.

In the ATRA treatment groups, no difference in expression levels for *aldh1a2* was observed, but for all *cyp26* genes a profound up-regulation of gene expression proportional to the concentration of the chemical was seen (Figure 5 A). In the DEAB treatment groups upregulation of *aldh1a2* was noted at higher concentrations, however, this was not statically significant. However, the *cyp26* genes did not respond significantly to DEAB treatments (Figure 5 B).

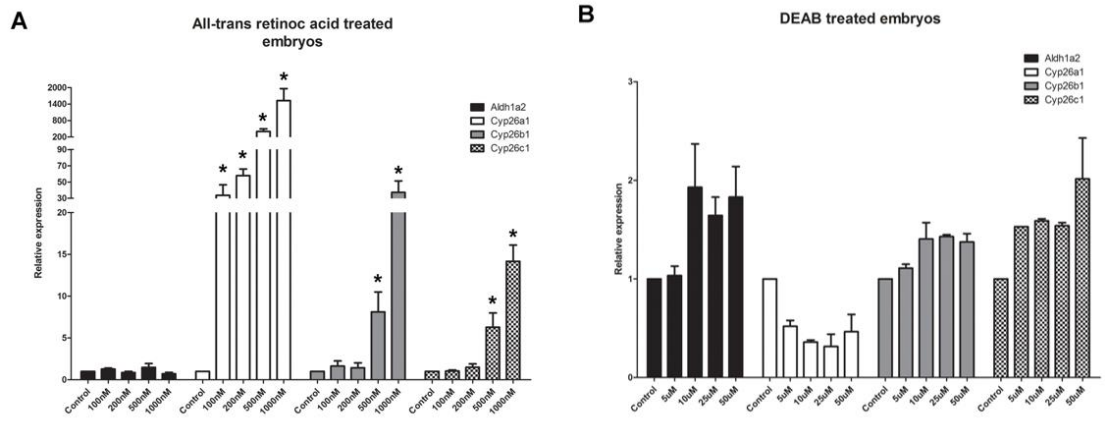


**Figure 5. Expression of retinoic acid biosynthesis and degradation enzymes genes in embryos treated with all-trans-retinoic acid and DEAB.** Treatment with retinoic acid of embryos from stage 14 onwards for 24 hours. Treatments with retinoic acid (A) showed no differences for the *aldh1a2*, but the Cyp26 genes were significantly upregulated at higher concentrations. Treatments with DEAB (B) had no strong effects, but at higher concentrations *aldh1a2*, *cyp26b1* and *cyp26c1* were slightly up-regulated, while *cyp26a1* was down-regulated. Asterisks indicates a significant difference ($p < 0.05$) after Student's t-test comparing the expression of control and treatment.

In mammals, retinoic acid is the main factor that induces the first meiosis in female [36]. We performed long-term treatments of medaka embryos (from stage 29 to 10 days after hatching, dah). Due to a high toxicity of the drugs, only the Citral

treatments allowed us to maintain the embryos alive until 30 dah. In female embryos, we saw a small delay of expression of *dmc1* (meiosis marker) in all stages, being significantly lower at 20 dah (Figure 6 A). At 30 dah, the expression levels showed no difference to the control animals. By analyzing the morphology of the gonads between the treated and the control, the only differences were observed at 20 dah, where the oocytes are in a more advanced stage of maturation and the size of the organ seems to be bigger in the control (Figure 6 B-G).

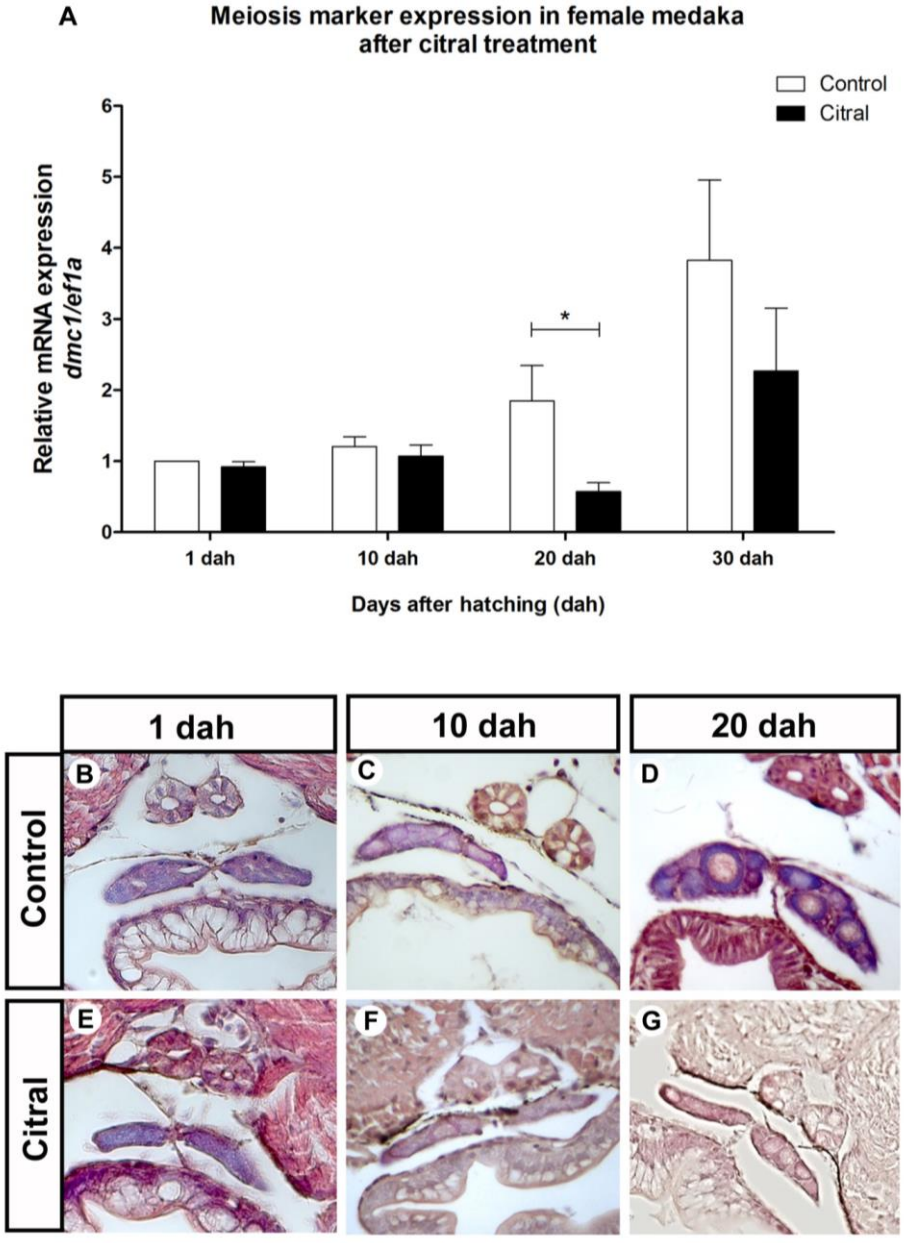


**Figure 6. Expression of the meiosis marker *dmc1* and gonad morphology after treatment with Citral in female medakas.** Treatment with Citral of female medaka embryos resulting in a lower meiosis marker (*dmc1*) expression (A). The morphology of the gonad comparing control (B-D) and treatment (E-G) shows only an observable difference at 20 dah. Asterisk indicates a significant difference ($p < 0.05$) after Student's t test comparing the expression of male and female gonads.

To assess a possible role of RA in the adult gonad, we performed treatments of testis explants (Figure 7). After three days of treatment no effect in expression levels of *dmc1* for those drugs that inhibit RA synthesis (Citral and DEAB) was recorded. Treatments with ATRA, AM580 and Talarozole (CYP26s inhibitor) are expected to stimulate the RA pathway. For AM580, we found a significant decrease in the expression of *dmc1*, while the other two drugs had no effect. Interestingly, combined treatment of testis explants with both AM580 and Talarozole led to a three fold significant upregulation of *dmc1* in comparison to the explants treated with AM580 alone.

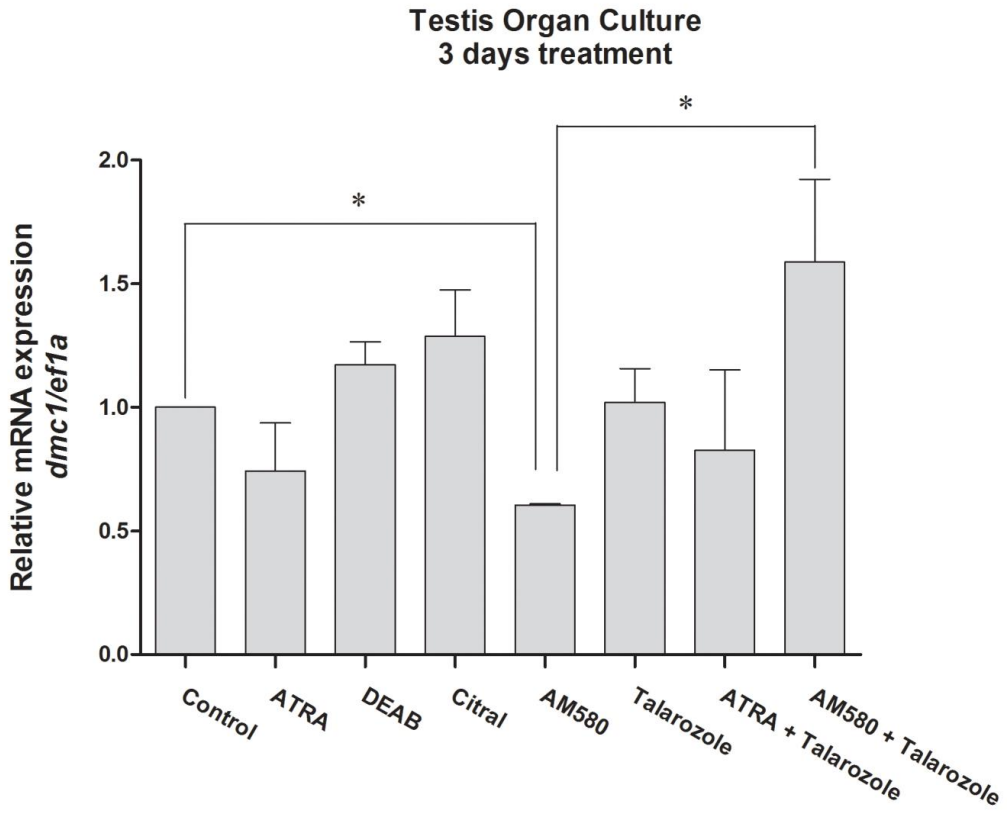


**Figure 7. Expression of meiosis marker *dmc1* gene after treatment of testis explant culture.** Treatment with AM580 resulting in downregulation of *dmc1* after 3 days of treatment. A small rescue is observed be treating the testis with both AM580 and Talarozole (CYP26 inhibitor). Asterisks indicates a significant difference ($p < 0.05$) after Student's t test comparing the expression of male and female gonads.

## 4. Discussion

The cognate role of RA in germ cells of mammals is to induce the cells to enter into the first meiosis. The timing of this event in the two different sexes is regulated by degradation of retinoic acid in the male gonad through CYP26B1 [15-17, 34, 36]. However, involvement of RA in germ cell fate and meiosis entry in fish is not well known. In medaka, some information on the expression pattern of RA biosynthesis enzyme genes during development has been reported, but the gonad had not been studied [42, 52]. Medaka is a well suited model species to study sex determination [43]. As a first step towards an understanding of the role of RA in sex determination and sexual development, we analyzed the RA metabolism gene expression patterns during development and in gonads of the adult fish. To obtain evidence for a functional role, we also monitored RA transcriptional control activity by using a RA responsive reporter gene expression system and did treatments with RA agonists and antagonists.

### 4.1 RA responsiveness signal observed distant from *aldh1a2* expression source.

The identification of the genes involved in RA metabolism and the possibility to examine the consequences of manipulating and visualizing RA signaling in well-characterized model organisms can lead to a better understanding of the roles of endogenous RA in sex differentiation and meiosis in both sex. We observed that often the source of RA production and its site of action are uncoupled, as it is typical for a diffusible morphogen. [40]. For instance in the retina of the medaka *aldh1a2* is expressed equally in the ventral and dorsal retina, like described in zebrafish, but only the ventral retina seems to be strongly responsive to the RA, [45, 53]. In the gonad at embryonic stage 33, RA is synthesized, but the RA responsiveness is exclusively in the nephric duct. This pattern was already described in zebrafish where during early kidney formation the RA signal is required for patterning the rostral/caudal domain of the renal progenitor cell, however the retinoic acid does not come from the pronephros itself but from the paraxial mesoderm adjacent to the kidney [54, 55]. In mammals, the *aldh1a2* gene is not expressed in the developing gonad, but the mesonephros is the source of RA [34, 36].

Taking these results together, it is likely that in the case of teleost, different than mammals, the RA that will act in the germ cell or in the gonad in general is derived from the gonad itself but not from the mesonephros, like what was proposed by Rodriguez-Mari et al. [40]. They suggest that this model is consistent with the fact that the mesonephros in zebrafish lies distant from the gonad and does not contact the gonad during the critical time of sex determination. Our data also confirm the hypothesis of an effect of RA in kidney development, despite its absence of production in the nephric ducts in fish [54, 55]. However, contrary to all other vertebrates, teleost are the only group that have a complete separation of the urinary and gonadal tubule systems [56]. However, the conclusion that only teleost then need such an early expression of *aldh1a2* in the gonad, does not hold true, since chicken also presents this pattern [38]. Thus, it appears that expression of *aldh1a2* in the mesonephros, but not in the early gonad, is a characteristic that is not conserved in all vertebrates.

## 4.2 Treatment with RA and DEAB indicate negative feedback loop regulating endogenous RA levels.

RA can exist as three stereometric isomers, all-trans-RA, 13-cis-RA and 9-cis-RA. Of these, all-trans-RA is believed to be the primary biologically active isomer. The biological effects of RA are mainly mediated by all-trans-RA binding to nuclear RARs. The RARs bind to their DNA through response elements RAREs, which depending of the presence or absence of the ligand (RA), regulate the transcription of multiple target genes [57, 58]. An in-silico promoter analyzes of zebrafish *cyp26a1* gene showed the presence of two canonical RARE that are also conserved in mouse [59]. The promoter region of medaka *cyp26a1* also contains these two canonical RAREs (Supplementary Figure 4). Certain tissues express *cyp26a1* in an apparent RA-independent manner [60], however it was demonstrated that endogenous RA is involved in controlling the expression of *cyp26a1* in cells within or adjacent to the presumptive hindbrain during gastrulation of zebrafish [61]. Treatment of embryos, tissues, and cells with exogenous RA strongly induces expression of *cyp26a1* in a time and concentration-dependent manner [61-63]. This was also observed in the all-trans-RA treated medaka embryos, where higher concentrations of RA correlated with higher expression of *cyp26a1*. This observation was also true for *cyp26b1* and *cyp26c1,* but only with the highest concentrations. However, a strong up-regulation of *cyp26a1* was already observed at

the lower concentrations of RA, meaning that the *cyp26a1* gene, which is preferentially expressed in female gonads, is most sensitive to RA-treatment induced regulation. Treatment with DEAB showed expression difference only for *cyp26a1*, where the gene was significantly down-regulated. It appears that the level of *cyp26a1* is responding directly and most sensitive to the levels of exogenous and endogenous RA.

## 4.3 RA regulates meiosis in adult gonad but not at the sex determination stage.

During gonad development of medaka, the entry into meiosis occurs first in females (around hatching) and later in males (around 20 days after hatching) [64, 65]. We detected responsiveness to RA in the germ cells of medaka around stage 30. Notably, this is at least 6 days before the first sign of meiosis entry in females can be observed. The window of RA expression coincidently matches with the critical period where sex can be reverted in female embryos to develop as fertile XX males [66]. This period is characterized by expression of the male sex determining gene *dmrt1bY* and is then followed by a mitotic arrest of the PCGs in the male gonad [67], and by the change of type I to type II proliferation of germ cells in female gonads [68]. However, at the hatching stage, which is characterized by meiosis entry in females, we did not detect any RA responsiveness of the reporter construct.

Long-term treatment with Citral showed that the entry of meiosis can be slightly delayed, but effects of inhibition of RA synthesis become only evident at later stages (20 dah), long after the first entry into meiosis. Unexpectedly, we noticed an early responsiveness to RA in the 12XRARE transgenic line considerably earlier than the first meiosis in females. As this responsiveness is seen in both sexes, it is unlikely that it has any function related to sex determination. One possibility is that it may be a general feature of PGC development. On the other hand, expression of other genes at this time has been reported, but these show sex specificity [3, 49, 65, 68, 69]. Future experiments employing conditional knockout of these genes might help to resolve the question on the functional meaning or these expression. Recent work in Tilapia showed that treatments with an inhibitor of RA synthesis or disruption of *aldh1a2* leads to a delay in entry of meiosis, similar to our results with medaka [41]. However, it cannot be excluded that the drugs cause an unspecific delay of development of the hatchlings.

