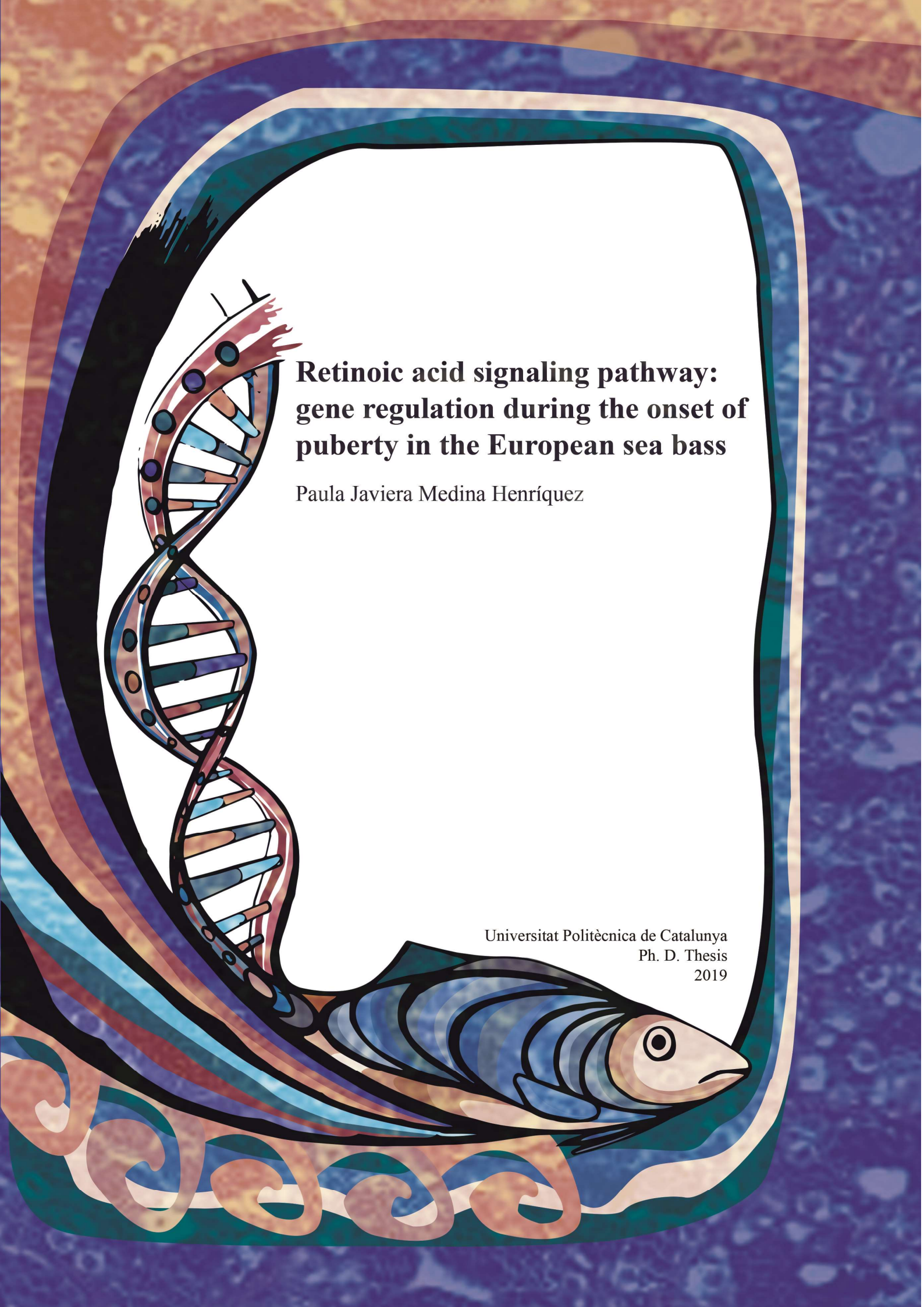

Retinoic acid signaling pathway: gene regulation during the onset of puberty in the European sea bass

Paula Javiera Medina Henríquez

Universitat Politècnica de Catalunya
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Ruta de señalización del ácido retinoico: regulación génica durante el inicio de la pubertad en la lubina Europea

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Esta Tesis ha sido realizada en el Departamento de Recursos Marinos Renovables del Instituto de Ciencias del Mar de Barcelona (ICM-CSIC). Parte de la experimentación también se ha llevado a cabo en el Instituto de Acuicultura Torre de la sal (IATS-CSIC), a través de una estrecha colaboración con el grupo de Fisiología de la Reproducción de Peces. La financiación económica para la experimentación se ha recibido a través de los siguientes Proyectos de Investigación:

- Mejora de la producción en acuicultura mediante el uso de herramientas biotecnológicas CSD2007-00002 (AQUAGENOMICS).
- Bases moleculares, celulares y endocrinas de la pubertad y del desarrollo gonadal en lubina (*Dicentrarchus labrax*). Desarrollo de tecnologías ambientales, hormonales, y de manipulación genética para su control PROMETEO/2010/003 (REPROBASS).
- Regulación Hormonal y desarrollo del eje hipotalámico-hipofisario-gonadal durante la diferenciación sexual y la pubertad en peces teleósteos PIE 200930I037(TERMOBASS).
- Regulación endocrina y paracrina de la diferenciación sexual y el desarrollo gonadal en la lubina AGL2011-28890 (REPROSEX).
- Acción complementaria al proyecto: regulación endocrina y paracrina de la diferenciación sexual y desarrollo gonadal en la lubina (REPROSEX).
- Avances en el control de la pubertad y ciclo reproductor de la lubina y su aplicación tecnológica a otras especies de peces PROMETEO II/2014/051 (REPOBASS II).
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- Blázquez, M., Medina, P., Crespo, B., Gómez, A., and Zanuy, S. (2017). Identification of conserved genes triggering puberty in European sea bass males (*Dicentrarchus labrax*) by microarray expression profiling. BMC Genomics 18, 441.
- Blázquez, M., Crespo, B., Medina, P., Gómez, A. y Zanuy, S. (2011). Bases moleculares del inicio de la pubertad en machos de lubina. Libro de Actas del XIII Congreso Nacional de Acuicultura. El equilibrio con el Medio Ambiente. (M. Villaroel, L. Reig, C. Almansa, eds.) ISBN 978-84-937611-9-6, pp. 57-58.
- Medina, P., Gómez, A., Zanuy, S., Bláquez, M. (2014). Testicular organ culture for the study of sea bass spermatogenesis. Advances in Comparative Endocrinology, vol VII. Public. Univ. Barcelona (I. Navarro, J. Gutiérrez y E. Capilla, eds.) ISBN: 974-84-475-3851-5, pp. 145-148.

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A mi hijo, Vicente Antai Padilla Medina

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Abbreviation list.

11-KT:	11-Ketotestosterone
4-OH-RA:	4-Hydroxy retinoic acid
4-oxo-RA	4-oxo-Retinoic acid
9cis-RA:	9cis retinoic acid
ADH:	Alcohol- dehydrogenase
AGN193109:	Retinoid analog
ALDH:	Aldehyde-dehydrogenase
APROMAR:	Asociación Empresarial de Acuicultura de España
atRA:	All-trans retinoic acid
BCMO1:	Beta-Carotene 15,15'-MonoOxygenase
BMS-204493:	RARα activity inhibitor
BPG:	Brain-pituitary-gonad axis
CF:	Correction factor
CRQ:	Correction factor relative quantity
cDNA:	Copy DNA
cRNA:	Copy RNA
DEAB:	Diethylaminobenzaldehyde
DEGs:	Differentially expressed genes
DEPC:	Diethyl pyrocarbonate
DNA:	Deoxyribonucleic acid
dNTP:	Nucleoside triphosphate
dpc:	Days post coitum
dpf:	days post fertilization
dph:	days post hatching
E2:	2 Estradiol
EIA:	Enzyme Immune Assay
FAO:	Food and Agriculture Organization (United Nations)
FSH:	Follicle-stimulating hormone
g:	Centrifuge force

G1:	Gap 1 phase of the cell cycle
G0:	G zero phase of the cell cycle
GnRH:	Gonadotropin releasing hormone
GTH-I:	Gonadotropin 1
GTH-II:	Gonadotropin 2
LH:	Luteinizing hormone
LRAT:	Lecytin-retinol transferase gene name
MAPK:	Mitogen-Activated Protein Kinases
MIS:	Meiosis inducing substance
MPS:	Meiosis preventing substance
mRNAs:	Messenger RNA
Mtons:	Million tons
NAD:	Nicotinamide adenine dinucleotide
NADP:	Nicotinamide adenine dinucleotide phosphate
NCBI:	National Center for Biotechnology Information
NRQ:	Normalized relative quantity
P:	Statistic P value
P:C:I:	Phenol: Chloroform: Isoamilalcochol
PAF:	Paraformaldehyde
PCA:	Principal Component Analysis
PCR:	Polymerase chain reaction
PGC:	Primordial germ cell
qPCR:	Quantitative real time polymerase chain reaction
RA:	Retinoic acid
RAL:	Retinaldehyde
RARE:	Retinoic acid response elements
RDH:	Retinol dehydrogenase
RE:	Retinil ester
RIN:	RNA integrity number
ROL:	Retinol

RQ:	Target gene relative quantity
rRNA:	Ribosomal RNA
SAC:	Spindle assembly checkpoint
SEM:	Standard error of the mean
SBR:	Sea bass ringer solution
SSC:	Saline-sodium citrate
tg:	Target gene
TGF:	Transforming growth factor
TTR:	Transthyretin
TGD:	Teleost genome duplication
UAB:	Autonomous University of Barcelona
UTR:	Untraslated region
WGD:	Whole genome duplication
Wnt:	Wingless/Integrated transduction pathway

Gene abbreviations and names mentioned in this thesis are described in each corresponding chapter.

Gene and protein nomenclature used in this thesis*

Species symbols	Nomenclature guideline
Human gene symbols	Designated by italicized upper-case Latin letters or numbers.
Human protein symbols	Designated by non italic uppercase letters or numbers.
Mouse and rat gene symbols	Begin with an uppercase letter, followed by all lowercase letters / numbers.
Mouse and rat protein symbols	Non italic uppercase letters or numbers.
Avian species gene symbols	Designated by italicized upper-case Latin letters or numbers
Avian species protein symbols	Designated by non italic upper-case letters or numbers.
Fish and amphibian genes	Gene symbols are three or more lowercase letters and are also italicized.
Fish and amphibian proteins	The protein symbol is the same as the gene symbol, but non-italic and the first letter is uppercase.

Examples		
Species	**Gene symbol**	**Protein symbol**
Human	*CYP26A1*	CYP26A1
Mouse	*Cyp26a1*	CYP26A1
Chicken	*CYP26A1*	CYP26A1
Sea bass or frog	*cyp26a1*	Cyp26a1

*Based on: HUGO Gene nomenclature committee (www.genenames.org), Mice genomics informatics (www.informatics.jax.org), Chicken gene nomenclature consortium (birdbase.org), Zebrafish model organism database (zfin.ogr), Gene nomenclature guidelines Xenbase (www.xenbase.org).

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ABSTRACT.

European sea bass (*Dicentrarchus labrax*) aquaculture is a thriving industry in Spain where it reached about 23,500 t and a market value of approximately 133 million euros. However, most of the sea bass farms produce a high percentage of males (70-90%) since the rearing temperature is increased during the initial stages of development to speed up development and growth. This is a problem because in this species, generally, females exhibit higher growth rates than males. This situation is aggravated as nearly 30% of these males reach puberty precociously during the first year of life, before attaining commercial size. Puberty is accompanied by a decrease in growth rates as energy diverts towards gonadal growth instead of somatic growth. In addition, it comes along with muscle waste and losses in the organoleptic properties of the meat. Because of these biological responses to captive breeding, there is a great need to understand the reproductive process and its control under intensive production conditions. The main objective of this thesis is to increase the knowledge of European sea bass reproduction. It is focussed on the role of the retinoic acid (RA) signaling pathway during early development and puberty.

Using a costume-made sea bass oligomicroarray we found several groups of genes that were differentially expressed in testis during the onset of male puberty. One of these groups included genes that belong to the RA signaling pathway. RA is the active form of vitamin A and is known to be essential for the onset of meiosis in tetrapods, although its role in fish is yet to be confirmed. These results prompted us to deepen on the possible role of the RA signaling pathway in gonad development and gametogenesis. An *in silico* analysis allowed us to describe for the first time the structure, phylogeny and evolutionary history of several genes and proteins involved in the synthesis and degradation of RA in the European sea bass. After an exhaustive histological study of gonad development, we could accurately identify specific stages of ovarian and testicular differentiation in this species. The expression of the main genes of the RA signaling pathway in specific stages of gonad development confirmed its role in the onset of puberty. Finally, an *in vitro* culture system of testicular explants from juvenile prepubertal fish and of testicular preparations from adult fish were set up to study the role of this pathway in the onset of puberty in sea bass males. The functional responses of genes related to RA transport, synthesis, degradation and receptor signaling were evaluated in the presence of stimulators and an inhibitors of the pathway and in different meiosis scenarios.

The results show that in the sea bass: a) the onset of meiosis coincides with an increase of 11KT levels and involves several pathways, including RA signaling; b) the enzymes related to RA synthesis and degradation have all the structural features to fulfil their specific functions; c) there is a well conserved evolutionary history of the enzymes involved in RA synthesis and degradation; d) the absence of *stra8*, a meiosis gatekeeper present in vertebrates and also in some fish, suggests that RA signaling in this species does not occur through the transduction of this particular gene; e) the genes involved in the RA signaling pathway are evolutionarily well conserved and play an important role in gonad development and gametogenesis; f) the expression dynamics of *cyp26a1* during gonad development makes it an excellent molecular marker for the onset of meiosis.

This thesis increases the understanding of the early molecular and endocrine events leading to puberty in the European sea bass by characterizing the RA signaling pathway. Moreover, it provides new questions and opens a novel research line to study the role of RA in puberty.

RESUMEN.

La acuicultura de la lubina Europea (*Dicentrarchus labrax*) es una industria pujante en España que alcanza unas 23.500 t y un valor de mercado cercano a 133 millones de euros. Sin embargo, la mayoría de las piscifactorías produce un elevado porcentaje de machos (70-90%) ya que, para acelerar el desarrollo y el crecimiento, se aumentan las temperaturas de cultivo durante las primeras etapas de vida. Esto supone un problema en la lubina ya que generalmente las hembras presentan mayores tasas de crecimiento que los machos y que el 30% de estos alcanza la pubertad precozmente durante el primer año de vida, antes de llegar a la talla comercial. Además, la pubertad está acompañada por una disminución de las tasas de crecimiento somático que implica un desgaste muscular y pérdidas en la calidad organoléptica de la carne. Debido a estas respuestas biológicas, existe una gran necesidad de comprender el proceso reproductivo y su control en condiciones intensivas de producción. El objetivo general de esta tesis es contribuir al conocimiento de la reproducción de la lubina europea, y se centra en el estudio de la ruta de señalización del ácido retinoico (RA) durante estados tempranos del desarrollo y la maduración sexual.

La utilización de un oligomicroarray de lubina, indicó la existencia de varios genes con expresión diferencial al inicio de la pubertad en machos. Entre ellos seleccionamos un grupo que incluía genes de la vía de señalización del RA, la forma activa de la vitamina A, fundamental para el inicio de la meiosis en tetrápodos. Un análisis *in silico* nos permitió describir por primera vez, la estructura, filogenia y la historia evolutiva de genes y proteínas implicados en la síntesis y degradación del RA. Tras un exhaustivo estudio histológico del desarrollo gonadal pudimos identificar con exactitud estados específicos de la diferenciación ovárica y testicular. El estudio de la expresión de los principales genes de la ruta de señalización del RA en estados específicos del desarrollo gonadal sirvió para demostrar su papel en el inicio de la pubertad. Finalmente, se puso a punto un sistema de cultivo *in vitro* de explantes testiculares de individuos prepúberes tanto juveniles como adultos para el estudio funcional y la determinación del papel de la ruta de señalización del RA en el inicio de la pubertad en machos. Con este fin se evaluó la respuesta funcional de los genes relacionados con el transporte, síntesis, degradación y señalización del RA ante la presencia de estimuladores e inhibidores de la vía y en diferentes escenarios de meiosis.

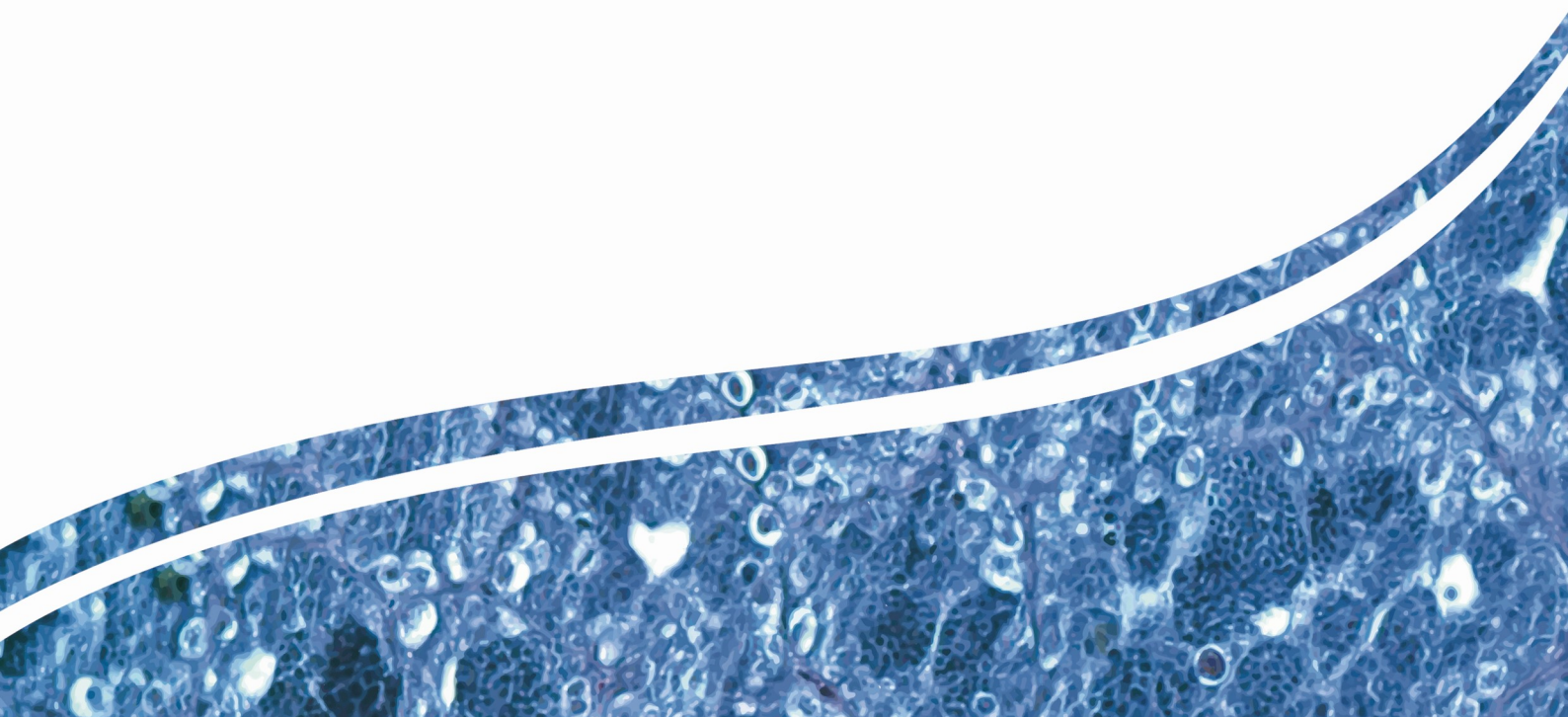
Los resultados de la tesis demuestran que en la lubina: a) el inicio de la meiosis coincide con un aumento en los niveles de 11KT y que involucra varias rutas, entre ellas la de señalización

del RA; b) las enzimas relacionadas con la síntesis y degradación del RA poseen todas las características estructurales para poder cumplir su función; c) existe una historia evolutiva bien conservada de las enzimas implicadas en la síntesis y degradación del RA, d) al igual que en otros teleósteos, la ausencia de *stra8*, marcador específico de meiosis en tetrápodos, sugiere que la señalización del RA en esta especie no ocurre a través de la transducción de este gen en particular; e) los genes implicados en la vía de señalización del RA están conservados, igual que en otros vertebrados, y juegan un papel relevante en el desarrollo gonadal y la gametogénesis, f) la dinámica de expresión del gen *cyp26a1* durante el desarrollo gonadal le convierte en un excelente marcador molecular del inicio de la meiosis.

Esta tesis contribuye al conocimiento del inicio de la pubertad en la lubina al caracterizar por primera vez la ruta de señalización del RA en esta especie y al proponer herramientas moleculares para su estudio. Además, aporta nuevas preguntas y abre una importante línea de investigación para el estudio del RA en la pubertad.

General Introduction.

The general introduction of this thesis is based in two theoretical frameworks; the first one describes the state of the art of the European sea bass reproduction research and the second one describes the state of the art of the Retinoic Acid signaling pathway in reproduction (Fig. 1). Thesis hypothesis and objectives are presented at the end of this section.



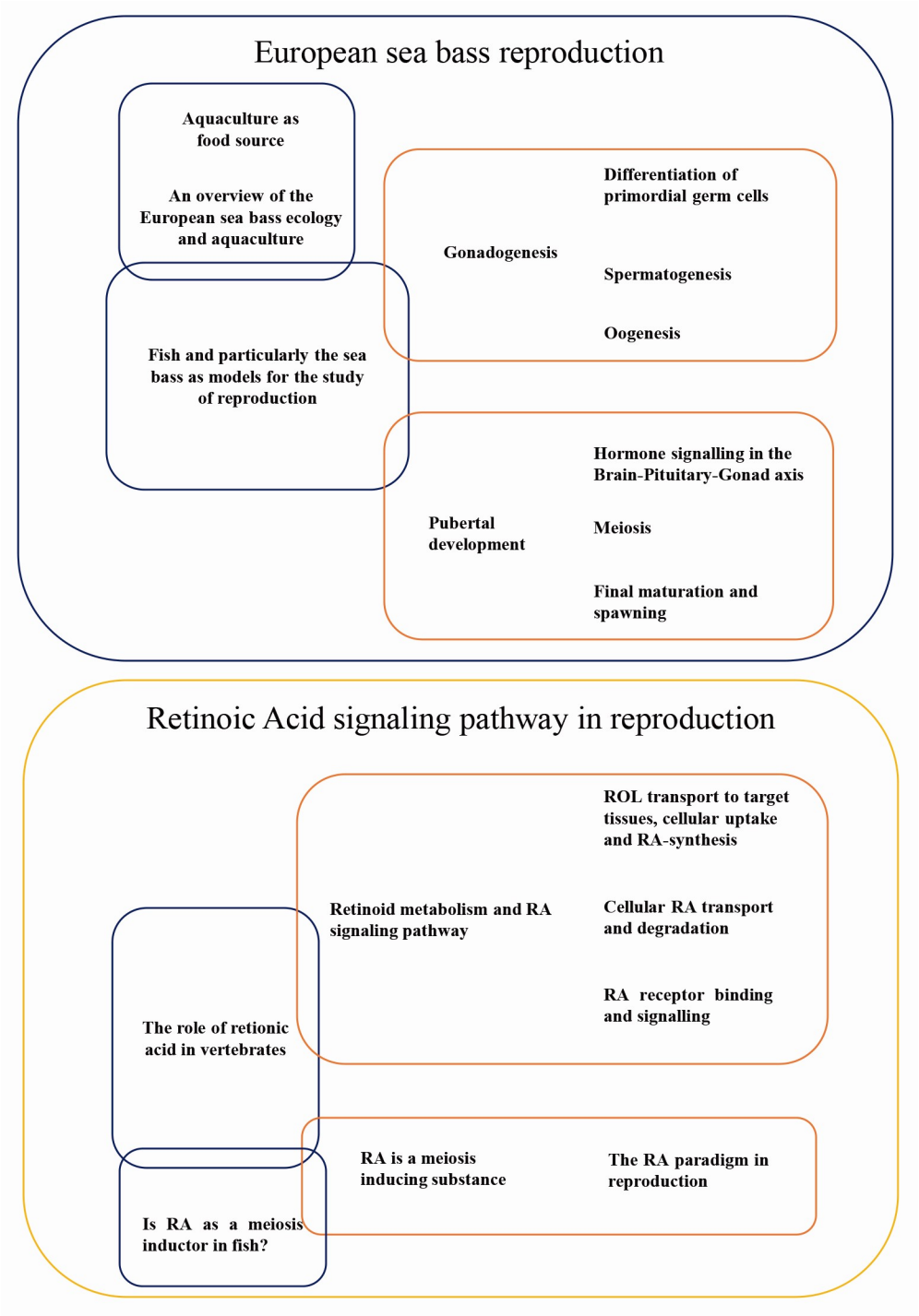


Figure 1: Graphical abstract of the theoretical framework preceding this study.

1.-Aquaculture as food source

Food industry is facing one of the most important challenges in human history. FAO estimates that, by 2050, food production will undergo a 70% increase from the total production of 2010 in order to meet the future nutritional needs of over 9,600 million people. To support this increase, traditional technologies will not be enough, since it will not only be a matter of food quantity, but food safety must be ensured as well, while protecting the environment and social welfare. This approach is part of the "blue growth" strategies that support sustainability in the marine and maritime sectors as a whole, and concerns fisheries, aquaculture, ecosystem services, trade and social protection of coastal communities (FAO, 2018). Fisheries and aquaculture are complementary activities that contribute to the world food production industry as the main sources of protein and polyunsaturated fatty acids. Fisheries focuses on catching, processing and selling aquatic organisms that belong to natural stocks, while aquaculture focuses on the enhancement of production yield by the intervention of at least one part of the productive cycle of aquatic organisms. The main difference between them is that aquaculture stocks are traceable and have individual or corporate ownership.

The success of modern aquaculture for a given species includes the control of its reproduction, an in-depth knowledge of its biology, technological innovations and the development of safe and quality food products (FAO 2018). According to the European commission of maritime affairs and fisheries (2014), in the EU alone, aquaculture had a value of 1,633 million euros and employed directly 90,464 people. This industry not only has a large potential for the development of healthy, safe, and competitive food products, but it also has the potential for the expansion of different areas such as environmental services related to the industry that range from water quality to residues management; energy production, like microalgae production of biodiesel (Mata et al., 2010); or natural new generation cosmetics, pharmaceuticals and biomaterials (Dan et al., 2012; Hasan et al., 2013). Even though aquaculture is the fastest increasing sector in food production, there is still a great deal of challenges and opportunities for further innovation and growth. Indeed, initiatives like Horizon 2020 programmes for research funding and innovation, attempt to maximize blue growth within a sustainable framework including the economic development, while ensuring social inclusion and environment protection. There is a need for research in the domestication of new species that include, nutrition and food development, growth enhancement, stress, health and disease control, reproduction and breeding programs (http://eshorizonte2020.cdti.es/index.asp?MP=87&MS=716&MN=2)

The latest APROMAR report (APROMAR 2018) indicates that world aquaculture production ascended to 110.2 million tons (mt) in 2016, with a value of over 194,778 million

euros and has surpassed global capture fisheries, that account for 93.7 mt. In 2016 Spain was the member state of the EU with the greatest aquaculture volume production, 283,831 t (22.0% of the EU total), followed by the United Kingdom with 194,492 t (15,0%) and France with 166,640 t

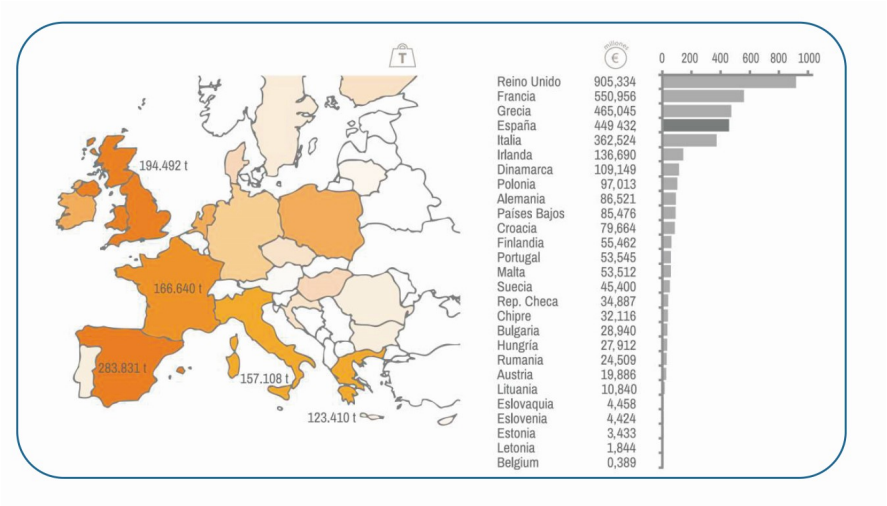


Figure 2: Aquaculture production values for the European Union. Source: APROMAR

(12.9%). In terms of production value however, Spain comes in fourth, with 449.5 million euros (12.1% of the total value) having the UK the highest production value of 905.3 million euros (24.3%) followed by France with 550.9 million euros (14.8%) and Greece with 465.0 million euros (12.5%) (Fig. 2). The main fin fish species in Spanish aquaculture were the sea bream (*Sparus aurata*), the rainbow trout (*Oncorhynchus mykiss*) and the European sea bass (*Dicentrarchus labrax*). This last one reached 23,445 tons and a production value of 132.9 million euros, although still additional imports from France, Italy and Greece are needed to meet the local demand (APROMAR, 2018). The successful control of reproduction is an important limiting factor for the maintenance of an organism in captivity. But, the effectiveness of this control requires a profound knowledge of the basic processes that regulate the onset and completion of the reproductive cycle.

2.- An overview of the European sea bass ecology and aquaculture

The European sea bass, *Dicentrarchus labrax*, is a teleost fish of the Order Perciforms that belongs to the Moronidae family (Fig. 3). The ecology of this species has been studied in detail by (Pickett and Pawson, 1994) and (Pérez-Ruzafa and Marcos, 2014) and is described as a eurythermic and euryhaline fish frequently found in coastal and estuarine zones of temperate waters. The thermal tolerance of this species ranges from 5 to 32ºC (Pérez-Ruzafa and Marcos, 2014) whereas the tolerance to salinity ranges from 0 to 60 ppt (Jensen et al., 1998). Its geographical distribution ranges from the English Channel (oriental Atlantic Ocean), to the Baltic Sea and from Norway to Morocco. Larvae are planktonic and at the juvenile stage they move to

estuaries and brackish waters and keep aggregated forming schools. At the adult stage, they become more gregarious, are demersal and appear usually distributed in shallow waters. In wild populations, the first sexual maturation occurs from two to four years of life and includes three to four spawning events in each annual breeding cycle during the winter season in Mediterranean populations, and can be extended up to June in Atlantic populations (FAO, 2012). One of the main problems of its production is associated with the reproductive process and the close interactions with somatic growth. Briefly, reproduction is accompanied by a decrease in growth rates as energy diverts towards gonadal growth instead of somatic growth (Hill et al., 2008). Furthermore, culture conditions can induce physiological changes that compromise reproductive success by diminishing fertility, egg quality, or even causing sterility in the most extreme instances (Carrillo et al., 2009). Most of the European sea bass farms produce extremely high percentages of males (70-90%) (Carrillo et al., 2010), since in order to speed up larval development and growth, the rearing temperature during early developmental stages, including the first 60 days of life is higher than the one fish experience in the wild (Piferrer et al., 2005). Indeed, it has been shown for different commercial strains that temperatures over 17ºC during larval rearing and nursing increase the number of males (Mylonas et al., 2005; Navarro-Martín et al., 2009a). In addition, the attainment of commercial size in fish farms (around 400-500 g) coincides with the onset of male puberty. Females, on the other hand, reach puberty at the third year of life and can grow bigger than males during the second year (Carrillo et al., 1995). These biological features represent a product quality concern, as puberty comes along with muscle waste, lower growth rates and losses in flesh quality (Carrillo et al., 1995; Carrillo, 2009; Taranger et al., 2010) resulting in a decreased market value. This is aggravated by the fact that nearly 30% of these males mature precociously before the first year of life, bringing up about 18% decrease in growth (Felip et al., 2008), while precociousness in females can occur during the second year of life, before reaching commercial size (Carrillo et al., 2015; Zanuy et al., 2001).

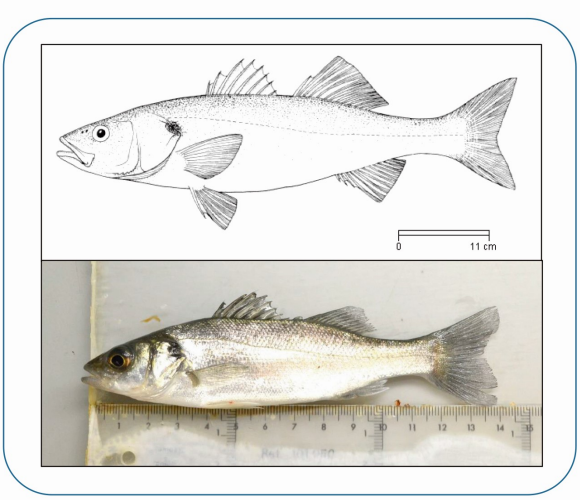


Figure 3. The European sea bass *Dicentrarchus labrax*. On top, an illustration of an adult individual (source: fishbase). At the bottom a photograph of a juvenile specimen.

As a consequence of these biological responses to captivity, it becomes of paramount importance to fully understand the reproductive process and its control under culture conditions,

in order to meet production needs without economic losses. Research topics such as sex determination, sex differentiation and manipulation of sex ratios, allow to set production protocols that enhance sex specific traits. For example, an all-female production of the European sea bass can show a greater growth scope than a mixed-sex culture. Moreover, the full knowledge of the onset of puberty might allow to set protocols to delay it in species such as the European sea bass or set the basis to hasten maturation in species that take many years to reach sexual maturity and are needed to mature fast (Okuzawa, 2002). In both scenarios the control of reproduction could lead to a reduction in production costs bringing about innovation in production processes.

Past research in European sea bass reproduction contains excellent examples of successful technological transfer to the aquaculture industry. The knowledge of circadian rhythms allowed for a continuous supply of eggs and larvae, in non-reproductive seasons, by the manipulation of photoperiod and temperature. Briefly, the sea bass presents a restricted spawning during three months during the winter season, with temperatures around 12ºC and 9-10 hours of light and 15-14 hours of darkness. If these conditions are delayed for three months in only four groups of fish, it would result in an all-year round spawning (Carrillo et al., 2009) (Fig. 4). This statement is the basis for aquaculture projections of total fish production volume, and used nowadays as industrial protocols.

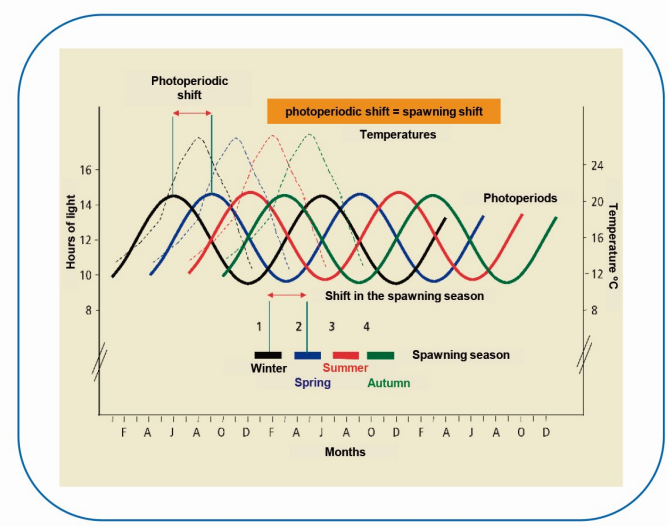


Figure 4. Four groups in correlative delays of photoperiod and temperature. Changes in the environmental factors proportionally shifts the reproductive season. Since the European sea bass spawning season lasts 3 months, a year round spawning is obtained (adapted from Carrillo et al., 2009).

Another example shows how basic research on the control of sex ratios resulted in a patented method to maximize the number of females in intensive culture conditions by rearing sea bass at low temperatures (between 13 and 17ºC) during the first 64 days of life (Navarro-Martín et al., 2009a; Piferrer et al., 2008). The technological transfer of this method allowed for a non-invasive, non-pharmacological industrial protocol, which uses temperature control in hatchery conditions to maximize the proportion of the sex with higher growth rates. Nowadays, this method is being used successfully in European sea bass farms in Spain and Turkey.

3.- Fish and particularly the sea bass as models for the study of reproduction

Fish are the most abundant class of vertebrates and have the longest evolutionary history. They feature diverse reproductive strategies including gonochorism, hermaphroditism, and unisexuality (Olivotto et al., 2017). This high plasticity allows some species to change phenotypic sex during life, a trait recognised as an evolutionary advantage to increase their reproductive success (Diotel et al., 2010), making fish an excellent model for the comparative study of reproduction. This diversity is governed by a highly flexible neuroendocrine system, influenced by environmental variables and seasonality that affect sex differentiation and sexual maturation (Blázquez et al., 1998). In this regard, temperature is considered as the main environmental factor altering sex differentiation in teleosts. This effect was first described in natural populations of the Atlantic silverside, *Menidia menidia*, (Conover and Kynard, 1987). It was later found in fish species reared in captivity for which high rearing temperatures, in general terms, had a masculinizing effect on sex ratios (Ospina-Alvarez and Piferrer 2008). Rearing density also affects sex differentiation in several fish species including the Siamese fighting fish, *Betta splendens*, (Eberhardt, 1943), the stickleback, *Gasterosteus aculeatus*, (Lindsey, 1962), the European eel, *Anguilla Anguilla*, (Degani and Kushnirov, 1992) or the zebrafish, *Danio rerio*, (Ribas et al 2017). Another example is the periodicity of these variables affecting puberty. In this regard, photoperiod acts in circannual endogenous rhythms controlling gating mechanisms or critical time windows for the onset of puberty that depend on the physiological state of the animal (Carrillo et al., 1994; Taranger et al., 2010). These features allow for the study of external factors that alter internal responses in reproduction. Indeed, common and divergent patterns between fish and tetrapods can contribute to the understanding of this process in other vertebrates.

The European sea bass, is a fully domesticated marine fish species capable to complete the reproductive cycle and spawn spontaneously in captivity with high fecundity and survival rates (Teletchea and Fontaine, 2014). Selective breeding programmes aimed at the improvement of growth, resistance to diseases and increase in product quality have also been developed for this species (Migaud et al., 2013) making it a good model for basic and applied studies of vertebrate reproduction. The European sea bass is a gonochoristic species, meaning that it exhibits separate sexes. As in most teleosts, no readily distinguishable sex chromosomes have been found in karyotype preparations in this species. It exhibits a polygenic sex determination system (Vandeputte et al., 2007) that can be influenced by the effect of the rearing temperature (Palaiokostas et al., 2015; Piferrer et al., 2005). This means that the final sex ratios in a population depend on both genetic inheritance and the thermal regime during a thermosensitive period located during larval development and early ontogenesis (Blázquez et al., 2009). Male-skewed

sex ratios in farmed populations can be obtained by increasing the temperature of the rearing water (Navarro-Martín et al., 2009a), or by adding androgens to the diet during early development (Blázquez et al., 2001; Blázquez et al., 1995; Blázquez and Saillant, 2019; Navarro-Martin et al., 2009b). Manipulation of sex determination by the induction of polyploidy, gynogenesis and androgenesis show that these technologies could be advantageous in the production of large sized fish and useful for the creation of genomic tools for this species, such as linkage maps and genome sequencing (reviewed in Felip and Piferrer, 2019).

In addition, a plethora of basic and applied studies on European sea bass reproduction support a theoretical framework for further research on this field (reviewed in Carrillo et al. 2015). Moreover, there has been quite an expansion on the generation of molecular tools thanks to the recent full genome sequencing (Tine et al., 2014). To mention a few, Alvarado et al. (2013) examines expression profiles of the kisspeptin system in the hypothalamus and pituitary of adult European sea bass during different gonadal stages, suggesting the key role of this neuroendocrine signal in the regulation of germ cell proliferation. Escobar et al. (2013) raised antibodies against the precursor of kisspeptins in order to map the kisspeptin system and correlate the expression of kisspeptins and their receptors. Crespo et al. (2013) set the goal to search for molecular markers of the early events preceding the onset of precocious puberty, that is, genes differentially expressed during precocious maturation. Zapater et al. (2018) described the localization of the three Esr subtypes in ovarian follicles and their regulation by gonadotropins. Finally, a species-specific microarray, has uncovered sex-specific transcriptomic profiles, identifying a number of differentially expressed genes in male and female gonads (Ribas et al., 2019).

Next, we present a theoretical framework based on the European sea bass reproduction, keeping in mind that there are still many issues to be addressed in order to understand and control the full reproduction process in culture conditions.

3.1.- Gonadogenesis

Gonadogenesis is the process responsible for the final formation of the gonad. It involves sex determination and sex differentiation (Blázquez et al., 2008; Devlin and Nagahama, 2002; Kobayashi et al., 2013; Olivotto et al., 2017). The plasticity of both mechanisms allows fish to exhibit a wide variety of reproductive strategies as a product of their life traits (De Mitcheson and Liu, 2008; Erisman et al., 2013).

Sex determination can be described as the genetic and environmental processes that result in the acquirement of the male or female sex of a given organism (Devlin and Nagahama, 2002). Initial studies in the European sea bass suggested that a XX/XY genetic sex determination system

could be operating in this species, based on the different pattern of distribution of heterochromatin in homologue chromosomes from pair 24 (Cano et al., 1996). It was later suggested, after analysing the results of the progeny from sex reversed males, that the most likely system could be a ZZ/ZW system (Blázquez et al., 1999). In parallel, it was shown that environmental factors, particularly temperature, was modulating and even overriding sex determination in this species (Blázquez et al., 1999; Blázquez et al., 1998). This laid the foundations for the presence of temperature-dependent sex determination (TSD) and was later followed by others leading to the same conclusion (Vandeputte and Piferrer., 2019 for references). Current knowledge reveals that European sea bass exhibits polygenic sex determination, with temperature influences (Piferrer et al., 2005a; Vandeputte et al., 2007; Vandeputte and Piferrer., 2019).

Sex differentiation occurs in fish during early development and consists on the transformation of an undifferentiated gonadal primordium into ovaries and testis (Devlin and Nagahama 2002). Several patterns of sex differentiation can be found in fish including gonochorism, hermaphroditism and unisexuality (Olivotto et al., 2017). Gonochorism is the most abundant and is characterized by the presence of separated sexes meaning that the undifferentiated gonadal primordium develops into an ovary or a testis and, during life, they only produce mature gametes of one of the sexes (Devlin and Nagahama, 2002; Olivotto et al., 2017). The European sea bass is a *differentiated gonochoristic* species. Moreover, sex differentiation is more dependent on length than on age (Blázquez and Saillant, 2019; Blázquez et al., 1998) and sex-related size differences are established before histological differentiation of the gonads (Koumoundouros et al., 2002; Papadaki et al., 2005; Saillant et al., 2002; Blázquez and Saillant, 2019). In addition, sex differentiation is influenced by environmental conditions (Vandeputte et al., 2007) and the temperature of the rearing water is confirmed as the main environmental cue responsible for the high male proportions found under intensive farming conditions (Blazquez et al., 1998; Díaz and Piferrer, 2015; Koumoundouros et al., 2002; Mylonas et al., 2005; Navarro-Martín et al., 2009a; Navarro-Martín et al., 2011; Pavlidis et al., 2000; Saillant et al., 2003; Sfakianakis et al., 2013). Moreover, exogenously administered sex steroids are capable to affect the normal course of sex differentiation in this species by acting through the cytochrome p450 aromatase (Piferrer and Blazquez, 2005). Experimental studies found high expression levels of *cyp19a1a*, the gene that codes for gonadal aromatase, by the onset of female differentiation as opposed to the low levels found during male differentiation, revealing this gene as a suitable molecular marker for early ovarian differentiation in this species (Blázquez et al., 2008). Indeed, an epigenetic mechanism involved in the methylation of the aromatase promoter seems to be responsible of the effect of the high rearing temperature in the modulation of sex differentiation in this species (Navarro-Martín

et al., 2011). Nevertheless, the conserved genetic, endocrine and environmental regulations, that influence sex determination and sex differentiation, are far from being fully understood.

3.2.- Differentiation of primordial germ cells

Primordial germ cell (PGC) can be defined as the undifferentiated stem cell capable to differentiate towards spermatozoa or oocytes. PGCs in the European sea bass can be identified by electron microscopy and through the expression of the *vasa* gene, a molecular marker implicated in the transduction of mRNAs involved in the differentiation of gonadal-specific cell types (Blázquez et al., 2011; Nishimura and Tanaka, 2014). Under culture conditions, gonads remain histologically undifferentiated during most of the first year of life (Roblin and Bruslé, 1983, Blázquez and Saillant, 2019). PGCs can be histologically identified as early as 25 days post fertilization (dpf), start migrating and colonizing the gonadal ridges at 35 dpf, and rapidly proliferate between 50 and 100 dpf (Roblin and Bruslé, 1983; Blázquez et al., 2011).

3.3.-Gametogenesis

Gametogenesis is a biological process that results in the formation of mature gametes, i.e., spermatozoa (spermatogenesis in males) and oocytes (oogenesis in females). Completion of the first full gametogenesis in a given organism results in the attainment of puberty. Gametogenesis involves functional and morphological changes towards cell specialization integrated by sex specific hormone signals that are, in turn, activated by environmental cues. Germ cells enter meiosis to form the gametes that transmit exactly half of the already recombined genetic information to the next generation. Sertoli and Leydig cells in testis, and follicle layers in ovaries give the structural support and produce paracrine factors that secure the maintenance and regulation of gametogenesis until the time of maturation. This process is repeated at each reproductive cycle and is sex specific.

3.3.1.- Spermatogenesis

After a specific number of self-renewal mitotic divisions, the European sea bass type A spermatogonia goes through mitotic divisions to form a short living type B spermatogonia. Type B spermatogonia enters the first meiotic division to form primary spermatocytes, also known as type I spermatocytes (preleptotene). After that, the second meiotic division gives rise to secondary spermatocytes (type II spermatocytes) with a haploid number of chromosomes. Later, during spermiation, secondary spermatocytes are transformed into spermatids that finally mature to form

the spermatozoids (Begtashi et al., 2004; Carrillo, 2009) (Fig. 5). For a detailed description of the different testicular stages in the European sea bass see Table 1.

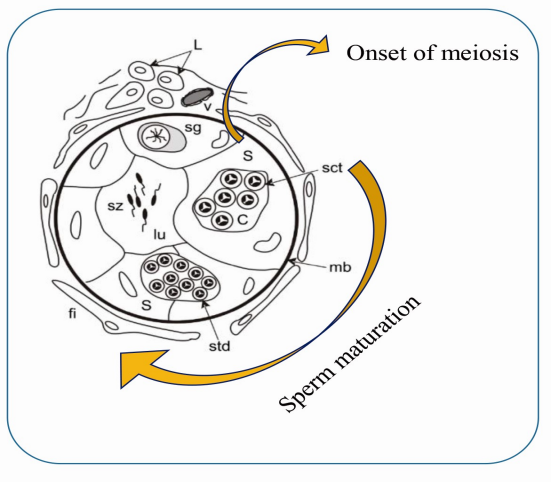


Figure 5. Schematic representation of fish spermatogenesis. Lobular testis organization during gonad development: basal membrane (mb), blood vessels (v), fibroblasts (fi) and the steroid producing Leydig cells (L). Cysts are formed by Sertoli cells (S) in close interaction with germ cells in different stages of development: spermatogonia (sg) transform into spermatocytes through meiosis, following through sperm maturation, specialitization into spermatids (std) and spermatozoa (sz) with no further proliferation. By the end of maturation, spermatozoa will remain in the lumen (lu) until spawning.

Table 1: Stages of maturation in European sea bass testis. Adapted from Begtashi et al., 2004.

Maturity stage	Morphological features
Stage I. Immature (Immature differentiated testis)	Enhanced mitotic proliferation of spermatogonia and formation of the seminiferous lobules. Cysts of spermatogonia are formed through meiosis from each type A spermatogonia.
Stage II. Early recrudescence (Early maturing testis)	Following a series of mitosis, the cysts become filled with late type A spermatogonia, distributed at the outer part of the lobule, which undergo the last mitotic division (type B spermatogonia) from which the preleptoneme spermatocytes will emerge, organized in cyst of primary spermatocytes and localized at the most internal part of the testis.
Stage III. Mid recrudescence (Early maturing testis)	Lobule size increases considerably. Abundant cysts containing primary and secondary spermatocytes are present which indicate that germ cells have entered the first meiotic division. Certain number of late type A spermatogonia and cysts of spermatids (second meiosis) are also observed.
Stage IV. Late recrudescence (Maturing testis)	All types of germ cells can be recognized and all germinal types of cells are observed. Spermatids engage into spermiogenesis and locate in the inner part of the lobule.
Stage V. Full spermiating testis (Maturing testis)	After spermiogenesis completion, the sperm is released into the seminiferous duct. Fish at this stage were considered as running
Stage VI. Post spawn (Regressed testis)	As spawn progresses, the lobules shrink and septa become greatly thickened. Residual spermatozoa remain for some weeks although spermatogenesis has ceased.

The name of the stages and cell type nomenclature used by Zanuy et al. (1999) and Rodriguez et. al (2001) appear between parenthesis.

3.3.2.- Oogenesis

As in other fish species, oogenesis in the European sea bass is organized in several stages including mitotic proliferation, primary growth, vitellogenesis and maturation (Mayer et al., 1988). Nevertheless, it is an artificial assignment of events, since oogenesis is a dynamic process that makes difficult to identify the beginning or the end of each step (Lubzens et al., 2010). The mitotic phase is characterized by the differentiation of PGCs into oogonia, which go through a species-specific number of self-renewal divisions before entering meiosis and form the primary oocytes. The central part of the teleosts ovary is a germinal epithelium that form lamellae in which the follicles develop and settle (Zanuy et al., 2009; Lubzens et al., 2010). Primary growth occurs during the formation of the ovarian follicle (Fig. 6 top) formed by the oocyte, the granulosa cells, the basement membrane, and the theca cells. Between the surface of the oocyte and the granulosa cells there is an acellular layer, the *zona radiata* or eggshell (Arukwe and Goksøyr, 2003). Theca cells provide steroid precursors, and granulosa cells produce steroidal mediators under the influence of Fsh and luteinizing hormone (Lh) (Senthilkumaran et al., 2004).

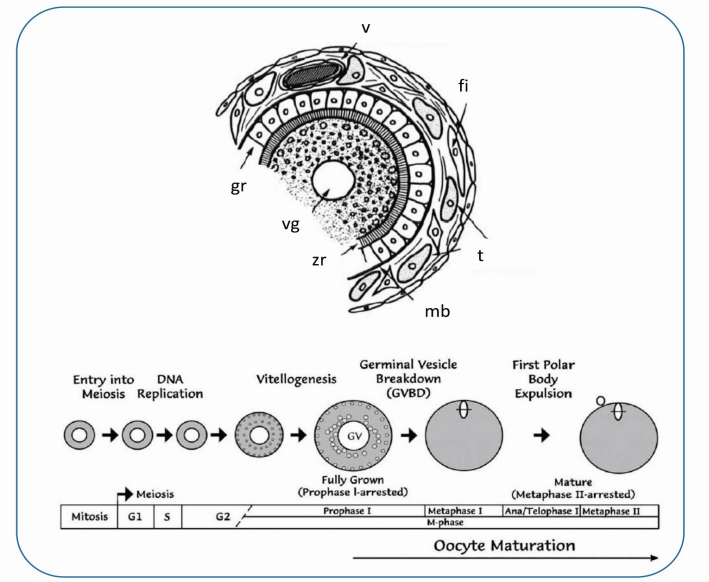


Figure 6: Top: Ovarian follicle in early vitellogenesis. Blood vessels (v), Fibroblast (fi), theca cells (t), Base membrane (mb), granulosa cells (gr), zona radiata (zr), germinal vesicle or nucleus (vg) granulosa cell (gr).

Bottom: Stages of fish oocyte development, meiosis events have a differential timing since meiosis is arrested twice during oocyte formation: at prophase I and at metaphase II. Meiosis is only completed at the time of fertilization. (Adapted from Nagahama, 1983 and Lubzens et al., 2010).

Once the follicles are formed, oogenesis progresses through secondary growth and vitellogenesis. The first lipid vesicles and cortical alveoli appear in the cytoplasm of primary oocytes. Meiosis starts but it is quickly arrested at the diplotene stage of prophase. This is followed by the uptake of vitellogenin, synthesized by the liver and its storage in the oocytes. This process is under gonadotropic control (see below section 3.4.1. Hormone signaling in the BPG axis), stimulating the synthesis of E_2 in follicular cells. Vitellogenesis results in a massive oocyte growth, during which nutritional reserves, as well as rRNAs and mRNAs, gather for

further use during fertilization and embryonic development. Meiosis is resumed after vitellogenesis but it is arrested again at metaphase II, until fertilization is completed. During this time, lipid vacuoles and yolk granules containing the vitellum coalesce, and the germinal vesicle migrates to the periphery of the oocyte, where germinal vesicle breakdown takes place. Completion of the first meiotic division gives rise to the first polar body, which degenerates, and to a large mature oocyte, ready to be ovulated, that largely increased in size through hydration. Ovulation is completed when the oocyte is released into the oviduct (Babin et al., 2007; Carrillo, 2009; Lubzens et al., 2010) (Fig. 6 bottom). For a detailed description of the different stages of ovarian development in the European sea bass refer to Table 2.

Table 2: Stages of maturity of the European sea bass ovary adapted from Mayer et al. (1988)

Stage of maturity	Morphological features
I Immature	Well-spaced ovigerous fold orientated towards the centre of the ovary, containing both oogonia (8-16μm) and primary oocytes at both the chromatin nucleolus (20-35μm) and perinucleolus stage (30-120μm); oogonia generally occur in nests of 4-8 cells in the stroma of the ovigerous folds
II Developing virgin or spent/recovering	Oogonia still present, both singly and in nests; spaces still appear between the ovigerous folds; primary oocytes at all stages are present, especially late perinucleolus stage oocytes (60-120μm); atretic follicles still present in spent/recovering.
III Developing (early)	Ovigerous folds fill the ovarian cavity; majority of primary oocytes at late perinucleolus stage (60-120μm); a number of the larger primary oocytes have started the secondary growth phase and show small lipid vesicles in the cytoplasm, i.e., lipid vesicle Stage I oocytes (110-160 μm); nucleus starts to appear convoluted.
IV Developing (late)	Oocytes at primary yolk granule stage (260-440μm) containing yolk granules accumulating in the outer cortex and with lipid vesicles starting to coalesce in the inner cortex; a prominent zona radiata is present; nucleus very convoluted.
V Gravid	Oocytes at the tertiary yolk granule stage (530-800μm) predominate; protein yolk granules spread throughout the cortex and large lipid vesicles (10-100μm) continue to coalesce and migrate centripetally; prominent zona radiata and follicle granulosa cells now appear cuboidal or columnar.
VI Running	Oocytes at both tertiary yolk granule stage (530-800μm) and hyaline stage (1100-1150μm)
VII Spent	Irregular convoluted ovigerous folds containing large atretic follicles and primary oocytes (peak period of oogenesis) at both the chromatin nucleolus (20-35μm) and perinucleolus stage (30-120μm)

Average oocyte diameter within parenthesis.

3.4.- Pubertal development

Pubertal development includes all the physiological processes by which an individual attains sexual competence for the first time and is capable to reproduce successfully, integrating life history traits, external stimuli, and internal cues (Ball and Wade, 2013; Okuzawa, 2002). As previously stated, it is not easy to assign a starting point to this developmental process, since it is not a consecutive turn of events, but a dynamic an interdependent process. Nevertheless, for definition purposes, we can consider that pubertal development starts after sex differentiation, when genetic, endocrine and environmental cues activate sex specific responses of germ cell maturation and somatic cell differentiation, and ends by the time the first gametogenic event (spermiation or ovulation) takes place and gametes are released for fertilization (Taranger et al., 2010). This process occurs every reproductive season with individuals engaging in repetitive cycles of PGCs proliferation, growth and differentiation (Mylonas et al., 2010).

In European sea bass males, meiosis occurs for the first time around the second year of life, and results in the attainment of puberty and the first gonad maturation (Begtashi et al., 2004). In females, meiosis starts during oogonia differentiation, followed by an arrest during oocyte growth, resumed back at maturity, only to arrest shortly and resume again by the time of fertilization (Lubzens et al., 2010; Mayer et al., 1988). The group-synchronous ovary type allows sea bass to have different populations of oocytes at different stages of development at the same time, and multiple ovulations could be possible in a matter of weeks during the spawning season (Asturiano et al., 2002).

Gonad maturation can only prosper if specific cues are available in appropriate environmental scenarios. As already stated, photoperiod and temperature regimes have been shown to modulate this process in wild and cultured European sea bass populations. These environmental conditions will ultimately affect puberty as they activate internal cues in the brain-pituitary-gonad (BPG) axis that trigger the initiation of the hormone signaling cascade that induce gametogenesis and final maturation.

3.4.1.- Hormone signaling in the Brain-Pituitary-Gonad axis

Sexual reproduction is a tightly regulated process that comprises different life stages, and is driven by a sequence of events occurring at a precise time along the BPG axis (Fig. 7). In the European sea bass, signaling occurs through consecutive and interdependent events that result in hormone synthesis. In the brain, kisspeptins activate the BPG axis stimulating the secretion of Gonadotropin Releasing Hormone (GnRH) from the GnRH neurons (Felip et al., 2009). Current evidence supports that kisspeptins are key elements that link environmental stimuli, steroid

feedback and metabolic factors (Tena-Sempere, 2006). In mammals, GnRH is secreted to the portal system and transported to the pituitary where it activates gonadotropin release. In teleosts, GnRH acts directly on the gonadotropic cells of the pituitary, where it activates gonadotropin release (Carrillo, 2009). Two major gonadotropins are produced and secreted in vertebrates; FSH, also known in fish as GtH-I, that stimulates gametogenesis (Okuzawa, 2002); and LH, known in fish as GTH-II, that participates in gonad maturation (Prat et al., 1996). The relative importance of these two hormones in pre-and early puberty in fish seems to vary within species (Okuzawa, 2002). In salmonids, Fsh regulates early spermatogenesis and vitellogenesis (Babin et al., 2007; Swanson et al., 1991). However, in the European sea bass, Fsh is in charge of germ cell proliferation, triggering spermatogenesis via androgen production, and regulating spermatogenesis-related genes (Mazón et al., 2014).

Gonadal sex steroids, i.e., androgens, estrogens and progestogens induce germ cells to enter meiosis. Their feedback interactions also determine different stages of gametogenesis and oogenesis (Fig. 7). Estrogens are products of androgen metabolism catalysed by the p450 aromatase enzyme complex, encoded by the *Cyp19* gene (Conley and Hinshelwood, 2001). In tetrapods, a single copy of *Cyp19* can be found in each haploid genome, while teleosts exhibit two different copies of this gene *cyp19a1a* and *cyp19a1b*. These genes encode two structurally different proteins, P450aromA and P450aromB, mainly expressed in gonads and brain, respectively and with similar catalytic properties (Piferrer and Blazquez, 2005). Enzyme activity of both aromatase isoforms is correlated with sex steroid levels, due to auto regulatory loops of estrogens and aromatizable androgens that in turn are controlled by aromatase gene expression levels (Blázquez et al., 2008; Diotel et al., 2010; Uno et al., 2012).

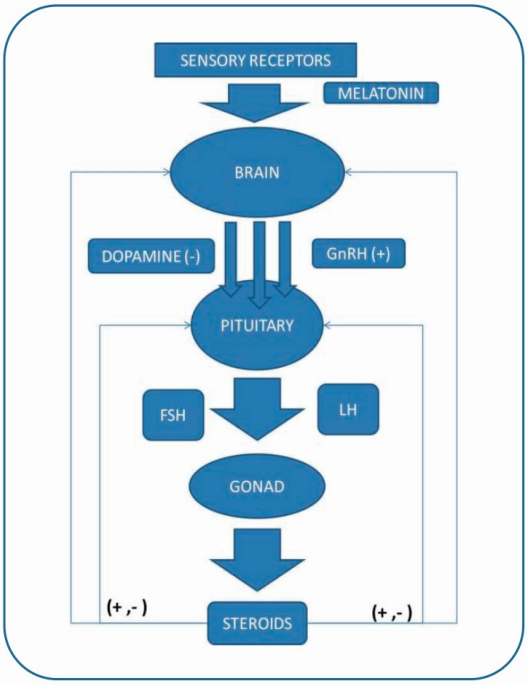


Figure 7. The brain-pituitary–gonad axis. Environmental variables stimulate melatonin secretion at hypothalamic level which, in turn, produces dopamine and Gonadotropin releasing hormone (GnRH). These hormones will stimulate, in a positive or negative feedback loop, the secretion of sex steroids that will regulate gametogenesis and final gonad maturation.

3.4.2.- Meiosis

The onset of meiosis can be considered as one of the events leading to puberty since it is only acquired when the whole process has been completed and the animal is sexually competent to reproduce for the first time. It is very likely that the mechanisms involved in the onset of puberty are similar to those involved in the onset of successive gonad maturation events because in fish, germ cells in males and females proliferate in each reproductive cycle as opposed to female mammals. During gametogenesis, diploid germ cells reduce by half their genetic material through meiosis resulting in the formation of the haploid gametes. It is a highly conserved process among eukaryotes, driven by a specialized cycle of DNA replication (reviewed in Ohkura, 2015).

Meiosis proceeds in two consecutive stages, each one consisting of four different phases (Fig.8). *Prophase I*, involves condensation, alignment and association of homologous chromosomes that allows for the recombination or "*crossing over*" of genetic material between non-sister chromatids (reviewed in Nasmyth, 2001). During *metaphase I*, homologous chromosomes line up perpendicular to the spindle and, at *anaphase I*, the homologous sets get pulled apart and move to opposite poles. Finally, at *telophase I* each chromosome set reaches its respective pole and the cell membrane closes giving rise to two independent cells. These newly formed cells are diploid, but their genetic material is not identical to that of the paternal cells due to the recombination of genetic material between homologous chromosomes during crossing over (Feng et al., 2014). Meiosis II enters a second round of cell division but with no further DNA replication. Finally, haploid gametes are formed; in the male four spermatozoa, and in the female only one functional oocyte and genetic material as polar bodies (Bowles and Koopman, 2007).

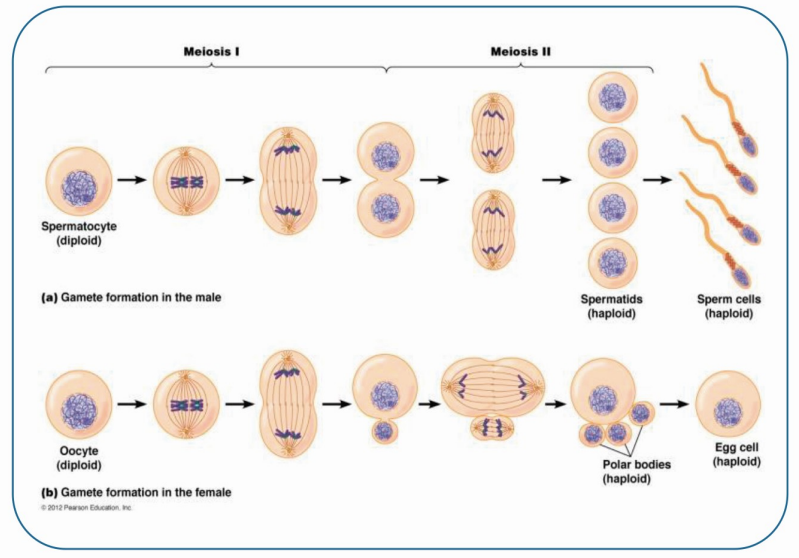


Figure 8. Schematic stages of meiosis progression during gametogenesis in males and females. A single germ cell can form four haploid spermatozoids or only one functional haploid oocyte.

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Meiosis is achieved through a highly coordinated transcription of numerous genes (Harigaya and Yamamoto, 2007), but the cues that cause germ cells to enter meiosis have a sex-specific origin and vary greatly among taxa. In mammals, specifically in male mice, primordial germ cells differentiate as T-prospermatogonia that remain arrested in the G1/G0 stage of the cell cycle (McLaren,1984; Mc Laren and Southee 1997). Meiosis is resumed at the onset of puberty, around a week after birth, and occurs continuously in adult stages (Swain, 2006). In female mice, germ cells enter meiotic prophase I and become oogonia at around 13.5 days post coitum. Before birth, oogonia enter a prolonged arrest stage, known as dictyate that lasts just before ovulation, time when the first meiotic division is completed. This gives way to the second meiotic division that remains arrested at metaphase II and is completed just after fertilization (Speed, 1982). In fish, differences in the timing of meiosis respond to different life traits. An interesting example can be found in male salmon for which the age of sexual maturation is influenced by genetic and environmental factors (Powers 1986). For example, Chinook salmon, *Oncorhynchus tshawytscha,* can attain sexual maturation within a wide time period that expands between 0 to 7 years of age, with meiosis arrested several months prior to final maturation (Shearer et al., 2006). During this period, fish are able to "evaluate" their developmental potential in terms of growth, size, and the level of energy stores or a combination of these factors. The result of this evaluation allows them to either continue the developmental pathway leading to maturity or to arrest maturation until the following season (Silverstein et al., 1998).

3.5.- Final maturation and spawning

In fish, the full competence of the BPG axis allows for the completion of gonad maturation and the appearance of secondary sex characteristics, behaviour, and reproductive fitness (Schulz and Goos, 1999). All these variables are integrated through highly efficient sensory systems that perceive environmental and social stimuli and translate them into physiological responses. This information modulates the synthesis of hypothalamic regulators and pituitary gonadotropins (Fsh and Lh) that in turn control gonad development and reproduction (Carrillo, 2009).

In European sea bass males, testosterone and 11KT reach their highest levels from December to March (reproductive season) (Prat et al., 1990; González and Piferrer, 2003), concomitant to an increase of the gonadosomatic index (Felip et al., 2008) and of spermiating fish (Prat et al., 1990). In females, seasonal variations of E_2 occur during vitellogenesis and peak at post vitellogenic stages, from December to January. High E_2 levels are needed to maintain oocyte viability until environmental conditions stimulate the surge of Lh, thus inducing final maturation (from January to March) (Prat et al., 1990; Mañanós et al., 1997; Asturiano et al., 2000; Asturiano

et al., 2002; González and Piferrer, 2003). In addition, high progestogen levels were found during postvitellogenesis and final maturation (Asturiano et al., 2002). Understanding the role of androgens, estrogens, and progestogens in the different stages of sexual development has made possible to induce gonad maturation and spawning, controlling the reproductive process in several cultured fish species (reviewed in Zohar and Mylonas, 2001).

Despite this solid framework in sea bass reproduction, there are still many issues to be addressed. Next, we set the theoretical basis for the study of the role of RA in sea bass reproduction.

4.- The role of retinoic acid (RA) in vertebrates

Vitamin A is not naturally synthesized and it can only be incorporated through the diet (Li et al., 2014). The main sources of vitamin A are in the form of retinol (ROL) or retinyl esters, and also as provitamin A, which are the carotenoids present in plants (Furr and Clark, 1997). After ingestion, both vitamin A and provitamin A are converted to retinal (RAL) and finally to RA, its active form (Chung and Wolgemuth, 2004). The final intracellular RA concentration is determined by its synthesis, degradation, transport and cellular uptake. Furthermore, intracellular RA levels regulate the timing and specificity of its signaling (Chambon, 1994; Ross et al., 2000), controlling the expression of several genes, coding for transcription factors, enzymes, structural proteins, cell surface receptors, neurotransmitters, neuropeptide hormones and growth factors (Albalat, 2009). RA exerts pleiotropic functions in a wide number of biological processes such as axial and regional patterning of the nervous system (McCaffery et al., 2003), embryogenesis (Reijntjes et al., 2004), body patterning (Marill et al., 2003), organ development (Campo-Paysaa et al., 2008), skeletal development (Spoorendonk et al., 2008), cell differentiation (Niederreither and Dolle, 2008), apoptosis of cancer cells (Liu et al., 1996), and alcohol metabolism (Molotkov and Duester, 2002). In reproduction, RA is known to play a fundamental role in the transition from mitosis to meiosis in mouse developing ovary, and to regulate meiotic progression in pubertal testis (Koubova et al., 2006). Moreover, the same substance that is in charge of RA degradation, cytochrome p450 enzyme CYP26, could act as a meiosis inhibiting factor in prepubertal testis (Bowles et al., 2006; Childs et al., 2011; Koubova et al., 2006). The RA signaling pathway is well conserved throughout evolution and the RA machinery is a common genetic feature among diverse lineages of metazoans and thus no longer considered a chordate innovation (Albalat, 2009). The role of the RA-signaling pathway in tetrapod reproduction has been demonstrated in several taxa and nowadays it is considered crucial in the onset of meiosis (Griswold et al., 2012). These studies show that RA is not only involved in SpgA differentiation

(Pellegrini et al., 2008), but it is also essential for meiosis onset (Griswold et al., 2012). However, studies of the role of RA on the control of spermatogenesis and oogenesis in fish are still scarce.

4.1.- Retinoid metabolism and RA signaling pathway

Vitamin A cannot be synthesized *de novo* and is acquired from the diet in the form of retinyl esters (RE), ROL, provitamin A and carotenoids (such as ß-carotene) (Furr and Clark, 1997; Li et al., 2014; Vieira, 1998). Dietary ß-carotenes are absorbed in the intestine and metabolized to form aggregates of lipoproteins, known as chylomicrons, for the secretion into the lymphatic system and transport to the adipose tissue and lipid rich organs such as the liver (Fierce et al., 2008). They are cleaved by ß-carotene-15,15`-monooxygenase (BCMO1) to produce two molecules of all-trans retinaldehyde (also known as retinal, RAL) (Fig. 9). This enzyme can cleave all-trans and 9-cis ß-carotene, yielding a 1:1 mixture of all-trans and 9-cis retinal (Furr and Clark, 1997). These molecules act as an intermediate between their respective ROL and RA isomers, since they can be reversibly converted into ROL, through the action of reductase enzymes (RDH11-14), or irreversibly oxidized into RA (Albalat, 2009). ROL can also be stored in cytosolic lipid droplets of specialized liver cells, as retinyl ester (RE), due to the action of lecithin- ROL transferase (LRAT). RE can be hydrolysed back to ROL by the action of retinyl ester hydrolases (REH) and released into the circulation, thus ensuring a constant supply to peripheral tissues under conditions where dietary retinoids are not available (Kumar et al., 2012; Li et al., 2014; Schreiber et al., 2012) (Fig. 10A).

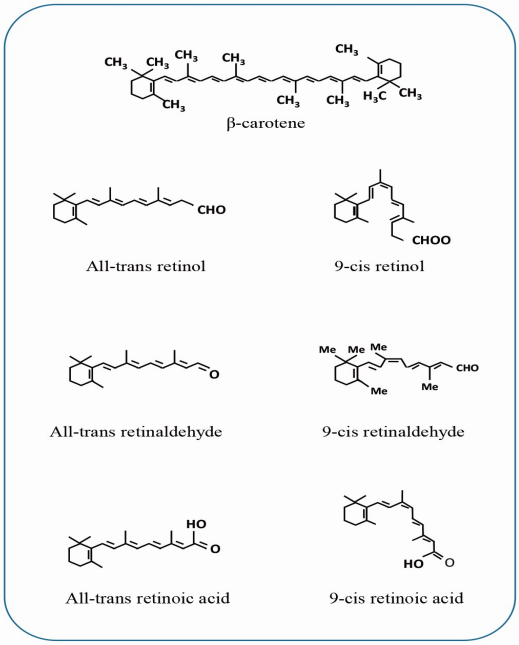

Figure 9. Chemical structure of all-trans and 9-cis isoforms of the retinoids involved in the RA signaling pathway. Modified form Gesto et al. (2012).

4.1.1.- ROL transport to target tissues, cellular uptake and RA synthesis

ROL is released into the blood system, bound to ROL-binding protein 4 (RBP4) forms a complex that in turn joins to transthyretin in a 1:1 ratio, which stabilizes it and reduces renal filtration through the glomeruli (Funkenstein, 2001). Within this complex, ROL is protected from enzymatic damage and oxidation during transport. The membrane receptor STRA6 (Stimulated

by Retinoic Acid protein 6) mediates ROL cellular intake and its transport through the membrane. STRA6 is involved in the catalytic cleavage of ROL from RBP4, resulting in ROL uptake, storage and cellular metabolism in retinoid dependent extra-hepatic tissues like skin, choroid plexus, placenta and testis (Amengual et al., 2014; Muenzner et al., 2013; Ruiz et al., 2012) (Fig. 10B). It is found in blood-organ barriers, such as the pigment epithelium of the retina and also in Sertoli cells (Schmitt and Ong, 1993). Within the cell, free ROL can bind to cellular retinoid binding protein (CRBP), thus making it soluble, physiologically available and protected from unspecific reactions (Kelly and von Lintig, 2015).

