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Study of genetic factors and temperature influence on sex determination and differentiation in turbot

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CERTIFY THAT

The manuscript entitled "Study of genetic factors and temperature influence on sex determination and differentiation in turbot", presented by Diego Robledo Sánchez to opt for the Ph.D. degree, has been realized under their supervision, it is considered concluded and they authorize the presentation to the competent Committee.

And for the record, they hereby sign.

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Abstract

Sex, as intuitive and simple as it may seem to us, poses some of the most interesting and complex questions when studying life. Sex is an intrinsic characteristic of most eukaryote species which eventually has led to the appearance of two differentiated adult phenotypes or sexes, males and females. This distinction rules a huge part of our lives and is the origin of important evolutionary processes based on intra-sex competition or inter-sex conflict due to sexual antagonism. Furthermore, sex is an important character for a plethora of species involved in human activities, for example in aquaculture many fish species present sex size dimorphisms where one sex grows faster than the other, and so knowing how sex is determined in each species is of the outmost interest. Traditionally, sex determination has been considered a cascade process with a master gene at the top, but recent findings have suggested that, instead, it might be a network process where different genetic and environmental factors can alter gonad fate, which in turn would be connected with a huge number of different sex determination mechanisms in vertebrates, especially in poikiloterms. In this new view of sex, the different players involved in sex differentiation gain relevance and their study may help us understanding how the fate of the gonad is determined. In this work, we have studied sex differentiation in turbot, a flatfish with a marked sex dimorphism where females grow faster than males. This species presents genetic sex determination, but also temperature effects on sex ratios have been reported, which seem to be family-dependant. Our aim was to study sex differentiation in turbot to gain knowledge about how sex is determined in this species and also in a broader sense in fish. This work consists of expression studies in turbot gonads using two different techniques: real-time PCR and microarrays. First of all, the real time PCR technique was setup for gonad development studies in turbot. The different methods available for reference gene stability calculation and efficiency determination were assessed. Then, using this information we performed an extensive expression study on turbot sex differentiation ranging from undifferentiated to differentiated gonads at three different temperatures. We found that the first molecular signs of sex differentiation are observed at 90 days post fertilization and that three genes, *cyp19a1a*, *amh* and *vasa*, can be used to sex turbot at this stage. Furthermore, the expression of genes involved in germ cell development pointed towards their involvement in early sex differentiation and possibly sex determination. Temperature effects on sex differentiation were also assessed in this study. A higher proportion of females was obtained at cold temperatures and several genes showed temperature dependant expression changes. Finally, to complete our study, we also performed a microarray analysis in turbot gonad samples from undifferentiated individuals to male and female juveniles. Female gonads were found to be more different from undifferentiated gonads than those of males, requiring the regulation of a large number of genes and the involvement of different processes including epigenetic mechanisms. Furthermore, the involvement of known sex differentiation genes and previously unrelated genes in sex differentiation was observed. This study has widened our knowledge on sex differentiation in turbot in particular and in fish in general, helping to understand the role of many genes involved in sex differentiation across the whole vertebrate taxa and pointing towards other genes which have been connected with sex for the first time. Our data suggest that a network model might be more accurate to explain sex determination in turbot, where the environment can interact with genetic factors and modify gonad fate.

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Resumen

El sexo, aunque parezca un concepto intuitivo y simple, presenta algunas de las cuestiones biológicas más interesantes y complejas. El sexo es una característica intrínseca de la mayoría de eukariotas que eventualmente condujo a la aparición de dos fenotipos adultos diferenciados conocidos como sexos, machos y hembras. Esta distinción gobierna buena parte de nuestras vidas y es el origen de importantes procesos evolutivos basados en la competición entre individuos del mismo sexo o en el conflicto entre sexos debido a fenómenos de antagonismo sexual. El sexo es un carácter importante para diversas actividades humanas. Por ejemplo, muchos peces cultivados presentan dimirfismo sexual en el que uno de los sexos crece más rápido que el otro, y por lo tanto es interesante conocer como es determinado el sexo en cada especie. Tradicionalmente, la determinación sexual ha sido considerada un proceso en cascada con un gen maestro en la cima, pero a partir de descubrimientos recientes se ha sugerido que este proceso puede responder a un modelo en red en el que diferentes factores genéticos y ambientales interaccionan para determinar el destino de la gónada., lo cual estaría conectado con el gran número de mecanismos de determinación sexual en vertebrados, sobretodo en organismos poikilotermos. En este nuevo escenario, los diferentes factores involucrados en la diferenciación gonadal cobran importancia y su estudio puede ayudar a entender cómo se decide el destino de la gónada. En este trabajo hemos estudiado la diferenciación sexual en el rodaballo, un pez plano con un marcado dimorfismo sexual en el que las hembras crecen más rápido que los machos. Esta especie presenta determinación sexual genética, pero también se han detectado efectados de la temperatura sobre las proporciones sexuales en ciertas familias. Nuestro objetivo era estudiar la diferenciación sexul del rodaballo para conocer cómo se establece el sexo en esta especie y contribuir al conocimiento de cómo se produce la diferenciación sexual en peces. Este trabajo consiste en estudios de expresión en gónada de rodaballo utilizando dos técnicas distintas: PCR en tiempo real y microarrays. Primero, se puso a punto la técnica de PCR en tiempo real para estudios de desarrollo gonadal en rodaballo. Los distintos métodos para determinar los genes de referencia y el cálculo de la eficiencia fueron analizados. Después, utilizando esta información se desarrolló un amplio estudio de la diferenciación sexual en rodaballo, utilizando gónadas desde undiferenciadas hasta diferenciadas tomadas a tres temperaturas diferentes. Los primeros signos de diferenciación sexual fueron encontrados a 90 días post fertilización y tres genes, *cyp19a1a*, *amh* y *vasa*, pueden utilizarse para establecer el sexo de los rodaballos en este estadio. Además, la expresión de genes relacionados con las células germinales apunta a que deben tener un papel en la diferenciación sexual y posiblemente también en la determinación. También se estudiaron los efectos de la temperatura, encontrándose una mayor proporción de hembras a bajas temperatura y efectos en al expresión de varios genes. Finalmente, para completar nuestro estudio, se analizaron muestras de gónada de rodaballo desde estadios indiferenciados hasta machos y hembras juveniles mediante microarrays. Se encontró que la gónada femenina se diferencia más de la gónada indiferenciada que la masculina, requiriendo la regulación de un mayor número de genes y la acción de diferentes procesos incluyendo mecanismos epigenéticos. Este estudio ha ampliado nuestro conocimiento sobre la diferenciación sexual en el rodaballo en particular y en peces en general, ayudándonos a entender el papel de muchos genes involucrados en la diferenciación sexual en los vertebrados y apuntando a otros genes conectados con el sexo por primera vez. Nuestros datos sugieren que un modelo de red sería más preciso para explicar la determinación sexual en el rodaballo, donde el ambiente puede interactuar con factores genéticos y modificar el destino de la gónada.