In adult testis of mouse, expression of RA-synthesis genes was found in the germ cells, Sertoli cells and Leydig cells [70]. Expression of *aldh1a1* starts after the onset of *Sry*, which implies a male-specific source of RA in the process of testis differentiation [71]. In zebrafish, no gonadal expression of *aldh1a3* was observed and *aldh1a2* was detected in the undifferentiated gonad and in the adult gonad, in Sertoli cells for testis and in the somatic interstitial cells for the ovary. Medaka *aldh1a2* is also expressed in the adult gonad of both sexes, where higher expression occurs in testis than in ovary. This suggests that a bias in levels of RA is important still in the adult gonad. In mammals, a difference between male and female was also observed. This is in agreement with the findings in fish. Medaka *aldh1a2* is the only member of the *aldh1* gene family, which combines the roles of *aldh1a2* and *aldh1a3* of zebrafish [42]. Thus, gene expression is restricted to this single paralog in medaka and may explain the high differences of *aldh1* expression between male and female. One may speculate that if there is an early testis differentiation specific function of *aldh1a2* in medaka, which might be maintained in the adult, like for most male sex genes [3].

In mouse, only *cyp26b1* regulates the timing of meiosis onset. The undifferentiated gonads (11.5 dpc) express *cyp26b1* at low levels in both male and female. After expression of *Sry* in male gonad (12.5) levels of *cyp26b1* are increased in the testis, and at the same period the levels of *cyp26b1* are reduced in the ovary. The *cyp26b1* expression in the early testis is important to prevent the germ cells from undergoing meiosis, arresting them in $G_0/G_1$ phase of the cell cycle until birth, where the cells resume proliferation. The expression of the *cyp26* genes is restricted to the peritubular myoid cells of juvenile testis, which surround and isolate seminiferous tubules from the rest of the body. In females, RA acts in the early ovary, leading the cells into meiosis and oocyte development by activating *stra8* [16, 37]. In zebrafish, the gonads of both sexes pass through a transitional stage acquiring a juvenile ovary-like gonad with immature oocytes. In females, these oocytes continue to develop and reinforce the development of mature ovaries, but in males, oocytes die by apoptosis and the gonads then become testis [72]. Hence, no sexual dimorphic onset of meiosis can be recognized.

In zebrafish, Cyp26a1 is the main RA-degrading enzyme in the adult gonad [40], instead of Cyp26b1 as in mammals. The function of meiosis blocking for *cyp26a1*

in zebrafish appeared to be conserved, since this gene was found to be only expressed in those somatic cells that are not surrounding meiotic cells. In females, the expression is restricted to the oocytes arrested in meiosis. Our data in medaka show that *cyp26a1* has higher expression levels in ovary, where it specifically localizes to the early oocytes, in agreement with the findings in zebrafish. However, our data from the 12XRARE transgenic reporter line showed that RA is acting also in oocytes that already have resumed meiosis. Thus, *cyp26a1* is up-regulated in oocytes that entered prophase-I meiotic arrest, and is downregulated when meiosis is resumed [40] These data are in agreement with the hypothesis that *cyp26a1* acts as a meiosis inhibitor.

However, a role of *cyp26a1* in meiosis arrest may be sexually dimorphic in zebrafish and medaka: in the testis, Sertoli cells protect spermatogonia to enter meiosis, and in the ovary, *cyp26a1* is expressed in the early oocytes (late stage 1B) that are arrested in diplotene [40]. This fact together with the high levels of *aldh1a2* in testis leads us to infer that there is a sex specific role for RA in male, in addition to meiosis induction. On the other hand, in the ovary it has to be strongly repressed because it would boost meiosis entry of germ cells. Together with the fact that *aldh1a2* has a specific Leydig cell expression, we hypothesize that RA may be not only related to meiosis, but also to the maintenance of the male gonad identity. In the adult testis of medaka, interestingly, we also found sexually dimorphic RA responsiveness. In males, the response to RA is observed in Leydig cells, type A spermatogonia and Sertoli cells close to the RA-responsive spermatogonia. The tip of the germinal epithelium lobe of medaka testis is the region to which type A spermatogonia are restricted [69], and those cells are pre-meiotic cells [73]. This may indicate that RA is acting in the germ cells by inducing gametogenesis and consequently meiosis.

The role of RA in reproduction was already reported for different animals [15, 74]. Experiments in mouse show that RA plays an important role in initiation of spermatogenesis and in proliferation and differentiation of Sertoli cells [75, 76]. In testis explants culture, we find evidence that RA may interfere with meiosis rather than initiating it. A significant decrease of meiosis marker was observed after the treatment with the RARα agonist rather than up-regulation of the meiosis marker. This explanation would be supported by the observation in mammals that the entry of meiosis occurs only after 9 days of the induction by RA. After this induction, the

undifferentiated spermatogonia go into differentiation, followed by 5 cycles of division and just then undergoes meiosis. In the case of medaka, differentiating spermatogonia within a cyst are connected to each other through cytoplasmic bridges and clonally divide 9–10 times before entering meiosis [77]. In our experiments, we provided a 3-day treatment, meaning it is likely the germ cells that were induced by RA still did not enter meiosis. However, this does not explain the down-regulation of the meiosis markers after treatment.

The counteraction of the RA metabolism enzymes puts a note of caution to all treatment experiments. Considering that RA signaling is a complex and delicate pathway, it could mean that treatments with antagonists and agonists might not reflect the physiological situation and allow us to infer effects of endogenous RA. The down-regulation of meiosis marker in our organ culture experiments, and a not clear induction of meiosis by RA treatments, can be related to the strong up-regulation of the degrading enzymes after induction be exogenous RA.

## 5. Conclusions

Our findings allow us to suggest a complementary hypothesis for the control of meiosis entry through RA, adding to what was proposed for zebrafish in the adult gonad. In female medaka, like in zebrafish, it is conceivable that RA has the function to release the oocytes (later 1B) that were arrested in meiosis during oocyte maturation [78, 79]. This is partly because of the early signal to RA responsiveness in the later 1B oocytes. In males, on other hand, entry of meiosis is probably related to a direct action of RA in the germ cells. However, an indirect role of RA in meiosis and gametogenesis cannot be excluded, since Sertoli cells and Leydig cells also present responsiveness in adult testis. This is in line with the hypothesis proposed by McLean et al [80], that RA induces one or several so far unidentified factors in Sertoli cells that are secreted and initiate meiosis in the germ cells.

In the embryo, we find no evidence that RA is essential for the first entry of meiosis in medaka, contradicting what is known in mammals and other vertebrates. One possible explanation may come from the fact that the majority of teleost, from which we have information so far, do not have a *stra8* orthologous gene. In Tilapia, it was shown that RA is important, but not necessary, for the entry of meiosis [41]. On the

other hand, in catfish, it seems that this process is similar to mammals, since this species possesses a *stra8* gene [81, 82]. Since both medaka and Tilapia do not have the *stra8* gene, the commonality of both fish lacking this important mediator of RA action is in line with the absence of an effect during the sex determination period. However, many other explanations for a difference in the mechanisms that regulate the entry into the first meiosis exist and further experiments with an unbiased approach should help to find the molecules that are instrumental in this process.

## 6. Acknowledgements

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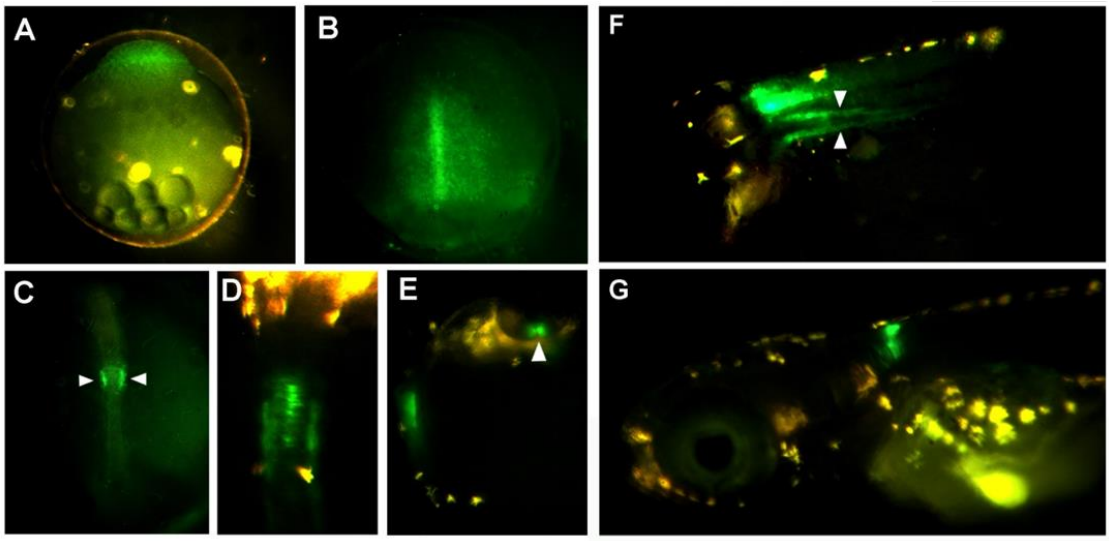
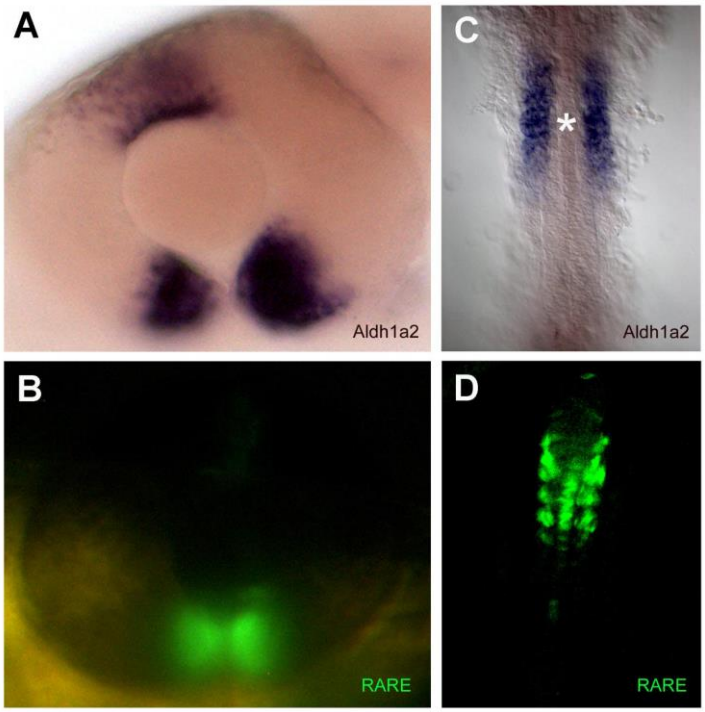
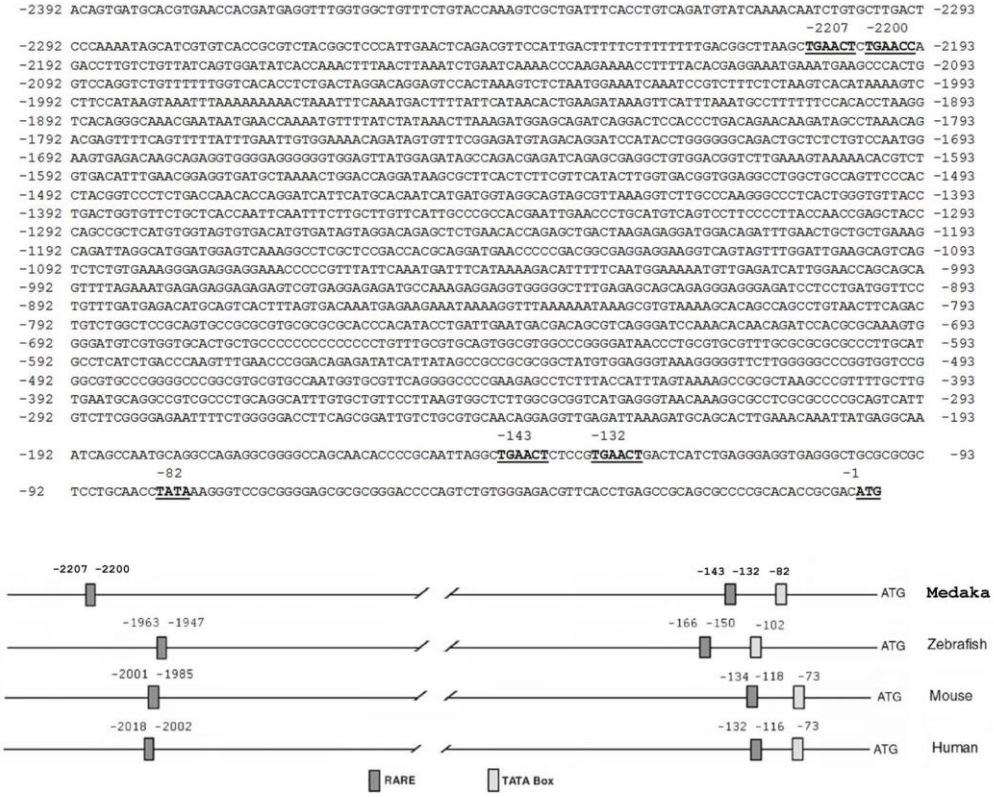
## 7. Supplementary figures




**Supplementary Figure 1. Spatial and temporal expression patterns of 12X Retinoic Acid Responsive Element (RARE) transgenic line during medaka development.** (A) Early blastula stage with ubiquitous RA-responsiveness. (B) Late gastrula stage with ubiquitous responsiveness on the embryonic shield. (C) Stage 18 with responsiveness on the lateral plate mesoderm (arrowhead). (D) Stage 23 (12 somite stage) responsiveness in the somites (until 5-6), neural tube and notochord. (E) Stage 27 showing responsiveness in the retina, strongly at the ventral part (arrowhead). (F) Stage 33 with pronephros responsiveness clearly recognizable (arrowheads). (G) Hatching stage, with responsiveness of the hindbrain being more prominent compared to other tissues.




**Supplementary Figure 2. Comparison between *aldh1a2* expression and responsiveness to retinoic acid in medaka embryo.** (A) *in situ* hybridization for *alh1a2* with ventral and dorsal expression domains in the retina. (B) RA responsiveness in the ventral retina. (C) *in situ* hybridization for *alh1a2* showing expression in the first four somites. (D) GFP expression in the first four somites and the neural tube of a *tg(12xRARE:GFP)* embryo. The asterisk indicates the region where RA action is not restricted to its source of synthesis.




**Supplementary Figure 3. Sequence of medaka promotor region of *cyp26a1*.** RA responsive elements (RARE) in the *cyp26a1* medaka promoter region. Those elements are conserved in the promoter of this gene in different species. Promoters sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov). Scheme adapted from ref. [59].

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# Chapter 3

## Analyzes of Expression Regulation of Sex Determination Genes by the Retinoic Acid Pathway in Medaka

**Article in Preparation**

## Abstract

Sex determination (SD) is a highly diverse and complex mechanism, and in fish, even within groups of closely related species a wide spectrum of different systems can be found. SD in vertebrates is characterized by a difference in the timing of initiation of the first meiosis. In vertebrates, the main molecule responsible for germ cell sex identity and meiotic timing is the retinoic acid (RA). Meiosis initiation occurs first in female and later in male. Thus, SD is intimately related to the responsiveness of the germ cells to undergo meiosis in a sex-specific manner. In this study, we used a fish model species for sex determination, the Japanese medaka (*Oryzias latipes*) to investigate the connection between RA and the sex determination pathway. Treatments with RA pathway activators and inhibitors in spermatogonial stem cells (SG3) show that RA can induce differentiation of the germ cells into adipocytes; however, no meiosis could be induced. The disruption of the RA degrading enzyme genes *cyp26a1* generated only homozygotes males, but not through sex-reversal process. Activation of RA pathway by exogenous drug treatments show upregulation of *dmrt1bY* in early embryonic stages, and *dmrt1a* and *amh* in later stages. After induction with ATRA, luciferase activity of *dmrt1bY* promoter show increase of activity in HEK293 cells, but a decrease in the case of SG3 cells. My findings show that RA can regulate the sex determination pathway through overexpressing male related genes that are also related to germ cell differentiation.

## 1. Introduction

Sex determination is the decision whether the bipotential gonadal primordium will become a testis or an ovary [1, 2]. This complex and tightly controlled developmental process is characterized by a difference in the timing of meiotic initiation [3, 4]. In all so far studied vertebrates, initiation meiotic occurs earlier in females than in males, and retinoic acid (RA) signaling takes a crucial role in this process [4-7].

RA is a molecule derived from vitamin A responsible for activation of different genes during development, being important for the formation of the anterior-posterior axis, and proper development of many different organs and biological systems in many species [8]. RA is small, polar and diffusible, and the concentration levels are fine-tuned by the balance between its synthesis and its oxidative degradation [9, 10]. The main enzymes involved in the synthesis of RA are the retinaldehyde dehydrogenases (RALDHs) and the enzymes responsible for its degradation are the cytochrome P450 family 26 (CYP26s) [8, 11-14].