The first step in RA synthesis consists of an oxidation mediated by alcohol dehydrogenase that reversibly transforms ROL into RAL (Duester, 2001). Subsequently, aldehyde dehydrogenase enzymes (ALDHs) catalyze a non-reversible oxidation that transforms RAL in RA, making ALDHs the ultimate RA synthesis mediators (Duester, 2001) (Fig. 10C). ALDHs

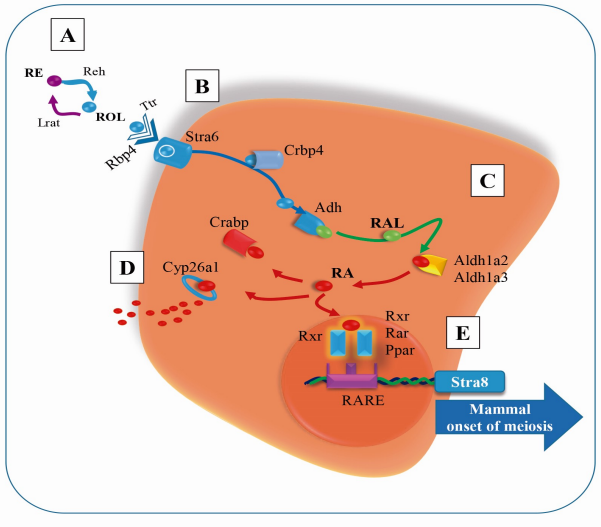

Figure 10. Schematic representation of the RA metabolic pathway key components in A) liver storage, B) transport, C) synthesis, D) degradation and E) RA signaling. Retinil ester (RE), Retinol (ROL), Retinil hydrolase (Reh), Lecytin-retinol transferase (Lrat), Transthyretin (Ttr), Retinol binding protein 4 (Rbp4), alcohol dehydrogenase (Adh), retinal (RAL), aldehyde dehydrogenase (Aldh), retinoic acid (RA), cellular retinoic acid binding protein (Crabp), Cytochrome P450 Family 26 (Cyp26), retinoid nuclear receptor (Rxr), retinoic acid nuclear receptor (Rar), peroxisome-proliferator activated nuclear receptor (Ppar), retinoic acid response elements (RARE), stimulated by retinoic acid protein 8 (Stra8).

exhibit a broad substrate specificity and are able to oxidize several highly reactive aliphatic and aromatic aldehydes derived from exogenous or endogenous precursors. Specifically, enzymes of the ALDH1a family present in the cytosol use all-trans retinal and 9-cis retinal as the main substrates to synthesize RA (Muzio et al., 2012). In tetrapods, there are three members of this family namely ALDH1a, ALDH1a2 and ALDH1a3 with a high specificity for RA synthesis, and are expressed in tissues with high retinoid content (Pittlik et al., 2008). In fish, however, Aldh1a1 is missing (Cañestro et al., 2009).

4.1.2.- Cellular RA transport and degradation

RA is highly unstable and toxic, therefore, once synthesized it remains bound to a cellular RA binding protein (CRABP) that modulates its steady-state concentration and thus, its bioavailability. It competes in affinity with RA nuclear receptors and, although the functions are

not fully understood, biochemical and genetic data evidence that the higher the level of CRABP in the cytoplasm, the less sensitive the cell is to a given external concentration of *all-trans* RA atRA (Gudas, 1994). This suggest that there is a RA binding/release dynamic that regulates the amount of free RA within the cell. It also seems to be in charge to deliver RA directly to nuclear receptors for signaling and thereby to alter cellular responses (Fiorella and Napoli, 1991; Napoli, 2012). The intracellular RA availability is also regulated by members of the CYP26 family in charge of its degradation and involved in the detoxification and metabolism of various substances (Uno et al., 2012). CYP26A1 and CYP26B1 use all-trans RA as specific substrate, whereas CYP26C1 has more affinity for 9-cis RA (Thatcher and Isoherranen, 2009). They transform RA into more polar compounds easier to eliminate from the cell such as 4-OH-RA and 4-oxo-RA (Ross and Zolfaghari, 2011) (Fig. 10D). The RA level may be determined indirectly by the balance between its synthesis and degradation mediated by ALDH and CYP26 family enzymes (Piprek et al., 2013; Sakai et al., 2001). Their expression dynamics can create an uneven RA distribution that direct organogenesis patterning (Duester, 2008) and, in the case of gonad development, they can inhibit the onset of meiosis in a sex specific manner (Koubova et al., 2006).

4.1.3.- RA receptor binding and signaling

Once RA is formed it can exert paracrine and autocrine actions. In the paracrine model, RA is generated in one cell and taken up by a target tissue, where it binds to its receptor inducing an effect, or to a non-target tissue where RA is metabolized (Duester, 2008). In the autocrine model, RA is synthesized, bound to its receptor and metabolized in the same cell (Napoli, 1996). Evidence of both models apply under specific circumstances during fetal development or adult life (Thatcher and Isoherranen, 2009).

The RA nuclear receptors (RARs), namely RAR and RXR, are ligand inducible transcriptional activators with three isoforms each; alpha, beta and gamma, encoded by separate genes that work as heterodimers forming multiple combinations. RARs are activated by atRA and 9cis-RA, whereas RXRs are activated only by 9cis-RA (Bastien and Rochette-Egly, 2004; Kurokawa et al., 1995). Moreover, RXRs are also heterodimers for other nuclear receptors, including; thyroid hormone receptor, vitamin D receptor and peroxisome proliferator-activated receptor (PPAR) which particularly, also has three isoforms (alpha, beta and gamma) (Niederreither and Dolle, 2008). The ligand of the RXR/PPAR heterodimer remains controversial, although a crosstalk between their specific ligands (RA and long chain fatty acids) has been proposed, evidencing an influence of retinoid signaling and lipid metabolism (Ziouzenkova and Plutzky, 2008).

Signaling occurs when RA binds to its nuclear receptor heterodimeric-couple and, in turn to specific binding site, known as retinoic acid response elements (RARE) (Ziouzenkova and Plutzky, 2008), that regulate the transcription of more than 500 genes (Balmer and Blomhoff, 2002) (Fig. 10E). In some cases, ligand free RAR- RXR complexes bind to RAREs and repress gene transcription using large proteins endowed with enzymatic activities, which maintain chromatin in a condensed and repressed state, until RA is available again (Weston et al., 2003). The roles of the RA signaling pathway's key components are summarized in Table 3.

Table 3: Key components of the RA signaling pathway in mice model

Protein name in mice model	Abbreviation	Function
Retinol Binding protein 4	RBP4 Formerly known as RBP	Retinol (ROL) bound to RBP4 is the main circulating retinoid (during fasting) (Blaner, 2007). Associated to transthyretin (TTR) seems to increase the ROL- RBP4 complex stability to prevent ROL loss during kidney filtration, and restricts free partitioning into the intercellular space outside the vascular system (Funkenstein, 2001). In murine systems, it is secreted from various tissues, mainly from ROL store organs including testis, specifically Sertoli cells (Davis and Ong, 1992). In oviparous animals it is thought to deliver retinol to the yolk (Funkenstein, 2001).
Stimulated by retinoic acid protein 6	STRA6	Member of the stimulated by retinoic acid group of proteins and a highly expressed transmembrane protein in blood-organ barriers (Bouillet et al., 1997). It acts as a pore which transports RA directionally between extra and intracellular RBPs (Kelly and von Lintig, 2015).
Aldehyde dehydrogenase family 1a2 and 1a3	ALDH1A2 and ALDH1a3 (also known as RALDH2 and RALDH3)	They are in charge of oxidizing aldehydes (specifically retinal), into their respective carboxylic acid forms, modulating several cell functions like: cell differentiation, proliferation, survival as well as response to oxidative stress (Muzio et al., 2012).
Cellular Retinoic acid binding protein II	CRABPII	Binds all-trans RA with lower affinity than CRABPI. Its transcription seems to be induced by RA and could act as a buffer to control spatiotemporally RA concentration, thus modifying the intracellular level of available RA bound to nuclear receptors signaling (Lampron et al., 1995).
Cytocrome p450 family 26a1	CYP26a1	Member of the P450 superfamily, catabolizes RA into polar metabolites such as 4-OH-RA, 18-OH-RA, and 5, 8-epoxy-RA (Uno et al., 2012). It plays important roles regulating pattern formation in embryogenesis (Reijntjes et al., 2004), and the development of embryonic germ cells. In mice it regulates the entry of male germ cell entry into meiosis (Li et al., 2009).
Retinoid nuclear receptor alpha	RXRα	Nuclear receptor for 9-*cis*-retinoic acid and a variety of dietary lipids like the docosahexaenoic acid (DHA), it is an ubiquitous partner for several members of nuclear receptors and an obligate partner for all-trans RA receptor, RAR α, ß and γ (Chawla et al., 2001).

Retinoic acid nuclear receptor alpha	RARα	Acting together to RXR α, ß and γ as an heterodimer, they recognize RA response elements, and signals the *Stra8* gene transcription in a ligand receptor manner (Feng et al., 2014).
Peroxisome proliferator-activated receptor gamma	PPARγ	Activated by poly unsaturated fatty acids and other arachidonic acid metabolites to promote fat storage (Chawla et al., 2001). Studies have shed new light on how retinoids are involved in ppar responses (Ziouzenkova and Plutzky, 2008), linking retinoid metabolism to fat storage.
Retinoic acid response elements	RARE	A pair of direct repeats within the regulatory DNA region of genes that are RA inducible
Stimulated by retinoic acid protein 8	STRA8	Recognized as a tetrapod meiosis gatekeeper, it is required for premeiotic DNA replication and the subsequent events of meiotic prophase, including chromosome condensation, cohesion, synapsis, and recombination (Anderson et al., 2008).

4.2.- RA is a meiosis inducing substance

For many years, a number of studies have reported the existence of active diffusible substances that regulate germ cell differentiation. These studies suggest that sex-differences in the timing of meiosis, particularly in mice, are induced by the presence of a meiosis inducing substance (MIS) in females, and a meiosis preventing substance (MPS) in males (McLaren, 2003).

A landmark study using mouse *in vitro* gonad explant cultures, showed that meiosis could be induced in morphologically undifferentiated male germ cells, after co-culturing them with adult ovaries that contained germ cells in meiotic prophase (Byskov and Saxén, 1976). The study suggested that an unknown substance, present in adult ovaries, was able to induce meiosis in testicular PCGs. However, meiotic germ cells in fetal ovary explants co cultured with fetal male whole testis explants could not reach the diplotene stage of meiotic prophase, suggesting that an unknown substance present in the testis could be inhibiting meiosis in ovaries (Byskov and Saxén, 1976). The study concluded that MIS was not a sex steroid, based on previous studies demonstrating that the administration of different sex steroids to developing mammalian gonads failed to induce meiosis (Young, 1961). The general hypothesis in the late1970's was that MIS and MPS might be chemically related, suggesting that in the differentiated testis, MIS could be catabolically inactivated and converted into MPS (Byskov, 1979).

An independent and different line of research starting during the late 1920's and early 1930's, showed a direct effect of vitamin A on rat reproduction as a result of the administration of vitamin A-deficient diets (Evans, 1928; Mason, 1933; Wolbach and Howe, 1925). Vitamin A deficiency resulted in gross systemic atrophy in rats, such as detachment and destruction of the epididymis in males, and cornification of the vaginal epithelium in females. Later studies showed

that ROL metabolism generates retinal in order to induce the synthesis of visual pigments, and that an additional irreversible oxidation to RA is involved in growth and tissue maintenance (Baxter, 1952). This simplified pathway was first reported by (Dowling and Wald, 1960) and it stands essentially unchanged nowadays (reviewed in Clagett-Dame and Knutson, 2011). During the 1990's (Van Pelt and De Rooij, 1991) demonstrated that exogenous RA was capable to induce spermatogonial proliferation and differentiation in vitamin A-deficient rats. Briefly, RA injections to rats fed with a vitamin A-deficient diet resulted in the full transition of spermatogonia to elongated spermatids, concluding that RA is the active form of vitamin A and is involved in spermatogenesis.

Only recently, research on meiosis and on the effect of vitamin A in reproduction found a common ground when it was demonstrated that RA was capable to activate the onset of meiosis, and that CYP26 was found to inhibit meiosis by degrading RA in both male and female gonads (Koubova et al., 2006). This has been confirmed in several animal models including mice (Bowles et al., 2006), birds (Smith et al., 2008), and amphibians (Wallacides et al., 2009). In humans, the influence of RA on meiosis could only be proven in fetal ovaries, while in fetal testis other mechanisms than that of cyp26-mediated catabolism of RA may be responsible for preventing germ cells from entering meiosis (Childs et al., 2011).

In fish, there are only a few studies dealing with the role of RA in meiosis and its relevance for reproduction. In the model species zebrafish, it was shown that RA levels were high in bipotent gonads, and therefore *cyp26* expression was low (Rodríguez-Marí et al., 2013). This is consistent with the early ovarian-phase (regardless of their final sex), typical of undifferentiated gonochoristic fish (Devlin and Nagahama, 2002; Olivotto et al., 2017), and common to all zebrafish specimens (Liew and Orbán, 2013; Wang and Orban, 2007). The study performed by Rodríguez-Marí et al. (2013) revealed sex dimorphic expression of *cyp26* during gonad differentiation, coinciding with final sex commitment. In early differentiating ovaries, by the onset of meiosis, RA levels where high and *cyp26* expression was low. However, at the time of oocyte development, coinciding with meiosis arrest, RA levels were low and *cyp26* expression was high. Conversely, in early differentiating testes, RA levels were low due to the upregulation of *cyp26* while an increase of RA levels, concomitant with a downregulation of *cyp26* occurred during meiosis progression by the time of spermatogenesis (Rodríguez-Marí et al., 2013). Studies in medaka, a primary gonochoristic (the undifferentiated gonad develops directly into an ovary or a testis), did not find evidence for the role of RA in the initiation of meiosis in embryos but reported sexually dimorphic expression of RA metabolizing enzymes, with a higher expression in ovaries from adult fish, suggesting an important role in the resumption of meiosis at the end of

oocyte maturation (Adolfi et al., 2016). Taken together, the importance of RA in the onset of meiosis differs among fish species and its role in reproduction is yet to be fully addressed.

4.3.- How does RA induce meiosis?

In mice, RA is able to induce meiosis in a paracrine manner, its metabolism takes place in somatic cells, while signaling occurs in premeiotic germ cells that respond by expressing the Stimulated by Retinoic Acid gene 8 (*Stra8*) in gonad somatic cells (Feng et al., 2014). RA acts as a ligand to the RAR-RXR heterodimer that binds to the RARE sequence upstream of *Stra8* gene (Bowles and Koopman, 2007) inducing its transcription. However, the variables involved in its regulation and how it functions in the initiation of meiosis, are unknown. RA could act on somatic cells, that send a secondary signal to induce meiosis, thus involving some intermediate transcription factor (Balmer and Blomhoff, 2002; Bowles and Koopman, 2007; Feng et al., 2014). *Stra8* encodes a glutamic acid-rich protein with no homology to other known proteins (Feng et al., 2014). It is required for premeiotic DNA replication, and induces the first round of meiosis in a sex specific timing, i.e., during embryonic development in females, and by the onset of puberty in male mice (Anderson et al., 2008; Koubova et al., 2006). Conversely, in *Stra8*-null mice, germ cells are unable to initiate meiosis (Baltus et al., 2006). This meiosis initiation model has been described for many tetrapods (Griswold et al., 2012), however, its implication in fish is only recently being addressed. It seems that *stra8* was lost from the teleost lineage, based on its absence on the zebrafish genome and other available reference teleost genomes (Rodríguez-Marí et al., 2013; Feng et al., 2015). However, these results have been challenged by the discovery of an *stra8* ortholog in several teleosts (Dong et al., 2013; Pasquier et al., 2016). The results suggest that two distinct pathways are present in the teleost lineage; a *stra8* independent and a *stra8* dependent one, both relying on RA to initiate meiosis (Feng et al., 2015; Li et al., 2016).

New evidence seems to point at another meiosis initiation pathway modulated by Rec8. Just as in S*tra8,* the *Rec8* gene displays RAR binding sites in the promoter region, thus supposedly activated by RA. REC8 is a cohesin, required for the completion of sister chromatid cohesion, proper synapsis and chiasmata formation and segregation. Its activation occurs in parallel and independently from the *Stra8* function and is conserved between the sexes (Koubova et al., 2014). Evidence shows that germ cells in STRA8 deficient fetal ovaries express REC8, nevertheless, it seems that STRA8 is not required for final mitotic division but it is for premeiotic DNA replication and prophase. Notwithstanding, REC8 is not required for premeiotic DNA replication and germ cells can progress into meiotic prophase without it (Baltus et al., 2006). The *rec8* gene is present in all sequenced genomes of teleost (Feng et al., 2015) rising new questions about the influence of RA in fish development and reproduction.

4.4.- The RA paradigm in reproduction

The prominent role of RA in reproduction has been demonstrated in several tetrapod taxa, and the case of RA as a meiosis inducing substance is reviewed in Griswold et al. (2012). The last decade of research on this topic has driven to major conclusions that nowadays establish a solid paradigm in development:

(i) The onset of meiosis is blocked by dietary retinoid deficiency

In male murine models that do not efficiently store RA, vitamin A deprivation decreased the expression of *Stra8* and caused meiotic failure, resulting in spermatogonic arrest, blocking the transition between undifferentiated to differentiated spermatogonia (Li et al., 2011). Once vitamin A, ROL or RA intake is restored in the diet, meiosis is resumed synchronously (Huang and Hembree, 1979). Large doses of RA alone can also resume meiosis, suggesting that RA and not its precursor is the active factor (Li et al., 2011; Van Pelt and De Rooij, 1991). Furthermore, in female mice, a vitamin A deficient diet resulted in germ cells failing to induce *Stra8* expression and enter meiosis, remaining undifferentiated. In addition, the administration of vitamin A moderately deficient diets resulted in 30% of the females starting meiosis when compared with 75% of females starting meiosis after administration of vitamin A complete diets (Li et al., 2009). These results show that there is a dose dependent requirement for RA to initiate meiosis.

(ii) RA induces the expression of the meiotic gatekeeper gene Stra8.

Several studies report an upregulation of *Stra8* in adult testis and in the fetal ovary before meiosis entry (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006; Trautmann et al., 2008). On the other hand, *Stra8*-knockout mice completed meiotic replication (s-phase of the cell cycle), but failed to complete prophase (first stage of meiosis), making *Stra8* a key player for the stable commitment of the meiotic cycle (Mark et al., 2008). Moreover, *Stra8* was identified as an RA responsive gene in embryonic carcinoma cell line P19 in which the *Stra8* expression increased within 2 hours of exposure to RA (Bouillet et al., 1995).

(iii) Meiosis is triggered by exogenous retinoids.

Treatment of embryonic rat ovarian explants with RA or RARα agonist induced meiosis entry (Livera et al., 2000). Exogenous RA stimulated meiosis in germ cells from embryonic testis by the upregulation of meiosis markers including *Stra8*, *Scp3*, or *Dmc1* (Bowles et al., 2006; Koubova et al., 2006; Trautmann et al., 2008). Exogenous RA can induce embryonic testis to enter meiosis, but it cannot restore meiosis in *Stra8*-null fetal ovaries (Koubova et al., 2006),

suggesting that the main role of RA is to induce *Stra8,* which condensate the chromatin for the progression of meiosis prophase.

(iv) Meiosis is triggered by endogenous retinoids.

Studies focussed on the effect of endogenous retinoids are commonly oriented to inhibit the action of CYP26B1, the major RA degradation enzyme in mammals. When using ketoconazole, a potent but nonspecific CYP inhibitor, the expression of *Stra8*, *Scp3* and *Dmc1* was upregulated (Koubova et al., 2006). The same conclusion was drawn when using R115866, a more specific CYP26 inhibitor (Snyder et al., 2010). However, ketoconazole and BMS204493, a RAR antagonist, were unable to induce meiosis in an *ex vivo* gonad culture system (Koubova et al., 2006). These studies demonstrate that the expression of *Stra8* and the entry into meiosis require RAR signaling and that high CYP26 levels are blocking meiosis.

(v) Meiosis is inhibited by RA receptor antagonists and stimulated by agonists.

RARs are expressed in germ cells in testis and ovary cultures (Bowles et al., 2006; Morita and Tilly, 1999). Synthetic RAR antagonists (BMS-204493, AGN193109) inhibit meiosis and the expression of *Stra8*, *Scp3*, and *Dmc1* in mouse ovary cultures. Furthermore, when cultured with RAR agonist it is able to stimulate *Stra8* expression through all RAR isotypes (Koubova et al., 2006).

(vi) Meiosis is blocked by ALDH inhibitors.

Bis-(dichloroacetyl)-diamines (BDAD) are well known spermatogenesis inhibitors. They act by inhibiting the biosynthesis of RA from ROL and block *Stra8* expression in embryonic, neonatal and adult mouse testes (Hogarth et al., 2011). Moreover, the inhibition of ALDH1a2 by BDAD, in rabbit testis, resulted in low RA levels, impaired spermatogenesis and infertility (Amory et al., 2011). Furthermore, another potent ALDH inhibitor, citral, blocks meiosis in mouse, and human fetal tissues (Le Bouffant et al., 2010; Pietruszko et al., 1999).

(vii) RA is present at the right place at the right time to drive meiosis.

RA was found in fetal mouse ovary, yet not in testis of a matching state, when using a transgenic mouse model that expressed beta-galactosidase under the control of an RA response element (RARE-hsplacZ). Although the mice reporter assay revealed possible sites of action for RA, it could not show where it was actually synthesized. A screening of different tissues revealed that the mesonephros, rather than the gonads, synthesizes RA (Bowles et al., 2006; Snyder et al., 2010). These results suggest that RA diffuses into the adjacent gonad to stimulate *Stra8*

expression and meiosis in germ cells. Histochemical studies showed that Cyp26B1 co-localized with STRA8 in germ cells exhibiting a non-uniform distribution. These results suggest that the uneven presence of RA in different subsets of spermatogonia is due to a non-uniform distribution of CYP26 enzymes, resulting in asynchronous spermatogenesis (Snyder et al., 2010).

(viii) RA stimulates meiosis in other non-mammalian models.

RA stimulates meiosis in several tetrapod models such as birds (Smith et al., 2008), amphibians (Wallacides et al., 2009) and in fish (Feng et al., 2015; Li et al., 2016). Moreover, components of the RA machinery, i.e. enzymes, binding proteins, and nuclear receptors have also been identified in several invertebrates although their role in reproduction has not been confirmed (Albalat, 2009).

(ix) There are other pathways required for meiosis that are activated by RA.

In mice, RA activates the transcription of *Rec8* to influence meiosis (Koubova et al., 2014). Furthermore, in fish models, a *stra8* independent signaling of RA that induces meiosis has been reported in tilapia (Feng et al., 2015).

Despite all the evidences derived from the previous conclusions, still many questions need to be answered. Research implicating other substances, rather than RA, as the sole meiosis inductor (Kumar et al., 2011), or the Stra8 as the only pathway that stimulates meiosis (Koubova et al., 2014) is a must. Moreover, what is the role of the RA signaling pathway in marine fish?. Is there an influence of the RA signaling pathway during gonad development and the onset of puberty in the European sea bass?

General hypothesis.

Mendelsohn et al. (1994) raised one fundamental question regarding retinoid signaling: how can a simple compound such as RA be capable to induce so many diverse and complex responses?. More than a century of research has contributed to the creation of a paradigm on the RA signaling pathway for tetrapod gonad development. Even though it has been challenged (Kumar and Thompson, 2012), a plethora of evidences support the hypothesis that RA is the ultimate meiosis inducing substance in tetrapods (Griswold et al., 2012). The conserved machinery involving RA, synthesis, transport, receptor signaling and degradation is also present during embryogenesis in invertebrates (Albalat, 2009) and during fish reproduction (Rodríguez-Marí et al., 2013), although its role in gametogenesis has not been proved. The hypothesis that drives this study is:

The retinoic acid machinery is conserved in the European sea bass and plays a role in gonad development.

Objectives.

Main objective.

The main objective of the present thesis is to contribute to the general knowledge of the European sea bass (*Dicentrarchus labrax*) reproduction by assessing the RA signaling pathway, known to be essential for the onset of meiosis in tetrapods, while searching for transcriptomic tools that could mark important events during gonad development, including sex differentiation and puberty in males and females.

Specific objectives

1.- To compare the European sea bass transcriptome in testis before and right at the start of puberty.

2.- To identify potential genes or pathways that could be used as molecular markers for puberty.

3.- To characterize the Aldh1a family in the European sea bass describing the protein moiety, phylogenic origin and evolutionary history.

4.- To characterize Cyp26a1 in the European sea bass describing the protein moiety, phylogenetic origin and evolutionary history.

5.- To assess gene expression levels of key components of the RA signaling pathway in different tissues.

6.- To study the expression patterns of key components of the RA signaling pathway in male and female gonads during sex differentiation and onset of puberty.

7.- To investigate *in vitro* functional responses of key components of the RA signaling pathway in European sea bass testis explants.

REFERENCE LIST

Adolfi, M. C., Herpin, A., Regensburger, M., Sacquegno, J., Waxman, J. S., and Schartl, M. (2016). Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. *Scientific reports* **6**, 34281.

Albalat, R. (2009). The retinoic acid machinery in invertebrates: ancestral elements and vertebrate innovations. *Mol Cell Endocrinol* **313**, 23-35.

Alvarado, M. V., Carrillo, M., and Felip, A. (2013). Expression of kisspeptins and their Receptors, gnrh-1/gnrhr-II-1a and gonadotropin genes in the brain of adult male and female European sea bass during different gonadal stages. *Gen Comp Endocrinol* **187**, 104-116.

Amengual, J., Zhang, N., Kemerer, M., Maeda, T., Palczewski, K., and von Lintig, J. (2014). STRA6 is critical for cellular vitamin A uptake and homeostasis. *Human Molecular Genetics*.

Amory, J. K., Muller, C. H., Shimshoni, J. A., Isoherranen, N., Paik, J., Moreb, J. S., Amory, D. W., Evanoff, R., Goldstein, A. S., and Griswold, M. D. (2011). Suppression of Spermatogenesis by Bisdichloroacetyldiamines Is Mediated by Inhibition of Testicular Retinoic Acid Biosynthesis. *Journal of Andrology* **32**, 111-119.

Anderson, E. L., Baltus, A. E., Roepers-Gajadien, H. L., Hassold, T. J., de Rooij, D. G., van Pelt, A. M., and Page, D. C. (2008). Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci U S A* **105**, 14976-80.

APROMAR. 2018. Acuicultura en España. 2018. Access through: http://www.apromar.es/sites/default/files/2018/APROMAR_Informe_ACUICULTURA_2018.pdf.

Arukwe, A., and Goksøyr, A. (2003). Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative Hepatology* **2**, 4.

Asturiano, J., Sorbera, L., Ramos, J., Kime, D., Carrilo, M., and Zanuy, S. (2000). Hormonal regulation of the European sea bass reproductive cycle: an individualized female approach. *Journal of Fish Biology* **56**, 1155-1172.

Asturiano, J. F., Sorbera, L. A., Ramos, J., Kime, D. E., Carrillo, M., and Zanuy, S. (2002). Group-synchronous ovarian development, spawning and spermiation in the European sea bass (Dicentrarchus labrax L.) could be regulated by shifts in gonadal steroidogenesis. *Scientia Marina* **66**, 273-282.

Babin, P. J., Cerdà, J., and Lubzens, E. (2007). "The fish oocyte: from basic studies to biotechnological applications," Springer.

Balmer, J. E., and Blomhoff, R. (2002). Gene expression regulation by retinoic acid. *J Lipid Res* **43**, 1773-808.

Baltus, A. E., Menke, D. B., Hu, Y.-C., Goodheart, M. L., Carpenter, A. E., de Rooij, D. G., and Page, D. C. (2006). In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet* **38**, 1430-1434.

Ball, G. F., and Wade, J. (2013). The value of comparative approaches to our understanding of puberty as illustrated by investigations in birds and reptiles. *Hormones and Behavior* **64**, 211-214.

Bastien, J., and Rochette-Egly, C. (2004). Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**, 1-16.

Baxter, J. G. (1952). Synthesis and Properties of Vitamin A and Some Related Compounds. *In* "Fortschritte der Chemie Organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products/Progrès Dans La Chimie Des Substances Organiques Naturelles" (L. Zechmeister, ed.), pp. 41-87. Springer Vienna, Vienna.

Begtashi, I., Rodríguez, L., Moles, G., Zanuy, S., and Carrillo, M. (2004). Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (Dicentrarchus labrax, L.). I. Morphological aspects. *Aquaculture* **241**, 539-559.

Billard, R., Fostier, A., Weil, C., and Breton, B. (1982). Endocrine control of spermatogenesis in teleost fish. Canadian Journal of Fisheries and Aquatic Sciences 39, 65-79.

Blázquez, M., Bosma, P. T., Fraser, E. J., Van Look, K. J. W., and Trudeau, V. L. (1998). Fish as models for the neuroendocrine regulation of reproduction and growth. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **119**, 345-364.

Blázquez, M., Carrillo, M., Zanuy, S., and Piferrer, F. (1999). Sex ratios in offspring of sex-reversed sea bass and the relationship between growth and phenotypic sex differentiation. *Journal of Fish Biology* **55**, 916-930.

Blázquez, M., Felip, A., Zanuy, S., Carrillo, M., and Piferrer, F. (2001). Critical period of androgen-inducible sex differentiation in a teleost fish, the European sea bass. *Journal of Fish Biology* **58**, 342-358.

Blázquez, M., González, A., Mylonas, C. C., and Piferrer, F. (2011). Cloning and sequence analysis of a vasa homolog in the European sea bass (Dicentrarchus labrax): Tissue distribution and mRNA expression levels during early development and sex differentiation. *Gen Comp Endocrinol* **170**, 322-333.

Blázquez, M., González, A., Papadaki, M., Mylonas, C., and Piferrer, F. (2008). Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (Dicentrarchus labrax). *Gen Comp Endocrinol* **158**, 95-101.

Blázquez, M., Navarro-Martín, L., and Piferrer, F. (2009). Expression profiles of sex differentiation-related genes during ontogenesis in the European sea bass acclimated to two different temperatures. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* **312**, 686-700.

Blázquez, M., Piferrer, F., Zanuy, S., Carrillo, M., and Donaldson, E. M. (1995). Development of sex control techniques for European sea bass (Dicentrarchus labrax L.) aquaculture: effects of dietary 17 α-methyltestosterone prior to sex differentiation. *Aquaculture* **135**, 329-342.

Blázquez, M., and Saillant, E. (2019). Morphological and endocrine aspects of sex differentiation in the European sea bass and implications for sex control in aquaculture. In: Sex Control in aquaculture. Vol I (H.P Wang, F. Piferrer, S.L. Chen, and Z.G.Shen, eds.). pp. 327-346.

Blázquez, M., Zanuy, S., Carrillo, M., and Piferrer, F. (1998). Effects of rearing temperature on sex differentiation in the European sea bass (Dicentrarchus labrax L.). *Journal of Experimental Zoology* **281**, 207-216.

Bouillet, P., Oulad-Abdelghani, M., Vicaire, S., Garnier, J.-M., Schuhbaur, B., Dollé, P., and Chambon, P. (1995). Efficient Cloning of cDNAs of Retinoic Acid-Responsive Genes in P19 Embryonal Carcinoma Cells and Characterization of a Novel Mouse Gene, Stra1 (Mouse LERK-2/Eplg2). *Dev Biol* **170**, 420-433.

Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M., and Rossant, J. (2006). Retinoid signaling determines germ cell fate in mice. *Science* **312**, 596 - 600.

Bowles, J., and Koopman, P. (2007). Retinoic acid, meiosis and germ cell fate in mammals. *Development* **134**, 3401-11.

Byskov, A. G. (1979). Regulation of meiosis in mammals. *In* "Annales de Biologie Animale Biochimie Biophysique", Vol. 19, pp. 1251-1261. EDP Sciences.

Byskov, A. G., and Saxén, L. (1976). Induction of meiosis in fetal mouse testis in vitro. *Dev Biol* **52**, 193-200.

Campo-Paysaa, F., Marletaz, F., Laudet, V., and Schubert, M. (2008). Retinoic acid signaling in development: tissue-specific functions and evolutionary origins. *Genesis* **46**, 640-56.

Cano, J., Pretel, A., Melendez, S., Garcia, F., Caputo, V., Fenocchio, A., and Bertollo, L. (1996). Determination of early stages of sex chromosome differentiation in the sea bass Dicentrarchus labrax L.(Pisces: Perciformes). *Cytobios (United Kingdom)*.

Cañestro, C., Catchen, J. M., Rodriguez-Mari, A., Yokoi, H., and Postlethwait, J. H. (2009). Consequences of lineage-specific gene loss on functional evolution of surviving paralogs: ALDH1A and retinoic acid signaling in vertebrate genomes. *PLoS Genet* **5**, e1000496.

Carrillo, M. (2009). La Reproducción de los peces: aspectos básicos y sus aplicaciones en acuicultura. *Cerebro y reproducción en peces. Bases Neurales y Neuroendocrinas. Ed. fundación Observatorio español para la Acuicultura, Madrid*.

Carrillo, M., Begtashi, I., Rodríguez, L., Marin, M. C., and Zanuy, S. (2010). Long photoperiod on sea cages delays timing of first spermiation and enhances growth in male European sea bass (Dicentrarchus labrax). *Aquaculture* **299**, 157-164.

Carrillo M, Zanuy S, Prat F, Cerda J, Ramos J, Mañanós E, Bromage NJ. Sea bass (*Dicentrarchus labrax*). In: Bromage NR, Roberts RJ (Ed.), Broodstock management and egg and larval quality. Blackwell Science Ltd, Oxford 1995; pp. 138-168.

Carrillo, M., Bromage, N., Zanuy, S., Serrano, R., and Prat, F. (1989). The effect of modifications in photoperiod on spawning time, ovarian development and egg quality in the sea bass (Dicentrarchus labrax L.). *Aquaculture* **81**, 351-365.

Carrillo, M., Espigares, F., Felip, A., Escobar, S., Molés, G., Rodríguez, R., Alvarado, M. V., Gómez, A., and Zanuy, S. (2015). Updating control of puberty in male European sea bass: A holistic approach. *Gen Comp Endocrinol* **221**, 42-53.

Carrillo, M., Zanuy, S., Felip, A., Bayarri, M. J., Moles, G., and Gomez, A. (2009). Hormonal and environmental control of puberty in perciform fish: the case of sea bass. *Ann N Y Acad Sci* **1163**, 49-59.

Carrillo, M., Zanuy, S., Prat, F., Cerda, J., Mananos, E., Bromage, N., Ramos, J., and Kah, O. (1994). Nutritional and Photoperiodic Effects On Hormonal Cycles and Quality of Spawning in Sea Bass (Diceatrarchus Labrax L.). *Netherlands Journal of Zoology* **45**, 204-209.

Clagett-Dame, M., and Knutson, D. (2011). Vitamin A in reproduction and development. *Nutrients* **3**, 385-428.

Conley, A., and Hinshelwood, M. (2001). Mammalian aromatases. *Reproduction* **121**, 685-695.

Conover, D. O., and Kynard, B. E. (1984). Field and laboratory observations of spawning periodicity and behavior of a northern population of the Atlantic silverside, Menidia menidia (Pisces: Atherinidae). *Environmental Biology of Fishes*, *11*(3), 161-171

Crespo, B., Gómez, A., Mazón, M. J., Carrillo, M., and Zanuy, S. (2013). Isolation and characterization of Ff1 and Gsdf family genes in European sea bass and identification of early gonadal markers of precocious puberty in males. *Gen Comp Endocrinol* **191**, 155-167.

Chambon, P. (1994). The retinoid signaling pathway: molecular and genetic analyses. *Seminars in Cell Biology* **5**, 115-125.

Chawla, A., Repa, J. J., Evans, R. M., and Mangelsdorf, D. J. (2001). Nuclear Receptors and Lipid Physiology: Opening the X-Files. Science 294, 1866-1870.

Childs, A. J., Cowan, G., Kinnell, H. L., Anderson, R. A., and Saunders, P. T. (2011). Retinoic Acid signaling and the control of meiotic entry in the human fetal gonad. *PLoS One* **6**, e20249.

Chung, S. S., and Wolgemuth, D. J. (2004). Role of retinoid signaling in the regulation of spermatogenesis. *Cytogenet Genome Res* **105**, 189-202.

Dan, Z., Qiu-kuan, W., ZHANG, H.-y., Yuan-ping, L., and ZHANG, L.-l. (2012). Moisture Absorption, Moisture Retention and Anti-oxidantive Activity of Crude Extract of Kelp (Laminaria japonica) and Sodium Alginate [J]. *Fisheries Science* **6**, 011.

De Mitcheson, Y. S., and Liu, M. (2008). Functional hermaphroditism in teleosts. *Fish and Fisheries* **9**, 1-43.

Degani, G., and Kushnirov, D. (1992). Effects of 17β-Estradiol and Grouping on Sex Determination of European Eels. *The Progressive Fish-Culturist* **54**, 88-91.

Devlin, R. H., and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191-364.

Díaz, N., and Piferrer, F. (2015). Lasting effects of early exposure to temperature on the gonadal transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination. *BMC Genomics* **16**, 679.

Diotel, N., Le Page, Y., Mouriec, K., Tong, S. K., Pellegrini, E., Vaillant, C., Anglade, I., Brion, F., Pakdel, F., Chung, B. C., and Kah, O. (2010). Aromatase in the brain of teleost fish: expression, regulation and putative functions. *Front Neuroendocrinol* **31**, 172-92.

Dong, R., Yang, S., Jiao, J., Wang, T., Shi, H., Zhou, L., Zhang, Y., and Wang, D. (2013). Characterization of Stra8 in Southern catfish (Silurus meridionalis): evidence for its role in meiotic initiation. *BMC Molecular Biology* **14**, 11.

Dowling, J. E., and Wald, G. (1960). The biological function of vitamin A acid. *Proceedings of the National Academy of Sciences* **46**, 587-608.

Duester, G. (2001). Genetic dissection of retinoid dehydrogenases. *Chemico-Biological Interactions* **130–132**, 469-480.

Duester, G. (2008). Retinoic Acid Synthesis and Signaling during Early Organogenesis. *Cell* **134**, 921-931.

Eberhardt, K. (1943). Geschlechtsbestimmung und -Differenzierung bei Betta Splendens Regan I. *Zeitschrift für Induktive Abstammungs- und Vererbungslehre* **81**, 363-373.

Erisman, B. E., Petersen, C. W., Hastings, P. A., and Warner, R. R. (2013). Phylogenetic perspectives on the evolution of functional hermaphroditism in teleost fishes. *Integrative and comparative biology* **53**, 736-754.

Escobar, S., Servili, A., Espigares, F., Gueguen, M.-M., Brocal, I., Felip, A., Gómez, A., Carrillo, M., Zanuy, S., and Kah, O. (2013). Expression of kisspeptins and kiss receptors suggests a large range of functions for kisspeptin systems in the brain of the European sea bass. *PLoS One* **8**, e70177.

European commision of maritime affairs and fisheries (2014). Safeguarding the future of our seas, generating new prosperity. Luxembourg: Publications Office of the European Union. Print.

Evans, H. M. (1928). The effects of inadequate vitamin A on the sexual physiology of the female. *Journal of Biological Chemistry* **77**, 651-654.

FAO. (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome. Licence: CC BY-NC-SA 3.0 IGO.

FAO. (2012). FAO fact sheet, fisheries and aquaculture in Europe nº57 august 2012.

Felip, A., Zanuy, S., and Carrillo, M. (2006). Comparative analysis of growth performance and sperm motility between precocious and non-precocious males in the European sea bass (Dicentrarchus labrax, L.). *Aquaculture* **256**, 570-578.

Felip, A., Zanuy, S., Muriach, B., Cerdá-Reverter, J. M., and Carrillo, M. (2008). Reduction of sexual maturation in male Dicentrarchus labrax by continuous light both before and during gametogenesis. *Aquaculture* **275**, 347-355.

Felip, A. and Piferrer, F. (2019). The Induction of Polyploidy, Gynogenesis, and Androgenesis in the European Sea Bass. In Sex Control in Aquaculture, Volume I, First Edition. Edited by Han-Ping Wang, Francesc Piferrer, Song-Lin Chen, and Zhi-Gang Shen.

Felip, A., Zanuy, S., Pineda, R., Pinilla, L., Carrillo, M., Tena-Sempere, M., and Gómez, A. (2009). Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. *Mol Cell Endocrinol* **312**, 61-71.

Feng, C.-W., Bowles, J., and Koopman, P. (2014). Control of mammalian germ cell entry into meiosis. *Mol Cell Endocrinol* **382**, 488-497.

Feng, R., Fang, L., Cheng, Y., He, X., Jiang, W., Dong, R., Shi, H., Jiang, D., Sun, L., and Wang, D. (2015). Retinoic acid homeostasis through aldh1a2 and cyp26a1 mediates meiotic entry in Nile tilapia (Oreochromis niloticus). *Scientific reports* **5**, 10131.

Fierce, Y., de Morais Vieira, M., Piantedosi, R., Wyss, A., Blaner, W. S., and Paik, J. (2008). In vitro and in vivo characterization of retinoid synthesis from β-carotene. *Archives of Biochemistry and Biophysics* **472**, 126-138.

Fiorella, P. D., and Napoli, J. (1991). Expression of cellular retinoic acid binding protein (CRABP) in Escherichia coli. Characterization and evidence that holo-CRABP is a substrate in retinoic acid metabolism. *Journal of Biological Chemistry* **266**, 16572-16579.

Funkenstein, B. (2001). Developmental expression, tissue distribution and hormonal regulation of fish (Sparus aurata) serum retinol-binding protein. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **129**, 613-622.

Furr, H. C., and Clark, R. M. (1997). Intestinal absorption and tissue distribution of carotenoids. *The Journal of nutritional biochemistry* **8**, 364-377.

González, A., and Piferrer, F. (2003). Aromatase activity in the European sea bass (Dicentrarchus labrax L.) brain. Distribution and changes in relation to age, sex, and the annual reproductive cycle. *Gen Comp Endocrinol* **132**, 223-230.

Griswold, M. D., Hogarth, C. A., Bowles, J., and Koopman, P. (2012). Initiating meiosis: the case for retinoic acid. *Biol Reprod* **86**, 35.

Gudas, L. J. (1994). Retinoids and vertebrate development. *Journal of Biological Chemistry* **269**, 15399-15402.

Handel, M. A., and Schimenti, J. C. (2010). Genetics of mammalian meiosis: regulation, dynamics and impact on fertility. *Nat Rev Genet* **11**, 124-136.

Harigaya, Y., and Yamamoto, M. (2007). Molecular mechanisms underlying the mitosis–meiosis decision. *Chromosome Research* **15**, 523-537.

Hasan, J., Crawford, R. J., and Ivanova, E. P. (2013). Antibacterial surfaces: the quest for a new generation of biomaterials. *Trends in Biotechnology* **31**, 295-304.

Hill, J. W., Elmquist, J. K., and Elias, C. F. (2008). Hypothalamic pathways linking energy balance and reproduction. *American Journal of Physiology-Endocrinology and Metabolism* **294**, E827-E832.

Hogarth, C. A., Evanoff, R., Snyder, E., Kent, T., Mitchell, D., Small, C., Amory, J. K., and Griswold, M. D. (2011). Suppression of Stra8 expression in the mouse gonad by WIN 18,446. *Biol Reprod* **84**, 957-965.

Huang, H. F. S., and Hembree, W. C. (1979). Spermatogenic response to vitamin A in vitamin A deficient rats. *Biol Reprod* **21**, 891-904.

Jensen, M. K., Madsen, S. S., and Kristiansen, K. (1998). Osmoregulation and salinity effects on the expression and activity of Na+, K+-ATPase in the gills of European sea bass, Dicentrarchus labrax(L.). *The Journal of experimental zoology* **282**, 290-300.

Kagawa, H., Young, G., Adachi, S., and Nagahama, Y. (1982). Estradiol-17β production in amago salmon (Oncorhynchus rhodurus) ovarian follicles: Role of the thecal and granulosa cells. *Gen Comp Endocrinol* **47**, 440-448.

Kelly, M., and von Lintig, J. (2015). STRA6: role in cellular retinol uptake and efflux. *Hepatobiliary Surgery and Nutrition* **4**, 229-242.

Kobayashi, Y., Nagahama, Y., and Nakamura, M. (2013). Diversity and Plasticity of Sex Determination and Differentiation in Fishes. *Sexual Development* **7**, 115-125.

Koubova, J., Hu, Y.-C., Bhattacharyya, T., Soh, Y. Q. S., Gill, M. E., Goodheart, M. L., Hogarth, C. A., Griswold, M. D., and Page, D. C. (2014). Retinoic Acid Activates Two Pathways Required for Meiosis in Mice. *PLoS Genet* **10**, e1004541.

Koubova, J., Menke, D., Zhou, Q., Capel, B., Griswold, M., and Page, D. (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci USA* **103**, 2474 - 9.

Koumoundouros, G., Pavlidis, M., Anezaki, L., Kokkari, C., Sterioti, A., Divanach, P., and Kentouri, M. (2002). Temperature sex determination in the European sea bass, Dicentrarchus labrax (L., 1758) (Teleostei, Perciformes, Moronidae): Critical sensitive ontogenetic phase. *Journal of Experimental Zoology* **292**, 573-579.

Kumar, R., and Thompson, E. B. (2012). Folding of the glucocorticoid receptor N-terminal transactivation function: dynamics and regulation. *Mol Cell Endocrinol* **348**, 450-6.

Kumar, S., Chatzi, C., Brade, T., Cunningham, T. J., Zhao, X., and Duester, G. (2011). Sex-specific timing of meiotic initiation is regulated by Cyp26b1 independent of retinoic acid signaling. *Nature communications* **2**, 151.

Kumar, S., Sandell, L. L., Trainor, P. A., Koentgen, F., and Duester, G. (2012). Alcohol and aldehyde dehydrogenases: Retinoid metabolic effects in mouse knockout models. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1821**, 198-205.

Kurokawa, R., Söderström, M., Hörleinf, A., Halachmi, S., Brown, M., Rosenfeld, M. G., and Glass, C. K. (1995). Polarity-specific acti retinoic acid receptors determined by a co-repressor. *Nature* **377**, 5.

Lampron, C., Rochette-Egly, C., Gorry, P., Dolle, P., Mark, M., Lufkin, T., LeMeur, M., and Chambon, P. (1995). Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal. Development 121, 539-548.

Le Bouffant, R., Guerquin, M. J., Duquenne, C., Frydman, N., Coffigny, H., Rouiller-Fabre, V., Frydman, R., Habert, R., and Livera, G. (2010). Meiosis initiation in the human ovary requires intrinsic retinoic acid synthesis. *Hum Reprod* **25**, 2579-90.

Li, H., MacLean, G., Cameron, D., Clagett-Dame, M., and Petkovich, M. (2009). Cyp26b1 expression in murine Sertoli cells is required to maintain male germ cells in an undifferentiated state during embryogenesis. *PLoS One* **4**, e7501.

Li, H., Palczewski, K., Baehr, W., and Clagett-Dame, M. (2011). Vitamin A deficiency results in meiotic failure and accumulation of undifferentiated spermatogonia in prepubertal mouse testis. *Biol Reprod* **84**, 336-341.

Li, M., Feng, R., Ma, H., Dong, R., Liu, Z., Jiang, W., Tao, W., and Wang, D. (2016). Retinoic acid triggers meiosis initiation via stra8-dependent pathway in Southern catfish, Silurus meridionalis. *Gen Comp Endocrinol* **232**, 191-198.

Li, Y., Wongsiriroj, N., and Blaner, W. S. (2014). The multifaceted nature of retinoid transport and metabolism. *Hepatobiliary Surgery and Nutrition* **3**, 126-139.

Liew, W. C., and Orbán, L. (2013). Zebrafish sex: a complicated affair. *Briefings in functional genomics* **13**, 172-187.

Lindsey, C. C. (1962). Experimental study of meristic variation in a population of threespine sticklebacks, *Gasterosteus aculeatus*. *Canadian Journal of Zoology* **40**, 271-312.

Liu, Y., Lee, M. O., Wang, H. G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X. (1996). Retinoic acid receptor beta mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Molecular and Cellular Biology* **16**, 1138-49.

Livera, G., Rouiller-Fabre, V., Valla, J., and Habert, R. (2000). Effects of retinoids on the meiosis in the fetal rat ovary in culture. *Mol Cell Endocrinol* **165**, 225-231.

Lubzens, E., Young, G., Bobe, J., and Cerdà, J. (2010). Oogenesis in teleosts: How fish eggs are formed. *Gen Comp Endocrinol* **165**, 367-389.

Maack, G., and Segner, H. (2003). Morphological development of the gonads in zebrafish. *Journal of Fish Biology* **62**, 895-906.

Marill, J., Idres, N., Capron, C. C., Nguyen, E., and Chabot, G. G. (2003). Retinoic Acid Metabolism and Mechanism of Action: A Review. *Current Drug Metabolism* **4**, 1-10.

Mark, M., Jacobs, H., Oulad-Abdelghani, M., Dennefeld, C., Féret, B., Vernet, N., Codreanu, C.-A., Chambon, P., and Ghyselinck, N. B. (2008). STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature chromosome condensation. *Journal of Cell Science* **121**, 3233-3242.

Mason, K. E. (1933). Differences in testis injury and repair after vitamin A-deficiency, vitamin E-deficiency, and inanition. *American Journal of Anatomy* **52**, 153-239.

Mata, T. M., Martins, A. A., and Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews* **14**, 217-232.

Mayer, I., Shackley, S. E., and Ryland, J. S. (1988). Aspects of the reproductive biology of the bass, Dicentrarchus labrax L. I. An histological and histochemical study of oocyte development. *Journal of Fish Biology* **33**, 609-622.

Mazón, M. J., Gómez, A., Yilmaz, O., Carrillo, M., and Zanuy, S. (2014). Administration of Follicle-Stimulating Hormone In Vivo Triggers Testicular Recrudescence of Juvenile European Sea Bass (Dicentrarchus labrax). *Biol Reprod* **90**, 6, 1-10.

McCaffery, P. J., Adams, J., Maden, M., and Rosa-Molinar, E. (2003). Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen. *European Journal of Neuroscience* **18**, 457-472.

McLaren, A. (1981). The fate of germ cells in the testis of fetal Sex-reversed mice. *Journal of Reproduction and Fertility* **61**, 461-467.

McLaren, A. (2003). Primordial germ cells in the mouse. *Dev Biol* **262**, 1 - 15.

McLaren, A., Mittwoch, U., and Josso, N. (1995). Germ cells and germ cell sex [and discussion]. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **350**, 229-233.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., and Mark, M. (1994). Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120**, 2749-2771.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M. P., and Carrillo, M. (2013). Gamete quality and broodstock management in temperate fish. *Reviews in Aquaculture* **5**, S194-S223.

Molotkov, A., and Duester, G. (2002). Retinol/Ethanol Drug Interaction during Acute Alcohol Intoxication in Mice Involves Inhibition of Retinol Metabolism to Retinoic Acid by Alcohol Dehydrogenase. *Journal of Biological Chemistry* **277**, 22553-22557.

Morita, Y., and Tilly, J. L. (1999). Segregation of retinoic acid effects on fetal ovarian germ cell mitosis versus apoptosis by requirement for new macromolecular synthesis 1. *Endocrinology* **140**, 2696-2703.

Muenzner, M., Tuvia, N., Deutschmann, C., Witte, N., Tolkachov, A., Valai, A., Henze, A., Sander, L. E., Raila, J., and Schupp, M. (2013). Retinol-Binding Protein 4 and Its Membrane Receptor STRA6 Control Adipogenesis by Regulating Cellular Retinoid Homeostasis and Retinoic Acid Receptor α Activity. *Molecular and Cellular Biology* **33**, 4068-4082.

Muzio, G., Maggiora, M., Paiuzzi, E., Oraldi, M., and Canuto, R. A. (2012). Aldehyde dehydrogenases and cell proliferation. *Free Radic Biol Med* **52**, 735-46.

Mylonas, C. C., Anezaki, L., Divanach, P., Zanuy, S., Piferrer, F., Ron, B., Peduel, A., Ben Atia, I., Gorshkov, S., and Tandler, A. (2005). Influence of rearing temperature during the larval and nursery periods on growth and sex differentiation in two Mediterranean strains of Dicentrarchus labrax. *Journal of Fish Biology* **67**, 652-668.

Mylonas, C. C., Fostier, A., and Zanuy, S. (2010). Broodstock management and hormonal manipulations of fish reproduction. *Gen Comp Endocrinol* **165**, 516-34.

Nagahama, Y. (2002). Endocrine regulation of gametogenesis in fish. *International Journal of Developmental Biology* **38**, 217-229.

Nagahama, Y. (1983). 6 The Functional Morphology of Teleost Gonads. Fish physiology 9, 223-275.

Nagahama, Y., and Yamashita, M. (1989). Mechanisms of synthesis and action of 17α, 20β-dihydroxy-4-pregnen-3-one, a teleost maturation-inducing substance. *Fish Physiol Biochem* **7**, 193-200.

Napoli, J. L. (1996). Retinoic acid biosynthesis and metabolism. *The FASEB Journal* **10**, 993-1001.

Napoli, J. L. (2012). Physiological insights into all-trans-retinoic acid biosynthesis. *Biochim Biophys Acta* **1821**, 152-67.

Nasmyth, K. (2001). Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. *Annual review of genetics* **35**, 673-745.

Navarro-Martín, L., Blázquez, M., Viñas, J., Joly, S., and Piferrer, F. (2009a). Balancing the effects of rearing at low temperature during early development on sex ratios, growth and maturation in the European sea bass (Dicentrarchus labrax).: Limitations and opportunities for the production of highly female-biased stocks. *Aquaculture* **296**, 347-358.

Navarro-Martin, L., Blazquez, M., and Piferrer, F. (2009b). Masculinization of the European sea bass (Dicentrarchus labrax) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. *Gen Comp Endocrinol* **160**, 3-11.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., and Piferrer, F. (2011). DNA Methylation of the Gonadal Aromatase (cyp19a) Promoter Is Involved in Temperature-Dependent Sex Ratio Shifts in the European Sea Bass. *PLoS Genet* **7**, e1002447.

Niederreither, K., and Dolle, P. (2008). Retinoic acid in development: towards an integrated view. *Nat Rev Genet* **9**, 541-53.

Nishimura, T., and Tanaka, M. (2014). Gonadal Development in Fish. *Sexual Development* **8**, 252-261.

Ohkura, H. (2015). Meiosis: an overview of key differences from mitosis. *Cold Spring Harbor perspectives in biology* **7**, a015859.

Okuzawa, K. (2002). Puberty in teleosts. *Fish Physiol Biochem* **26**, 31-41.

Olivotto, I., Leu, M., Blázquez, M.(2017). Life cycles in marine ornamental species-fishes as a case study. In: Calado, R., Olivotto, I., Oliver, M. P., & Holt, G. J. (Eds.). Marine ornamental species aquaculture. New Jersey: John Wiley & Sons.

Ospina-Álvarez, N., and Piferrer, F. (2008). Temperature-Dependent Sex Determination in Fish Revisited: Prevalence, a Single Sex Ratio Response Pattern, and Possible Effects of Climate Change. *PLoS One* **3**, e2837.

Palaiokostas, C., Bekaert, M., Taggart, J. B., Gharbi, K., McAndrew, B. J., Chatain, B., Penman, D. J., and Vandeputte, M. (2015). A new SNP-based vision of the genetics of sex determination in European sea bass (Dicentrarchus labrax). *Genetics Selection Evolution* **47**, 1.

Papadaki, M., Piferrer, F., Zanuy, S., Maingot, E., Divanach, P., and Mylonas, C. C. (2005). Growth, sex differentiation and gonad and plasma levels of sex steroids in male- and female-dominant populations of Dicentrarchus labrax obtained through repeated size grading. *Journal of Fish Biology* **66**, 938-956.

Pasquier, J., Cabau, C., Nguyen, T., Jouanno, E., Severac, D., Braasch, I., Journot, L., Pontarotti, P., Klopp, C., and Postlethwait, J. H. (2016). Gene evolution and gene expression after whole genome duplication in fish: the PhyloFish database. *BMC Genomics* **17**, 1.

Pavlidis, M., Koumoundouros, G., Sterioti, A., Somarakis, S., Divanach, P., and Kentouri, M. (2000). Evidence of temperature-dependent sex determination in the European sea bass (Dicentrarchus labrax L.). *Journal of Experimental Zoology* **287**, 225-232.

Pawson, M., Pickett, G., and Witthames, P. (2000). The influence of temperature on the onset of first maturity in sea bass. *Journal of Fish Biology* **56**, 319-327.

Pérez-Ruzafa, Á., and Marcos, C. (2014). Ecology and distribution of Dicentrarchus labrax (Linnaeus 1758). *Biology of European Sea Bass*, 1.

Pickett, G. D., and Pawson, M. G. (1994). "Sea Bass: Biology," Springer Science & Business Media.

Pietruszko, R., Abriola, D. P., Izaguirre, G., Kikonyogo, A., Dryjanski, M., and Ambroziak, W. (1999). Aldehyde Inhibitors of Aldehyde Dehydrogenases. *In* "Enzymology and Molecular Biology of Carbonyl Metabolism 7" (H. Weiner, E. Maser, D. W. Crabb and R. Lindahl, eds.), pp. 79-87. Springer US, Boston, MA.

Piferrer, F. (2013). Epigenetics of sex determination and gonadogenesis. *Developmental Dynamics* **242**, 360-370.

Piferrer, F., and Blazquez, M. (2005). Aromatase distribution and regulation in fish. *Fish Physiol Biochem* **31**, 215-26.

Piferrer, F., Blázquez, M., and Navarro-Martin, L. (2008). "Método para el control térmico de la proporción de sexos en la lubina."

Piferrer, F., Blazquez, M., Navarro, L., and Gonzalez, A. (2005). Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (Dicentrarchus labrax L.). *Gen Comp Endocrinol* **142**, 102-10.

Piferrer, F., and Guiguen, Y. (2008). Fish gonadogenesis. Part II: molecular biology and genomics of sex differentiation. *Reviews in Fisheries Science* **16**, 35-55.

Piprek, R. P., Pecio, A., Laskowska-Kaszub, K., Kloc, M., Kubiak, J. Z., and Szymura, J. M. (2013). Retinoic acid homeostasis regulates meiotic entry in developing anuran gonads and in Bidder's organ through Raldh2 and Cyp26b1 proteins. *Mech Dev* **130**, 613-627.

Pittlik, S., Domingues, S., Meyer, A., and Begemann, G. (2008). Expression of zebrafish aldh1a3 (raldh3) and absence of aldh1a1 in teleosts. *Gene Expr Patterns* **8**, 141-7.

Prat, F., Sumpter, J. P., and Tyler, C. R. (1996). Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductivecycle in male and female rainbow trout (Oncorhynchus mykiss). *Biol Reprod* **54**, 1375-1382.

Reijntjes, S., Gale, E., and Maden, M. (2004). Generating gradients of retinoic acid in the chick embryo: Cyp26C1 expression and a comparative analysis of the Cyp26 enzymes. *Developmental Dynamics* **230**, 509-517.

Ribas, L., Valdivieso, A., Díaz, N., & Piferrer, F. (2017). Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response. *Journal of Experimental Biology*, *220*(6), 1056-1064.

Ribas, L., Crespo, B., Sanchez-Baizan, N., Xavier, D., Kuhl, H., Rodriguez, J. M., Diaz, N., Boltana, S., MacKenzie, S., and Moran, F. (2019). Characterization of the European Sea Bass (Dicentrarchus labrax) Gonadal Transcriptome During Sexual Development. *Marine Biotechnology*, 1-15.

Roblin, C., and Bruslé, J. (1983). Ontogenèse gonadique et différenciation sexuelle du Loup Dicentrarchus labrax, en conditions d'élevage. *Reproduction Nutrition Développement* **23**, 115-127.

Rodríguez-Marí, A., Cañestro, C., BreMiller, R. A., Catchen, J. M., Yan, Y.-L., and Postlethwait, J. H. (2013). Retinoic Acid Metabolic Genes, Meiosis, and Gonadal Sex Differentiation in Zebrafish. *PLoS One* **8**, e73951.

Ross, A. C., and Zolfaghari, R. (2011). Cytochrome P450s in the regulation of cellular retinoic acid metabolism. *Annu Rev Nutr* **31**, 65-87.

Ross, S. A., McCaffery, P. J., Drager, U. C., and De Luca, L. M. (2000). Retinoids in Embryonal Development. *Physiological Reviews* **80**, 1021-1054.

Ruiz, A., Mark, M., Jacobs, H., Klopfenstein, M., Hu, J., Lloyd, M., Habib, S., Tosha, C., Radu, R. A., Ghyselinck, N. B., Nusinowitz, S., and Bok, D. (2012). Retinoid Content, Visual Responses, and Ocular Morphology Are Compromised in the Retinas of Mice Lacking the Retinol-Binding Protein Receptor, STRA6. *Investigative Ophthalmology & Visual Science* **53**, 3027-3039.

Saillant, E., Fostier, A., Haffray, P., Menu, B., Laureau, S., Thimonier, J., and Chatain, B. (2003). Effects of rearing density, size grading and parental factors on sex ratios of the sea bass (Dicentrarchus labrax L.) in intensive aquaculture. *Aquaculture* **221**, 183-206.

Saillant, E., Fostier, A., Haffray, P., Menu, B., Thimonier, J., and Chatain, B. (2002). Temperature effects and genotype-temperature interactions on sex determination in the European sea bass (Dicentrarchus labrax L.). *Journal of Experimental Zoology* **292**, 494-505.

Sakai, Y., Meno, C., Fujii, H., Nishino, J., Shiratori, H., Saijoh, Y., Rossant, J., and Hamada, H. (2001). The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo. *Genes & development* **15**, 213-225.

Schmitt, M. C., and Ong, D. E. (1993). Expression of cellular retinol-binding protein and lecithin-retinol acyltransferase in developing rat testis. *Biol Reprod* **49**, 972-979.

Schreiber, R., Taschler, U., Preiss-Landl, K., Wongsiriroj, N., Zimmermann, R., and Lass, A. (2012). Retinyl ester hydrolases and their roles in vitamin A homeostasis(). *Biochim Biophys Acta* **1821**, 113-123.

Schultz, D. R., Perez, N., Tan, C. K., Mendez, A. J., Capo, T. R., Snodgrass, D., Prince, E. D., and Serafy, J. E. (2005). Concurrent levels of 11-ketotestosterone in fish surface mucus, muscle tissue and blood. *Journal of Applied Ichthyology* **21**, 394-398.

Schulz, R. W., de Franca, L. R., Lareyre, J. J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R. H., and Miura, T. (2010). Spermatogenesis in fish. *Gen Comp Endocrinol* **165**, 390-411.

Schulz, R. W., and Goos, H. J. T. (1999). Puberty in male fish: concepts and recent developments with special reference to the African catfish (Clarias gariepinus). *Aquaculture* **177**, 5-12.

Senthilkumaran, B., Yoshikuni, M., and Nagahama, Y. (2004). A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. *Mol Cell Endocrinol* **215**, 11-18.

Sfakianakis, D. G., Papadakis, I. E., Papadaki, M., Sigelaki, I., and Mylonas, C. C. (2013). Influence of rearing temperature during early life on sex differentiation, haemal lordosis and subsequent growth during the whole production cycle in European sea bass Dicentrarchus labrax. *Aquaculture* **412**, 179-185.

Shearer, K., Parkins, P., Gadberry, B., Beckman, B., and Swanson, P. (2006). Effects of growth rate/body size and a low lipid diet on the incidence of early sexual maturation in juvenile male spring Chinook salmon (Oncorhynchus tshawytscha). *Aquaculture* **252**, 545-556.

Silverstein, J. T., Shearer, K. D., Dickhoff, W. W., and Plisetskaya, E. M. (1998). Effects of growth and fatness on sexual development of chinook salmon (Oncorhynchus tshawytscha) parr. *Canadian Journal of Fisheries and Aquatic Sciences* **55**, 2376-2382.

Smith, C., Roeszler, K., Bowles, J., Koopman, P., and Sinclair, A. (2008). Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. *BMC Developmental Biology* **8**, 85.

Snyder, E. M., Small, C., and Griswold, M. D. (2010). Retinoic Acid Availability Drives the Asynchronous Initiation of Spermatogonial Differentiation in the Mouse. *Biol Reprod* **83**, 783-790.

Speed, R. (1982). Meiosis in the foetal mouse ovary. *Chromosoma* **85**, 427-437.

Spoorendonk, K. M., Peterson-Maduro, J., Renn, J., Trowe, T., Kranenbarg, S., Winkler, C., and Schulte-Merker, S. (2008). Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. *Development* **135**, 3765-74.

Swain, A. (2006). Sex Determination: Time for Meiosis? The Gonad Decides. *Current Biology* **16**, R507-R509.

Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biol Reprod* **44**, 29-38.

Taranger, G. L., Carrillo, M., Schulz, R. W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F. A., Dufour, S., Karlsen, O., Norberg, B., Andersson, E., and Hansen, T. (2010). Control of puberty in farmed fish. *Gen Comp Endocrinol* **165**, 483-515.

Teletchea, F., and Fontaine, P. (2014). Levels of domestication in fish: implications for the sustainable future of aquaculture. *Fish and Fisheries* **15**, 181-195.

Tena-Sempere, M. (2006). GPR54 and kisspeptin in reproduction. *Human Reproduction Update* **12**, 631-639.

Thatcher, J. E., and Isoherranen, N. (2009). The role of CYP26 enzymes in retinoic acid clearance. *Expert Opin Drug Metab Toxicol* **5**, 875-86.

Tine, M., Kuhl, H., Gagnaire, P.-A., Louro, B., Desmarais, E., Martins, R. S. T., Hecht, J., Knaust, F., Belkhir, K., and Klages, S. (2014). European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nature communications* **5**.

Trautmann, E., Guerquin, M.-J., Duquenne, C., Lahaye, J.-B., Habert, R., and Livera, G. (2008). Retinoic acid prevents germ cell mitotic arrest in mouse fetal testes. *Cell cycle* **7**, 656-664.

Uno, T., Ishizuka, M., and Itakura, T. (2012). Cytochrome P450 (CYP) in fish. *Environ Toxicol Pharmacol* **34**, 1-13.

Van Pelt, A. M. M., and De Rooij, D. G. (1991). Retinoic Acid Is Able to Reinitiate Spermatogenesis in Vitamin A-Deficient Rats and High Replicate Doses Support the Full Development of Spermatogenic Cells. *Endocrinology* **128**, 697-704.

Vandeputte, M., Dupont-Nivet, M., Chavanne, H., and Chatain, B. (2007). A Polygenic Hypothesis for Sex Determination in the European Sea Bass Dicentrarchus labrax. *Genetics* **176**, 1049-1057.

Vandeputte, M., Piferrer, F. (2019). Genetic and environmental components of sex determination in the European sea bass. In: Sex Control in aquaculture, Vol I (H.P Wang, F. Piferrer, S.L. Chen, and Z.G.Shen, eds.). pp. 307-325.

Vieira, A. V. (1998). Retinoid Endocrinology. *In* "Fat-Soluble Vitamins" (P. J. Quinn and V. E. Kagan, eds.), pp. 29-51. Springer US, Boston, MA.

Wallacides, A., Chesnel, A., Chardard, D., Flament, S., and Dumond, H. (2009). Evidence for a conserved role of retinoic acid in urodele amphibian meiosis onset. *Dev Dyn* **238**, 1389-98.

Wang, X. G., and Orban, L. (2007). Anti-Mullerian hormone and 11 beta-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. *Dev Dyn* **236**, 1329-38.

Weston, A. D., Blumberg, B., and Underhill, T. M. (2003). Active repression by unliganded retinoid receptors in development: less is sometimes more. *The Journal of Cell Biology* **161**, 223-228.

Wolbach, S. B., and Howe, P. R. (1925). Tissue changes following deprivation of fat-soluble a vitamin. *The Journal of Experimental Medicine* **42**, 753-777.

Young, W. C. (1961). The hormones and mating behavior. Sex and internal secretions, 2, 1173-1239.

Zanuy, S., Carrillo, M., Felip, A., Rodríguez, L., Blázquez, M., Ramos, J., and Piferrer, F. (2001). Genetic, hormonal and environmental approaches for the control of reproduction in the European sea bass (Dicentrarchus labrax L.). *Aquaculture* **202**, 187-203.

Zanuy, S., Carrillo, M., Rocha, A., & Molés, G. (2009). II Regulación y control hormonal del proceso reproductor de los teleósteos. La reproducción de los peces: aspectos básicos y sus aplicaciones en acuicultura. OESA, CSIC, MMAMRN, Madrid, España, 97-172.

Zapater, C., Molés, G., Muñoz, I., Pinto, P. I., Canario, A. V., and Gómez, A. (2018). Differential involvement of the three nuclear estrogen receptors during oogenesis in European sea bass (Dicentrarchus labrax). *Biol Reprod* **100**, 757-772.

Ziouzenkova, O., and Plutzky, J. (2008). Retinoid metabolism and nuclear receptor responses: New insights into coordinated regulation of the PPAR-RXR complex. *FEBS Lett* **582**, 32-8.

Zohar, Y., and Mylonas, C. C. (2001). Endocrine manipulations of spawning in cultured fish: from hormones to genes. *Aquaculture* **197**, 99-136.

Chapter 1.

The aim of this chapter was to compare the European sea bass testicular transcriptome before and right at the start of puberty in order to identify potential genes or pathways that could be used as molecular markers for this key reproductive developmental process (Fig. 1).

Testis samples in two different stages of development were chosen based on a histological study and a biochemical analysis of sex steroids in plasma. Samples were classified as: early differentiated (stage I) or as early recrudescence (stage II). The mRNA was extracted, purified and hybridized to a costume made oligonucleotide high-density European sea bass microarray to analyse differentially expressed genes (DEGs). Subsequently, gene names, synonyms and gene functions were assigned to each DEG.

Microarray hybridizations were validated through quantitative real time polymerase chain reaction (qPCR) comparing the expression of 14 genes with different functions in samples from individual testis at stage I and samples at stage II. A qualitative tissue-distribution analysis of these DEGs and other possible additional markers was also included in the study. Finally, preliminary trials for the localization of the expressing sites of some of these molecular markers, including cyp26a1 and rxrα, were conducted in testicular samples and assessed by in situ hybridization.

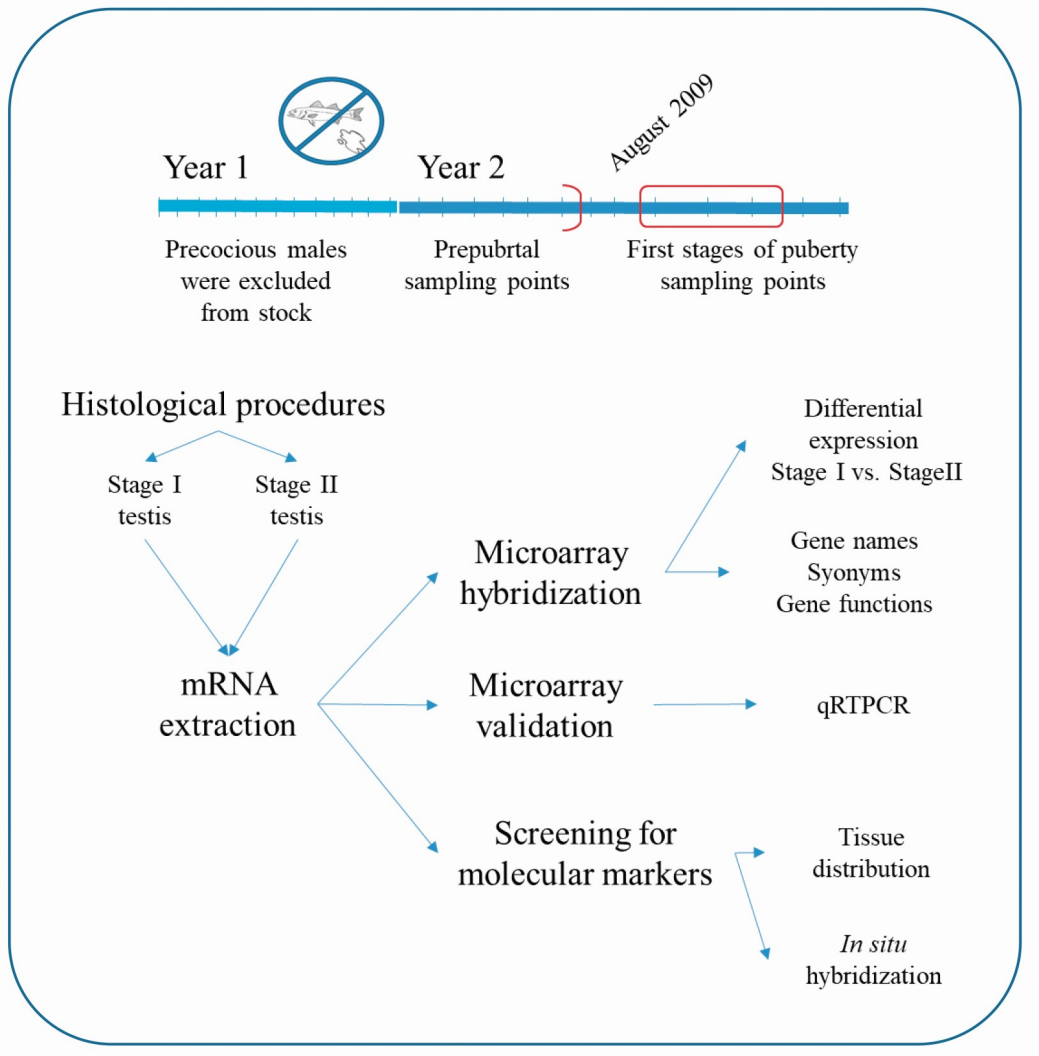

Figure 1. Schematic representation of the experimental design from Chapter 1.

Identification of conserved genes triggering puberty in European sea bass males by microarray expression profiling

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ABSTRACT

Spermatogenesis is a complex process characterized by the activation and/or repression of a number of genes in a spatio-temporal manner. Pubertal development in males starts with the onset of the first spermatogenesis and implies the division of primary spermatogonia and their subsequent entry into meiosis. This study is aimed at the characterization of genes involved in the onset of puberty in European sea bass, and constitutes the first transcriptomic approach focused on meiosis in this species. Sea bass testes collected at the onset of puberty (first successful reproduction) were grouped in stage I (resting stage), and stage II (proliferative stage). Transition from stage I to stage II was marked by an increase of 11ketotestosterone (11KT), the main fish androgen, whereas the transcriptomic study resulted in 315 genes differentially expressed between the two stages. The onset of puberty induced 1) an up-regulation of genes involved in cell proliferation, cell cycle and meiosis progression, 2) changes in genes related with reproduction and growth, and 3) a down-regulation of genes included in the retinoic acid (RA) signaling pathway. The analysis of GO-terms and biological pathways showed that cell cycle, cell division, cellular metabolic processes, and reproduction were affected, consistent with the early events that occur during the onset of puberty. Furthermore, changes in the expression of three RA

nuclear receptors point at the importance of the RA-signaling pathway during this period, in agreement with its role in meiosis. The results contribute to boost our knowledge of the early molecular and endocrine events that trigger pubertal development and the onset of spermatogenesis in fish. These include an increase in 11KT plasma levels and changes in the expression of several genes involved in cell proliferation, cell cycle progression, meiosis or RA-signaling pathway. Moreover, the results can be applied to study meiosis in this economically important fish species for Mediterranean countries, and may help to develop tools for its sustainable aquaculture.