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Introduction

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1. Sex

The distinction between sexes, males and females, becomes obvious to everyone very soon after childbirth and accompanies us throughout our lifetime. This distinction is present in every human culture and that is very clearly seen in our languages. Furthermore, maleness and femaleness have been present as fundamental symbolic elements in several civilizations, for example in the Greek myth of creation through Gaia and Uranus, or in the Taoism as yin (female) and yang (male). Great thinkers along our history have spent their time thinking about sex and sexes. Sex is important and is intrinsic to our existence.

From a scientific point of view, sex is a biological phenomenon where two specialized cells, called gametes, which come from two adult individuals, combine their genetic material to produce off-spring. Sex usually involves the fusion of two haploid gametes from two individuals with differential reproductive roles, males and females. In this process, the genetic material of both parental individuals merges in the resulting zygote, appearing new genetic combinations which were not present in none of the progenitors. Even in those species which do not present differentiated sexes (isogamy) complementary gametes must still meet. Difficulties finding suitable partners have been connected with the origin of hermaphrodites, species where both male and female reproductive organs are present in a single individual. Sex is exclusive of eukaryotes and is widespread all along the phylum. An estimated 99% of eukaryote species reproduce sexually (Otto, 2009).

The sex of an organism is defined by the gametes produced in specialized organs, the gonads. We speak of males and females when their gametes are different (anisogamy). Males are characterized by small and mobil gametes called spermatozoa, while females present large and motionless gametes called eggs or ova. The existence of separate sexes implies different reproductive roles for males and females, which in turn is the origin of an evolutionary mechanism, sexual selection, which consists in the competence to reproduce between individuals of the same sex (Scharer et al. 2012).

Traditionally a two-fold cost of sex when compared to asexual reproduction has been hypothesized since a male and a female are required to produce offspring, while asexual reproduction only requires one individual. So, why did sex arise and why has it been maintained along the evolution of eukaryotes?

1.1. Origin and maintenance of sex

To dive into the depths of the origin of life and evolution is to enter the fields of speculation, and so sex origin is still today a matter of debate. The origin of sex must have occurred very soon during the first stages of life on the Earth, in parallel to the origin of eukaryotes, since sex is present exclusively in this group and spread through all its branches, its mechanisms being conserved throughout the whole group. These facts suggest that sex is likely to have appeared only once around 1.5 billion years ago (Javaux et al. 2001). It is not a simple task to explain the origins of sex, however it must be irremediably linked to the origin

of meiosis. However, what happened first? Were there cell fusions occurring when meiosis appeared as a mechanism to reduce ploidy (Cavalier-Smith, 2010)? Or was meiosis present already as a mechanism of DNA repair using other DNA from other cells (Bernstein et al. 2011)? Are the mechanisms of meiosis and bacterial conjugation somewhat connected? These, as many other questions regarding the very beginning of life, are difficult to answer and so the origin of sex is far from being resolved. None of the hypothesis is supported by enough empirical evidence to rise above the others.

Furthermore, the maintainance of sex in the vast majority of eukaryotes also poses some questions, since sex costs largely outweight those of asexual reproduction: sex is slower, requires outcrossing, two different sexes and may break beneficial gene combinations (Lehtonen et al. 2012). However, this cost might not have been such when sex first arised, for example in isogamy ancestors the two-fold cost of producing males would not be present. At the origin sex could have been an almost cost-free action and, since very few lineages have renounced to sex, it must have very strong evolutionary benefits.

One of the oldest explanations for sex maintenance in eukaryotes was Weismann's at the end of the 19th century: sex creates variation on which selection can act (Weismann, 1886). However, sex and recombination destroy well adapted genetic combinations created by evolution during the adaptation to an environment, so reducing the overall fitness of the offspring when compared to the well adapted parents. The most extended hypothesis to explain why sex has been maintained and expanded in eukaryotes is that sex would be favored, in general terms, when individuals are not adapted to their environments, or, said in a different way, in unfavorable environmental conditions (Otto, 2009). An experiment in a rotifer species with populations which favor sex or asexual reproduction showed that sex is preferred when the environment is changed. In this situation the offspring produced by sex reproduction would present a higher fitness variation, producing some very well-fitted individuals which would disproportionately contribute to future generations, carrying with them also alleles which favor sex reproduction (Becks and Agrawal, 2012). Sex was probably facultative during the first steps of eukaryote evolution and, as a result of rapid changing environments and strong selection in small populations, it probably became constitutive and, eventually, asexual reproduction was lost. At that point, sexual reproduction was the only option, and all the later appearing disadvantages were irrelevant. We were stuck with sex.