The importance of RA in meiosis entry has been widely studied in mammals, but this role was also described in other vertebrates [15-17]. In mice, RA makes primordial germ cells (PGCs) enter meiosis in female by inducing *Stra8* (*stimulated by retinoic acid gene 8*) expression and initiates oogenesis, while testis produce the CYP26 enzyme that destroys RA and delays entry into meiosis in male [18, 19]. Therefore, the factors that are regulating the expression of *Cyp26b1* are sex-specific. In testes, *Cyp26b1* is up-regulated by the transcription factor SF1 in Leydig cells and by SF1 and SOX9 in Sertoli cells. In ovaries, this expression is suppressed by the female-specific transcription factor FOXL2 [20].

The medaka (*Oryzias latipes*), which has XY-XX chromosomal system, contains a well characterized master sex determination gene in the Y-chromosome, *dmrt1bY*. This gene is a duplication of the autosomal version of the maleness-related gene *dmrt1a* [21, 22]. In fish, the role of RA in meiosis is still unclear, since most studies are incomplete and lack functional data [1, 23-25]. In addition to this high complexity, no sequence of *stra8* was found so far in most teleost genomes including medaka.

In medaka, the number of germ cells is around the same in both XY and XX embryos. In female, the germ cells start to proliferate and enter meiosis around the hatching stage. In male, only at around 15 to 20 dph (days post hatching) the germ cells resume proliferation and become spermatogonia [26]. In **Chapter 2**, I provide evidence that RA is implicated in meiosis regulation in adult gonad. However, I could not find clear evidence of RA been involved in the first meiosis. Moreover, the absence of RA responsiveness at the germ cell level at the time of the first meiosis entry motivated me to ask the following questions: is there any role of RA in sex determination? In addition, what controls the initiation of meiosis if not RA?

An indication may come from the role of *dmrt1bY*, which was shown to be involved not only in the determination and differentiation of the gonadal soma in male, but to be also involved in cell cycle arrest of the PGCs specifically in G2 [27]. Interestingly sex-related genes classically described as sex determination genes are also involved in gametogenesis [28-31]. This indicates that sex determination genes can also be involved in regulating the time of meiosis entry through inhibition of the first meiosis.

To analyze a possible connection of the mechanism of sex determination and meiosis entry I used the well-characterized model species medaka. Here, I showed that the activation of RA pathway in early embryos leads to an increase in expression of important male sex-related genes, indicating a straight correlation between RA and sex determination pathways in medaka.

## 2. Materials and methods

### 2.1 Animals

Laboratory reared medaka (*Oryzias latipes*, Class Actinopterygii, order Beloniformes, family Adrianichthyidae) were used. For detailed description of this model species and its features see [32]. All experiments were performed with fish of the Carbio strain. The animals were kept under standard photoperiod cycle of 14 hr/10 hr light/dark at 26°C (±1°C). Eggs were collected 1-2 hr after starting the light cycle and raised at 26°C in Danieau's medium (17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)2$, 1.5 mM Hepes, pH 7.2). The stages of development were identified according to Iwamatsu (2004) [33].

### 2.2 Production of aldh1a2, cyp26a1 and cyp26b1 reporter transgenic fish

For generation of *aldh1a2* and *cyp26a1* transgenic line, the meganuclease protocol [34] was used. The *ISceI::Aldh1a2::mCherry* and *ISceI::Cyp26a1::mCherry* plasmids was obtained by replacing the promoter driven reporter cassette of *ISceI::mCherry* vector with the medaka *alh1a2* and *cyp26a1* promoter region. Briefly, the promoters of *aldh1a2* and *cyp26a1* genes fragments were obtained by cloning 800 bp and 3,0 kb 5' upstream of the transcription start of the genes respectively into *ISceI::mCherry* vector. Restriction enzyme recognition site was added to the primers and used for the PCR amplification using medaka genomic DNA as template. All oligonucleotide sequences are provided in Table 1. The quality of the PCR fragments was confirmed by DNA sequencing. Plasmid DNA was injected at a concentration of 100 ng/µl together with the *I-SceI* meganuclease enzyme (0,5 unit/µL in 0,5 x *I-SceI* buffer), through the chorion into the cytoplasm of the one-cell stage embryo. Embryos were kept at 28.5°C until hatching. The mCherry positive fish were selected as putative founder fish, raised to sexual maturity, mated to wild type partners and tested for germline transmission.

Bacterial artificial chromosome clone encompassing medaka *cyp26b1* (ola1-199K19), genomic regions were obtained from NRBP Medaka (http://www.shigen.nig.ac.jp/medaka/). A BAC transgenic method using homologous recombination was employed to generate the reporter construct as previously described

[35, 36]. The following primers were used to amplify *eGFP* fragments for homologous recombination into the different BAC clones: BAC-Cyp26b1-GFP-Fw: CTTTTGAGCACATCCATCTCCAGATTCTTCTGCTCCATCATGCTGTTCGAC AGCGATATCATTTCTGTCGCCTTAAAG, BAC-Cyp26b1-GFP-Rv: GCTGCTGGGACACCACCAGCAGCAGCGCCATGGACACCAGGCAGGCGGC CAGGAACAAACGACCCAACACCGTGCG. After homologous recombination, the generated fragments were inserted into the BAC clones in frame downstream of the translation initiation site of the targeted genes.

Microinjection of DNA was performed as described previously [37] using BAC clone DNA at a concentration of 50-100 ng/mL. Adult $G_0$ fish were then screened for fluorescence and positive individuals were raised to adulthood. Positive $G_0$ fish were mated to each other and the offspring were again sorted for fluorescence.

For imaging embryos, hatchlings or tissues were mounted with 1-2% low melting temperature agarose. Confocal pictures and image stacks were acquired using a Nikon C1 (eclipse Ti) confocal laser scanning microscopy and the NIS elements AR software.

## 2.3 *In vivo* drugs treatments

Treatments and dilutions of the drugs were made in Danieau's medium. From the dome stage (stage 14) onwards, the embryos were treated with *all-trans*-retinoic acid (ATRA) at 1 µM and kept in the dark for 24 hours (stage 22) to see the effect of the treatments in the early stages.

To investigate an effect on sex related genes regulation, I made long-term treatments from stage 29 until 1 day after hatching (dah) of AM580 (10 nM) and ATRA (10nM), with medium changes every 2 days. Specimen were collected at 1dah and genotyped for sex by PCR amplification of the Y-linked male determining gene *dmrt1bY* using the genomic DNA as template.

## 2.4 Disruption of cyp26a1 by TALEN

The genomic sequence of *cyp26a1* (Ensembl gene number ENSORLG00000014516) was retrieved from the Ensembl medaka genome browser (http://www.ensembl.org/Oryzias_latipes). The construct TALEN expression vectors

(left, pCS2TAL3DDD, and right, pCS2TAL3RRR, with both vectors containing the respective TALE fragment, the *Fok*I cleavage domain, and other necessary components) were developed and provided by Dr. David J. Grunwald, Department of Human Genetics, University of Utah, Salt Lake City, UT 84112, USA. The TALEN target sites of *cyp26a1* were designed in the second exon, with the right binding site located at the junction of exon 2 and intron 2. The *cyp26a1* TALEN recognition sequences were left TALEN 5' –TCTCCAACATGCACGGAT- 3' and right TALEN 5' –TGGAGACTCACCTTTTT- 3'. Between the binding sites, an 18-bp spacer is included, where the *Fok*I nuclease cuts.

The *in vitro* transcription of the TALEN was carried out with the Sp6mMESSAGEmMACHINE Kit (Ambion). The resulting mRNA was purified using Phenol/Chloroform-Extraction and then quantified using NanoDrop-2000 (Thermo Scientific). The left arm and right arm mRNA of each TALEN pair was then mixed at a molar ratio of 1:1, with the final concentration in the mixture of 100 ng/µL mRNA of each arm, and stored at -80°C until use. About 200 to 600 pg of the mRNA mixture was directly microinjected into medaka embryos at the one-cell stage. The injected embryos were cultivated at 26°C and 10 animals collected at stage 1 dah to extract DNA for mutation efficiency analysis.

## 2.5 Disruption of cyp26a1 and cyp26b1 by CRISPR/Cas9

To study the functions of *cyp26a1* and *cyp26b1* in meiosis initiation of germ cells and sex determination, I used the CRISPR/Cas9 technology to generate *cyp26a1* and *cyp26b1* mutant medaka lines. For synthesis of Cas9 mRNA, the plasmid pCS2-nCas9n was digested at 37°C with *Not*I (Thermo Fisher). The PCR product was resolved in 0,8% agarose gel, and the fragment was purified using GenElute™ Gel Extraction Kit (Sigma-Aldrich). The purified fragment was used as template for synthesis of Cas9 mRNA using the Sp6 mMessage Machine kit (Ambion).

For designing the CRISPR guideRNAs (gRNA), target regions of the genes *cyp26a1* and *cyp26b1* were searched using the ZiFiT Targeter (http://zifit.partners.org/ZiFiT/). For each gene two gRNA were chosen by generating a long fragment deletion facilitating the screening process. The oligonucleotides were first submitted to an Oligo Annealing reaction using T4-DNA-ligase by heating the

reaction at 95°C and decreasing 5°C every 2 min until 25°C. Later the annealed oligonucleotides were sub-cloned in the pDR274 plasmid at the *Bsa*I (NEB) restriction enzyme site and transformed into bacteria *E. coli* DH5α. The plasmids containing the oligonucleotides were linearized at 37°C using *Dra*I (Thermo Fisher), and later purified using GenElute™ PCR Clean-Up kit (Sigma-Aldrich). The linearized fragments were used as template for the gRNA synthesis reaction with the T7 Maxi Script Kit (Ambion). The resulting gRNAs were purified using Phenol/Chloroform-Extraction and quantified using NanoDrop-2000 (Thermo Scientific).

The two gRNAs for each gene and the Cas9 mRNA were co-injected into one-cell stage embryos of medaka at a concentration of 100 ng/µl and 200 ng/µl, respectively. Ten injected embryos were collected after injection at hatching stage. The genomic DNA was extracted from injected and wild type embryos, and used for mutation efficiency analysis.

## 2.6 Genotyping of embryos and adult fish

To determine the sex of embryos and adult fish and the presence and absence of mutations, genomic DNA was extracted. Caudal fin clip of the adult fish or tissue from whole larvae were isolated and incubated for 1 hour at 95°C in 100 µL of Base Solution (25 mM NaOH, 0,2 mM EDTA, pH=12) and shaking. The solution was cooled down on ice, 100 µL of Neutralization Solution (40mM Tris-HCl pH=5.0) added and vortexed. 2 µL of the total volume was used in a 25µL PCR reaction. The PCR products were resolved in 1% agarose gel.

To analyze the genotypic sex, a pair of primers (Dmrt1andbY_Fw03 and Dmrt1andbY_Rv02) was used that amplifies a fragment of both *dmrt1a (*1100 bp*)* and *dmrt1bY (*900 bp*)*, yielding one PCR product (*dmrt1a*) in XX genotypes, and two PCR products (*dmrt1a* and *dmrt1bY*) in XY genotypes. To detect *cyp26a1* TALEN mutants, primers were designed flanking the region where the mutations are expected (Exon2). PCR product were purified using GenElute™ Gel Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions and sequenced using the PCR amplification primers.

To screen the mutant CRISPR fish, a piece of tail fin was clipped from each individual, and genomic DNA was extracted as described above. Target genomic loci were amplified using the primers cyp26a1-KO-F/R for *cyp26a1* and cyp26b1-KO-F/R for *cyp26b1*. For both genes, heterozygotes mutant present two PCR products, the wild type *locus* being the product with higher molecular weight, homozygotes produce only the low molecular weight PCR product.

## 2.7 Cell culture

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum, and maintained at 37°C, 5% $CO_2$ with 100% humidity. SG3 cells were cultured as previously described [38]. Cells were grown at 28°C on gelatin-coated wells in ESM4 medium consisting of Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g/L) with HEPES (20 mM, pH 7.8) supplemented with 15% fetal calf serum, glutamine (4 mM), nonessential amino acids (0.1 mM), antibiotics, sodium pyruvate (1 mM), sodium selenite (2 nM), 2-mercaptoethanol (100 mM), basic fibroblast growth factor (10 ng/mL), medaka embryo extract (1 embryo/mL), and 1% trout serum.

To induce gametogenesis in SG3, the cells were grown at full confluence without subculture for 2 weeks, as described [38]. To observe an influence of retinoic acid on the induction of meiosis, the cells were cultured in medium containing Citral (1mM), DEAB (5µM), AM580 (1nM and 10nM). Medium was changed every 3 days. The attached cells were seeded at two time points, 1 week (d7) and 2 weeks (d14) after full confluence. The control cells were seeded only at 2 weeks' time point. At each medium change, the detached cells were also seeded, in case some meiotic cell being present in the supernatant.

## 2.8 Luciferase reporter assays

To analyze transcriptional regulation of *dmrt1bY*, a 9220 bp fragment upstream of the *dmrt1bY* open reading frame (ORF) was isolated and cloned into pBSII-ISceI plasmid (pBSII-ISceI::9KbDmrt1bYprom plasmid). Subsequently, *Gaussia* luciferase gene from pGLuc-basic (New England Biolabs) plasmid was inserted. The pBSII-ISceI::3KbDmrt1bYprom::GLuc and pBSII-ISceI::6KbDmrt1bYprom::GLuc plasmids were constructed the same way removing 5' fragments of the 9220 bp

Dmrt1bY promoter region using restriction enzyme digestion and re-ligation. All plasmid information is described in Ref. [39].

Transient transfections in HEK 293 and SG3 cells were done at 80% confluence by a polyethylenimine-based procedure and electroporation, respectively. For the transfection of one well of a 24-well plate, 0,2 µg of the respective pBSII-ISceI::Dmrt1bYprom::GLuc reporter constructs was used. To normalize the *Gaussia* activity, cells were co-transfected with 0,5 µg firefly luciferase expressing plasmid (ptkLUC+). After 16–18 h (d1), medium was changed. On d2, cells were incubated for 24 h and with 1µM ATRA or DMSO for control. On d3, cells were harvested in 100 µl of 1 X PLB (Promega). *Gaussia* luciferase activity was quantified using the Dual-Luciferase® Reporter Assay System from Promega and the TriStar LB941microplate multimode reader (Berthold Technologies). Experiments for which error bars are shown result from at least three replicates and error bars represent the standard error of the mean.

## 2.10 Western blot analysis

Cells were harvested and lysed in lysis buffer (20 mM Hepes, pH 7.8, 500 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 0.1% sodium deoxycholate, 0.5% Nonidet P40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 200 µM $Na_3VO_4$, 1 mM PMSF and 100 mM NaF). Then, 40–60 µg of protein was resolved by SDS/PAGE and analyzed by immunoblotting. The medaka anti-scp3 antibody was kindly provided by Dr. Ricardo Benavente, Würzburg. Anti-β-actin antibodies were purchased from Santa Cruz Biotechnology. As positive control, testis samples were used.