INTRODUCTION

Puberty in fish, as in other vertebrates, comprises the developmental process during which an immature individual acquires for the first time the ability to undergo sexual reproduction (Carrillo et al., 2015; Schulz et al., 2010). In teleost males, puberty is tightly regulated and implies the proliferation and division of spermatogonia (mitotic phase), their subsequent entry into meiosis with the appearance of spermatocytes (meiotic phase), and the final formation of the spermatids and the haploid mature spermatozoa (Nóbrega et al., 2009; Schulz et al., 2010). A species-specific number of genetically determined divisions characterize the mitotic phase (Nóbrega et al., 2009), whereas the meiotic phase remains under the influence of the retinoic acid (RA) signaling pathway (Adolfi et al., 2016; Feng et al., 2015). Somatic Sertoli cells are important players during spermatogenesis, exhibiting a high mitotic activity, particularly at the beginning of each seasonal cycle (Schulz et al., 2010), and are essential for the proliferation and differentiation of germ cells (Griswold, 1998). Mitosis and meiosis reveal thus as key processes for the onset of puberty in vertebrates. Meiosis is of particular importance since it implies the recombination and reduction of the genetic material, essential to ensure the correct formation of gametes, and therefore guarantees the reproduction and maintenance of the species.

Spermatogenesis is marked by the functional stimulation of the brain-pituitary-gonad (BPG) axis, responsible for its neuroendocrine control (Zohar et al., 2010). The brain is the central organ that integrates the circuits that sense the internal and external stimuli and secretes different neuropeptides that control the production of gonadotropins from the pituitary. It is generally accepted that gonadotropins (follicle stimulating hormone; Fsh and luteinizing hormone; Lh), and androgens are the main internal stimuli for vertebrate spermatogenesis. Both gonadotropins become activated by a number of factors among which the metabolic status of the individual, in

terms of body size and visceral fat content, or the photoperiod, are worth mentioning (Carrillo et al., 2015; Escobar et al., 2016; Schulz et al., 2010; Taranger et al., 2010). 11ketotestosterone (11KT) is the main androgen in fish and plays an important role in the progression of spermatogenesis (Schulz and Miura, 2002). It is involved in the proliferation of spermatogonia towards meiosis (Miura and Miura, 2003) and mediates the action of several factors produced by Sertoli cells like antimüllerian hormone (Amh) and insulin-like growth factors (Igfs) at the start of the cycle (Rolland et al., 2009). In addition, Fsh has been shown to stimulate 11KT production in several fish species (Chauvigne et al., 2012; Garcia-Lopez et al., 2009; García-Lopez et al., 2010; Skaar et al., 2011). In fact, Fsh receptor is present not only in Sertoli cells but also in Leydig cells, the somatic cells with steroidogenic capability, as shown in Senegalese sole (Chauvigne et al., 2012) African catfish (Garcia-Lopez et al., 2009) and zebrafish (Crespo et al., 2016; García-Lopez et al., 2010; Skaar et al., 2011).

The European sea bass (*Dicentrarchus labrax*) is an important fish species for marine aquaculture that after intense research on its reproductive function has become a model for both basic and applied research. A number of studies focused on the endocrine control of reproduction shed light on the process and aided to develop protocols for its control in captivity (reviewed by Carrillo et al., 2015). Increased growth rates under intensive culture resulted in precocious puberty in about 20-30% of males by the end of the first year of life (Begtashi et al., 2004), something that in normal conditions occurs during the second year (Zanuy et al., 2001). As in other farmed fish, precocious maturation is one of the main drawbacks for its culture (Taranger et al., 2010), resulting in important economic losses since by the time of marketing during the second year, precocious males exhibit a smaller size than that of normal-maturing males (Felip et al., 2006; Felip et al., 2008). Several studies have shown that this problem can be partially solved by a well-planned strategy of photoperiod control (Begtashi et al., 2004; Felip et al., 2008; Rodríguez et al., 2005; Rodrıguez et al., 2004), although clearly, more work is needed to understand why males mature precociously. Brain factors including kisspeptins (Alvarado et al., 2013; Escobar et al., 2013; Felip et al., 2009), gonadotropin-releasing hormones (GnRHs) (González-Martínez et al., 2004; González-Martínez et al., 2002), leptin and their receptors (Escobar et al., 2016) have been characterized in this species. Regarding pituitary gonadotropins, both Fsh and Lh receptors have been cloned (Rocha et al., 2009), and different assays are currently available to measure Fsh and Lh levels in plasma and pituitary (Mateos et al., 2006; Molés et al., 2011; Molés et al., 2012). Moreover, recombinant sea bass gonadotropins have been used as a biotechnological approach in gene therapy for assisted reproduction (Mazón et al., 2014; Mazón et al., 2015). In addition, secretion patterns of sex steroids (Prat et al., 1990), sex steroid receptors, and several

steroidogenic enzymes (Blázquez et al., 2008; Rocha et al., 2009) during the seasonal cycle have also been reported.

Despite all the previous knowledge, little is known about the molecular machinery that triggers puberty in sea bass males, apart from a study reporting the possible involvement of several genes in the early events preceding gonadal maturation (Crespo et al., 2013). The recent availability of several molecular resources, including a partially annotated sea bass genome database (Tine et al., 2014) gave us the opportunity to study this process using a high throughput strategy. The aim of the present work is to compare the transcriptome from sea bass testes before and right at the start of puberty, and to identify potential genes and pathways involved in the process. This will boost our knowledge of the onset of pubertal development from a basic perspective and will help to implement tools for the improvement of a sustainable aquaculture.

MATERIALS AND METHODS

Animals, rearing conditions and samplings

European sea bass hatched in April 2008 at the Ecloserie Marine de Gravelines (EMG) in the North of France and were grown there until 100 days post hatching (dph) when they were transported to our aquaria facilities at the Institute of Aquaculture Torre la Sal (IATS), a research centre belonging to the Spanish National Research Council (CSIC) in the Spanish Mediterranean coast. The facilities were approved for animal experimentation by the Ministry of Agriculture and Fisheries and by the Department of Fisheries from the Generalitat Valenciana (certificate number ES120330001055). Fish were reared in 2,000 l round fiberglass tanks under natural conditions of photoperiod and temperature until the end of the experiment. In December 2008, coinciding with the first breeding season in this species, fish were subjected to abdominal massage to check for the presence of sperm. This allowed us to eliminate males exhibiting precocious puberty that could interfere with our results. These procedures were repeated every 20 days for a period of about three months. Prior to the start of the second breeding season (August 2009), coinciding with sea bass normal puberty, a sampling procedure was designed to obtain sea bass testes covering the first stages of spermatogenesis. Samplings (15 fish per sampling point) were performed every 10 days starting in mid-August and finishing by the end of October. At each sampling point, fish were anesthetized with 2-phenoxyethanol (0.2 $ml \cdot l^{-1}$) and blood was taken from the caudal vein for plasma sex-steroid measurements. Fish were subsequently sacrificed by quickly severing their spinal cord and the central part of the gonads were dissected for histology

whereas the rest was kept at -80ºC for further RNA extractions needed for the analyses including microarray hybridizations, validations, and tissue specific expression studies. *In situ* hybridization was performed in two-year old males (sampled in October 2011), born and reared at the IATS under the same conditions as the other fish. In all cases, fish were treated in agreement with the Spanish regulations (Royal Decree Act 53/2013) and the European legislation (2010/63 EU) concerning the protection of animals used for experimental and other scientific purposes. All steps were taken to reduce animal suffering.

Histological analysis

After dissection, the central part of the testes was immediately fixed overnight in 4% formaldehyde: 1% glutaraldehyde in phosphate buffered saline (PBS; (McDowell and Trump, 1976)). Tissues were washed in PBS and dehydrated in an increasing series of ethanol 70-96%. Samples were embedded in glycol methacrilate resin (Technovit 7100; Heraeus, Kulzer, Germany), sectioned at 3-4 μm, and stained as in (Bennett et al., 1976). The stages of testicular development and the type and abundance of germ cells in each stage were assessed according to (Begtashi et al., 2004) and (Espigares et al., 2015), respectively. For *in situ* hybridization, the fixative used was slightly different and consisted of 4% paraformaldehyde (PAF), 0.1M phosphate buffer, 5% picric acid solution at 4ºC (see procedures for *in situ* hybridization in Additional Methodology).

Steroid analysis by Enzyme Immune Assay (EIA)

Plasma 11KT levels were determined by enzyme immune assay (EIA) in 20 fish selected from each developmental stage, using the protocol by (Rodríguez et al., 2000). Briefly, antibodies were used at a final concentration of 1:200,000 and the tracer (Cayman chemicals, MI, USA) was diluted at 1:50 Ellman Units (UE)/ml (used at 0.1042 EU/ml). The sensitivity of the assay was around 0.003 ng/ml (Bi/B0 = 90%) and half displacement (Bi/B0 = 50%) occurred around 0.03 ng/ml (slope = -1.018). The inter-assay coefficient of variation (n = 2 plates) was 1.72%.

RNA isolation and cDNA synthesis

For hybridizations and real-time validations, testes (approx. 50-100 mg) were homogenized in Trizol (Invitrogen, Carlsbad, CA) using the FastPrep® Instrument (Qbiogene, Inc., Carlsbad, CA), a tissue homogenizer with ceramic spheres as a lysing matrix. Total RNA was extracted from the lysate with the PureLink™ RNA mini Kit (Invitrogen), following the manufacturer's instructions. Briefly, RNA was phase separated, washed, and finally eluted in

DEPC water. For the tissue-distribution study, telencephalon, hypothalamus, cerebellum, spleen, gills, head kidney, kidney, liver, testis, ovary, heart and gut were homogenized in a thioglycerol-based buffer included in the Maxwell® 16 LEV simplyRNA tissue kit (Promega, Madison, WI). The homogenates were used for RNA isolation with the Maxwell® 16 instrument (Promega) following the manufacturer's instructions that include a DNase treatment. Nevertheless, an additional test was done on the RNAs to discard any possible DNA contamination. For microarray hybridizations RNA quality was assessed with a Bioanalyzer 2100 (RNA 6000 Nano LabChip kit Agilent, Spain) and only RNAs with RIN values higher than 8.5 were used. For other downstream applications such as quantitative real time PCR (qPCR) or conventional PCR (tissue expression study), RNA quantification was done with a Nanodrop 2000c (Thermo Scientific, Wilmington, DE) and stored at -80ºC until further cDNA synthesis. Total RNA (3µg) was reverse transcribed to cDNA with Superscript III (Invitrogen) and random hexamers following the manufaturer's instructions. Protection of RNA from ribonucleases during cDNA synthesis was done by including 40 units of RNAse inhibitors (RNasin, Promega). The reaction was inactivated at 70ºC for 15 min.

Microarray hybridization and analysis

RNA labelling, hybridizations, and scanning were performed at the Autonomous University of Barcelona (UAB). Total RNA (100ng) was amplified and Cy3-labeled with One-Color Microarray Gene Expression Analysis (Low Input Quick Amp Labelling kit, Agilent) along with One-Color RNA SpikeIn Kit (Agilent) following the manufacturer's instructions. The resulting cRNA was purified (RNeasy mini spin columns; Qiagen), quantified with a Nanodrop ND-1000 and checked with a Bioanalyzer 2100 as previously described. Amplified samples (1.65µg per sample) were hybridized to a custom oligonucleotide high-density sea bass microarray (Agilent 4 × 44 K design format; http://www.agilent.com/) containing 60-mer oligonucleotides with a linker directly spotted on glass slides using the Agilent's SurePrint Tecnology.

Three samples from each testicular stage, selected after histological examination and 11KT plasma levels, were used for microarray hybridizations, each of them consisting of a pool RNAs from six males. The pools were used as biological replicates and thus independent samples for microarray hybridizations. In addition, and since each microarray plate can hold up to four samples (4 x 44 design), one sample from each stage was randomly chosen and hybridized in both plates as a quality control to check for possible inter-plate hybridization differences. The probes contained in the microarray (GEO accession number GPL13443) cover 13,199 unique sequences

of *Dicentrarchus labrax* that include 6,275 annotated transcripts, each with 3 specific probes, and 6,924 ESTs with 1 probe/target sequence. Assuming that a typical diploid teleost genome is expected to have 26-28 thousand protein coding loci, the microarray used for the study should cover about half of the genes of the species. Hybridizations were done at 65 °C for 17 h (GE Hybridization Kit; Agilent). Washes were conducted as recommended by the manufacturer using Agilent's Gene Expression Wash Pack with stabilization and drying solution and arrays were scanned with a G2505B (Agilent). Several quality control features and spot intensities were extracted with Agilent's Feature Extraction software v10.4. Finally, data were analyzed with GeneSpring software v10.1. Percentile shift normalization was used to adjust all spot intensities in the array (percentile target = 75).

Principal Component Analysis (PCA) was used as a quality control on samples and allowed to decrease the number of false positives before the statistical analysis. Normalized data were filtered by comparison of the standard deviation expression among groups (filter by expression). Statistical analyses were performed on filtered data using a t-test. Significant differences in the transcriptomic profile between early stages of spermatogenesis (data filtered at a fold change (FC) expression of 2) were set at $p < 0.01$. The corresponding study was deposited at the Gene Expression Omnibus (GEO-NCBI) database under the accession number GSE47400 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=exgnmkgcfbwnhkp&acc=GSE47400) and the platform that validates the microarray can be found at GPL13443).

Gene annotation and enrichment analysis

The web-based tools Genecards (www.genecards.org), Uniprot (www.uniprot.org) and AMIGO (amigo.geneontology.org) were used to assign gene names, synonyms and functions to the differentially expressed genes (DEGs) found after microarray hybridizations. The annotation of the sequences was manually curated, improving the accuracy of the information obtained from the microarray used for this study. A further improvement was added implementing the Blast2Go software (Conesa et al., 2005) that enriched the number of GO-term annotations. A list containing all genes included in our custom-made microarray was used as a reference set to evaluate the enrichment in GO-terms in the subset of DEGs. The resulting data were analyzed with Fisher's exact test with multiple testing correction of the false discovery rate. In addition, annotated DEGs were ascribed to functional biological pathways using the Kyoto Encyclopaedia of Genes and Genomes (http://genome.jp/kegg/kegg2.html) and the altered metabolic pathways were assessed.

Array validation by quantitative real-time PCR (qPCR)

EST sequences of the DEGs were used as a query in Blast searches against the sea bass genome and GeneBank databases in order to position the selected DEGs in their corresponding genes. Primers for the amplification of the DEGs were designed in areas covering intron-exon boundaries to check for genomic contamination using Primer 3 (http://frodo.wi.mit.edu/primer3/).

Primers (Thesis Extended Methodology Table 1) were checked by conventional PCR and the amplified fragments sequenced to verify their identities. qPCR analyses were performed with an iCycler iQ™ (BioRad Labs., Inc.) using SYBR® Green (PCR Master Mix; Applied Biosystems). PCR reactions were run in triplicate in optically clear 96-well plates in a final 20 µl volume containing 10 µl of 2x Sybr Green Master mix, 10 pmol of each primer and 5 µl of diluted cDNA (1:50 for the target genes or 1:500 for the reference gene). Cycling parameters included an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and annealing-extension at 60-72 °C for 1 min ending with an extension at 72 °C for 1 min. A final temperature dissociation step was done to ensure the presence of just one product. qPCR data were collected with iCycler™ iQ optical system software (v. 3.0, BioRad). The cycle threshold (Ct) was calculated as the average of three replicates per sample.

Gene expression analyses were conducted using the Q-Gene core module (Muller et al., 2002). Briefly, for each gene the amplification efficiency (E) was calculated from the slope of the linear correlation between Cts and the logarithm of the amount of serially diluted RNA, used as a standard, following the equation $E = 10^{(-1/slope)}$. E values for the different genes were within the range of 93.5-101.8%. Values were normalized (normalized expression; NE) to the constitutively expressed reference gene *18S rRNA* in each sample (n = 8 individual fish per stage and gene) according to the equation $NE = (E_{ref})^{Ctref}/(E_{target})^{Cttarget}$. *18S rRNA* was considered a good reference gene since it exhibited the best bestkeeper index when comparing different developmental stages (Mitter et al., 2009). In addition, the expression of this gene remains constant in many physiological conditions such as differentiation and proliferation (Fuster et al., 2002) making it a suitable reference gene for this study.

Tissue specific expression

The expression of the selected DEGs was assessed in different tissues including telencephalon, hypothalamus, cerebellum, spleen, gills, head kidney, kidney, liver, testis, ovary, heart and gut. PCR reactions were performed with an initial denaturation of 5 min at 94 ºC, and then 34 cycles with the following characteristics: denaturation at 94 ºC for 30 s, annealing at 60

ºC for 30 s, and extension at 72ºC for 30 s. A final extension of 2 min at 72 ºC was added at the end of the 34 cycles.

In situ hybridization probe synthesis

Sense and antisense mRNA probes were labelled with DIG RNA labelling mix (Roche Diagnostic Co., Indianapolis, Indiana, USA) by *in vitro* transcription with SP6 and T7 RNA polymerases (Promega Co. Madison, WI, USA). Information on primers and restriction enzymes is detailed in Additional Table 1. The riboprobes were quantified with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA) and stored at -80ºC for future in situ hybridizations (check detailed protocol in Additional Methodology at the end of this chapter).

In situ hybridization

The *in situ* hybridization procedure was carried out according to Escobar et al. (2013), (detailed protocol in Additional Methodology). Briefly, testis samples from 2 year-old males (n=4), were fixed overnight in 4% PAF, dehydrated and embedded in paraffin. Adjacent transversal sections (6µm) were mounted onto poly-L-lysine–coated slides and kept at 4ºC. Samples were de-waxed, re-fixed in 4%PAF, and treated with proteinase K. After treatment, samples were post-fixed in 4% PAF and rinsed with SSC. Hybridizations were performed overnight in a solution containing the DIG labelled riboprobes previously synthesized. On the next day, slides were processed for immunodetection by an overnight incubation in alkaline phosphatase-conjugated sheep Fab fragment antibodies to digoxygenine (Roche, Indianapolis, IN, USA) diluted 1:2000. Fluorescent detection was done after incubation at RT for 2 h with HNPP (2-hydroxy-3-naphtoic acid-2`-phenylanilide phosphate) in HPP/fastRED solution (Roche). Finally, slides were mounted with Vecta Shield hard set mounting medium containing 4',6-diamidino-2-phenilindone (DAPI). To confirm the specificity of the probes, parallel series of slides were hybridized with their corresponding sense riboprobes. Sections were analyzed with a UV light microscope and images captured in a ProGres3 camera using ProGres software. Sample slides were stored at 4ºC, protected from light and humidity.

Statistical analyses

Student's t-test for hormonal analysis, microarray hybridizations and gene expression levels was used to reveal significant differences between stage I and stage II. In all cases, significant differences were accepted at $p < 0.05$.

RESULTS

Sample selection: histological and hormonal classification.

Sea bass testes were histologically classified according to their stage of spermatogenesis (Begtashi et al., 2004; Espigares et al., 2015). Since the study was focussed on the onset of spermatogenesis, only testes in stage I and stage II were used. Briefly, stage I, corresponded to an immature testis, and was characterized by the presence of type A spermatogonia located within the seminiferous lobules (Fig. 2A). Stage II, corresponded to testis in a proliferative phase and was characterized by the presence of type A spermatogonia, abundant cysts of type B

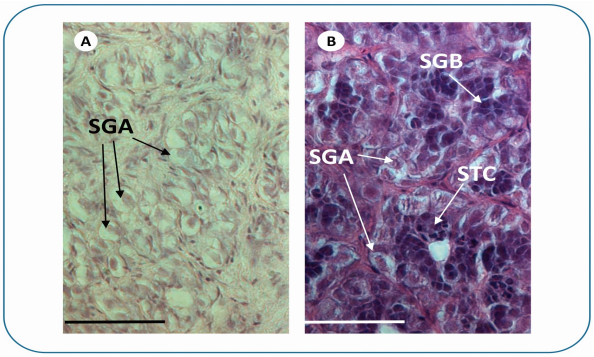

Figure 2. Photomicrographs of testicular developmental stage I and stage II in European sea bass testis. A) Sexually immature testis in stage I characterized by the presence of type A spermatogonia (SGA) within the seminiferous lobules. B) Early recrudescent testis in stage II characterized by the presence of some SGA, abundant cysts of type B spermatogonia (SGB) and some cysts of type I spermatocytes (STC). Scale bar represents 50µm.

spermatogonia and some cysts of type I spermatocytes (Fig. 2B). Plasma samples from males previously classified by histology as stage I (n=20) or stage II (n=20) were used to check their levels of 11KT. The results showed that 11KT was a suitable marker to discriminate between males in stage I and stage II with significantly higher levels in the latter than the former (Fig. 3).

Based on this result, testes from 18 males in stage I (11KT levels ranging between 0.35 and 0.87 ng/ml), and 18 males in stage II (11KT levels ranging between 2.18 and 3.64 ng/ml)

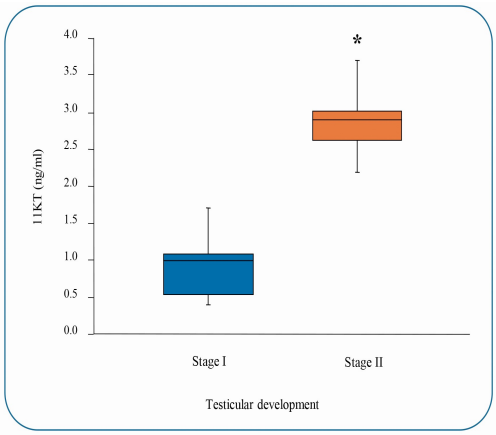

Figure 3. Box-and-whiskers plots of 11 ketotestosterone (11KT) plasma levels in sea bass males during early stages of puberty. 11KT levels were measured at two different stages of pubertal development: stage I (sexually immature testes) and stage II (early recrudescent testes). Box represents upper and lower quartiles and whiskers represent maximum and minimum observed values. Horizontal line represents the median value (0.99 ng/ml for stage I and 2.90 ng/ml for stage II). The asterisk denotes statistical differences between both groups after a student t-test ($p < 0.05$); $n = 20$ in each experimental group.

were selected for further RNA extractions and microarray hybridizations. For that purpose, the RNAs from the 18 males of each testicular stage were randomly divided and pooled into six groups, three corresponding to stage I and the other three to stage II. The pools of each stage consisted of the RNA from six different fish of that particular stage up to 18 fish per stage. The pools were used as biological replicates and thus independent samples for microarray hybridizations.

Microarray hybridizations

Changes in gene expression during the onset of spermatogenesis (stage I versus stage II) were assessed with a sea bass specific microarray previously described and validated (GPL13443). The study identified 315 DEGs between the two spermatogenic stages (FC > 2), among which 162 corresponded to functionally annotated genes whereas the remaining 153 were non-annotated sequences. When comparing their expression, a similar number of DEGs were found to be upregulated and downregulated (150 upregulated versus 165 downregulated; see Additional table 2 for a list of all DEGs and Additional table 3 for a glossary of the genes involved in cell proliferation, reproduction, growth and RA-signaling pathway with particular mention in this study). A PCA showed the spatial distribution of the microarray data and revealed the presence of two clear clusters, one corresponding to stage I testes and the other one to stage II testes (Fig. 4). Component 1 explained 92.43% of the variation whereas component 2 was responsible for 5.13% of the variation. In addition, a heatmap representation of the DEGs grouped fish according to their stage of testicular development (Fig. 5).

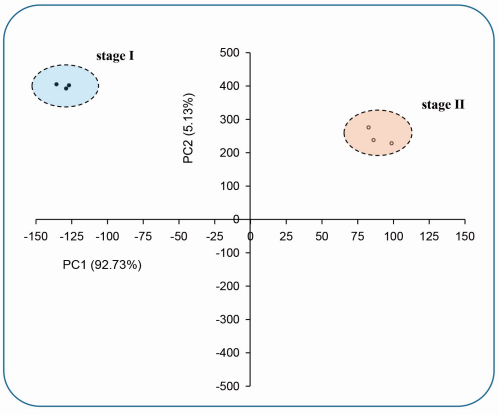

Figure 4. Principal component analysis of the results from the microarray hybridizations. Each data point corresponds to a pool of RNAs from testis of six different fish. Black circles correspond to RNAs from testis in stage I and white circles to RNAs from testis in stage II. Numbers between brackets represent the percentage of variation explained by each component; i.e. component 1 (PC1) and component 2 (PC2).

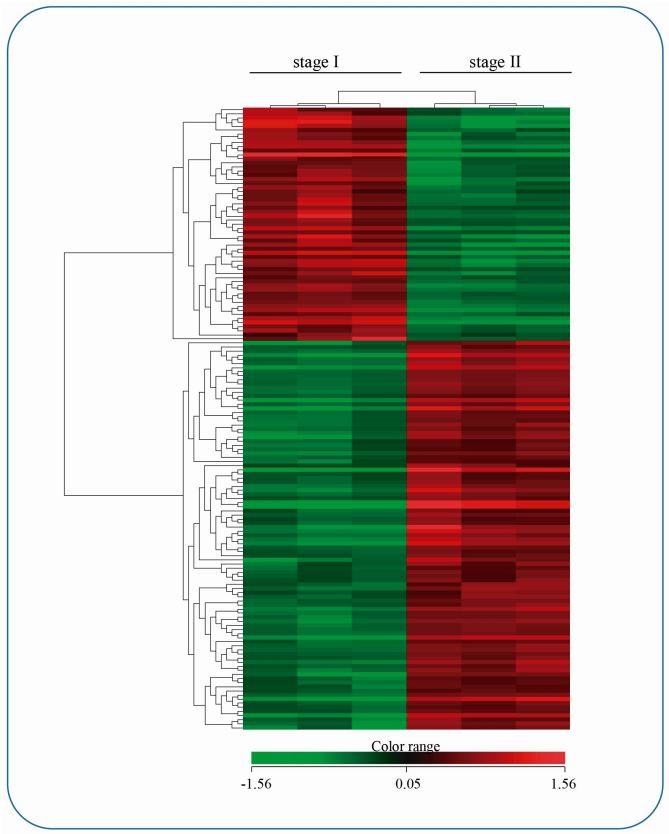

Figure 5. Hierarchical heat map of sea bass annotated ESTs differentially expressed during early stages of puberty. The individual genes are pictured horizontally showing their relative expression values across all replicates of the different stages of pubertal development (three replicates per stage) that are represented in each column. The colour scheme is calibrated to the log2 expression values with red representing higher transcript abundance and green lower transcript abundance. The heatmap displays only DEGs (corresponding to 152 annotated genes) with significantly different expression values ($p<0.05$) between stage I and stage II and a log2 fold change value greater than two (Pearson correlation). The scale bar shows Z-score values.

A Gene Ontology (GO) study of the DEGs of the microarray resulted in a distribution among the three main functional categories including biological processes (Fig. 6A) with a high presence of genes involved in cell division, cell cycle, cell differentiation and cytoskeleton organization typical of the increased cell proliferating activity during early testicular recrudescence. In addition, genes involved in growth, reproduction, metabolism and catabolism were also differentially expressed. Regarding the molecular function (Fig. 6B), binding, enzymatic activity, and transport were differentially regulated. As for the cell component (Fig. 6C), a majority of the processes appeared taking place in the nucleus and were linked to the protein complex. These results were supported by a GO enrichment analysis that resulted in the identification of several DEGs in the major functional categories undergoing changes throughout sea bass spermatogenesis. Three main subsets were apparent, one including several biological processes focused on reproduction, cell cycle, cell division, chromosome segregation and cellular metabolic processes; a functional subset related to binding; and finally a cellular component category mainly related to processes taking place in the nucleus. The fact that cell cycle processes occur mainly in the nucleus suggests that both subsets are mechanistically related and are involved in cell division and progression. The analysis of the affected biological pathways during the onset of pubertal development (Additional Table 4) indicated that 15 of the DEGs (>2.0 FC) were involved in metabolic pathways mainly

related to nucleotide, amino acid and lipid metabolism and retinol metabolism. Pathways involved in cellular processes mainly cell cycle, meiosis, and DNA replication and repair were also affected, and included 13 DEGs. Other group of DEGs was involved in different signaling pathways and the last group includes pathways related with the endocrine system.

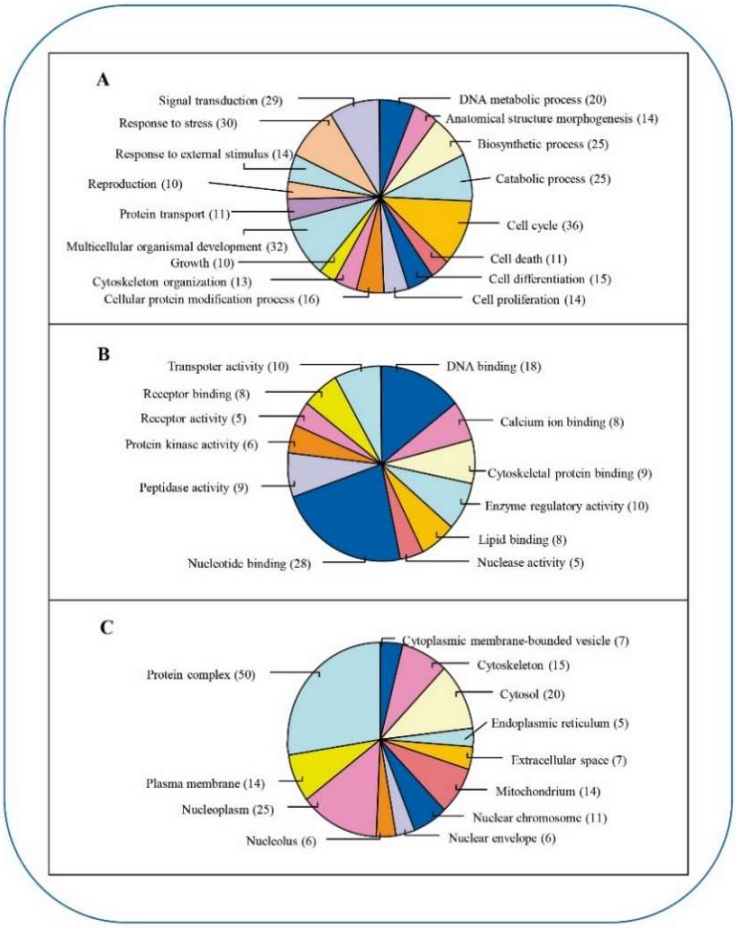

Figure 6. Distribution by GO-terms of the differentially expressed genes (DEGs) during the onset of sea bass puberty. The multi-level pie graph classified the DEGs according to their GO-terms in three main categories, including: A; biological processes (cutoff value = 10 sequences), B; molecular function (cutoff value = 5 sequences), and C; cell component (cutoff value = 5 sequences). The number of genes found in each GO-term appear written between parentheses.

Microarray validations

All the results obtained after the microarray hybridizations were validated by qPCR. We have to keep in mind that the use of long oligo-based microarrays (60-mers) has been shown to have a high sensitivity for detection but a lower specificity (Slegtenhorst-Eegdeman et al., 1995) and therefore it could have a reduced ability to discriminate between similar transcripts produced by the same locus, paralogs or similar members of large gene families. For this reason, we have

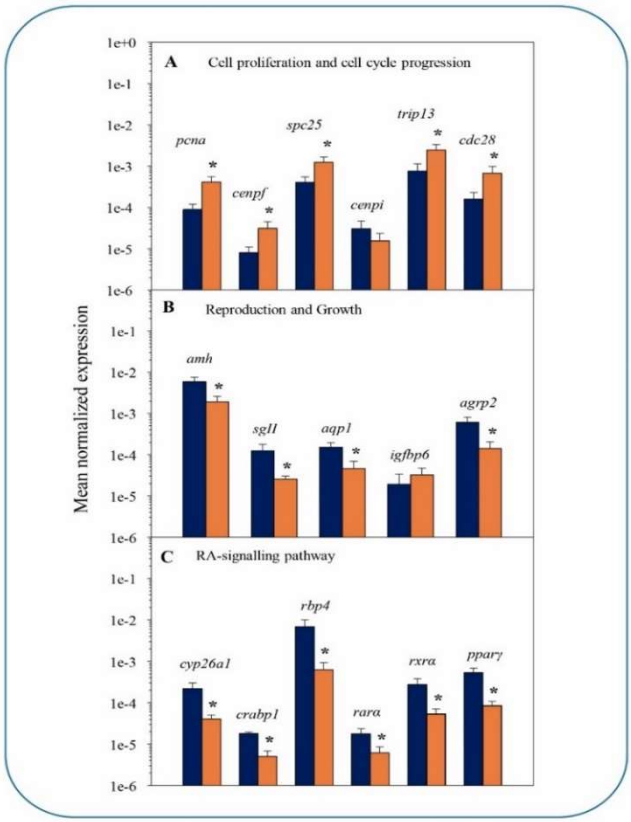

Figure 7. qPCR results for genes differentially expressed in the microarray during the onset of puberty in the sea bass. Genes were selected according to their relevance in different reproductive events. A) Genes involved in cell proliferation and cell cycle progression; proliferating cell nuclear antigen (*pcna*), centromere protein I (*cenpi*), spindle pole body component 25 (*spc25*), centromere protein f (*cenpf*), thyroid hormone receptor interactor 13 (*trip13*), and cdc28 protein kinase (*cdc28*). B) Genes involved in reproduction and growth; antimüllerian hormone (*amh*), aquaporin 1 (*aqp1*), secretogranin II (*sgII*), agouti-related protein 2 (*agrp2*), insulin-like growth factor binding protein 6 (*igfbp6*). C) Genes involved in the RA signaling pathway: RA-metabolizing enzyme cytochrome P450 26a1 (*cyp26a1*), retinol binding protein 4 (*rbp4*), RA-binding protein (*crabp1*). This group includes three RA-nuclear receptors, RA receptor alpha (*rarα*), retinoid X receptor alpha (*rxrα*), and peroxisome proliferator-activated receptor gamma (*pparγ*). Blue bars represent stage I testis and orange bars represent stage II testis. The stage-specific expression levels were normalized to those of the constitutively expressed *18S rRNA* gene in each sample. Expression data are shown as mean normalized expression + SEM. Y-axis is represented in logarithmic scale for easier visualization. Asterisks indicate statistically significant differences ($p < 0.05$) between stage I and stage II after a student-t test.

not only validated the microarray results by quantifying gene expression, but we have also cloned and sequenced several full length DEGs to make sure that the oligos detected in the microarray, in fact correspond to the sequences they represent. The relative differential expression was assessed for 14 transcripts. Six of them (*pcna*, *cenpi*, *spc25*, *cenpf*, *trip13*, *cdc28*), were included in a group of genes with special relevance in cell proliferation and cell cycle progression (Fig. 7A). Five transcripts (*aqp1*, *amh*, *sgII*, *agrp2, igfbp6*) were included in the group of genes with relevance in reproduction and growth (Fig. 7B). The remaining three transcripts (*cyp26a1*, *rbp4*, *crabp1*) were located in the group of genes involved in the RA-signaling pathway (Fig. 7C). In

addition, to gain more insight on the importance of the RA-signaling pathway in meiosis, the expression of three more transcripts, corresponding to RA-nuclear receptors (*rarα*, *rxrα* and *pparγ*) was studied in stage I and stage II testis (Fig. 7C). The stage-specific expression levels were normalized to those of the constitutively expressed *18S rRNA* gene in each sample. As expected from the microarray hybridizations, in the cell proliferation and cell cycle progression group, five of the selected DEGs used for qPCR validation showed an upregulation in stage II testes; *pcna* showed the highest differences in expression, while *cenpi* did not show any significant change (Table 1). Also consistent with the microarray, the reproduction and growth related genes, showed a significant decrease of expression in stage II testes. However, the downregulation of *igfbp6* could not be confirmed by qPCR (Table 1). The RA related group (including the nuclear receptors *rarα*, *rxrα*, *pparγ*, not found in the microarray), showed a downregulation during the transition from stage I to stage II, being *rpb4* the one with the highest expression differences (Table 1). In general, the comparison of both methods revealed a good correlation between them (Fig. 8).

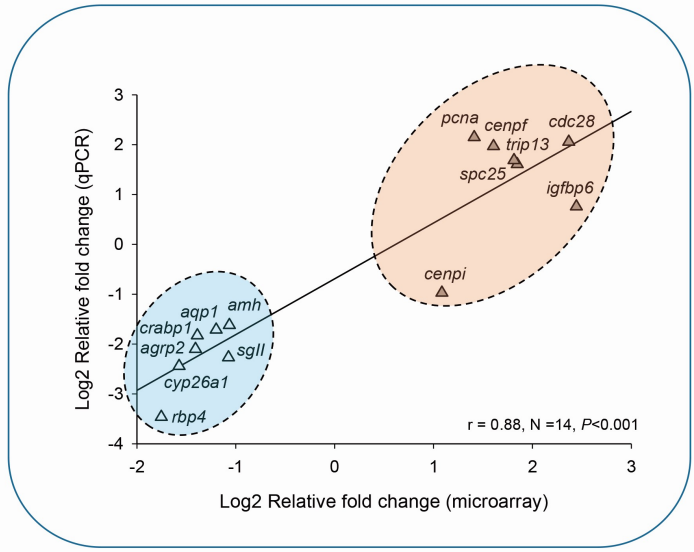

Figure 8. Correlation between microarray and qPCR results. Fold change (FC) induction values of *rbp4*, *cyp26*, *agrp2*, *crabp1*, *aqp1*, *amh*, *sgII*, *cenpi*, *spc25*, *trip13*, *igfbp6*, *cdc28*, *cenpf*, and *pcna* gene transcripts are plotted as log2 values of the relative fold change. The X-axis represent microarray data whereas the Y axis corresponds to qPCR data. The regression line and the corresponding r coefficient are also represented. For complete gene names see Fig. 7 caption.

The FC difference in expression examined by microarray hybridization and by qPCR was always in the same direction except for *cenpi* and *igfbp6* for which the results from the qPCR failed to reproduce those from the microarray, since the differences between both stages found by qPCR were not statistically different ($p = 0.339$ and $p = 0.454$, respectively). Other than this minor disagreement, the microarray results were validated by the qPCR. It is worth mentioning that in a number of genes, the microarray results exhibited lower differences between the two developmental stages than those found from the qPCR, indicating that this particular microarray

may represent an underestimate of the extent of differential expression during sea bass spermatogenesis (Table 1).

Table 1. Microarray versus qPCR fold change (FC) results for 14 genes in the sea bass transcriptome affected during early stages of pubertal development

		Microarray			qPCR		
Gene group	Gene abbreviation	Regulation *	FC	FC adjusted p-value	Regulation*	FC	FC p-value (t-test)
Cell proliferation and cell cycle progression	*pcna*	up	2.66	0.0014	up	4.43	0.016
	cenpf	up	3.04	0.0011	up	3.91	0.043
	spc25	up	3.60	0.0004	up	3.05	0.003
	cenpi	up	2.12	0.0015	no change	0.51	0.339
	trip13	up	3.51	0.0005	up	3.22	0.032
	cdc28	up	5.16	0.0003	up	4.15	0.000
Reproduction and growth	*amh*	down	2.09	0.0041	down	3.07	0.007
	sgII	down	2.10	0.0094	down	4.82	0.047
	aqp1	down	2.29	0.0034	down	3.28	0.045
	igfbp6	up	5.44	0.0089	no change	1.69	0.454
	agrp2	down	2.65	0.0099	down	4.29	0.039
RA signaling	*cyp26a1*	down	2.97	0.0098	down	5.43	0.029
	crabp1	down	2.62	0.0008	down	3.55	0.000
	rbp4	down	3.37	0.0005	down	11.02	0.003

*Regulation = FC value in stage II compared with stage I (stage II/stage I)
Upregulated = up; downregulated = down.

Tissue specific expression

In order to assess the tissue specific expression of the selected DEGs and the three extra genes, we performed a qualitative tissue distribution study by conventional PCR (Fig. 9). Among the cell proliferation genes, *pcna* and *spc25* were ubiquitously expressed whereas *cenpi* expression was mainly restricted to gonads, with highest levels in ovary. Regarding the genes involved in reproduction and growth, *aqp1* was expressed at similar levels in all the tissues studied. *sgII* expression was found in all tissues but with higher levels in pituitary, cerebellum, kidney and testis. The expression of *amh* was highest in gonads and undetectable in telencephalon and pituitary whereas *igfbp6* expression was restricted to the gills, liver, testis and gut. Finally, the genes involved in RA-signaling pathway were expressed at similar levels in all tissues except *cyp26a1* that showed highest expression in gonads and *crabp1* that was mostly expressed in head kidney and at lower levels in dorsal kidney, gills and testis.

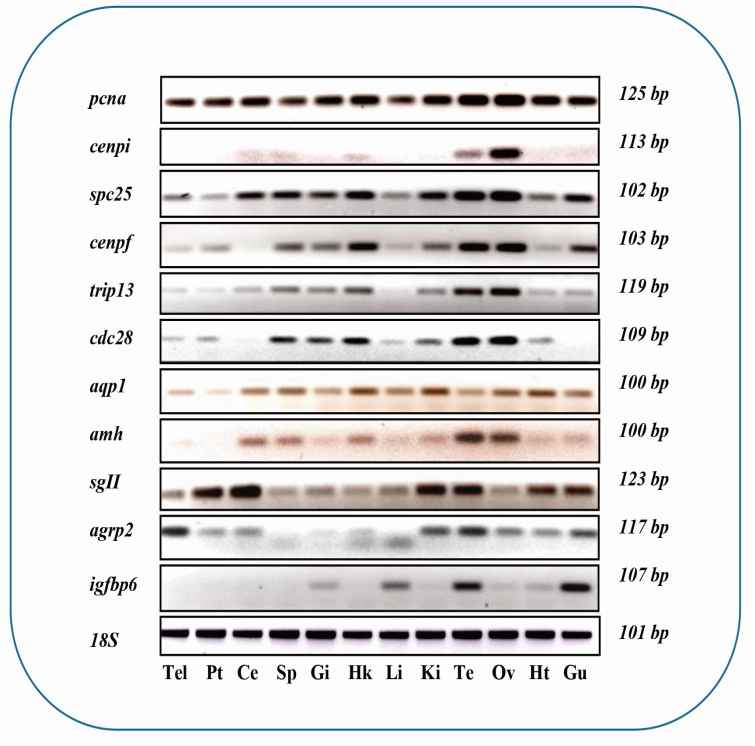

Figure 9. Tissue distribution of sea bass transcripts involved in different biological processes during sea bass early puberty. Biological processes include: a) cell proliferation and cell cycle progression (*pcna*, *cenpi*, *spc25*, *cenpf*, *trip13*, and *cdc28*); b) reproduction (*aqp1*, *amh*, *sgII*, *agrp2*, and *igfbp6*. The expression was detected by RT-PCR in different tissues including telencephalon (Tel), pituitary (Pt), cerebellum (Ce), spleen (Sp), gills (Gi), head kidney (Hk), liver (Li), posterior kidney (Ki), testis (Te), ovary (Ov), heart (Ht), and gut (Gu). 18S ribosomal RNA (*18S*) was used internal control to check for the integrity of the cDNA template. Information about genes is included in Additional table 3.

In situ hybridization of *cyp26a1* and *rxrα* in European sea bass testis

In situ hybridization studies were performed to locate the expression sites of *rxrα* and *cyp26a1* in sea bass testis since both of them are important genes of the RA signaling pathway. The *vasa* and *star* probes were used as control for spermatogonia and Leydig cells, respectively (Cao et al., 2012; Kusakabe et al., 2006). Our results showed a strong and clear signal for *vasa* in spermatogonia, which decreased in cysts containing type I spermatocytes (Fig. 10). Unfortunately, no signal for *star* could be found and no clear or consistent signal in the outer layer of the testicular lobules could enable us to identify putative Leydig cells. Moreover, only apoptotic zones were hybridized to the star antisense probe (data not shown).

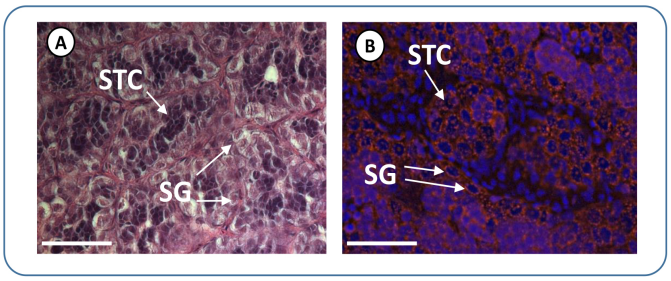

Figure 10. Transverse sections showing *in situ* hybridization of *vasa* in stage II testis. A) Section of the studied area stained with H&E showing spermatogonia (SG) and spermatocytes (STC). B) Germ cells expressing *vasa* (SG and STC) are marked with fluorescent FastRED dye (red signal). Nuclei are dyed with DAPI (blue signal). Bar=50µm.

The *rxrα* expressing cells were detected only within testicular lobules and were identified as germ cells since the signal, although weak, shared the same localization than that of *vasa* that was used as a control of germ cells (Fig. 11).

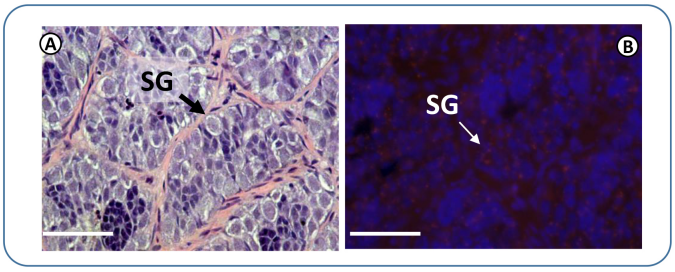

Figure 11. Transverse sections showing *in situ* hybridization of *rxrα* in stage II testis. A) Section of the studied area stained with H&E showing spermatogonia (SG). B) Germ cells expressing *rxrα* (SG) are marked with fluorescent FastRED dye (weak red signal). Nuclei are dyed with DAPI (blue signal). Bar=50µm.

Regarding *cyp26a1*, the signal appeared close to the somatic cells, between and inside the testicular lobules. However, the signal was discrete and not consistent in all the lobules (Fig. 12).

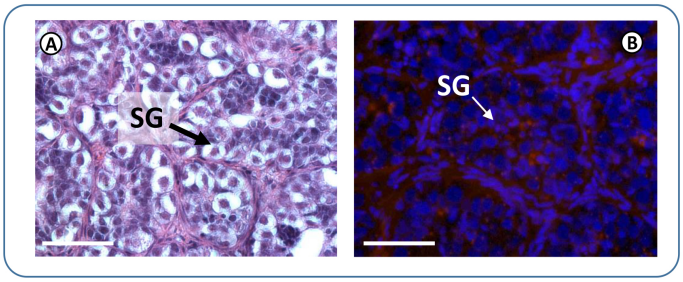

Figure 12. Transverse sections showing in situ hybridization of *cyp26a1* in stage II testis. A) Section of the studied area stained with H&E; spermatogonia (SG). B) Germ cells expressing *cyp26a1* (SG) are marked with fluorescent FastRED dye (weak red signal). Nuclei are dyed with DAPI (blue signal). Bar=50µm.

DISCUSSION

The present study contributed to identify differences in gene expression during the early stages of pubertal development in European sea bass males using a custom-made microarray. However, and despite the fact that the differences observed at the transcriptome and the steroid level are related to puberty (first successful reproduction), it is possible that similar changes could be found in successive reproductive seasons since they mark the transition between quiescence

and the start of gametogenesis. The use of long oligo-based microarrays has been shown to have a higher sensitivity for detection but a lower specificity (Relógio et al., 2002) and therefore could have a reduced ability to discriminate between similar transcripts produced by the same locus, paralogs or similar members of large gene families. To circumvent this problem, we cloned the full-length sequence of several selected DEGs and then validated the results with qRT-PCR obtaining a good correlation between both methods. In addition, the study revealed the complete sequences of some transcripts for the first time in the sea bass, adding contrasted information to the microarray that was based on EST sequences. However, for other DEGs that were not annotated in the microarray, we could not find any match to reveal their identities in any of the databases searched including Genebank, Ensembl, and Uniprot. The different stages of spermatogenesis (stage I and stage II) used for the study were classified by histology and their corresponding 11KT levels were further confirmed by EIA, demonstrating that the increase of circulating 11KT marks the initiation of pubertal development. A similar result was found in other teleosts including eel (Miura et al., 1991), goldfish (Kobayashi et al., 1991), zebrafish (Skaar et al., 2011) or trout (Rolland et al., 2013) and 11KT measured from the mucus of carps was suitable to differentiate males from females (Schultz et al., 2007; Schultz et al., 2005). However, no correlation between 11KT and gonad developmental stage in either male or female carp could be found (Schultz et al., 2005). Our results open the possibility to explore the use of 11KT as a non-lethal marker for the onset of puberty in this species helping to manage the fish farms stocks to separate precocious from non-precocious sea bass males.

The response of the transcriptome during early spermatogenesis is reflected in the affected biological pathways found after the GO study. Among these pathways we found that cell proliferation, cell cycle, DNA replication and repair, and meiosis progression were preferentially affected during the onset of male puberty. The spindle assembly checkpoint (SAC) is a control mechanism of dividing cells that ensures the correct segregation of chromosomes by blocking cell cycle progression until kinetochores are properly connected to the spindle (Lara-Gonzalez et al., 2012). In our study, several genes coding for SAC proteins (*bub3*) and SAC protein regulators (*mad2l1bp* and *ttk*), were upregulated in stage II testes. Moreover, a group of up regulated genes, such as *ndc80*, *spc25*, *aurkb* and *cdc28*, which are needed for SAC activity, were also found in this study (Additional table 2). *ndc80* and *spc25* code for essential proteins of the Ndc80 complex, needed for SAC activity (Kline-Smith et al., 2005) while *aurkb* (aurora kinase b) controls kinetochore orientation during meiosis (Monje-Casas et al., 2007). Furthermore, defects in *cdc28* function result in delays in the exit from mitosis and in meiosis impairment among others (Mendenhall and Hodge, 1998). Another group of transcripts coding for centromere proteins,

namely *cenph*, *cenpi* or *cenpf*, were also upregulated. In the case of *cenpi*, in addition to its role in centromere formation, it is involved in the response of gonadal tissues to Fsh (Slegtenhorst-Eegdeman et al., 1995). The upregulation of *cenpi* in stage II coincides with the initiation of the gradual increase in Fsh plasma levels in sea bass during early spermatogenesis (Carrillo et al., 2015; Molés et al., 2012). This is in line with the role of Fsh inducing germ cell proliferation and marks the onset of spermatogenesis through the activation of spermatogenesis-related genes (Mazón et al., 2014). Several transcripts like *sycp2* and *syce1*, coding for proteins of the synaptonemal complex and *trip13*, required for the completion of meiosis (Li and Schimenti, 2007), were upregulated in stage II testes. Moreover, *pcna*, essential for DNA replication and a molecular marker of dividing cells (Smith et al., 2008), also increased during the onset of spermatogenesis in agreement with the active mitosis of spermatogonia typical of this period (Begtashi et al., 2004). The role of *pcna* in the proliferation of germ cells has been described in several teleosts and is currently used in a number of fish species as a marker of spermatogenesis progression (Schulz et al., 2010). All these results are supported by the analysis of affected biological pathways (Additional Table 4) that showed higher expression of genes involved in cellular processes, particularly those involved in cell cycle, meiosis and DNA replication and repair. Other signaling pathways involved in testicular development such as Wnt, MAPK, hedgehog and TGF beta (Windley and Wilhelm, 2016) were altered during early puberty in sea bass. Altogether, the upregulation of the above mentioned genes is indicative of an active period of mitosis, reflect the need for a tight control of the correct division of the cells, and constitutes an indicator for the progression of meiosis typical of early stages of spermatogenesis.

The tissue distribution study reinforces the importance of genes like *pcna* and *spc25* that appeared ubiquitously expressed due to their role during the G1 phase of the cell cycle (Franco et al., 2010; Moldovan et al., 2007; Satisbury, 1995). However, *cenpi* expression was mainly restricted to gonads, with highest levels in ovary. This result is in line to its function, since *cenpi* encodes for a centromere protein critical for accurate chromosome alignment and clustering during meiosis (Unhavaithaya and Orr-Weaver, 2013). Regarding *trip13*, it shows a ubiquitous expression with a higher intensity in gonads. This is in agreement with its function as it allows for the evaluation of correct chromosome synapsis (homologous chromosome recognition) and the self-destruction of defective cells (Li and Schimenti, 2007). The *cdc28* function, together with cycline-kinase proteins, is to allow the transition from one cell cycle stage to another (Strich, 2004). Our results showed a more specific expression in *cdc28* expression with low band intensity in cerebral tissues, higher band intensity in gonads and no expression at all in gut, suggesting a higher activity in prepubertal gonads than in other tissues.

A second group of DEGs includes transcripts involved in reproduction and growth. The anti müllerian hormone (*amh*) is involved in gonad development and steroidogenesis in vertebrates and induces the regression of müllerian ducts in mammals during male embryogenesis (Josso et al., 2006). Although fish do not have müllerian ducts, *amh* homologues have been identified in several teleosts (Pfennig et al., 2015), suggesting evolutionary conserved functions for this gene. The role of Amh as a meiosis inhibiting factor was first shown in eels (Miura et al., 2002) and recently in zebrafish (Skaar et al., 2011), induced by the increase of circulating 11KT that blocked *amh* expression facilitating spermatogenesis completion. In teleosts, *amh* has a key role in early testicular maturation with highest levels in pre-spermatogenic testis and lowest during spawning (Wu et al., 2010). In the European sea bass, administration of recombinant Fsh induced spermatogonial proliferation and differentiation into spermatocytes, due to the increase of 11KT levels and the concomitant suppression of *amh* expression (Mazón et al., 2014). Moreover, *amh* mRNA and protein expression was detected in Sertoli cells of prepubertal sea bass, and the signal decreased during spermatogenesis (Rocha et al., 2009). In addition, we also found that *amh* expression was highest in gonads when compared to the rest of tissues studied according to its role as a paracrine regulator of gonad maturation (Wuertz et al., 2007). Our results showed a decrease of *amh* levels during early spermatogenesis in agreement with its role as an inhibitor of spermatogenesis progression. This is supported by the increase of *pcna*, *cenpi* and 11KT levels in the same testicular stage. Likewise, a downregulation of *amh* during the reproductive cycle has been found in the testicular transcriptome of rainbow trout (Rolland et al., 2009) and in precocious Atlantic salmon (Guiry et al., 2010), further demonstrating that the inhibitory effect of Amh on the onset of puberty can be extended to all fish species so far studied. In addition, the study of the biological pathways affected during the onset of puberty, also show the importance of *amh* in several signaling pathways including those of TGF beta, hippo and cAMP (Windley and Wilhelm, 2016).

Our results also show a low *sgII* expression at the time of the onset of spermatogenesis, in agreement to the low Lh levels found in the European sea bass at this stage of gonad development (Carrillo et al., 2015; Rocha et al., 2009). Despite the few studies available for *sgII* in fish, other than those in goldfish (Blázquez et al., 1998; Zhao et al., 2009; Zhao et al., 2010), it is known that secretogranin, the protein encoded by *sgII*, is widely distributed in secretory granules of neurons and endocrine cells (Blázquez and Shennan, 2000). Secretogranin is the precursor of secretoneurin, a bioactive neuropeptide capable to induce Lh secretion, therefore stimulating reproduction (Blázquez and Shennan, 2000; Zhao et al., 2009; Zhao et al., 2010). This is reinforced by our results showing highest *sgII* expression in testis and in brain tissues, including

pituitary, cerebellum and kidney. It would be very interesting to determine whether *sgII,* and therefore secretoneurin, also increase during later stages of spermatogenesis, coinciding with the surge of Lh, to experimentally test this hypothesis.

Earlier studies in sea bream point at the relevance of aquaporin 1 (Aqp1) in fish reproduction due to its role in water intake during oocyte hydration prior to spawning (Fabra et al., 2006) and in the activation of sperm motility during the last stages of spermatogenesis (Boj et al., 2015; Zilli et al., 2009). A previous study in European sea bass acclimated to freshwater showed a downregulation of *aqp1 e*xpression in intestine epithelial cells, suggesting a role in water absorption (Giffard-Mena et al., 2007). Additionally, SsAQP1o a member of the aquaporin family that functions as an oocyte-specific osmotic regulator has been found in gilthead seabream, *Sparus aurata*, (Tingaud-Sequeira et al., 2008). We found that *aqp1* was ubiquitously expressed at similar levels in all the tissues studied evidencing the importance of *aqp1* maintaining water homeostasis in all tissues. In addition, our results also show that *aqp1* levels remained low during early spermatogenesis, in agreement with its prominent role in sperm maturation during the last stages of spermatogenesis.

The agouti-related protein 2 (AGRP2) is an orexigenic peptide with a key role in the regulation of energy balance in mammals (Girardet and Butler, 2014) and fish (Guillot et al., 2016). In this regard, our study shows that the lipid metabolism pathway where *agrp2* was included was affected during the early stages of pubertal development further supporting its role in energy balance. Furthermore, a direct link between leptin, the most powerful orexigenic neuropeptide in fish (Volkoff et al., 2005), and the AGRP system has been suggested in sea bass males (Escobar et al., 2016). Moreover, abundant *Agrp* expression was found in mouse pachytene-spermatocytes and immunohistochemistry revealed that Agrp co-localized with Scp3, a meiotic-specific protein of the synaptonemal complex (Ilnytska et al., 2009). Although *agrp2* has been characterized in sea bass testis (Agulleiro et al., 2014), this is the first time its involvement in spermatogenesis is suggested, possibly due to the specific energy requirements during spermatogenesis and the decrease in food intake. This is in agreement with the downregulation of *agrp2* in sea bass brain after long-term fasting (Agulleiro et al., 2014) and its decrease in testis during early spermatogenesis (present study), and link the appetite and growth system with reproduction (Reinecke, 2009).

In addition, a transcriptomic study of trout testis revealed that Fsh administration induced the increase of *igfbp6* (Sambroni et al., 2013). This strong Fsh-induced upregulation was present during early spermatogenesis, including germ cell proliferation and meiosis, and was associated

to the effect of the Igf-signaling pathway on spermatogenesis progression (Sambroni et al., 2013). Our results also show a clear upregulation of *igfbp6*, coinciding with the first stages of pubertal development and the increase of 11KT plasma levels.

Finally, a group of DEGs associated with the RA-signaling pathway were differentially expressed in the sea bass transcriptome during the early stages of spermatogenesis. Two transcripts coding for binding proteins, one in charge of ROL transport (*rbp4*) through the blood stream and another one (*crabp1*) in charge of the translocation of RA to the nucleus of the target cells (Schug et al., 2007) were differentially expressed. In addition, *cyp26a1*, responsible for the degradation of intracellular RA and essential for the maintenance of RA homeostasis (White et al., 1996), was affected. The decrease of *cyp26a1* in stage II is associated with a decrease in the translocation and transport of RA brought about by the downregulation of *rbp4* and *crabp1*, in order to maintain the homeostasis of RA that otherwise, and at high levels can be toxic for the cell (Chung and Wolgemuth, 2004). In addition, ROL metabolism was one of the affected metabolic pathways found in the present study. It seems thus plausible that in the European sea bass, the suppression of RA degradation and the concomitant increase in the availability of RA could be partially responsible for triggering the onset of meiosis. In zebrafish testes, *cyp26a1* was expressed in germ cells entering meiosis, while in females, a downregulation was found in oocytes during meiosis resumption (Rodríguez-Marí et al., 2013). Likewise, in medaka, RA was found to act directly on Sertoli cells, Leydig cells, and pre-meiotic germ cells with a decrease of *cyp26a1* expression by the time of meiosis resumption, whereas in ovaries, RA-transcriptional activity is highest in meiotic oocytes (Adolfi et al., 2016). In addition, *in vivo* Fsh-injection to pre-spermatogenic zebrafish males induced the onset of spermatogenesis and resulted in changes of several enzymes involved in the RA signaling pathway, including a decrease in *cyp26a1* expression, although no effect was found after *ex vivo* culture of pre-spermatogenic testes with Fsh (Crespo et al., 2016). Moreover, the administration of an inhibitor of RA synthesis in combination with a deficient diet of vitamin A (a precursor of RA) to adult zebrafish also induced a downregulation of *cyp26a1*, most likely to increase intracellular RA levels, although spermatogenesis was still disrupted, and fertility compromised (Pradhan and Olsson, 2015). The above mentioned studies suggest that a decrease in *cyp26* expression is associated with the onset of spermatogenesis and the initiation of meiosis.

To gain more insight on the importance of the RA signaling pathway in meiosis we focussed on the cellular expression sites of *rxrα* and *cyp26a1* in testicular samples. The *in situ* hybridization results show *rxrα* localization within the testicular lobules, specifically in the germ

cells, since the signal, although weak, co-localized with that of *vasa*, an important maternal regulator of primordial germ cells (Cardinali et al., 2004) widely used to discriminate PGCs in fish (Olsen et al., 1997; Yoon et al., 1997). In addition, high *vasa* expression levels have been detected during germ cell proliferation in the European sea bass (Blázquez et al., 2011). Moreover, strong *vasa* expression has been found in spermatogonia of fish such as tilapia (Kobayashi et al., 2000), gibel carp (Xu et al., 2005), zebrafish (Leal et al., 2009) and gilthead sea bream (Cardinali et al., 2004), further confirming germ cell-specific espression of this gene. In addition, in mice, *Rxrα* transcripts are specifically detected in round spermatids located close to the epithelium of the luminal side of the seminiferous tubules during late maturation indicating its expression in cells of the germinal lineage (Kastner et al., 1996; Vernet et al., 2006).

Regarding the main cell type for expression of *cyp26a1* we could localize it closer to the somatic cells located between the testicular lobules and also inside of them although it was not consistent in all testicular lobules. In fish, *cyp26a1* has been detected in adult testes of zebrafish as a subset of cells in a site expected for Leydig cells, although the presence of germ cells expressing *cyp26a1* could not be discarded (Rodríguez-Marí et al., 2013). In agreement with our results, *cyp26a1* signal could not be detected in medaka testis (Adolfi et al., 2016). It is well accepted that the cytochrome p450 hydrolase enzymes are expressed in cells that may generate a catabolic barrier to RA action. This compartmentalization could modulate, in a paracrine manner, cell proliferation and spermatogenesis (Vernet et al., 2006). Our microarray results show that *cyp26a1* expression reaches lowest levels in stage II, precisely the stage used for the *in situ* hybridization study. It could thus be that using samples in a different developmental stage may enable the exact identification of the specific expression sites. A similar scenario could be responsible for the results obtained for *star*, a regulator of steroid production during cholesterol transport across the mitochondrial membrane (Stocco and Clark, 1996) used as a molecular marker for Leydig cells in *Senegalese sole* (Chauvigne et al., 2012). We could not find a consistent signal for this marker within the outer layer of the testicular lobules that enabled us to identify putative Leydig cells. In the European sea bass, the expression of *star* strongly correlates with the expression of *lhr*, the gene encoding Lh receptor (Rocha et al., 2009), linked to final maturation and spermiation (Kagawa et al., 1998; Miwa et al., 1994; Planas and Swanson, 1995; Swanson et al., 1991). Since we have used testis in stage II for the *in situ* hybridization study, coinciding with early stages of spermatogenesis and thus low *star* levels (Rocha et al.2009), this could explain the absence of a clear detectable signal for this transcript that would allow us to identify Leydig cells.

CONCLUSION

To the best of our knowledge, this is the first transcriptomic study focussed on the early stages of puberty, and aimed at the identification of molecular and endocrine signals triggering the start of the initial spermatogenic wave in European sea bass. Increases in androgen plasma levels, particularly 11KT, mark the transition between testicular stage I and stage II. This opens the possibility to explore the use of 11KT in the management of sea bass stocks in aquaculture farms to separate precocious from non-precocious males. The study improved the annotation of different genes of the microarray and helped to increase the knowledge of several mechanisms and biological pathways involved in early stages of puberty. Altogether, the study showed that the onset of spermatogenesis is characterized by the activation of genes involved in cell cycle progression and division including mitosis and meiosis. The differential expression of several components of the RA signaling pathway suggests their important role in the onset of meiosis but further *in situ* hybridization experiments are clearly needed to verify the cell-specific expression sites of specific molecular markers for the onset of meiosis. This work lays the foundation for an in-depth study of the RA signaling pathway and its role in the onset of meiosis in fish. A future increase in the sequencing of the European sea bass gonad transcriptome and the use of RNA-seq technologies will help to shed light on the molecular pathways involved in relevant aspects of the reproductive process of this economically important species and will aid to develop comparative studies on gonadal differentiation and maturation in teleosts.

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REFERENCE LIST

Adolfi, M. C., Herpin, A., Regensburger, M., Sacquegno, J., Waxman, J. S., and Schartl, M. (2016). Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. *Scientific reports* **6**, 34281.

Agulleiro, M. J., Cortés, R., Leal, E., Ríos, D., Sánchez, E., and Cerdá-Reverter, J. M. (2014). Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (Dicentrarchus labrax). *Gen Comp Endocrinol* **205**, 251-259.

Alvarado, M. V., Carrillo, M., and Felip, A. (2013). Expression of kisspeptins and their Receptors, gnrh-1/gnrhr-II-1a and gonadotropin genes in the brain of adult male and female European sea bass during different gonadal stages. *Gen Comp Endocrinol* **187**, 104-116.

Begtashi, I., Rodríguez, L., Moles, G., Zanuy, S., and Carrillo, M. (2004). Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (Dicentrarchus labrax, L.). I. Morphological aspects. *Aquaculture* **241**, 539-559.

Bennett, H. S., Wyrick, A. D., Lee, S. W., and McNeil, J. H. (1976). Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. *Stain Technology* **51**, 71-97.

Blázquez, M., Bosma, P. T., Chang, J. P., Docherty, K., and Trudeau, V. L. (1998). γ-Aminobutyric Acid Up-Regulates the Expression of a Novel Secretogranin-II Messenger Ribonucleic Acid in the Goldfish Pituitary 1. *Endocrinology* **139**, 4870-4880.

Blázquez, M., González, A., Mylonas, C. C., and Piferrer, F. (2011). Cloning and sequence analysis of a vasa homolog in the European sea bass (Dicentrarchus labrax): Tissue distribution and mRNA expression levels during early development and sex differentiation. *Gen Comp Endocrinol* **170**, 322-333.

Blázquez, M., González, A., Papadaki, M., Mylonas, C., and Piferrer, F. (2008). Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (Dicentrarchus labrax). *Gen Comp Endocrinol* **158**, 95-101.

Blázquez, M., and Shennan, K. I. (2000). Basic mechanisms of secretion: sorting into the regulated secretory pathway. *Biochemistry and Cell Biology* **78**, 181-191.

Boj, M., Chauvigné, F., Zapater, C., and Cerdà, J. (2015). Gonadotropin-Activated Androgen-Dependent and Independent Pathways Regulate Aquaporin Expression during Teleost (Sparus aurata) Spermatogenesis. *PLoS One* **10**, e0142512.

Cao, M., Yang, Y., Xu, H., Duan, J., Cheng, N., Wang, J., Hu, W., and Zhao, H. (2012). Germ cell specific expression of Vasa in rare minnow, Gobiocypris rarus. *Comp Biochem Physiol A Mol Integr Physiol* **162**, 163-70.

Cardinali, M., Gioacchini, G., Candiani, S., Pestarino, M., Yoshizaki, G., and Carnevali, O. (2004). Hormonal Regulation of Vasa-Like Messenger RNA Expression in the Ovary of the Marine Teleost Sparus aurata. *Biol Reprod* **70**, 737-743.

Carrillo, M., Espigares, F., Felip, A., Escobar, S., Molés, G., Rodríguez, R., Alvarado, M. V., Gómez, A., and Zanuy, S. (2015). Updating control of puberty in male European sea bass: A holistic approach. *Gen Comp Endocrinol* **221**, 42-53.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674-3676.

Crespo, B., Gómez, A., Mazón, M. J., Carrillo, M., and Zanuy, S. (2013). Isolation and characterization of Ff1 and Gsdf family genes in European sea bass and identification of early gonadal markers of precocious puberty in males. *Gen Comp Endocrinol* **191**, 155-167.

Crespo, D., Assis, L. H. C., Furmanek, T., Bogerd, J., and Schulz, R. W. (2016). Expression profiling identifies Sertoli and Leydig cell genes as Fsh targets in adult zebrafish testis. *Mol Cell Endocrinol* **437**, 237-251.

Chauvigne, F., Verdura, S., Mazon, M. J., Duncan, N., Zanuy, S., Gomez, A., and Cerda, J. (2012). Follicle-stimulating hormone and luteinizing hormone mediate the androgenic pathway in Leydig cells of an evolutionary advanced teleost. *Biol Reprod* **87**, 35.

Chung, S. S., and Wolgemuth, D. J. (2004). Role of retinoid signaling in the regulation of spermatogenesis. *Cytogenet Genome Res* **105**, 189-202.

Escobar, S., Rocha, A., Felip, A., Carrillo, M., Zanuy, S., Kah, O., and Servili, A. (2016). Leptin receptor gene in the European sea bass (Dicentrarchus labrax): Cloning, phylogeny, tissue distribution and neuroanatomical organization. *Gen Comp Endocrinol* **229**, 100-111.

Escobar, S., Servili, A., Espigares, F., Gueguen, M.-M., Brocal, I., Felip, A., Gómez, A., Carrillo, M., Zanuy, S., and Kah, O. (2013). Expression of kisspeptins and kiss receptors suggests a large range of functions for kisspeptin systems in the brain of the European sea bass. *PLoS One* **8**, e70177.

Espigares, F., Rocha, A., Molés, G., Gómez, A., Carrillo, M., and Zanuy, S. (2015). New insights into the factors mediating the onset of puberty in sea bass. *Gen Comp Endocrinol* **224**, 176-185.

Fabra, M., Raldúa, D., Bozzo, M. G., Deen, P. M. T., Lubzens, E., and Cerdà, J. (2006). Yolk proteolysis and aquaporin-1o play essential roles to regulate fish oocyte hydration during meiosis resumption. *Dev Biol* **295**, 250-262.

Felip, A., Zanuy, S., and Carrillo, M. (2006). Comparative analysis of growth performance and sperm motility between precocious and non-precocious males in the European sea bass (Dicentrarchus labrax, L.). *Aquaculture* **256**, 570-578.

Felip, A., Zanuy, S., Muriach, B., Cerdá-Reverter, J. M., and Carrillo, M. (2008). Reduction of sexual maturation in male Dicentrarchus labrax by continuous light both before and during gametogenesis. *Aquaculture* **275**, 347-355.

Felip, A., Zanuy, S., Pineda, R., Pinilla, L., Carrillo, M., Tena-Sempere, M., and Gómez, A. (2009). Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. *Mol Cell Endocrinol* **312**, 61-71.

Feng, R., Fang, L., Cheng, Y., He, X., Jiang, W., Dong, R., Shi, H., Jiang, D., Sun, L., and Wang, D. (2015). Retinoic acid homeostasis through aldh1a2 and cyp26a1 mediates meiotic entry in Nile tilapia (Oreochromis niloticus). *Scientific reports* **5**, 10131.

Franco, A., Jouaux, A., Mathieu, M., Sourdaine, P., Lelong, C., Kellner, K., and Heude Berthelin, C. (2010). Proliferating cell nuclear antigen in gonad and associated storage tissue of the Pacific oyster Crassostrea gigas: seasonal immunodetection and expression in laser microdissected tissues. *Cell Tissue Res* **340**, 201-10.

Fuster, G., Vicente, R., Coma, M., Grande, M., Felipe, A., and Pinto Garcia, V. (2002). One-step reverse transcription polymerase chain reaction for semiquantitative analysis of mRNA expression. *Methods and findings in experimental and clinical pharmacology* **24**, 253-260.

Garcia-Lopez, A., Bogerd, J., Granneman, J. C., van Dijk, W., Trant, J. M., Taranger, G. L., and Schulz, R. W. (2009). Leydig cells express follicle-stimulating hormone receptors in African catfish. *Endocrinology* **150**, 357-65.

García-Lopez, A., de Jonge, H., Nobrega, R. H., de Waal, P. P., van Dijk, W., Hemrika, W., Taranger, G. L., Bogerd, J., and Schulz, R. W. (2010). Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology* **151**, 2349-2360.

Giffard-Mena, I., Boulo, V., Aujoulat, F., Fowden, H., Castille, R., Charmantier, G., and Cramb, G. (2007). Aquaporin molecular characterization in the sea-bass (Dicentrarchus labrax): the effect of salinity on AQP1 and AQP3 expression. *Comp Biochem Physiol A Mol Integr Physiol* **148**, 430-44.

Girardet, C., and Butler, A. A. (2014). Neural melanocortin receptors in obesity and related metabolic disorders. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **1842**, 482-494.

González-Martínez, D., Madigou, T., Mañanos, E., Cerdá-Reverter, J. M., Zanuy, S., Kah, O., and Muñoz-Cueto, J. A. (2004). Cloning and expression of gonadotropin-releasing hormone receptor in the brain and pituitary of the European sea bass: an in situ hybridization study. *Biol Reprod* **70**, 1380-1391.

González-Martínez, D., Zmora, N., Mañanos, E., Saligaut, D., Zanuy, S., Zohar, Y., Elizur, A., Kah, O., and Muñoz-Cueto, J. A. (2002). Immunohistochemical localization of three different prepro-GnRHs in the brain and pituitary of the European sea bass (Dicentrarchus labrax) using antibodies to the corresponding GnRH-associated peptides. *Journal of Comparative Neurology* **446**, 95-113.