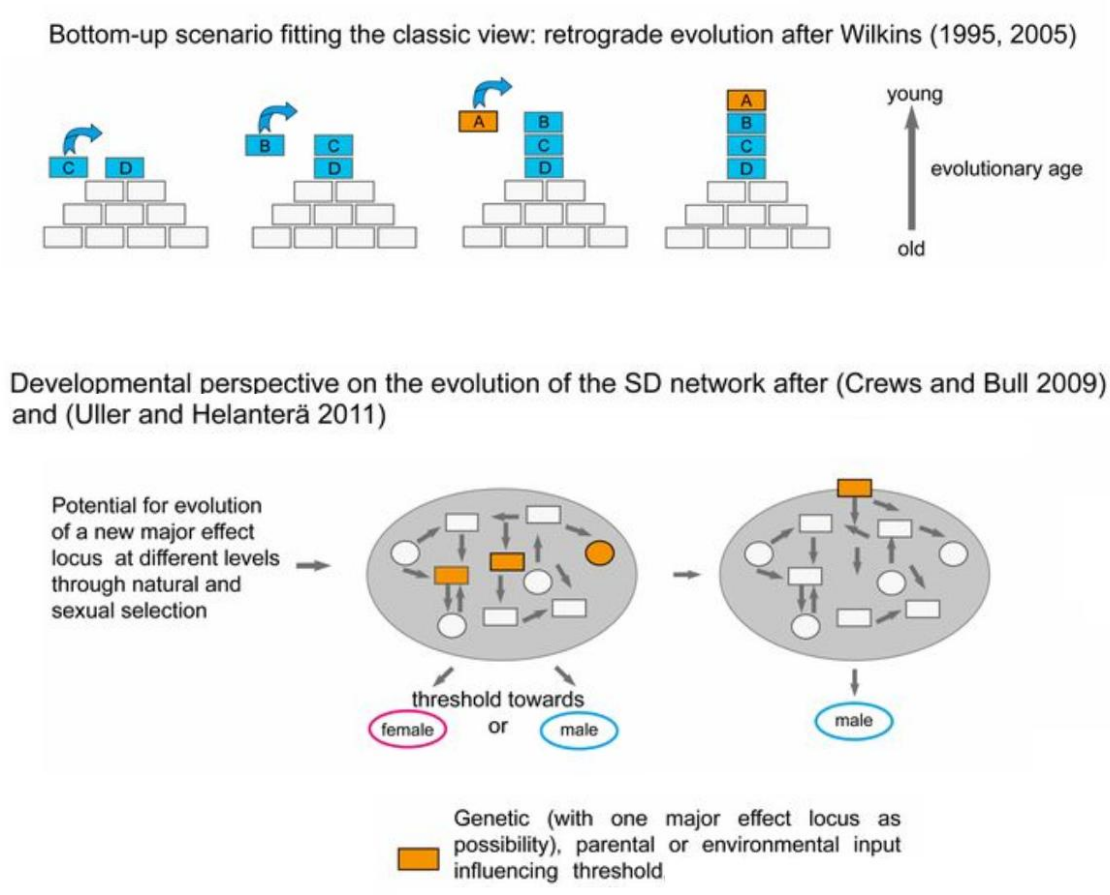
1.2. Important concepts related to sex

Sex can be very different between distinct eukaryote groups: plants, fungus or animals present their own peculiarities. Reproductive strategies are diverse, ranging from unisexuality, only one sex and reproductions occurs by parthenogenesis; to hermaphroditism, male and female gonads are present in the same individuals, either sequential or simultaneously, with or without autofecundation; and gonochorism, separate sexes.

We will mainly focus on sex in vertebrate animals along this thesis, which present anisogamy with differentiated male and female gametes produced in differentiated male and

female gonads, named testis and ovaries respectively. Testis and ovaries usually develop from a bipotential undifferentiated gonad, although in sequencial hermaphrodites a testis might develop from an ovary and the other way around. The process leading to the development of male or female phenotype, including gonad development and the secondary sex characteristics related to morphology, physiology and behavior is known as **sex differentiation** (Penman and Piferrer, 2008). However, prior to sex differentiation, a decision has to be made on whether to develop as male or as female. The process which establishes sex in an individual is known as **sex determination**. The sex determination mechanisms vary, and they have been classically divided into two main categories: genetic sex determination (GSD) and environmental sex determination (ESD). These represent the extremes among a range of intermediate sex determination options where genotype and environment might have different weights (Penman and Piferrer, 2008; Heule et al. 2014).

Figure 1. Two different models for sex determination and differentiation



Modified from Heule et al. (2014).