## 2.11 Real time quantitative RT-PCR

Organs of adult males and females and whole embryos of different developmental stages were collected. Total RNA was extracted from 3 pools of adult fish tissues (n=4) or whole embryos (n=20) using the TRIZOL reagent (Invitrogen) according to the supplier's recommendation. After DNase treatment, reverse transcription was done with 2 µg total RNA using RevertAid First Strand Synthesis kit (Fermentas) and random primers. Real-time quantitative PCR was carried out with SYBR Green reagent and amplifications were detected with a *Mastercycler® ep realplex* (Eppendorf). All results are averages of at least three independent RT reactions

from three independent RNA preparations. Transcript levels of the target genes were normalized against the medaka elongation factor-1 alpha (*ef1a*) gene. The ΔCt values presented as means ± standard error of the mean (SEM), were analyzed by one-way ANOVA, Tukey's and Student's t test. A significance level of $P < 0.05$ was used for all tests.

| Oligo | Sequence | Purpose |
|---|---|---|
| Dmrt1andbY_Fw03 | 5'-AGTGCTCCCGCTGCCGGAAC-3' | Sex genotyping |
| Dmrt1andbY_Rv02 | 5'-ACAAATCCCAAGCTCCTCCT-3' | Sex genotyping |
| Cyp26a1_TALEN_Fw | 5'-GGAGCACAGGCTCGTGTC-3' | TALEN screeening |
| Cyp26a1_TALEN_Rv | 5'-GTCCGTCCTGATCTGCTCC-3' | TALEN screeening |
| Cyp26a1_CRISPR_Fw | 5'-CCCGCAATTAGGCTGAACTC-3' | CRISPR screeening |
| Cyp26a1_CRISPR_Rv | 5'- CTGAAGGGCACATCAATGGG -3' | CRISPR screeening |
| Cyp26b1_CRISPR_Fw | 5'-GGCAGGCACTCAGCAGTATT-3' | CRISPR screeening |
| Cyp26b1_CRISPR_Rv | 5'-CGTTGCCCCTAGTAGCTCTT-3' | CRISPR screeening |
| Cyp26a1_gRNA1_Ol1 | 5'-TAGGGCCATGTCGCGGTGTGCG-3 | guideRNA cloning |
| Cyp26a1_gRNA1_Ol2 | 5'-AAACCGCACACCGCGACATGGC-3 | guideRNA cloning |
| Cyp26a1_gRNA2_Ol1 | 5'-TAGGCCCGGGACTCGTGCGTGC-3 | guideRNA cloning |
| Cyp26a1_gRNA2_Ol2 | 5'-AAACGCACGCACGAGTCCCGGG-3 | guideRNA cloning |
| Cyp26b1_gRNA1_Ol1 | 5'-TAGGTGCTAGCGCTGGCGGTGG-3 | guideRNA cloning |
| Cyp26b1_gRNA1_Ol2 | 5'-AAACCCACCGCCAGCGCTAGCA-3 | guideRNA cloning |
| Cyp26b1_gRNA2_Ol1 | 5'-TAGGTGGCTAGCTCGAAGCGAC-3 | guideRNA cloning |
| Cyp26b1_gRNA2_Ol2 | 5'-AAACGTCGCTTCGAGCTAGCCA-3 | guideRNA cloning |
| DMT1m | 5'-TCCGGCTCCACAGCGGTC-3' | qRT-PCR (*dmrt1a*) |
| DMT1r | 5'-TCCGCAATCAGCTTGCATTTGG-3' | qRT-PCR (*dmrt1a*) |
| DMTYc2 | 5'-CTGGAAAGACTGCCAGTGCTT-3' | qRT-PCR (*dmrt1bY*) |
| DMTYb | 5'-AGACAGAGGATTGGGGCTGG-3' | qRT-PCR (*dmrt1bY*) |
| Amh_RT_Fw | 5'-GACCTCCCAGCATCCTCTTT-3' | qRT-PCR (*amh*) |
| Amh_RT_Rv | 5'-GAAAAGCAGAAGTGGCGTCA-3' | qRT-PCR (*amh*) |
| Ef1a-F | 5'-GCCCCTGGACACAGAGACTTCATCA-3' | qRT-PCR (*ef1a*) |
| Ef1a-R | 5'-AAGGGGGCTCGGTGGAGTCCAT-3' | qRT-PCR (*ef1a*) |

**Table 1 –Sequence of the oligos used in the present study**

# 3. Results

## 3.1 Drugs treatment effects in SG3 cells

Medaka cells SG3 cells were previously reported to be able to undergo meiosis and complete gametogenesis *in vitro*. [38] To see a possible regulation of meiosis in those cells, I performed drug treatments with inhibitors (Citral and DEAB) and an inducer (AM580) of the retinoic acid pathway.

Culturing the cells under the same conditions as described in Ref. [38], no protein presence of SCP3 could be observed. Considering that the RA pathway could induce

gametogenesis in SG3 through initiation of meiosis, treatments with the agonist of RAR (AM580) and the inhibitors of RALDH were performed. Analyses of the meiosis maker protein Sycp3 could not show any induction of meiosis *in vitro* (Figure 1A). Comparing the morphology of the cells after 14 days without drug (Figure 1B) and with drug, I observed that the cells treated with AM580 underwent differentiation to adipocytes (Figure 1C), like earlier described [40]. In cells cultured without drugs, I could observe detaching cells forming tetrads and agglomeration of cells that was thought to be spermatocytes.

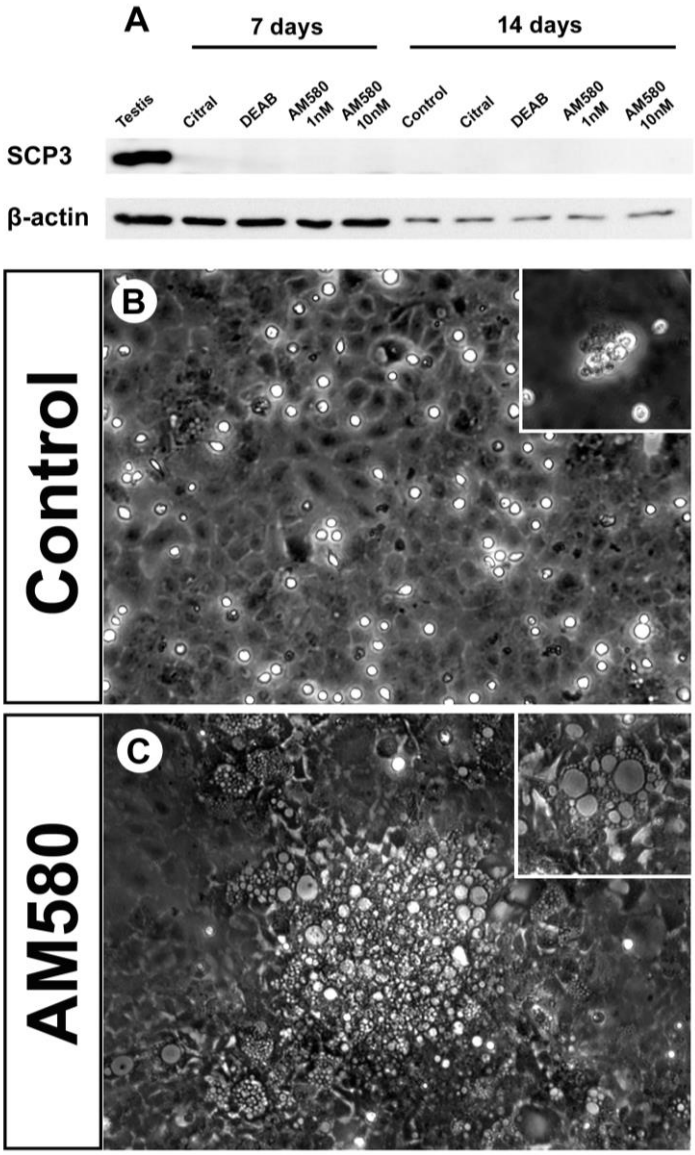



**Figure 1. Treatments with RA signaling drugs do not induce meiosis *in vitro*.** SG3 cells were cultured until full confluence than collected after 7 days and 14 days in the presence or absence of drugs. (A) Western blotting showing the presence of SCP3 in the positive control (testis) and absence in all treatment conductions, including in the negative control. (B) Negative control of SG3 cells at 14 days; detached cells forming an agglomerate. (C) Cells treated with AM580 (10nM) for 14 days, showing an adipocytes-like phenotype with abundant lipid vesicles.

## 3.2 Expression of RA metabolism genes during the development of medaka

In order to follow in detail the dynamics of expression of the main retinoic acid metabolism genes, I produced transgenic reporter lines for *aldh1a2*, *cyp26a1* and *cyp26b1*. For all the transgenic lines, during early embryonal stages high expression signal were observed. The first *aldh1a2* expression was detected at the mid-gastrula stage (stage 15) (Supplementary Figure 1). During neurula stage, *aldh1a2* has complementary expression domain in relation to *cyp26a1* and *cyp26b1*. Here, *aldh1a2* shows expression in the posterior half of the embryos (Figure 2 A-C), while both *cyp26a1* and *cyp26b1* expression are located at the anterior part (Figure 2 D-I). At later stages of embryogenesis, unfortunately, no signal was detectable for all three transgenic (more information in Appendix 4). The lack of expression could be due to a possible insufficient promoter fragment, where important regulatory elements are missing.

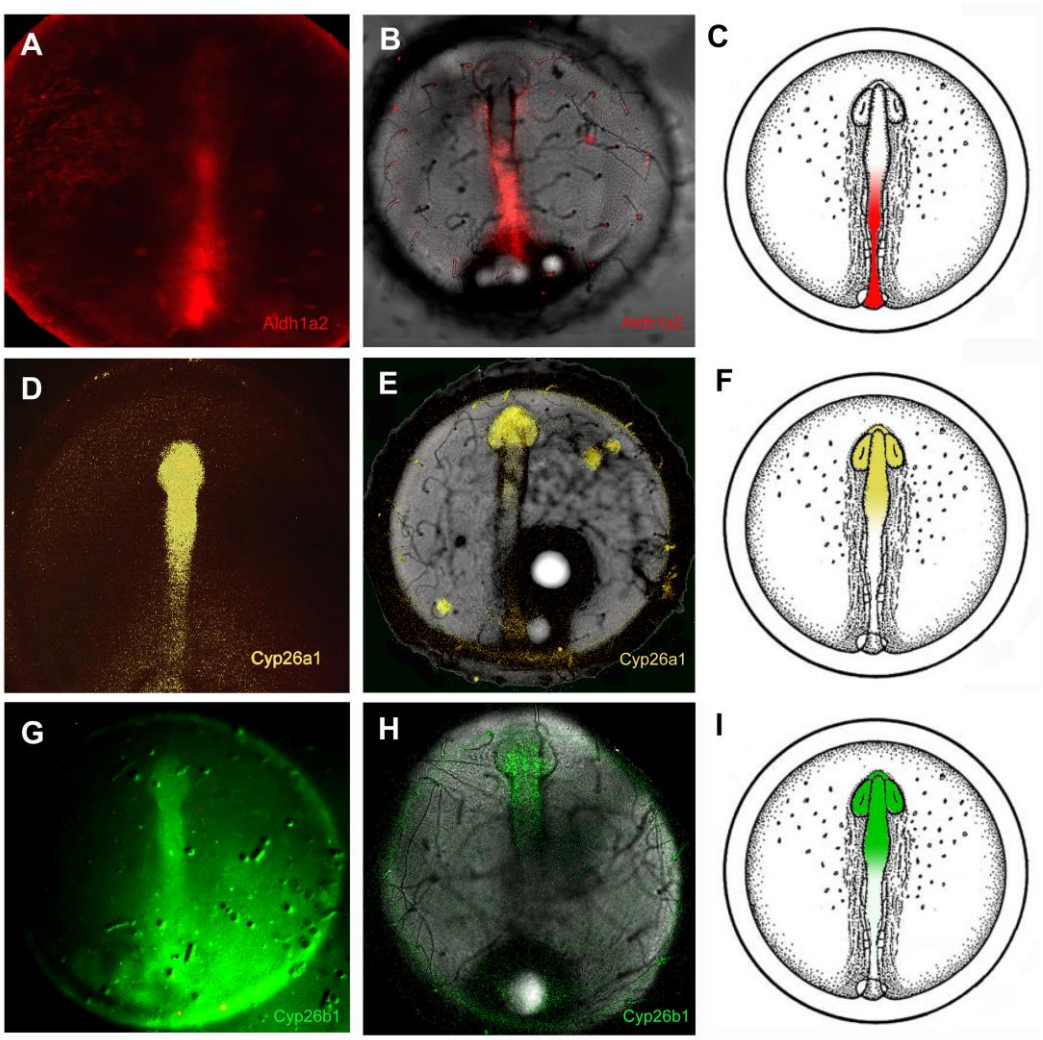


**Figure 2. Early expression pattern of *aldh1a2*, *cyp26a1* and *cyp26b1* in medaka embryos.** *aldh1a2* (A and B) presents a posterior expression pattern in the embryo during the early stages of development (stage 17). At this same stage *cyp26a1* (D and E) and *cyp26b1* (G and H) have an anterior expression domain. The expression of *aldh1a2* is complementary to *cyp26a1* and *cyp26b1* expression, represented schematically in C, F and I. Schematic drawings of embryos adapted from Ref.[33].

## 3.3 Disruption of *cyp26a1* by TALEN

In **Chapter 2**, I showed that *cyp26a1* is differently expressed at gonad level in medaka, been stronger in females than in males. To evaluate a possible role of *cyp26a1* in the timing of meiosis entry and consequently in sex determination/differentiation, I generated knockout *cyp26a1* medaka.

To evaluate whether the injected *cyp26a1* TALENs indeed induces mutations, I amplified the expected target site from $F_0$ embryos and adult founders male and female, and sequenced the 544bp PCR product (Figure 3A). I produced three different mutant lines (Figure 3B) with deletions at the target site. However, only the Δ5 mutation encodes a protein with a predicted compromised function (frameshift, premature termination), while the other two would have an in-frame deletion of two or three aa that still could lead to fully functional enzyme (Figure 3 C). All three mutants present deletion or substitution in the P450 superfamily domain, however, the Δ5 mutation generates a shorter protein that lacks the Cytochrome P450 cysteine heme-iron ligand signature.

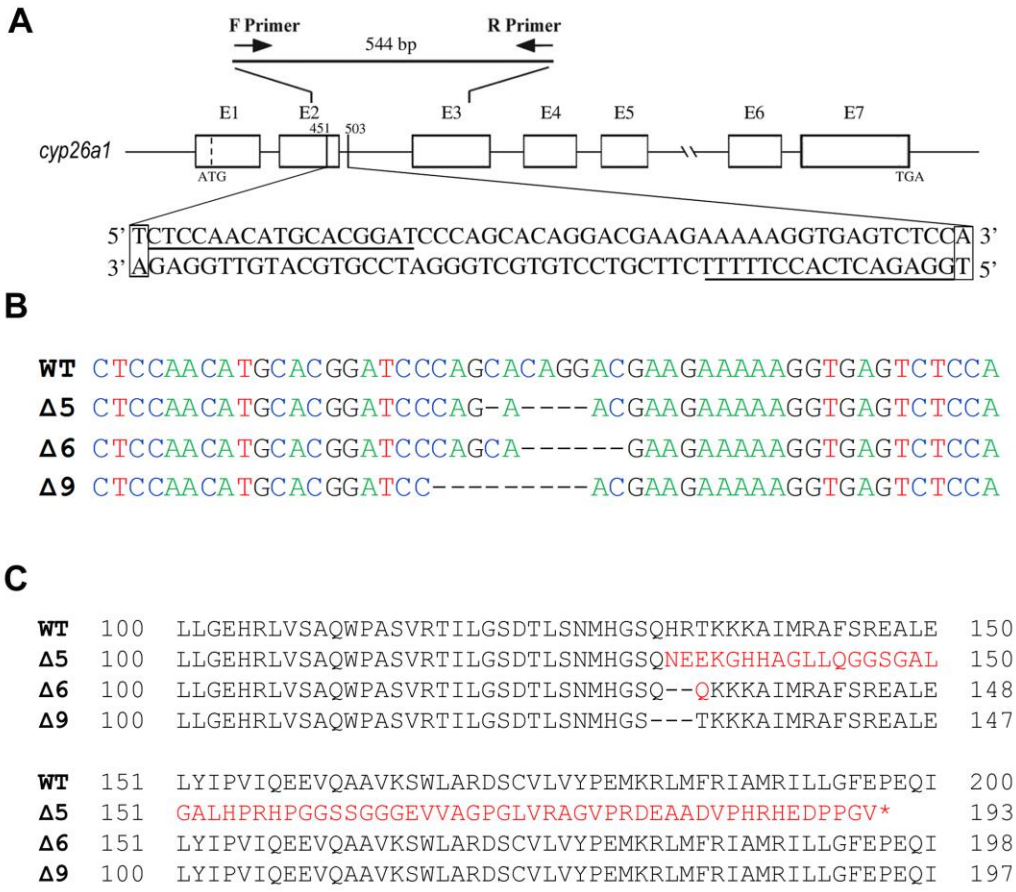



**Figure 3. Genomic structure of the medaka *cyp26a1* gene and mutation sequences induced by TALENs.** (A) The *cyp26a1* TALENs were designed to target the second exon of the gene. The right binding site was located at the junction of exon 2 and intron 2. Underlined bases indicate the left and right recognition sequences of the TALENs. Forward and reverse primers were designed to amplify the fragment for sequencing. (B) Wildtype and mutant *cyp26a1*sequences. (C) Predicted proteins sequences. Amino acid substitutions are labeled in red. Deletions are indicated by dashes. E, exon; F, forward; R, reverse; WT, wild-type.

In the process of establishing stable mutant lines, I did not obtain homozygous female for all three mutants. However, heterozygote females are viable and fertile. In the case of males, both heterozygote and homozygote mutants are viable and fertile (Table 2)

| Sex | Heterozygote | Homozygote |
|---|---|---|
| Male | 7 | 5 |
| Female | 10 | 0 |

**Table 2 – Number of homozygotes and heterozygotes mutants for *cyp26a1* by TALEN.** The numbers are the total amount of animals combining all stable three mutant lines.

## 3.4 Disruption of *cyp26a1* and *cyp26b1* by CRISPR/Cas9

The transgenic lines show that *cyp26a1* and *cyp26b1* have the same pattern of expression in the early embryos, suggesting that the disruption of one of these genes can still allow the normal development of the embryo. Another study shows that CYP26A1 and CYP26B1 are qualitatively similar RA hydroxylases with overlapping profiles in in human tissues [41]. In **Chapter 2**, I showed that *cyp26b1* is ubiquitously expressed in adult organs, and *cyp26a1* seems to have a more specific profile, especially with a strong expression in ovary in comparison with testis. My expression analyzes (Figure 2 D-I) of both *cyp26a1* and *cyp26b1* showed a possible compensatory function between them. To exclude the possibility that *cyp26b1* may overtake the role of *cyp26a1* in the gonad, I generated full knockout of both *cyp26a1* and *cyp26b1* genes.