Griswold, M. D. (1998). The central role of Sertoli cells in spermatogenesis. *In* "Seminars in cell & developmental biology", Vol. 9, pp. 411-416. Elsevier.

Guillot, R., Cortés, R., Navarro, S., Mischitelli, M., García-Herranz, V., Sánchez, E., Cal, L., Navarro, J. C., Míguez, J. M., and Afanasyev, S. (2016). Behind melanocortin antagonist overexpression in the zebrafish brain: a behavioral and transcriptomic approach. *Hormones and Behavior* **82**, 87-100.

Guiry, A., Flynn, D., Hubert, S., O'Keeffe, A. M., LeProvost, O., White, S. L., Forde, P. F., Davoren, P., Houeix, B., and Smith, T. J. (2010). Testes and brain gene expression in precocious male and adult maturing Atlantic salmon (Salmo salar). *BMC Genomics* **11**, 211.

Ilnytska, O., Sözen, M. A., Dauterive, R., and Argyropoulos, G. (2009). Control Elements in the Neighboring ATPase Gene Influence Spatiotemporal Expression of the Human Agouti-Related Protein. *Journal of Molecular Biology* **388**, 239-251.

Josso, N., Picard, J. Y., Rey, R., and di Clemente, N. (2006). Testicular anti-Mullerian hormone: history, genetics, regulation and clinical applications. *Pediatric endocrinology reviews: PER* **3**, 347-358.

Kagawa, H., Tanaka, H., Okuzawa, K., and Kobayashi, M. (1998). GTH II but not GTH I induces final maturation and the development of maturational competence of oocytes of red seabreamin vitro. *Gen Comp Endocrinol* **112**, 80-88.

Kastner, P., Mark, M., Leid, M., Gansmuller, A., Chin, W., Grondona, J. M., Decimo, D., Krezel, W., Dierich, A., and Chambon, P. (1996). Abnormal spermatogenesis in RXR beta mutant mice. *Genes & development* **10**, 80-92.

Kline-Smith, S. L., Sandall, S., and Desai, A. (2005). Kinetochore–spindle microtubule interactions during mitosis. *Current opinion in cell biology* **17**, 35-46.

Kobayashi, M., Aida, K., and Stacey, N. E. (1991). Induction of testis development by implantation of 11-ketotestosterone in female goldfish. *Zoological Science* **8**, p389-393.

Kobayashi, T., Kajiura-Kobayashi, H., and Nagahama, Y. (2000). Differential expression of vasa homologue gene in the germ cells during oogenesis and spermatogenesis in a teleost fish, tilapia, Oreochromis niloticus. *Mech Dev* **99**, 139-142.

Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., and Young, G. (2006). Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. *Journal of Endocrinology* **189**, 541-554.

Lara-Gonzalez, P., Westhorpe, F. G., and Taylor, S. S. (2012). The spindle assembly checkpoint. *Current Biology* **22**, R966-R980.

Leal, M. C., Cardoso, E. R., Nóbrega, R. H., Batlouni, S. R., Bogerd, J., França, L. R., and Schulz, R. W. (2009). Histological and Stereological Evaluation of Zebrafish (Danio rerio) Spermatogenesis with an Emphasis on Spermatogonial Generations 1. *Biol Reprod* **81**, 177-187.

Li, X., and Schimenti, J. C. (2007). Mouse pachytene checkpoint 2 (trip13) is required for completing meiotic recombination but not synapsis. *PLoS Genet* **3**, e130.

Mateos, J., Mañanós, E. L., Swanson, P., Carrillo, M., and Zanuy, S. (2006). Purification of luteinizing hormone (LH) in the sea bass (Dicentrarchus labrax) and development of a specific immunoassay| Purificación de la hormona luteinizante (LH) en la lubina (Dicentrarchus labrax) y desarrollo de un inmunoensayo específico.

Mazón, M. J., Gómez, A., Yilmaz, O., Carrillo, M., and Zanuy, S. (2014). Administration of Follicle-Stimulating Hormone In Vivo Triggers Testicular Recrudescence of Juvenile European Sea Bass (Dicentrarchus labrax). *Biol Reprod* **90**, 6, 1-10.

Mazón, M. J., Molés, G., Rocha, A., Crespo, B., Lan-Chow-Wing, O., Espigares, F., Muñoz, I., Felip, A., Carrillo, M., and Zanuy, S. (2015). Gonadotropins in European sea bass: endocrine roles and biotechnological applications. *Gen Comp Endocrinol* **221**, 31-41.

McDowell, E., and Trump, B. (1976). Histologic fixatives suitable for diagnostic light and electron microscopy. *Archives of pathology & laboratory medicine* **100**, 405-414.

Mendenhall, M. D., and Hodge, A. E. (1998). Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews* **62**, 1191-1243.

Mitter, K., Kotoulas, G., Magoulas, A., Mulero, V., Sepulcre, P., Figueras, A., Novoa, B., and Sarropoulou, E. (2009). Evaluation of candidate reference genes for QPCR during ontogenesis and of immune-relevant tissues of European seabass (Dicentrarchus labrax). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **153**, 340-347.

Miura, T., and Miura, C. (2003). Molecular control mechanisms of fish spermatogenesis. *Fish Physiol Biochem* **28**, 181-186.

Miura, T., Miura, C., Konda, Y., and Yamauchi, K. (2002). Spermatogenesis-preventing substance in Japanese eel. *Development* **129**, 2689-2697.

Miura, T., Yamauchi, K., Takahashi, H., and Nagahama, Y. (1991). Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (Anguilla japonica). *Proceedings of the National Academy of Sciences* **88**, 5774-5778.

Miwa, S., Yan, L., and Swanson, P. (1994). Localization of two gonadotropin receptors in the salmon gonad by in vitro ligand autoradiography. *Biol Reprod* **50**, 629-642.

Moldovan, G.-L., Pfander, B., and Jentsch, S. (2007). PCNA, the Maestro of the Replication Fork. *Cell* **129**, 665-679.

Molés, G., Gómez, A., Carrillo, M., Rocha, A., Mylonas, C. C., and Zanuy, S. (2011). Determination of Fsh Quantity and Bioactivity During Sex Differentiation and Oogenesis in European Sea Bass1. *Biol Reprod* **85**, 848-857.

Molés, G., Gómez, A., Carrillo, M., and Zanuy, S. (2012). Development of a homologous enzyme-linked immunosorbent assay for European sea bass FSH. Reproductive cycle plasma levels in both sexes and in yearling precocious and non-precocious males. *Gen Comp Endocrinol* **176**, 70-78.

Monje-Casas, F., Prabhu, V. R., Lee, B. H., Boselli, M., and Amon, A. (2007). Kinetochore orientation during meiosis is controlled by Aurora B and the monopolin complex. *Cell* **128**, 477-490.

Muller, P. Y., Janovjak, H., Miserez, A. R., and Dobbie, Z. (2002). Short technical report processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* **32**, 1372-1379.

Nóbrega, R. H., Batlouni, S. R., and França, L. R. (2009). An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. *Fish Physiol Biochem* **35**, 197-206.

Olsen, L. C., Aasland, R., and Fjose, A. (1997). A vasa-like gene in zebrafish identifies putative primordial germ cells. *Mech Dev* **66**, 95-105.

Pfennig, F., Standke, A., and Gutzeit, H. O. (2015). The role of Amh signaling in teleost fish–multiple functions not restricted to the gonads. *Gen Comp Endocrinol* **223**, 87-107.

Planas, J. V., and Swanson, P. (1995). Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) in vitro. *Biol Reprod* **52**, 697-704.

Pradhan, A., and Olsson, P.-E. (2015). Inhibition of retinoic acid synthesis disrupts spermatogenesis and fecundity in zebrafish. *Gen Comp Endocrinol* **217**, 81-91.

Prat, F., Zanuy, S., Carrillo, M., de Mones, A., and Fostier, A. (1990). Seasonal changes in plasma levels of gonadal steroids of sea bass, Dicentrarchus labrax L. *Gen Comp Endocrinol* **78**, 361-373.

Reinecke, M. (2009). Insulin-like growth factors and fish reproduction. *Biol Reprod* **82**, 656-661.

Relógio, A., Schwager, C., Richter, A., Ansorge, W., and Valcárcel, J. (2002). Optimization of oligonucleotide-based DNA microarrays. *Nucleic Acids Research* **30**, e51-e51.

Rocha, A., Zanuy, S., Carrillo, M., and Gómez, A. (2009). Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. *Gen Comp Endocrinol* **162**, 265-275.

Rodríguez-Marí, A., Cañestro, C., BreMiller, R. A., Catchen, J. M., Yan, Y.-L., and Postlethwait, J. H. (2013). Retinoic Acid Metabolic Genes, Meiosis, and Gonadal Sex Differentiation in Zebrafish. *PLoS One* **8**, e73951.

Rodríguez, L., Begtashi, I., Zanuy, S., and Carrillo, M. (2000). Development and validation of an enzyme immunoassay for testosterone: Effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass (Dicentrarchus labrax, L.) at puberty. *Fish Physiol Biochem* **23**, 141-150.

Rodríguez, L., Begtashi, I., Zanuy, S., and Carrillo, M. (2005). Long-term exposure to continuous light inhibits precocity in European male sea bass (Dicentrarchus labrax, L.): hormonal aspects. *Gen Comp Endocrinol* **140**, 116-125.

Rodrıguez, L., Carrillo, M., Sorbera, L. A., Zohar, Y., and Zanuy, S. (2004). Effects of photoperiod on pituitary levels of three forms of GnRH and reproductive hormones in the male European sea bass (Dicentrarchus labrax, L.) during testicular differentiation and first testicular recrudescence. *Gen Comp Endocrinol* **136**, 37-48.

Rolland, A. D., Lardenois, A., Goupil, A.-S., Lareyre, J.-J., Houlgatte, R., Chalmel, F., and Le Gac, F. (2013). Profiling of androgen response in rainbow trout pubertal testis: relevance to male gonad development and spermatogenesis. *PLoS One* **8**, e53302.

Rolland, A. D., Lareyre, J. J., Goupil, A. S., Montfort, J., Ricordel, M. J., Esquerre, D., Hugot, K., Houlgatte, R., Chalmel, F., and Le Gac, F. (2009). Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. *BMC Genomics* **10**, 546.

Sambroni, E., Lareyre, J.-J., and Le Gac, F. (2013). Fsh controls gene expression in fish both independently of and through steroid mediation. *PLoS One* **8**, e76684.

Satisbury, J. L. (1995). Centrin, centrosomes, and mitotic spindle poles. *Current opinion in cell biology* **7**, 39-45.

Schug, T. T., Berry, D. C., Shaw, N. S., Travis, S. N., and Noy, N. (2007). Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. *Cell* **129**, 723-733.

Schultz, D. R., Perez, N., Mendez, A. J., Snodgrass, D., Serafy, J. E., Prince, E. D., Crow, W. A., and Capo, T. R. (2007). Tracking gender factors in fish surface mucus: temporal patterns in individual Koi (Cyprinus carpio). *Journal of Applied Ichthyology* **23**, 184-188.

Schultz, D. R., Perez, N., Tan, C. K., Mendez, A. J., Capo, T. R., Snodgrass, D., Prince, E. D., and Serafy, J. E. (2005). Concurrent levels of 11-ketotestosterone in fish surface mucus, muscle tissue and blood. *Journal of Applied Ichthyology* **21**, 394-398.

Schulz, R. W., de Franca, L. R., Lareyre, J. J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R. H., and Miura, T. (2010). Spermatogenesis in fish. *Gen Comp Endocrinol* **165**, 390-411.

Schulz, R. W., and Miura, T. (2002). Spermatogenesis and its endocrine regulation. *Fish Physiol Biochem* **26**, 43-56.

Skaar, K. S., Nobrega, R. H., Magaraki, A., Olsen, L. C., Schulz, R. W., and Male, R. (2011). Proteolytically activated, recombinant Anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology* **152**, 3527-3540.

Slegtenhorst-Eegdeman, K. E., Post, M., Baarends, W. M., Themmen, A. P. N., and Grootegoed, J. A. (1995). Regulation of gene expression in Sertoli cells by follicle-stimulating hormone (FSH): cloning and characterization of LRPR1, a primary response gene encoding a leucine-rich protein. *Mol Cell Endocrinol* **108**, 115-124.

Smith, C., Roeszler, K., Bowles, J., Koopman, P., and Sinclair, A. (2008). Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. *BMC Developmental Biology* **8**, 85.

Stocco, D. M., and Clark, B. J. (1996). Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews* **17**, 221-244.

Strich, R. (2004). Meiotic DNA replication.

Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biol Reprod* **44**, 29-38.

Taranger, G. L., Carrillo, M., Schulz, R. W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.-A., Dufour, S., Karlsen, Ø., Norberg, B., Andersson, E., and Hansen, T. (2010a). Control of puberty in farmed fish. *Gen Comp Endocrinol* **165**, 483-515.

Tine, M., Kuhl, H., Gagnaire, P.-A., Louro, B., Desmarais, E., Martins, R. S. T., Hecht, J., Knaust, F., Belkhir, K., and Klages, S. (2014). European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nature communications* **5**.

Tingaud-Sequeira, A., Chauvigne, F., Fabra, M., Lozano, J., Raldua, D., and Cerda, J. (2008). Structural and functional divergence of two fish aquaporin-1 water channels following teleost-specific gene duplication. *BMC Evol Biol* **8**, 259.

Unhavaithaya, Y., and Orr-Weaver, T. L. (2013). Centromere proteins CENP-C and CAL1 functionally interact in meiosis for centromere clustering, pairing, and chromosome segregation. *Proceedings of the National Academy of Sciences* **110**, 19878-19883.

Vernet, N., Dennefeld, C., Guillou, F., Chambon, P., Ghyselinck, N. B., and Mark, M. (2006). Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells. *The EMBO journal* **25**, 5816-5825.

Volkoff, H., Canosa, L. F., Unniappan, S., Cerda-Reverter, J. M., Bernier, N. J., Kelly, S. P., and Peter, R. E. (2005). Neuropeptides and the control of food intake in fish. *Gen Comp Endocrinol* **142**, 3-19.

White, J. A., Guo, Y.-D., Baetz, K., Beckett-Jones, B., Bonasoro, J., Hsu, K. E., Dilworth, F. J., Jones, G., and Petkovich, M. (1996). Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. *Journal of Biological Chemistry* **271**, 29922-29927.

Windley, S. P., and Wilhelm, D. (2016). Signaling Pathways Involved in Mammalian Sex Determination and Gonad Development. *Sexual Development* **9**, 297-315.

Wu, G. C., Chiu, P. C., Lyu, Y. S., and Chang, C. F. (2010). The expression of amh and amhr2 is associated with the development of gonadal tissue and sex change in the protandrous black porgy, Acanthopagrus schlegeli. *Biol Reprod* **83**, 443-53.

Wuertz, S., Gessner, J., Kirschbaum, F., and Kloas, W. (2007). Expression of IGF-I and IGF-I receptor in male and female sterlet, Acipenser ruthenus--evidence for an important role in gonad maturation. *Comp Biochem Physiol A Mol Integr Physiol* **147**, 223-30.

Xu, H., Gui, J., and Hong, Y. (2005). Differential expression of vasa RNA and protein during spermatogenesis and oogenesis in the gibel carp (Carassius auratus gibelio), a bisexually and gynogenetically reproducing vertebrate. *Developmental Dynamics* **233**, 872-882.

Yoon, C., Kawakami, K., and Hopkins, N. (1997). Zebrafish vasa homologue RNA is localized to the cleavage planes of 2-and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development* **124**, 3157-3165.

Zanuy, S., Carrillo, M., Felip, A., Rodríguez, L., Blázquez, M., Ramos, J., and Piferrer, F. (2001). Genetic, hormonal and environmental approaches for the control of reproduction in the European sea bass (Dicentrarchus labrax L.). *Aquaculture* **202**, 187-203.

Zhao, E., Basak, A., Wong, A. O., Ko, W., Chen, A., Lopez, G. C., Grey, C. L., Canosa, L. F., Somoza, G. M., Chang, J. P., and Trudeau, V. L. (2009). The secretogranin II-derived peptide secretoneurin stimulates luteinizing hormone secretion from gonadotrophs. *Endocrinology* **150**, 2273-82.

Zhao, E., Hu, H., and Trudeau, V. L. (2010). Secretoneurin as a hormone regulator in the pituitary. *Regulatory peptides* **165**, 117-122.

Zilli, L., Schiavone, R., Chauvigne, F., Cerda, J., Storelli, C., and Vilella, S. (2009). Evidence for the involvement of aquaporins in sperm motility activation of the teleost gilthead sea bream (Sparus aurata). *Biol Reprod* **81**, 880-8.

Zohar, Y., Muñoz-Cueto, J. A., Elizur, A., and Kah, O. (2010). Neuroendocrinology of reproduction in teleost fish. *Gen Comp Endocrinol* **165**, 438-455.

CHAPTER 1 ADDITIONAL METHODOLOGY

In situ hybridization probe synthesis

Specific primers were designed for *in situ* hybridization of *cyp26a1, rxrα*, *vasa* and *star* genes. Primers for each gene were designed using primer 3 software (Additional table 1). A conventional PCR was performed for each gene, using a pool of four cDNA samples from, 2 year-old male testis as template. The following amplification conditions were used: initial 5 min at 94ºC for a starting denaturation step, followed by 40 cycles consisting on: 30 sat 94ºC for the cycle denaturation step, 30 s at 60ºC for the primer annealing step, and 1.5 min at 72ºC for the extension step, followed by a final 7 min at 72ºC as the final extension step. In order to avoid unspecific amplification products, a secondary amplification was performed using purified PCR products (E.Z.N.A.® Gel Purification kit, Omega Biotek, Norcross, GA, USA) as templates and the same conditions as before.

The band of the corresponding size (Additional table 1) was cut out of an agarose gel, purified with E.Z.N.A.® Gel Purification kit (Omega Biotek, Norcross, GA, USA), and quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The PCR products were cloned using pGEM-T easy vector kit (Promega) in *E. coli* competent cells following manufacturer's instructions. A mini preparation (miniprep) was carried out to screen for colonies that included the plasmid with the corresponding insert (described in miniprep section). Plasmids were digested using SpeI and AaTII (Biolabs New England, USA) restriction enzymes at 37ºC during 3 hours for *cyp26a1* and *rxrα* probes respectively, and for *vasa* and *star*

control probes EcoRI (Biolabs New England, USA) was used according to the manufacturer's instructions. Transcripts were visualized in agarose gel electrophoresis and bands of the proper size were cut out and purified using P:C:I purification technique (described in Phenol: Chloroform: Isoprolylalcohol DNA purification technique section). The DNA was quantified in NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and sequenced with universal primers by the IBMCP sequencing service, in Valencia, Spain.

A maxiprep was carried out following the manufacturer`s instructions (maxiprep Pure Link High pure plasmid max prep kit (Sigma-Aldrich, St Louis, MO, USA), using colonies that had included the correct sequence insert. Briefly, cells were grown overnight at 37ºC and harvested by centrifuging at 4000x*g* for 10 min, resuspended and lysated for 5 min using the buffers included in the kit. The mixture was precipitated and loaded in a Filter Maxi column, washed, and the DNA eluted out of the column. DNA was precipitated with isopropanol and centrifuged at 16000*xg* during 30 min at 4ºC. The pellet was washed on 100% ethanol, centrifuged, and dried. Finally, samples were diluted in 200µl nuclease free water, and DNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The plasmids were digested using the same restriction enzymes mentioned for the minipreps. Transcripts of the correct size were visualized in agarose gel electrophoresis and bands of the proper size cut out and purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences Ltd, Buckinghamshire, England), according to manufacturer's instructions.

Antisense and sense mRNA probes were obtained using the purified plasmids as template for *in vitro* transcription with SP6 and T7 RNA polymerases (Promega Co. Madison, WI, USA). Finally, probes were labelled with DIG RNA labelling mix (Roche Diagnostic Co., Indianapolis, Indiana, USA) (Additional table 1). Briefly, a 2 h incubation at 37ºC or 42ºC was carried out following manufacturer's instructions, followed by a 30 min at 37ºC DNAse treatment. The reaction was stopped with EDTA and samples purified in Nucleospin RNA clean-up kit (Macherey-Nagel Co., Diiren, Germany). Synthesized probes were quantified (NanoDrop 2000 spectrophotometer Thermo Scientific, Wilmington, DE, USA) and stored at -80ºC for *in situ* hybridizations.

In situ **hybridization**

The *rxrα* and *cyp26a1*genes were selected as putative molecular markers for germ cells and Sertoli cells, respectively, based upon: a significant decrease of expression that did not reach

undetectable levels, a high fold change (FC), their particular tissue distribution and the sites of expression found in other species (Adolfi et al., 2016; Rodríguez-Marí et al., 2013). In order to localize the expression of *cyp26a1* and *rxrα* mRNA in the testis, an *in situ* hybridization was performed using *vasa* and *star* genes as control for expression in germ cells and Leidig cells, respectively.

Testis samples of four 2 year-old males, were fixed overnight in 4% PAF solution, 0.1M phosphate buffer and 5% picric acid. Samples were dehydrated, in increasing concentrations of ethanol DEPC solution, embedded in paraffin (Thermo Scientific, Wilmington, DE, USA) and cut transversally in six parallel series at a 6µm. Sections were mounted onto poly-L-lysine–coated slides and kept under RNases-free environment. One series of 3 or 4 sections was used for the antisense probe, other for the sense probe (negative control), and one for a conventional histology procedure described above, stained with hematoxilin-eosin standard protocol (Bennett et al., 1976).

The *in situ* hybridization procedure was carried out according to (Escobar et al., 2013) technique modified from (Servili et al., 2011). Paraffin was removed submersing samples in Ottix plus (Diapath, Italy) twice in 10 min baths at room temperature. Sample re-hydration was performed through decreasing concentrations of ethanol and 2 final PBS (0.1Mphosphate buffer, pH7.4) baths, followed by fixation in 4% PAF buffered solution during 20 min. Sections were incubated with proteinase k during 5 min at room temperature (10mg/mlin 50nM Tris-HCL, pH8, 0.5mM EDTA), rinsed, and post-fixed in 4% PAF. Slides were rinsed twice in saline-sodium citrate (SSC) 2X at room temperature.

Antisense probe hybridizations were performed overnight at 65ºC in a RNase-free humid chamber using 100µl of hybridization buffer (2X SSC; 2.5% dextran sulphate; 50% deionized formamide; 5X Denhardt's solution; 50µg/ml of yeast tRNA, pH8.0;4mM EDTA), which included 0.5µg/ml of the DIG-labeled probe.

After hybridization, sections were rinsed twice in 2X SSC at 65ºC, followed by 2 additional rinses of 2xSSC/50% formamide over 30 min, and finally 0.2X and 0.1X SSC at room temperature during 15 min each. Sections were incubated for 10 min in 100mMtris-HCl buffer, 150mMNaCl, pH7.5), blocked during 30 min in the same solution by adding 0.5% blocking agent and 0.2% triton-100. Followed by an overnight incubation at room temperature in alkaline phosphatase-conjugated sheep fab fragments antibodies to digoxigenin diluted 1/2000 (Roche Diagnostic Co., Indianapolis, IN, USA).

On the next day sections were incubated for fluorescent detection with HNPP (2-hydroxy-3-naphtoic acid-2`-phenylanilide phosphate) in HPP/fastRED solution (Roche Diagnostic Co., Indianapolis, Indiana, USA) for 3 h. PBS and PBS/tween washes were carried out for 10 min each. Finally, slides were coversliped with Vecta Shield Hard Set mounting medium containing 4',6-diamidino-2-phenilindone (DAPI). To confirm probe specificities, parallel series of slides were hybridized with their corresponding sense counterparts and these hybridizations yielded no signal.

Sections were observed under UV light microscope and images captured in a ProGres3 camera and ProGres software. Sample slides were stored at 4ºC, protected from light and humidity.

Miniprep

At least 10 well identified transformed colonies (check cloning procedures) were inoculated overnight in liquid LB amp^+ culture medium (10mg/ml triptone, 5g/ml yeast extract, 10mg/ml NaCl, p7.0, 1µl/ml ampicillin (Sigma-Aldrich, St Louis, MO, USA) at 37ºC. Bacterial cultures were pelleted (6000rpm) and supernatant eliminated, a solution of 0.2N NaOH, 1%SDS was added and incubated on ice during 5 min. A solution of AcK (5M Ac-3M K) was added and incubated for 10 min for cell lysis. A 13000rpm centrifugation separated the debris and the supernatant reserved. DNA from the supernatant was precipitated with 0.6ml isopropanol and centrifuged at 13,000rpm during 15 min at 4ºC. The remaining pellet was dried at room temperature and resuspended in DNAse free water.

Phenol: Chloroform: Isoprolylalcohol DNA purification technique.

DNA from the minipreps was purified by the P:C:I (Sigma-Aldrich, St Louis, MO, USA) technique, following manufacturer's instructions. Briefly 100µl of P:C:I were vortexed along with 100µl sample. After a 2 min centrifugation (13,000rpm), supernatant was reserved and 100µl of chloroform added. Samples were centrifuged at 13,000rpm for 2 min and 250µl of cold 100% ethanol were added. Samples were incubated during 1 h at -80ºC and centrifuged at 13,000rpm for 30 min at 4ºC. Supernatant was eliminated and cold 75% ethanol added. Samples were vortexed and centrifuged at 13,000rpm for 10 min at 4ºC. Afterwards the supernatant was eliminated and the pellet dried at room temperature. Finally, DNA was resuspended in 20µl of DNAse free water.

REFFERENCE LIST

Adolfi, M. C., Herpin, A., Regensburger, M., Sacquegno, J., Waxman, J. S., and Schartl, M. (2016). Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. *Scientific reports* **6**, 34281.

Bennett, H. S., Wyrick, A. D., Lee, S. W., and McNeil, J. H. (1976). Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. *Stain Technology* **51**, 71-97.

Escobar, S., Servili, A., Espigares, F., Gueguen, M.-M., Brocal, I., Felip, A., Gómez, A., Carrillo, M., Zanuy, S., and Kah, O. (2013). Expression of kisspeptins and kiss receptors suggests a large range of functions for kisspeptin systems in the brain of the European sea bass. *PLoS One* **8**, e70177.

Rodríguez-Marí, A., Cañestro, C., BreMiller, R. A., Catchen, J. M., Yan, Y.-L., and Postlethwait, J. H. (2013). Retinoic Acid Metabolic Genes, Meiosis, and Gonadal Sex Differentiation in Zebrafish. *PLoS One* **8**, e73951.

Servili, A., Le Page, Y., Leprince, J., Caraty, A., Escobar, S., Parhar, I. S., Seong, J. Y., Vaudry, H., and Kah, O. (2011). Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. *Endocrinology* **152**, 1527-1540.

CHAPTER 1 ADDITIONAL TABLES

Additional Table 1. Primers for the amplification of riboprobes used for *in situ* hybridization

Gene name	**Restriction enzyme / RNApol**	**Primers (5'→3')**	**Probe size (bp)**
cyp26a1	Antisense: AaTII7/SP6 Sense: spI/ T7	Fw: ATGGCTCTGAGCACACTACTGG Rv: ACTTTGAGCTGTTTCCTAGAGCA	1642
rxrα	Antisense: spI/T7 Sense: AaTII/ SP6	Fw: ATGCACCCATCTCTGCTCA Rv: GGACAGGTTCTCCATCCAAA	1465
vasa	Antisense: spI/T7 Sense: aTII/ SP6	Fw: CAGGGAGACTGTTGGATGTG Rv: CTTGGACAGATCCTCCCTGT	945
star	Antisense: spI/T7 Sense: AaTII/ SP6	Fw: ATGCTGCCTGCAACCTTC Rv: CAGCAGGCATGAGCCATCT	860

Additional Table 2. A table containing all the DEGs found in the microarray during the onset of European sea bass puberty. Available in Blázquez et al., 2017 additional files on-line version. The searchable excel file contains the probe ID and primary accession numbers assigned in the custom European sea bass microarray to all differentially expressed genes. The p-value and adjusted p-value are also included, as well as the fold change and the regulation (stage I versus stage II) obtained after comparison of sea bass testes during the onset of puberty. The annotation information is given in the gene description column that reported the presence of 153 non-annotated sequences from a total of 315 differentially expressed genes, and in the gene description column containing a brief explanation of each gene function. Finally, a column with the assigned GO-terms is also included (excel format, .xls). (XLS 144 kb).

Also accessible through the following link:
https://www.dropbox.com/s/ww3m3gclr4zkizs/Chapter%201%20additional%20table%202.xls?dl=0

Additional Table 3. Glossary of genes involved in cell proliferation, reproduction, growth and RA-signaling pathway with particular mention in the study

Gene abbreviation	Gene name
agrp2	Agouti-related protein 2
amh	Anti-Mullerian hormone
aqp1	Aquaporin 1
aurkb	Aurora kinase b
bub3	Mitotic checkpoint protein bub3
cdc28	Cell division control protein 28
cenpf	Centromere protein F
cenph	Centromere protein H
cenpi	Centromere protein I
crabp1	Cellular retinoic acid binding protein 1
cycl1	Cylicin 1
cyp26a1	Cytochrome P450, family 26, subfamily A, member 1
igfbp6	Insulin-like growth factor binding protein 6
mad2l1bp	Mitotic arrest deficient-like 1 (mad2l1) binding protein
ndc80	Ndc80 kinetochore complex component
pcna	Proliferating cell nuclear antigen
pparγ	Peroxisome proliferator activated receptor γ
rarα	Retinoic acid receptor alpha
rbp4	Retinol binding protein 4
rxrα	Retinoid X receptor gamma
sgII	Secretogranin II
spc25	Spc25, kinetochore complex component
sycp2	Synaptonemal complex protein 2
trip13	Thyroid hormone receptor interactor 13
ttk	ttk protein kinase

Additional Table 4. Affected KEGG pathways at the onset of European sea bass puberty

Pathway name	Genes involved
Metabolic pathways	
Nucleotide metabolism	*nme3, pold3, cad, tyms,* ***nt5e****, dtymk*
Retinol metabolism	***cyp26a1***
Lipid metabolism	***pnpla2****, gpd1,* ***agrp2****, ptges*
Amino acid metabolism	*odc1, pycr2,* ***smox****, ass1, cad, shmt1,* ***pah***
Cellular processes	
Cell cycle	*bub3, mcm3, pcna, ttk, ccnd2, mad2l1bp, cdc28, aurkb, ndc80, spc25*
Meiosis	*mcm3, cdc28, dmc1, mad2l2, sycp2, syce1, cenph, cenpi, cenpf, ndc80, spc25*
DNA replication and repair	*mcm3, pold3, fen1, pcna, rfc3*
Focal adhesion	***actb****, ccnd2,* ***thbs1***
Genetic information processing	
FoxO signaling pathway	***tgfbr1****,* ***stat3****, plk4, ccnd2*
Hippo signaling pathway	***amh, tgfbr1****, ccnd2,* ***actb***
Jak-STAT signaling pathway	***stat3, socs3****, ccnd2*
TGF beta signaling pathway	***amh, tgfbr1, thbs1***
MAPK signaling pathway	*gpd1, cdc28,* ***tgfbr1***
TNF signaling pathway	***socs3***
Toll-like receptor signaling pathway	*ctsk*
Wnt signaling pathway	*cacybp, ccnd2*
PI3K-Akt signaling pathway	*ccnd2,* ***thbs1***
cAMP signaling pathway	*adcyap1,* ***amh***
Hedgehog signaling pathway	*ccnd2, ihh*
Rap1 signaling pathway	*actb, thbs1*
Endocrine system	
Prolactin signaling pathway	***stat3, socs3****, ccnd2*
Renin secretion	***aqp1***
Thyroid hormone signaling pathway	***actb***

Downregulated genes appear in bold type and upregulated genes appear in normal type.

Chapter 2.

The microarray hybridizations showed several groups of genes with differential expression between stage I and stage II testis, interestingly, one of them belonged to the RA signaling pathway known to initiate meiosis in tetrapods and in some freshwater fish. The synthesis (aldh1a family members), degradation (cyp26 family) and meiosis signaling (stra8) of RA are three key members of the RA metabolic pathway that were cloned, sequenced and further compared with counterparts from other species and taxa by an in silico analysis, in order to characterize them for the first time in the sea bass. The search of aldh1a2, aldh1a3, cyp26a1, as well as the stra8 gene, a tetrapod meiosis gatekeeper, in several taxa, was done by performing a BLAST of counterparts from close evolutionary species against the European sea bass genome. An amino acid alignment enabled us to compare between taxa the proteins (enzymes) resulting from each gene. The phylogenetic analysis showed the evolutionary history of each enzyme while the synteny analysis allowed for the physical co-localization of the studied genes in their chromosomes and the comparison of conserved arrangements within the genomic neighbourhood in other species (Fig. 1). Finally, the obtained sequences of aldh1a2, aldh1a3 and cyp26a1 were submitted to the NCBI database.

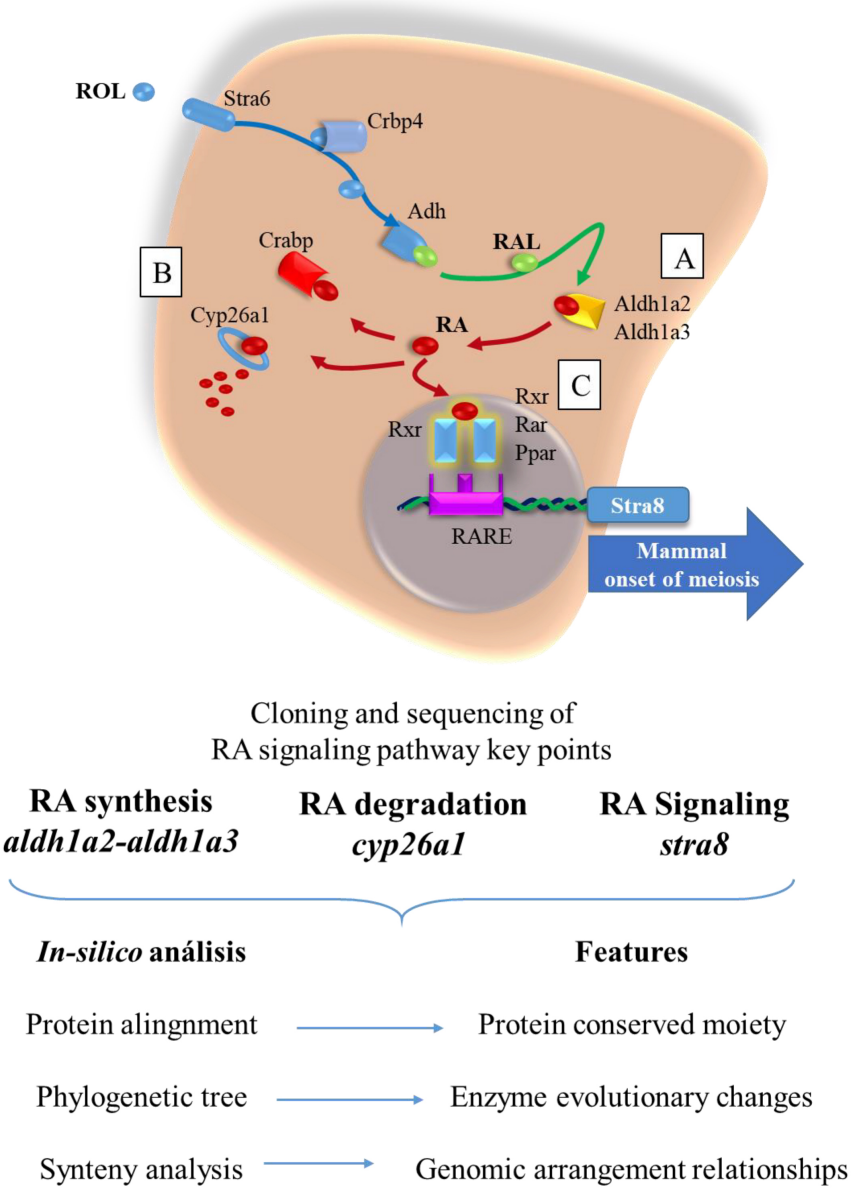

Figure 1. Schematic representation of the experimental design from Chapter 2. Three key points of the RA signaling pathway were selected for cloning and sequencing. A) represents the RA synthesis process, B) represents the RA degradation process and C) represents the *stra8* signaling process of meiosis.

Chapter 2. Characterization of the genes encoding the main enzymes involved in the synthesis (*aldh1a2, aldh1a3*), degradation of the RA (*cyp26a1*) and the meiosis gatekeeper *stra8* in the European sea bass.

Characterization of the genes encoding the main enzymes involved in the synthesis (*aldh1a2*, *aldh1a3*), degradation of the RA (*cyp26a1*) and the meiosis gatekeeper *stra8* in the European sea bass.

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ABSTRACT

Aldehyde dehydrogenases, specifically those belonging to the aldh1a subfamily, are known to metabolize retinal to retinoic acid (RA) while cyp26 family members are responsible for the inactivation of RA through its catabolism into more polar compounds. Both are key players maintaining RA homeostasis, a molecule that functions as a ligand for RA nuclear receptors which, in turn, regulate the expression of several genes. Indeed, this transcriptional activation is required for vertebrate meiosis in general, although the role in fish meiosis is yet to be fully understood. In this line, the presence of RA activates the transcription of *stra8* a gene involved in the onset of meiosis in tetrapods. This study is aimed at the cloning and characterization of *aldh*, *cyp26*, *and stra8* genes in European sea bass. We confirm the absence of *aldh1a1* and *stra8* genes, while *aldh1a2*, *aldh1a3* and *cyp26a1* were identified and characterized in the European sea bass genome. The study of their nucleotide sequences showed the presence of all the conserved domains typical of Aldhs and of cytochrome p450 enzymes. The phylogenetic analysis of the aldh family in vertebrates suggests that a common ancestor to tetrapods and teleost already exhibited both *aldh1a2* and *aldh1a3* genes. The phylogenetic analysis of the Cyp26 family in vertebrates confirms the identity of Cyp26a1 in the European sea bass and suggest an independent functionalization of its coding gene prior to the two rounds of genome duplication in vertebrates. The study of synteny revealed a well-conserved genomic neighbourhood around *aldh1a2* genes from different taxa. Moreover, the absence of *stra8* in the genome, as well as the conserved

genomic neighbourhood found in other taxa, suggest a *stra8* independent signaling for RA during sea bass meiosis.

INTRODUCTION

The *ALDH* superfamily genes code for a group of enzymes that irreversibly oxidize aldehydes to their corresponding carboxylic acids (Muzio et al., 2012). Aldhs can be found in the *Archaea*, *Eubacteria* and *Eukarya* taxonomic groups, reinforcing their importance throughout evolution (Alnouti and Klaassen, 2008). Moreover, their transcription occurs in a wide variety of tissues (Jackson et al., 2011) and play different roles in many biological processes, including tolerance to osmotic stress in plants (Gao and Han, 2009), vertebrate embryogenesis, body patterning, or organ development among others (Gudas, 1994; Napoli, 2012; Ross et al., 2000). The family catalyses the oxidation of aldehydes to their corresponding carboxylic acids which affect cellular homeostasis and physiological functions. Specifically, the ALDH1 subfamily is involved in the oxidation of RAL and the formation of RA that activate the nuclear receptors RAR or RXR (Jackson et al., 2011). In tetrapods, the gene family is constituted by three members namely *Aldh1a1*, *Aldh1a2*, and *Aldh1a3*, mainly located in the cytosol. In teleosts, the absence of *aldh1a1* seemed to be demonstrated (Cañestro et al., 2009; Pittlik et al., 2008) until recently, that it was challenged by the discovery of *aldh1a1* in tilapia, *Oreochromis niloticus* and also in zebra mbuna, *Mylandia zebra*, and red zuma, *Haplochromis nyererei*, (Holmes, 2015). The study suggests a restricted distribution of this gene among several African teleost species (Holmes, 2015). Moreover, the recent finding of the three genes in spotted gar discards the possibility that *aldh1a1* could be a lobe-finned innovation (Braasch et al., 2015) as previously suggested (Cañestro et al., 2009).

The CYP superfamily consists of a large number of p450 enzymes, which typically catalyse monooxygenase reactions involving oxygen and an equivalent number of electrons (Uno et al., 2012). Among these members we focussed on the study of the cyp26 family, due to its role in the degradation of RA into more polar compounds easier to be excreted from the cell (Lutz et al., 2009; Napoli, 1996). Several members of this family have been reported including cyp26a1, cyp26b1, and cyp26c1 (Blázquez et al., 2017). In higher vertebrates, the differential expression of *Cyp26b1* is in charge of the sex-specific timing of meiotic onset (Ross and Zolfaghari, 2011). However, in zebrafish and tilapia *cyp26a1*, rather than *cyp26b1*, is the main RA-degradation gene expressed during gonad development (Feng et al., 2015; Rodríguez-Marí et al., 2013).

Another important component of the RA signaling pathway, at least in tetrapods, is *Stra8* (the stimulated by retinoic acid 8), a gene activated by the presence of RA in the cell that has been associated with the initiation of meiosis in tetrapods. In this regard, a lack of *stra8* has been reported in several fish species (Pasquier et al., 2016; Rodríguez-Marí et al., 2013). Despite these findings, a *stra8* homolog could be identified in Southern catfish, *Silurus meridionalis,* (Dong et al., 2013) and other fish species (Pasquier et al., 2016), suggesting that in fish two different mechanisms, one dependent and the other one independent of the presence of *stra8*, might regulate the RA-mediated entry into meiosis (Feng et al., 2015; Li et al., 2016).

The European sea bass, *Dicentrarchus labrax,* is one of the main representatives of European aquaculture and an important biological model for basic and applied research in fish reproduction. Furthermore, the recent annotation of its genome represents an important tool to study the RA signaling pathway in marine fish. The present study is aimed at the molecular characterization of several key players involved in the maintenance of RA homeostasis, a molecule that functions as a ligand for RA nuclear receptors which, in turn, regulates the expression of several genes. This study reports the characterization of the European sea bass Aldh1a proteins and genes in order to gain more insight on the synthesis of RA during early gonad development. Indeed, this transcriptional activation is required for vertebrate meiosis in general, although the role in fish meiosis is yet to be fully understood.

MATERIALS AND METHODS

Animals and sampling procedures

Animals were obtained from the European sea bass stock kept at the Experimental Aquarium and Chamber Facility (ZAE) at the Institute of Marine Sciences belonging to the Spanish National Research Council (ICM-CSIC) in Barcelona (41°23'N; 2°11E), where fish were reared according to standard procedures for this species aquaculture. Details about rearing conditions and sampling procedures are thoroughly described in Chapter 3.

RNA isolation and cDNA synthesis

Testis samples at 200, 250 and 300 dph were pooled together before RNA extraction in order to reduce individual expression variability. These sampling times were selected based on the high expression levels of both *aldhs* during these developmental stages (Chapter 3; Medina et al., 2019). Total RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA. USA), following

the manufacturer's instructions. Briefly, each sample was homogenized in Trizol®, isopropanol precipitated, washed in 75% ethanol, and eluted in DEPC water. Total RNA concentration and quality were measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Willmington, DE) at 230nm, and checked in 1% agarose gel electrophoresis. A DNase I (Thermo Scientific, Willmington, DE) treatment was used to remove genomic DNA from RNA preparations following manufacturer's instructions. Total RNAs (200 ng) were reverse transcribed into cDNA from each sample using Superscript III (Invitrogen, Carlsbad, CA. USA) and random hexamers according to the manufacturer's protocol. A final step of 15 min at 70ºC was included to inactivate the transcriptase. A PCR for *r18s* was performed to check for cDNA degradation or contamination and verified through electrophoresis. The resulting cDNAs were stored at -20ºC and further used as a template to amplify, clone and sequence the complete coding regions of *aldh1a2*, *aldh1a3 and cyp26a1.*

Cloning and sequencing of *aldh1a2, aldh1a3* and *cyp26a1*

The genes of interest were localized along the European sea bass genome (Tine et al., 2014) and their structure and base composition visualized using the Apollo Genome Annotation and Curation Tool program (1.10.0 version) and the Artemis program. Using Primer3 software (http://primer3.ut.ee), specific primers were designed in 3'- and 5'- untranslated flanking regions (UTRs) to amplify their full-coding sequences (Table 1). A single pool of cDNAs from testis at 200, 250 and 300 dph was used as a template for PCR amplification. Cycling parameters included 5 min at 94ºC, followed by 25 cycles consisting on 30 s at 94ºC, 30 s at 58ºC and 2 min at 72ºC. Finally, a 7 min at 72ºC final extension step was included. Fragments of the expected size were cloned into a bacterial vector using the pGEM-T easy cloning kit (Promega, Madison WI, USA), according to the manufacturer's instructions. Briefly, a ligation reaction was performed to insert the purified fragment into a plasmid vector (pGEM-T easy). The vector was then incorporated into competent bacterial cells (DH5α) for transformation and grown in LB bacto-agar (Sigma-Aldrich, St. Louis, MO, USA) plates containing X-gal and IPTG (Promega, Madison WI, USA). Several colonies were selected, grown in liquid LB and finally sequenced with an automatic ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA), using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The identity of the clones was confirmed by comparing their sequences with those found in the European sea bass genome database (Tine et al., 2014).

Table 1. Primers for the amplification of European sea bass *aldh1a2*, *aldh1a3* and *cyp26a1* complete coding sequences

Gene name	***Primer sequence (5'→3')***
aldh1a2	Fw: CAGAGAGAGAGAGACGGC Rv: GGAAGATGCTACCTCCTCCC
aldh1a3	Fw: AGACGGAGCAGACAGACTGG Rv: TAGCCTGATGGAATGGGAAG
cyp26a1	Fw: ATGGCTCTGAGCACACTACTGG Rv: ACTTTGAGCTGTTTCCTAGAGCA

Forward primers (Fw) were located in the 5'UTR region whereas reverse primers (Rv) were located in the 3'UTR region of their respective gene sequences.

Evolutionary relationships of *aldh1a* and *cyp26* family members in sea bass

A TBLASTN search (Altschul et al., 1997) was conducted to identify *aldh1a1*, *aldh1a2* and *aldh1a3* sequences in the European sea bass genome database (Tine et al., 2014), using the protein sequences from *Mus musculs* (accession number NP_038495.2), *Oreochromis niloticus* (accession number: AGM75104.1) and *Takifugu rubripes* (accession number: NP_00153657.1) as queries. Based on the ESTs of the microarray (Chapter 1; Blázquez et al., 2017), the full sequence of European sea bass *cyp26a1* was localized along the genome.

In order to search for the conserved moieties of the ALDH1A subfamily and the CYP26 family, and confirm the identity of the cloned sequences, ALDH1A2, ADH1A3 and CYP26A1 amino acid sequences of different species were obtained from public databases (accession numbers appear listed in Additional Tables 1 and 2) and aligned using the ClustalW2 software package (Thompson et al., 2002) with default settings. These sequences were compiled from NCBI GeneBank or the Ensembl database.

Phylogenetic relationships within the Aldh1a family and the Cyp26 family were assessed including protein sequences (only complete coding sequences) found in mammals, reptiles, birds, amphibians, and fish (accession numbers appear listed in Additional Tables 3 and 4). For the phylogenetic tree, the distances were computed with the Poisson correction method (Zuckerkandl and Pauling, 1965) and the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) after a bootstrap test using 1000 replicates. The phylogenetic analysis was carried out in MEGA v.4 (Tamura et al., 2007).

The conserved synteny was analyzed by comparing the genomic neighbourhood surrounding *Aldh1a2* and *Aldh1a3* genes from different taxa, and their corresponding genomic

arrangement in the European sea bass. The genomic neighbourhood was established as conserved tandem genes upstream and downstream the studied target genes. Information about gene organization was extracted from the Ensembl Genome Project, the Genomicus genome browser (DYOGEN group v79.01) and using the Artemis comparison tool (Release 13.0.0 1) for the European sea bass genome database (gene ID numbers appear in Additional Table 5, while information about the genes conforming *aldh1a2 and aldh1a3* genomic neighbourhood is included in Additional Table 6 and 7, respectively). We could not perform a synteny analysis for *cyp26a1* since this gene has been coupled to unanchored scaffolds and gathered randomly in a virtual unit of the European sea bass genome database, therefore, it was not possible to accurately assess its location. In this case, we performed a synteny analysis of several vertebrates including fish and tetrapods (gene ID numbers appear in Additional Table 8, while information about the genes conforming *CYP26A1* genomic neighbourhood appear included in Additional Table 9). Furthermore, an *in silico* study of the 5' upstream 1,500 bp of the flanking promoter sequence using MatInspector and (Promo) Transfac v.8.3 was used to identify the presence of putative binding sites for transcription factors that could be involved in the activation or repression of *cyp26a1* transcription.

European seabass *stra8* search and sequence analysis

To identify the presence of *stra8* in European sea bass, a query was made using *stra8* sequences from different vertebrates including human (accession number: AAP47163.1), Chinese softshell turtle (accession number: XP_025041836.1) and spotted gar (accession number: ANC96758.1). The sequences were used as blast queries against the sea bass genome database to check whether *stra8* was annotated in this species. A synteny analysis of *STRA8* in tetrapods and fish (gene ID numbers appear in Additional Table 10, while information about the genes conforming *STRA8* genomic neighbourhood appear included in Additional Table 11) was carried out with available information from genome assemblies found in Ensembl (http://www.ensembl.org) and from the European sea bass genome database (Tine et al., 2014). The genomic neighbourhood was established including the most conserved genes surrounding *STRA8* in tetrapods. In order to check if this conserved genomic neighbourhood in tetrapods was also present in fish, a search for the genomic location of each gene was conducted in other teleosts.

RESULTS

Cloning of *aldh1a2* and *aldh1a3* genes, protein structure and phylogenetic analysis

After a TBLASTN search, we were unable to find *aldh1a1* within the sea bass genome database or in any other publicly available fish sequence databases. Regarding aldh1a2 and aldh1a3, the resulting nucleotide sequences shared 100% identity to those found in the European sea bass genome (Tine et al., 2014). Sequence comparisons with other fish species revealed that they shared a high percentage of identity, particularly with tilapia, *Oreochromis niloticus* (96.3%) (Table 2) and Fugu, *Takifugu rubripes* (85.5%) (Table 3), respectively.

Table 2. Percentage of ALDH1A2 sequence identities among several vertebrates

	Human	**Opossum**	**Zebrafinch**	**Sea bass**	**Zebrafish**	**Cod**	**Tilapia**
Human	100						
Opossum	97.49	100					
Zebrafinch	94.58	94.78	100				
Sea bass	**82.43**	**82.05**	**81.04**	**100**			
Zebrafish	79.34	78.57	77.56	**85.14**	100		
Cod	78.14	77.76	76.36	**87.23**	82.01	100	
Tilapia	82.05	81.85	81.04	**96.33**	84.36	85.69	100

Accession numbers in Additional table 1.

The cDNA isolated for *aldh1a2* contained an open reading frame (ORF) 1,557 bp long. It is organized in 13 exons that encode a 518 amino acid protein, with a theoretical PI of 5.61 and 56.52kDa molecular weight. The complete coding sequence was deposited in GenBank under the accession number KR534500. Regarding *aldh1a3*, it contains an ORF 1,536 bp long organized in 13 exons that encode a 511 amino acid protein, with a theoretical PI of 6.17 and a 56.52kDA molecular weight. The complete coding sequence was deposited in GenBank with the accession number KR534501.

Table 3. Percentage of Aldh1a3 sequence identities among several vertebrates

	Human	Opossum	Anole lizard	Zebra Finch	Spotted gar	Sea bass	Zebrafish	Cod	Fugu
Human	100								
Opossum	88.67	100							
Anole lizard	76.18	78.35	100						
Zebra Finch	86.13	89.26	81.10	100					
Spotted gar	74.76	75.54	69.49	76.13	100				
Sea bass	**70.20**	**71.96**	**65.48**	**71.57**	**77.45**	**100**			
Zebrafish	69.86	71.04	64.76	71.43	75.73	**79.65**	100		
Cod	69.22	70.00	65.68	71.18	77.45	**83.92**	78.43	100	
Fugu	66.47	67.25	61.74	67.06	73.14	**85.52**	72.99	76.47	100

Accession numbers in Additional table 1.

The deduced amino acid sequences of both Aldhs exhibited all the structural features of a functional oxidative enzyme (Fig.2). (a) the Rossmann fold motif, an amino acid arrangement that gives the capability to use NAD or NADP as a cofactor, was highly conserved between both Aldhs in all aligned species and is represented in the sea bass as the G^{263}STEVG (aldh1a2), and G^{257}STEVG (aldh1a3) (Fig. 3 and 4, respectively). (b) the bridging domain or "glutamic acid active site motif" was found in the sea bass as LELGGKN being E the active site in 286 (Aldh1a2) and 280 (Aldh1a3) positions (Fig. 3 and 4, respectively). This same pattern was found in all of the aldh1a3-aligned species, nevertheless, a change of the N amino acid (Asparagine), for an S amino acid (Serine) was found in Aldh1a2 of zebrafish and tetrapods. (c) the "catalytic domain" or cysteine active site was found as FFNAGQCC^{320}TAGS in the sea bass Aldh1a2 (Fig. 3) and as FYNQGQCC^{314}TAAS in Aldh1a3 (Fig. 4). The C residues, in 320 and

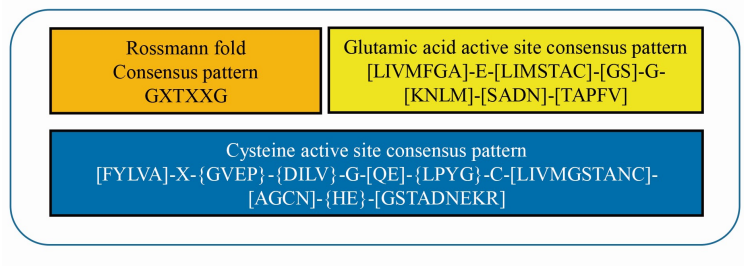

Figure 2. Consensus patterns for the different conserved sites representative of the Aldh family. Rossmann fold structure in orange box (Liu et.al 1997), glutamic acid active site motif in light yellow box (prosite code PS0070. https://prosite.expasy.org). Cysteine active site motif in blue box (prosite code PS00687.https://prosite.expasy.org). Amino acids between brackets [ ] are accepted at this position. Amino acids between curly brackets { } are not accepted at this position.

314 positions, were conserved in all studied sequences nevertheless, there was group defining differences in the 4th (Aldh1a2) and 11th (Aldh1a3) positions of the consensus patterns (Fig. 3 and Fig 4, respectively).

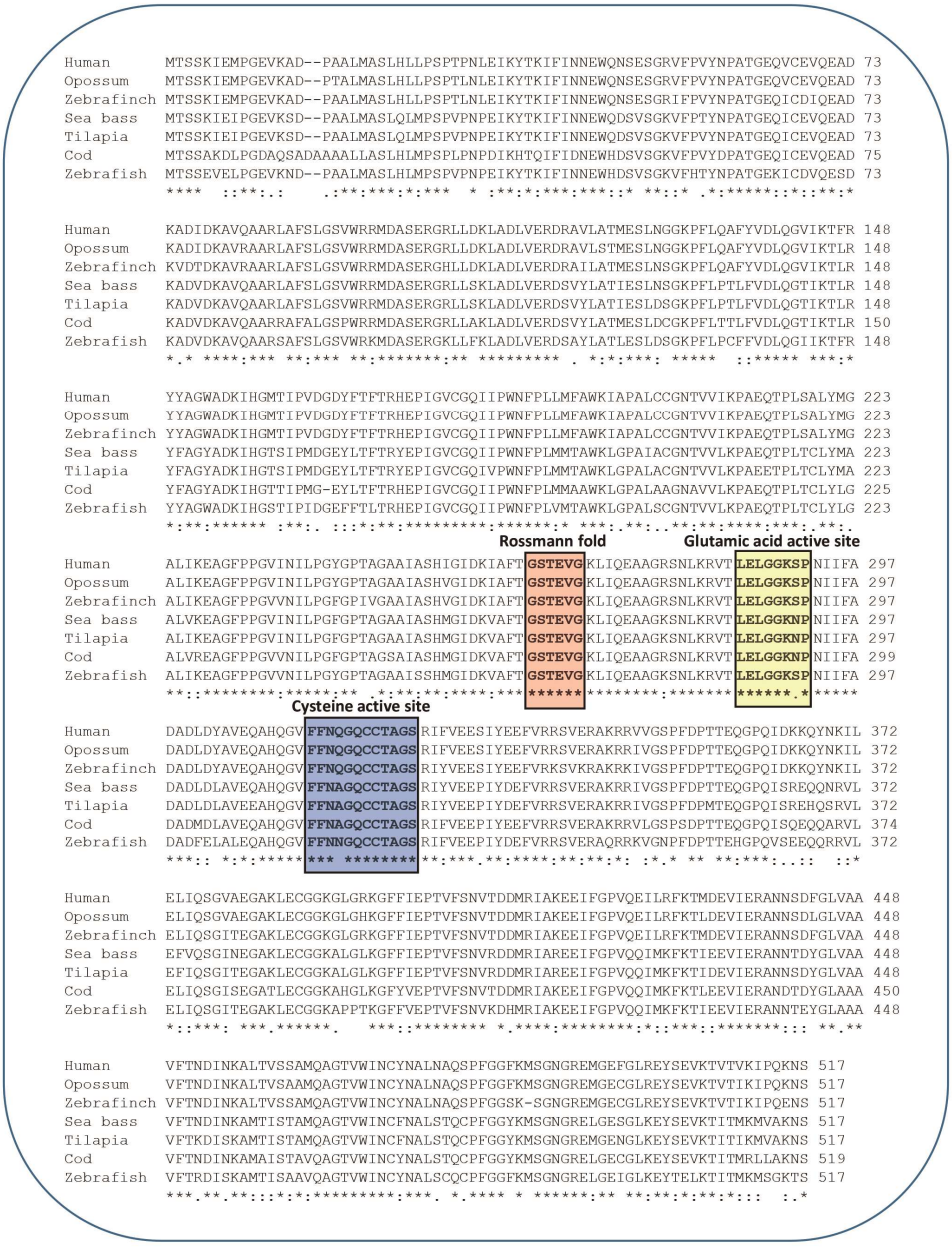

Figure 3. Multiple amino acid sequence alignment of ALDH1A2 proteins. The clustalW2 analysis includes several species such as human, opossum, zebrafinch, European sea bass (sea bass), tilapia, cod, and zebrafish. The Rossmann fold motif is represented in an orange box, the glutamic acid active site in a yellow box, and the cysteine active site in a blue box. Accession numbers appear listed in additional table 1.

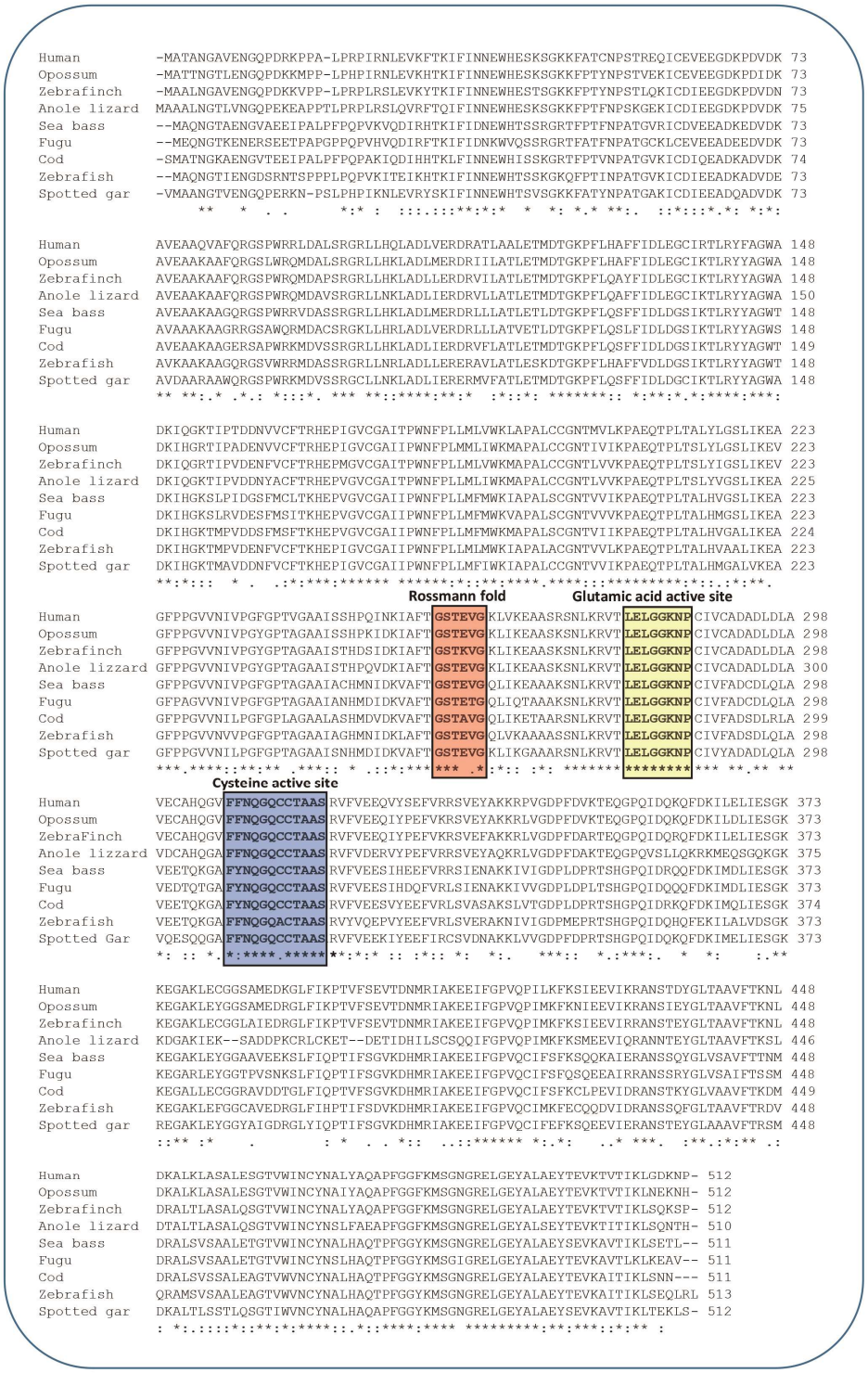

Figure 4. Multiple amino acid sequence alignment of ALDH1A3 proteins. The clustalW2 analysis includes several species such as human, opossum, zebrafinch, anole lizard, European sea bass (sea bass), fugu, cod, zebrafish and spotted gar. The Rossmann fold motif is represented in an orange box, the glutamic acid active site in a yellow box, and the cysteine active site in a blue box. Accession numbers are listed in additional table 1.

The consensus tree showed two main branches, one containing Aldh1a2 proteins and the other one Aldh1a3 proteins. Each branch, was grouped in two clades corresponding either to fish or tetrapods, this last one showing a much slower nucleotide divergence (Fig. 5). European sea bass Aldh1a2 and Aldh1a3 sequences were grouped with other teleosts and shared the highest homology with Fugu (85.52%).

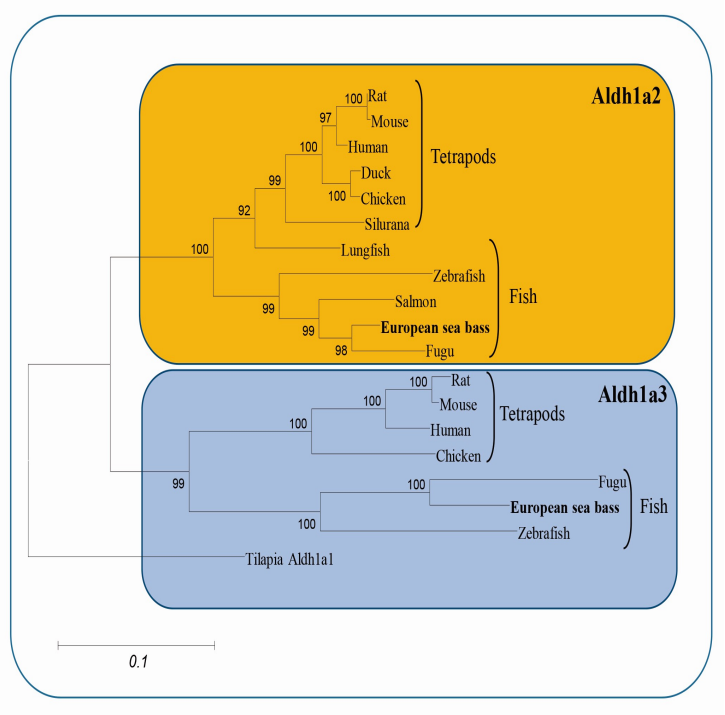

Figure 5. Phylogenetic tree of ALDH1A2 and ALDH1A3 proteins in different vertebrates. The tree was constructed using the Neighbor-joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of times each branching was obtained out of the 1000 bootstraps is shown next to the nodes. The evolutionary distances were computed using the Poisson correction method. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al 2007). An aldh1-like form from tilapia was used as outgroup to root the tree. The scale bar represents the nucleotide substitution per site. Accession numbers appear listed in additional table 3.

Synteny analysis of sea bass *aldhs*

The synteny analysis showed that both genes are located in the same chromosome (ligation group LG5), revealing a well-conserved genomic neighbourhood (Fig. 6). We found the presence of an unusual form of gene organization at the *adh1a2* vicinity, the tandem *polr2m*, *gcom1* and *myzap* (genID:145781), absent in other teleosts but present in tetrapods (described as *grinl1a* complex in Chinese turtle and zebra finch). In fact, only *polr2m* was found in fish precisely downstream *aldh1a2*. (Fig. 6A). Regarding the genomic neighbourhood surrounding the *aldh1a3* gene, it appears well conserved among tetrapods, and although in modern fish taxa some genes are missing, the immediate tandem arrangement remains conserved (Fig. 6B).

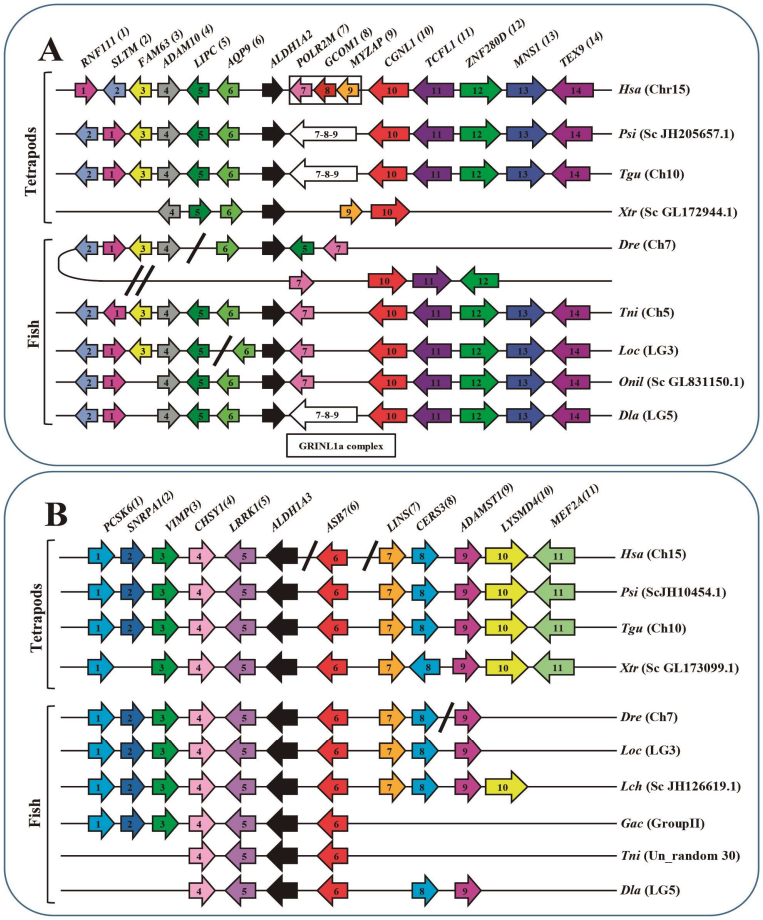

Figure 6. Comparative synteny of (A) *ALDH1A2* and (B) *ALDH1A3* in tetrapods and fish. Gene name abbreviations are shown on top of the figure. Continuous horizontal lines represent regions of the same chromosome. Simple transverse bars between two genes indicate they are not adjacent. Double transverse bars represent large chromosome regions between two genes. Each gene is represented by a coloured arrow with its identifying number. The direction of the arrow indicates the orientation of the transcription unit with respect to *aldh1a2 or aldh1a3*. The chromosome (Ch) number, ligation group (Lg) or scaffold (Sc) number where each gene cluster is located appears between brackets. Accession numbers, genome assemblies, and abbreviations are compiled in Additional table 5. Information about the genes conforming *aldhs* genomic neighbourhood appears in additional tables 6 and 7.

Cloning of *cyp26a1* gene and protein structure analysis

The results found after microarray hybridizations (Chapter 1; Blázquez 2017) showed that a decrease in *cyp26a1* expression was associated with the onset of spermatogenesis and the initiation of meiosis and prompted us to clone and obtain the full length of its cDNA. The interest was derived from its prominent role maintaining the homeostasis of intracellular RA levels (White et al., 1997) and because RA is known to be essential for the onset of meiosis in several vertebrates (Bowles and Koopman, 2007; Rodríguez-Marí et al., 2013; Smith et al., 2008; Wallacides et al.,

CYP FAMILY Consensus pattern or P450 signature
[FW]-[SGNH]-X-[GD]-{F}-[RKHPT]-{P}-C-[LIVMFAP]-[GAD])

Figure 7. Consensus pattern for the CYP family (prosite code PDOC00081. https://prosite.expasy.org). Cysteine active site motif in orange box. Amino acids between brackets [ ] are accepted at this position. Amino acids between curly brackets { } are not accepted at this position.

2009). The cDNA isolated for sea bass *cyp26a1* contained an ORF 1,178 bp long, was flanked by a 500 bp 3'UTR region and was deposited in the GenBank under the accession number KJ187657. The deduced amino acid sequence encodes a protein 488 amino acid long with a theoretical PI of 9.04 and a calculated molecular weight of 55.49 kDa that includes the consensus pattern of the Cyp family, or "P450 signature" that allows monoxigenase reactions (Meunier et al., 2004) (Fig. 7). The 10 amino acid residue is represented as the FGGGSRMC^{434}VG sequence with an invariant cysteine (in the 434 position), which is an iron-heme binding site, in charge of substrate catalysis. This structure was conserved among Cyp26a1 of other studied teleost, except in tilapia where there is an isoleucine (I) in 435 position instead of the conserved valine (V) (Fig. 8). A Genbank search resulted in the identification of several full-length sequences for Cyp26 proteins in teleosts and tetrapods, including amphibians, reptiles, birds and mammals.

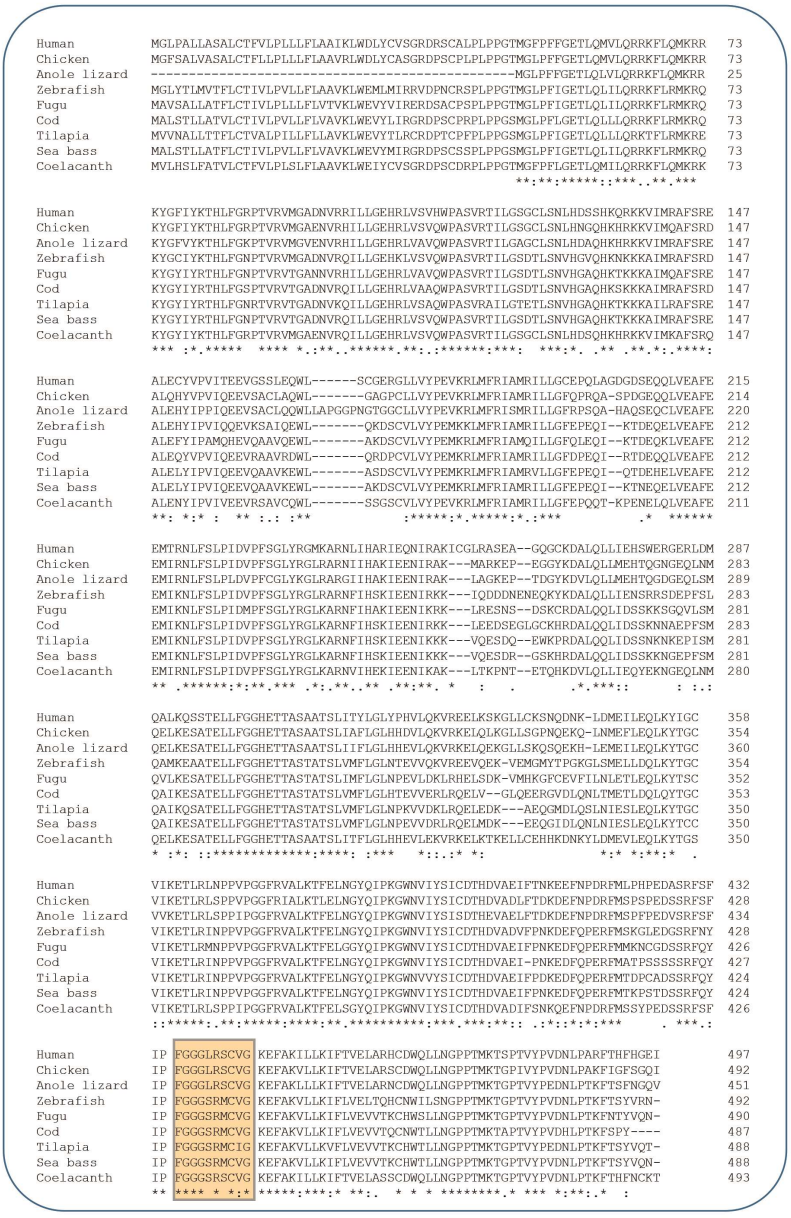

Figure 8. Multiple amino acid sequence alignment of the CYP26A1 proteins. The clustalw analysis includes several species such as human, chicken, anole lizard, zebrafish, fugu, cod, tilapia, European sea bass and coelacanth. The conserved active site of the CYP family (or P450 signature) is represented in an orange box. Accession numbers appear listed in additional table 2.