Traditionally, sex studies have considered sex determination and sex differentiation as two different processes. However, this vision has been questioned in the last years. The classic vision is that sex determination mechanisms are placed on the top of a sex differentiation cascade controlling wether a male or female differentiation cascade occurs. However, lately the whole process of sex determination and differentiation has started to be seen as a network structure instead of a lineal cascade (Figure 1) (Heule et al. 2014). In this sense, some authors have suggested that sex determination and the initial steps of sex differentiation should not be viewed as two separate processes, but as a continuous one where several factors interact and control the development of a male or a female depending on expression thresholds of particular genes, cell proliferation, hormonal levels and environmental cues (Crews and Bull, 2009; Uller and Helanterä, 2011; Schwanz et al. 2013; Heule et al. 2014).

1.3. Sex determination genes and sex chromosomes

As a consequence of the traditional view of sex with a determinant factor atop of the differentiation cascade, sex studies have usually been focused in finding a factor acting as a "switch", leading sex development towards the formation of a male or a female phenotype (Wilson and Makova, 2009; Martínez et al, 2014). If sex is determined genetically, this factor would be a gene, which is known as the sex determination gene. We also speak of sex chromosomes as those which are involved in sex determination. In a genetic sex determination system with a single sex determination gene, there would be only a pair of sex chromosomes, the ones harboring that gene. Commonly, the sex determination gene is present (or absent) in one of the chromosomes of the sexual pair, determining the absence or presence of this chromosome the sex of the individual. Usually, recombination is suppressed between sex chromosomes, which leads to their differentiation and eventually may result in heteromorphic chromosomes.

The appearance of recombination suppression and heteromorphic sex chromosomes might be explained by sexual antagonism. If the sex determination gene is next to another gene or allele favorable to one sex but detrimental to the other, natural selection will act to suppress recombination (Bull, 1983; Rice, 1987). Recombination suppression would be the first and critical step in the evolution of differentiated sex chromosomes: only through this process can emerging sex chromosomes keep favorable genetic combinations to solve the sexual conflict (Schartl, 2004). Some sexual antagonisms can lead to morphologic sex dimorphic phenotypes, involving different colors, shapes or sizes for example, which in turn may make one of the sexes more appealing for some human activities.

The most common sex chromosome systems are XX/XY and ZZ/ZW, where males and females, respectively, are the heterogametic sex. But other type of chromosome systems exist, for example XX/X0, where females have two sex chromosomes and males only one, or also multiple sex chromosome systems with more than two sex chromosomes per individual, like platypus, the loach fish (Saitoh, 1989) and several neotropical fish species (Almeida-Toledo

and Foresti, 2001), or the tiger beetles (Galián et al. 2002). In any case, XX/XY and ZZ/ZW systems are largely majority (Bachtrog et al. 2011).

The supression of recombination affects mainly to the sex chromosome specific of the heterogametic sex (Y or W), since the other chromosome (X or Z) still maintains recombination in homozygous state (XX or ZZ individuals). This suppression of recombination does not usually affect the whole chromosome, but the region of variable length surrounding the sex determination gene. Currently, the most accepted hypothesis is that the suppression of recombination is initially achieved by chromosomal inversions (Wimmer et al. 2005; Lemaitre et al. 2009). In the absence of recombination, the sex differential region is always in heterozygotic state which causes deleterious mutations, deletions, insertions, transposable elements and repeated sequences to accumulate in the Y and W chromosomes in a process called "asexual decay". This process is also characterized by the accumulation of heterochromatin and length reduction due to the loss of genetic material (Vallender and Lahn, 2004), which in turn reinforces the suppression of recombination. In this situation, sex chromosome heteromorphisms are expected to become visible at cytological level. However, some species with strong genetic sex determination systems do not show cytogenetically heteromorphic chromosomes. For example in some bird species, with clearly ancient sex determination genes, sex chromosomes are indistinguishable (Charlesworth and Mank, 2010). Supression of recombination should always lead to chromosomal degeneration, so the absence of heteromorphism can be either because the sex chromosomes are young or because there is not suppression of recombination. The fact is that suppression of recombination is not observed in every sex chromosome system (Charlesworth and Mank, 2010).

2. Sex determination in vertebrates

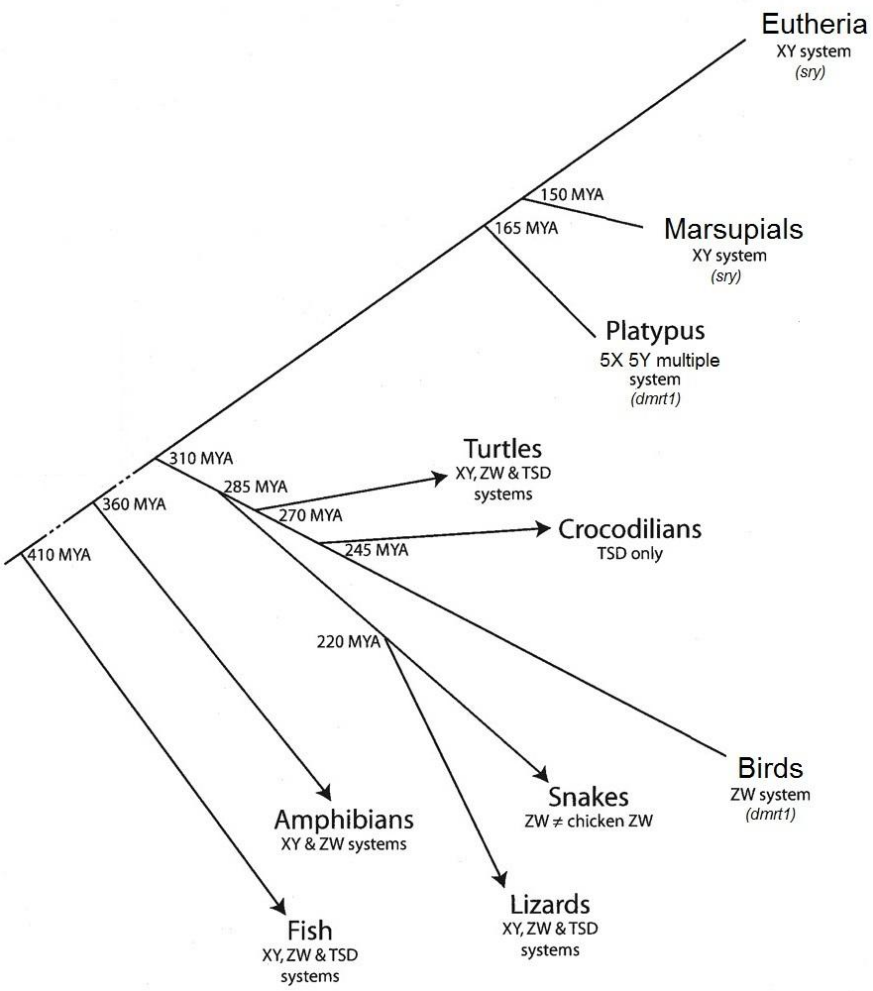
Vertebrates currently present around 64,000 species which can be divided in two big groups: fish and tetrapods, which diverged ~410 million years ago and present quite different sex determination systems (Figure 2).