Fertilized eggs co-injected with gRNA and Cas9 mRNA were grown until hatching and used for CRISPR/Cas9 effect analysis. The primers used for mutant screening were designed to generate different size fragments from the wild-type and the mutant allele. The *cyp26a1* the wild-type fragment is 1159 bp long and the mutant allele corresponds to 302 bp (Figure 4 A). For *cyp26b1,* the wild-type allele is 1582 bp long and the mutant around 624 bp (Figure 4 B). All fragments were purified and sequenced.

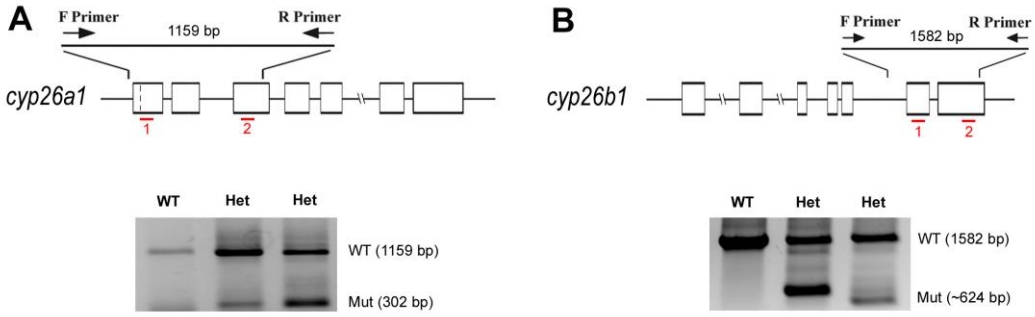



**Figure 4. Disruption of medaka *cyp26a1* (A) and *cyp26b1* (B) by CRISPR/Cas9 in F0 generation.** Gene structure and the target sites of gRNA (red underlined) are shown. After injection, hatched larvae were randomly selected to extract their genomic DNA for PCR amplification. For both genes, the wild-type allele present the bigger size and the mutated allele the lower. F, forward; Mut, mutant allele; R, reverse; WT, wild-type allele.

In the process of establishing stable mutant lines, like for the mutants produced with TALEN technology, I did not obtain females (XX) homozygous for the mutant *cyp26a1* gene in the injected fish, while homozygous male were fertile and developed normally (Table 3). In the case of *cyp26b1* genotypic knockout, no sex bias was observed, and both male and female homozygous mutants were fertile and developed normally (Table 3).

| | Cyp26a1 | | Cyp26b1 | |
|---|---|---|---|---|
| **Sex** | **Heterozygote** | **Homozygote** | **Heterozygote** | **Homozygote** |
| Male | 4 | 2 | 1 | 1 |
| Female | 12 | 0 | 3 | 1 |

**Table 3 – Number of homozygotes and heterozygotes mutants for *cyp26a1* and *cyp26b1* by CRISPR/Cas9.** The numbers are the total amount of animals combining all F0 mutant.

## 3.5 Expression of sex determination genes after induction of the RA pathway

Since the entry of meiosis occurs first in female and only thereafter in male, this can suggests that RA metabolism can play a role on the sex determination and differentiation genes. Thus, I performed treatments of medaka embryos at different time points with ATRA and AM580 to activate the RA pathway. From the treated embryos, I analyzed the expression levels of three genes known to be involved in sex determination or gonad differentiation.

Treatments with ATRA during early stages of embryos from the

BACdmrt1bY::GFP reporter line showed a ubiquitous induction in the GFP signal at late neurula stages, but no induction was observed in later stages. However, long-term treatments (stage 29 until 1 dah) of BACdmrt1a::GFP transgenic fish with ATRA resulted in a specific induction of expression in the somatic gonad at hatching stage for both sexes after treatments (Figure 5 A). Gene expression levels male-related sex determination genes were determined from whole embryos after treatment with AM580 starting on stage 29 until 1 day after hatching (dah) (Figure 5 B). The *dmrt1bY* expression levels were unaffected in males (female lack this gene). However, *amh* and *dmrt1a* showed significantly increased mRNA levels in both sexes.

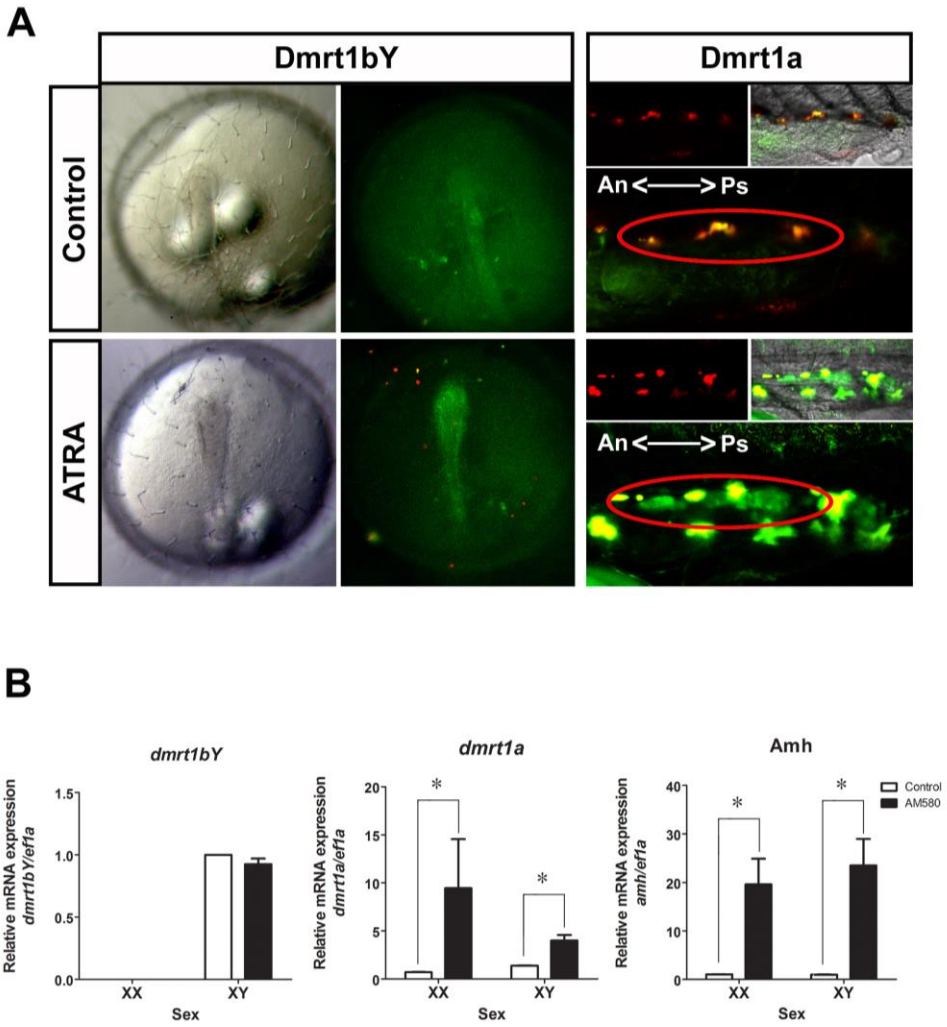



**Figure 5. Regulation of sexual development genes after RA pathway activation.** (A) Treatments with ATRA in early stages of BACdmrt1bY::GFP transgenic line increase GFP signal ubiquitously. Long-term treatments of BACdmrt1a::GFP clearly show gonad (red circle) specific induction of *dmrt1a* at 1 dah. (B) Expression levels of *dmrt1bY*, *dmrt1a* and *amh* at 1 dah stage after long-term treatments with AM580. No effect on dmrt1bY expression is observed, however, significant upregulation of *dmrt1a* and *amh* occurred in embryos of both sexes. Values are expressed as arbitrary mRNA units normalized against the expression levels of *ef1a* amplified from the same template and relative to the average expression of control male and female embryos. Asterisk indicates a significant difference ($p < 0.05$) after Student's t test comparing the expression between control and AM580 treatments

## 3.6 Effect of ATRA on *dmrt1bY* promoter activity

Because RA treatment in early stages showed upregulation of the male sex determination gene, *dmrt1bY*. I asked whether RA has a direct effect on the *dmrt1bY* promoter, I analyzed 3 kb, 6 kb and 9 kb fragments upstream from the transcription start of the pGLuc-basic reporter vector. At least eight putative retinoic acid responsive elements (RARE) are predicted in the promotor (http://jaspar.binf.ku.dk/), five of which are located inside the 6 kb fragment. (Figure 6 A).

After transient transfection into HEK 293 cells, the basal *dmrt1bY* promoter activity was highest for the 3 kb fragment, followed by the 9 kb and the 6 kb fragment. After ATRA treatment, I observed an increase of luciferase activity in all constructs for HEK 293 cells. In SG3 cells, the 3 kb fragment also had the strongest basal activity; however, the 6 kb and 9 kb both presented equally low luciferase signal. After the ATRA treatment, the activity of luciferase was decreased for all promoter fragments in SG3 cells (Figure 6 B).

Quantifying the fold changes in luciferase activity comparing control (DMSO) and ATRA treated cells, revealed that in HEK 293 cells the increase was constant (around 1,7 folds) in all promoter fragments. For SG3 cells, the decrease of activity was stronger in the 3 kb fragment (0,5 fold), followed by the 6 kb (0,75 fold) while the 9 kb showed no regulation after ATRA treatment (Figure 6 C). This result indicates that RA has an opposite function comparing germ line and somatic cells.

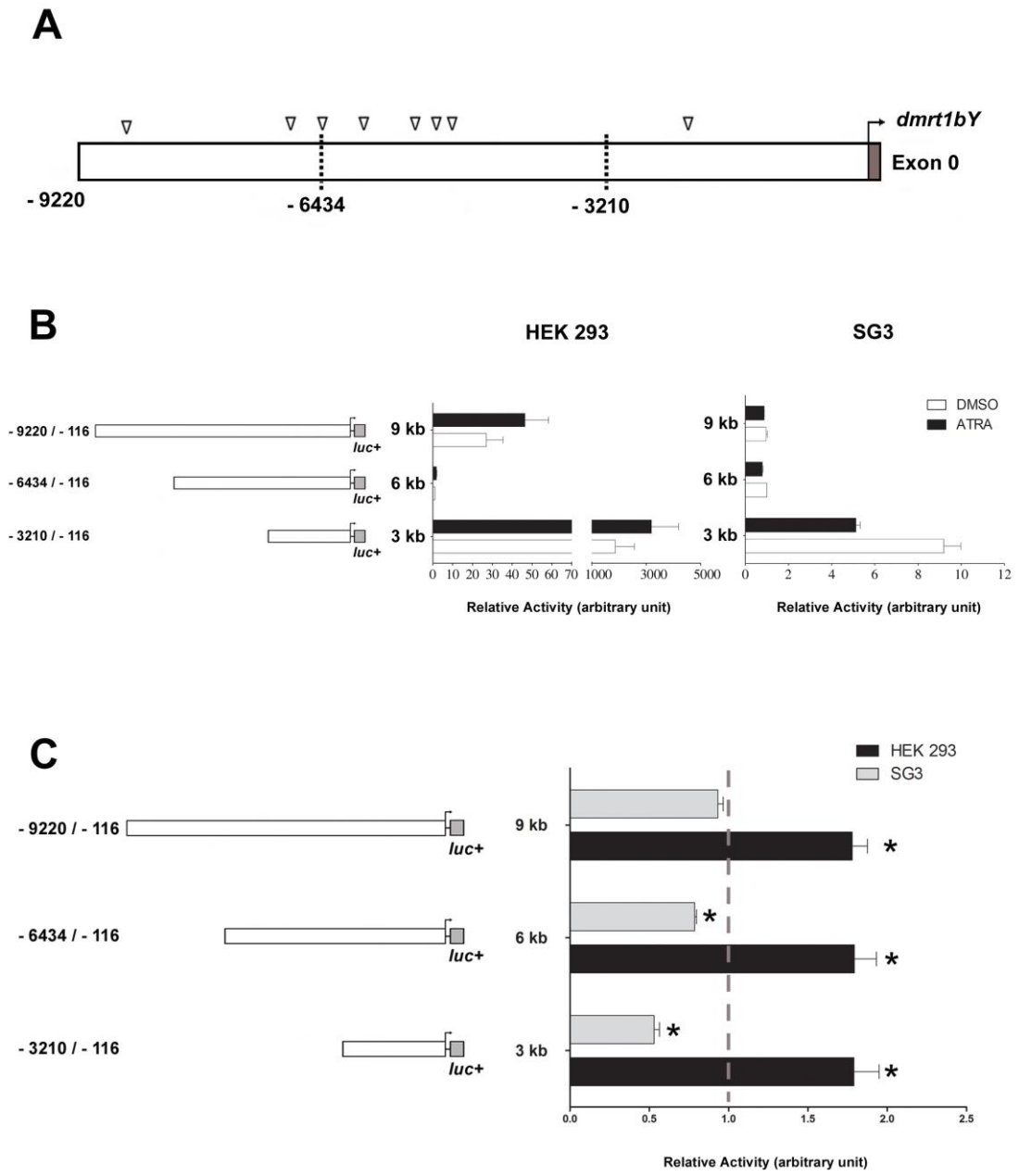



**Figure 6. Transcriptional activity of *dmrt1bY* promoter in HEK 293 and SG3 cells after ATRA treatment.** Activity of the *dmrt1bY* fragments was determined using pGLuc-basic luciferase reporter vectors and normalized co-transfecting with firefly luciferase ptkLUC+ reporter vector. (A) Schematic representation of the *dmrt1bY* promoter indicating location of putative RAREs (arrowhead) and the promoter fragments (dashed lines). (B) Luciferase activity in controls (DMSO, higher in 3 kb fragment and lower in the 6kb for both cell lines) and after ATRA treatment (increased in all promotor fragments for HEK 293 cells, and decreased in SG3). Values are arbitrary units normalized the against 6 kb DMSO fragment luciferase activity (*Gaussia*/Firefly). (C) Normalized luciferase activity (against DMSO treated) of each fragment (dashed gray line). In HEK 293 cells, all *dmrt1bY* promoter fragments showed significant increase of activity after ATRA treatment. In SG3 cells, the 3 kb and 6 kb fragments presented significant decrease of activity. Asterisks indicate significant differences ($p < 0.05$, Student's t test).

# 4. Discussion

## 4.1 RA pathway activation does not induce meiosis in SG3 cells.

To study and recapitulate spermatogenesis *in vitro* is very important to understand the molecular mechanism behind gametogenesis. For this process a strong interaction has been demonstrated between germ cells and somatic cells, and the process of sperm formation is mainly regulated by this crosstalk [31, 42]. SG3 cells have been reported as the only *in vitro* model in fish, so far, to generate sperm from stable line of spermatogonial stem cell [38]. However, no protein analyzes was done in this work, and the main supporting data are based on gene expression of meiosis markers and cell morphology.

In my PhD work, I was not able to detect the meiosis marker protein SCP3 under exactly the same conditions as described in Ref [38]. As Vitamin A is known to be required for production of fertile sperm in male mammals [43], I then performed different drug treatments *in vitro* expecting that the meiosis marker SCP3 would be observed as result of a possible meiosis inducing effect of RA. However, no SCP3 protein was detected. Recently, RA was shown to have a role in the initiation of spermatogenesis and differentiation of type A spermatogonia into type A1 spermatogonia of mice [44]. In fish, the first evidence of RA being crucial for meiosis initiation was recently reported in *Silurus meridionalis* [45]. In the **Chapter 2**, I showed that responsiveness to RA is present in the undifferentiated spermatogonia, but also in the Sertoli cells close to those spermatogonia. The fact that induction of meiosis *in vitro* was not observed could be related to the absence of Serloli cells in the cell culture. In mice in cultured neonatal testes and in isolated gonocytes/spermatogonia *in vitro* it was shown that, germ cells could respond to RA independently from the presence of Sertoli cells. Whether the differentiation triggered by RA in this somatic cell-free system can lead to formation of late differentiating spermatogonia and primary spermatocytes is not clear [46]. However, I cannot exclude the possibility that after several passages of culture, the SG3 cells could have lost the capacity of undergo meiosis. This puts a note of caution to the interpretation of RA not inducing meiosis *in vitro*.

Our group described earlier the plasticity of the SG3 cells by differentiating these cells into 4 different ectodermal and mesodermal somatic cells by ectopic expression of lineage specific transcription factors. In addition, it was shown that ATRA could

induce the differentiation of SG3 into adipocytes [40]. I confirmed that activating RA pathway, this time with the RARα agonist, induces formation of adipocytes. This indicates that, despite the failure of meiosis induction, SG3 cells can respond to the exogenous treatments independent from Sertoli cells.

## 4.2 Disruption of *cyp26a1* and *cyp26b1* indicates no effect on sexual development

In vertebrates, the levels of RA are tuned by the balance between synthesis, by the RALDH enzymes, and degradation, by CYP26 enzymes. This balance creates a gradient of RA in the embryo, been important for the anterior-posterior axis formation [9, 14, 47, 48]. I observed that in early medaka embryo the anterior-posterior axis shows complementary expression of the *aldh1a2* and the *cyp26*s genes indicating that this feature is also conserved in teleost like in other vertebrates [8, 9].