Phylogenetic analysis and promoter study of *cyp26a1*

The phylogenetic analysis showed that the sea bass protein was evolutionarily closer to Cyp26a1 proteins, while it was more distant from other Cyp26b1 and Cyp26c1 proteins (Fig. 9 and Additional Table 4 for accession numbers). The consensus tree had three main branches, one containing all A1 sequences, another one including B1 sequences and the remaining with C1 sequences. The European sea bass sequence clustered together in the A1 group further supporting its identity. Comparisons of the deduced amino acid sequence with other full-length Cyp26a1 in different fish species revealed that the highest homology was shared with stickleback (94.4%) and the lowest with zebrafish (82.4%) (Table 4). These slight differences among fish support that Cyp26a1 structure in teleosts is highly conserved. A study of the 5' flanking sequence (1,500 bp upstream of the first ATG) of the *cyp26a1* gene showed the presence of binding sites for different

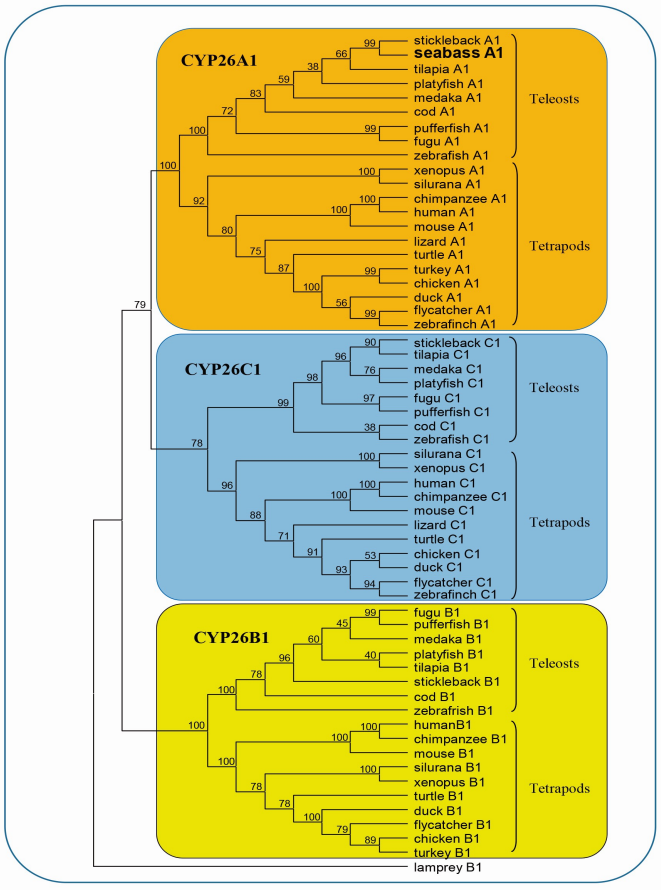

Figure 9. Phylogenetic tree of CYP26 family proteins. The tree was constructed using the Neighbor-Joining method. The consensus tree inferred from 1000 bootstrap replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of times each branching was obtained out of the 1000 bootstraps is shown next to the nodes. The evolutionary distances were computed using the Poisson correction method. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Lamprey Cyp26b1 was used as outgroup to root the tree. Accession numbers appear listed in additional table 4.

transcription factors among which is worth mentioning RA-nuclear receptors (Rxr, Rar, and Ppar), steroid receptors, and several elements involved in cell cycle regulation (Additional Table 12).

Table 4. Percentage of Cyp26a1 sequence identity among several vertebrates

	Human	Mouse	Chicken	Frog	Sea bass	Stickleback	Zebrafish	Platy fish
Human	100							
Mouse	93.96	100						
Chicken	80.28	81.30	100					
Frog	68.65	69.60	71.97	100				
Sea bass	**68.65**	**67.62**	**69.34**	**64.34**	**100**			
Stickleback	68.11	67.49	69.21	64.89	**94.44**	100		
Zebrafish	68.50	68.29	70.61	64.20	**82.38**	81.07	100	
Platyfish	67.08	66.67	6940	64.90	**88.32**	86.83	76.64	100

Accession numbers in Additional table 2.

Genomic arrangement surrounding *cyp26a1*

A synteny analysis of *cyp26a1* in the European sea bass could not be performed since the available genome data base grouped this gene in a virtual ligand group formed by various left-

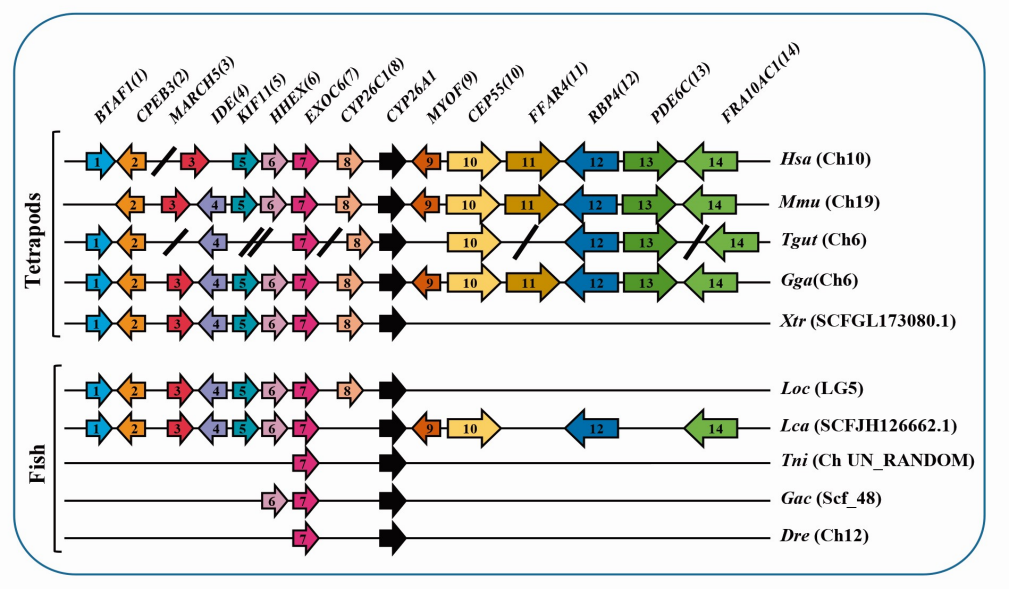

Figure 10. Comparative synteny of *CYP26A1* genes in tetrapods and fish. Gene name abbreviations are shown on top of the figure and a number between brackets is used to identify each gene. Continuous horizontal lines represent regions of the same chromosome. Simple transverse bars between two genes indicate they are non-adjacent. Double transverse bars represent large chromosome regions between two genes. Each gene is represented by a coloured arrow with its identifying number. The direction of the arrow indicates the orientation of the transcription unit with respect to *Cyp26a1*. The chromosome (Ch) number, ligation group (Lg) or scaffold (Sc) number in which each gene cluster is located appears between brackets. Accession numbers, genome assemblies, and abbreviations are compiled in additional table 8. Information of the genes conforming *Cyp26a1* genomic neighbourhood appear included in additional table 9.

over sequences (Tine et al., 2014). Nevertheless, the comparison of the genomic neighbourhood surrounding *Cyp26a1* among other tetrapods, revealed the presence of a highly conserved region (Fig. 10), in which *Cyp26a1* and *Cyp26c1* are also tandem genes. A tandem of *cyp26a1* and *cyp26c1* genes was found within the same chromosome in the ancient spotted gar and the coelacanth, nevertheless such feature was not found in the modern teleost.

European sea bass *stra8* sequence search

The *stra8* genomic neighbourhood was well conserved among tetrapods (Fig. 11). However, in available fish genomes, *stra8* was only found in the ancient holostei spotted gar (*Lepisosteus oculatus*), showing a non-conserved synteny when compared to tetrapods. It is worth noting that the spotted gar appeared before the emergence of teleosts and therefore, prior to the teleost-specific whole genome duplication. No significant hits for European sea bass *stra8* could

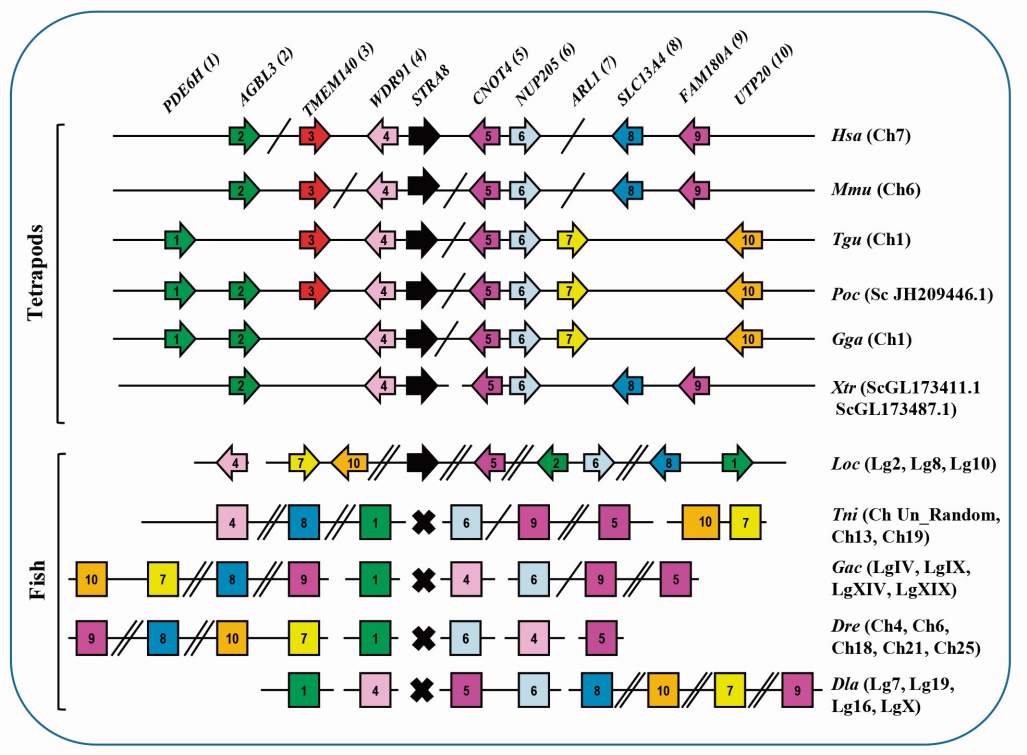

Figure 11. Comparative synteny of *STRA8* genes in tetrapods and fish. Gene name abbreviations are shown on top of the figure and a number between brackets is used to identify each gene. Continuous horizontal lines represent regions of the same chromosome. Simple transverse bars between two genes indicate they are not adjacent. Double transverse bars represent large chromosome regions between two genes. Each gene is represented by a coloured arrow with its identifying number. The direction of the arrow indicates the orientation of the transcription unit with respect to *Stra8.* The chromosome (Ch) number, ligation group (Lg) or scaffold (Sc) number in which each gene cluster is located appears indicated between brackets. The black cross represents the absence of the gene. In the particular case of fish where *stra8* has not been found in their genomes, the different neighbouring genes were represented with a box, instead of an arrow, and thus no orientation is depicted in the figure. Accession numbers, genome assemblies, and abbreviations are compiled in additional table 10. Information about the genes conforming *Stra8* genomic neighbourhood appear included in additional table 11.

be found using several available vertebrate sequences after a blast search against the genome database. Moreover, the conserved genome arrangement found in tetrapods was scattered in different chromosomes or chromosomal regions in fish (Fig. 11). Similarly, no *stra8* hits were found in any of the modern teleosts for which the genome has been sequenced including tetraodon (*Tetraodon nigroviridis*), stickleback (*Gasterosteus aculeatus*), and zebrafish (*Danio rerio*).

DISCUSSION

The present study reports for the first time the isolation, cloning and sequencing of European sea bass *aldh1a2*, *aldh1a3 and cyp26a1*. All of them appear linked to the regulation of RA metabolism, either involved in its synthesis (Aldhs) or its degradation (Cyp26a1) (Adolfi et al., 2016; Feng et al., 2015; Uji et al., 2006), therefore with a prominent role in meiosis initiation (Griswold et al., 2012).

Both cloned *aldhs* contained the three signature motifs that characterize the ALDH family and give them a functional oxidative role. First, the Rossman fold motif, the amino acid arrangement that gives the capability to use NAD or NADP as a cofactor, is usually found as GXGXXG, in which the first glycine is an invariant amino acid in several dehydrogenase families (Lesk, 1995; Perozich et al., 1999). As also reported in other teleosts, the European sea bass presents a variant of this motif (GX**T**XXG), although it still retains the capability to interact with the nicotine amide ring (Liu et al., 1997). Second, the bridging domain, was also found to be highly conserved in both protein sequences. In the particular case of aldh1a2, a change of an Asparagine for a Serine was found in zebrafish and in tetrapods, although the protein retains its function. Moreover, in both aldhs, the glutamic acid active site exhibits the GG motif that marks the boundary between the coenzyme-binding and the catalytic domains and is essential for the catalytic reaction (Hempel et al., 2001). Third, the catalytic domain for which the C residues, in 320 and 314 positions, were conserved in all studied sequences and respond to its function as an active site with catalytic activity in all ALDH family proteins (Perozich et al., 1999). These three motifs (the Rossman fold, the glutamic acid active site, and the cysteine active site) have been recently described in tilapia and compared to mouse Aldh1a1 and Aldh1a2, enabling for the identification of key residues that contribute to catalysis, structure and function, suggesting a well conserved structure throughout vertebrate evolution and teleost divergence (Holmes, 2015).

The phylogenetic tree was divided in two main clusters that separated Aldh1a2 from Aldh1a3 proteins. Moreover, within each cluster, a clade grouping tetrapods and another one

grouping fish were present. This was supported by the high bootstrap values (values between 92-100%), thus confirming the identity of European sea bass *aldh1a2* and *aldh1a3* cloned sequences, placing them within the teleosts. By rooting the tree to tilapia Aldh1a1 sequence, we can suggest a putative common ancestor to both subfamilies and, due to a shorter Aldh1a3 branch, an ancestor prior to the appearance of Aldh1a2. A similar situation has been reported in zebrafish where Aldh1a2 and Aldh1a3 proteins grouped in sister clades (Pittlik et al., 2008), suggesting that both genes were already present in a common ancestor of tetrapods and teleosts. Moreover, other phylogenetic studies support the hypothesis that claims for the idea that a*ldh1a2* was the ancestral gene from which *aldh1a1* and *aldh1a3* arose after sequential gene duplication, in most vertebrate genomes, before the tetrapod and teleost lineages diverged (Cañestro et al., 2009; Holmes, 2015).

In an attempt to understand the evolutionary history of *aldh1a2*, *aldh1a3, cyp26a1* and *stra8* we performed a study of synteny that revealed the physical co-localization of their genomic neighbourhoods. As in other vertebrates, the European sea bass *aldh1a* family members are found within the same chromosome. Indeed, both genes are located in the ligation group LG5, with *aldh1a2* starting in base pair 13785600 and *aldh1a3* in base pair 32347020, and exhibit a well conserved genomic neighbourhood. This is a common feature for the aldh1a-like vertebrate genes that appeared generally located within the same chromosome suggesting this could be a representative family trait, originated by an ancestral aldh-like gene that underwent sequential gene duplication generating additional *ALDH1A* genes (Holmes, 2015).

An unusual form of gene organization was found at the *adh1a2* vicinity in the European sea bass, the *grinl1a*, formed by a 3-gene tandem unit including *polr2m*, *gcom1* and *myzap*, and also present in tetrapods including humans, zebrafinch, and the Chinese soft-shell turtle but absent in the teleosts studied so far. In the African clawed frog, *Xenopus tropicalis*, only the *myzap* gene located in the proximity of *aldh1a2* is conserved form the tandem. In other teleosts, only *polr2m* appears well conserved and located tightly close to *aldh1a2* while in the zebrafish only *polr2m* could be found with the presence of two copies one of them in a distant downstream region of the same chromosome than *aldh1a2* (Ch7). This tandem represents a natural occurring read-through transcription element called complex transcription unit (Prakash et al., 2010; Roginski et al., 2018; Roginski et al., 2004) that produces three sets of transcripts and proteins from two neighbouring genes transcribed in the same direction (Kim et al., 2012; Roginski et al., 2018). In humans it conjoins two apparently discrete genes into a third combined gene (Roginski et al., 2004). The characterization of the *grinl1a* complex in the European sea bass is an interesting matter that deserves further study. In this regard, combined transcripts provide evidence for an unusual form

of gene organization and expression, which increases the extractable information content of a segment of genomic DNA and constitutes a potentially significant mechanism to increase the proteome of a given genome (Roginski et al., 2004).

The *aldh1a3* genomic neighbourhood was well conserved in all the studied tetrapods and in ancient fishes like the spotted gar and the coelacanth. However, in modern teleosts some genes are missing from the arrangement, maybe due to gene loss or translocations during evolution (Cañestro et al., 2009). Taken together, the highly conserved synteny of both genes in tetrapods and in teleosts suggest that the *aldh1a* family originated during the first whole genome duplication (WGD). The subsequent teleost genome duplication (TGD) retained *aldh1a2* and *aldh1a3* in the same chromosome, although the surrounding genomic arrangement diverged (Holmes, 2015).

The pivotal role of Cyp26 maintaining intracellular RA homeostasis prompted us to clone, sequence, and study its phylogenetic relationships with other counterparts from the family in other species and taxa. The primary structure of the protein includes the consensus pattern of the Cyp family, or "P450 signature" that allows monoxigenase reactions (Meunier et al., 2004). The 10 amino acid residue is represented as the FGGGSRMC^{434}VG sequence with an invariant cysteine (in 434 position), which is an iron-heme binding site, in charge of substrate catalysis. This structure was conserved among Cyp26a1 proteins of other teleosts, except in tilapia where there is an isoleucine (I) in 435 position instead of the conserved valine (V). The alignment of the deduced protein sequence with other Cyp26 proteins available from other vertebrates confirmed its identity as Cyp26a1 revealing slight differences in homology among teleosts, further supporting their high conservation due to its pivotal role controlling RA levels (Rodríguez-Marí et al., 2013; Thatcher and Isoherranen, 2009). The tree shows the presence of a common ancestor cyp26 protein, strengthening the hypothesis of an independent functionalization of its coding gene prior to the two rounds of genome duplication in vertebrates (Rodríguez-Marí et al., 2013). It seems that the conserved genomic region surrounding *cyp26a1* has been scattered during the duplication of the teleost genome, since *cyp26a1* and *cyp26c1* are not in the same neighbourhood and not even in the same chromosome, suggesting that *cyp26* family arose before the WGD in teleost fish (Catchen et al., 2009). Furthermore, conserved synteny and expression analyses in zebrafish reveal a lineage-specific subfunctionalization of vertebrate genome duplication, showing that *cyp26a1* and its paralog in tetrapods *Cyp26b1*, became the primary genes encoding RA degradation enzymes in an independent manner after genome duplication (Rodríguez-Marí et al., 2013). Moreover, the study of the promoter showed the presence of binding sites for cell cycle regulators and for RA nuclear receptors including Ppar, Rxr and Rar, and also DR1 and DR5 sites

(RA-responsive elements) indicating the role of RA, via the interaction with its nuclear receptors, in the regulation of *cyp26a1* transcription in the sea bass. A similar result has been shown for the *cyp26a1* promoter in zebrafish (Hu et al., 2008; Loudig et al., 2000) and medaka (Adolfi et al., 2016), although the development of functional studies is clearly needed to confirm the capability of RA to induce the regulation of *cyp26a1* in the sea bass.

The timing and regulation of meiosis in tetrapods differ dramatically between sexes. However, there is a common conserved feature in male and female gonads; the *Stra8* mediated premeiotic DNA replication (Anderson et al., 2008). In mice, *Stra8* is required for the onset of meiosis in embryonic ovaries (Baltus et al., 2006; Menke et al., 2003) and in juvenile testis (Anderson et al., 2008). Nevertheless, this gene could have been lost in teleosts because it is absent in a number of fish genomes including tetraodon, fugu, medaka, tilapia and zebrafish (Feng et al., 2015; Rodríguez-Marí et al., 2013). This was challenged by the discovery of an *stra8* ortholog in Southern catfish (Dong et al., 2013). The study stated that *stra8* could be difficult to identify in other fish species either because of a very low sequence similarity or because of incomplete genome sequencing. Our results also show an absence of *stra8* and of the conserved tetrapod genomic neighbourhood. Nevertheless, the RA signaling pathway seems to be completely functional during puberty, suggesting a role in meiosis in a *stra8* independent manner.

The European sea bass genome has been sequenced to about 30x coverage using a combination of three independent sequencing technologies including whole-genome shotgun, mate pair and BAC-end sequencing (Tine et al., 2014) and recently improved with the addition of RNAseq data (Chaves-Pozo et al., 2017). It represents one of the highest-quality genomes available for an aquaculture fish species thus it seems very unlikely that the absence of *stra8* could be due to incomplete sequencing. However, it cannot be completely excluded that the low sequence similarity (less than 30%) could be responsible for the difficulty in its identification. Recent evidence showed the presence of *stra8* homologs in several teleosts belonging to different families (Pasquier et al., 2016), but not in zebrafish or any Acanthomorpha (Rodríguez-Marí et al., 2013). The study concluded that a single *stra8* paralog was retained after the teleost-specific whole genome duplication (Hoegg et al., 2004; Taylor et al., 2003). Therefore, it was suggested that *stra8* was lost in Acanthomorpha, the largest group of teleosts where the European sea bass is included, and independently in the Cypriniform lineage due to a lineage-specific loss event (Pasquier et al., 2016).

In teleosts, RA might play a critical role in meiotic initiation through two different signaling pathways: a *stra8*-dependent and a *stra8*-independent one, although both possibilities

rely on the final RA balance (Dong et al., 2013; Feng et al., 2015; Li et al., 2016). Recent evidence showed that *Stra8* is not the only meiosis-inducing gene activated by RA, and experiments with *Cyp26b1* and *Stra8* mutant mice reveal *Rec8* as a new RA target gene (Koubova et al., 2014). Its regulation occurs independently but in the same temporal and spatial manner as *Stra8*. Both genes play critical roles during early meiotic processes, suggesting that RA-induced meiosis may follow two independent pathways (Koubova et al., 2014). However, further functional analyses are needed to elucidate the ultimate role of RA triggering puberty in the European sea bass.

CONCLUSION

The present study reports for the first time the characterization of the Aldh1a family and Cyp26a1 in the European sea bass. The studied proteins have all the specific structural features needed for their function. The evolutionary relationships of the Aldh1a family confirm the absence of Aldh1a1 in European sea bass and support the current hypothesis that a common ancestor of tetrapods and teleost exhibited both *aldh1a2* and *aldh1a3* genes. In addition, we add evidence to the current hypothesis that suggests the Aldh1a family is not a tetrapod innovation, but it originated during the first WGD and that the subsequent TGD retained this gene family in the same chromosome, despite the divergences found in the surrounding genomic arrangement. The evolutionary relationships of Cyp26a1 also support the evidence of a common ancestor for the Cyp26 family, strengthening the hypothesis of an independent gene functionalization prior to the two rounds of genome duplication in vertebrates. In addition, the absence of *stra8* and a conserved genomic neighbourhood in the European sea bass genome points to a gene loss in the ray finned lineage during the second TGD and suggests that RA signaling in this species does not occur through the transduction of this particular meiosis gatekeeper, as it has been demonstrated in tetrapods and other fish.

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REFERENCE LIST

Adolfi, M. C., Herpin, A., Regensburger, M., Sacquegno, J., Waxman, J. S., and Schartl, M. (2016). Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. *Scientific reports* **6**, 34281.

Alnouti, Y., and Klaassen, C. D. (2008). Tissue distribution, ontogeny, and regulation of aldehyde dehydrogenase (Aldh) enzymes mRNA by prototypical microsomal enzyme inducers in mice. *Toxicological Sciences* **101**, 51-64.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402.

Anderson, E. L., Baltus, A. E., Roepers-Gajadien, H. L., Hassold, T. J., de Rooij, D. G., van Pelt, A. M., and Page, D. C. (2008). Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci U S A* **105**, 14976-80.

Baltus, A. E., Menke, D. B., Hu, Y.-C., Goodheart, M. L., Carpenter, A. E., de Rooij, D. G., and Page, D. C. (2006). In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet* **38**, 1430-1434.

Blázquez, M., Medina, P., Crespo, B., Gómez, A., and Zanuy, S. (2017). Identification of conserved genes triggering puberty in European sea bass males (Dicentrarchus labrax) by microarray expression profiling. *BMC Genomics* **18**, 441.

Bowles, J., and Koopman, P. (2007). Retinoic acid, meiosis and germ cell fate in mammals. *Development* **134**, 3401-11.

Braasch, I., Gehrke, A. R., Smith, J. J., Kawasaki, K., Manousaki, T., Pasquier, J., Amores, A., Desvignes, T., Batzel, P., and Catchen, J. (2015). The spotted gar genome illuminates vertebrate evolution and facilitates human-teleost comparisons. Nature genetics 47, 427.

Cañestro, C., Catchen, J. M., Rodriguez-Mari, A., Yokoi, H., and Postlethwait, J. H. (2009). Consequences of lineage-specific gene loss on functional evolution of surviving paralogs: ALDH1A and retinoic acid signaling in vertebrate genomes. *PLoS Genet* **5**, e1000496.

Catchen, J. M., Conery, J. S., and Postlethwait, J. H. (2009). Automated identification of conserved synteny after whole-genome duplication. *Genome Research*.

Chaves-Pozo, E., Valero, Y., Esteve-Codina, A., Gómez-Garrido, J., Dabad, M., Alioto, T., Meseguer, J., Esteban, M. Á., and Cuesta, A. (2017). Innate Cell-Mediated Cytotoxic Activity of European Sea Bass Leucocytes Against Nodavirus-Infected Cells: A Functional and RNA-seq Study. *Scientific reports* **7**, 15396.

Dong, R., Yang, S., Jiao, J., Wang, T., Shi, H., Zhou, L., Zhang, Y., and Wang, D. (2013). Characterization of Stra8 in Southern catfish (Silurus meridionalis): evidence for its role in meiotic initiation. *BMC Molecular Biology* **14**, 11.

Feng, R., Fang, L., Cheng, Y., He, X., Jiang, W., Dong, R., Shi, H., Jiang, D., Sun, L., and Wang, D. (2015). Retinoic acid homeostasis through aldh1a2 and cyp26a1 mediates meiotic entry in Nile tilapia (Oreochromis niloticus). *Scientific reports* **5**, 10131.

Gao, C., and Han, B. (2009). Evolutionary and expression study of the aldehyde dehydrogenase (ALDH) gene superfamily in rice (Oryza sativa). *Gene* **431**, 86-94.

Griswold, M. D., Hogarth, C. A., Bowles, J., and Koopman, P. (2012). Initiating meiosis: the case for retinoic acid. *Biol Reprod* **86**, 35.

Gudas, L. J. (1994). Retinoids and vertebrate development. *Journal of Biological Chemistry* **269**, 15399-15402.

Hempel, J., Kuo, I., Perozich, J., Wang, B.-C., Lindahl, R., and Nicholas, H. (2001). Aldehyde dehydrogenase. *European Journal of Biochemistry* **268**, 722-726.

Hoegg, S., Brinkmann, H., Taylor, J., and Meyer, A. (2004). Phylogenetic Timing of the Fish-Specific Genome Duplication Correlates with the Diversification of Teleost Fish. *Journal of Molecular Evolution* **59**, 190-203.

Holmes, R. S. (2015). Comparative and evolutionary studies of vertebrate ALDH1A-like genes and proteins. *Chemico-Biological Interactions* **234**, 4-11.

Hu, P., Tian, M., Bao, J., Xing, G., Gu, X., Gao, X., Linney, E., and Zhao, Q. (2008). Retinoid regulation of the zebrafish cyp26a1 promoter. *Developmental dynamics: an official publication of the American Association of Anatomists* **237**, 3798-3808.

Jackson, B., Brocker, C., Thompson, D. C., Black, W., Vasiliou, K., Nebert, D. W., and Vasiliou, V. (2011). Update on the aldehyde dehydrogenase gene (ALDH) superfamily. *Human Genomics* **5**, 283-303.

Kim, D.-S., Kim, D.-W., Kim, M.-Y., Nam, S.-H., Choi, S.-H., Kim, R. N., Kang, A., Kim, A., and Park, H.-S. (2012). CACG: A database for comparative analysis of conjoined genes. *Genomics* **100**, 14-17.

Koubova, J., Hu, Y.-C., Bhattacharyya, T., Soh, Y. Q. S., Gill, M. E., Goodheart, M. L., Hogarth, C. A., Griswold, M. D., and Page, D. C. (2014). Retinoic Acid Activates Two Pathways Required for Meiosis in Mice. *PLoS Genet* **10**, e1004541.

Lesk, A. M. (1995). NAD-binding domains of dehydrogenases. *Current Opinion in Structural Biology* **5**, 775-783.

Li, M., Feng, R., Ma, H., Dong, R., Liu, Z., Jiang, W., Tao, W., and Wang, D. (2016). Retinoic acid triggers meiosis initiation via stra8-dependent pathway in Southern catfish, Silurus meridionalis. *Gen Comp Endocrinol* **232**, 191-198.

Liu, Z. J., Sun, Y. J., Rose, J., Chung, Y. J., Hsiao, C. D., Chang, W. R., Kuo, I., Perozich, J., Lindahl, R., Hempel, J., and Wang, B. C. (1997). The first structure of an aldehyde dehydrogenase reveals novel interactions between NAD and the Rossmann fold. *Nature structural biology* **4**, 317-326.

Loudig, O., Babichuk, C., White, J., Abu-Abed, S., Mueller, C., and Petkovich, M. (2000). Cytochrome P450RAI (CYP26) promoter: a distinct composite retinoic acid response element underlies the complex regulation of retinoic acid metabolism. *Molecular endocrinology* **14**, 1483-1497.

Lutz, J. D., Dixit, V., Yeung, C. K., Dickmann, L. J., Zelter, A., Thatcher, J. E., Nelson, W. L., and Isoherranen, N. (2009). Expression and functional characterization of cytochrome P450 26A1, a retinoic acid hydroxylase. *Biochem Pharmacol* **77**, 258-68.

Medina, P., Gómez, A., Zanuy, S., and Blázquez, M. (2019). Involvement of the retinoic acid signaling pathway in sex differentiation and pubertal development in the European sea bass Dicentrarchus labrax. Heliyon 5, e01201-e01201.

Menke, D. B., Koubova, J., and Page, D. C. (2003). Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. *Dev Biol* **262**, 303-312.

Meunier, B., de Visser, S. P., and Shaik, S. (2004). Mechanism of Oxidation Reactions Catalyzed by Cytochrome P450 Enzymes. *Chemical Reviews* **104**, 3947-3980.

Muzio, G., Maggiora, M., Paiuzzi, E., Oraldi, M., and Canuto, R. A. (2012). Aldehyde dehydrogenases and cell proliferation. *Free Radic Biol Med* **52**, 735-46.

Napoli, J. L. (1996). Retinoic acid biosynthesis and metabolism. *The FASEB Journal* **10**, 993-1001.

Napoli, J. L. (2012). Physiological insights into all-trans-retinoic acid biosynthesis. *Biochim Biophys Acta* **1821**, 152-67.

Pasquier, J., Cabau, C., Nguyen, T., Jouanno, E., Severac, D., Braasch, I., Journot, L., Pontarotti, P., Klopp, C., and Postlethwait, J. H. (2016). Gene evolution and gene expression after whole genome duplication in fish: the PhyloFish database. *BMC Genomics* **17**, 1.

Perozich, J., Nicholas, H., Wang, B.-C., Lindahl, R., and Hempel, J. (1999). Relationships within the aldehyde dehydrogenase extended family. *Protein Science* **8**, 137-146.

Pittlik, S., Domingues, S., Meyer, A., and Begemann, G. (2008). Expression of zebrafish aldh1a3 (raldh3) and absence of aldh1a1 in teleosts. *Gene Expr Patterns* **8**, 141-7.

Prakash, T., Sharma, V. K., Adati, N., Ozawa, R., Kumar, N., Nishida, Y., Fujikake, T., Takeda, T., and Taylor, T. D. (2010). Expression of Conjoined Genes: Another Mechanism for Gene Regulation in Eukaryotes. *PLoS One* **5**, e13284.

Rodríguez-Marí, A., Cañestro, C., BreMiller, R. A., Catchen, J. M., Yan, Y.-L., and Postlethwait, J. H. (2013). Retinoic Acid Metabolic Genes, Meiosis, and Gonadal Sex Differentiation in Zebrafish. *PLoS One* **8**, e73951.

Roginski, R. S., Lau, C. W., Santoiemma, P. P., Weaver, S. J., Du, P., Soteropoulos, P., and Yang, J. (2018). The human GCOM1 complex gene interacts with the NMDA receptor and internexin-alpha. *Gene* **648**, 42-53.

Roginski, R. S., Mohan Raj, B. K., Birditt, B., and Rowen, L. (2004). The human GRINL1A gene defines a complex transcription unit, an unusual form of gene organization in eukaryotes. *Genomics* **84**, 265-276.

Ross, A. C., and Zolfaghari, R. (2011). Cytochrome P450s in the regulation of cellular retinoic acid metabolism. *Annu Rev Nutr* **31**, 65-87.

Ross, S. A., McCaffery, P. J., Drager, U. C., and De Luca, L. M. (2000). Retinoids in Embryonal Development. *Physiological Reviews* **80**, 1021-1054.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.

Smith, C., Roeszler, K., Bowles, J., Koopman, P., and Sinclair, A. (2008). Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. *BMC Developmental Biology* **8**, 85.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599.

Taylor, J. S., Braasch, I., Frickey, T., Meyer, A., and Van de Peer, Y. (2003). Genome duplication, a trait shared by 22,000 species of ray-finned fish. *Genome Research* **13**, 382-390.

Thatcher, J. E., and Isoherranen, N. (2009). The role of CYP26 enzymes in retinoic acid clearance. *Expert Opin Drug Metab Toxicol* **5**, 875-86.

Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2002). Multiple Sequence Alignment Using ClustalW and ClustalX. *In* "Current Protocols in Bioinformatics". John Wiley & Sons, Inc.

Tine, M., Kuhl, H., Gagnaire, P.-A., Louro, B., Desmarais, E., Martins, R. S. T., Hecht, J., Knaust, F., Belkhir, K., and Klages, S. (2014). European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nature communications* **5**.

Uji, S., Suzuki, T., and Kurokawa, T. (2006). Molecular cloning and expression of retinoic-acid synthesizing enzyme raldh2 from Takifugu rubripes. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* **1**, 133-138.

Uno, T., Ishizuka, M., and Itakura, T. (2012). Cytochrome P450 (CYP) in fish. *Environ Toxicol Pharmacol* **34**, 1-13.

Wallacides, A., Chesnel, A., Chardard, D., Flament, S., and Dumond, H. (2009). Evidence for a conserved role of retinoic acid in urodele amphibian meiosis onset. *Dev Dyn* **238**, 1389-98.

White, J. A., Beckett-Jones, B., Guo, Y.-D., Dilworth, F. J., Bonasoro, J., Jones, G., and Petkovich, M. (1997). cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450 (CYP26). *Journal of Biological Chemistry* **272**, 18538-18541.

Zuckerkandl, E., and Pauling, L. (1965). Evolutionary divergence and convergence in proteins. *In* "Evolving genes and proteins", pp. 97-166. Elsevier.

CHAPTER 2 ADDITIONAL TABLES

Additional Table 1. Accession numbers of ALDH1A2 and ALDH1A3 protein sequences

Species	ALDH1A2	ALDH1A3
Human	NP_003879.2	NP_000684.2
Opossum	XP_001368154.1	XP_016280156.1
Anole lizard		XP_003226545.2
Zebra Finch	NP_001070153.1	XP_002199949.1
Tilapia	NP_001298257.1	
Cod	ENSGMOP00000016730.1	ENSGMOP00000018554
Zebrafish	AAL26232.1	NP_001038210.1
Fugu		NP_001153657.1
Spotted gar		XP_006628723.1
Sea bass	ANA57439.1	ANA57440.1

Additional Table 2. Accession numbers of CYP26A1, CYP26B1 and CYP26C1 protein sequences

Species	**CYP26A1**	**CYP26B1**	**CYP26C1**
Homo sapiens	NP_000774	NP_063938	NP_899230
Anolis carolinensis	ENSACAT00000006924		ENSACAP00000006744
Gallus gallus	NP_001001129	XP_426366	XP_421678
Latimeria chalumnae	ENSLACT00000018096	ENSLACT00000017367	
Oreochromis niloticus	ENSONIT00000009134.1	ENSONIP00000000575	ENSONIP00000019469
Gadus morhua	ENSGMOT00000014287	ENSGMOT0000005622	ENSGMOTO00000001784
Danio rerio	NP_571221.2	NP_997831	NP_001025122
Takifugu rubripes	ENSTRUP00000004613	ENSTRUP00000031925	ENSTRUP00000032385
Dicentrarchus labrax	AHY95171.1		

Additional Table 3. Accession numbers used to infer the phylogenetic trees for ALDH1A2 and ALDH1A3 proteins

Species	**ALDH1A2**	**ALDH1A3**
Human	NP_003879.2	NP_000684.2
Mouse	NP_033048.2	NP_444310.3
Rat	NP_446348.2	EDM08431.1
Duck	ADY68767.1	
Chicken	NP_990326.1	NP_990000.1
Silurana	NP_001039196.1	
Spotted gar	ADX94806.1	
Zebrafish	AAL26232.1	NP_001038210.1
Salmon	ACI33975.1	
Fugu	BAE20172.1	NP_0012038210.1
Sea bass	ANA57439.1	ANA57440.1
Tilapia ALDH1A1	XP_025767670.1	

Additional Table 4. Accession numbers used to infer the phylogenetic tree for CYP26A1, CYP26B1 and CYP26C1 proteins

Species	**CYP26A1**	**CYP26B1**	**CYP26C1**
Human	NP_000774	NP_063938	NP_899230
Mouse	NP_031837	NP_780684	NP_001171184.1
Chimpanzee	ENSPTRP00000044339	ENSPTRP00000020680	ENSPTRP00000004819
Lizard	ENSACAT00000006924		ENSACAP00000006744
Turtle	ENSPSIP00000020213	ENSPSIT00000014991.1	ENSPSIP00000020108
Chicken	NP_001001129	XP_015141554.1	XP_421678
Zebrafinch	ENSTGUP00000009117		ENSTGUP00000009124
Flycatcher	ENSFALP00000008250	ENSFALP00000011370	ENSFALP00000008251
Turkey	ENSMGAP00000009264	ENSMGAP00000014077	
Duck	ENSAPLP00000002315	ENSAPLP00000007277	ENSAPLP00000002568
Silurana	NP_001016147	ENSXETP00000046568	ENSXETP00000039433
Xenopus	NP_001088938	ACF33501	NP_001089956
Sea bass	AHY95171		
Stickleback	ENSGACP00000020277	ENSGACP00000024870	ENSGACP00000014662
Tilapia	ENSONIT00000009134.1	ENSONIP00000000575	ENSONIP00000019469
Platyfish	ENSXMAP00000014170	ENSXMAP00000000560	ENSXMAT00000030892.1
Medaka	NP_001265772	ENSORLP00000004308	ENSORLT00000002542.2
Cod	ENSGMOT00000014287	ENSGMOT00000005622	ENSGMOT00000001784.1

Additional Table 5. Ensembl databases and accession numbers of genes used for *ALDH1A* family synteny analysis

Species (Abbreviation)	**Ensembl database**	**ALDH1A2**	**ALDH1A3**
Homo sapiens (Hsa)	GRCh38	ENSG00000128918	ENSG00000184254
Taenopygia guttata (Tgut)	taeGut3.2.4	ENSTGUG00000006178	ENSTGUG00000008854
Pelodiscus sinensis (Psi)	PelSin_1.0	ENSPSIG00000017126	ENSPSIG00000014175
Xenopus laevis (Xla)	JGI4.2	ENSXETG00000010865	ENSXETG00000018751
Lepistosteus oculatus (Loc)	LepOcu1	ENSLOCG00000013574	ENSLOCG00000012733
Latimeria chalumnae (Lch)	LatCha1		ENSLACG00000017897
Danio rerio (Dre)	Zv9	ENSDARG00000053493	ENSDARG00000076933
Tetraodon nigroviridis(Tni)	TETRAODON 8.0	ENSTNIG00000006520	ENSTNIG00000006694
Oreochromis niloticus (Onil)	Orenil 1.0	ENSONIG00000005813	
Gasterosteus aculeatus(Gac)	BROAD S1		ENSGACG00000013986
*Dicentrarchus labrax (Dla)**	dicLab v1.0c	KR534500	KR534501

*NCBI database accession numbers.

Additional Table 6. List of names, symbols and function of genes that appear in *ALDH1A2* synteny analysis

Gene name	Gene symbol	Function
ADAM Metallopeptidase Domain 10	*ADAM10*	Involved in the cleavage of many proteins including tnf-alpha and e-cadherin.
Aquaporin 9	*AQP9*	Encodes a protein that allows passage of a broad range of non-charged solutes and also stimulates urea transport and osmotic water permeability.
Cingulin Like 1	*CGNL1*	Encodes a protein that localizes to both adherens and tight cell-cell junctions and mediates junction assembly and maintenance by regulating the activity of the small GTPases rhoa and rac1.
MINDY Lysine 48 Deubiquitinase 1	*FAM63* or *MINDY1*	Encodes for a hydrolase that can specifically remove lys-48-linked conjugated ubiquitin from proteins.
GRINL1A Complex Locus 1	*GCOM1*	May encode proteins that share sequence identity with the upstream gene product or with both the upstream and downstream gene products.
Lipase C, Hepatic Type	*LIPC*	The product of this gene has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake.
Meiosis Specific Nuclear Structural 1	*MNS1*	Encodes a protein highly similar to the mouse meiosis-specific nuclear structural 1 protein.
Myocardial Zonula Adherens Protein	*MYZAP*	Encodes a protein that is abundantly expressed in cardiac tissue.
RNA Polymerase II Subunit M	*POLR2M*	Encodes a subunit of a specific form of RNA polymerase II termed pol II(G).
Transcription Factor 12	*TCFL12*	DNA binding transcription factor activity and protein dimerization activity.
Testis Expressed 9	*TEX9*	No data available.
Zinc Finger Protein 280D	*ZNF280D*	Encodes a protein that may function as transcription factor.

Compiled from GeneCards® database at www.genecard.org.

Additional Table 7. List of names, symbols and function of genes that appear in *ALDH1A3* synteny analysis

Gene name	Gene symbol	Function
ADAM Metallopeptidase With Thrombospondin Type 1 Motif 17	*ADAMTS17*	Involved in cell-cell and cell-matrix interactions, including fertilization, muscle development, and neurogenesis.
Ankyrin Repeat And SOCS Box Containing 7	*ASB7*	Acts as a bridge between specific substrate-binding domains and the more generic proteins that comprise a large family of E3 ubiquitin protein ligases.
Ceramide Synthase 3	*CERS3*	Involved in the regulation of sphingolipid synthesis by catalysing the formation of ceramides from sphingoid base and acyl-coa substrates.
Chondroitin Sulfate Synthase 1	*CHSY1*	Play critical roles in the biosynthesis of chondroitin sulphate, a glycosaminoglycan involved in many biological processes including cell proliferation and morphogenesis.
Lines Homolog 1	*LINS*	Posttranscriptional regulator of genes involved in developmental timing and self-renewal in embryonic stem cells.
Leucine Rich Repeat Kinase 1	*LRRK1*	Encodes a multi-domain protein that is a leucine-rich repeat kinase and a GDP/GTP binding protein.
Lysm Domain Containing 4	*LYSMD4*	No data available.
Myocyte Enhancer Factor 2[a]	*MEFZA*	The protein encoded by this gene is a DNA-binding transcription factor that activates many muscle-specific, growth factor-induced, and stress-induced genes.
Proprotein Convertase Subtilisin/Kexin Type 6	*PCSK6*	Encodes a protease that process protein and peptide precursors trafficking through regulated or constitutive branches of the secretory pathway.
Small Nuclear Ribonucleoprotein Polypeptide A'	*SNRPA1*	Contributes to the binding of stem loop IV of U2 snRNA.
Selenoprotein S	*VIMP*	Encodes a protein involved in the degradation of misfolded proteins in the endoplasmic reticulum, and may also have a role in inflammation control.

Compiled from GeneCards® database at www.genecard.org.

Additional Table 8. Ensembl accession numbers used for *CYP26A1* synteny analysis

Species (Abreviation)	**Ensemble database**	**Ensembl number**
Homo sapiens (Hsa)	GRCh38p5	ENSG00000095596
Mus musculus (Mmu)	GRCm38.p4	ENSMUSG00000024987
Gallus gallus (Gga)	Galgal4	ENGAL00000006729
Taenopygia guttata (Tgut)	taeGut3.2.4	ENSTGUG00000008850
Xenopus tropicalis (Xtr)	JGI4.2	ENSXETG00000018184
Lepisosteus oculatus (Loc)	LepOcu1	ENSLOCG00000006966
Latimeria chalumnae (Lch)	LatCha1	ENSLACG00000015829
Tetraodon nigroviridis (Tni)	TETRAODON 8.0	ENSTNIG00000004668
Gasterosteus aculeatus (Gac)	BROAD S1	ENSGACG00000015370
Danio rerio (Dre)	Zv9/GRCz10	ENSDARG00000033999
*Dicentrarchus labrax (Dla)**	dicLab v1.0c	KJ187657

*NCBI database accession numbers.

Additional Table 9. List of names, symbols and gene function that appear in *CYP26A1* synteny analysis

Gene name	Gene symbol	Function
B-TFIID Tata-box Binding Protein Associated Factor 1	*BTAF1*	The product of this gene is a part of the B-TFIID complex that is required for transcription initiation of genes by RNA polymerase II.
Centrosomal Protein 55	*CEP55*	Related to the cytoskeletal signaling pathway.
Cytoplasmic Polyadenylation Element Binding Protein 3	*CPEB3*	Related to the oocyte meiosis pathway.
Exocyst Complex Component 6	*EXOC6*	Essential for vesicular traffic from the Golgi apparatus to the cell surface in yeast.
Free Fatty Acid Receptors	*FFAR*	Involved in the metabolic regulation of insulin secretion.
FRA10A Associated CGG Repeat 1	*FRA10AC1*	The protein encoded by this gene is a nuclear phosphoprotein of unknown function.
Hematopoietically Expressed Homeobox	*HHEX*	Involved in developmental processes.
Insulin Degrading Enzyme	*IDE*	This gene encodes a zinc metallopeptidase that degrades intracellular insulin.
Kinesin Family member 11	*KIF11*	Encodes a motor protein required for establishing a bipolar spindle during mitosis.
Membrane Associated Ring-ch-type Finger 5	*MARCH5*	Encodes for a ubiquitin ligase of the mitochondrial outer membrane.
Myoferlin	*MYOF*	The protein encoded by this gene is a type II membrane protein that is structurally similar to dysferlin.
Phosphodiesterase 6C	*PDE6C*	Associated with cone dystrophy type 4 (COD4).
Retinol Binding Protein 4	*RBP4*	Codes for the protein belongs to the lipocalin family and is the specific carrier for retinol (vitamin A alcohol) in the blood.

Compiled from GeneCards® database at www.genecard.org.

Additional table 10. Ensembl accession numbers used for *STRA8* synteny analysis

Species (Abreviation)	**Ensembl database**	**STRA8 ID number**
Homo sapiens (Hsa)	GRCh38	ENSG00000146857
Mus musculus (Mmu)	GRCm38.p3	ENSMUSG00000029848
Taenopygia guttata (Tgut)	taeGut3.2.4	ENSTGUG00000009183
Pelodiscus sinensis (Psi)	PelSin_1.0	ENSPSIG00000013824
Gallus gallus (Gga)	Galgal4	ENSGALG00000011722
Xenopus tropicalis (Xtr)	JGI4.2	ENSXETG00000020235
Lepisosteus oculatus (Loc)	LepOcu1	ENSLOCG00000016111
Tetraodon nigroviridis (Tni)	TETRAODON8.0	Gene not found
Gasterosteus aculeatus (Gac)	BROADS1	Gene not found
Danio rerio (Dre)	Zv9	Gene not found
Dicentrarchus labrax (Dla)	dicLab v1.0c	Gene not found

Additional table 11. List of names, symbols and function of genes that appear in *STRA8* synteny analysis

Gene name	Gene symbol	Function
ATP/GTP Binding Protein Like 3	*AGBL3*	Encodes a metallocarboxypeptidase that mediates deglutamylation and deaspartylation of target proteins such as tubulins.
ADP Ribosylation Factor Like Gtpase 1	*ARL1*	Encodes a protein involved in the regulation of intracellular vesicular membrane trafficking. Important for the function of the golgi apparatus.
CCR4-NOT Transcription Complex Subunit 4	*CNOT4*	Involved in protein ubiquitination.
Family With Sequence Similarity 180 Member A	*FAM180A*	Osteoblastic specific gene.
Nucleoporin 205	*NUP205*	Encodes a subunit of the nuclear pore complex involved in the active transport of proteins and RNAs between the nucleus and cytoplasm.
Solute Carrier Family 13 Member 4	*SLC13A4*	Involved in the transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds.
Transmembrane Protein 140	*TMEM140*	Encodes a transmembrane protein and contains transcription factor binding sites for pparg in its promoter.
UTP20, Small Subunit Processome Component	*UTP20*	Involved in 18S pre-rrna processing.
WD Repeat Domain 91	*WDR91*	Mutations in this gene are associated with retinitis pigmentosa, a degenerative disease that causes vision impairment.

Compiled from GeneCards® database at www.genecard.org.

Additional Table 12. Transcription factor binding sites along the European sea bass cyp26a1 promoter sequence (1500 bp)

TF family information	Matrix information	Start	End	Anchor position	Strand	Core similarity	Matrix similarity	Sequence
SWI/SNF related nucleophosphoproteins with a RING finger DNA binding motif	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	11	21	16	+	0.96	0.97	tcACATttgcg
Vertebrate TATA binding protein factor	Avian C-type LTR TATA box	24	40	32	-	0.75	0.78	tggtatgGAAGcccatt
RXR heterodimer binding sites	**Bipartite binding site of VDR/RXR heterodimers, DR5 sites**	**38**	**62**	**50**	**-**	**0.81**	**0.80**	**atgctggttacactcAGGGcactgg**
Estrogen response elements	Estrogen-related receptor alpha	65	87	76	+	0.76	0.87	agtcAAAGtcatggaattttaca
RXR heterodimer binding sites	**Constitutive androstane receptor / retinoid X receptor heterodimer, DR4 sites**	**65**	**89**	**77**	**+**	**0.75**	**0.77**	**agtcaaAGTCatggaattttacatc**
TALE homeodomain class recognizing TG motifs	Binding site for monomeric Meis1 homeodomain protein	137	153	145	-	1.00	0.99	cggcaacTGTCaccgtt
Peroxisome proliferator-activated receptor	**PPAR/RXR heterodimers, DR1 sites**	**159**	**181**	**170**	**+**	**0.79**	**0.78**	**tcccaccggggtgaAGCTcaggg**
Estrogen response elements	Estrogen-related receptor alpha	420	442	431	-	1.00	0.88	acgcAAGGttatccccgggctac
Vertebrate steroidogenic factor	SF1 steroidogenic factor 1	429	443	436	-	1.00	0.97	aacgCAAGgttatcc
Nuclear receptor subfamily 2 factors	Nuclear hormone receptor TR2, DR5 binding sites	486	510	498	-	1.00	0.77	cgggttcaaacttgGGTCagcaaag
RXR heterodimer binding sites	**Retinoic acid receptor / retinoid X receptor heterodimer, DR5 sites**	**488**	**512**	**500**	**-**	**1.00**	**0.89**	**agcggGTTCaaacttgggtcagcaa**

Cell cycle regulators: Cell cycle homology element	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	498	510	504	+	1.00	0.96	aagtTTGAacccg
HOX - PBX complexes	Meis homeobox 1	616	632	624	+	1.00	0.85	aagcaGATTgatagaaa
Peroxisome proliferator-activated receptor	**Peroxisome proliferator-activated receptor gamma**	**641**	**663**	**652**	**+**	**0.89**	**0.89**	**caagcaggggcAGAGctgaggt**
DM domain-containing transcription factors	Doublesex and mab-3 related transcription factor 1	662	682	672	+	1.00	0.77	gttcccggccgcaTTGTtaca
DM domain-containing transcription factors	Doublesex and mab-3 related transcription factor 3	670	690	680	+	1.00	0.90	ccgcattgtTACActgaaaca
RXR heterodimer binding sites	**VDR/RXR Vitamin D receptor RXR heterodimer, DR3 sites**	**703**	**727**	**715**	**+**	**0.79**	**0.88**	**gaaggaaggggattGGGGccatatc**
HOX - PBX complexes	Meis homeobox 1	751	767	759	+	1.00	0.86	attccGATTtaaactca
RXR heterodimer binding sites	**Highly conserved DR1 element selected by LXRbeta/RXR heterodimers**	**763**	**787**	**775**	**-**	**1.00**	**0.71**	**attttaaagGGTCaaacttctgagt**
Nuclear receptor subfamily 2 factors	Apolipoprotein AI regulatory protein 1, NR2F2, DR1 sites	768	792	780	-	1.00	0.84	tttaaattttaaagGGTCaaacttc
HOX - MEIS1 heterodimers	Meis1b and Hoxa9 form heterodimeric binding complexes on target DNA	774	788	781	+	1.00	0.81	TGACcctttaaaatt
HOX - PBX complexes	Meis homeobox 1	824	840	832	-	1.00	0.88	gaaatGATTcaacctgt
SWI/SNF related nucleophosphoproteins with a RING finger DNA binding motif	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	844	854	849	-	1.00	0.98	caCCATgtgga

Cell cycle regulators: Cell cycle homology element	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	866	878	872	-	1.00	0.96	gcatTTGAacctg
Motif composed of binding sites for pluripotency or stem cell factors	Composed binding site for Oct4, Sox2, Nanog, Tcf3 (Tcf7l1) and Sall4b in pluripotent cells	870	888	879	+	1.00	0.79	ttcaaatGCATctaaatca
Vertebrate TATA binding protein factor	Mammalian C-type LTR TATA box	912	928	920	+	1.00	0.98	gcctaTAAAataatagg
SOX/SRY-sex/testis determinig and related HMG box factors	HMGA family of architectural transcription factors (HMGA1, HMGA2)	923	947	935	+	1.00	0.90	aataggaaatgAATGaatgaataaa
SOX/SRY-sex/testis determinig and related HMG box factors	HMG box-containing protein 1	930	954	942	+	1.00	1.00	aatgaatgAATGaataaactttgag
RXR heterodimer binding sites	**Bipartite binding site of VDR/RXR heterodimers, DR5 sites**	**1100**	**1124**	**1112**	**+**	**1.00**	**0.77**	**gcggggcttaagccaAGGTaatgag**
Vertebrate steroidogenic factor	SF1 steroidogenic factor 1	1109	1123	1116	+	1.00	0.96	aagcCAAGgtaatga
Estrogen response elements	Estrogen-related receptor alpha	1110	1132	1121	+	1.00	0.88	agccAAGGtaatgagtgtgtaaa
SOX/SRY-sex/testis determinig and related HMG box factors	SRY (sex determining region Y)-box 6	1146	1170	1158	+	1.00	0.99	tggcaACAAaggcgtcttgggacgc
SOX/SRY-sex/testis determinig and related HMG box factors	SRY-box containing gene 2	1162	1186	1174	-	1.00	0.94	ccgagACAAtggcagtgcgtcccaa
Testis-specific bHLH-Zip transcription factors	Spermatogenic Zip 1 transcription factor	1184	1194	1189	+	1.00	0.95	cGGAGggaacc

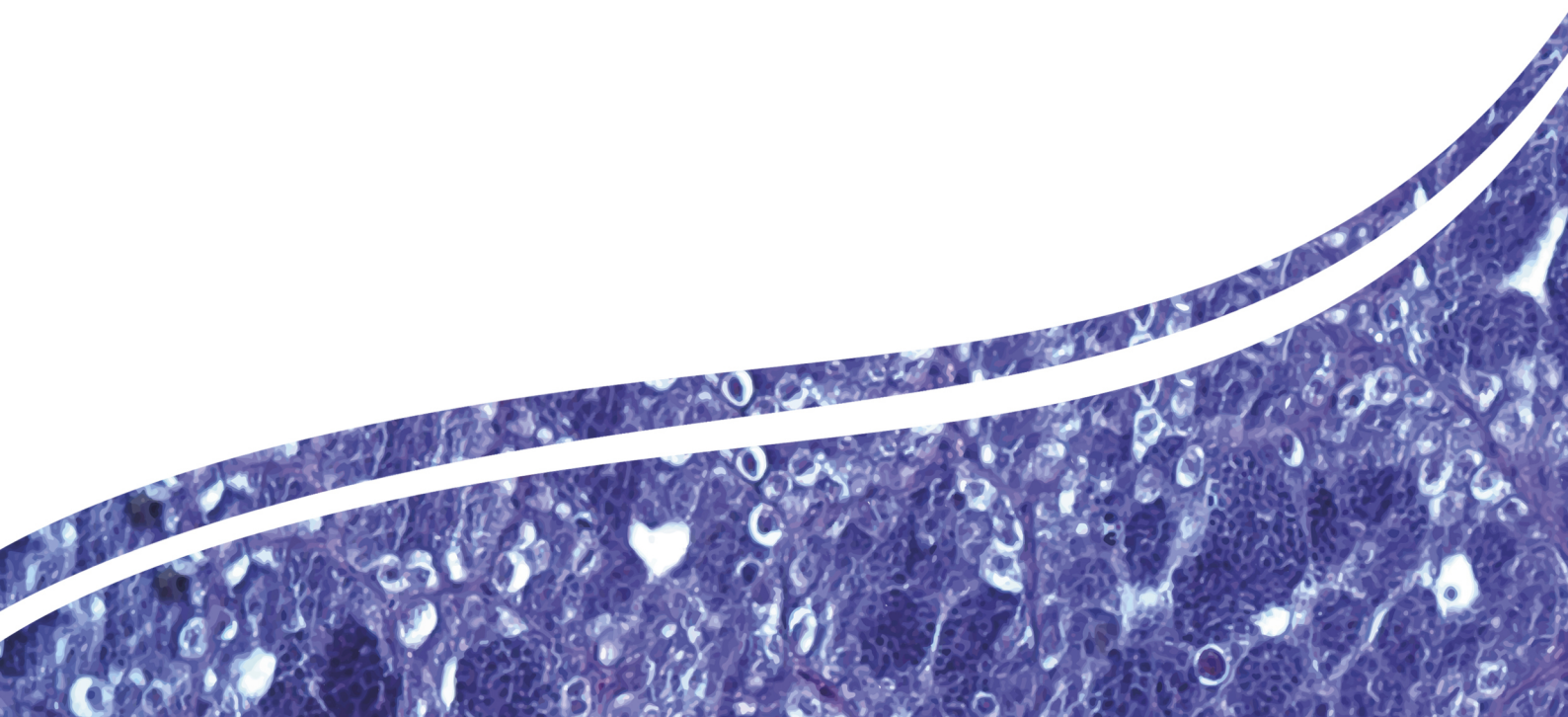
Peroxisome proliferator activated receptor homodimers	**Pal3 motif, bound by a PPAR-gamma homodimer, IR3 sites**	**1183**	**1205**	**1194**	**-**	**0.76**	**0.69**	**gaaAAGGtgatggttccctccga**
Peroxisome proliferator-activated receptor	**Peroxisome proliferator-activated receptor gamma, DR1 sites**	**1193**	**1215**	**1204**	**-**	**1.00**	**0.87**	**atttcactgagaAAAGgtgatgg**
Cell cycle regulators: Cell cycle dependent element	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)	1216	1228	1222	+	1.00	0.88	ccgcCGCGgattg
AHR-arnt heterodimers and AHR-related factors	DRE (dioxin response elements), XRE (xenobiotic response elements) bound by AHR/ARNT heterodimers	1222	1246	1234	+	1.00	0.97	cggattgtctGCGTgcaacaggagg
Vertebrate steroidogenic factor	Alpha (1)-fetoprotein transcription factor (FTF), liver receptor homologue-1 (LRH-1)	1260	1274	1267	-	1.00	0.97	gttcCAAGggccgca
SWI/SNF related nucleophosphoproteins with a RING finger DNA binding motif	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	1272	1282	1277	-	1.00	0.99	cgCCATttgtt
Cell cycle regulators: Cell cycle dependent element	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)	1274	1286	1280	-	0.84	0.88	gcttCGCCatttg
SOX/SRY-sex/testis determinig and related HMG box factors	SRY-box containing gene 3	1268	1292	1280	+	1.00	0.95	ttggaaCAAAtggcgaagcaaggca
Myc associated zinc fingers	MYC-associated zinc finger protein related transcription factor	1306	1318	1312	+	1.00	0.89	ggaggcGGGGccg
E2F-myc activator/cell cycle regulator	E2F transcription factor 4, p107/p130-binding protein	1305	1321	1313	+	1.00	0.98	cggagGCGGggccgagc

SOX/SRY-sex/testis determinig and related HMG box factors	SRY (sex determining region Y)-box 9 homodimer	1330	1354	1342	-	0.80	0.78	ttcacTCAAagttcatctttaattg
Nuclear receptor subfamily 2 factors	Hepatic nuclear factor 4alpha, DR1 sites	1334	1358	1346	-	1.00	0.97	ttagttcactCAAAgttcatcttta
Peroxisome proliferator-activated receptor	**Peroxisome proliferator-activated receptor gamma, DR1 sites**	**1337**	**1359**	**1348**	**-**	**1.00**	**0.84**	**attagttcactcAAAGttcatct**
RXR heterodimer binding sites	**Retinoic acid receptor / retinoid X receptor heterodimer, DR5 sites**	**1336**	**1360**	**1348**	**-**	**1.00**	**0.97**	**aattaGTTCactcaaagttcatctt**
AP1, Activating protein 1	Activator protein 1	1344	1356	1350	+	1.00	0.88	ctttGAGTgaact
v-ERB and RAR-related orphan receptor alpha	RAR-related orphan receptor alpha2	1342	1364	1353	-	0.75	0.86	gataaattaGTTCactcaaagtt
DM domain-containing transcription factors	Doublesex and mab-3 related transcription factor 5	1370	1390	1380	-	1.00	0.83	gcagcctGTTAccttctcctc
Cell cycle regulators: Cell cycle dependent element	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)	1388	1400	1394	+	1.00	0.90	tgccCGCGgtctt
Vertebrate TATA binding protein factor	Mammalian C-type LTR TATA box	1405	1421	1413	+	1.00	1.00	gcctaTAAAagcgcagg
RXR heterodimer binding sites	**Bipartite binding site of VDR/RXR heterodimers, DR5 sites**	**1453**	**1477**	**1465**	**-**	**1.00**	**0.78**	**ggagcggtgcagctcAGGTgaagct**
TALE homeodomain class recognizing TG motifs	Binding site for monomeric Meis1 homeodomain protein	1481	1497	1489	+	1.00	0.97	aaccgccTGTCactgca

Chapter 2. Characterization of the genes encoding the main enzymes involved in the synthesis (*aldh1a2, aldh1a3*), degradation of the RA (*cyp26a1*) and the meiosis gatekeeper *stra8* in the European sea bass.

Chapter 3.

Once the major enzymes of the RA signaling pathway were confirmed, characterized and cloned, the aim was set to search for a possible influence in early differentiation and meiosis during male and female puberty. An exhaustive histological study was carried out to establish stages of maturation in different developmental periods including: sexual differentiation, onset of meiosis in testis, and full development in ovary (since meiotic division is arrested and resumed several times only to finish by the time of fertilization). Nine representative genes of the RA pathway (rbp4, stra6, aldh1a2, aldh1a3, rarα, rxrα, pparγ, crabp1 and cyp26a1) were selected to quantify their relative expression at each sampling point through qPCR, thus creating and comparing expression patterns throughout the study (Fig. 1). In addition, two control genes (cyp19a1 and amh) were used as a control to further verify the sex in histologically undifferentiated fish and also as a marker for the assignment of the developmental stage of the fish previously assessed by histology.



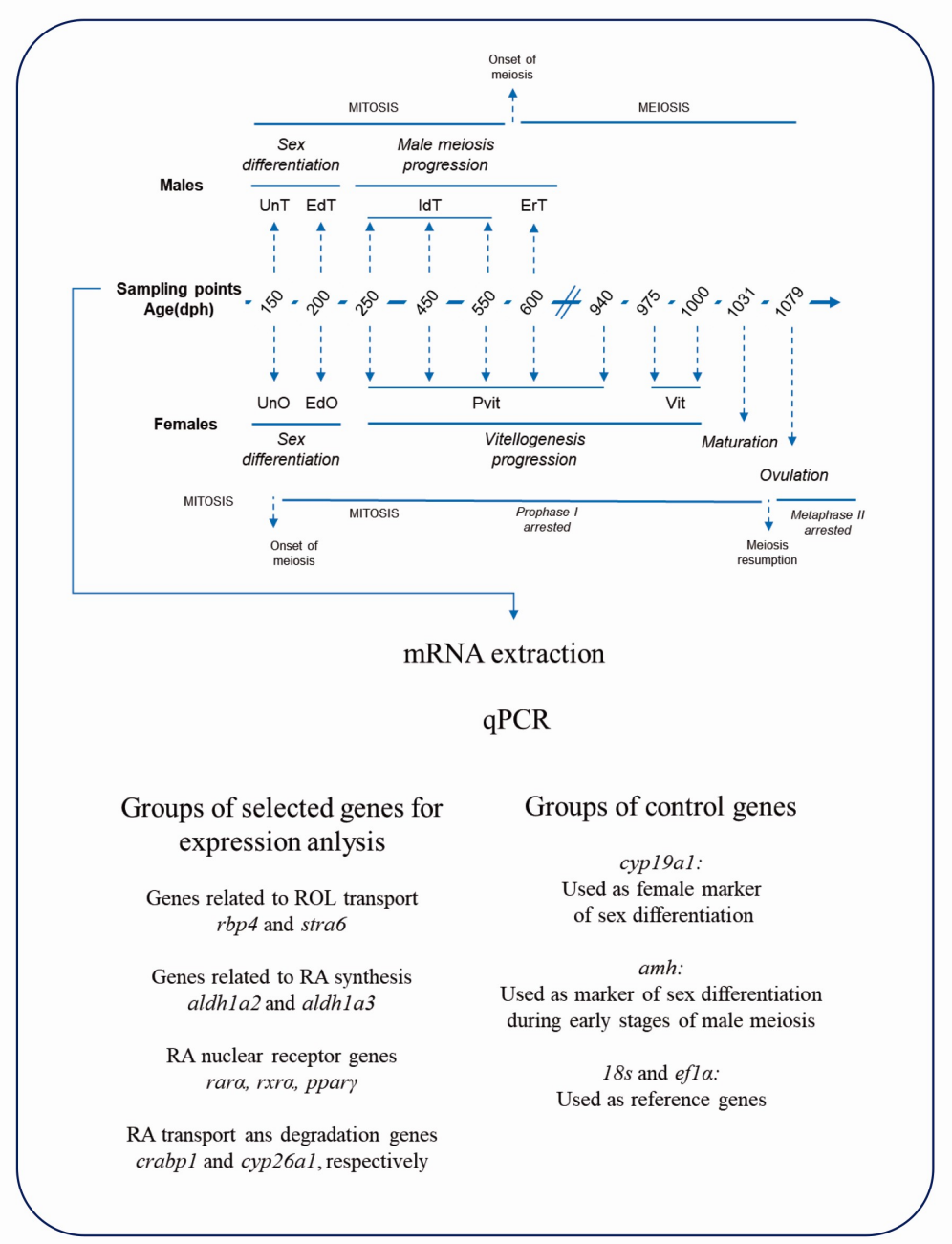


Figure 1. Experimental design depicting the sampling time points represented as days post hatching (dph) during the different gonad developmental stages covered by the study. Vertical arrows link the different sampling points with their developmental stage and their corresponding period (in italics) during puberty in European sea bass. Abbreviations: undifferentiated ovary (UnO), undifferentiated testis (UnT), early differentiated testis (EdT), immature differentiated testis (IdT), early recrudescent testis (ErT), previtellogenesis (Pvit), vitellogenesis (Vit), maturation, and ovulation.

Involvement of the retinoic acid signaling pathway in sex differentiation and pubertal development in the European sea bass

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ABSTRACT

Retinoic Acid (RA) is a vitamin A derivative present in many biological processes including embryogenesis, organ development and cell differentiation. The RA signaling pathway is essential for the onset of meiosis in tetrapods, although its role in fish reproduction needs further evidence. This study reports the expression profiles of several genes involved in this pathway during sex differentiation and the first reproductive season in European sea bass (*Dicentrarchus labrax*) gonads. The assessed genes are representative of several steps of the pathway including retinol transport, RA synthesis, nuclear receptors, RA transport and degradation. The results show that, these genes were overexpressed during early gonad development and their expression decreased during meiosis progression in males and during vitellogenesis in females. Specifically, a decrease of *cyp26a1*, involved in RA degradation, together with an increase of *aldh1a2* and *aldh1a3*, in charge of RA-synthesis, might ensure the availability of high RA levels at the time of meiosis in males and females. Taken together, our results might help to understand the role of RA signaling in teleost gonad development.

INTRODUCTION

Retinoic acid (RA) is the active form of vitamin A, and exerts pleiotropic functions in many biological processes such as differentiation of the nervous system (McCaffery et al., 2003), embryogenesis (Reijntjes et al., 2004), body patterning (Marill et al., 2003), organ and skeletal development (Campo-Paysaa et al., 2008; Spoorendonk et al., 2008) or cell differentiation (Niederreither and Dolle, 2008). More than a century of research has built up a solid framework concluding that the RA signaling pathway is essential for the onset of meiosis in tetrapods (Griswold et al., 2012). Briefly, this pathway involves the initial formation of a complex for retinol (ROL) bloodstream transport, which includes the ROL binding protein-4 (Rbp4). Once in the target tissues, ROL enters the cell by interacting with the trans-membrane receptor Stra6 (stimulated by RA protein 6) (Albalat, 2009; Bowles and Koopman, 2007), expressed in Sertoli cells and other blood organ-barrier tissues (Amengual et al., 2014). Depending on the needs, ROL can either remain bound to Stra6, or be transformed into RA by two tandem oxidations. The first oxidation is mediated by an alcohol dehydrogenase and results in the synthesis of retinal (RAL), whereas the second one is mediated by aldehyde dehydrogenases (Aldhs) (Napoli, 2012). RA is highly unstable and toxic and can either bind to an intracellular protein (Crabp), in order to keep it soluble, or be degraded by Cyp26 enzymes into more polar compounds easier to clear out from the cell (Thatcher and Isoherranen, 2009).

RA signals are transduced by RA nuclear receptors (Rars) and retinoid receptors (Rxrs), which can hetero- or homodimerize to regulate gene expression. Each receptor consists of three isoforms and their dimerization results in multiple combinations (Chawla et al., 2001). *In vivo*, RA exists mostly as the all-trans isomeric conformation and only trace amounts of the 9-cis isomer can be found. Although both isomers are capable to bind to the ligand binding domain of Rar, Rxr has higher affinity for the 9-cis isomer (Heyman et al., 1992). Moreover, Rxrs are also heterodimeric partners of other nuclear receptors, including thyroid hormone receptor, vitamin D receptor, and peroxisome proliferator-activated receptor (Ppar) (Chawla et al., 2001). The activated ligand/receptor complex binds to RA response elements (RAREs) that regulate the transcription of over 500 genes (Balmer and Blomhoff, 2002). Among them, it is worth mentioning *Stra8* (stimulated by RA gene 8), required for premeiotic DNA replication and the entry in prophase I of germ cells, making it essential for meiosis progression in tetrapods (Griswold et al., 2012).

In mouse, chicken and newt models, the onset of meiosis is induced by the balance between the synthesis and degradation of RA in a species- and sex-specific manner (Bowles et al., 2009; Smith et al., 2008; Wallacides et al., 2009). In mouse, meiosis starts before birth in

females, and by the time of puberty in males (Koubova et al., 2006), whereas in chicken the involvement of RA in the onset of meiosis has only been proven for females (Smith et al., 2008). In newt, the onset of meiosis in females occurs during larval life while in males it starts during metamorphosis (Wallacides et al., 2009), bringing about changes in the expression of key players of the RA signaling pathway. All these evidences support the role of RA signaling in the timing of the onset of meiosis in tetrapods, however, its role in gonad development and reproduction in fish still needs to be addressed. In this regard, a lack of *stra8* has been reported in several fish species (Pasquier et al., 2016; Rodríguez-Marí et al., 2013) and also in the European sea bass (Chapter 2; Medina et al., 2019). Despite these findings, a *stra8* homolog could be identified in Southern catfish, *Silurus meridionalis* (Dong et al., 2013), and other fish species (Pasquier et al., 2016), suggesting that in fish two different mechanisms, one dependent and the other one independent of the presence of *stra8*, might regulate the RA-mediated entry into meiosis (Feng et al., 2015; Li et al., 2016).

The European sea bass (*Dicentrarchus labrax*) is an excellent model to study the role of the RA signaling pathway in fish reproduction. The gonochoristic status of this marine teleost (Piferrer et al., 2005) allows for the possibility to provide a detailed sex-specific assessment of different components of the pathway during gonad development. The endocrine changes during its reproductive cycle are well known (Carrillo et al., 2015; Mañanós et al., 1997; Prat et al., 1990; Rocha et al., 2009) and different markers of sex differentiation (Blázquez et al., 2008; Blázquez et al., 2009; Díaz and Piferrer, 2015) and sexual maturation (Blázquez et al., 2017; Crespo et al., 2013) have been identified. This highly valued aquaculture species exhibits a high incidence of precocious puberty, particularly in males, with about 20-30% of them maturing by the end of the first year of life (Begtashi et al., 2004), as opposed to normal maturation that occurs by the end of the second year (Zanuy et al., 2001), influencing negatively growth, animal welfare and final product value (Carrillo et al., 2015; Espigares et al., 2015). The problems associated with precocious puberty have resulted in the development of production protocols to alleviate these negative effects (Begtashi et al., 2004; Carrillo et al., 2015; Felip et al., 2008; Rodríguez et al., 2005). The role of RA signaling in European sea bass reproduction is not known, and could represent a source of genetic markers during the attainment of puberty. Recently, a microarray expression profiling study identified 315 genes differentially expressed in testis by the time of meiosis among which, a group of them were involved in the RA signaling pathway (data included in Chapter 1; Blázquez et al., 2017). The present study is aimed to describe the expression profiles of several key genes of the RA signaling pathway during sex differentiation and puberty in order to identify possible molecular markers triggering meiosis in the European sea bass.