2.1. Sex in tetrapods

There are four big groups of tetrapods: mammals, birds, reptiles and amphibians. While mammals and birds present highly conserved sex determination systems, the other two groups do not.

Mammalian sex chromosomes, and especially the human ones, are the most studied and represent the model which has been used to study other vertebrates and to draw hypothesis about the evolution of sex determination. Placental mammals have a XX/XY sex determination system originated 165 million years ago (MYA), with a sex determination gene

Figure 2: Tetrapod phylogeny and their respective sex determination systems



Modified from Veyrunes et al. (2008).

named *sry* (*sox* region in Y) which arose by shuffling of the HMG box of the transcription factor SOX3 (*sox3*) and another gene linked to the X chromosome, microprocessor complex subunit DGCR8 (*dgcr8*) (Sato et al. 2010). Marsupial mammals have the same *sry*-XY sex determination system but their chromosomes lack a region added 105 MYA to placental mammals' sex chromosomes (Veyrunes et al. 2008). On the contrary monotremes, platypus and echidna, have a multiple chromosome sex determination system, composed of five X chromosomes and five or four Y chromosomes respectively (Grützner et al. 2004). These chromosomes do not show homology with the sex chromosomes of other mammals nor do they bear the *sry* gene (Ferguson-Smith and Rens, 2010).

All birds share the same ZZ/ZW genetic determination system which appeared around 120 MYA (van Tuinen and Hedges, 2001). The sex determination gene is *dmrt1*, which is absent in the W chromosome and works in a dosage-dependant manner, two *dmrt1* copies are necessary for the development of a male gonad (Smith et al. 2009). Most birds present heteromorphic sex chromosomes but some groups, like the ratites, have homomorphic sex

chromosomes with a single small differential region (Ellegren, 2000; Shetty et al. 2002). Interestingly, the avian Z chromosome shares homology with the monotreme sex chromosomes, which may suggest that the common mammalian ancestor had a ZW sex determination system similar to that of birds (Veyrunes et al. 2008). *Dmrt1*, the sex determination gene in birds, is present in the monotreme sex chromosomes, but it does not seem to be the sex determination gene, which is still unknown (Ferguson-Smith and Rens, 2010).

Reptiles exhibit a great variation of sex determining systems. Environmental sex determination is predominant and probably the ancestral state from which ZW and XY systems have evolved repeatedly (Janzen and Phillips, 2006; Organ and Janes, 2008). Temperature sex determination is mostly conserved in crocodiles and turtles, where incubation temperature determines sex. In crocodiles high temperatures usually promotes the development of males, while in turtles high temperatures produce females. In lizards, temperature and genetic (ZW and XY) sex determination systems are present and in several species sex determination is controlled both by genetic signals and temperature effects (Quinn et al. 2007; Radder et al. 2008). There are around five thousand species of lizards (Uetz and Hošek, 2014) and the karyotype of approximately a thousand has been obtained of which less than two hundred present heteromorphic sex chromosomes (Ezaz et al. 2009). Yet, highly conserved old sex determining systems exist in some reptile lineages. For example iguanas present a broadly conserved XY sex determination system with homologous sex chromosomes originated between 123 and 73 MYA (Rovatsos et al. 2014). Also snakes present a conserved ZZ/ZW genetic sex determination system originated 40 MYA with sex chromosomes ranging from completely homomorphic to strongly heteromorphic depending on the family (Vicoso et al. 2013). Recently, a small lizard was discovered, G*ekko hokouensis*, which presents a ZZ/ZW system and the gene content of the Z chromosome is homologous to that of the Z chromosome of birds, included *dmrt1* (Kawai et al. 2009). However, since *Gekko hokouensis* chromosomes seem to be of recent origin it is thought that both systems do not share a common origin, but rather that the same chromosome pair was independently selected for sex determination in both lineages (Kawai et al. 2009). The ZZ/ZW bird sex determination system is believed to have evolved independently to that of reptiles (Kawai et al. 2007).