Disruption of both *cyp26a1* and *cyp26b1* had no effect on the development of the embryos. This result, together with the same expression pattern of both *cyp26a1* and *cyp26b1* in early stages, indicates those genes have a redundancy in the early embryos like showed in zebrafish [49]. The *cyp26b1* homozygous mutants were fertile. Both sexes developed normally and were undistinguishable to wildtype animals. However, in the *cyp26a1* mutant homozygotes, only XY males could be obtained. This could be due to the fact that disruption of *cyp26a1* is lethal for females but not for male individuals. My results suggest that, disrupting the main RA degrading enzymes have no effect on the sex determination, but that *cyp26a1* may to be important for female survival, or off target mutations could lead to segregation distortion due to the CRISPR/Cas9 technical effect. However, the low sample size and the lack of stable CRISPR line puts a note of caution to the results with the mutants. The production of *cyp26a1* deficiency in Tilapia and catfish did not affect normal development of the animals but was connected to an earlier initiation of meiosis for both XX and XY fish [45, 50]. Future experiments in medaka with both cyp26 mutants for an eventual effect on the timing of meiosis entry thus need to be performed.

## 4.3 Exogenous activation of RA pathway upregulates male-related genes.

The network and factors involved in the sex determination are shown to be more complex and diverse than was observed in the last 20 years. The diversity of sex determination mechanisms is particularly evident in species of the genus *Oryzias*. *O. luzonensis*, a closely related species to *O. latipes*, lack the master male sex determination gene (*dmrt1bY*), but acquired *gsdfY* as new sex determination gene. In more distantly related *Oryzias* species, even other genes operate as male determiners [51, 52]. On the contrary, to this molecular diversity, in vertebrates there is a straight correlation between the sex of the animal and meiosis entry of the PGCs. In both sexes, the number of PGCs is the same until late embryogenesis. Then PGCs start to proliferate in female embryos first and enter meiosis immediately afterwards. In males, the PGCs stay in a quiescent status and only later PGCs resume proliferation and become spermatogonia [6, 16, 53, 54]. Therefore, to characterize the relation between sex-related genes and meiosis entry and a possible role of retinoic acid in this process, I performed treatments with RA agonists and analyzed possible changes in the sex determination pathway.

My results showed that activation of the RA pathway upregulates *dmrt1bY* during very early stages in the entire embryo. This is much prior to any development of the primordial gonad and no specific upregulation was observed in PGCs. Thus, this might be not effective for sexual development and more related to background expression effects. Only later expression of *dmr1bY* becomes cell type-specific. It is first detected in clustered germ cells located within the lateral plate mesoderm at stage 28 until stage 30-31, but a function of this early PGC expression is still unknown [55]. Expression in the somatic cells of the gonadal primordium is then observed at stage 33. Experiments showed that *dmrt1bY* has an important role in mitotic arrest of male PGCs especially in G2 phase [27, 56]. This function was related to the expression of *dmrt1bY* in the somatic cells at stage 33, but a longer lasting effect on mitosis arrest from *dmrt1bY* expression in the PCG at early stages cannot be excluded. The luciferase results for *dmrt1bY* promoter indicated that this promoter fragment shows basal expression in spermatogonial stem cell SG3 and even human embryonic kidney cells HEK293. RA treatments revealed increased promoter activity in HEK cells in the SG3,

it can be decreased. This indicates that probably RA may have an antagonistic role in germ cells and somatic cells on *dmrt1bY* levels. Interestingly, *in vivo* during male gonadal development a switch from PGC to somatic expression occurs with *dmrt1bY* being downregulated in the germ cells and concomitantly upregulated in the somatic cells [55]. It is tempting to speculate that RA may be involved in this process.

At the sex determining stage, *dmrt1* and *amh* expression was induced by RA treatment in both sexes. Both genes are classically known as male-related sex determination genes. Contrary to *dmrt1bY*, the *dmrt1a* gene showed strongly increased mRNA levels in both male and female hatchlings at the hatching stage after RARα activation during late embryonic development. Importantly, this upregulation occurs specifically in the gonad. The *dmrt1a* gene is known to have a main role in testis differentiation and maintenance [1, 57]. Treatments with cortisol and high temperature in medaka embryos resulted in sex-reversed XX males, where *dmrt1a* most probably takes over the role of *dmrt1bY* in the early gonad male sex determination [23, 25, 58]. These data were confirmed analyzing the BACdmrt1a::GFP transgenic expression at gonad level (Peter Fischer, personal communication).

The expression profile of *dmrt1a* in medaka fish has so far been documented to commence only about 10 dah, when the male gonad differentiation process starts, and exclusively in somatic cells of the gonad [3]. By analyzing the expression of *dmrt1a* in early embryos, I was able to detect PGC expression signal as early as stage 25/26 (Supplementary Figure 2), disappearing around stage 31/32. This *dmrt1a* expression in the germ cells goes together with the early expression of *dmrt1bY*, suggesting that *dmrt1a* can play the same role of *dmrt1bY* in mitosis arrest of the PGCs. The early expression of *dmrt1a* in both male and female PGC indicates that this early germ cell expression may have no role in sex determination under physiological condition. In mammals, loss *Dmrt1* causes spermatogonia to precociously exit the spermatogonial differentiation or meiosis. In mice, DMRT1 blocks testicular RA signaling from activating genes normally involved in female sex determination, allowing Sertoli cells to participate in RA signaling without being sexually reprogrammed [28]. DMRT1 restricts RA responsiveness by repressing *Stra8* transcription preventing meiosis and promoting spermatogonial development in adult testis in mice [30, 59, 60]. In fish, the levels of *dmrt1* is regulated depending of the maturation cycle of the gonad, with an

extreme decrease of expression after spermiation [61, 62]. The induction by ATRA of *dmrt1a* in the gonad of medaka for both sexes leads me to suggest that the treated embryos could have developed to female-to-male sex reversed fish. Unfortunately, long-term treatments with AM580 are extremely lethal for the embryos, which makes it difficult to observe an effect on gonad development at later stages. Future *in vitro* experiments on the regulation of *dmrt1a* promoter activity will help to elucidate if RA has a direct effect on the regulation of this gene.

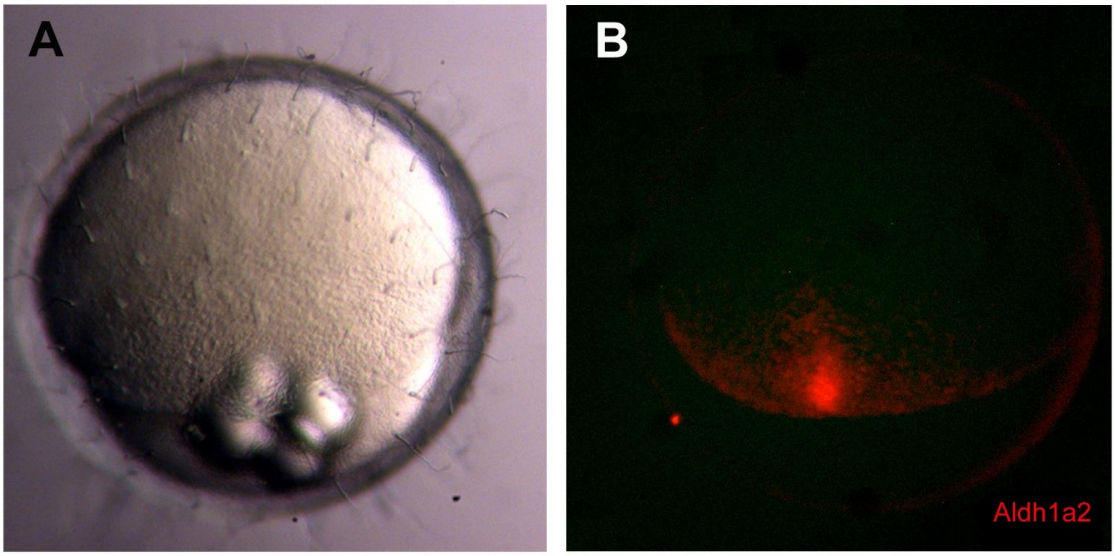
Overexpression of *amh* in both sexes after AM580 treatment can be also correlated to the blocking of germ cell differentiation. In mammals, AMH is classically known to be involved in the regression of the female reproductive primordium (Müllerian duct) in males [63, 64]. However, in teleost the Müllerian duct does not exist, however the *amh* gene is detected in both female and male developing gonads [65, 66]. Medaka, which are mutant for AMH type II receptor (*amhrII)* have an excess of germ cells and show male-to-female sex reversal. Analyzes of the phenotype of this mutant, called *hotei*, show that AMH signaling acts in the supporting cells of the undifferentiated gonad to promote proliferation of the mitotically active germ cells [67]. The germ cells in the gonadal primordia are characterized according to their type of division: type I and type II. The type I division correspond to a self-renewal division, while in type II division, germ cells synchronously and successively divide and subsequently enter meiosis [3, 26]. Hence, *amh* is important to regulate the proliferation of type I germ cells and the loss of this signaling results in hyperproliferation of germ cells [67]. Our results show that RA pathway activation leads to overexpression of *amh*, however, it is unknown in which cell type(s) this occurs. Future studies with expression localization of *amh* after RA treatment should help to uncover whether Amh signaling in the gonad is under RA regulation.

## 5. Conclusions

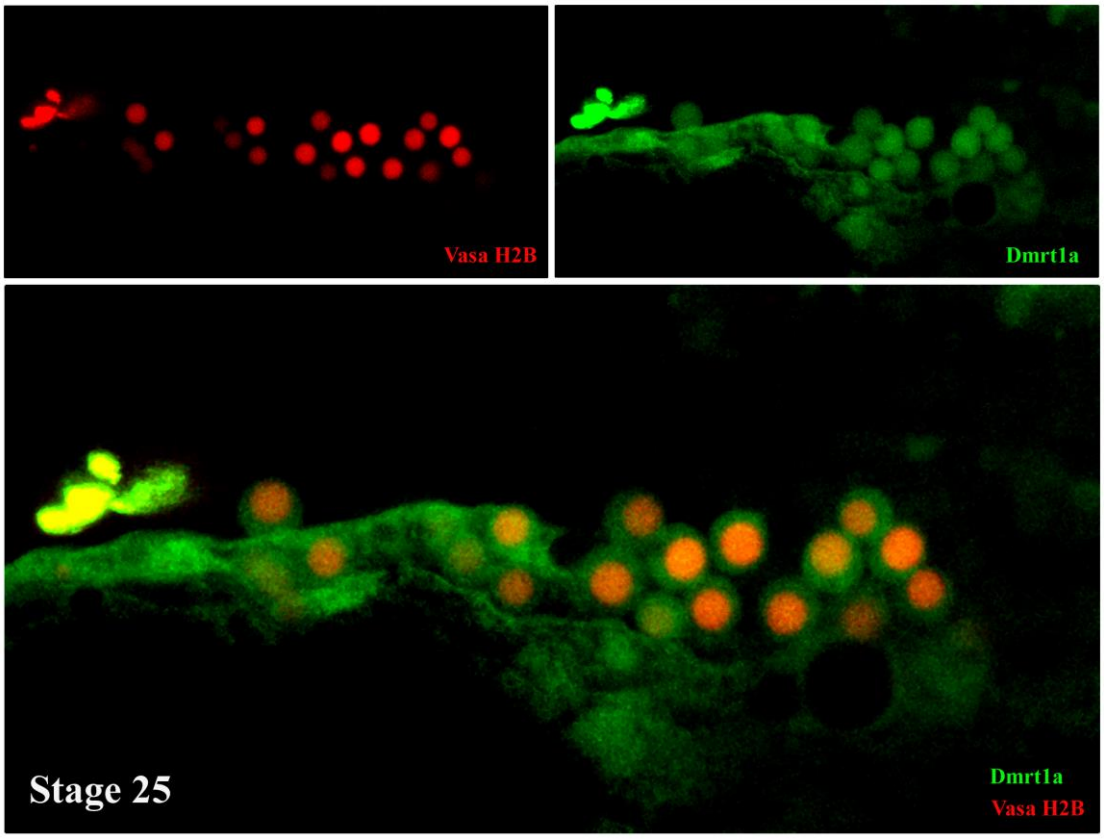
My findings show an unexpected role of RA in regulating sex determination genes. RA pathway activation leads to the regulation of genes not only related to sex determination, but also to gametogenesis and meiosis entry. This makes me to suggest that, in medaka, RA can directly control germ cell differentiation not through *stra8* regulation, but by activating sex determination genes related in gametogenesis and entry in meiosis at SD stages. RA may have an indirect effect on meiosis in medaka,

but in this case, acting as an inhibitor. In addition, I showed that the RA pathway can have a role in isolated spermatogonial germ cells, but the presence of the supporting cells are still important for the process of gametogenesis.

## 6. Supplementary figures




**Supplementary Figure 1. Aldh1a2 transgenic line early expression.** Expression of *aldh1a2* (Red) in embryo of medaka. (A) Embryo under normal light stage 15. (B) Embryo showing signal at the shield of the gastrulating embryo (stage 15).




**Supplementary Figure 2. Early expression of *dmrt1a* in the germ cells.** Double transgenic embryos (BACdmrt1a::GFP::Vasa::H2B::mCherry) show strong expression of *dmrt1a* (green) in vasa positive (Red) germ cells at stage 25 of medaka.

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# Chapter 4

## Integrative Discussion and Conclusions

# 1. Integrative discussion

In multicellular organisms the formation of the gametes is a key event for the production of future generations [1]. In this process the germ cells take two crucial decisions, a temporal one, namely when meiosis entry happens, and lineage decision to develop either to sperm or egg. In high vertebrates, the cell cycle decision between mitosis and meiosis, which takes place in both sexes, might be considered as distinct from the sperm/egg [2]. However, the timing of the mitosis/meiosis decision and features of meiosis itself are often sex-specific, suggesting a close relationship between the mitosis/meiosis and sperm/egg decisions [2]. Furthermore, in all vertebrates, the meiotic initiation is among the first signs of sexual dimorphism in the ovary, suggesting somatic cues for these two main germ cell decisions [3, 4]. In mammals, the mechanism leading to meiosis entry is reasonably well understood, and the critical molecule in the induction of meiosis entry is retinoic acid (RA) [5]. RA is a polar diffusible molecule related to diverse developmental processes and organogenesis [6]. At the germ cell level, RA induces expression of the main meiosis regulator STRA8 (stimulated by retinoic acid gene 8) [7]. However, not much is known about STRA8 and without a better understanding of this gene, it is premature to conclude a conserved mechanism in other phylogenetic groups. There are some indications of a main role of RA in meiosis induction also in other tetrapod [8, 9]. However, phylogenetic analyses of vertebrates show that *stra8* is absent from the genomes the vast majority of teleost fish, adding a question mark for the importance of RA in meiosis induction in this group of vertebrate. *Silurus meridionalis* is, so far, the only teleost species in which *stra8* gene was isolated and described [10]. In this species, like in mammals, RA was shown to be involved in the process of first meiosis entry and adult gametogenesis [11]. In Nile tilapia (*Oreochromis niloticus*), *stra8* is absent but RA was shown be important to regulate the timing of meiosis entry, while it appeared not necessary for the gametogenesis process [12].

## 1.1 Retinoic acid regulates meiosis entry in adult gonads in s*tra8* independent fish medaka.

In addition to what was shown in tilapia, in **Chapter 2** I showed in medaka that the responsiveness of RA is not restricted to its site of synthesis in the adult gonad and during gonadal development for both sexes. Interestingly, the expressions of *aldh1a2* and

*cyp26a1* are sexually dimorphic, with *aldh1a2* being higher in testis, and *cyp26a1* higher in ovary. This dimorphism is also reflected in the localization of both transcripts and the responsiveness to RA in adult gonads for both sexes.

In ovary, the cells that express *aldh1a2* are in the somatic part of the gonad, namely the parenchymal cells and are not belonging to ovarian follicular cell types (granulosa and theca cells). On the contrary, *cyp26a1* expression occurs in the germ line, specifically in the early oocytes that are in meiotic arrest. In zebrafish it was hypothesized that the function of *cyp26a1* in those cells is to arrest the oocytes in prophase-I, because the signal disappears when meiosis is resumed [13]. In medaka, not only the expression of *cyp26a1* is stronger in the meiosis-arrested oocytes, but also the responsiveness is stronger in the later oocytes that are resuming meiosis (Figure 1).