MATERIAL AND METHODS

Fish and rearing conditions

Freshly fertilized sea bass eggs were obtained from the Institute of Aquaculture Torre la Sal (Castellón, Spain) and immediately transported to our experimental aquaria facilities (ZAE) at the Institute of Marine Sciences in Barcelona (41°23'N; 2°11E). Egg incubation and fish rearing were performed according to standard procedures for sea bass aquaculture (Morretti, 1999). Briefly, eggs were placed in 600 L tanks shortly after fertilization (end of March). At four days post hatching (dph), stocking densities were reduced by half and divided into two 600 L tanks in order to create a male-enriched group and a female-enriched group. The male enriched group was obtained following a previously described protocol (Navarro-Martin et al., 2009), taking advantage of the fact that in this gonochoristic fish species, genotype and temperature contribute to the final sex ratios (Piferrer et al., 2005). Briefly, at 5 dph, the temperature of the rearing water was increased (0.5 ºC/day) up to 20 ºC and kept constant until 60 dph when it was brought back to natural conditions for the rest of the experiment. However, it is not possible in this species to obtain a female-enriched population just by thermal manipulations. Therefore, in order to increase the probability to find females in the samplings, fish were fed a commercial pelleted sea bass food containing 10 $mg \cdot kg^{-1}$ estradiol (E_2) using the alcohol evaporation method adapted to this species (Blázquez et al., 1998). This food was administered *ad libitum*, twice a day, for a short 27-day period (between 93 and 120 dph), covering the labile period of sex differentiation (Navarro-Martín et al., 2009; Piferrer et al., 2005), switching back to untreated food at 120 dph. Since European sea bass does not exhibit any external sexual dimorphism, the generation of male- and female- enriched groups helped us to increase the number of males or females selected at each sampling time, thus reducing the number of animals sacrificed to comply with the 3Rs principle. This treatment was shown to render females with no differences from untreated females in this species (Blázquez et al., 1998; Navarro-Martin et al., 2009). Moreover, pioneer studies in fish focussed on the clearance kinetics of exogenous sex steroids showed that, regardless of the route of administration, the hormones were eliminated from the body in a matter of days (reviewed in (Piferrer, 2001)). Fish were reared and sacrificed according with the Spanish regulations (Royal Decree Act 53/2013), the European legislation concerning the protection of vertebrates used for experimental and other scientific purposes (2010/63 EU).

Gonad development and sampling intervals

Males from the male-enriched group were sampled from August 2012 (150 dph) to November 2013 (600 dph) including different representative stages of sex differentiation and

early puberty. Females from the E_2-treated group were sampled from August 2012 (150 dph) to March 2015 (1079 dph), for a total of 11 sampling points (see Fig. 1 for experimental design). In each sampling, fish were anesthetized in 0.2% phenoxyethanol, assessed for standard length (± 0.1 cm) and weight (± 0.001g) and sacrificed by severing their spinal cord. Gonads were removed and a fraction of each one was fixed in 4% buffered paraformaldehyde (PAF) for histological assessment of the developmental stage. The rest was snap-frozen in liquid nitrogen and kept at -80 °C for further gene expression analysis.

Histological procedures

Fixed gonads were dehydrated in an increasing ethanol series, embedded in Histoplast (Thermo Scientific), cut at 5 μm, and stained with haematoxylin-eosin. The exact developmental stage was determined by light microscopy following the criteria described in (Begtashi et al., 2004; Espigares et al., 2015) for testes and in (Asturiano et al., 2000; Mayer et al., 1988) for ovaries. Briefly, four developmental periods were defined (Fig. 1). First, *sex differentiation* which includes the transition from undifferentiated gonads until the histological visualization of a testis (early differentiated testis) or an ovary (early differentiated ovary). Since no histological sex-related features were observed in the undifferentiated gonads, the assignment of sex was based on *cyp19a1a* and *amh* expression levels (Additional Figure 1). *cyp19a1a* has been used as molecular marker for sex assignment in the European sea bass with higher expression in future females than in males (Blázquez et al., 2008; Blázquez et al., 2009; Díaz and Piferrer, 2015), while in other teleosts the opposite pattern has been shown for *amh* (Baron et al., 2005; Fernandino et al., 2008; Maugars and Schmitz, 2008). Second, *male meiosis progression* which includes the transition from an immature differentiated testis to an early recrudescent testis. Third, *vitellogenesis progression* which covers the transition from a previtellogenic ovary to the onset of vitellogenesis. Fourth, *final ovarian maturation* which includes the last physiological transformations in the oocyte prior to spawning and ranges from maturation to ovulation. A brief resumption of meiosis occurs in oocytes by the time of ovulation and becomes arrested again until fertilization, when meiosis is finally completed with the extrusion of the second polar body (Lubzens et al., 2010; Mayer et al., 1988). Six males and six females in the same stage of gonad development were selected at every sampling point up to 600 dph. From 600 dph onwards, only females (n = 6 at each sampling point) were taken.

RNA extraction and cDNA synthesis

Total RNA was extracted with TRIzol® (Invitrogen™) following the manufacturer's instructions and eluted in nuclease free water. RNA concentration and quality were measured

using Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and checked in 1% agarose gel electrophoresis. In order to compare between samples, total RNAs (200 ng) were transcribed into cDNA using Superscript III (Invitrogen) and random hexamers according to the manufacturer's protocol. The resulting cDNAs were used as a template to amplify the genes of interest using real-time quantitative polymerase chain reaction (qPCR) for gene expression analysis or conventional PCR for tissue distribution studies.

Primer design and quantitative real-time PCR assays

To ease the understanding of the RA signaling pathway, the studied genes were classified into four different groups, according to their putative role along the pathway a) a ROL transport and cellular intake-related group including the stimulated by retinoic acid gene 6 (*stra6*) and the retinoid binding protein 4 (*rbp4*); b) a group of RA synthesis-related genes including the aldehyde dehydrogenase family1a2 (*aldh1a2*) and the aldehyde dehydrogenase family 1a3 (*aldh1a3*); c) a group of genes involved in RA-signaling that encoded for nuclear receptors, including retinoic acid receptor alpha (*rarα*), retinoid receptor alpha (*rxrα*), and peroxisome proliferator-activated receptor gamma (*pparγ*); d) a group of genes involved in RA transport and degradation that included the retinoic acid binding protein 1 (*crabp1*) and the RA degrading gene cytochrome P450 26a1 (*cyp26a1*). In addition, gonadal aromatase (*cyp19a1a*) and the anti-müllerian hormone (*amh*) were used as markers of sex differentiation at early developmental stages. Gene sequences were obtained from annotated public databases, or predicted with a blast search on the European sea bass genome (Tine et al., 2014). Primers were designed using Primer3 software (http://primer3.ut.ee), all of them featured similar melting temperatures and were located in intron-exon boundaries to check for possible genomic contamination. Amplification efficiencies (*E*) were calculated using a linear regression between a cDNA dilution series of the mean cycle quantification (Cq) plotted against the log amount input cDNA, where $E = 10^{(-1/\text{slope})}$. Regression slopes resulted in values around -3.3 and *E* values around 2.0. In all cases, linear correlations between the mean Cq and the cDNA dilution were equal or higher than 0.96 (Thesis Extended Methodology Table 1).

Amplifications (qPCR) were performed with a 7300 real-time PCR System (Applied Biosystems) using 2 µl of EvaGreen qPCR Mix Plus (Cultek Molecular Bioline, Madrid) according to the manufacturer's instructions. The reaction included 500 nM of each primer, and 1 µl of a 1:10 dilution of first strand cDNA as template in a 10µl final volume. Samples were run in triplicate in optically clear 96-well plates and for each plate, technical and biological replicates were added in order to compare between runs. Cycling parameters were 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Finally, a dissociation step at 95 °C

for 15 s, 60 °C for 1 min and 95 °C for 15 s was included. Expression values were calculated using the qBase quantification method (Hellemans et al., 2007). Briefly, this method is a modification of the ΔΔCt classic model that takes into account multiple reference genes and gene specific amplification efficiencies, as well as the errors on all measured parameters along the entire calculation. Furthermore, an inter-run calibration algorithm allows for the correction of differences among runs. With this purpose, two reference genes, *18S rRNA* and *ef1a,* previously shown to be the most stable housekeeping genes in this species (Mitter et al., 2009) and routinely used in our group for studies of gonad development (Rocha et al., 2009) were used to normalize inter-run measurements (Further information is available in Thesis extended methodology). For the first time in this species, the use of a correction factor calculated from the expression of two reference genes allowed us to compare all the studied genes in two different tissues (testis and ovaries), during different developmental stages that otherwise would not have remained stable along the experiment.

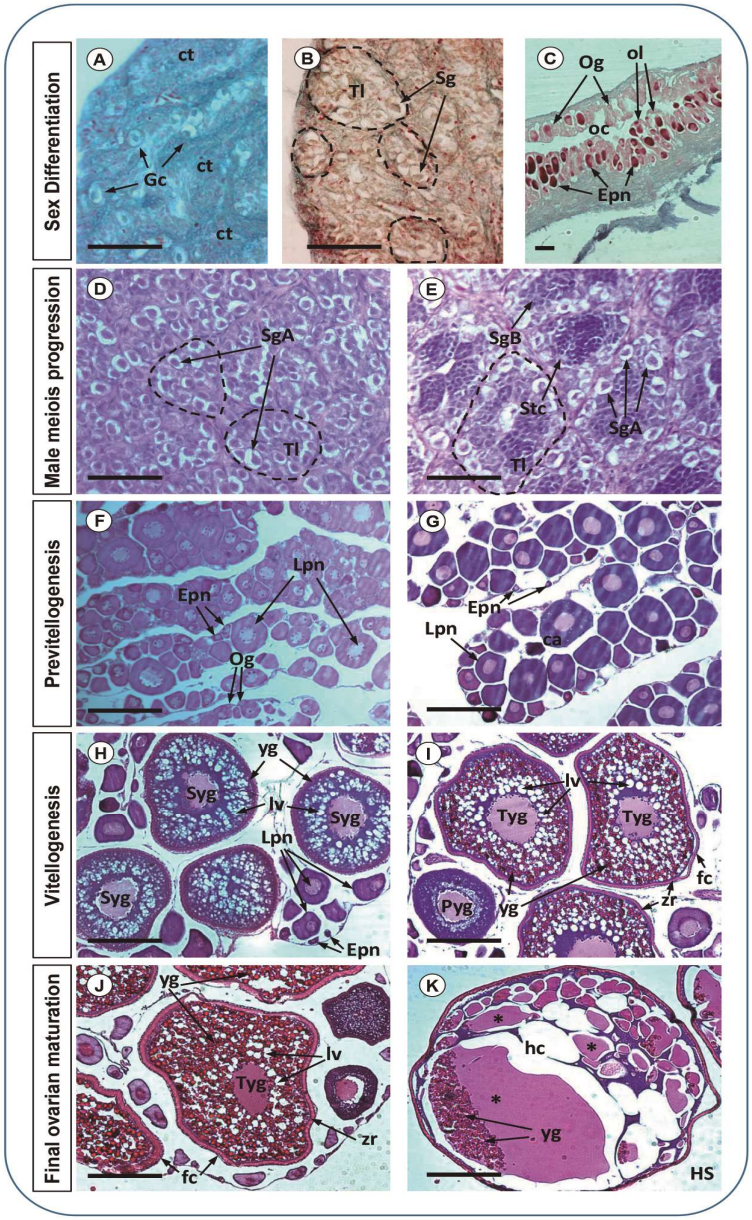
Tissue specific expression

The expression of the RA signaling pathway genes was assessed in different tissues including optic tectum, hypotalamus, telencephalon, pituitary, cerebellum, heart, muscle, fat, skin, kidney, head kidney, liver, spleen, gut, eye, testis, ovary. The expression was measured in two different sets of tissues and therefore, some transcripts (*rbp4*, *rarα*, *rxrα*, *pparγ*, *crabp1,* and *cyp26a1*) were only assessed in telencephalon, pituitary, cerebellum, heart, kidney, head kidney, liver, spleen, gut, testis, and ovary while transcripts of *stra6*, *aldh1a2*, and *aldh1a3* were assessed in all the tissues. A conventional PCR was performed using pooled cDNAs from each tissue of three different fish (n=3) and the following conditions: an initial denaturation of 5 min at 94 ºC, and then 34 cycles with a denaturation step at 94 ºC for 30 s, an annealing step at 60 ºC for 30 s, and an extension step at 72ºC for 30 s. A final extension step was included of 2 min at 72 ºC at the end of the 34 cycles.

Statistical analysis of data

Statistical analyses were performed with Statistica (StatSoft, Tulsa, OK, USA) software. All data were previously tested for normality (Shapiro-Wilk test) and log transformed when needed to ensure the homoscedasticity of variances. Variation of relative gene expression within sexes was assessed by a two-way ANOVA and was expressed as normalized relative quantities (Hellemans et al., 2007). Tukey post hoc test (multiple comparisons) was used to check for statistical differences ($P < 0.05$).

RESULTS



Gonad development

The male-enriched group resulted in a 70% male: 30% female ratio after the temperature treatment while an all-female group resulted after E_2 treatment. At 150 dph, total body length (TL) ranged between 7.3 cm and 9.9 cm. The histological study revealed a common *Undifferentiated stage* in testis and ovaries (Fig. 2A). Sex-related differences appeared at about 200 dph; at a TL 10.1-10.7 cm in males and 10-12.4 cm in females. *Early differentiated testes* (Fig. 2B) showed well-developed testicular lobules with spermatogonia as the predominant cell type and *Early differentiated ovaries* (Fig. 2C) were organised in ovarian lamellae with

Figure 2. Histological sections of European sea bass gonads at different stages of development. The stages include sexual differentiation, covering from an undifferentiated gonad- 150 dph with the presence of isolated germ cells immersed in connective tissue (A) to an early differentiated testis- 200 dph with the organisation of the gonad in testicular lobules (B) or an early differentiated ovary-200 dph with a central ovarian cavity and the organisation of ovarian lamellae (C). Male meiosis progression, ranging from an immature differentiated testis- 250 dph (D) to a recrudescent testis-600 dph (E). Vitellogenesis progression, covering from a previtellogenic ovary 250 dph (F-G) to a full vitellogenic ovary-940 dph. (H-I). Final ovarian maturation, including the transition between a mature ovary-1031 dph with the prevalence of oocytes in tertiary yolk granule stage (J) and ovulation-1079 dph with the appearance of oocytes in hyaline stage (K). Dotted lines encircle individual testicular lobules. Abbreviations: germ cell (Gc), connective tissue (ct), spermatogonia (Sg), type A spermatogonia (SgA), type B spermatogonia (SgB), type I spermatocytes (Stc), Tl (testicular lobule), ovarian cavity (oc), ovarian lamellae (ol), oogonia (Og), early perinucleolar oocyte (Epn), late prinucleolar oocyte (Lpn), primary yolk granule stage oocyte (Pyg), secondary yolk granule stage oocyte (Syg), tertiary yolk granule stage oocyte (Tyg), hyaline stage oocyte (HS), yolk granule (yg), lipid vesicle (lv), zona radiata (zr), follicular cells (fc), hydrated cytoplasm (hc). Asterisks represent coalesced yolk granules. In all photographs, the scale bar equals 50μm.

oogonia (8-16 μm diameter) and perinucleolar oocytes (30μm diameter). As gonad development progressed, *immature differentiated testes* (250, 450 and 550 dph; TL 11-17 cm) showed spermatogonia A as a predominant cell type (Fig. 2D). Testicular development was assessed until *early recrudescent testes* (600 dph; TL 11.5-19.5 cm) showing cysts of spermatogonia A and B and a small number of type I spermatocytes (Fig. 2E). In females, the *early differentiated* stage was followed by a long *previtellogenic stage* that lasted up to the third year of life and was assessed at five sampling points (250, 450, 550, 600, 940 dph; TL 10.7-31.5 cm). The *previtellogenic ovary* exhibited isolated oogonia and a few oocytes in the transition from early to late perinucleolar stage (60-120 μm diameter; Fig. 2F-G). The *vitellogenic ovary* (975 and 1000 dph; TL 23-38.5 cm), was characterized by an increase in the number of late perinucleolar oocytes in secondary growth phase (>120 μm diameter) and the presence ofdition, oocytes at secondary yolk granule stage (440-530 μm diameter) and some at tertiary yolk granule stage (530-800 μm diameter) were also present (Fig. 2I). At oocytes at primary yolk granule stage (260-440 μm diameter; Fig. 2H). In ad the *oocyte maturation* stage (1031 dph; TL 23.2-30.5 cm), ovaries showed a prevalence of oocytes at tertiary yolk granule stage (Fig. 2J). *Ovulation* (1079 dph; TL 24.7-34 cm) was marked by the presence of oocytes at tertiary yolk granule stage and hyaline stage (1100-1150 μm diameter; Fig. 2K).

Molecular markers of early sex differentiation (*cyp19a1a* and *amh*)

cyp19a1a and *amh* were used as proxies to discriminate between males and females at 150 dph, before histological sex differentiation was completed. At 150 dph, the low *cyp19a1a* expressors found in the temperature-treated group were unequivocally classified as males and the high expressors as females (Additional Figure 1A). These values were correlated with high *amh* and low *amh* levels, respectively (Additional Figure 1B). It is worth mentioning that only fish with low *cyp19a1a* and high *amh* expression levels were classified as males (undifferentiated males) and used for the analysis. All fish in the E_2-treated group exhibited high *cyp19a1a* and low *amh* expression and were classified as females. This was confirmed at the end of the experiment since a 100% female population was found in that group (not a single male was found in any of the samplings performed during the three years that lasted the experiment). In agreement with a previous study (Chapter 1; Blázquez et al., 2017), we also found a sharp decrease of *amh* during the transition between late differentiated and early recrudescent testis (Additional Figure 2) that was used to confirm meiosis initiation.

Gene expression patterns of RA signaling pathway genes during development

ROL transport and cellular intake-related genes (*rbp4* and *stra6*)

Higher *rbp4* levels were generally found in males than in females with the highest values during differentiation and in immature differentiated testis. At the end of this stage, coinciding with early recrudescence, a significant decrease was observed (Fig. 3A). Conversely, no changes during sex differentiation and previtellogenesis were found in females, although a significant decrease occurred at vitellogenesis, remaining low during maturation and slightly increasing by the time of ovulation (Fig. 3A). Regarding *stra6*, mRNA levels were higher in females than in males, opposite to what has been shown for *rbp4*, except in histologically undifferentiated gonads where the lowest levels were found for both sexes (Fig. 3B). A significant increase in *stra6* was found during gonad differentiation ($P < 0.001$) reaching up to a hundred fold in ovaries. Expression peaked in immature testis and previtellogenic ovaries, followed by a decrease in early recrudescent testis, coinciding with the onset of male meiosis, and throughout vitellogenesis, reaching the lowest values during maturation and ovulation.

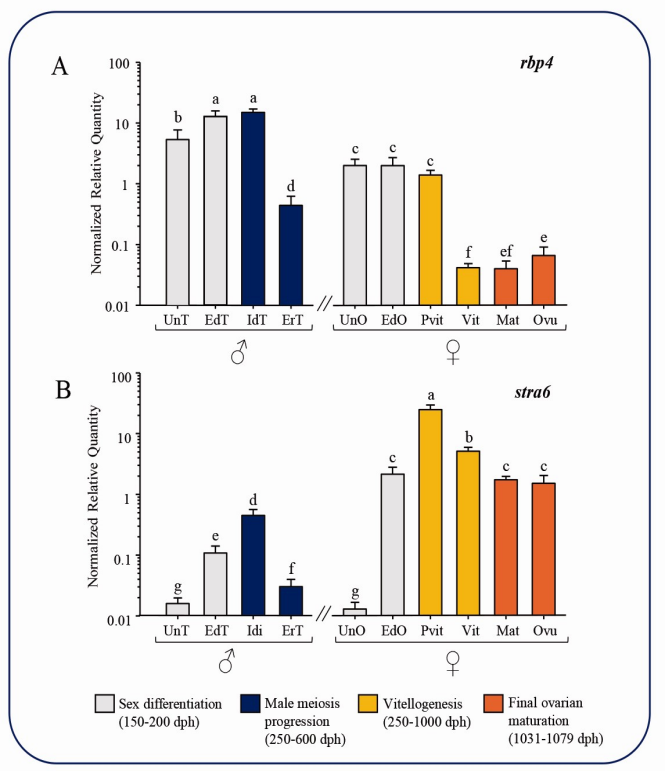


Figure 3. Expression patterns of the RA-signaling pathway transport and cellular intake-related genes, (A) *rbp4* and (B) *stra6* in male and female European sea bass gonads. Grey bars represent gonad differentiation which includes undifferentiated testis (UnT) or undifferentiated ovary (UnO), and early differentiated testis (EdT) and ovary (EdO). Blue bars represent male meiosis progression i.e., the transition from immature differentiated testis (IdT) to early recrudescent testis (ErT). Yellow bars represent the vitellogenic process and include the transition from previtellogenesis (Pvit) to vitellogenesis (Vit). Orange bars represent final maturation and include maturation (Mat) and ovulation (Ovu). Samples were analyzed by quantitative real-time fluorescent PCR. Expression data are shown as the normalized relative quantities + SEM of six samples run in triplicate and plotted on a logarithmic scale for easier visualization. Values were normalized to those of the constitutively expressed *18S* rRNA gene corrected with the expression of *ef1a* amplified from the same reverse transcribed template. Different letters denote significant differences after a Tukey test ($p < 0.05$).

RA synthesis-related genes (*aldha1a2*, *aldh1a3*)

Similar expression patterns were found in both *aldhs*, progressively increasing from undifferentiated gonads until immature differentiated testis and previtellogenic ovaries (Fig. 4A-B). A significant decrease of the two *aldhs* was found in early recrudescent testis. In females, *aldhs* decreased during vitellogenesis and maturation and increased back again during the transition between maturation and ovulation especially for *aldh1a3*.

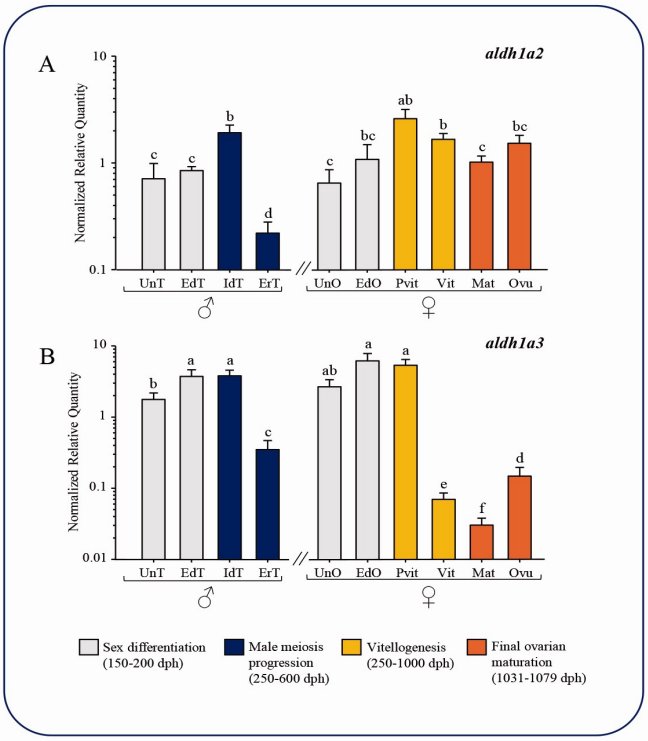


Figure 4. Expression patterns of RA synthesis related genes, (A) *aldh1a2* and (B) *aldh1a3* in male and female European sea bass gonads. Check legend from figure 3 for further details.

Nuclear receptor genes (*rarα*, *rxrα*, *pparγ*)

Undifferentiated females exhibited lower *rarα* and *rxrα* expression than males at the same stage while similar levels were found in both sexes by the end of gonad differentiation (Fig. 5A-B). In males, the highest *rarα, rxrα* and *pparγ* levels were found in immature differentiated testis followed by a decrease during early recrudescence, returning to values close to those found during sex differentiation. In females, *rarα, rxrα* and *pparγ* were highest in previtellogenic ovaries with a marked decrease during vitellogenesis and maturation and remaining low during ovulation with the exception of *pparγ* that increased again by the time of ovulation to similar levels than those found in previtellogenic ovaries (Fig. 5C).

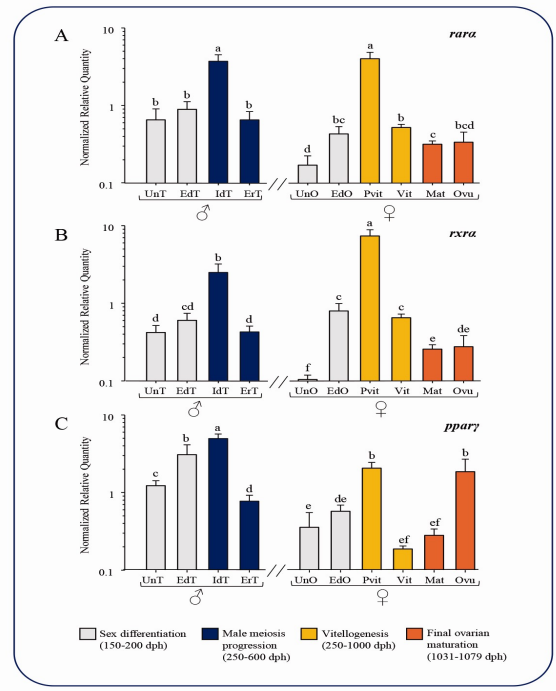


Figure 5. Expression patterns of RA nuclear receptor genes, (A) *rarα*, (B) *rxrα* and (C) *pparγ* in male and female European sea bass gonads. Check legend from figure 3 for further details.

RA transport- and degradation-related genes (*crabp1*, *cyp26a1)*

No changes in *crabp1* expression were found during male and female gonad differentiation, although a significant decrease occurred in early recrudescent testis. The highest values of *crabp1* were found in previtellogenic ovaries, which decreased again during vitellogenesis and remained low during maturation and ovulation (Fig. 6A).

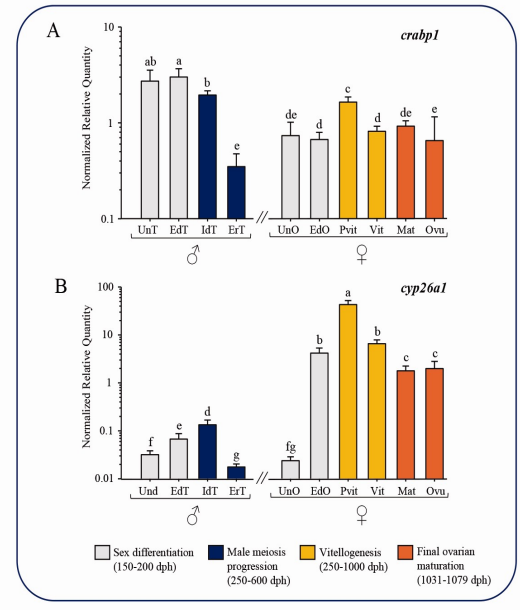


Figure 6. Expression patterns of RA transport and degradation related genes, (A) *crabp1* and (B) *cyp26a1* in male and female European sea bass gonads. Check legend from figure 3 for further details.

The expression of *cyp26a1* was characterized by an increase at the end of gonad differentiation in both sexes (Fig. 6B). Interestingly, in females, this increase reached up to a hundredfold in previtellogenic ovaries whereas in males a marked *cyp26a1* decrease was found in early recrudescent testis. Furthermore, vitellogenesis progression was characterized by a decrease in the expression of *cyp26a1* whereas no significant changes were found during maturation and ovulation.

Tissue distribution of the RA signaling pathway genes in the European sea bass

Our results show a ubiquitous presence of *rbp4* in the studied tissues, however the *stra6* receptor, was more specific, and its expression appears restricted to optic tectum, pituitary, kidney, gut, eye and gonads (Fig. 7A). The RA synthesis genes, *aldh1a2* and *aldh1a3* were expressed all the studied tissues (Fig. 7B). The nuclear receptors were also expressed in all tissues although showing similar band intensities (Fig. 7C). The RA related group of genes showed a higher tissue specificity in c*rabp1*, expressed primarily in head kidney and at lower levels in dorsal kidney, gills and testis, whereas c*yp26a1* expression was highest in gonads (Fig. 7D).

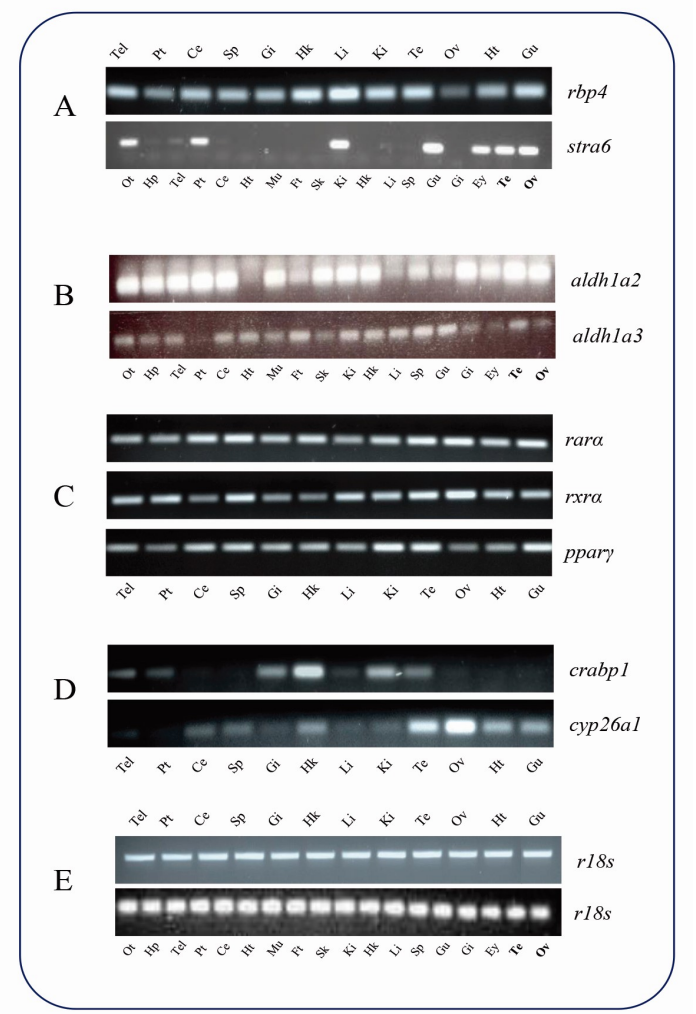


Figure 7. Tissue distribution of sea bass transcripts involved in the RA signaling pathway during sea bass early puberty including: A) ROL related genes, B) RA synthesis genes, C) RA nuclear receptor genes, D) RA related genes, in tissues such as: telencephalon (Tel), pituitary (Pt), cerebellum (Ce), spleen (Sp), gills (Gi), head kidney (Hk), liver (Li), posterior kidney (Ki), testis (Te), ovary (Ov), heart (Ht), and gut (Gu), Optic tectum (Ot), Hypotalamus (Hp), Muscle (Mu), Fat (Ft), Skin (Sk), Eye (Ey). E)18S ribosomal RNA (*r18S*) was used as a positive internal control to check for the integrity of the cDNA template. Since the experiment was performed on two different sets of tissues, upper panel of *r18S* represent the internal control for the expression of *rbp4*, *rarα*, *rxrα*, *pparγ*, *crabp1,* and *cyp26a1*, whereas the lower panel of *r18S* is the internal control for the expression of *stra6*, *aldh1a2*, and *aldh1a3*.

DISCUSSION

The present study provides the first comprehensive analysis of the expression patterns of several key components of the RA signaling pathway during gonad differentiation and the onset of puberty in male and female European sea bass. In the particular case of females, the study covers until their first maturation at the end of the third year of age, when meiosis I was completed. In agreement with previous studies, the use of *cyp19a1a* allowed us to discriminate between males and females before histological sex differentiation has occurred (Blázquez et al., 2008; Blázquez et al., 2009; Díaz and Piferrer, 2015). Regarding *amh*, this is the first time that it is successfully used as molecular marker for testicular differentiation in this species and is confirmed as a marker of the onset of meiosis previously reported in the European sea bass (Blázquez et al., 2017). Altogether, the results can be relevant for other teleosts since the availability of retinoids in the eggs is essential for proper embryonic development in fish (He et al., 2009; Lubzens et al., 2010). Furthermore, our results suggest the involvement of RA in the timing of male and female meiotic initiation, probably using an *stra8* independent signaling pathway (Koubova et al., 2014).

In mammals, retinoids are transferred from the dam to the fetus via the placenta (Quadro et al., 2004), whereas in oviparous vertebrates, they remain stored in the egg during vitellogenesis (Levi et al., 2008). ROL binds to Rbp4 and is transported from the liver to the peripheral tissues where it binds to the membrane receptor Stra6 and gets incorporated inside the target cells (Kawaguchi et al., 2007). In mice, *Stra6* is expressed in Sertoli cells and provides nutrients, including vitamin A, to support spermatogenesis (Bouillet et al., 1997). In fish, in addition to the liver, *rbp4* expression has also been found in gonads, including testicular Sertoli cells (Funkenstein, 2001) and ovary (Levi et al., 2008; Sammar et al., 2001), suggesting that retinoid transport and storage are important for reproduction. We found a ubiquitous expression of *rbp4* in all the studied tissues, however the expression of its receptor *stra6,* was restricted to brain tissues (optic tectum), pituitary, kidney, gut, eye and gonads. This was somehow striking since a similar tissue expression was expected for *rbp4* and *stra6*, as the binding protein and the membrane receptor function as counterparts to supply ROL to the cell (Ross, 1993). However, it seems that not only stra6 can aid in ROL uptake, since RBPR2, a novel ROL receptor has been found in mammals. This could explain why the liver and other tissues can uptake vitamin A without the presence of Stra6 (Alapatt et al., 2013). Our results show highest *rbp4* and *stra6* expression levels in late differentiated testis, suggesting a need of ROL within the testicular environment in the stages previous to the onset of meiosis, time when the expression of *rbp4* and *stra6* decreased again, probably due to the exhaustion of ROL reserves by their transformation into RAL and RA.

In fish ovaries, ROL can remain bound to Stra6, or become transformed into RAL, the main retinoid in fish eggs (Devlin and Nagahama, 2002; Irie and Seki, 2002; Lubzens et al., 2010), entering the oocyte together with vitellogenin as part of the HDL plasma fraction (Lubzens et al., 2010). In this study, the high *rbp4* and *stra6* levels found in previtellogenic ovaries, just before the start of vitellogenesis, suggest that an increase of intracellular ROL transport and uptake is required to supply the needs of the oocyte during this process. A similar result was found in zebrafish where the expression of *stra6* was higher in vitellogenic than in non-vitellogenic ovaries (Levi et al., 2012), although in trout the highest *stra6* levels were recorded in ovaries of juvenile fish (Levi et al., 2008). During vitellogenesis progression, we found a significant decrease of *rbp4 and stra6* expression, coinciding with the natural increase in E_2 plasma levels in this species (Mañanós et al., 1997; Prat et al., 1996; Rocha et al., 2009). Since vitellogenin synthesis is regulated by E_2 levels, it seems plausible that in the European sea bass retinoid metabolism could also be mediated by the E_2 content.

In vertebrates, RA level is set by the balance between its synthesis by Aldhs and its degradation by Cyp26 enzymes (Duester, 2008). At least three genes code for different members of the family of RA synthesizing enzymes, namely *Aldh1A1*, *Aldh1A2*, and *Aldh1A3* (Holmes, 2015). In teleosts, the general trait is the absence of *aldh1a1* (Canestro et al., 2009; Pittlik et al., 2008) and in some species *aldh1a3* has also been lost during evolution (Feng et al., 2015; Holmes, 2015). Our results show that *aldh1a2* and *aldh1a3* are present in European sea bass, indicating that the two might be needed for the irreversible oxidation of RAL into RA in the gonads (Napoli, 2012; Rodríguez-Marí et al., 2013; Uji et al., 2011). Moreover, both genes were ubiquitously expressed, according to their tightly controlled function in the overall production of RA (Griswold et al., 2012). Nevertheless, since in other teleosts *Aldh1a3* is either not expressed in the gonads (Rodríguez-Marí et al., 2013) or even absent in their genomes (Feng et al., 2015; Holmes, 2015), its role in meiosis initiation needs to be further studied.

In tetrapods, *Cyp26b1* is downregulated by the time germ cells enter meiosis, with clear sex-related differences in its timing of expression (Koubova et al., 2006; Smith et al., 2008; Wallacides et al., 2009) while in fish, *cyp26a1* seems to be a meiosis preventing factor (Feng et al., 2015; Lau et al., 2013; Li et al., 2016; Rodríguez-Marí et al., 2013). In tilapia, *cyp26a1* and *aldh1a2* are the main regulators of RA levels with high *aldh1a2* and low *cyp26a1* expression at the time of meiosis (Feng et al., 2015). Indeed, loss of gene function by CRISPR/Cas9 resulted in delayed meiosis in *aldh1a2* deficient females while meiotic initiation was advanced in *cyp26a1* deficient males, confirming the key role of both genes in the onset of meiosis in tilapia (Feng et al., 2015). Moreover, in Southern catfish (Li et al., 2016) and zebrafish (Rodríguez-Marí et al.,

2013), an increase in *aldh1a2* expression was found at the time of meiosis in both sexes, further involving RA in meiosis regulation. Our results also show a clear downregulation of *cyp26a1* in early recrudescent testis, coinciding with the onset of meiosis, as previously reported by our group using a microarray expression profiling approach (Chapter 1; Blázquez et al., 2017). This downregulation was preceded by high expression of *aldhs* in late differentiated testis, prior to the onset of meiosis. Moreover, an increase of *aldhs* concomitant with a downregulation of *cyp26a1* were found during the transition between maturation and ovulation in females, time when meiosis resumption takes place, similar to what has been reported in zebrafish (Rodríguez-Marí et al., 2013) and medaka (Adolfi et al., 2016), further implicating this enzyme in meiosis progression in the European sea bass. These studies evidence the sex-specific regulation of RA levels at the time of meiosis in different fish models. Moreover, since *cyp26a1* represents an endpoint in RA metabolism, we suggest that its downregulation in male and female gonads could be used as a suitable molecular marker for the initiation of fish meiosis.

RA synthesis and lipid metabolism are linked in a complex regulatory mechanism where RAL has a key role. Indeed, Rxr/Ppar heterodimers bind to peroxisome proliferator response elements in the promoters of different genes involved in lipid metabolism and lipogenesis, acting as transcriptional regulators (Chawla et al., 2001; Ziouzenkova and Plutzky, 2008). RAL inhibits *Rxr-Pparγ* activation in mice (Ziouzenkova and Plutzky, 2008), while in human dendritic cells the activation of *PPARγ* upregulated *ALDH1A2* expression, resulting in increased RA levels (Szatmari et al., 2006). We found that the RA nuclear receptors were expressed in all the tissues studied, reflecting the general actions and the importance of RA in numerous biological processes throughout evolution (Albalat, 2009) and its involvement in the proliferation and differentiation of many cell types (Mark et al., 2006). The present study shows that the highest expression levels of nuclear receptors (*rarα*, *rxrα* and *pparγ*) occur right before the onset of meiosis in males, while in females, the increase during meiosis resumption is only true for *pparγ*. These changes could be induced in response to the higher RA availability driven by the upregulation of *aldhs* and the decrease of *cyp26a1* during the same periods. Moreover, the increase of *pparg* expression concomitant to that of *aldh1a3* by the time of ovulation suggest a tight coordination between lipid homeostasis/energy metabolism and reproduction in European sea bass similar to what has been reported in reproductive tissues in mice (Froment et al., 2006). Indeed, a recent study showed that the lipid metabolism pathway was affected during the onset of puberty in European sea bass males, with several genes involved in the regulation of energy balance being altered, probably due to the specific energy requirements and the decrease in food intake during this reproductive stage (Blázquez et al., 2017).

Rar/Rxr heterodimers are responsible for *in vivo* RA signal transduction, using mainly atRA as ligand (Brelivet et al., 2012). We also show similar expression patterns for *rarα* and *rxrα,* in both sexes, indicating their heterodimeric interdependence. The regulation of this ligand-dependent model will determine the transcription of the right RA-target gene at the precise time, suggesting a tight control of RA signaling during gonad development in European sea bass. Our data suggest that the high levels of RA synthesis (*aldh1a2*, *aldh1a3*) and of RA signaling (*rarα, rxrα, pparγ*) genes found prior to the onset or by the resumption of meiosis in males and females, respectively could be triggering the transcription of other RA-induced genes involved in gonad maturation. At these stages we also found high levels of *crabp1* in both sexes consistent with its role as spatiotemporal "buffer" that protects the cell from an excess of RA, either by keeping it bound in the cytosol, facilitating its degradation by Cyp26s (Fiorella and Napoli, 1991; Won et al., 2004), or by delivering it to the nucleus where it binds to and activates RA receptors (Takase et al., 1986). The present study revealed that the expression of *crabp1* was prominent in head kidney and lower in dorsal kidney, gills and testis. Moreover, *cyp26a1*showed highest expression in gonads. Studies in mice reveal that there is a dynamic expression pattern between these two genes in a time and tissue dependent manner, that facilitate the delivering of RA from the cytosol in the nucleus and the RA clearance, reducing RA availability in the cell. (Ross and Zolfaghari, 2011; Thatcher and Isoherranen, 2009). Likewise, in a previous study (Chapter 1; Blázquez et al., 2017), high *crabp1* expression was found in premeiotic European sea bass males followed by a downregulation at the time of meiosis (Blázquez et al., 2017), suggesting its role in RA availability during this process. Although Crabps are essential for hindbrain patterning in zebrafish since they compensate for changes in RA production (Cai et al., 2012), their involvement in RA signaling in mouse is not crucial (Lampron et al., 1995). In the present study, the possible increase of RA content in the gonads driven by high levels of *aldhs* in premeiotic stages might give the signal to maintain high levels of *crabp1* and *cyp26a1* in order to keep physiological intracellular RA levels and avoid possible toxic effects of an RA excess (Chung and Wolgemuth, 2004).

In females, the highest expression of RA synthesis genes, RA receptor genes, and RA binding proteins occurred in previtellogenic ovaries and decreased as vitellogenesis progressed. Expression remained low during maturation and ovulation except for *pparg* and the RA synthesis genes *aldh1a2* and *aldh1a3* that slightly increased by the time of ovulation in line with the higher RA requirements needed to complete meiosis during that developmental period. We also found high *amh* in testes prior to the onset of meiosis, in agreement with its role as a potent inhibitor of the synthesis of 11-ketotestosterone, the main androgen in fish (Borg, 1994), that characterizes the stages previous to the onset of puberty in European sea bass males (Blázquez et al., 2017;

Mazón et al., 2014). Moreover, a progressive increase of estrogen levels was observed in vitellogenic ovaries, remaining high during the transition between maturation and ovulation, prior to meiosis resumption (Mañanós et al., 1997; Prat et al., 1990; Rocha et al., 2009). Altogether, our results suggest a close link between steroidogenic enzymes and RA related genes by the time of meiosis, supported by the increase of RA availability in the gonad prior to meiosis driven by the increase of *aldh1a2* and *aldh1a3* and the decrease of *cyp26a1* in both sexes. A summary figure (Additional Figure 3) compiles the results on the expression of genes involved in the RA signaling pathway of European sea bass males and females.

CONCLUSION

To the best of our knowledge this is the first study trying to address the involvement of the RA signaling pathway in the regulation of key events during the reproductive process in male and female European sea bass. In summary, the highest expression levels of genes involved in the RA signaling pathway are found during the stage previous to meiosis initiation or resumption in testis and ovaries, respectively. We hypothesize that during the premeiotic stage, fish might be increasing the RA levels within the gonadal milieu needed to switch on meiosis by inducing an upregulation of its synthesis, transport, and signaling. *cyp26a1* levels are also kept high in order to prevent an early entry into meiosis, in agreement with the role of this enzyme as the meiosis inhibiting factor in vertebrates. The decrease of *cyp26a1* expression at the time of male meiosis and also during maturation/ovulation, when meiosis is resumed in females, will induce the increase of gonadal RA levels reaching a threshold to trigger meiosis. This decrease of *cyp26a1* could be used as a molecular marker for the onset of meiosis in European sea bass. Taken together our results suggest an increase of RA bioavailability and a subsequent signaling through nuclear receptors by the time of meiosis.

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REFFERENCE LIST

Adolfi, M. C., Herpin, A., Regensburger, M., Sacquegno, J., Waxman, J. S., and Schartl, M. (2016). Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. Scientific reports 6, 34281.

Alapatt, P., Guo, F., Komanetsky, S. M., Wang, S., Cai, J., Sargsyan, A., Rodríguez Díaz, E., Bacon, B. T., Aryal, P., and Graham, T. E. (2013). Liver Retinol Transporter and Receptor for Serum Retinol-binding Protein (RBP4). Journal of Biological Chemistry 288, 1250-1265.

Albalat, R. (2009). The retinoic acid machinery in invertebrates: ancestral elements and vertebrate innovations. Mol Cell Endocrinol 313, 23-35.

Amengual, J., Zhang, N., Kemerer, M., Maeda, T., Palczewski, K., and von Lintig, J. (2014). STRA6 is critical for cellular vitamin A uptake and homeostasis. Human Molecular Genetics.

Asturiano, J., Sorbera, L., Ramos, J., Kime, D., Carrilo, M., and Zanuy, S. (2000). Hormonal regulation of the European sea bass reproductive cycle: an individualized female approach. Journal of Fish Biology 56, 1155-1172.

Balmer, J. E., and Blomhoff, R. (2002). Gene expression regulation by retinoic acid. J Lipid Res 43, 1773-808.

Baron, D., Houlgatte, R., Fostier, A., and Guiguen, Y. (2005). Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 73, 959-66.

Begtashi, I., Rodríguez, L., Moles, G., Zanuy, S., and Carrillo, M. (2004). Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (Dicentrarchus labrax, L.). I. Morphological aspects. Aquaculture 241, 539-559.

Blázquez, M., González, A., Papadaki, M., Mylonas, C., and Piferrer, F. (2008). Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (Dicentrarchus labrax). Gen Comp Endocrinol 158, 95-101.

Blázquez, M., Medina, P., Crespo, B., Gómez, A., and Zanuy, S. (2017). Identification of conserved genes triggering puberty in European sea bass males (Dicentrarchus labrax) by microarray expression profiling. BMC Genomics 18, 441.

Blázquez, M., Navarro-Martín, L., and Piferrer, F. (2009). Expression profiles of sex differentiation-related genes during ontogenesis in the European sea bass acclimated to two different temperatures. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 312, 686-700.

Blázquez, M., Zanuy, S., Carrillo, M., and Piferrer, F. (1998). Structural and functional effects of early exposure to estradiol-17β and 17α-ethynylestradiol on the gonads of the gonochoristic teleost Dicentrarchus labrax. Fish Physiol Biochem 18, 37-47.

Borg, B. (1994). Androgens in teleost fishes. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 109, 219-245.

Bouillet, P., Sapin, V., Chazaud, C., Messaddeq, N., Décimo, D., Dollé, P., and Chambon, P. (1997). Developmental expression pattern of Stra6, a retinoic acid-responsive gene encoding a new type of membrane protein. Mech Dev 63, 173-186.

Bowles, J., Feng, C.-W., Knight, D., Smith, C. A., Roeszler, K. N., Bagheri-Fam, S., Harley, V. R., Sinclair, A. H., and Koopman, P. (2009). Male-specific expression of Aldh1a1 in mouse and chicken fetal testes: Implications for retinoid balance in gonad development. Developmental Dynamics 238, 2073-2080.

Bowles, J., and Koopman, P. (2007). Retinoic acid, meiosis and germ cell fate in mammals. Development 134, 3401-11.

Brelivet, Y., Rochel, N., and Moras, D. (2012). Structural analysis of nuclear receptors: from isolated domains to integral proteins. Mol Cell Endocrinol 348, 466-73.

Cai, A. Q., Radtke, K., Linville, A., Lander, A. D., Nie, Q., and Schilling, T. F. (2012). Cellular retinoic acid-binding proteins are essential for hindbrain patterning and signal robustness in zebrafish. Development 139, 2150-2155.

Campo-Paysaa, F., Marletaz, F., Laudet, V., and Schubert, M. (2008). Retinoic acid signaling in development: tissue-specific functions and evolutionary origins. Genesis 46, 640-56.

Cañestro, C., Catchen, J. M., Rodriguez-Mari, A., Yokoi, H., and Postlethwait, J. H. (2009). Consequences of lineage-specific gene loss on functional evolution of surviving paralogs: ALDH1A and retinoic acid signaling in vertebrate genomes. PLoS Genet 5, e1000496.

Carrillo, M., Espigares, F., Felip, A., Escobar, S., Molés, G., Rodríguez, R., Alvarado, M. V., Gómez, A., and Zanuy, S. (2015). Updating control of puberty in male European sea bass: A holistic approach. Gen Comp Endocrinol 221, 42-53.

Crespo, B., Lan-Chow-Wing, O., Rocha, A., Zanuy, S., and Gómez, A. (2013). foxl2 and foxl3 are two ancient paralogs that remain fully functional in teleosts. Gen Comp Endocrinol 194, 81-93.

Chawla, A., Repa, J. J., Evans, R. M., and Mangelsdorf, D. J. (2001). Nuclear Receptors and Lipid Physiology: Opening the X-Files. Science 294, 1866-1870.

Chung, S. S., and Wolgemuth, D. J. (2004). Role of retinoid signaling in the regulation of spermatogenesis. Cytogenet Genome Res 105, 189-202.

Devlin, R. H., and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208, 191-364.

Díaz, N., and Piferrer, F. (2015). Lasting effects of early exposure to temperature on the gonadal transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination. BMC Genomics 16, 679.

Dong, R., Yang, S., Jiao, J., Wang, T., Shi, H., Zhou, L., Zhang, Y., and Wang, D. (2013). Characterization of Stra8 in Southern catfish (Silurus meridionalis): evidence for its role in meiotic initiation. BMC Molecular Biology 14, 11.

Duester, G. (2008). Retinoic Acid Synthesis and Signaling during Early Organogenesis. Cell 134, 921-931.

Espigares, F., Rocha, A., Molés, G., Gómez, A., Carrillo, M., and Zanuy, S. (2015). New insights into the factors mediating the onset of puberty in sea bass. Gen Comp Endocrinol 224, 176-185.

Felip, A., Zanuy, S., Muriach, B., Cerdá-Reverter, J. M., and Carrillo, M. (2008). Reduction of sexual maturation in male Dicentrarchus labrax by continuous light both before and during gametogenesis. Aquaculture 275, 347-355.

Feng, R., Fang, L., Cheng, Y., He, X., Jiang, W., Dong, R., Shi, H., Jiang, D., Sun, L., and Wang, D. (2015). Retinoic acid homeostasis through aldh1a2 and cyp26a1 mediates meiotic entry in Nile tilapia (Oreochromis niloticus). Scientific reports 5, 10131.

Fernandino, J. I., Hattori, R. S., Kimura, H., Strussmann, C. A., and Somoza, G. M. (2008). Expression profile and estrogenic regulation of anti-Mullerian hormone during gonadal development in pejerrey Odontesthes bonariensis, a teleost fish with strong temperature-dependent sex determination. Dev Dyn 237, 3192-9.

Fiorella, P. D., and Napoli, J. (1991). Expression of cellular retinoic acid binding protein (CRABP) in Escherichia coli. Characterization and evidence that holo-CRABP is a substrate in retinoic acid metabolism. Journal of Biological Chemistry 266, 16572-16579.

Froment, P., Gizard, F., Defever, D., Staels, B., Dupont, J., and Monget, P. (2006). Peroxisome proliferator-activated receptors in reproductive tissues: from gametogenesis to parturition. Journal of Endocrinology 189, 199-209.

Funkenstein, B. (2001). Developmental expression, tissue distribution and hormonal regulation of fish (Sparus aurata) serum retinol-binding protein. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 129, 613-622.

Griswold, M. D., Hogarth, C. A., Bowles, J., and Koopman, P. (2012). Initiating meiosis: the case for retinoic acid. Biol Reprod 86, 35.

He, C., Wang, C., Li, B., Xie, F., Chen, Y., and Zuo, Z. (2009). Tissue-specific and embryonic expression of the retinoid X receptors in Sebastiscus marmoratus. Comp Biochem Physiol B Biochem Mol Biol 154, 221-8.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8, R19.

Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., and Thaller, C. (1992). 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 68, 397-406.

Holmes, R. S. (2015). Comparative and evolutionary studies of vertebrate ALDH1A-like genes and proteins. Chemico-Biological Interactions 234, 4-11.

Irie, T., and Seki, T. (2002). Retinoid composition and retinal localization in the eggs of teleost fishes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 131, 209-219.

Kawaguchi, R., Yu, J., Honda, J., Hu, J., Whitelegge, J., Ping, P., Wiita, P., Bok, D., and Sun, H. (2007). A Membrane Receptor for Retinol Binding Protein Mediates Cellular Uptake of Vitamin A. Science 315, 820-825.

Koubova, J., Hu, Y.-C., Bhattacharyya, T., Soh, Y. Q. S., Gill, M. E., Goodheart, M. L., Hogarth, C. A., Griswold, M. D., and Page, D. C. (2014). Retinoic Acid Activates Two Pathways Required for Meiosis in Mice. PLoS Genet 10, e1004541.

Koubova, J., Menke, D., Zhou, Q., Capel, B., Griswold, M., and Page, D. (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc Natl Acad Sci USA 103, 2474 - 9.

Lampron, C., Rochette-Egly, C., Gorry, P., Dolle, P., Mark, M., Lufkin, T., LeMeur, M., and Chambon, P. (1995). Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal. Development 121, 539-548.

Lau, E.-L., Lee, M.-F., and Chang, C.-F. (2013). Conserved sex-specific timing of meiotic initiation during sex differentiation in the protandrous black porgy Acanthopagrus schlegelii. Biol Reprod 88, 150.

Levi, L., Levavi-Sivan, B., and Lubzens, E. (2008). Expression of genes associated with retinoid metabolism in the trout ovarian follicle. Biol Reprod 79, 570-7.

Levi, L., Ziv, T., Admon, A., Levavi-Sivan, B., and Lubzens, E. (2012). Insight into molecular pathways of retinal metabolism, associated with vitellogenesis in zebrafish. American Journal of Physiology-Endocrinology and Metabolism 302, E626-E644.

Li, M., Feng, R., Ma, H., Dong, R., Liu, Z., Jiang, W., Tao, W., and Wang, D. (2016). Retinoic acid triggers meiosis initiation via stra8-dependent pathway in Southern catfish, Silurus meridionalis. Gen Comp Endocrinol 232, 191-198.

Lubzens, E., Young, G., Bobe, J., and Cerdà, J. (2010). Oogenesis in teleosts: How fish eggs are formed. Gen Comp Endocrinol 165, 367-389.

Mañanós, E. L., Zanuy, S., and Carrillo, M. (1997). Photoperiodic manipulations of the reproductive cycle of sea bass (Dicentrarchus labrax) and their effects on gonadal development, and plasma 17ß-estradiol and vitellogenin levels. Fish Physiol Biochem 16, 211-222.

Marill, J., Idres, N., Capron, C. C., Nguyen, E., and Chabot, G. G. (2003). Retinoic Acid Metabolism and Mechanism of Action: A Review. Current Drug Metabolism 4, 1-10.

Mark, M., Ghyselinck, N. B., and Chambon, P. (2006). Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu. Rev. Pharmacol. Toxicol. 46, 451-480.

Maugars, G., and Schmitz, M. (2008). Gene expression profiling during spermatogenesis in early maturing male Atlantic salmon parr testes. Gen Comp Endocrinol 159, 178-87.

Mayer, I., Shackley, S. E., and Ryland, J. S. (1988). Aspects of the reproductive biology of the bass, Dicentrarchus labrax L. I. An histological and histochemical study of oocyte development. Journal of Fish Biology 33, 609-622.

Mazón, M. J., Gómez, A., Yilmaz, O., Carrillo, M., and Zanuy, S. (2014). Administration of Follicle-Stimulating Hormone In Vivo Triggers Testicular Recrudescence of Juvenile European Sea Bass (Dicentrarchus labrax). Biol Reprod 90, 6, 1-10.

McCaffery, P. J., Adams, J., Maden, M., and Rosa-Molinar, E. (2003). Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen. European Journal of Neuroscience 18, 457-472.

Mitter, K., Kotoulas, G., Magoulas, A., Mulero, V., Sepulcre, P., Figueras, A., Novoa, B., and Sarropoulou, E. (2009). Evaluation of candidate reference genes for QPCR during ontogenesis and of immune-relevant tissues of European seabass (Dicentrarchus labrax). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 153, 340-347.

Morretti, A. (1999). "Manual on hatchery production of seabass and gilthead seabream," Food & Agriculture Org.

Napoli, J. L. (2012). Physiological insights into all-trans-retinoic acid biosynthesis. Biochim Biophys Acta 1821, 152-67.

Navarro-Martin, L., Blazquez, M., and Piferrer, F. (2009). Masculinization of the European sea bass (Dicentrarchus labrax) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. Gen Comp Endocrinol 160, 3-11.

Navarro-Martín, L., Blázquez, M., Viñas, J., Joly, S., and Piferrer, F. (2009). Balancing the effects of rearing at low temperature during early development on sex ratios, growth and maturation in the European sea bass (Dicentrarchus labrax).: Limitations and opportunities for the production of highly female-biased stocks. Aquaculture 296, 347-358.

Niederreither, K., and Dolle, P. (2008). Retinoic acid in development: towards an integrated view. Nat Rev Genet 9, 541-53.

Pasquier, J., Cabau, C., Nguyen, T., Jouanno, E., Severac, D., Braasch, I., Journot, L., Pontarotti, P., Klopp, C., and Postlethwait, J. H. (2016). Gene evolution and gene expression after whole genome duplication in fish: the PhyloFish database. BMC Genomics 17, 1.

Piferrer, F. (2001). Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197, 229-281.

Piferrer, F., Blazquez, M., Navarro, L., and Gonzalez, A. (2005). Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (Dicentrarchus labrax L.). Gen Comp Endocrinol 142, 102-10.

Pittlik, S., Domingues, S., Meyer, A., and Begemann, G. (2008). Expression of zebrafish aldh1a3 (raldh3) and absence of aldh1a1 in teleosts. Gene Expr Patterns 8, 141-7.

Prat, F., Sumpter, J. P., and Tyler, C. R. (1996). Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductivecycle in male and female rainbow trout (Oncorhynchus mykiss). Biol Reprod 54, 1375-1382.

Prat, F., Zanuy, S., Carrillo, M., de Mones, A., and Fostier, A. (1990). Seasonal changes in plasma levels of gonadal steroids of sea bass, Dicentrarchus labrax L. Gen Comp Endocrinol 78, 361-373.

Quadro, L., Hamberger, L., Gottesman, M. E., Colantuoni, V., Ramakrishnan, R., and Blaner, W. S. (2004). Transplacental delivery of retinoid: the role of retinol-binding protein and lipoprotein retinyl ester. American Journal of Physiology-Endocrinology and Metabolism 286, E844-E851.

Reijntjes, S., Gale, E., and Maden, M. (2004). Generating gradients of retinoic acid in the chick embryo: Cyp26C1 expression and a comparative analysis of the Cyp26 enzymes. Developmental Dynamics 230, 509-517.

Rocha, A., Zanuy, S., Carrillo, M., and Gómez, A. (2009). Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. Gen Comp Endocrinol 162, 265-275.

Rodríguez-Marí, A., Cañestro, C., BreMiller, R. A., Catchen, J. M., Yan, Y.-L., and Postlethwait, J. H. (2013). Retinoic Acid Metabolic Genes, Meiosis, and Gonadal Sex Differentiation in Zebrafish. PLoS One 8, e73951.

Rodríguez, L., Begtashi, I., Zanuy, S., and Carrillo, M. (2005). Long-term exposure to continuous light inhibits precocity in European male sea bass (Dicentrarchus labrax, L.): hormonal aspects. Gen Comp Endocrinol 140, 116-125.

Ross, A. (1993). Cellular metabolism and activation of retinoids: roles of cellular retinoid-binding proteins. The FASEB Journal 7, 317-327.

Ross, A. C., and Zolfaghari, R. (2011). Cytochrome P450s in the regulation of cellular retinoic acid metabolism. Annu Rev Nutr 31, 65-87.

Sammar, M., Babin, P. J., Durliat, M., Meiri, I., Zchori, I., Elizur, A., and Lubzens, E. (2001). Retinol binding protein in rainbow trout: molecular properties and mRNA expression in tissues. Gen Comp Endocrinol 123, 51-61.

Smith, C., Roeszler, K., Bowles, J., Koopman, P., and Sinclair, A. (2008). Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. BMC Developmental Biology 8, 85.

Spoorendonk, K. M., Peterson-Maduro, J., Renn, J., Trowe, T., Kranenbarg, S., Winkler, C., and Schulte-Merker, S. (2008). Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. Development 135, 3765-74.

Szatmari, I., Pap, A., Rühl, R., Ma, J.-X., Illarionov, P. A., Besra, G. S., Rajnavolgyi, E., Dezso, B., and Nagy, L. (2006). PPARγ controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. The Journal of Experimental Medicine 203, 2351-2362.

Takase, S., Ong, D. E., and Chytil, F. (1986). Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus. Archives of Biochemistry and Biophysics 247, 328-334.

Thatcher, J. E., and Isoherranen, N. (2009). The role of CYP26 enzymes in retinoic acid clearance. Expert Opin Drug Metab Toxicol 5, 875-86.

Tine, M., Kuhl, H., Gagnaire, P.-A., Louro, B., Desmarais, E., Martins, R. S. T., Hecht, J., Knaust, F., Belkhir, K., and Klages, S. (2014). European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. Nature communications 5.

Uji, S., Kurokawa, T., Hashimoto, H., Kasuya, T., and Suzuki, T. (2011). Embryogenic staging of fugu, Takifugu rubripes, and expression profiles of aldh1a2, aldh1a3 and cyp26a1. Development, growth & differentiation 53, 715-725.

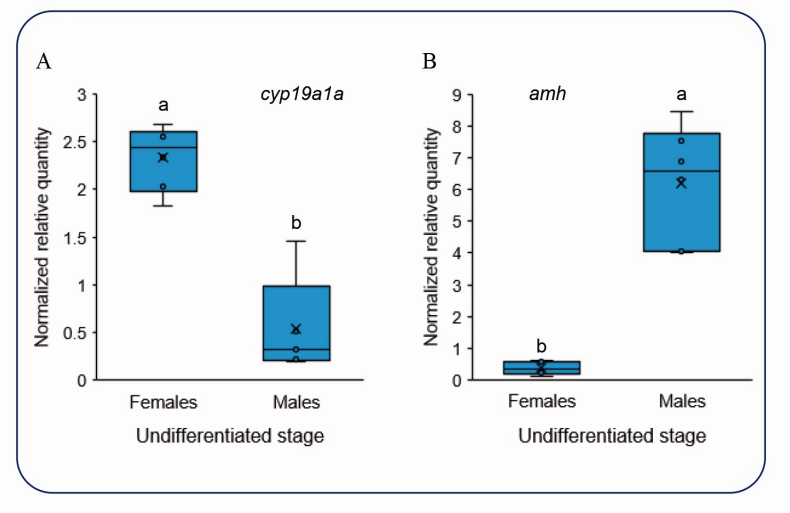
Wallacides, A., Chesnel, A., Chardard, D., Flament, S., and Dumond, H. (2009). Evidence for a conserved role of retinoic acid in urodele amphibian meiosis onset. Dev Dyn 238, 1389-98.

Won, J. Y., Nam, E.-C., Yoo, S. J., Kwon, H. J., Um, S. J., Han, H. S., Kim, S. H., Byun, Y., and Kim, S. Y. (2004). The effect of cellular retinoic acid binding protein-I expression on the CYP26-mediated catabolism of all-trans retinoic acid and cell proliferation in head and neck squamous cell carcinoma. Metabolism 53, 1007-1012.

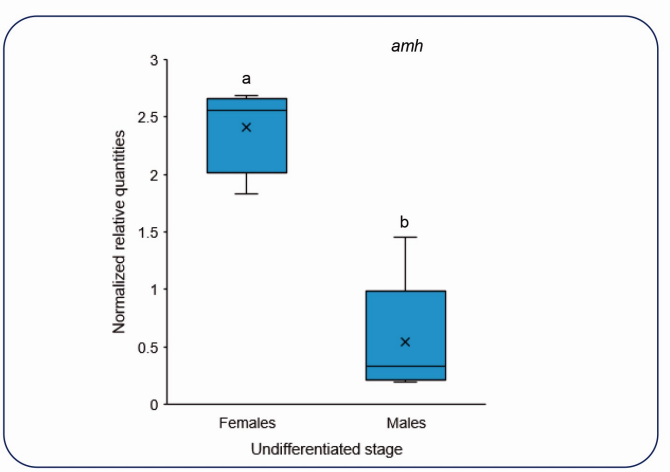
Zanuy, S., Carrillo, M., Felip, A., Rodríguez, L., Blázquez, M., Ramos, J., and Piferrer, F. (2001). Genetic, hormonal and environmental approaches for the control of reproduction in the European sea bass (Dicentrarchus labrax L.). Aquaculture 202, 187-203.

Ziouzenkova, O., and Plutzky, J. (2008). Retinoid metabolism and nuclear receptor responses: New insights into coordinated regulation of the PPAR-RXR complex. FEBS Lett 582, 32-8.

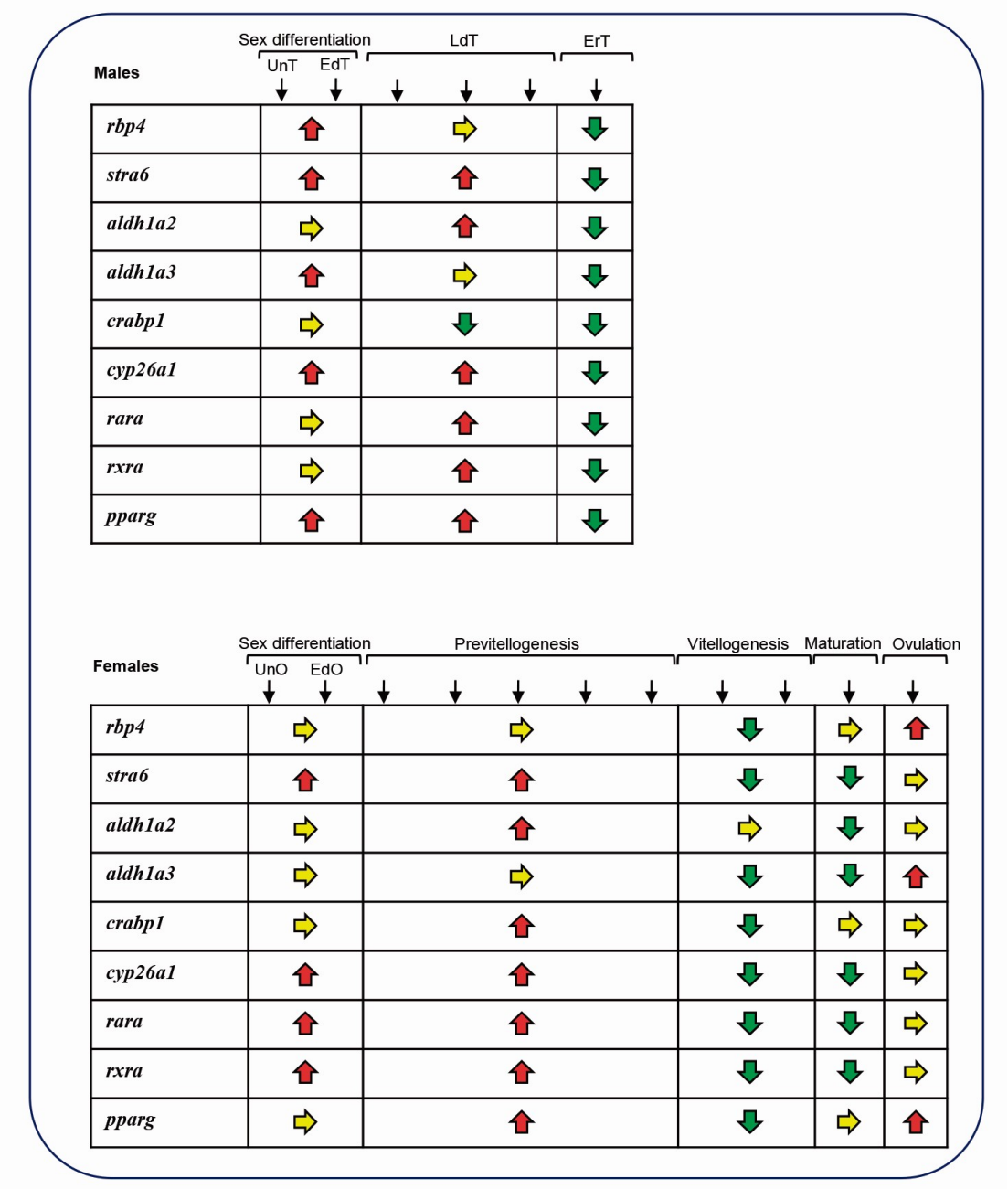
CHAPTER 3 ADDITIONAL FIGURES



Additional Figure 1. Expression levels of marker genes of sex differentiation in histologically undifferentiated European sea bass gonads at 150 dph. (A) gonadal aromatase (*cyp19a1a*), (B) anti-müllerian hormone (*amh*). Samples were analyzed by quantitative real-time fluorescent PCR. Expression data are shown as the normalized relative quantities of six samples run in triplicate and normalized to those of the constitutively expressed *18S* rRNA gene previously corrected with the expression of *ef1a* amplified from the same reverse transcribed template. Data are represented in box-and-whisker plots where boxes represent upper and lower quartiles and whiskers show maximum and minimum values. The horizontal line represents the median (2.56 for females and 0.33 for males for *cyp19a1a*, and 0.35 for females and 6.31 for males for *amh*) and the cross inside the box the average of the group (2.41 for females and 0.54 for males for *cyp19a1a*, and 0.4 for females and 5.95 for males for *amh*). Circles inside the plots represent expression data from each individual fish. Different letters denote statistical differences between both groups after a student t-test ($p < 0.05$).



Additional Figure 2. Gene expression levels of anti-müllerian hormone (*amh*) during early stages of sexual maturation in European sea bass males. Samples were analyzed by quantitative real-time fluorescent PCR. Expression data are shown as the normalized relative quantities of six samples run in triplicate and normalized to those of the constitutively expressed *18S* rRNA gene previously corrected with the expression of *ef1a* amplified from the same reverse transcribed template. Data are represented in box-and-whisker plots where boxes represent upper and lower quartiles and whiskers show maximum and minimum values. The horizontal line represents the median (31.79 for late differentiated testis and 9.88 for early recrudescent testis) and the cross inside the box the average of the group (32.89 for late differentiated testis and 10.12 for early recrudescent testis). Circles inside the plots represent expression data from each individual fish. Different letters denote statistical differences between both groups after a student t-test ($p < 0.05$).



Additional Figure 3. Schematic diagram including a simplified summary of the results on the expression levels of key genes involved in the RA signaling pathway during the first reproductive cycle in European sea bass males and females. For each given gene, upright red arrows indicate upregulation, horizontal yellow arrows indicate no change and downright green arrows indicate downregulation. Black arrows stand for the different sampling points within each representative developmental period. The change in expression for every gene in a given developmental stage is calculated by comparison with the preceding stage. During sex differentiation, the comparisons are done between the undifferentiated and the early differentiated stages. UnT = undifferentiated testis, EdT = early differentiated testis, LdT = late differentiated testis, ErT =early recrudescent testis, UnO = undifferentiated ovary, EdO = early differentiated ovary.

Chapter 4.

After confirming that the RA signaling pathway has a role during gonad development, our aim was to investigate the possible functional responses of key components of this pathway. With this purpose, in vitro experiments focussed on the induction and inhibition of RA synthesis in different meiosis scenarios were designed using gonads of prepubertal (550 and 570 dph) and adult European sea bass males (1190 dph). Testicular cultures were performed using two different procedures depending on the size of the gonad that is subsequently based on the size/age of the fish (Fig.1). The first procedure allowed to quantify gene expression in whole gonad explants from prepubertal fish. This technique maintains the gonad environment intact, making the culture conditions as close as possible as those found in vivo (Leal et al., 2009; Miura et al., 1991). The second procedure allowed to have a representative whole gonad explants from large adult testis and to minimize culture medium (Planas et al., 1993). Prepubertal (550dph) and adult (1190dph) explants were treated with all-trans RA (atRA), diethylaminobenzaldehyde (DEAB), 11KT or Fsh in order to find functional responses in the expression of RA signaling pathway genes. Expression levels were determined for different groups of genes including the retinol (ROL) transport group (rbp4 and stra6), the RA synthesis and degradation group (aldh1a2, aldh1a3 and cyp26a1), the RA nuclear receptors group (rarα, rxrα and pparγ) and a group of marker genes involved in mitotic and proliferative events (amh and pcna) using qPCR. In addition, the stage of development was assessed through histology for each individual.