Most amphibians present genetic sex determination (Eggert, 2004) and usually homomorphic sex chromosomes, but the type and gene content differs significantly between the different species (Uller and Helanterä, 2011; Mawaribuchi et al. 2012). Frogs and toads mainly show ZW sex determination systems, while XY systems are the most common in salamanders (Schmid et al. 1991, Smith and Voss, 2009). The different chromosomal systems of the present amphibians are believed to have arisen independently along evolution (Nakamura, 2009). *Xenopus laevis* shows a ZZ/ZW sex determination system and its sex determining gene is the only one known in amphibians, *dm-w*, which is homologous to the avian *dmrt1* and acts as an antagonist inhibiting the formation of a male gonad, causing instead ovary development (Yoshimoto et al. 2008). *Dm-w* must have evolved recently, since

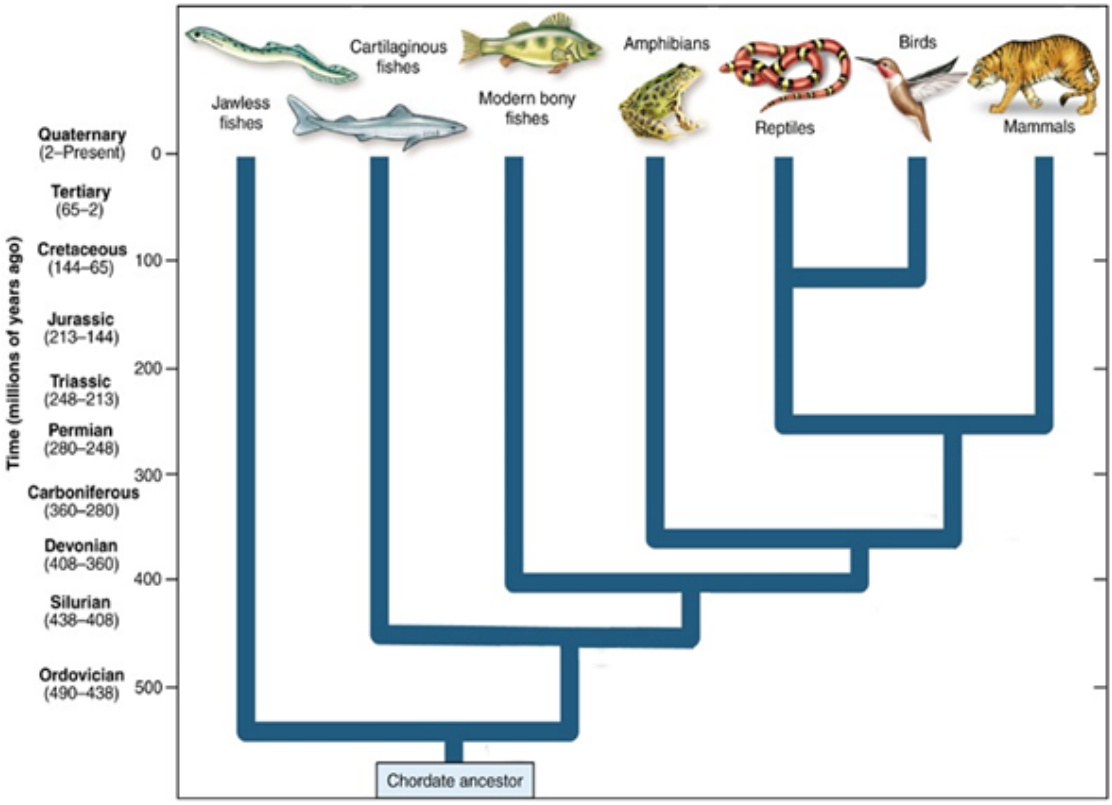
it is not present in other nearby species like *Xenopus tropicalis* (Uno et al. 2008). Several changes between ZW and XY systems have occurred in amphibians (Hillis and Green, 1990). One of the most extreme cases was observed in *Rana rugosa*, where sex determination has evolved twice, and both XY and ZW populations exist inhabiting different Japanese islands with an inter-breeding area harboring a mixture of all sex chromosomes (Ogata et al. 2003). It has been shown that the X chromosome is homologous to the W and the Y is homologous to the Z (Uno et al. 2008).

Both mammalian and avian sex determination systems are consistent with a cascade model, with *sry* and *dmrt1* on the top as sex determination master switches. Mammals and birds are endotherms, maintaining constant body temperatures throughout all their lives. On the contrary, reptiles and amphibians body temperature depends on the environment being more variable during their life and also more diverse between species. This is also true for their sex determination systems which contrast with the high conservation of the other two groups. However, as we mentioned, there are also some groups of reptiles with conserved sex determination systems, but also reptiles try to maintain their body temperature constant through external heat sources, while amphibians do not, and also conserved sex determination systems have not been discovered among them. There seems to be a connection between body temperature control and sex. Homeotermy appeared twice independently in birds and mammals and this might be critical for the fixation of their sex determination systems. Fish live in the widest range of environmental conditions and are poikilotherms as amphibians so, what is the situation in the last group of vertebrates?

2.2. Sex in fish

Fish is a paraphyletic group which comprises three different groups: jawless fish, cartilagous fish and bony fish (Figure 3). Among these, bony fish or teleosts form the largest group with over 26.000 species (Nelson, 2006) and also the best studied. Teleosts present a great variety of sex determination mechanisms. Sex can be determined by genetic or environmental factors, or by a combination of both (Devlin and Nagahama, 2002). Genetic sex determination includes single gene sex determination systems and polyfactorial systems with several genes and different chromosomes (Penman and Piferrer, 2008). Furthermore, fish also present every type of reproductive strategy, ranging from hermaphroditism with two reproductive organs in the same individual to gonochorism or unisexual species which reproduce by parthenogenesis (Devlin and Nagahama, 2002). The common fish ancestor is thought to have presented separate sexes (Smith, 1975), since hermaphroditism is rare in this group (Devlin and Nagahama, 2002) and restricted to the extremes of the fish phylogeny, which suggests a polyphyletic origin (Mank et al. 2006). However, it is difficult to discriminate between an ancestor with genetic or environmental sex determination since nowadays the sex determination mechanism of only a few species is well known. Also, many changes in sex determination have occurred along evolution, even between closely related species (Volff et al. 2007).