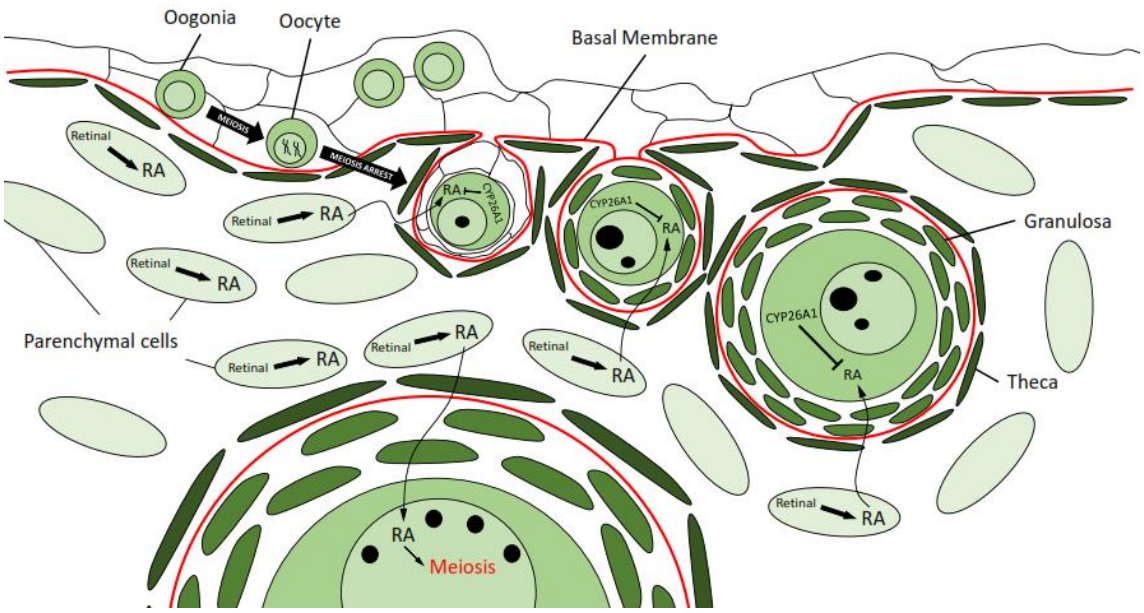


**Figure 1. Schematic image of RA metabolism, regulation and action in adult ovary.** The expression of *aldh1a2* is restricted to parenchymal cells surrounding the ovarian follicles. RA diffuses to the oocytes and is degraded by CYP26A1 enzyme in the early oocytes in meiosis arrest. In the later oocytes, the expression of *cyp26a1* disappears, allowing the oocyte to strongly respond to RA and complete meiosis.

In adult male gonads, the expression of *aldh1a2* is also restricted to the somatic cells, being expressed in Leydig cells, which are important for steroidogenic hormones production [14]. The Leydig cells also present responsiveness to RA, suggesting a possible autocrine effect on those cells. Differently from ovary, the germ cells with the higher responsiveness to RA are the type A spermatogonia, which have not started the spermatogenesis process and, consequently, meiosis. Interestingly, the responsiveness to RA in Sertoli cells was observed only in the spermatocysts that are close to the type A

and type B spermatogonias, indicating a somatic contribution to the progress of gametogenesis. However, no *cyp26a1* could be detected, meaning that the regulation of RA action does not involve Cyp26a1. To investigate this further, I performed treatments of testes in organ culture (**Chapter 2**) expecting that RA pathway activation could lead to meiosis entry. However, I observed that in adult testis the treatments with the RARα agonist led to a decrease of meiosis. Since the CYP26s proteins do not degrade the agonist, it shows that the reduction of meiosis marker levels is due to a direct effect on the activation of the RA pathway. The question at that moment was to investigate if the spermatogonia could undergo meiosis in the absence of the supporting Sertoli cells. In **Chapter 3**, I performed several treatments in the spermatogonial stem cell line SG3 to try to induce meiosis in a scenario, where Sertoli cell absent. I could observe that spermatogonia are capable to respond to RA and go into differentiation to adipocyte, but not to undergo meiosis like observed previously [15]. The production of sperm and meiosis induction of SG3 was already reported by Hong et al. [16]. However, this effect was more readily observed when co-culturing SG3 cells with rainbow trout gonad somatic cells (RTG), indicating a strong contribution from somatic cells to germ cell gametogenesis. Still, the details of such a crosstalk between spermatogonia and Sertoli cells in meiosis induction are not clear. Treatments with exogenous *all-trans* retinoic acid (ATRA) and the agonist of RA receptor alpha (RARα) during different developmental stages showed upregulation of important male related genes, *amh* and *dmrt1a,* in both male and female early gonads (**Chapter 3**). This result adds a piece from the puzzle of the contribution of somatic cells in meiosis entry regulation. Both *amh* and *dmrt1* are not only genes related to formation of the somatic part of the testis, but are also implicated in gametogenesis regulation. In medaka, *amh* is important to regulate the proliferation of germ cells [17-19], and in zebrafish it was shown that AMH blocks the differentiation of spermatogonial stem cells to type A spermatogonia [20]. For *Dmrt1* in mice, it was shown that it restricts RA responsiveness by directly repressing *Stra8* transcription preventing meiosis and promoting spermatogonial development in adult testis [21, 22]. In medaka, it is known that both *dmrt1bY* and *dmrt1a* are important to control the cell cycle of the germ cells [23]. Putting all the pieces together, I hypothesize that in adult testis of medaka, RA acts in type A spermatogonia leading to germ cell proliferation and meiosis arrest through AMH and DMRT1 signals coming from the Sertoli cells (Figure 2). However, it is possible that RA action in type B spermatogonia can be the signal to induce meiosis only in those cells. Further experiments need to be performed to confirm this hypothesis.

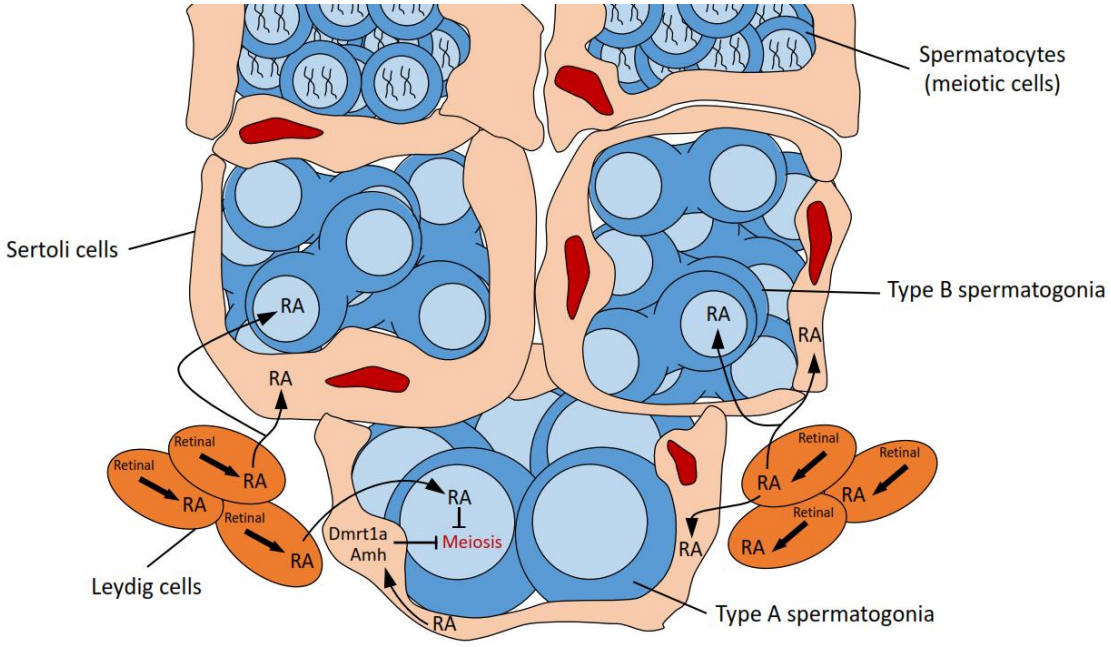


**Figure 2. Schematic image of RA metabolism, regulation and action in adult testis.** The expression of *aldh1a2* is restricted to Leydig cells in the testicular interstitial tissue. RA diffuses to the type A and type B spermatogonias and the Sertoli cells surrounding them. In type A spermatogonia, RA acts by inhibiting the meiosis and gametogenesis progression through Sertoli cells signals (Dmrt1 and Amh). Type B spermatogonia respond to RA, probably mediating meiosis entry.

## 1.2 RA does not mediate first meiosis induction in embryos of medaka.

Still, the whole process of meiosis regulation on adult gonad as discussed above does not answer the initial question, if RA is involved in the first meiosis, leading to gametogenesis and determining the sex of the germ cells. Even in STRA8-absent fish species, RA is shown to play a crucial and important role in meiosis induction [12, 13], meaning that RA probably activates a different pathway leading to meiosis regulation in a Stra8-independent manner. In mammals, the link between sex determination and RA pathways becomes apparent shortly after sex is determined. Experiments show that the co-expression of transcription factor genes for steroidogenic factor-1 (*Sf1*) and *Sox9* (*Sry*-related HMG box 9) upregulates *Cyp26b1* in the male gonad, inhibiting the action of RA in germ cells. *Sox9* is a gene related to testis differentiation that is expressed only after the expression of the male master sex determination gene *Sry*. Furthermore, the transcription factor Forkhead box L2 (FOXL2), related to ovarian differentiation, suppresses the upregulation of *Cyp26b1* by SF1 in ovaries [24]. In **Chapter 2**, I showed that in medaka the primordial germ cells (PGCs) present responsiveness to RA in both sexes (stage 30), before the sex determination period of male (stage 33) and long before the meiosis entry stage in female (around hatching). More importantly, exogenous treatments with different activators and inhibitors of the retinoic acid pathway showed

that meiosis could only be regulated after the sex was already determined and differentiation of the gonad as proceeded. In these experiments, I could show that RA is important to meiosis regulation only in the differentiated gonad, but it is not for the first meiosis induction.

Interestingly, I found as reported in **Chapter 3** that RA has an effect on expression of genes from the sex determination pathway. Hence, I hypothesize that the regulation of the first meiosis entry may be mainly through the sex determination genes, which can be regulated indirectly by RA. Recently, studies using BAC transgenic *dmrt1bY* reporter lines showed that the expression of this gene is much early than expected. From around stage 26 until stage 32, the expression of *dmrt1bY* is restricted to the PGCs of males. Later the signal gradually switched from germ cells to the somatic cells of the primordial gonad [25]. This result motivates to reconsider the timing of male sex determination in medaka; however, an early function of *dmrt1bY* in the PGCs is still unknown. In **Chapter 3** I performed expression analyses of *dmrt1a* using the BAC transgenic *dmrt1a* reporter line and I observed that *dmrt1a* in both sexes also presents an early expression in the PGCs around stage 26 until stage 32. However, the signal vanishes around stage 33, reappearing only around 10 days after hatching, then in the somatic cells parallel to the switch of *dmrt1bY*, during the time of male sex differentiation period [26-28] (Figure 3).

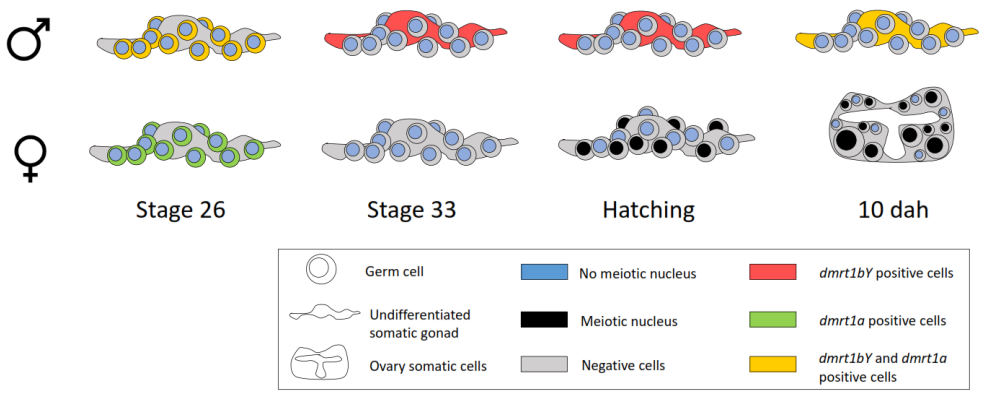


**Figure 3. Schematic image of *dmrt1a* and *dmrt1bY* expression during gonad development of medaka.** In early stages (between stage 26 and stage 32), *dmrt1bY* is expressed in the early PGCs. The same pattern was observed for *dmrt1a* in both sexes in early stages. Later, around stage 33, the expression of *dmrt1a* vanishes in both sexes, and *dmrt1bY* expression switches to the somatic cells. The female gonad show the first meiotic cells around hatching, while the male gonad maintains undifferentiated and expressing *dmrt1bY*. The expression of *dmrt1a* reappears in the somatic gonad around 10 days after hatching (dah) in males.

The expression pattern of *dmrt1bY* and *dmrt1a* during development of medaka is another evidence of *dmrt1a* and *dmrt1bY* sub-functionalization, where the early role of

*dmrt1a* in sex determining the somatic gonad is overtaken by *dmrt1bY* [29-31]. Due to the role of Dmrt1 in arresting the cells in mitosis and preventing meiosis, I hypothesize that this early expression of *dmrt1a* in both sexes and *dmrt1bY* in males maintains the PGCs in a quiescent status. In female, after the *dmrt1a* expression disappears, the germ cells are free to enter meiosis, but in males, *dmrt1bY* somatic expression maintains the meiosis inhibition. However, functional experiments on germ cells of *dmrt1a* and *dmrt1bY* expression are needed to understand if those genes are sufficient to regulate meiosis entry without an extra trigger.

## 2. Final remarks

With this thesis, I hope to have contributed to the field, especially in understanding the mechanism of meiosis regulation from an evolutionary perspective. Studies on this topic are normally focusing on a direct mechanism involving a pathway, in which retinoic acid induces the production of Stra8. So far, the initiation of meiosis could not be de-correlated from the presence of this factor. I showed for the first time a link between retinoic acid synthesis and responsiveness on the level of the gonads in medaka, demonstrating for RA a role in meiosis in adult gonads in the absence of *stra8.* Importantly, I also find that retinoic acid is not needed for the first meiosis entry in early embryos. This calls in the question the generalization of a role for retinoic acid in sexual development made from observations on mouse embryos. Transcriptomic studies are now ongoing in medaka and can be useful to identify potential target genes that are related to both sex determination and meiosis regulation. Taking in account the advantages of genome editing by CRISPR/Cas9 system, it seems promising to evaluate the role of the main sex-related genes in meiosis entry by complete depletion of some candidate genes.

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# Appendices

# Appendix 1 – FOXL2 localization in adult ovary of medaka.

**Publication**: Herpin A, Adolfi MC, Nicol B, Hinzmann M, Schmidt C, Klughammer J, Engel M, Tanaka M, Guiguen Y, Schartl M: *Divergent expression regulation of gonad development genes in medaka shows incomplete conservation of the downstream regulatory network of vertebrate sex determination*. **Mol Biol Evol** 2013, **30**:2328-2346.

By investigating the protein distribution of FOXL2 in medaka ovary, I defined a new subpopulation of theca cells, where ovarian-type *aromatase* transcriptional regulation appears to be independent of FOXL2 (Figure 1).

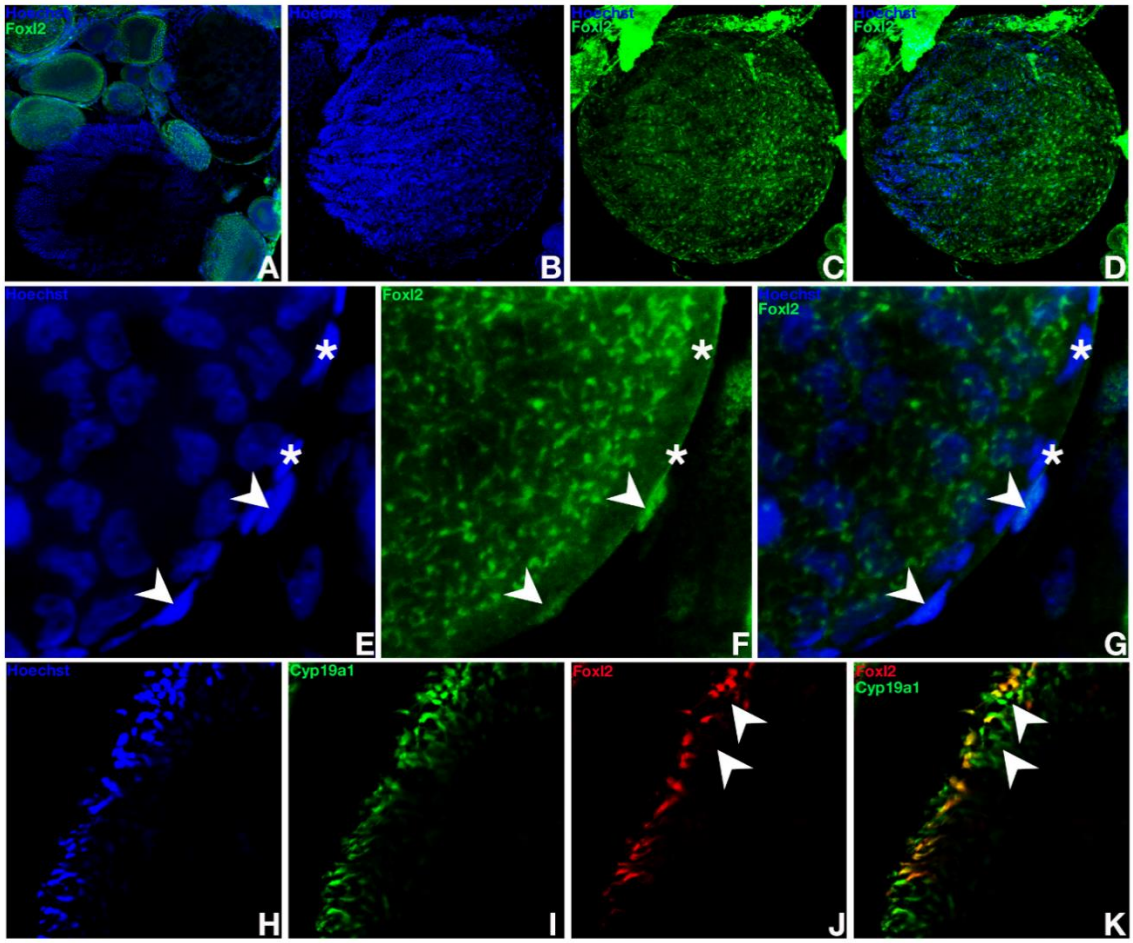


**Figure 1. Protein expression and localization of FOXL2 in the ovary.** FOXL2 immunostaining is present in the nuclei of the follicular cells of the previtellogenic and vitellogenic follicles (A). In vitellogenic follicles, FOXL2 protein is detected in all granulosa cells (B to D). Nuclear localization of FOXL2 in few theca cells (E to G). FOXL2 presence occurs only in a sub-population of theca cells (arrowheads versus asterisk in E to G) as shown by comparison with the thecal layer marker aromatase *cyp19a1* (H to K).