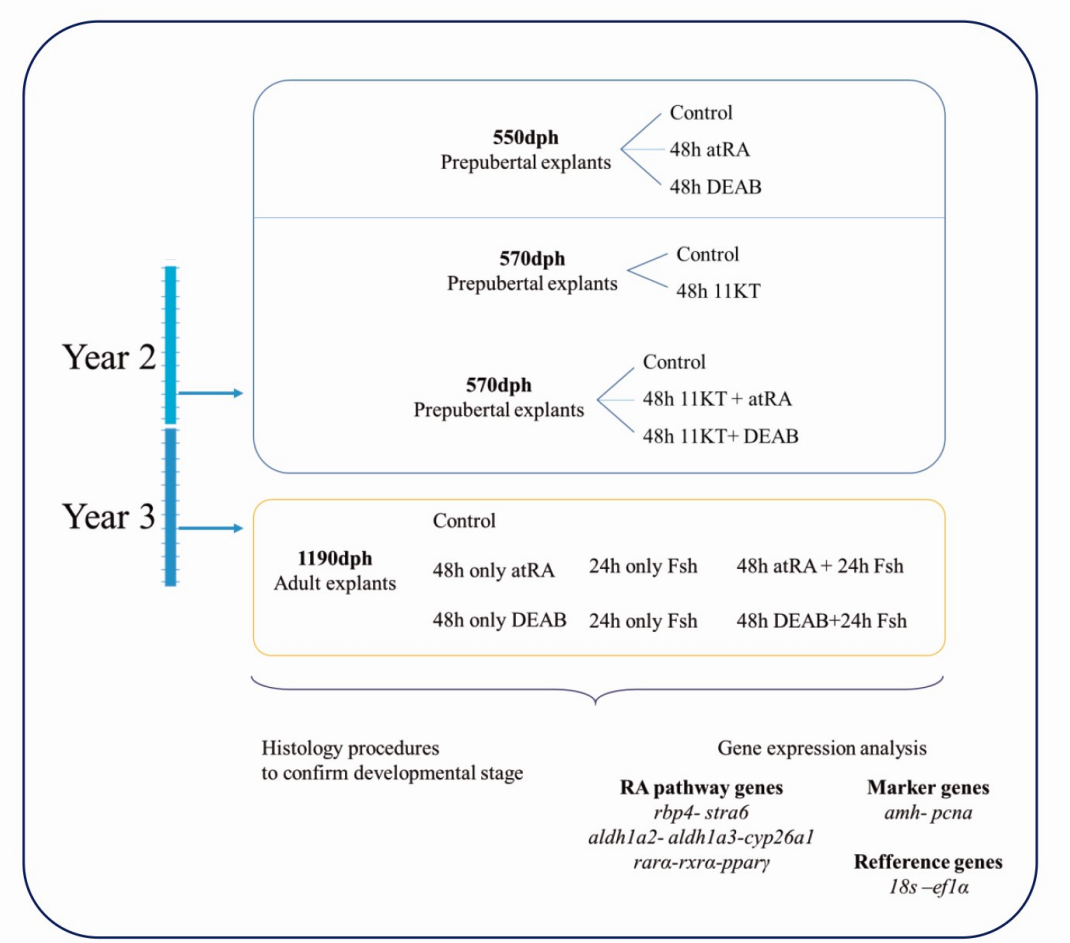


Figure 1. Schematic representation of Chapter 4 experimental design. Top blue box: experiments performed in testicular explant cultures from prepubertal fish. Orange box: experiment performed in cultures of testicular preparations from adult fish. Days post hatching (dph), All-trans RA (atRA), diethylaminobenzaldehyde (DEAB), 11Ketotestosterone (11KT), Follicle Stimulating Hormone (Fsh).

In vitro functional analysis of the expression of RA signaling pathway genes in European sea bass meiosis

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ABSTRACT

In mouse, chicken and newts, RA has been established as a paracrine signal involved in the onset of meiosis through the transcription of the *stra8* gene. Nevertheless, in fish, the involvement of RA in the onset of meiosis is still far from being fully understood. To gain a better understanding of the influence of RA in the onset of meiosis of the European sea bass, we have studied the *in vitro* functional gene expression responses to the RA signaling pathway agonist, all-trans retinoic acid (atRA) and to the RA synthase inhibitor (aldh inhibitor) 4-diethylaminobenzaldehyde (DEAB). The study includes two *in vitro* meiosis scenarios; the first one simulates, pharmacologically, an increase of 11-ketotestosterone (11KT) that occurs during the transition from immature differentiated testis to early recrudescent testis (Blázquez et al., 2017), and the second one, stimulates the endogenous synthesis of 11KT through a follicle stimulating hormone (Fsh) treatment (Mazón et al., 2014). Among the studied genes retinol transport genes (*rbp4* and *stra6*), RA synthesis and degradation genes (*aldh1a2*, *aldh1a3* and *cyp26a1*), RA nuclear receptors (*rarα, rxrα* and *pparγ*) were included. Our results show gene expression responses to an increase of exogenous RA and to different meiosis scenarios, confirming the functionality of the RA signaling pathway during meiosis. In particular, the upregulation of *cyp26a1* as a result of increased RA levels, suggest it could be used as a suitable molecular marker for the onset of meiosis.

INTRODUCTION

The RA metabolic machinery has been described in vertebrates and invertebrates, and is considered as a common tool for many biological processes (Albalat, 2009; Griswold et al., 2012; Mendelsohn et al., 1994; Napoli, 2012). Specifically, the role of RA in the transcription of S*tra8* gene to initiate meiosis, has been characterized in mouse (Bowles et al., 2009), chicken (Smith et al., 2008) and newt models (Wallacides et al., 2009). In fish, the involvement of RA in meiosis has been recently demonstrated using *in vivo* and *in vitro* systems, revealing species-related differences in *stra8* signaling (Rodríguez-Marí et al., 2013). In southern catfish, the role of RA in meiotic initiation operates via *stra8* signaling (Li et al., 2016). Conversely, in zebrafish the onset of meiosis is independent of *stra8* and sex-related differences in *cyp26a1* expression suggest a role for this gene in the differential progression of meiotic oocytes in both sexes (Rodríguez-Marí et al., 2013). In tilapia, RA is indispensable for meiotic initiation via *stra8* independent pathway, suggesting that the balance between *aldh1a2* and *cyp26a1* expression is also critical (Feng et al., 2015). In medaka, RA signaling (*stra8* independent) plays a key role in meiosis at later stages of gonad development rather than in embryonic sex determination (Adolfi et al., 2016).

Recent studies in the European sea bass describe the molecular structure of the enzymes involved in the synthesis and degradation of RA (Chapter 2). Moreover, *in vivo* expression patterns of key players of the RA signaling pathway during early stages of gonad development and meiosis initiation in males and females have also been reported (Chapter 3; Medina et al., 2019). However, no functional information is yet available to confirm their role in the onset of meiosis during gonad development. Most of the studies on sea bass gonad specific genes are limited to the analysis of their expression levels on dissected tissue samples or in gonad samples treated with specific hormones (Crespo et al., 2013; Molés et al., 2011b; Rocha et al., 2009). Regarding functional studies, *in vitro* systems allow to test specific responses of particular genes to different conditions, allowing for a direct study of each gene function. So far protocols for *in vitro* culture of the European sea bass have been based in primary cultures of mixed populations of testicular cells (Asturiano et al., 2000) or explants of minced testis (Molés et al., 2008). The aim of this study is to set up a relatively simple and cost-effective culture system (Leal et al., 2009) in order to have a better understanding of the role of RA signaling in the onset of meiosis in this species. With that purpose, we set up two different methods for short-term *in vitro* testicular cultures in order to characterize functional gene expression responses to the RA pathway agonist and RA synthase inhibitor in two meiosis scenarios; one of them characterized by an increase of 11KT levels (Blázquez et al., 2017) and another one by an increase of Fsh levels (Mazón et al., 2014; Molés et al., 2008).

MATERIALS AND METHODS

Animals and sampling

Animals hatched at the Institute of Aquaculture Torre la Sal (Castellón, Spain) and were immediately transported to our experimental aquaria facilities (ZAE) at the Institute of Marine Sciences (ICM-CSIC) in Barcelona (41°23'N; 2°11E). Rearing was performed according to standard procedures for sea bass aquaculture. A male-enriched group of fish was obtained following the procedures described in Chapter 3, which is adapted to this species and has been chosen specifically for its effectiveness, logistics and implementation at the ZAE facilities.

At each sampling point, fish were anesthetized in a 0.25% phenoxiehtanol (Sigma-Aldrich, St Louis, MO, USA) bath, provided with air flow, and sacrificed through a quick decapitation. The skin of the fish surface was cleaned and dried before opening the visceral cavity. Gonads were removed and quickly preserved in appropriate conditions for further histological and explant culture procedures. In all cases, fish were sacrificed according with the Spanish regulations (Royal Decree Act 53/2013), the European legislation concerning the protection of vertebrates used for experimental and other scientific purposes (2010/63 EU) and in accordance with the Society for Study of Reproduction's specific guidelines. All the recommendations were followed to reduce animal suffering.

Chemical treatments

All-trans retinoic acid (atRA; Sigma, St Louis, MO, USA) was dissolved in dimetylsulfoxide (DMSO; Fisher Scientific, Toronto, ON, Canada) that was used as a vehicle at a stock concentration of 160mM. Working aliquots at 160µM were stored at -80ºC for further treatments. Diethylaminobenzaldehyde (DEAB; Sigma), the inhibitor of aldhs enzymatic activity, was also prepared in DMSO at a 100mM stock solution concentration. Working aliquots at 1mM were stored at -80ºC until required. Previous trials showed that DMSO could be used as a vehicle since it did not have any effect on the expression of RA signaling genes (Additional Figure 1). 11KT (Sigma) was dissolved in 100% ethanol to a stock concentration of 100µM. Aliquots of recombinant sea bass follicle stimulating hormone (Fsh) were provided by Dr. Ana Gómez at the Institute of Aquaculture Torre la Sal (IATS-CSIC) at 7.6µg/ml and stored at-80ºC until needed. The hormone was produced using Chinese Hamster Ovary (CHO) cells as expression system. The protocol and validation are described in (Molés et al., 2011a; Molés et al., 2011b). Briefly, CHO cells were cultured in order to obtain a stable clone that expressed the pCMV-scFsh plasmid (Molés et al., 2011b). The hormone was produced according to the protocol described by (Schatz et al., 2003). Once stable clones were isolated, the cells and conditioned medium were screened

for the presence of scFsh. One selected clone expressing the hormone was grown in cell culture flasks. The cells were incubated for 9 days in specific culture media at 25ºC. Then, the culture supernatants containing the hormone were harvested and concentrated by ultrafiltration with centrifugal filter devices.

***In vitro* explant culture of prepubertal testis**

For prepubertal fish that exhibit small gonads, we used a method based on the testis culture system for Japanese eel (Miura et al., 1991) and zebrafish (Leal et al., 2009) adapted and modified for the European sea bass as follows. Briefly, after euthanasia, gonads were carefully removed and cleaned out of connective tissue. Testis were separated and placed for 2 min in ice-cold calcium-magnesium-free sea bass ringer solution (SBR) pH 7.4, consisting on 130mM NaCl, 5.0mM KCl (Sigma), 1.0mM Na_2HPO_4, 1.0mM $NaHCO_3$ (Labox, Spain), 25mM HEPES (Thermo Scientific, Wilmington, DE, USA), 5mM Glucose (Sigma), 1mM Na^+ pyruvate, 200U/ml penicillin and 200µg/ml streptomycin (Invitrogen, Carlsbad, CA. USA). Testicular explants (left and right gonads) were subsequently washed for 2 min in an ice-cold SBR solution containing 0.5% v/v bleach, thoroughly rinsed in SBR and finally placed in ice-cold basal culture medium pH 7.4. Basal culture medium consisted of Leivovitz L-15 medium (Invitrogen), supplemented with 1mg/l insulin, 5g/l bovine serum albumin (Sigma-Aldrich), 2.38g/l HEPES (Thermo Scientific) and 200U/l penicillin, 200µg/l streptomicin (Invitrogen), filter-sterilized at 0.2µml; pH was adjusted to 7.4. Explants were maintained in basal medium for about 1 h at 4ºC, on ice and protected from the light, before further culture procedures.

Flat-bottom plates (48 wells) were used as moulds to make 1.5% agarose cylinders (0.75µl). Once set, the agarose cylinders were carefully removed from the plates, transferred to 24 well plates, covered with a nitrocellulose membrane and pre-soaked overnight at 4ºC in 1ml basal medium before the initiation of explant cultures. On the following day, the medium was replaced with 1ml of fresh medium at room temperature. Left and right explants from the same fish were processed in parallel, carefully extended on top of the nitrocellulose membrane, one in the control well containing only basal medium, and the other one in the treated well containing the corresponding experimental treatment. (Fig. 2).

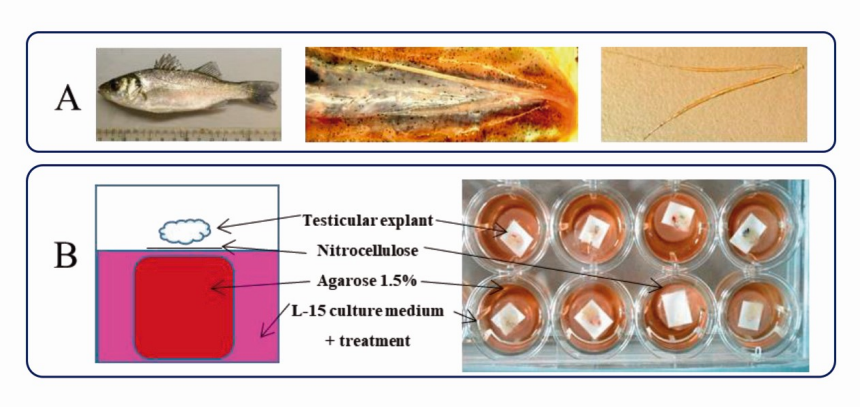

Figure 2. *In vitro* explant culture for juvenile testis samples. A) Photograph of a *D. labrax* male and gonads at 550dph. B) Example of a Culture Plate set up.

An initial study was performed to search for a functional response to atRA stimulation and DEAB inhibition on the expression of key genes from the RA signaling pathway. With this purpose, two groups of prepubertal testicular explants (*n*=8 each group), from immature differentiated fish (550 dph) were placed in 24 well culture plates, under dim light conditions, and subjected to the following treatments: Group 1: a) Control; only containing basal culture medium (1ml) and 1µl/ml DMSO, and b) atRA treatment that contained 1µM atRA resuspended in DMSO into the basal culture medium (1ml total volume). Group 2: a) Control; only containing basal culture medium (1ml) and 1µl/ml DMSO, and b) DEAB treatment that contained 5µM DEAB resuspended in DMSO into the basal culture medium (1ml total volume). Plates were carefully sealed, covered with aluminium foil and incubated in a dark humid chamber at 21ºC for a period of 48 hours. At the end of the incubation period, a portion of the explant was fixed in 2% PAF for further histological assessment and the other portion was snap frozen in liquid nitrogen and kept at -80ºC for further gene expression studies.

In the next studies, the addition of 11KT to the explant cultures was used to simulate the conditions that would be found in a meiosis onset scenario (Chapter 1; Blázquez et al., 2017) and because this androgen is known to stimulate spermatogenesis in immature fish (Leal et al., 2009; Mazón et al., 2014; Miura et al., 1991; Ohta et al., 2007). In a pilot experiment, we checked the effects of the addition of 11KT alone on testicular explants at an immature differentiated stage (*n*=3; 570 dph). The experimental design contained two groups subjected to the following treatments: a) control; containing basal culture media and 0.04% ethanol (as this is the vehicle needed to resuspend 11KT) for 48h, and b) 11KT treatment, containing 100nM 11KT in 0.04% ethanol.

In a subsequent study, testicular explants at immature differentiated stage (*n*=16, 570dph) were used to simulate a meiosis scenario, consisting of high 11KT levels, and to test the effects of atRA or DEAB. This group was divided into two sets giving rise to two studies. The first study (*n*=8) included two groups: a) control; containing 100nM 11KT, and b) a atRA-stimulated group that contained 100nM 11KT and 1μM atRA. The second study (*n*=8) included two groups: a) control; containing 100nM 11KT, and b) DEAB-stimulated group that contained 100nM 11KT and 5μM DEAB. This experimental design was repeated with the same groups, the same sample size, and identical conditions but in testicular explants at early recrudescent stage (*n*=16, 570dph).

In all treatments, incubations were performed at 21ºC in a humid chamber and in complete darkness. After completion of the different treatments, subsamples were taken for histology assessment and gene expression studies.

***In vitro* culture of testicular preparations from adult fish**

In vitro culture preparations based on the methodology reported by Planas et al. (1993), were performed to assess the functional response of adult three-year-old European sea bass males (two reproductive cycles completed). Briefly, whole testes from each fish were removed and placed in sea bass Ringer solution on ice, washed in an SBR 0.5% bleach solution and rinsed thoroughly on fresh SBR. A fine paste was made by carefully chopping the testis with a scalpel in a cold sterile petri dish. The preparation was washed twice with 5ml of ice-cold SBR, centrifuged at 600 rpm for 5 min at 4ºC, and filtered through a sterile 250μm nylon mesh. Finally, each single preparation was divided into four parts (about 20 mg each) and transferred into 48-well culture plates containing 250μl SBR as incubation media, and subjected to the different experimental treatments.

The experiment was aimed to test the effects of atRA or DEAB under a simulated meiosis scenario characterized by the presence of high Fsh levels. For this purpose, two different groups of testicular preparations from adult fish (1190dph) were set up, one group at the immature differentiated stage (*n*=8) and another one at early recrudescent stage (*n*=8). Both groups were processed under dim light conditions and subjected to the following treatments: The first group consisted of a) *control*; containing incubation media with 0.04% ethanol and 0.0001% DMSO for 48h, b) atRA; containing 1μM atRA for 48h, c) Fsh treatment; containing incubation media for the first 24h, followed by 24h stimulation with 300 ng/ml Fsh in ethanol, and d) atRA + Fsh treatment; consisting of 24h stimulation with 1μM atRA, followed by a 24h incubation with Fsh (300ng/ml). The second group consisted of a) *control*; containing incubation media with 0.04% ethanol and 0.0001% DMSO for 48h, b) DEAB; containing 5μM DEAB in DMSO for 48h, c)

Fsh treatment; containing incubation media for the first 24h, followed by 24h stimulation with 300ng/ml Fsh in ethanol, and d) DEAB + Fsh treatment; consisting of 24h stimulation with 5µM DEAB, followed by a 24h incubation with Fsh (300ng/ml). Incubations were performed over a rotating plate (100rpm) at 21ºC in a humid chamber and in complete darkness. After completion of the different treatments (48h), subsamples were taken for histology assessment and gene expression studies.

Regardless of the culture technique used i.e., intact testicular explants or testicular preparations, subsamples for histology were taken prior to the start of the different treatments in order to check and confirm their testicular developmental stage (see histological analysis). It is worth mentioning that treatments were "blind performed" without knowing *a priori* the developmental stage of the testis that could only be confirmed after histological examination when treatments had already finished. It was then when the different gonad samples could be grouped according to their stage of development for further gene expression studies.

Histological analysis

The exact developmental stage of the gonads used for the experiments was accurately verified by conventional histological analysis and light microscopy. Briefly, a small portion of one gonad from each fish was fixed overnight in 4% buffered paraformaldehyde. On the following day, samples were dehydrated in an increasing ethanol series, embedded in Histoplast (Thermo Scientific), cut at 5 µm, and stained with haematoxylin-eosin as described in previous chapters. The stage of gonad development was determined based on the criteria described in (Begtashi et al., 2004; Espigares et al., 2015). Briefly, two developmental stages were used for the studies: a) immature differentiated testis (IdT) mainly composed by spermatogonia A as the predominant cell type and b) early recrudescence testis (ErT) containing cysts of spermatogonia A and B and a small number of type I spermatocytes (Fig.3).

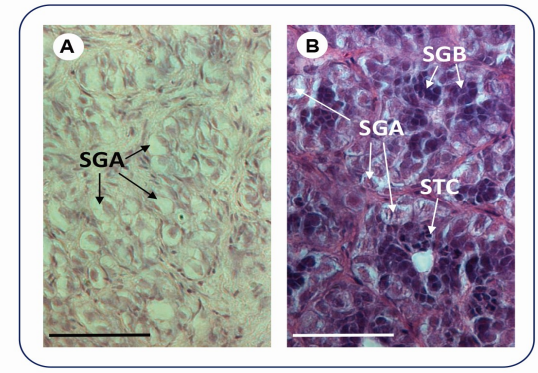

Figure 3. Photomicrographs European sea bass testis used for the different experiments. A) Immature differentiated testis (Stage I), contains type A spermatogonia (SGA) within the seminiferous lobules.

B) Early recrudescent testis (Stage II), contains type A spermatogonia (SGA), type B spermatogonia (SGB) and type I spermatocytes (SCT). Scale bar represents 50µm.

Gene expression studies

Total RNA was extracted using Trizol® Reagent (Invitrogen, Carlsbad, CA) and cDNA synthesized with SuperScript III (Invitrogen, Carlsbad, CA) following the procedures described in Chapter 3. The cDNA was then used as a template for qPCR, with primers for: *rbp4*, *stra6*, *aldh1a2*, *aldh1a3*, *cyp26a1, rarα, rxrα, pparγ*; two marker genes: *amh* and *pcna* and two refference genes: *18s* and *ef1α* (described in Thesis extended methodology Table 1). The qPCR reaction was carried out in a 7300 real-time PCR System (Applied Biosystems), using the following parameters: 2 min at 50ºC, 10 min at 95ºC, 40 amplification cycles consisting of 15 s of denaturation at 95ºC, 1 min alignment at 60ºC, a dissociation step (melting-curve analysis to check unspecific product) consisting of 95ºC during 15 s, and finally an extension step of 1min at 55ºC. All samples were run in triplicate and, for each 96 well plate (BioRad Labs., Inc.), technical and biological controls were added in order to compare between runs. For the analysis of gene expression, samples were grouped according to their corresponding developmental stage based on the results obtained from the histological study.

Statistical analysis of data

Data were normalized using the q-base methodology described in Thesis Extended Methodology and presented as the mean value + standard error of the mean (SEM). For comparisons, the expression levels of the different genes are presented as mRNA levels proportional to those of the control group (fold change) which were arbitrarily set as 1.

Differences in gene expression between control and treatments in prepubertal explants, were determined using the Student t-test. The combined treatments in adult testicular preparations**,** were assessed with a one-way ANOVA followed by a Tukey's post hoc test (multiple comparisons). For both tests, statistical differences were considered significant when $p < 0.05$.

RESULTS

Prepubertal explant cultures

In immature differentiated prepubertal explants, atRA, was able to increase the expression of several genes involved in the RA signaling pathway (Fig. 4). Regarding the group of genes related to ROL transport, *rbp4* was upregulated while no changes could be detected for *stra6.* Regarding the genes involved in RA synthesis, *aldh1a2* was upregulated when treated with atRA whereas no differences in *aldh1a3* expression were found. Nevertheless, atRA treatment was

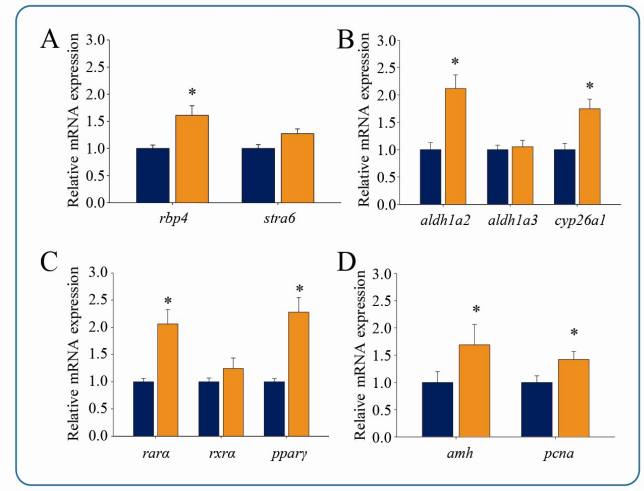

Figure 4. Effects of atRA (1µM) in the expression of several genes involved in the RA signaling pathway in testicular explants of prepubertal European sea bass at immature differentiated stage. A) ROL transport genes, B) RA synthesis and degradation genes, C) Nuclear receptor genes, D) Reproduction genes. The different experimental groups were represented as follows: blue bars (control group; n=8), and orange bars (atRA treated group; n=8). Expression values in the control group were arbitrarily set at 1 and those in the treated group were expressed as a proportion of the mean value in the *control* group (average + SEM). Asterisks indicate significant differences with the control group after a Student-T test (p<0.05).

capable to induce a significant increase of *cyp26a1* levels, and also changes in the RA nuclear receptors group as shown by the upregulation of *rarα* and *pparγ*. The expression of the marker genes, *amh* and *pcna*, was also upregulated after atRA treatment at this developmental stage. Treatment of prepubertal immature differentiated explants with DEAB resulted in the significant downregulation of *rbp4*, *pparγ* and *pcna* while expression of other genes was not altered (Fig. 5).

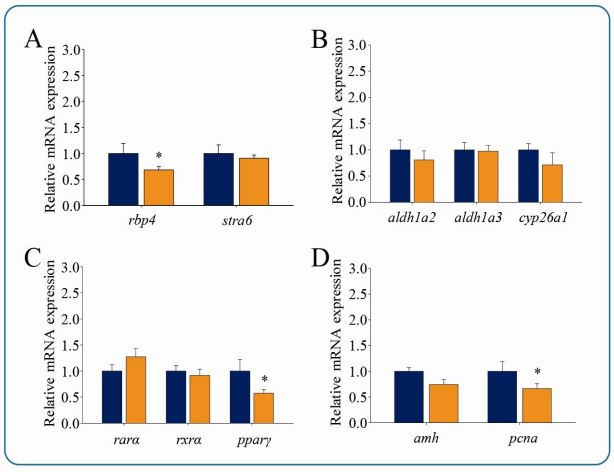

Figure 5. Effects of DEAB (5µM) in the expression of several genes involved in the RA signaling pathway in testicular explants of prepubertal European sea bass at immature differentiated stage. A) ROL transport genes, B) RA synthesis and degradation genes, C) Nuclear receptor genes, D) Reproduction genes. The different experimental groups were represented as follows: blue bars (control group; n=8), and orange bars (atRA treated group; n=8). Expression values in the control group were arbitrarily set at 1 and those in the treated group were expressed as a proportion of the mean value in the *control* group (average + SEM). Asterisks indicate significant differences with the control group after a Student-T test (p<0.05).

A pilot study to check the effects of exogenous 11KT in immature differentiated explants showed a significant downregulation of *rbp4*, *aldh1a3*, and *cyp26a1*, when compared with control explants cultured only with basal medium (Additional Figure 2).

Based on these results, we performed a complete study where testicular explants were treated with 11KT in combination with atRA (Fig. 6) or with DEAB (Fig. 7). In immature differentiated explants, *stra6, cyp26a1, rarα, rxrα,* and *pcna* exhibited an upregulation in response to the atRA+11KT combined treatment (Fig. 6 top). Moreover, when this treatment was assayed in early recrudescent explants, it resulted in the upregulation of *stra6*, *aldh1a3*, *cp26a1 and pcna* (Fig 6 bottom).

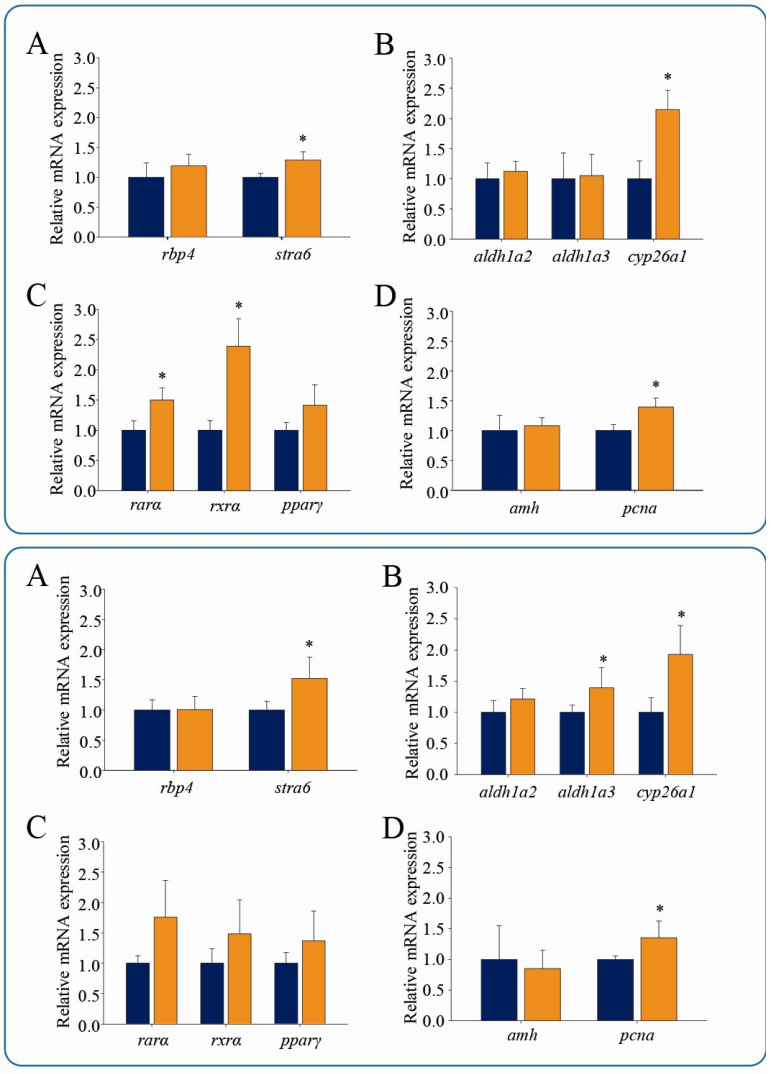

Figure 6. Effects of atRA (1µM) and a 11KT (100nM) combined treatment in the expression of genes involved in the RA signaling pathway in testicular explants of prepubertal European sea bass at immature differentiated stage (top panel) and early recrudescence stage (bottom panel). A) ROL transport genes, B) RA synthesis and degradation genes, C) Nuclear receptor genes, D) Reproduction genes. The different experimental groups were represented as follows: blue bars (control group; *n*=8), and orange bars (atRA or 11KT treated groups; *n*=8 each). Expression values in the control group were arbitrarily set at 1 and those in the treated group were expressed as a proportion of the mean value in the *control* group (average + SEM). Asterisks indicate significant differences with the control group after a Student-T test ($p<0.05$).

In the combined treatment of 11KT+DEAB, a significant upregulation was found only in *rarα* expression at immature differentiated stage (Fig. 7 top), while *stra6*, *cyp26a1, rarα* and *pcna* were upregulated in early recrudescent explants (Fig. 7 bottom).

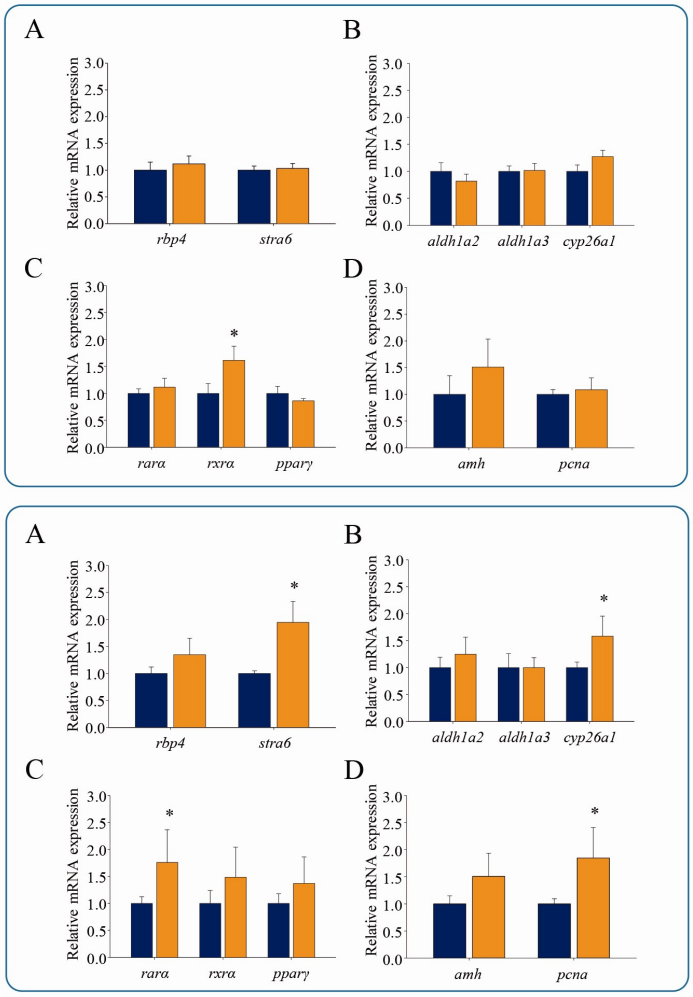

Figure 7. Effects of DEAB (5µM) and a 11KT (100nM) combined treatment in the expression of genes involved in the RA signaling pathway in testicular explants of prepubertal European sea bass at immature differentiated stage (top panel) and early recrudescence stage (bottom panel). A) ROL transport genes, B) RA synthesis and degradation genes, C) Nuclear receptor genes, D) Reproduction genes. The different experimental groups were represented as follows: blue bars (control group; n=8), and orange bars (DEAB or 11KT treated groups; n=8 each). Expression values in the control group were arbitrarily set at 1 and those in the treated groups were expressed as a proportion of the mean value in the *control* group (average + SEM). Asterisks indicate significant differences with the control group after a Student-T test (p<0.05).

Cultures of testicular preparations from adult fish

During the second reproductive cycle, testicular preparations at immature differentiated stage were incubated with atRA+FSH, (Fig. 8) while those at early recrudescence stage were incubated with DEAB+FSH (Fig. 9), in order to simulate a meiosis scenario with high androgen production via Fsh stimulation. A significant increase of *rbp4*, *aldh1a2*, *aldh1a3* and *cyp26a1* was found with atRA treatment (Fig. 8) while Fsh alone could only increase *aldh1a2* and *aldh1a3* expression. Nevertheless, the combined effect of atRA and Fsh resulted in changes in the expression of *rbp4, aldh1a3* and *cyp26a1*. Single DEAB treatment only affected *stra6*, and *rxrα*

while single Fsh treatment had no effect in any of the studied genes. Finally, the combination of DEAB and Fsh did not result in any change in gene expression (Fig. 9)

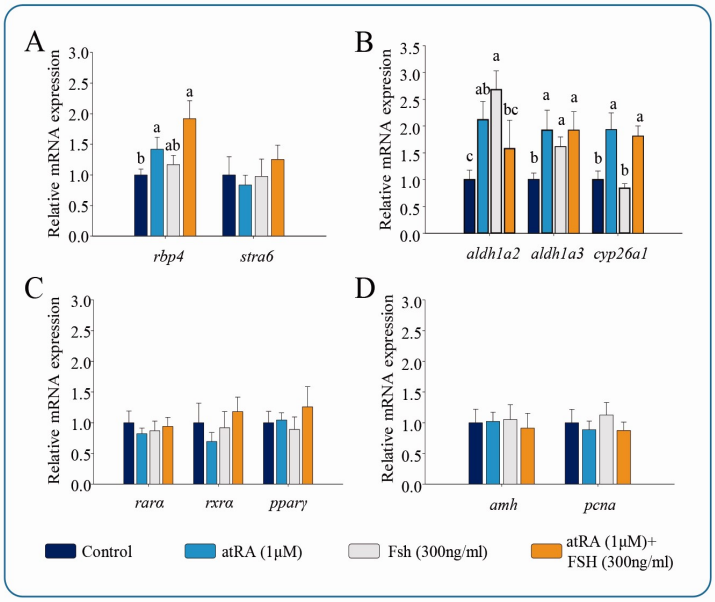

Figure 8. Effects of atRA and Fsh in the expression of genes involved in the RA signaling pathway in testicular preparations of European sea bass at immature differentiated stage. A) ROL transport genes, B) RA synthesis and degradation genes, C) Nuclear receptors genes, D) Reproduction genes. The different experimental groups were represented as follows (n=8 each): dark blue bars (*control* group), light-blue bars (atRA (1μM) treated group), grey bars (Fsh (300ng/ml) treated group), orange bars (combined 1μM atRA + 300ng/ml Fsh treated group). Expression values in the control group were arbitrarily set at 1 and those in the treated groups were expressed as a proportion of the mean value in the *control* group (average + SEM). Different letters indicate significant differences between groups after a Tukey test (p<0.05).

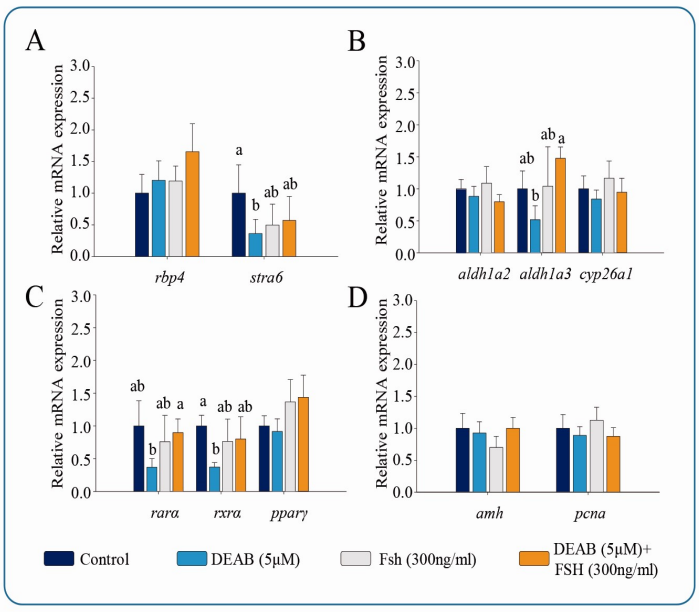

Figure 9. Effects of DEAB and Fsh in the expression of genes involved in the RA signaling pathway in testicular preparations of European sea bass at early recrudescent stage. A) ROL transport genes, B) RA synthesis and degradation genes, C) Nuclear receptor genes, D) Reproduction genes. The different experimental groups were represented as follows (n=8 each): dark blue bars (*control* group), light-blue bars (DEAB (5μM) treated group), grey bars (Fsh (300ng/ml) treated group), orange bars (combined 5μM DEAB + 300ng/ml Fsh treated group). Expression values in the control group were arbitrarily set at 1 and expression values in the treated groups were expressed proportional to the mean value in the *control* group (average + SEM). Different letters indicate significant differences between groups after a Tukey test (p<0.05).

DISCUSSION

Spermatogenesis is a complex process that involves a series of events including cell proliferation, cell differentiation, mitosis and meiosis until the final formation of the haploid spermatozoa (De Rooij and Griswold, 2012; Nóbrega et al., 2009; Schulz et al., 2010). Several hormones and steroids are known to be involved in spermatogenesis progression in fish, such as Fsh, Lh Amh, or 11KT (Schulz et al., 2010; Yaron and Levavi-Sivan, 2011). This thesis is aimed at the study of the onset of meiosis in the European sea bass, in particular at the role of the RA signaling pathway in the orchestration of this process. In previous studies (Chapters 1-3) we used a transcriptomic approach to describe the molecular structure and function of the main genes from the RA signaling pathway and to find their expression signatures (*in vivo*) during the onset of puberty in male and female European sea bass. Here, we continue to apply this approach using an *in vitro* system to determine whether the action of atRA and DEAB could bring about functional responses in the RA signaling pathway under two different meiosis scenarios. Studies in chicken showed that atRA can alter the expression of specific genes, inducing changes in several transcription factors, growth factors, and extracellular matrix proteins, resulting in embryonic stem cell differentiation towards the male germ cell lineage (Zhang et al., 2016). In mouse, atRA has been proven necessary for the progression of spermatogenesis *in vivo* (Silva et al., 2009) and for the *in vitro* proliferation and survival of PGCs (Tan et al., 2016). Our results showed that the functional response to atRA treatment in general is to increase the expression of several genes involved in the RA signaling pathway. Moreover, these changes are in accordance with the stage of gonad development. Conversely, the response to DEAB seems to depend on both the stage of gonad development and also the technique used, i.e., intact explant cultures or partially dispersed cells in testicular preparations.

Studies in mice revealed that STRA6 is involved in the bidirectional flux of ROL between its blood carrier protein RBP4 and the cellular retinoid binding protein 1, CRABP1 (Isken et al., 2008; Kawaguchi et al., 2007), playing a critical part in the internalisation of ROL across the blood-testis barrier (Kelly et al., 2016). *In vitro* experiments in mice showed that atRA induces *Stra6* expression while repressing that of *Rbp4* (Alapatt et al., 2013; Mercader et al., 2008). Likewise, our results also show an interesting expression dynamics between *rbp4* and *stra6*, independent of the stage of testicular development, the treatment, or the technique used, where changes in the expression of *rbp4* are never paralleled by changes in the expression of *stra6*, and vice versa. At the immature differentiated stage, regardless of the age or the technique used, atRA induced the upregulation of *rbp4* while the expression of *stra6* remained stable. Moreover, the upregulation of *stra6* only occurred when atRA was given in combination with 11KT, suggesting

that the exogenous supply of RA together with an increase of 11KT levels, typical of a natural scenario of meiosis onset, were capable to activate *stra6* in order to incorporate atRA into the cell. A similar situation was found in the combined treatment DEAB+11KT, reinforcing the idea of the importance of increased 11KT levels for the initiation of meiosis in this species. In this regard, studies in other fish species have shown that 11KT levels increase gradually as spermatogenesis progresses, decreasing by the time of spermiation (Miura et al., 1991; Cavaco et al., 1998; Rodríguez et al., 2001; Le Gac et al., 2008; Schulz et al., 2010), indicating that this androgen is an important endocrine signal for the initiation of testicular development. Furthermore, our results from Chapter 1 show that this increase marks the transition from immature differentiated to early recrudescent testis and is accompanied by the differential expression of 315 genes, broadening our knowledge of early molecular and endocrine events that trigger pubertal development in the European sea bass. In addition, the administration of Fsh, alone or in combination with atRA, was capable to upregulate *rbp4*. Similarly, FSH has been reported to promote retinol uptake in mouse ovaries *in vivo* and in follicular granulosa cells *in vitro*, suggesting a role for this hormone in RA signaling (Liu et al., 2018; Jiang et al 2018). Taken together, our results show that the functional response to atRA alone or in combination with 11KT or with Fsh is the upregulation of genes involved in ROL uptake (*rbp4* or *stra6*), revealing a tight control in ROL transport within the European sea bass testis by the time of meiosis.

The bioavailability of intracellular RA is based on a controlled equilibrium between its synthesis, by Aldhs, and its degradation, by Cyp26, into other inactive and readily excreted water-soluble products (reviewed by Teletin, 2017). Indeed, an increase in RA levels dictate germ cell differentiation and the entry into meiosis in female mouse germ cells (Koubova et al., 2006). Moreover, meiosis onset in males was raised by the cyclic action of *Aldhs*, and that of *Cyp26b1* that modulate RA bioavailability within the germ cell environment (Raverdeau et al., 2012; Bowles et al., 2006). Our results show a strong regulation of *aldhs* and *cyp26a1*, in response to their function that support their key role in the regulation of RA availability in different meiosis scenarios. In this regard, studies in tilapia showed an upregulation of *aldh1a2* and a downregulation of *cyp26a1* before the onset of meiosis in ovaries and testes (Feng et al., 2015). Moreover, in zebrafish males c*yp26a1* expression was already detected in the undifferentiated gonadal primordium, becoming more abundant during early sex differentiation and highly upregulated in the immature prepubertal testis (Rodríguez-Marí et al., 2013).

Previous studies in European sea bass males support the notion that an increase of endogenous RA takes place right by the onset of meiosis, together with changes in the expression of different genes involved in the RA signaling pathway, and coinciding with the first significant

increase of 11KT levels (Blázquez et al., 2017; Medina et al., 2019). The present study shows an upregulation of *aldh1a2* in prepubertal testicular explants stimulated with atRA and a downregulation of both *aldhs* in a simulated scenario of meiosis onset mediated by the administration of 11KT (statistically different only in *aldh1a3*). Furthermore, the administration of atRA or Fsh to testicular preparations of prepubertal fish, simulating a scenario of meiosis onset, resulted in the upregulation of both *aldhs*, suggesting that high RA levels are needed for the progression of meiosis during spermatogenesis. Likewise, *in vivo* Fsh injection triggered spermatogenesis onset in zebrafish by inducing an increase in RA synthesis (*aldh1a2* upregulation) and a decrease in RA degradation (*cyp26a1* downregulation) (Crespo et al 2016). However, no effects were found when *ex vivo* cultures of pre-spermatogenic testes were treated with Fsh (Crespo et al., 2016). Similarly, a lack of response in *aldh1a2* expression was found in zebrafish embryos after atRA administration (Adolfi et al., 2016), probably due to the fact that treatments took place during very early developmental stages. Our results consistently show that the upregulation of *aldhs* occurs concomitant with an increase of *cyp26a1* expression in almost each experimental condition. Likewise, medaka *ex vivo* treatment of embryos, tissues and cells with exogenous RA, induced a dose-dependent regulation of *cyp26a1* expression levels (Adolfi et al., 2016). This suggests that a higher RA clearance rate would be needed to regulate RA intracellular levels in a time- and dose-dependent manner, since an excess of RA is detrimental for health and cell maintenance in embryos and adults (Thatcher and Isoherranen, 2009).

DEAB is an inhibitor of RAL oxidation into RA by competing with the aldehyde substrate, although its exact mechanism of action is yet to be elucidated (Morgan et al., 2015). This compound has been used as an Aldh1 selective repressor in several studies (Alsop et al., 2008; Chute et al., 2006; Koppaka et al., 2012; Morgan et al., 2015; Reijntjes et al., 2007) and as an inhibitor of RA synthesis in different commercial kits (Luo et al., 2015). Indeed, DEAB acts as a selective inhibitor of Aldh1 enzymes by binding reversibly to the active site of the enzyme, not by blocking *aldhs* gene transcription (Morgan et al., 2015). This is supported by our results showing that DEAB did not change the expression of *aldhs* in any developmental stage, in agreement with its role as an enzyme inhibitor. Interestingly, we found a downregulation of *cyp26a1* in immature differentiated testicular explants after DEAB treatment. This suggests that to counteract the effect of Aldh1 inhibition, in order to reach again the metabolic *status quo* typical of a meiosis scenario, a downregulation of *cyp26a1* and thus a decrease of RA degradation is needed. In prepubertal Nile tilapia, the inhibition of *aldh1a2*, after the administration of a DEAB treated diet, resulted in a delayed meiotic initiation and a simultaneous downregulation of *cyp26a1* in order to restore endogenous RA levels (Feng et al., 2015). Moreover, the use of a Cyp26a1 inhibitor resulted in early meiotic initiation with an upregulation of *aldh1a2* (Feng et

al., 2015). Similarly, treatment of catfish fry with exogenous RA or with a cyp26a1 inhibitor advanced meiotic entry, while inhibition of Aldh1a2 enzyme activity after DEAB administration resulted in a delay of meiosis onset that could later be rescued by RA administration (Li et al., 2016). In addition, a downregulation of *cyp26a1* was found in medaka embryos treated with DEAB, while the opposite was shown after atRA administration (Adolfi et al., 2016).

In vitro studies in mice show that STRA6 mediates bidirectional ROL transport dictated by RBP4 that controls RAR activity and subsequent adipocyte differentiation (Muenzner et al., 2013). We could not find any consistent response of the RA nuclear receptor genes to the different treatments assayed, not even any clear link with other genes of the pathway. However, based on their function, it was plausible to expect an upregulation of *rarα* after atRA administration (Mark et al., 1999), or even that *rarα, rxrα* and *pparγ* would have exhibited similar expression patterns since one of the mechanisms by which retinoids carry out their functions, is by activating or repressing the transcription of specific genes through their interaction with rar/rxr nuclear receptors (Fields et al., 2007; Ziouzenkova and Plutzky, 2008). These different modes of regulation reveal high levels of complexity in the dynamics of retinoid-dependent transcription (Fields et al., 2007). Contrary to common expectations, we did not find a consistent expression pattern within the RA-receptor family, although the results could point at an interaction between *rbp4*, *stra6* and *rarα*. In this regard, an upregulation of *rarα* occurs together with changes in the expression of *rbp4* and *stra6* as a response to atRA administration. Studies in obese mice show that ROL can be delivered from hepatic stores to adipocyte precursor cells via RBP4 and STRA6, thus increasing RAR activity and adipocyte differentiation (Muenzner et al., 2013). Moreover, the activation of Aldh inhibits RXR- and PPAR-induced adipogenesis (Ziouzenkova and Plutzky, 2008). Indeed, *agrp2* –a gene coding for an orexigenic peptide with a key role in the regulation of energy balance in mammals (Girardet and Butler, 2014) and fish (Guillot et al., 2016)– was affected during early gonadal recrudescence in the European sea bass (Chapter 1), further indicating the importance for a correct control of lipid metabolism by the onset of meiosis. Taken together, our results suggest that membrane receptors *rbp4* and *stra6* and RA nuclear receptors may be involved in the intricate coordination of energy balance during meiosis and gametogenesis in this species.

Amh acts as a meiosis inhibiting factor by preventing spermatogenesis progression in several fish species including trout (Rolland et al., 2009), Atlantic salmon (Guiry et al., 2010), or European sea bass (Mazón et al., 2014; Rocha et al, 2016) among others. Notwithstanding, the *in vitro* experiments performed in this study could not find any effect in *amh* expression in the majority of the treatments assayed. However, a sharp decrease of *amh* during the transition

between late differentiated and early recrudescent testis has been recently reported in this species (Blázquez et al., 2017; Medina et al., 2019). In addition, several studies in fish have shown that increases in 11KT levels are able to downregulate *amh* expression in eel (Miura et al., 2002), zebrafish (Skaar et al., 2011), Atlantic salmon (Melo et al., 2015) and European sea bass (Blázquez et al., 2017; Rodríguez et al., 2019). One could speculate that the lack of effect on *amh* could be due to differences between the *in vivo* and the *in vitro* system or even to the different *in vitro* culture techniques used in the present study. Moreover, it could suggest that the treatments were not capable to induce significant changes in spermatogenesis progression, although they were able to modulate *pcna* expression, a gene involved in DNA synthesis and widely used as a marker of cell proliferation (Korfsmeier, 2002). Indeed, our results show an increase of *pcna* in juvenile fish exposed to atRA alone or in combination with 11KT. In that regard, the upregulation of *pcna* in spermatogonia would imply an increased mitotic activity, while the upregulation in spermatocytes would reflect increased meiotic activity. However, since the RT-PCR technique does not allow for the discrimination of the different cell types in the sample, we cannot infer whether this upregulation corresponds to mitosis or meiosis.

The results from all the different treatments appear summarized in Fig.10 and suggest that meiosis scenarios influence the RA pathway gene expression and that there is a positive correlation between genes involved in RA synthesis and degradation. The more is synthesized, the more is degraded to avoid an excess of RA within the cell and maintain intracellular homeostasis. Nevertheless, our results cannot reveal the intricate mechanism by which each of these genes contribute to the overall functioning of the RA signaling pathway in different meiosis scenarios.

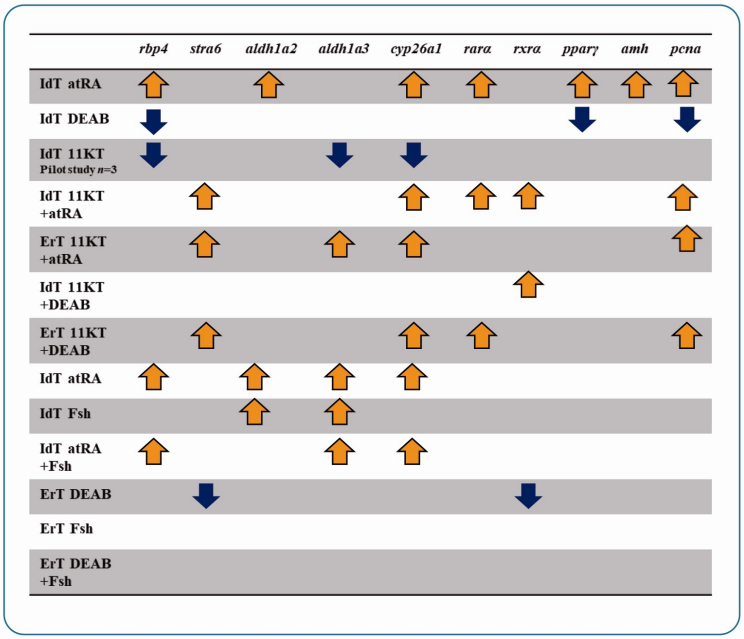

Figure 10. Schematic diagram including a simplified summary of the results on the expression change of key genes involved in the RA signaling pathway in testicular explants during different culture conditions. For each given gene, upright orange arrows indicate upregulation and downright blue arrows indicate downregulation. The change of expression for every gene in a given culture condition, is calculated by comparing control and treated explants. UnT = undifferentiated testis, EdT = early differentiated testis. 11KT =100nM 11Ketotestosterone, atRA= 1µM all-trans RA, DEAB = 5µM diethylaminobenzaldehyde, Fsh = 300ng/ml follicle stimulating hormone.

CONCLUSIONS

To the best of our knowledge, this is the first study aimed to reveal the functional role of the RA signaling pathway under different simulated *in vitro* meiosis scenarios in European sea bass males. Several genes involved in this pathway exhibit *in vitro* responses to atRA, DEAB, 11KT and Fsh according to their respective function, confirming that the RA metabolic machinery is active in testis during the onset and early stages of meiosis. Genes involved in ROL transport show a differential expression according to the need for ROL intake and the subsequent RA synthesis during meiosis, while the response of the nuclear receptor genes depends on the stage of gonad development. The expression dynamics of the RA synthesis and degradation genes, *aldh1a2*, *aldh1a3*, and *cyp26a1*, respectively, reinforce the idea that a tight control on the overall RA availability within the gonad is crucial during the onset of meiosis. In particular, the upregulation of *cyp26a1* as a response to the administration of atRA or during the natural increase of RA by the onset of meiosis. Furthermore, the downregulation of *cyp26a1* after the administration of 11KT is similar to that obtained during the natural increase of 11KT at the onset of meiosis. Our findings suggest that changes in the intracellular RA levels can be detected through the expression of *cyp26a1,* making it an excellent molecular marker for the initiation of meiosis in European sea bass males. However, further research on *cyp26a1* expression during the early events preceding the onset of puberty is needed to corroborate this hypothesis.

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REFFERENCE LIST

Adolfi, M. C., Herpin, A., Regensburger, M., Sacquegno, J., Waxman, J. S., and Schartl, M. (2016). Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. *Scientific reports* **6**, 34281.

Alapatt, P., Guo, F., Komanetsky, S. M., Wang, S., Cai, J., Sargsyan, A., Rodríguez Díaz, E., Bacon, B. T., Aryal, P., and Graham, T. E. (2013). Liver Retinol Transporter and Receptor for Serum Retinol-binding Protein (RBP4). *Journal of Biological Chemistry* **288**, 1250-1265.

Albalat, R. (2009). The retinoic acid machinery in invertebrates: ancestral elements and vertebrate innovations. *Mol Cell Endocrinol* **313**, 23-35.

Alsop, D., Matsumoto, J., Brown, S., and Van Der Kraak, G. (2008). Retinoid requirements in the reproduction of zebrafish. *Gen Comp Endocrinol* **156**, 51-62.

Asturiano, J. F., Sorbera, L. A., Zanuy, S., and Carrillo, M. (2000). Effects of polyunsaturated fatty acids and gonadotropin on prostaglandin series E production in a primary testis cell culture system for the European sea bass. *Journal of Fish Biology* **57**, 1563-1574.

Blázquez, M., Medina, P., Crespo, B., Gómez, A., and Zanuy, S. (2017). Identification of conserved genes triggering puberty in European sea bass males (Dicentrarchus labrax) by microarray expression profiling. *BMC Genomics* **18**, 441.

Bowles, J., Feng, C.-W., Knight, D., Smith, C. A., Roeszler, K. N., Bagheri-Fam, S., Harley, V. R., Sinclair, A. H., and Koopman, P. (2009). Male-specific expression of Aldh1a1 in mouse and chicken fetal testes: Implications for retinoid balance in gonad development. *Developmental Dynamics* **238**, 2073-2080.

Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M., and Rossant, J. (2006). Retinoid signaling determines germ cell fate in mice. *Science* **312**, 596 - 600.

Cavaco, J. E. B., Vilrokx, C., Trudeau, V. L., Schulz, R. d. W., and Goos, H. J. T. (1998). Sex steroids and the initiation of puberty in male African catfish (Clarias gariepinus). *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **275**, R1793-R1802.

Crespo, B., Zanuy, S., and Gómez, A. (2013). Development of an in vitro system for functional studies of ovarian follicular cells in European sea bass (Dicentrarchus labrax). *Cytotechnology* **65**, 273-286.

Crespo, D., Assis, L. H. C., Furmanek, T., Bogerd, J., and Schulz, R. W. (2016). Expression profiling identifies Sertoli and Leydig cell genes as Fsh targets in adult zebrafish testis. *Mol Cell Endocrinol* **437**, 237-251.

Chute, J. P., Muramoto, G. G., Whitesides, J., Colvin, M., Safi, R., Chao, N. J., and McDonnell, D. P. (2006). Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. *Proc Natl Acad Sci U S A* **103**, 11707-12.

De Rooij, D. G., and Griswold, M. D. (2012). Questions about spermatogonia posed and answered since 2000. *Journal of Andrology* **33**, 1085-1095.

Feng, R., Fang, L., Cheng, Y., He, X., Jiang, W., Dong, R., Shi, H., Jiang, D., Sun, L., and Wang, D. (2015). Retinoic acid homeostasis through aldh1a2 and cyp26a1 mediates meiotic entry in Nile tilapia (Oreochromis niloticus). *Scientific reports* **5**, 10131.

Fields, A. L., Soprano, D. R., and Soprano, K. J. (2007). Retinoids in biological control and cancer. *Journal of cellular biochemistry* **102**, 886-898.

Griswold, M. D., Hogarth, C. A., Bowles, J., and Koopman, P. (2012). Initiating meiosis: the case for retinoic acid. *Biol Reprod* **86**, 35.

Guiry, A., Flynn, D., Hubert, S., O'Keeffe, A. M., LeProvost, O., White, S. L., Forde, P. F., Davoren, P., Houeix, B., and Smith, T. J. (2010). Testes and brain gene expression in precocious male and adult maturing Atlantic salmon (Salmo salar). *BMC Genomics* **11**, 211.

Isken, A., Golczak, M., Oberhauser, V., Hunzelmann, S., Driever, W., Imanishi, Y., Palczewski, K., and von Lintig, J. (2008). RBP4 Disrupts Vitamin A Uptake Homeostasis in a STRA6-Deficient Animal Model for Matthew-Wood Syndrome. *Cell Metab* **7**, 258-268.

Kawaguchi, R., Yu, J., Honda, J., Hu, J., Whitelegge, J., Ping, P., Wiita, P., Bok, D., and Sun, H. (2007). A Membrane Receptor for Retinol Binding Protein Mediates Cellular Uptake of Vitamin A. *Science* **315**, 820-825.

Kelly, M., Widjaja-Adhi, M. A. K., Palczewski, G., and von Lintig, J. (2016). Transport of vitamin A across blood–tissue barriers is facilitated by STRA6. *The FASEB Journal* **30**, 2985-2995.

Koppaka, V., Thompson, D. C., Chen, Y., Ellermann, M., Nicolaou, K. C., Juvonen, R. O., Petersen, D., Deitrich, R. A., Hurley, T. D., and Vasiliou, V. (2012). Aldehyde Dehydrogenase Inhibitors: a Comprehensive Review of the Pharmacology, Mechanism of Action, Substrate Specificity, and Clinical Application. *Pharmacological Reviews* **64**, 520-539.

Korfsmeier, K. H. (2002). PCNA in the ovary of zebrafish (Brachydanio rerio, Ham.-Buch.). *Acta Histochem* **104**, 73-6.

Koubova, J., Menke, D. B., Zhou, Q., Capel, B., Griswold, M. D., and Page, D. C. (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci U S A* **103**, 2474-9.

Le Gac, F., Lareyre, J. J., MontFort, J., Esquerre, D., and Houlgatte, R. (2008). Transcriptional analysis of spermatogenesis regulation by sex steroids in trout. *Cybium* **32**, 119-121.

Leal, M. C., de Waal, P. P., Garcia-Lopez, A., Chen, S. X., Bogerd, J., and Schulz, R. W. (2009). Zebrafish primary testis tissue culture: an approach to study testis function ex vivo. *Gen Comp Endocrinol* **162**, 134-8.

Li, M., Feng, R., Ma, H., Dong, R., Liu, Z., Jiang, W., Tao, W., and Wang, D. (2016). Retinoic acid triggers meiosis initiation via stra8-dependent pathway in Southern catfish, Silurus meridionalis. *Gen Comp Endocrinol* **232**, 191-198.

Luo, M., Gates, K. S., Henzl, M. T., and Tanner, J. J. (2015). Diethylaminobenzaldehyde is a covalent, irreversible inactivator of ALDH7A1. *ACS chemical biology* **10**, 693-697.

Mark, M., Ghyselinck, N. B., Wendling, O., Dupé, V., Mascrez, B., Kastner, P., and Chambon, P. (1999). A genetic dissection of the retinoid signaling pathway in the mouse. *Proceedings of the Nutrition Society* **58**, 609-613.

Mazón, M. J., Gómez, A., Yilmaz, O., Carrillo, M., and Zanuy, S. (2014). Administration of Follicle-Stimulating Hormone In Vivo Triggers Testicular Recrudescence of Juvenile European Sea Bass (Dicentrarchus labrax). *Biol Reprod* **90**, 6, 1-10.

Medina, P., Gómez, A., Zanuy, S., and Blázquez, M. (2019). Involvement of the retinoic acid signaling pathway in sex differentiation and pubertal development in the European sea bass Dicentrarchus labrax. *Heliyon* **5**, e01201-e01201.

Melo, M. C., van Dijk, P., Andersson, E., Nilsen, T. O., Fjelldal, P. G., Male, R., Nijenhuis, W., Bogerd, J., de França, L. R., and Taranger, G. L. (2015). Androgens directly stimulate spermatogonial differentiation in juvenile Atlantic salmon (Salmo salar). *Gen Comp Endocrinol* **211**, 52-61.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., and Mark, M. (1994). Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120**, 2749-2771.

Mercader, J., Granados, N., Bonet, M. L., and Palou, A. (2008). All-Trans Retinoic Acid Decreases Murine Adipose Retinol Binding Protein 4 Production. *Cellular Physiology and Biochemistry* **22**, 363-372.

Miura, T., Miura, C., Konda, Y., and Yamauchi, K. (2002). Spermatogenesis-preventing substance in Japanese eel. *Development* **129**, 2689-2697.

Miura, T., Yamauchi, K., Takahashi, H., and Nagahama, Y. (1991). Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (Anguilla japonica). *Proceedings of the National Academy of Sciences* **88**, 5774-5778.

Molés, G., Gómez, A., Carrillo, M., Rocha, A., Mylonas, C. C., and Zanuy, S. (2011a). Determination of Fsh Quantity and Bioactivity During Sex Differentiation and Oogenesis in European Sea Bass1. *Biol Reprod* **85**, 848-857.

Molés, G., Gómez, A., Rocha, A., Carrillo, M., and Zanuy, S. (2008). Purification and characterization of follicle-stimulating hormone from pituitary glands of sea bass (Dicentrarchus labrax). *Gen Comp Endocrinol* **158**, 68-76.

Molés, G., Zanuy, S., Muñoz, I., Crespo, B., Martínez, I., Mañanós, E., and Gómez, A. (2011b). Receptor Specificity and Functional Comparison of Recombinant Sea Bass (Dicentrarchus labrax) Gonadotropins (Fsh and Lh) Produced in Different Host Systems1. *Biol Reprod* **84**, 1171-1181.

Morgan, C. A., Parajuli, B., Buchman, C. D., Dria, K., and Hurley, T. D. (2015). N,N-diethylaminobenzaldehyde (DEAB) as a substrate and mechanism-based inhibitor for human ALDH isoenzymes. *Chemico-Biological Interactions* **234**, 18-28.

Muenzner, M., Tuvia, N., Deutschmann, C., Witte, N., Tolkachov, A., Valai, A., Henze, A., Sander, L. E., Raila, J., and Schupp, M. (2013). Retinol-Binding Protein 4 and Its Membrane Receptor STRA6 Control Adipogenesis by Regulating Cellular Retinoid Homeostasis and Retinoic Acid Receptor α Activity. *Molecular and Cellular Biology* **33**, 4068-4082.

Napoli, J. L. (2012). Physiological insights into all-trans-retinoic acid biosynthesis. *Biochim Biophys Acta* **1821**, 152-67.

Nóbrega, R. H., Batlouni, S. R., and França, L. R. (2009). An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. *Fish Physiol Biochem* **35**, 197-206.

Ohta, T., Miyake, H., Miura, C., Kamei, H., Aida, K., and Miura, T. (2007). Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, Anguilla japonica. *Biol Reprod* **77**, 970-7.

Planas, J. V., Swanson, P., and Dickhoff, W. W. (1993). Regulation of testicular steroid production in vitro by gonadotropins (GTH I and GTH II) and cyclic AMP in coho salmon (Oncorhynchus kisutch). *Gen Comp Endocrinol* **91**, 8-24.

Raverdeau, M., Gely-Pernot, A., Féret, B., Dennefeld, C., Benoit, G., Davidson, I., Chambon, P., Mark, M., and Ghyselinck, N. B. (2012). Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. *Proceedings of the National Academy of Sciences* **109**, 16582-16587.

Reijntjes, S., Rodaway, A., and Maden, M. (2007). The retinoic acid metabolising gene, CYP26B1, patterns the cartilaginous cranial neural crest in zebrafish. *Int J Dev Biol* **51**, 351-60.

Rocha, A., Zanuy, S., Carrillo, M., and Gómez, A. (2009). Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. *Gen Comp Endocrinol* **162**, 265-275.

Rocha, A., Zanuy, S., and Gómez, A. (2016). Conserved anti-Müllerian hormone: anti-Müllerian hormone type-2 receptor specific interaction and intracellular signaling in Teleosts. Biol Reprod 94, 141, 1-13.

Rodríguez-Marí, A., Cañestro, C., BreMiller, R. A., Catchen, J. M., Yan, Y.-L., and Postlethwait, J. H. (2013). Retinoic Acid Metabolic Genes, Meiosis, and Gonadal Sex Differentiation in Zebrafish. *PLoS One* **8**, e73951.

Rodríguez, L., Begtashi, I., Zanuy, S., Shaw, M., and Carrillo, M. (2001). Changes in plasma levels of reproductive hormones during first sexual maturation in European male sea bass (Dicentrarchus labrax L.) under artificial day lengths. *Aquaculture* **202**, 235-248.

Rodríguez, R., Felip, A., Zanuy, S., and Carrillo, M. (2019). Advanced puberty triggered by bi-weekly changes in reproductive factors during the photolabile period in a male teleost fish, Dicentrarchus labrax L. *Gen Comp Endocrinol* **275**, 82-93.

Rolland, A. D., Lareyre, J. J., Goupil, A. S., Montfort, J., Ricordel, M. J., Esquerre, D., Hugot, K., Houlgatte, R., Chalmel, F., and Le Gac, F. (2009). Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. *BMC Genomics* **10**, 546.

Schatz, S. M., Kerschbaumer, R. J., Gerstenbauer, G., Kral, M., Dorner, F., and Scheiflinger, F. (2003). Higher expression of Fab antibody fragments in a CHO cell line at reduced temperature. *Biotechnology and bioengineering* **84**, 433-438.

Schulz, R. W., de Franca, L. R., Lareyre, J. J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R. H., and Miura, T. (2010). Spermatogenesis in fish. *Gen Comp Endocrinol* **165**, 390-411.

Silva, C., Wood, J. R., Salvador, L., Zhang, Z., Kostetskii, I., Williams, C. J., and Strauss, J. F. (2009). Expression profile of male germ cell-associated genes in mouse embryonic stem cell cultures treated with all-trans retinoic acid and testosterone. *Mol Reprod Dev* **76**, 11-21.

Skaar, K. S., Nobrega, R. H., Magaraki, A., Olsen, L. C., Schulz, R. W., and Male, R. (2011). Proteolytically activated, recombinant Anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology* **152**, 3527-3540.

Smith, C., Roeszler, K., Bowles, J., Koopman, P., and Sinclair, A. (2008). Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. *BMC Developmental Biology* **8**, 85.

Tan, H., Wang, J.-J., Cheng, S.-F., Ge, W., Sun, Y.-C., Sun, X.-F., Sun, R., Li, L., Li, B., and Shen, W. (2016). Retinoic acid promotes the proliferation of primordial germ cell–like cells differentiated from mouse skin-derived stem cells in vitro. *Theriogenology* **85**, 408-418.

Teletin, M., Vernet, N., Ghyselinck, N. B., and Mark, M. (2017). Roles of Retinoic Acid in Germ Cell Differentiation. Current Topics in Developmental Biology.

Thatcher, J. E., and Isoherranen, N. (2009). The role of CYP26 enzymes in retinoic acid clearance. *Expert Opin Drug Metab Toxicol* **5**, 875-86.

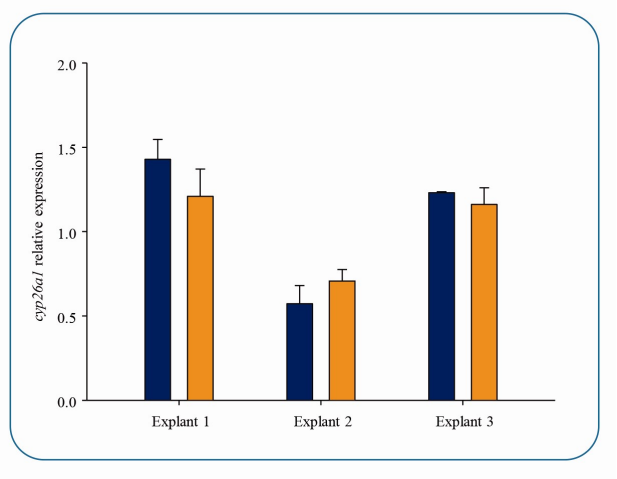
Wallacides, A., Chesnel, A., Chardard, D., Flament, S., and Dumond, H. (2009). Evidence for a conserved role of retinoic acid in urodele amphibian meiosis onset. *Dev Dyn* **238**, 1389-98.

Yaron, Z., and Levavi-Sivan, B. (2011). Endocrine regulation of fish reproduction. *Encyclopedia of fish physiology: from genome to environment* **2**, 1500-1508.

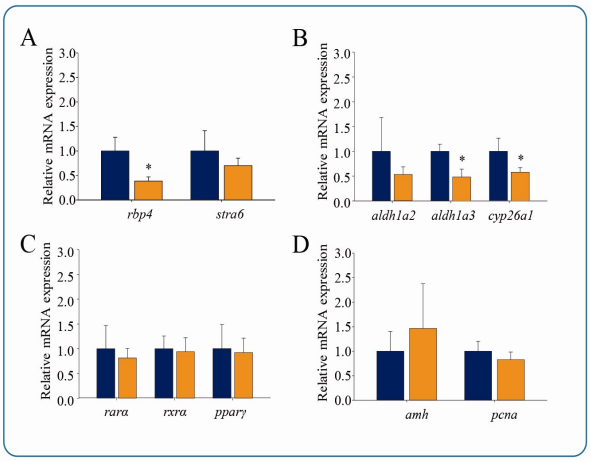
Zhang, Y., Wang, Y., Zuo, Q., Wang, X., Li, D., Tang, B., and Li, B. (2016). Selection of the Inducer for the Differentiation of Chicken Embryonic Stem Cells into Male Germ Cells In Vitro. *PLoS One* **11**, e0164664.

Ziouzenkova, O., and Plutzky, J. (2008). Retinoid metabolism and nuclear receptor responses: New insights into coordinated regulation of the PPAR-RXR complex. *FEBS Lett* **582**, 32-8.

CHAPTER 4 ADDITIONAL FIGURES



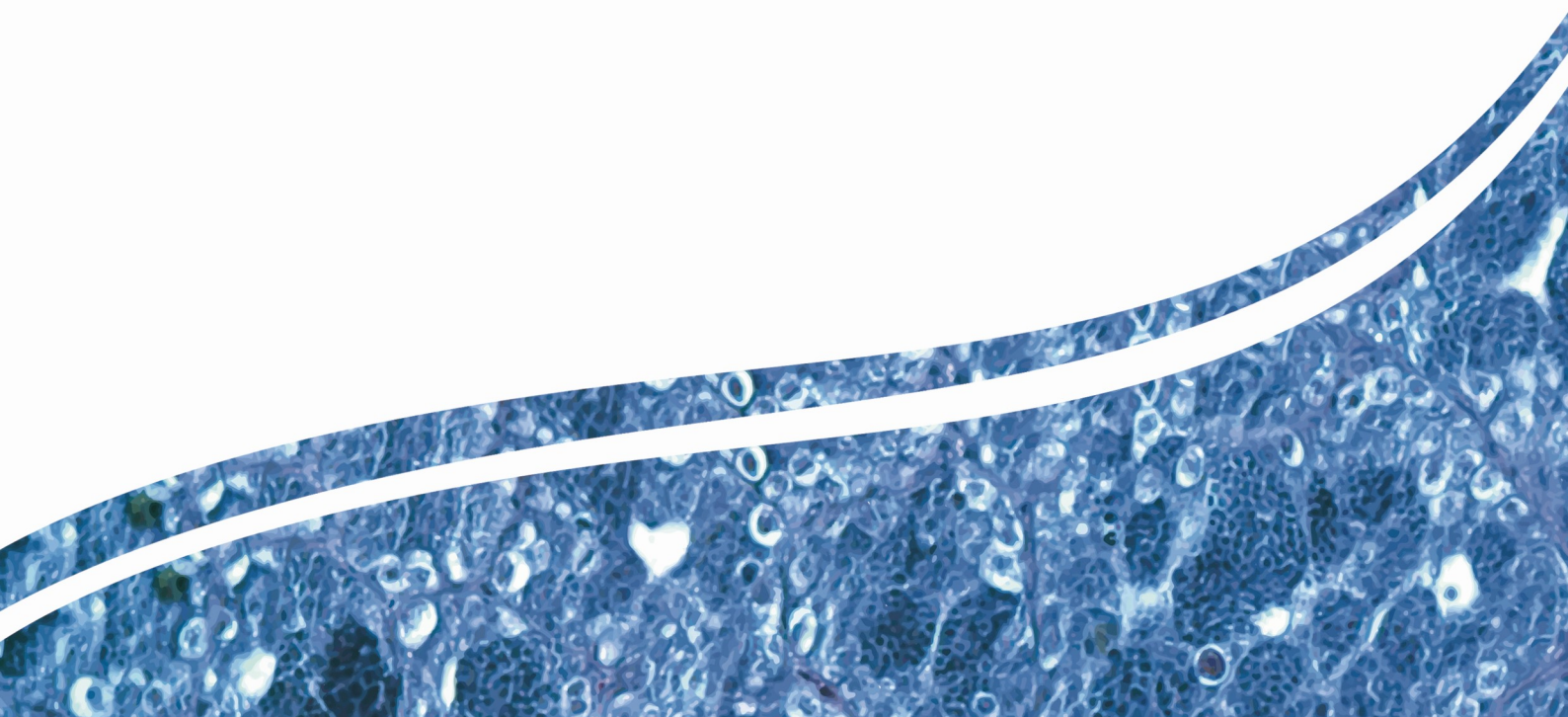
Additional Figure 1. Effects of DMSO (1µl/ml) in the expression of *cyp26a1* in testicular explants of prepubertal European sea bass at immature differentiated stage. Each explant corresponds to a single fish; one gonad was cultured in absence of DMSO (blue bars as control explant) and the other one in presence of DMSO (orange bars as treated explant). Bars represent the mean value (n=3) and error bar represents standard error of the mean (SEM). No significant differences were found between both groups after a Student t-test.