Figure 3. The fish group is paraphyletic as shown by the vertebrate phylogeny



Johnson and Losos, 2006

The control of sex determination and differentiation is very important for aquaculture industry. Nowadays, over 350 fish species are harvested in the world (FAO, 2014). There are several reasons to obtain single sex stocks for industry (Martínez et al. 2014). In some species growth sex dimorphisms exist, with either higher growth in males (tilapias) or, more frequently, females (flatfish, sea bass). In other species, sex maturation affects the organoleptic properties preferently in one sex (Piferrer et al. 2009). Furthermore, sex can be connected to characteristics like color or shape, associated with commercial value. Another particular case is that of the sturgeon since, in order to produce caviar, only females are productive.

2.2.1. Genetic sex determination in fish

Heteromorphic sex chromosomes have only been found in a 7% of the fish species studied (Penman and Piferrer, 2008; Oliveira et al. 2009). However, for most gonochoristic fish species, sex is genetically determined, either by one or several loci (Kikuchi and Hamaguchi, 2013). The most common sex determination systems are XX/XY and ZZ/ZW, but, as previously mentioned, other chromosome systems like XX/XO or XX/XY_1Y_2 have also been reported (Devlin and Nagahama, 2002). Given the economic importance of sex and

reproduction for aquaculture a big effort has been applied for understanding sex determination in fish. Yet, sex determination genes have only been identified in six species or groups of species: *Oryzias latipes* (*dmy*), *Oryzias luzonensis* (*gsdf*), *Oryzias dancena* (*sox3*) *Odonthesthes microlepidotus* (*amhY*), *Takifugu rubripes* (*amhr2*) and *Oncorhynchus mykiss* (*sdY*).

2.2.1.1. Medaka

The first sex determination gene discovered in fish was *dmy* in medaka (*Oryzias latipes*). Medaka is a freshwater fish from East of Asia popular in aquariums, which has been a pet in Japan since the 17th century. Medaka shows a XX/XY sex determination system with a sex determining gene *dmy*, a copy of *dmrt1*, present in chromosome Y. This Y chromosome arose around 10 MYA, being the youngest sex chromosome system discovered to date (Kondo et al. 2004). Sex chromosomes have been identified in eight *Oryzias* species. Surprisingly, seven different sex chromosome pairs have been found, five XX/XY and two ZZ/ZW (Tanaka et al. 2007). *Dmy*, besides *Oryzias latipes*, is the sex determining gene only in *Oryzias curvinotus* (Takehana et al. 2008). The *Oryzias* genus seems to offer a unique opportunity to study rapid transitions between different sex determination systems.

Recently, the sex determination gene was detected in other two species of this genus. *Oryzias luzonensis* shows a XX/XY system and the sex determining gene is *gsdf*, present in both chromosomes but with different alleles. A higher expression of the $gsdf^{Y}$ allele has been found and it has been associated to a mutation in its promoter for a steroidogenic factor 1 binding site, the gene responsible for the induction of *sry* in male mammals (Myosho et al. 2012). However, in another medaka species, *Oryzias dancena*, also XX/XY, *sox3* seems to be the sex determination gene and initiates testicular differentiation by upregulating the expression of *gsdf* (Takehana et al. 2014).

2.2.1.2. Patagonian pejerrey

Patagonian pejerrey (*Odonthesthes microlepidotus*) is an Argentinean freshwater fish with an XX/XY sex determination system (Strüssmann et al. 1997). Recently, an additional copy of the anti-müllerian hormone has been found in the male specific Y chromosome: *amhy* (Hattori et al. 2012). This additional *amh* copy drives male development. This was the first discovered case of a non transcription factor sex determining gene in fish.

2.2.1.3. Japanese puffer

Japanese puffer *(Takifugu rubripes)* is an Asian marine fish whose genome was the 2nd vertebrate one to be published (Aparicio et al. 2002). This fish has a XX/XY sex determination system (Kikuchi et al. 2007) and sex determination occurs due to a single SNP in the anti-müllerian hormone receptor 2 gene (*amhr2*) (Kamiya et al. 2012), which is conserved in other two closely related species (Kamiya et al. 2012). The X-associated *amhr2* codifies for a receptor variant with a reduced function, and so in XX individuals the *amh* cascade is not efficiently triggered, leading to female development. The sex determining region of *Takifugu rubripes* does not show reduction of recombination (Kamiya et al. 2012),

which might be more common than expected due to the low differentiation of sex chromosomes in fish (Devlin and Nagahama, 2002).