I could also demonstrate, that during the transition process if germ line steam cells to oocytes within the germinal cradle (*sox9b* positive cells), medaka FOXL2 protein is presence starts within the germ line steam cells and continues during meiosis until

oogenesis. In the following steps of oogenesis, the accompanying cells of the supporting layer progressively loose *sox9b* expression, while FOXL2 detection rises (Figure 2).

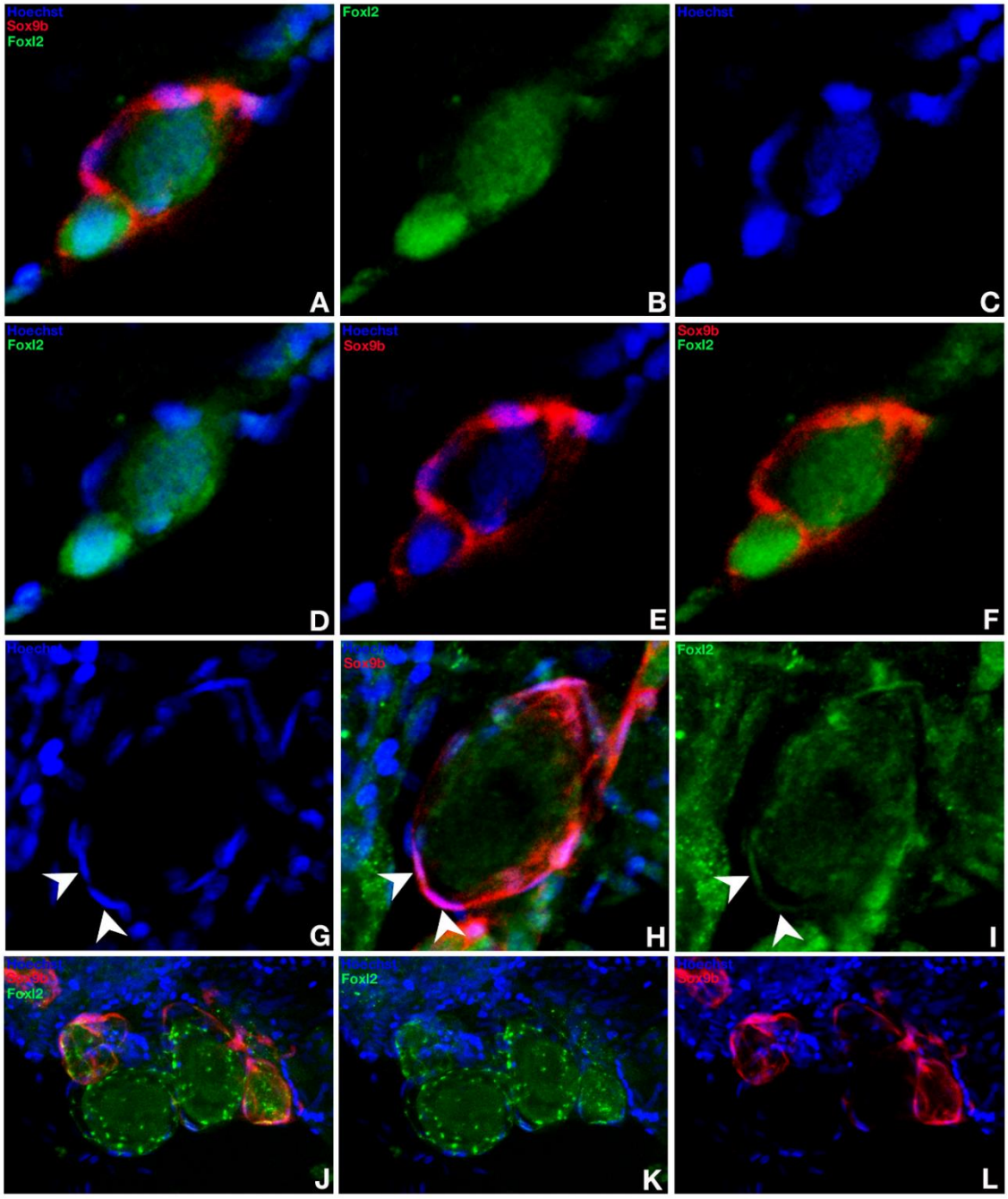


**Figure 2. FOXL2 protein localization in the ovarian cradle.** During development from germ line stem cells to oocytes within the germinal cradle, FOXL2 localization is first detected in the germ line stem cells and remains during meiosis until early oogenesis (A to L). Concomitantly, the accompanying somatic cells of the supporting layer progressively loose *sox9b* expression while FOXL2 presences rises (A to L).

## Appendix 2 – The reporter gene expression in the 12XRARE transgenic line is inducible and dependent of retinoic acid.

To analyze how modulation of RA signaling affects the responsiveness to RA, I performed treatments with both chemicals in the 12XRARE transgenic line (Figure 3 A-H). After treatment with high concentrations of RA, some new expression domains are observed especially in the encephalic region. I also observed a stronger expression and elongation of the domains anteriorly and posteriorly (Figure 3 C and G). In the fish treated with DEAB an almost complete ablation of RA reported expression was observed (Figure 3 D and H). Hence, the 12XRARE transgenic line generated in this work is RA inducible and RA dependent, making this line a trustable tool to analyze the effects of the RA during the gonadogeneses and germ cell fate.

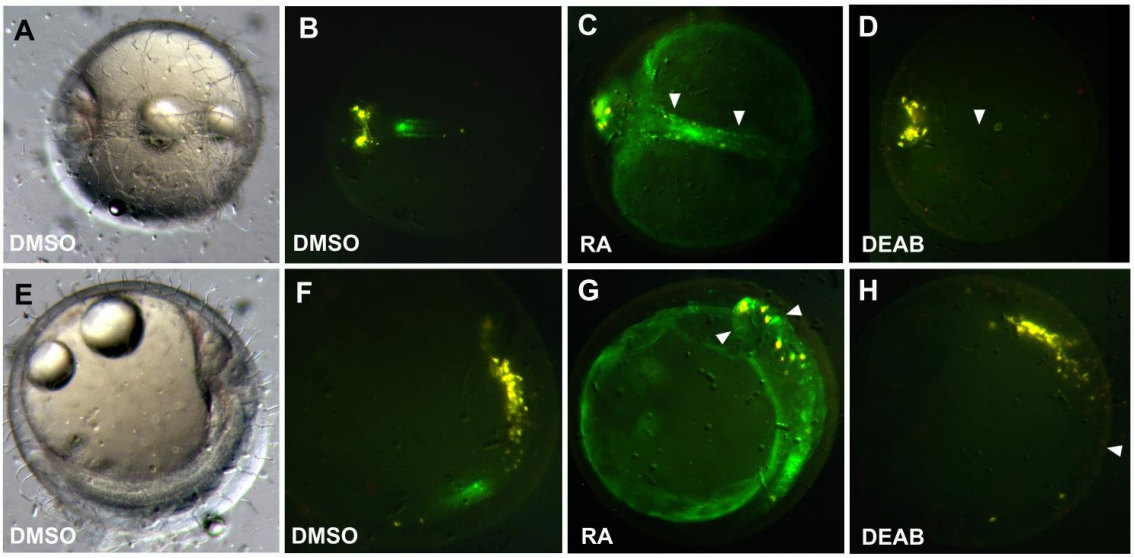


**Figure 3. 12XRARE transgenic signal after treatment with all-trans-retinoic acid and DEAB.** Treatment of embryos with 100 nM RA (C and G) starting at stage 14 induces transgene expression throughout the neural tube and neural retina by stage 23 (arrowhead) compared to the DMSO-treated control (A and B). Treatment of embryos with 5 µM DEAB (D and H), an RA synthase inhibitor, at the same stage significantly reduces transgene signal in the neural tube and abolishes it in all other regions of expression by stage 23 (arrow head) compared to DMSO-treated control (E and F).

## Appendix 3 – Early expression of 12XRARE shows no responsiveness to RA in germ cells.

To observe a possible responsiveness to RA in the germ cells in early embryos, I injected 12XRARE transgenic line eggs with *nanos*-mCherry mRNA and checked the expression. At late neurula stage (stage 18), responsiveness to RA is observed in the lateral plate mesoderm, and the germ cells are distributed along the anterior-posterior axis of the lateral plate mesoderm (Figure 4 A). At 9 somite stage (stage 22), the responsiveness is restricted to the firsts somite, the hindbrain and neural tube, and the germ cells are concentrated beneath the somite responsive to RA (Figure 4 B). In both stages, no responsiveness is observed in the germ cells.

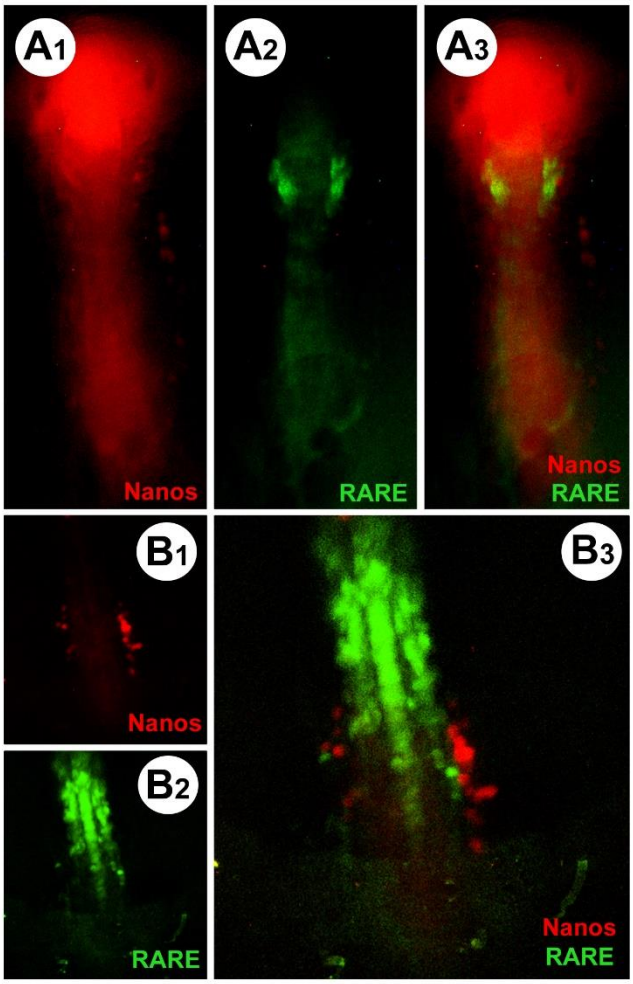


**Figure 4. Early expression of 12XRARE transgenic line injected with *nanos*-mCherry mRNA.** Green indicates responsiveness to RA and red indicates *nanos* positive germ cells. (A) Late neurula stage (stage 18). (B) 9 somite stage (stage 22).

Appendix 4 – Expression of *cyp26a1*, *cyp26b1* and *cyp26c1* during development of medaka.

I quantified the transcripts levels of all *cyp26* genes in different embryonic stages (Figure 5). Embryo sampling and expression analyses were performed like described in **Chapter 2** for *aldh1a2*.

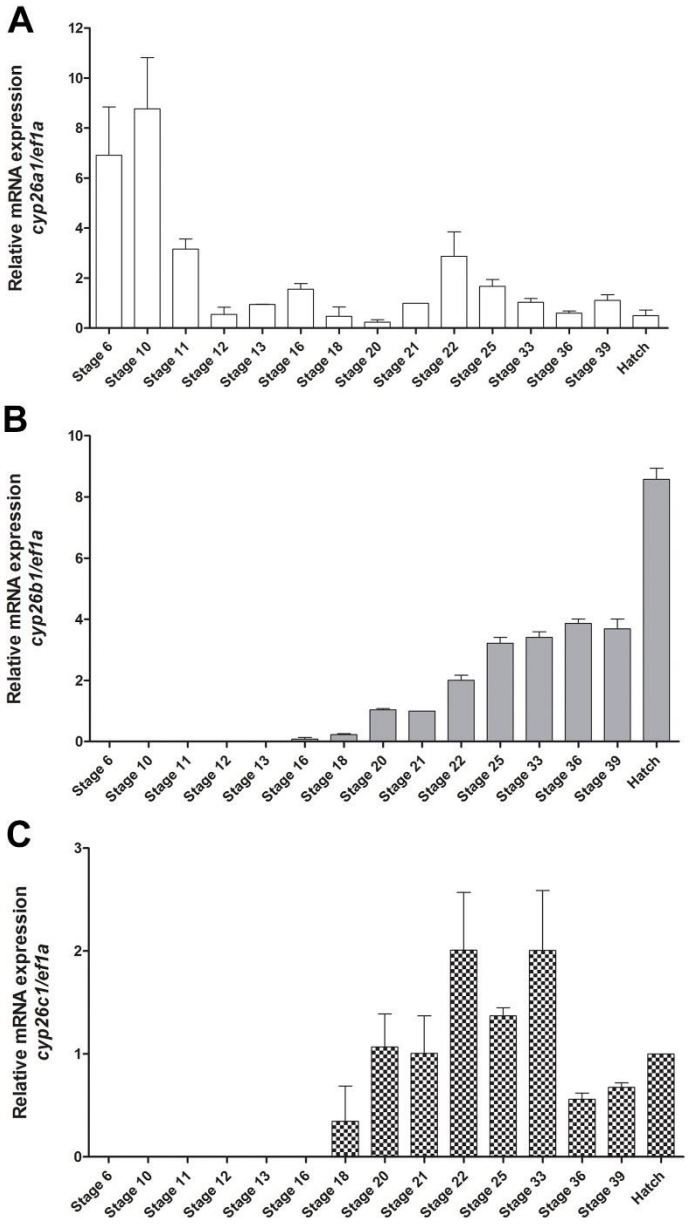


**Figure 5. Expression pattern of *cyp26a1*, *cyp26b1* and *cyp26c1* during the medaka development.** *Cyp26a1* (A), *cyp26b1* (B) and *cyp26c1* mRNA expression in embryos of medaka in different stages of development. Values are expressed as arbitrary units of mRNA normalized against the expression levels of ef1a amplified from the same template, relative to the expression observed in the stage 21 (*cyp26a1* and *cyp26b1*) and hatching stage (*cyp26c1*).

The expression of *cyp26a1* and *cyp26b1* show different and opposing profiles during the early stages (stages 6-20) and the later stages (stage 21 until hatching) of embryonic development. Expression of *cyp26a1* is high during very early development, and is abruptly downregulated after the midblasutla transition (stage 10), indicating mRNA maternal contribution of *cyp26a1*. At later stages, the levels are relatively low, with a small peak at stage 22 (Figure 5 A). The *cyp26b1* levels, in other hand, are low during early stages been upregulated at later stages, and having the highest expression at hatching (Figure 5 B). The expression levels of *cyp26c1* are detected starting from stage 18, been still observed at hatching (Figure 5 C).

## List of publications

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**Adolfi, M.C**.; Herpin, A.; Regensburger, M.; Sacquegno, J.; Waxman, J.S.; Schartl, M. Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. Submitted to *Scientific Reports*.

**Adolfi, M.C**.; Herpin, A.; Regensburger, M.; Schartl, M. Analyzes of expression regulation of sex determination genes by the retinoic acid pathway in medaka. *In preparation*.

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