Additional Figure 2. Pilot study of the effects of 11KT (100nM) in the expression of several genes involved in the RA signaling pathway in testicular explants of prepubertal European sea bass at immature differentiated stage. A) ROL transport genes, B) RA synthesis and degradation genes, C) Nuclear receptor genes, D) Reproduction genes. The different experimental groups were represented as follows: blue bars (control group; n=3), and orange bars (11KT treated group; n=3). Check legend from figure 4 for further details. Expression values in the control group were arbitrarily set at 1 and those in the treated group were expressed as a proportion of the mean value in the *control* group (average + SEM). Asterisks indicate significant differences with the control group after a Student-T test (p<0.05).

GENERAL DISCUSSION.

In the following discussion we interpret and explain the main results from this thesis, we justify our approach in each experiment and critically evaluate our findings.
Infographs are included within each chapter highlighting the findings.



Acording to a recent study undertaken by the European Parliament, aquaculture production in Europe is foreseeing to double by 2030 (FAO 2018). One of the main challenges facing the aquaculture industry is to help in the enormous task of feeding nine billion people by 2050. The study identifies major challenges on the long-term economic and ecologic impact that include: a) the reduction of potential interactions with wild fish stocks as a consequence of escapees, avoiding the negative effect that the genetic impact of escaped specimens may have on wild populations, and b) the need for selective and improved breeding to increase performance of the livestock. In this frame, the contribution of fish reproductive physiology is central for several aspects such as finding appropriate gene/pathway targets to solve problems of reproductive performance in captivity, a fact that hampers the successful progression of selection programs in a number of species.

The success in the production of a species relies, among other aspects, in the control of the reproduction. The basic knowledge of biological pathways such as spermatogenesis and oogenesis is crucial to develop new reproductive strategies destined to solve broodstock fertility problems such as precocious puberty (Carrillo, 2009). Indeed, an important challenge in aquaculture is the control of puberty, in particular its prevention or delay, as in captivity fish attain their first sexual maturation at an earlier time (precocious puberty). This trait is considered undesirable in many commercially farmed species, including the European sea bass, since it brings about reduced fish welfare with a lower resistance to infectious diseases, and adversely affects growth, feed utilisation and health, resulting in smaller specimens by the time of marketing, being necessary the development of protocols to delay or even inhibit this process (Taranger et al., 2010; Carrillo et al 2015). A better understanding of the effect of precocious puberty in animal performance is key to improve basic and applied aspects in aquaculture (Felip et al., 2006; Okuzawa, 2002; Trippel, 2003; Weltzien et al., 2004). In this regard, the identification of early markers of precocious puberty reveal as a valuable tool for fish farmers in order to provide a predictive method to discriminate between prepubertal fish that will mature in a given reproductive season from those that will not mature, long before the first signs of gonad recrudescence. Such a marker would allow for the early separation of precocious males from the stocks reducing economic loses in the aquaculture industry (Carrillo et al., 2015).

At the start of this thesis, the early detection of precocious maturation in cultured European sea bass was a challenge. The onset of meiosis was assessed by histological gonad staging, being impossible to detect it during the early stages preceding the onset of puberty (Crespo et al., 2013a; Maugars and Schmitz, 2008). Since then, several molecular markers for

testis precocity were identified in this species both in males and in females. Among them, *foxl3* and ancient *foxl2* paralog, with a high expression in testis (Crespo et al., 2013b). Using hemigonadectomy in sea bass juvenile males, it was possible to isolate *ff1b* (also known as *sf1* or *nr5a1*) a gene coding for a transcription factor involved in the onset of meiosis from males exhibiting precocious puberty (Crespo et al., 2013a). Another study carried out in immature juvenile females, revealed, after transcriptomic analysis, that *ncoa7* increased significantly in ovaries of females that attained precocious puberty, before any sign of vitellogenesis, when compared to those that remained immature (Crespo et al., 2013c). The *ncoa7* gene encodes a co-factor that binds to nuclear receptors such as the estrogen receptor 1, THRbeta, PPARgamma and RARalpha increasing their transcriptional activity (Shao et al. 2002). Its differential expression reveals that it could control early events before vitellogenesis. Recent studies in the European sea bass focussed on the kisspeptin system, as it controls the cyclic activity of the reproductive axis, also contributing to the general knowledge of the onset of puberty in this species (Alvarado et al., 2013; Escobar et al., 2016; Escobar et al., 2013; Espigares et al., 2015).

The research project behind this thesis was the development of markers that could contribute to the understanding of the rationale that supports the onset of puberty in the European sea bass and that can explain the high ratio of precocious males found in intensive culture conditions. The contribution to this body of research was done by assessing the RA signaling pathway, known to be essential for the onset of meiosis in tetrapods, while searching for molecular markers that detect transcriptomic changes of sex differentiation and onset of meiosis.

High throughput transcriptional studies (Fig 1.)

One of the goals of this thesis was to characterize the genes involved in the onset of puberty in European sea bass males, constituting the first transcriptomic approach focused on meiosis in this species (Chapter 1). The initial and crucial point was to have the right biological material for the comparisons. Previous histological studies in this species gave us the key to focus on the transition from stage I (immature differentiated) to stage II (recrudescence). Spermatogonia A is the predominant cell type in stage I testis and does not go through meiosis divisions. However, the appearance of spermatogonia B and primary spermatocytes in stage II testis are

indicative of the early stages of meiosis (Begtashi et al., 2004). Since it is feasible to identify these cell types by histology we could restrict the onset of meiosis to the transition between these two developmental stages (Figure 2, Chapter 2). Besides the histological characterization, we also checked plasma levels of sex-steroids including 11KT and testosterone as the main androgens and estradiol as the main estrogen. Among them, only 11KT was able to discriminate between stage I and stage II males with higher levels in stage II (Chapter 1), suggesting the use of this hormone as a non-lethal marker for the onset of puberty in European sea bass males (Blázquez et al., 2017). This can be a reliable indicator to regulate stocks in sea bass farms in order to reduce production costs, by separating fish that will mature precociously from the rest of the population.

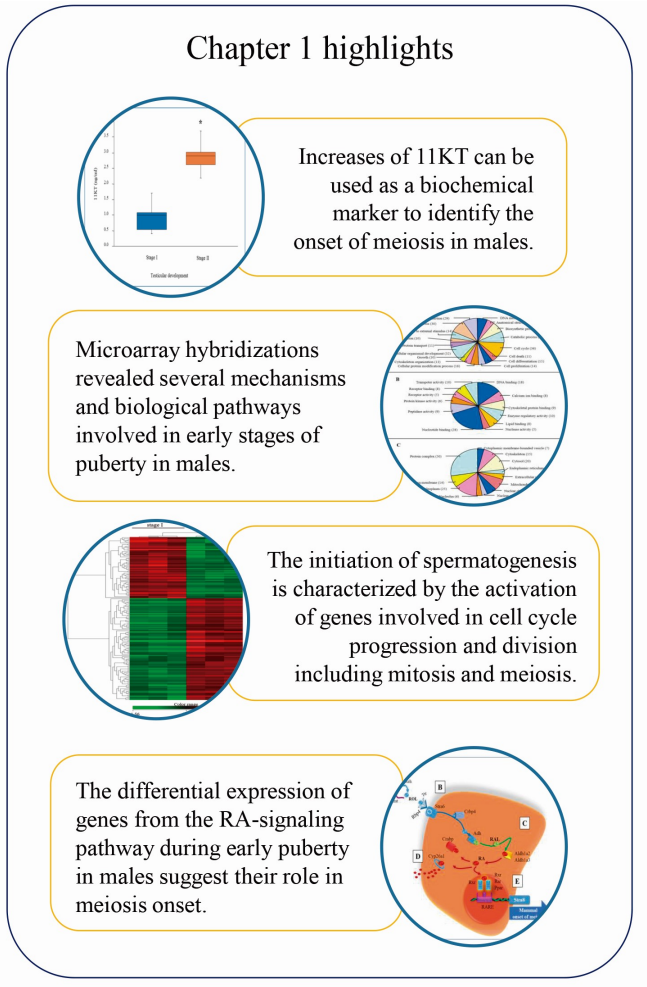


Figure 1: Infograph including the main results from Chapter 1

The microarray allowed for the identification of over 300 genes differentially expressed between both developmental stages, some of which could have potential applications for the study of reproduction. The onset of puberty was characterized by an upregulation of genes involved in cell proliferation, cell cycle and meiosis progression including genes coding for SAC proteins and SAC protein regulators (Kline-Smith et al., 2005), kinetochore-associated transcripts needed for SAC activity (Mendenhall and Hodge, 1998; Monje-Casas et al., 2007), transcripts coding for centromere proteins (Slegtenhorst-Eegdeman et al., 1995), genes coding for synaptotemal complex (Li and Schimenti, 2007) and genes from signaling pathways such as Wnt, MAPK, or TGFß (Josso et al., 2006). The upregulation of the above mentioned genes is indicative of an active period of mitosis, reflecting the need for a tight control of correct cell divisions, and

constitute an indicator for the progression of meiosis typical of this stage. We also found changes in genes involved in reproduction such as *amh* (Guiry et al., 2010; Rolland et al., 2009), *sgII* (Blázquez et al., 1998), and *aqp1* (Boj et al., 2015; Zilli et al., 2009), and others like *agrp2* that link early spermatogenesis to appetite and growth (Reinecke, 2009). Finally, it kept our attention the changes in genes involved in RA transport and degradation, e.g., *rbp4, crabp1* and *cyp26a1*, since RA was considered a key player in the onset of meiosis in tetrapods (Bowles et al., 2006; Bowles and Koopman, 2007; Smith et al., 2008; Wallacides et al., 2009). Indeed, for microarray validations we included genes coding for RA nuclear receptors, *rarα*, *rxrα*, and *pparγ*. The expression differences of these receptors between both developmental stages reinforced our initial hypothesis that the RA-signaling pathway was important during this period (Albalat, 2009).

To reveal the expression sites of some of the above mentioned RA-related genes, we performed an *in situ* hybridization study. Only testicular samples in stage II were used due to the availability of biological material by the time of the study. We chose *cyp26a1* due to the changes found during meiosis and because it is the end-point of the RA metabolic pathway (Ross and Zolfaghari, 2011). *cyp26a1* was localized close to the somatic cells between testicular lobules and also inside of them. Moreover, in zebrafish *cyp26a1* was found in Leydig cells, although its expression in germ cells could not be discarded (Rodríguez-Marí et al., 2013). We also chose *rxrα* since its expression was altered during meiosis, and because it functions as an obligated partner for *rarα* and *pparγ* (Bastien and Rochette-Egly, 2004; Chawla et al., 2001; Ziouzenkova and Plutzky, 2008). Although the signal was weak, *rxrα* expression was found in the germ cells as previously found in mouse (Kastner et al., 1996; Vernet et al., 2006).

In silico studies: sequencing, phylogeny and synteny (Fig.2)

Genes involved in the RA signaling pathway were selected to perform further in-depth studies due to its contrasted importance in the timing of meiosis in mammals (Bowles et al., 2006; Bowles and Koopman, 2007). The goal of Chapter 2 was to clone and sequence the main genes involved in RA metabolism (*aldh1a2, aldh1a3* and *cyp26a1*) and signaling (*stra8*), to describe their corresponding protein moiety, their phylogenetic origin and evolutionary history. In recent years, genome sequencing technologies have significantly increased the number of genes and proteins description (or prediction). The data generated by *in silico* studies have driven to a multidimensional analysis of many biological systems. Our *in silico* studies allowed to describe, for the first time in the European sea bass, the most important catalytic domains of key enzymes

in the RA signaling pathway. Furthermore, these data helped us to untangle the evolutionary history of these genes and proteins and answer whether *stra8* could be involved in the European sea bass onset of meiosis. The rationale behind the selection of these genes of the RA-signaling pathway was based on: *i) aldh1a2* and *aldh1a3* code for the enzymes involved in the direct synthesis of RA (Muzio et al., 2012); *ii) cyp26a1* controls RA concentration by metabolizing it into bio-inactive metabolites and is the end-point of the RA metabolic pathway (Ross and Zolfaghari, 2011); *iii) stra8* is required for pre meiotic DNA replication in tetrapods (Anderson et al., 2008; Baltus et al., 2006) and it could also have a role in teleosts (Dong et al., 2013). Our study confirmed the absence of *aldh1a1* in the European sea bass and support the current hypothesis that a common ancestor of tetrapods and teleost exhibited both *aldh1a2* and *aldh1a3* genes (Cañestro et al., 2009; Holmes, 2015). The cloned *aldhs* and their deduced proteins contained the three structural features of the Aldh family which give them a functional oxidative role; i) the Rossman fold motif, that gives the capability to use NAD or NADP as a cofactor (Lesk, 1995; Perozich et al., 1999); ii) the bridging domain (glutamic acid active site) essential for the catalytic reaction (Hempel et al., 2001); and iii) the catalytic domain (the cysteine active site) present in all ALDH family proteins (Perozich et al., 1999). The phylogenetic tree arrangement for Aldhs grouped Aldh1a2 and Aldh1a3 in two distinct branches, which in turn, grouped teleost sequences apart from tetrapod sequences. In agreement to current evidence in vertebrates (Cañestro et al., 2009; Holmes, 2015) and other fish (Pittlik et al., 2008), our results suggest a putative common ancestor to both subfamilies prior to the appearance of Aldh1a2. The synteny analysis confirms the current hypothesis that supports the *aldh1a* family originated during the

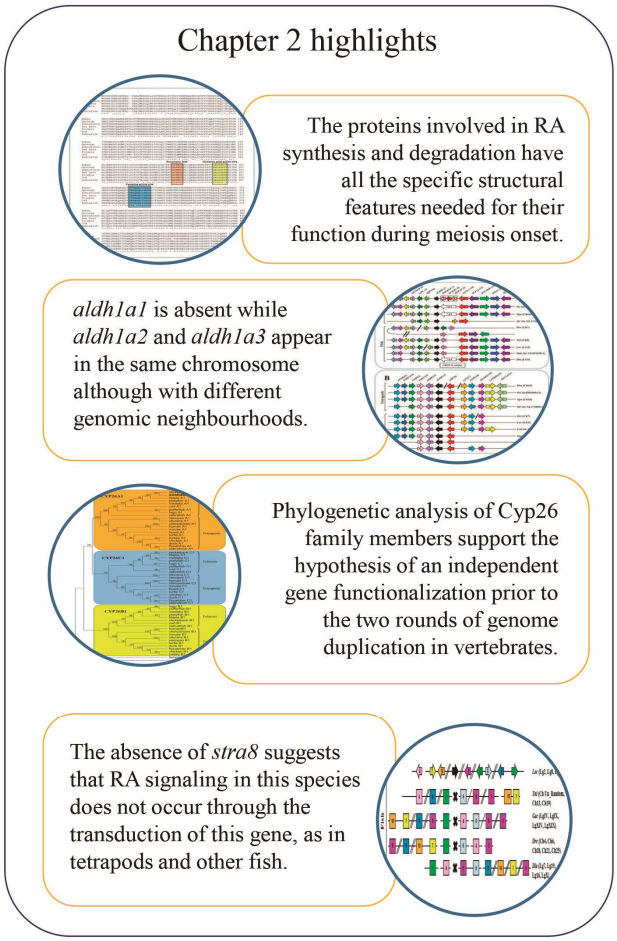


Figure 2: Infograph including the main results from Chapter 2

first whole genome duplication (WGD). The subsequent teleost genome duplication (TGD) retained *aldh1a2* and *aldh1a3* in the same chromosome, although the surrounding genomic arrangement diverged (Holmes, 2015).

The cloned *cyp26* gene and its deduced protein sequence showed the presence of the P450 signature that allows monoxigenase reactions to proceed (Meunier et al., 2004). The alignment of the deduced protein sequence with those from other vertebrates confirmed its identity as Cyp26a1. Notwithstanding, the slight differences in homology among teleosts, support their high conservation due to its pivotal role controlling RA levels (Rodríguez-Marí et al., 2013; Thatcher and Isoherranen, 2009). The phylogenetic tree shows the presence of a common ancestor, strengthening the hypothesis of an independent functionalization of its coding gene prior to the two rounds of genome duplication in vertebrates (Rodríguez-Marí et al., 2013). Furthermore, the synteny analysis confirmed that the conserved genomic region surrounding *cyp26a1* was scattered during the duplication of the teleost genome, suggesting that the *cyp26* family arose before the WGD in teleost fish (Catchen et al., 2009; Rodríguez-Marí et al., 2013).

The search for *stra8* or its conserved genome neighbourhood showed no significant hits in the European sea bass genome database. One could argue that this could be due low sequence similarity with other fish species or even to incomplete genome sequencing, rather than to the fact that it is not present in this species. However, the European sea bass genome is one of the highest quality genomes available for an aquaculture fish species. It has been sequenced to about 30x coverage with a combination of three different and independent technologies (Tine et al., 2014) and recently improved with the addition of RNAseq data (Chaves-Pozo et al., 2017). Therefore, it is very unlikely that the absence of *stra8* could be due to incomplete genome sequencing. Furthermore, many genes that belong to the conserved region surrounding tetrapod *STRA8*, in fish appear scattered in several regions of the genome, suggesting important evolutionary differences. Our results support the current evidence that a single *stra8* paralog was retained after the teleost-specific whole genome duplication (Hoegg et al., 2004; Taylor et al., 2003), suggesting that *stra8* was lost in Acanthomorpha, the largest group of teleosts where the European sea bass is included, and independently in the Cypriniform lineage due to a lineage-specific loss event (Pasquier et al., 2016).

In vivo gene expression studies (Fig. 3)

The RA synthesis and degradation machinery has all the structural features needed for their correct function during the onset of meiosis in European sea bass (Chapter 2). Nevertheless, in agreement with other fish species, we found that RA signaling does not occur through the canonical model accepted for tetrapods which states that *STRA8* is the gatekeeper of meiosis (Feng et al., 2014; Koubova et al., 2014; Koubova et al., 2006). At this stage, we set out to search for *in vivo* expression patterns of genes involved in the RA signaling pathway during gonad development and puberty in males and females (Chapter 3). This developmental interval included sex differentiation and meiosis onset in males, while in females it was extended up to the full ovarian maturation. Due to technical limitations, the direct measurement of RA levels in gonads was not feasible. Such measurement should differentiate all-trans and 9-cis RA to identify specific responses and includes the use of mass spectrometry, gas chromatography coupled to mass spectrometry or other high-throughput and expensive methods (Heaney et al., 2017). However, these techniques would only identify free RA, leaving bound-RA out of the total RA cell content. Alternatively, recent studies use gene expression levels as an indirect measurement of the amount of RA in the tissue (Bowles et al., 2009; Feng et al., 2015; Griswold et al., 2012; Rodríguez-Marí et al., 2013). Indeed, an upregulation of genes involved in RA synthesis -*aldhs*- together with a downregulation of genes involved in its degradation -*cyp26*- have been linked to an increase of RA levels (Adolfi et al., 2016). Moreover, even a small increase of *aldhs* could alter RA production inducing a biological response. In this regard, a study with testicular explants from *Aldh1a2*$^{-/-}$ knockout and *Aldh1a2*$^{-/-}$*/Aldh1a3*$^{-/-}$ double knockout mice showed an absence of RA in the developing ovary using a

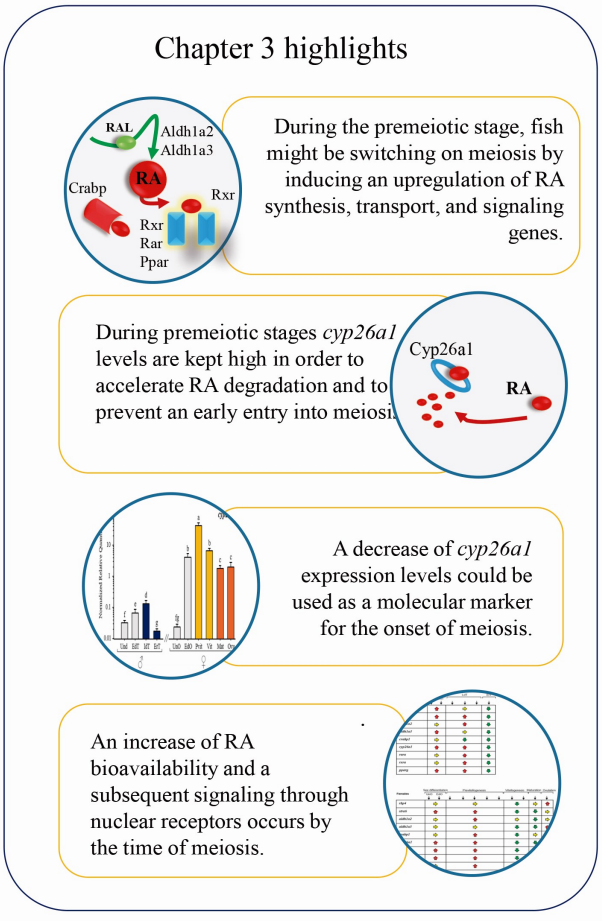


Figure 3: Infograph including the main results from Chapter 3

transgenic RARE-LacZ reporter mouse line (Kumar et al., 2011). Taken together all the previous evidences, we can safely assume that changes in RA content are driven by changes in *aldhs* and *cyp26a1* expression.

An enriched male group and an enriched female group were the origin of the biological material used for the *in vivo* and *in vitro* studies reported in Chapter 3 and Chapter 4, respectively. The fact that sex differentiation in European sea bass could be initially reprogramed by temperature (Navarro-Martín et al., 2009a) and sex steroids (Piferrer, 2001) allowed us to ensure that the fish used in the study will become males or females, regardless of their genetic component, and therefore minimize the number of animals sacrificed for the study. Indeed, this feature helped us to comply with the 3Rs principle since we could *a priori* know the future phenotypic sex of the animals before sex differentiation was completed. In fact, we found that only 30% of the animals present in the temperature-treated group were females, therefore an elevated number of animals would have been sacrificed in order to obtain all a statistically relevant number of females needed for the experiment in Chapter 3. It is important to keep in mind that the first full reproductive cycle in European sea bass females only occurs by the third year of age and that 11 samplings (from 150 dph to 1079 dph) were needed to cover it, as opposed to males for which the onset of the first meiosis occurs by the second year and thus only 6 samplings (from 150 dph to 600 dph) were done. For that reason, we included a short 27-day period treatment with E_2 during early stages of development (between 93 and 120 dph), knowing that this treatment renders females with no differences from untreated females in this species (Blázquez et al., 1998; Navarro-Martín et al., 2009b. Navarro-Martín et al., 2011; Díaz and Piferrer, 2015). Moreover, previous studies in fish focussed on the clearance kinetics of exogenously administered sex steroids demonstrated that they were eliminated from the body in a matter of days (reviewed in Piferrer, 2001), long before any of the samplings of the study took place.

Until now, the studies on the expression of different genes in European sea bass used non-normalized arbitrary input amounts and gene expression normalized to *18S* (only in males) separately to *ef1a* (only in females) (Alvarado et al., 2013; Rocha et al., 2009). The methods from Chapter 3 allowed for the comparison of gene expression levels between male and female samples and between different sampling points for the first time in this species. The algorithms for normalizing CT values into relative gene expression took into account each gene efficiency (Hellemans et al., 2007). Moreover, the reference genes (*18S* and *ef1a*) CT values were taken from all samples, including male and females, and used together in data normalization and data

inter run calibration, taking into account reference gene stability values and coefficient of variation (Hellemans et al., 2007). This method allowed to remove each sample specific non-biological variation and technical run to run variation between samples and represents an important achievement for gene expression comparison purposes.

The high *rbp4* and *stra6* expression levels found in late differentiated testis, are indicative for a need of ROL within the testicular environment by the onset of meiosis, time when their expression decreased again, probably due to the exhaustion of ROL reserves by their transformation into RAL and RA. During vitellogenesis progression, we found a significant decrease of *rbp4 and stra6* expression, coinciding with the natural increase in E_2 plasma levels in this species (Mañanós et al., 1997; Prat et al., 1996; Rocha et al., 2009). Since vitellogenin synthesis is regulated by E_2, it seems plausible that retinoid metabolism could also be mediated by the E_2 content. In tilapia, *cyp26a1* and *aldh1a2* are the main regulators of RA levels with high *aldh1a2* and low *cyp26a1* expression levels at the time of meiosis (Feng et al., 2015). We found a downregulation of *cyp26a1* in early recrudescent testis, coinciding with the onset of meiosis (Chapter 1 and Chapter 3) that was preceded by high expression of *aldhs* in late differentiated testis, prior to the onset of meiosis. Moreover, an increase of *aldhs* concomitant with a downregulation of *cyp26a1* was found during the transition between maturation and ovulation in females, time when meiosis resumption takes place, similar to what has been reported in zebrafish (Rodríguez-Marí et al., 2013) and medaka (Adolfi et al., 2016). Due to their function and putative role in the onset of meiosis, one might expect antagonistic expression dynamics between *aldhs* and *cyp26a1*, i.e., an increase of *aldhs* together with a decrease of *cyp26a1* and vice versa. Nevertheless, our findings suggest that the regulation of RA through its degradation is more potent that trough its synthesis. This was supported by previous studies in mutant mice showing that *Cyp26b1* regulated meiosis entry by reducing RA availability and not by RA synthesis through *Aldhs* (Kumar et al., 2011; Griswold et al., 2012). All these studies evidence a sex-specific regulation of RA levels at the time of meiosis in different fish models. Moreover, since *cyp26a1* represents an endpoint in RA metabolism, we suggest that its downregulation could be used as a suitable marker for the initiation of fish meiosis.

The highest expression levels of *rarα*, *rxrα* and *pparγ* occur right before the onset of meiosis in males, while in females, the increase during meiosis resumption is only true for *pparγ*. Moreover, the upregulation of *pparγ* concomitant to that of *aldh1a3* by the time of ovulation suggest a tight coordination between lipid homeostasis/energy metabolism and reproduction in European sea bass similar to what has been reported in reproductive tissues in mice (Froment et

al., 2006). Our data suggest that the high levels of RA synthesis (*aldh1a2*, *aldh1a3*) and of RA signaling (*rarα*, *rxrα, pparγ*) genes found prior to the onset or by the resumption of meiosis in males and females, respectively could be triggering the transcription of other RA-induced genes involved in gonad maturation.

To the best of our knowledge, this is the first thorough *in vivo*, long-term comparison of the expression of key players of the RA signaling pathway during the onset of meiosis in male and female fish. The study suggests the involvement of RA in the timing of male and female meiotic initiation, probably using a *stra8* independent signaling pathway (Koubova et al., 2014).

In vitro studies: testicular culture systems and gene expression (Fig. 4)

To support our findings from Chapter 3, we set out to demonstrate the functionality of the RA signaling pathway by its induction or inhibition in different meiosis scenarios using a controlled *in vitro* system. Experiments were performed only in testicular explants since the onset of meiosis in females is not restricted to a single histological stage. In this regard, meiosis in female fish has several cycles of arrest and resumption and only concludes at the time of fertilization (Lubzens et al., 2010 425), making unfeasible to simulate the *in vitro* conditions in our facilities.The experimental design (Chapter 4) was methodologically ambitious as it included several variables such as: *i)* stage of development, *ii)* age, *iii)* RA pathway agonist and antagonist treatments and, *iv)* two meiosis scenarios. Under these conditions, the study was aimed to assess the expression of eight genes related to the RA signaling pathway. Moreover, two different culture techniques were adapted to sea bass gonads and were specifically chosen for their effectiveness in other fish species, logistics and implementation in our experimental facilities. All these conditions added

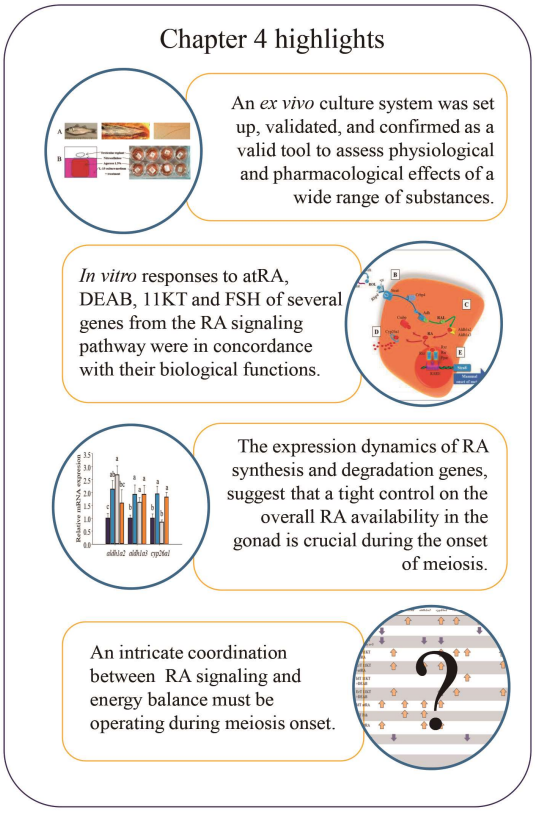


Figure 4: Infograph including the main results from Chapter 4

variability to the system and the conclusions but have the added value to constitute the first attempt for the study of meiosis using *in vitro* systems in this species. Treatments were "blind performed" without knowing *a priori* the gonad developmental stage that could only be confirmed after histological examination when treatments had already finished. It was then when the different samples were grouped according to their stage of development for further gene expression studies. Altogether, despite these technical difficulties, our results showed a response in the expression of several genes according to their function.

amh and *pcna* were used as markers to control the proper functioning of the *in vitro* systems, due to their respective functions in spermatogenesis progression and cell proliferation, respectively. We could not find changes in *amh* expression in the majority of the treatments assayed. However, the *in vivo* experiments reported a sharp decrease of *amh* during the transition between late differentiated and early recrudescent testis (Chapter 1 and Chapter 3). Moreover, treatments were able to modulate *pcna* expression, a gene widely used as a marker of cell proliferation (Korfsmeier, 2002) suggesting that changes in cell proliferation are indeed taking place in our *in vitro* system. It could then be that the lack of effect on *amh* is due to differences between the *in vivo* and the *in vitro* system or to the different *in vitro* culture techniques used in the study.

The functional response to atRA, alone or in combination with 11KT or with Fsh, was the upregulation of genes involved in ROL uptake (*rbp4* or *stra6*), revealing a tight control for ROL transport within the European sea bass testis by the time of meiosis and reinforcing the results from Chapter 3. Moreover, we found that the upregulation of *aldh1a2, aldh1a3* (RA synthesis) and *cyp26a1* (RA degradation) respond to atRA, regardless of the meiosis scenario considered. This supports the hypothesis which states that the regulation of RA levels through its degradation is far more potent that trough its synthesis. Such regulation has been described in tilapia (Feng et al., 2015) and zebrafish (Rodríguez-Marí et al., 2013). Moreover, increases in RA levels, either *in vivo* (Chapters 1 and 3) or *in vitro* (Chapter 4) are able to regulate *cyp26a1* expression. Therefore, we can conclude that *cyp26a1* can be used as a reliable molecular marker for the onset of meiosis in European sea bass males, also reinforcing the results from Chapter 1.

At least one of the mechanisms by which retinoids carry out their functions is by activating or repressing specific genes via the action of the RAR/RXR nuclear receptors (Fields et al., 2007; Ziouzenkova and Plutzky, 2008). Based on their function, we expected an upregulation of *rarα* as a response to atRA administration (Mark et al., 1999), or even similar expression patterns of *rarα, rxrα* and *pparγ*, since they function as heterodimers (Ziouzenkova

and Plutzky, 2008). However, and although we could not find such pattern, we noticed a consistent interaction between *rbp4*, *stra6* and *rarα* in response to atRA administration. Studies in obese mice show that ROL can be delivered from hepatic stores to adipocyte precursors cells via RBP4 and STRA6, thus increasing the amount of RAR activity and adipocyte differentiation (Muenzner et al., 2013). In addition, Aldh inhibits RXR and PPARc induced adipogenesis (Ziouzenkova and Plutzky, 2008) in mice. Taken together, we suggest that an intricate coordination between RA signaling and energy balance must be operating during the onset of meiosis, something already suggested in Chapter 1. Nevertheless, more studies are needed to demonstrate this hypothesis.

This thesis brings about new knowledge of the role of the RA signaling pathway during fish meiosis and opens new questions about its influence in puberty. Furthermore, biochemical and molecular tools were proposed as suitable markers of the onset of meiosis, such as 11KT and *cyp26a1*, although further experiments are needed to test these markers in early events of puberty.

REFFERENCE LIST

Adolfi, M. C., Herpin, A., Regensburger, M., Sacquegno, J., Waxman, J. S., and Schartl, M. (2016). Retinoic acid and meiosis induction in adult versus embryonic gonads of medaka. Scientific *reports* **6**, 34281.

Albalat, R. (2009). The retinoic acid machinery in invertebrates: ancestral elements and vertebrate innovations. *Mol Cell Endocrinol* **313**, 23-35.

Alvarado, M. V., Carrillo, M., and Felip, A. (2013). Expression of kisspeptins and their Receptors, gnrh-1/gnrhr-II-1a and gonadotropin genes in the brain of adult male and female European sea bass during different gonadal stages. *Gen Comp Endocrinol* **187**, 104-116.

Anderson, E. L., Baltus, A. E., Roepers-Gajadien, H. L., Hassold, T. J., de Rooij, D. G., van Pelt, A. M., and Page, D. C. (2008). Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci U S A* **105**, 14976-80.

Baltus, A. E., Menke, D. B., Hu, Y.-C., Goodheart, M. L., Carpenter, A. E., de Rooij, D. G., and Page, D. C. (2006). In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet* **38**, 1430-1434.

Bastien, J., and Rochette-Egly, C. (2004). Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**, 1-16.

Begtashi, I., Rodríguez, L., Moles, G., Zanuy, S., and Carrillo, M. (2004). Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (Dicentrarchus labrax, L.). I. Morphological aspects. *Aquaculture* **241**, 539-559.

Blázquez, M., Medina, P., Crespo, B., Gómez, A., and Zanuy, S. (2017). Identification of conserved genes triggering puberty in European sea bass males (Dicentrarchus labrax) by microarray expression profiling. *BMC Genomics* **18**, 441.

Boj, M., Chauvigné, F., Zapater, C., and Cerdà, J. (2015). Gonadotropin-Activated Androgen-Dependent and Independent Pathways Regulate Aquaporin Expression during Teleost (Sparus aurata) Spermatogenesis. *PLoS One* **10**, e0142512.

Bowles, J., Feng, C.-W., Knight, D., Smith, C. A., Roeszler, K. N., Bagheri-Fam, S., Harley, V. R., Sinclair, A. H., and Koopman, P. (2009). Male-specific expression of Aldh1a1 in mouse and chicken fetal testes: Implications for retinoid balance in gonad development. *Developmental Dynamics* **238**, 2073-2080.

Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M., and Rossant, J. (2006). Retinoid signaling determines germ cell fate in mice. *Science* **312**, 596 - 600.

Bowles, J., and Koopman, P. (2007). Retinoic acid, meiosis and germ cell fate in mammals. *Development* **134**, 3401-11.

Cañestro, C., Catchen, J. M., Rodriguez-Mari, A., Yokoi, H., and Postlethwait, J. H. (2009). Consequences of lineage-specific gene loss on functional evolution of surviving paralogs: ALDH1A and retinoic acid signaling in vertebrate genomes. *PLoS Genet* **5**, e1000496.

Carrillo, M. (2009). La Reproducción de los peces: aspectos básicos y sus aplicaciones en acuicultura. *Cerebro y reproducción en peces. Bases Neurales y Neuroendocrinas. Ed. fundación Observatorio español para la Acuicultura, Madrid*.

Carrillo, M., Espigares, F., Felip, A., Escobar, S., Molés, G., Rodríguez, R., Alvarado, M. V., Gómez, A., and Zanuy, S. (2015). Updating control of puberty in male European sea bass: A holistic approach. *Gen Comp Endocrinol* **221**, 42-53.

Catchen, J. M., Conery, J. S., and Postlethwait, J. H. (2009). Automated identification of conserved synteny after whole-genome duplication. *Genome Research*.

Crespo, B., Gómez, A., Mazón, M. J., Carrillo, M., and Zanuy, S. (2013a). Isolation and characterization of Ff1 and Gsdf family genes in European sea bass and identification of early gonadal markers of precocious puberty in males. *Gen Comp Endocrinol* **191**, 155-167.

Crespo, B., Lan-Chow-Wing, O., Rocha, A., Zanuy, S., and Gómez, A. (2013b). foxl2 and foxl3 are two ancient paralogs that remain fully functional in teleosts. *Gen Comp Endocrinol* **194**, 81-93.

Crespo, B., Gómez, A., Blázquez, M., Carrillo, M., Zanuy, S. (2013c). Early events of puberty in female European sea bass. 17th ICCE Barcelona, July 15-19. P-037.

Chaves-Pozo, E., Valero, Y., Esteve-Codina, A., Gómez-Garrido, J., Dabad, M., Alioto, T., Meseguer, J., Esteban, M. Á., and Cuesta, A. (2017). Innate Cell-Mediated Cytotoxic Activity of European Sea Bass Leucocytes Against Nodavirus-Infected Cells: A Functional and RNA-seq Study. *Scientific reports* **7**, 15396.

Chawla, A., Repa, J. J., Evans, R. M., and Mangelsdorf, D. J. (2001). Nuclear Receptors and Lipid Physiology: Opening the X-Files. *Science* **294**, 1866-1870.

Dong, R., Yang, S., Jiao, J., Wang, T., Shi, H., Zhou, L., Zhang, Y., and Wang, D. (2013). Characterization of Stra8 in Southern catfish (Silurus meridionalis): evidence for its role in meiotic initiation. *BMC Molecular Biology* **14**, 11.

Escobar, S., Felip, A., Zanuy, S., and Carrillo, M. (2016). Is the kisspeptin system involved in responses to food restriction in order to preserve reproduction in pubertal male sea bass (Dicentrarchus labrax)? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **199**, 38-46.

Escobar, S., Servili, A., Espigares, F., Gueguen, M.-M., Brocal, I., Felip, A., Gómez, A., Carrillo, M., Zanuy, S., and Kah, O. (2013). Expression of kisspeptins and kiss receptors suggests a large range of functions for kisspeptin systems in the brain of the European sea bass. *PLoS One* **8**, e70177.

Espigares, F., Rocha, A., Molés, G., Gómez, A., Carrillo, M., and Zanuy, S. (2015). New insights into the factors mediating the onset of puberty in sea bass. *Gen Comp Endocrinol* **224**, 176-185.

Felip, A., Zanuy, S., and Carrillo, M. (2006). Comparative analysis of growth performance and sperm motility between precocious and non-precocious males in the European sea bass (Dicentrarchus labrax, L.). *Aquaculture* **256**, 570-578.

Feng, C.-W., Bowles, J., and Koopman, P. (2014). Control of mammalian germ cell entry into meiosis. *Mol Cell Endocrinol* **382**, 488-497.

Feng, R., Fang, L., Cheng, Y., He, X., Jiang, W., Dong, R., Shi, H., Jiang, D., Sun, L., and Wang, D. (2015). Retinoic acid homeostasis through aldh1a2 and cyp26a1 mediates meiotic entry in Nile tilapia (Oreochromis niloticus). *Scientific reports* **5**, 10131.

Fields, A. L., Soprano, D. R., and Soprano, K. J. (2007). Retinoids in biological control and cancer. *Journal of cellular biochemistry* **102**, 886-898.

Froment, P., Gizard, F., Defever, D., Staels, B., Dupont, J., and Monget, P. (2006). Peroxisome proliferator-activated receptors in reproductive tissues: from gametogenesis to parturition. *Journal of Endocrinology* **189**, 199-209.

Griswold, M. D., Hogarth, C. A., Bowles, J., and Koopman, P. (2012). Initiating meiosis: the case for retinoic acid. *Biol Reprod* **86**, 35.

Guiry, A., Flynn, D., Hubert, S., O'Keeffe, A. M., LeProvost, O., White, S. L., Forde, P. F., Davoren, P., Houeix, B., and Smith, T. J. (2010). Testes and brain gene expression in precocious male and adult maturing Atlantic salmon (Salmo salar). *BMC Genomics* **11**, 211.

Heaney, L. M., Jones, D. J., and Suzuki, T. (2017). Mass spectrometry in medicine: a technology for the future? *Future science OA* **3**, FSO213-FSO213.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology* **8**, R19.

Hempel, J., Kuo, I., Perozich, J., Wang, B.-C., Lindahl, R., and Nicholas, H. (2001). Aldehyde dehydrogenase. *European Journal of Biochemistry* **268**, 722-726.

Hoegg, S., Brinkmann, H., Taylor, J., and Meyer, A. (2004). Phylogenetic Timing of the Fish-Specific Genome Duplication Correlates with the Diversification of Teleost Fish. *Journal of Molecular Evolution* **59**, 190-203.

Holmes, R. S. (2015). Comparative and evolutionary studies of vertebrate ALDH1A-like genes and proteins. *Chemico-Biological Interactions* **234**, 4-11.

Josso, N., Picard, J. Y., Rey, R., and di Clemente, N. (2006). Testicular anti-Mullerian hormone: history, genetics, regulation and clinical applications. *Pediatric endocrinology reviews: PER* **3**, 347-358.

Kastner, P., Mark, M., Leid, M., Gansmuller, A., Chin, W., Grondona, J. M., Decimo, D., Krezel, W., Dierich, A., and Chambon, P. (1996). Abnormal spermatogenesis in RXR beta mutant mice. *Genes & development* **10**, 80-92.

Kline-Smith, S. L., Sandall, S., and Desai, A. (2005). Kinetochore–spindle microtubule interactions during mitosis. *Current opinion in cell biology* **17**, 35-46.

Korfsmeier, K. H. (2002). PCNA in the ovary of zebrafish (Brachydanio rerio, Ham.-Buch.). *Acta Histochem* **104**, 73-6.

Koubova, J., Hu, Y.-C., Bhattacharyya, T., Soh, Y. Q. S., Gill, M. E., Goodheart, M. L., Hogarth, C. A., Griswold, M. D., and Page, D. C. (2014). Retinoic Acid Activates Two Pathways Required for Meiosis in Mice. *PLoS Genet* **10**, e1004541.

Koubova, J., Menke, D. B., Zhou, Q., Capel, B., Griswold, M. D., and Page, D. C. (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci U S A* **103**, 2474-9.

Kumar, S., Chatzi, C., Brade, T., Cunningham, T. J., Zhao, X., and Duester, G. (2011). Sex-specific timing of meiotic initiation is regulated by Cyp26b1 independent of retinoic acid signaling. *Nature communications* **2**, 151.

Lesk, A. M. (1995). NAD-binding domains of dehydrogenases. *Current Opinion in Structural Biology* **5**, 775-783.

Li, X., and Schimenti, J. C. (2007). Mouse pachytene checkpoint 2 (trip13) is required for completing meiotic recombination but not synapsis. *PLoS Genet* **3**, e130.

Lubzens, E., Young, G., Bobe, J., and Cerdà, J. (2010). Oogenesis in teleosts: How fish eggs are formed. Gen Comp Endocrinol 165, 367-389.

Mañanós, E. L., Zanuy, S., and Carrillo, M. (1997). Photoperiodic manipulations of the reproductive cycle of sea bass (Dicentrarchus labrax) and their effects on gonadal development, and plasma 17ß-estradiol and vitellogenin levels. *Fish Physiol Biochem* **16**, 211-222.

Mark, M., Ghyselinck, N. B., Wendling, O., Dupé, V., Mascrez, B., Kastner, P., and Chambon, P. (1999). A genetic dissection of the retinoid signaling pathway in the mouse. *Proceedings of the Nutrition Society* **58**, 609-613.

Maugars, G., and Schmitz, M. (2008). Gene expression profiling during spermatogenesis in early maturing male Atlantic salmon parr testes. *Gen Comp Endocrinol* **159**, 178-87.

Medina, P., Gómez, A., Zanuy, S., and Blázquez, M. (2019). Involvement of the retinoic acid signaling pathway in sex differentiation and pubertal development in the European sea bass Dicentrarchus labrax. *Heliyon* **5**, e01201-e01201.

Mendenhall, M. D., and Hodge, A. E. (1998). Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews* **62**, 1191-1243.

Meunier, B., de Visser, S. P., and Shaik, S. (2004). Mechanism of Oxidation Reactions Catalyzed by Cytochrome P450 Enzymes. *Chemical Reviews* **104**, 3947-3980.

Monje-Casas, F., Prabhu, V. R., Lee, B. H., Boselli, M., and Amon, A. (2007). Kinetochore orientation during meiosis is controlled by Aurora B and the monopolin complex. *Cell* **128**, 477-490.

Muenzner, M., Tuvia, N., Deutschmann, C., Witte, N., Tolkachov, A., Valai, A., Henze, A., Sander, L. E., Raila, J., and Schupp, M. (2013). Retinol-Binding Protein 4 and Its Membrane Receptor STRA6 Control Adipogenesis by Regulating Cellular Retinoid Homeostasis and Retinoic Acid Receptor α Activity. *Molecular and Cellular Biology* **33**, 4068-4082.

Muzio, G., Maggiora, M., Paiuzzi, E., Oraldi, M., and Canuto, R. A. (2012). Aldehyde dehydrogenases and cell proliferation. *Free Radic Biol Med* **52**, 735-46.

Navarro-Martín, L., Blázquez, M., Viñas, J., Joly, S., and Piferrer, F. (2009a). Balancing the effects of rearing at low temperature during early development on sex ratios, growth and maturation in the European sea bass (Dicentrarchus labrax).: Limitations and opportunities for the production of highly female-biased stocks. *Aquaculture* **296**, 347-358.

Navarro-Martin, L., Blazquez, M., and Piferrer, F. (2009b). Masculinization of the European sea bass (Dicentrarchus labrax) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. *Gen Comp Endocrinol* **160**, 3-11.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., and Piferrer, F. (2011). DNA Methylation of the Gonadal Aromatase (cyp19a) Promoter Is Involved in Temperature-Dependent Sex Ratio Shifts in the European Sea Bass. *PLoS Genet* **7**, e1002447.

Okuzawa, K. (2002). Puberty in teleosts. *Fish Physiol Biochem* **26**, 31-41.

Pasquier, J., Cabau, C., Nguyen, T., Jouanno, E., Severac, D., Braasch, I., Journot, L., Pontarotti, P., Klopp, C., and Postlethwait, J. H. (2016). Gene evolution and gene expression after whole genome duplication in fish: the PhyloFish database. *BMC Genomics* **17**, 1.

Perozich, J., Nicholas, H., Wang, B.-C., Lindahl, R., and Hempel, J. (1999). Relationships within the aldehyde dehydrogenase extended family. *Protein Science* **8**, 137-146.

Piferrer, F. (2001). Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture* **197**, 229-281.

Pittlik, S., Domingues, S., Meyer, A., and Begemann, G. (2008). Expression of zebrafish aldh1a3 (raldh3) and absence of aldh1a1 in teleosts. *Gene Expr Patterns* **8**, 141-7.

Prat, F., Sumpter, J. P., and Tyler, C. R. (1996). Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductivecycle in male and female rainbow trout (Oncorhynchus mykiss). *Biol Reprod* **54**, 1375-1382.

Reinecke, M. (2009). Insulin-like growth factors and fish reproduction. *Biol Reprod* **82**, 656-661.

Rocha, A., Zanuy, S., Carrillo, M., and Gómez, A. (2009). Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. *Gen Comp Endocrinol* **162**, 265-275.

Rodríguez-Marí, A., Cañestro, C., BreMiller, R. A., Catchen, J. M., Yan, Y.-L., and Postlethwait, J. H. (2013). Retinoic Acid Metabolic Genes, Meiosis, and Gonadal Sex Differentiation in Zebrafish. *PLoS One* **8**, e73951.

Rolland, A. D., Lareyre, J. J., Goupil, A. S., Montfort, J., Ricordel, M. J., Esquerre, D., Hugot, K., Houlgatte, R., Chalmel, F., and Le Gac, F. (2009). Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. *BMC Genomics* **10**, 546.

Ross, A. C., and Zolfaghari, R. (2011). Cytochrome P450s in the regulation of cellular retinoic acid metabolism. *Annu Rev Nutr* **31**, 65-87.

Shao, W., Halachmi, S., & Brown, M. (2002). ERAP140, a conserved tissue-specific nuclear receptor coactivator. Molecular and cellular biology, 22(10), 3358-3372.

Slegtenhorst-Eegdeman, K. E., Post, M., Baarends, W. M., Themmen, A. P. N., and Grootegoed, J. A. (1995). Regulation of gene expression in Sertoli cells by follicle-stimulating hormone (FSH): cloning and characterization of LRPR1, a primary response gene encoding a leucine-rich protein. Mol Cell Endocrinol 108, 115-124.

Smith, C., Roeszler, K., Bowles, J., Koopman, P., and Sinclair, A. (2008). Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. BMC Developmen*tal Biology* **8**, 85.

Taylor, J. S., Braasch, I., Frickey, T., Meyer, A., and Van de Peer, Y. (2003). Genome duplication, a trait shared by 22,000 species of ray-finned fish. *Genome Research* **13**, 382-390.

Thatcher, J. E., and Isoherranen, N. (2009). The role of CYP26 enzymes in retinoic acid clearance. *Expert Opin Drug Metab Toxicol* **5**, 875-86.

Tine, M., Kuhl, H., Gagnaire, P.-A., Louro, B., Desmarais, E., Martins, R. S. T., Hecht, J., Knaust, F., Belkhir, K., and Klages, S. (2014). European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nature communications* **5**.

Trippel, E. A. (2003). Estimation of male reproductive success of marine fishes. *Journal of Northwest Atlantic Fishery Science* **33**, 81-113.

Vernet, N., Dennefeld, C., Rochette-Egly, C., Oulad-Abdelghani, M., Chambon, P., Ghyselinck, N. B., and Mark, M. (2006). Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology* **147**, 96-110.

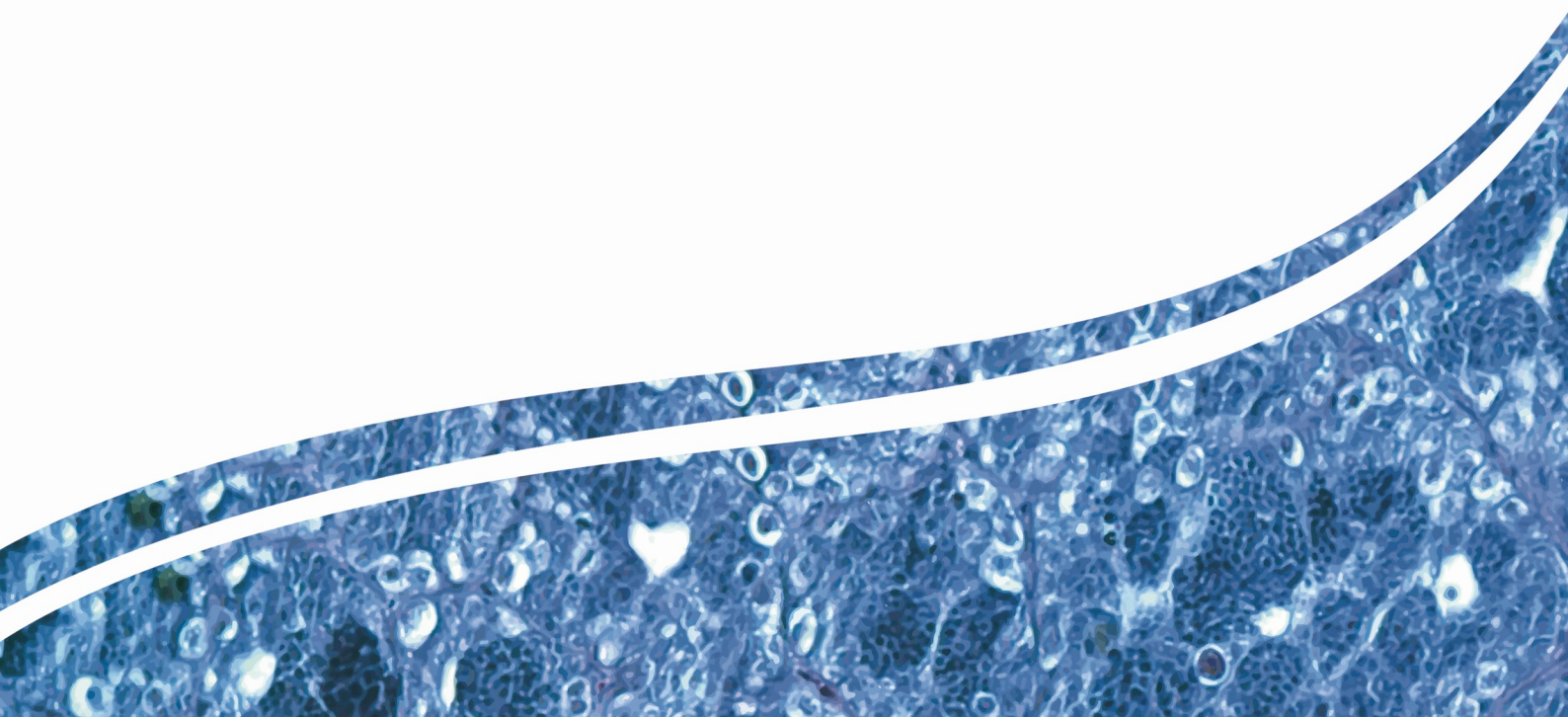
Wallacides, A., Chesnel, A., Chardard, D., Flament, S., and Dumond, H. (2009). Evidence for a conserved role of retinoic acid in urodele amphibian meiosis onset. *Dev Dyn* **238**, 1389-98.

Weltzien, F. A., Andersson, E., Andersen, O., Shalchian-Tabrizi, K., and Norberg, B. (2004). The brain-pituitary-gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). *Comp Biochem Physiol A Mol Integr Physiol* **137**, 447-77.

Zilli, L., Schiavone, R., Chauvigne, F., Cerda, J., Storelli, C., and Vilella, S. (2009). Evidence for the involvement of aquaporins in sperm motility activation of the teleost gilthead sea bream (Sparus aurata). *Biol Reprod* **81**, 880-8.

Ziouzenkova, O., and Plutzky, J. (2008). Retinoid metabolism and nuclear receptor responses: New insights into coordinated regulation of the PPAR-RXR complex. *FEBS Lett* **582**, 32-8.

General conclusions.



The general conclusions from the present PhD thesis are presented following the different Chapters in which the study has been divided and they are listed as follows:

CHAPTER 1

1.- Increases in androgen plasma levels, particularly 11KT, mark the transition between testicular stage I and stage II and can be used as a non-lethal marker for the onset of meiosis in the European sea bass.

2.- The study constitutes the first high throughput transcriptomic approach using microarray technologies focussed on meiosis in the European sea bass. The microarray was validated and the annotation of different genes was improved, increasing the knowledge of several mechanisms and biological pathways involved in early stages of puberty in this species.

3.- The differential expression of genes involved in cell cycle progression and division, and of several components of the RA signaling pathway during the onset of meiosis suggests their important role in early pubertal development.

CHAPTER 2

4.- The evolutionary relationships of the Aldh family confirm the absence of *aldh1a1* in the European sea bass and add evidence to the current hypothesis supporting that the Aldh1a family is not a tetrapod innovation, but it originated during the first WGD.

5.- The evolutionary relationships of Cyp26a1 support the evidence of a common ancestor for the Cyp26 family members strengthening the hypothesis of an independent gene functionalization prior to the two rounds of genome duplication in vertebrates.

6.- The absence of *stra8* and its conserved genomic neighbourhood in the European sea bass points to a gene loss in the ray finned lineage during the second TGD and suggests that RA signaling in this species does not occur through the transduction of this particular meiosis gatekeeper, as it has been demonstrated in tetrapods and other fish.

CHAPTER 3

7.- An increase of RA bioavailability and a subsequent signaling through nuclear receptors occurs by the time of meiosis. During the premeiotic stage, fish increase the RA levels within the gonadal milieu needed to switch on meiosis by inducing an upregulation of its synthesis, transport, and signaling.

8.- *cyp26a1* levels are kept high in order to accelerate RA degradation, to prevent an early entry into meiosis, and to avoid precocious puberty, in agreement with the role of this enzyme as the meiosis inhibiting factor in vertebrates.

9.- The decrease of *cyp26a1* expression by the onset of male meiosis and during maturation/ovulation, when meiosis is resumed in females, coincides with an increase of gonadal RA levels reaching a threshold to trigger meiosis making *cyp26a1* an excellent molecular marker for the onset of meiosis in European sea bass.

CHAPTER 4

10.-An *ex vivo* culture system has been set up, validated and proven successful for the study of early stages of European sea bass gonad development. This model can serve as a valid tool to assess physiological and pharmacological effects of a wide range of substances in fish gonads, and to study testicular development in teleosts.

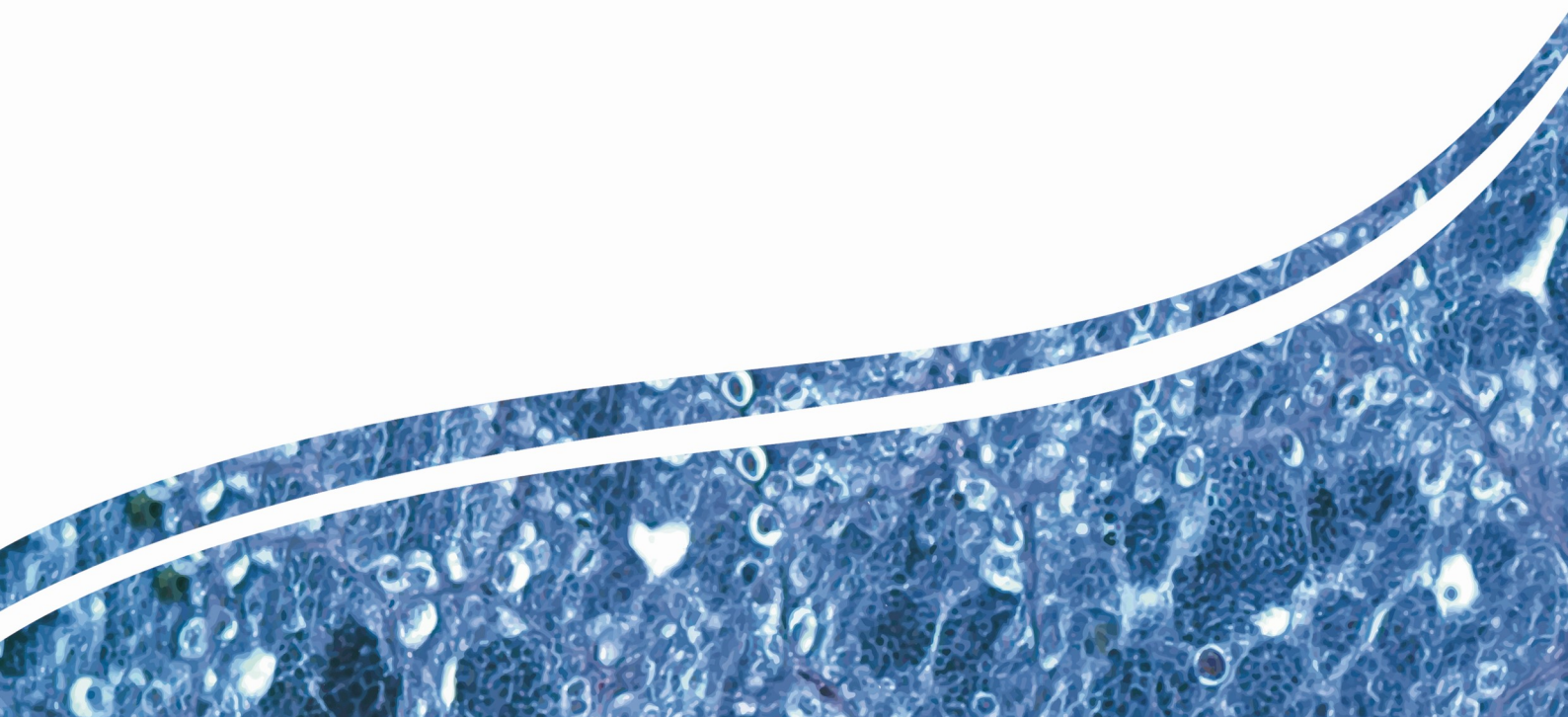
11.- Several genes involved in the RA signaling pathway exhibit *in vitro* responses to atRA, DEAB, 11KT and FSH according to their respective function, confirming that the RA metabolic machinery is active in testis during the onset and early stages of meiosis.

12.- The expression dynamics of the RA synthesis and degradation genes, *aldh1a2*, *aldh1a3*, and *cyp26a1*, respectively, demonstrate that a tight control on the overall RA availability within the gonad is crucial during the onset of meiosis.

Overall this thesis demonstrates that

The retinoic acid machinery is conserved in the European sea bass and plays a role in gonad development and puberty.

Thesis extended methodology.



Extended Methodology Table 1. Primers and features to calculate efficiency (E)

Gene	Primer sequence (5'→3')	Size (bp)	Slope	E	r2	Accession
pcna	Fwd: CCAAGGACGGAGTCAAGTTC Rev: CTGGACGGGTTCATTCATCT	125	-3.37	99	0.99	JQ755266
cenpi	Fwd: TCTGGCTAGGCTACGCTCTC Rev: GTGCTGTGTGGACAGCAACT	113	-3.24	101.8	0.99	KP739864
spc25	Fwd: CATTTGGGGATGGAGATACG Rev: GTCCTGATCCAAAGGGTTGA	102	-3.50	96.5	0.98	KP729615
cenpf	Fwd: GGCAGCTTGACAAGATCACA Rev: GAGAGACGGCACACTTCTCC	103	-3.45	97.3	0.96	KP765683
trip13	Fwd: GGGATTTGGGAGAGTCTGGT Rev: CGGTTCCATGAAATCAGGTT	119	-3.68	93.5	0.99	KP739862
cdc28	Fwd: GAGGAAGAGTGGAGGGGACT Rev: CCTTTGGGAGAGGTCTTCTG	109	-3.51	96.3	0.98	KP729616
igfbp6	Fwd: CCAACTGAAAACGTCCAAACG Rev: GGCGTCAAGACCTTTGATAAGTGTA	107	-3.33	99.9	0.98	KP739865
cyp19a1a	Fw: AGACAGCAGCCCAGGAGTTG Rv: TGCAGTGAAGTTGATGTCCAGTT	106	-3.30	2.01	0.99	AJ311177
amh	Fw: TGACTCCACTTCTGCTTTTCTCAT Rv: AGAAAGGAGGAGGTCTGTGAAGAG	100	-3.68	1.87	1.10	AM232701
pcna	Fw: CCAAGGACGGAGTCAAGTTC Rv: CTCGACGGGTTCATTCATCT	125	-3.37	1.98	1.01	JQ755266
rbp4	Fw: ACCCTGCCAAGTTCAGAATG Rv: GTGGACGGCGTAGTTATCGT	107	-3.44	1.95	1.03	KP739863
stra6	Fw: CTTGTCATGGACGCACTTTG Rv: AAGGAATGCCAAGCAAGCTA	124	-3.92	1.80	1.18	*D. labrax* genome
aldh1a2	Fw: GCAGACAAGGCTGATGTTGA Rv: TTCCTTGGAGGTCCACAAAC	214	-3.31	2.01	0.99	*D. labrax* genome
aldh1a3	Fw: CCTGGTGTCAGCAGTCTT Rv: ACCCTCCAAAGGGAGTCTGT	125	-3.19	2.06	0.96	*D. labrax* genome
rarα	Fw: CGCTAAACCGAACCCAGA Rv: CTTCTCGGCCTGTTCCAA	170	-3.28	2.02	0.99	KP749835
rxrα	Fw: CTGGTAGAGTGGGCCAAGAG Rv: GTTCTGTGAGCACCCTGTCA	226	-3.43	1.96	1.03	KP749834
pparγ	Fw: CAGATCTGAGGGCTCTGTCC Rv: CCTGGGTGGGTATCTGCTTA	186	-3.46	1.94	1.04	AY590303
crabp1	Fw: GCCACTTGGGAAACAGAAAA Rv: CATCGGCTCCAAAGATCAGT	124	-3.36	1.98	1.01	KP723829
cyp26a1	Fw: GCAGGAGCTGGTGGAAGCTT Rv: CCTTGCCTTCAGACCCCTGTA	120	-3.29	2.01	0.99	KJ187657
r18s	Fw: CCGCTTTGGTGACTCTAGATAACC Rv: CAGAAAGTACCATCGAAAGTTGATAGG	101	-3.30	2.00	0.99	AY831388
ef1α	Fw: AGATGCACCACGAGTCTCTGC Rv: GTTCTTGCTGGGTGGGTTC	128	-3.27	2.02	0.98	FM019753

qPCR data normalization

Data from the real-time qPCR assays were analysed using a modification of the classic ΔΔCt method that takes into account multiple reference genes and gene specific amplification efficiencies (Hellemans et al., 2007). This method has been supported by studies showing that appropriate normalization of RT-qPCR data with several stably expressed reference genes is essential to ensure accurate and reliable results (Bustin et al., 2009; Chang et al., 2010; Vandesompele et al., 2002). This model transforms quantification cycles into quantities using an exponential function based on the efficiency of the PCR reaction (for further details and formulas see Hellemans et al., 2007). The standard deviation (SD) and the coefficients of variation (CV) of the Cq values of *18s rRNA* and *ef1a*, the reference genes chosen for the study previously shown to be stable in this species (Mitter et al., 2009), were calculated for each sex and also for the total set of samples (including both sexes) to determine the most stable one (Pfaffl et al., 2004). Although both are suitable reference genes, *18S rRNA* was chosen as the main one since it consistently exhibited lower SD and CV values than *ef1a* in all conditions tested.

Gene	Males		Females		Both sexes	
	SD	CV%	SD	CV%	SD	CV%
18S rRNA	0.58	3.63	0.88	5.15	0.90	5.40
ef1a	1.20	5.76	1.40	6.87	1.31	6.34

Normalization factor using *18s rRNA* and *ef1a* as reference genes (Box1)

A ΔCq equation was used to determine the *18s rRNA* relative quantity for each sample, using *ef1a* as reference gene. The *18S rRNA* relative quantity (RQ) was then calculated using *18S rRNA* efficiency (*E*) and the corresponding ΔCq value (Hellemans et al., 2007; Formulas 9-12). Finally, the normalization factor was calculated as the geometric mean of *18S rRNA* RQ values of every sample and subsequently used for PCR corrections (Hellemans et al., 2007; Formulas 13-14).

Box 1

$$\Delta\mathrm{Cq} = \mathrm{Cq}\,(18S\ rRNA) - \mathrm{Cq}\,(ef1\mathrm{a})$$

$$18S\ rRNA\ RQ = \Delta\mathrm{Cq}^{(E\ 18S\ rRNA)}$$

$$18S\ rRNA\ NF = \ Geometric\ mean\ 18S\ rRNA\ RQ\ (all\ samples\ included)$$

Correction factor for each sample and corrected data (Box 2)

Data normalization of the target gene (tg) was obtained by dividing its relative quantity (tg RQ) by the reference gene normalization factor (calculated in box 1). A target gene specific correction factor (tg CF) was also calculated by taking into account the geometric mean of the normalized relative quantities (NRQ) from every sample. Finally, the corrected data was obtained by dividing the NRQ of the tg by the correction factor for each tg (tg CRQ). Final data were expressed as normalized relative quantities calculated as the mean of all samples of the same sex at each gonad developmental stage.

Box 2

$$\Delta\mathrm{Cq} = \mathrm{Cq}\,(18S\ rRNA) - \mathrm{Cq}\,(tg)$$

$$tg\ RQ = \Delta\mathrm{Cq}^{(E\ tg)}$$

$$tg\ NRQ = \frac{(tg\ RQ)}{(18S\ rRNA\ NF)}$$

$$tg\ CF = \ Geometric\ mean\ tg\ NRQ\ (all\ samples\ included)$$

$$tg\ CRQ = \frac{(tg\ NRQ)}{(tg\ CF)}$$

REFERENCES

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611-622.

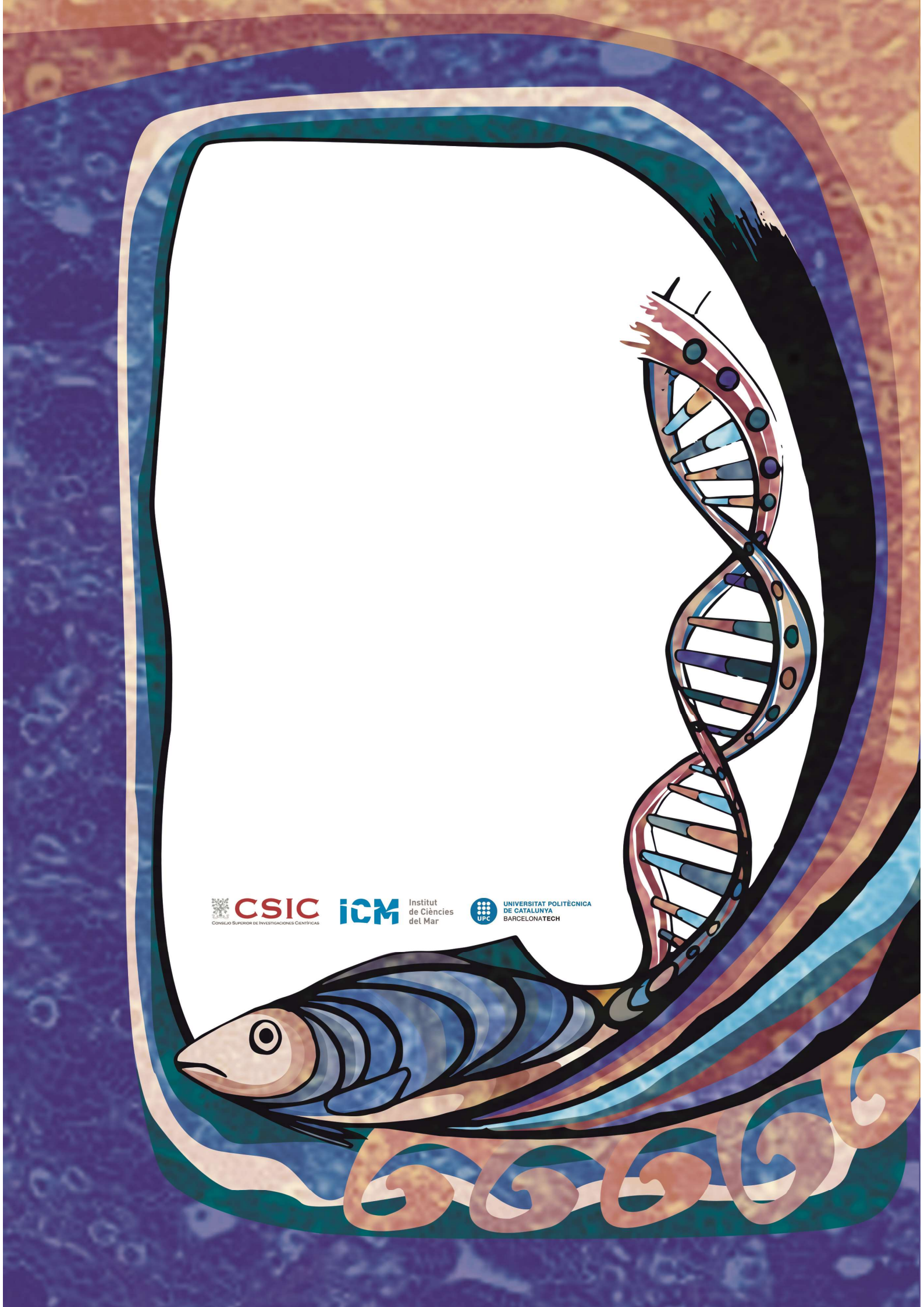
Chang, K.H., Mestdagh, P., Vandesompele, J., Kerin, M.J., Miller, N., 2010. MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. BMC cancer 10, 173.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.

Mitter, K., Kotoulas, G., Magoulas, A., Mulero, V., Sepulcre, P., Figueras, A., Novoa, B., Sarropoulou, E., 2009. Evaluation of candidate reference genes for QPCR during ontogenesis and of immune-relevant tissues of European seabass (*Dicentrarchus labrax*). Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 153, 340-347.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotech. Lett. 26, 509-515.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, research0034. 0031.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