2.2.1.4. Rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) is a salmonid fish inhabiting the North of the Pacific Ocean. This species has been cultured since the 19th century and its aquaculture production in 2012 was of 850,000 tones (FAO, 2014). Its sex determination system is XX/XY and the sex determining gene has been recently discovered and named *sdY* (Yano et al. 2012). This gene presents homology with the interferon regulatory factor 9 (*irf9*) and is the first sex determining gene discovered apparently not related to the sex differentiation network. *Irf9* is involved in the type I interferon response in mammals and, so, implicated in the immune response (Takaoka and Yanai, 2006). This study reveals that new genes, at first not obviously connected with sex differentiation, may be recruited as sex determining genes, further complicating the search for candidate sex determination genes in fish. The presence of *sdY* has been confirmed in fifteen species of salmonids, and associated with male sex in thirteen of them, but, surprisingly, the Y chromosomes in these salmonid species are not homologous, thus suggesting some kind of jumping or transposition of the SD gene (Yano et al. 2013).

2.2.1.5. Sablefish and Tongue sole

Two other strong candidates have been proposed as sex determining genes in teleost species. In the sablefish (*Anoplopoma fimbria*) male specific insertions have been detected in the *gsdf* promoter, which suggests an XX/XY system and *gsdf* as the sex determination gene (Rondeau et al. 2013). On the other hand, the tongue sole (*Cynoglossus semilaevis*) shows a ZZ/ZW sex determination system and *dmrt1* has been suggested as the sex determining gene, because it is found associated to sex and pseudogenized in the W chromosome (Chen et al. 2014). This sex determination system would be analogous to that of birds, where *dmrt1* determines the sex depending on its dosage.

2.2.2. Environmental sex determination in fish

Environmental sex determination has been traditionally assumed to be widely distributed in fish. As in amphibians or reptiles, the main sex determining environmental factor in fish is temperature. However, thermosensitivity in fish differs from that observed in reptiles, particularly because in fish monosexual populations are rare, even under extreme circumstances (Baroiller and D'Cotta, 2001). Furthermore, the influence of temperature on species with a genetically determined system have been largely reported in teleosts (Baroiller et al. 1999; Baroiller and D'Cotta, 2001; Baroiller and Guiguen, 2001) and most fish sex determination systems can be described as genetic with environmental influences. In most thermosensitive fish species, male-biased offsprings are obtained at high temperatures, and development of ovaries is induced at lower temperatures (Baroiller et al. 1999). However, it has been argued that the detected temperature effects on sex ratios in fish might not be

realistic since the used temperature ranges do not fit to the species natural habitat in many cases (Ospina-Álvarez and Piferrer, 2008). Other environmental factors like pH, hypoxia, population density or social interactions have also been reported to affect sex determination in some species (Guerrero-Estévez and Moreno-Mendoza, 2010).

Sex steroids seem to be heavily involved in the sex determination response to environmental conditions (Nakamura, 2010). Most studies point towards the enzyme complex aromatase, which is associated with the production of estrogens (Ramsey and Crews, 2009) and plays a central role in the sex differentiation of all non-mammalian vertebrates (Guiguen et al. 2010). Aromatase has also been suggested as a primary target for temperature sex determination in reptiles (Pieau and Dorizzi, 2004). Although its implication has not been demontrated in all the species with temperature sex determination and, consequently, other alternative target molecules may exist (Uller and Helanterä, 2011). Nonetheless, the most recurrent hypothesis is that high temperature inhibits the enzyme aromatase, possibly through its activators, the genes *foxl2* and *ftr1*, although the implication of heat-shock proteins has also been suggested (Luckenbach et al. 2009). Recently, methylation of the aromatase promoter was connected to temperature sex ratio shifts in European sea bass (*Dicentrarchus labrax*; Navarro-Martín et al. 2011), pointing towards an epigenetic mechanism controlling the expression of aromatase and sex proportions.

On the other hand, accumulating evidences suggest that the endocrine stress-axis may play a critical role in environmental sex determination. High temperatures have been reported to produce male-biased offsprings and to raise cortisol levels in medaka (*Oryzias latipes*; Hayashi et al. 2010), pejerrey (*Odontheshes bonariensis*; Hattori et al. 2009) and Japanese flounder (*Paralichthys olivaceus*; Yamaguchi et al. 2010). Furthermore, a male-skewed sex ratio has recently been found related to background color in southern flounder (*Paralichthys lethostigma*), and it has also been associated with higher cortisol levels (Mankiewicz et al. 2013). So, various environmental factors affecting sex determination might be acting through a common stress mechanism.

Yet, other mechanisms have been suggested to explain temperature effects on sex differentiation. Hayashi et al. (2010) proposed that the follicle stimulating hormone receptor (*fshr*) would be directly regulated by temperature and would be as well connected to germ cell proliferation. Fernandino et al. (2013) suggested a different temperature-mediated mechanism which would imply the over-expression of the steroidogenic enzyme hydroxysteroid (11-beta) dehydrogenase 2 (*hsd11b2*), which is involved in the synthesis of androgens like the 11-ketosterone.

In many species, environmental factors have effects on sexual determination which depend on the genetic background, and interactions between family and temperatura have been documented in several species (Vandeputte et al. 2007; Martínez et al. 2014). Temperature sensitivity can thus be a hereditary trait (Baroiller et al. 1999). In *Poeciliopsis lucida* exposure to high temperatures can alter the sexual proportion towards male only in sensitive lines (Schultz, 1993).

2.3. Sex determination systems: insights from vertebrates

Fish live in a great variety of habitats and their sex determination systems are highly heterogeneous. This variability is also shown by amphibians and reptiles to an extent (Valenzuela, 2008), and contrasts with the very conserved sex determination systems of mammals and birds, in parallel with a higher homeostasis along development, especially regarding temperature (Barske and Capel, 2008). Furthermore, in some reptiles, amphibians and fish, sex determination systems seem to evolve rapidly, leading to closely related species showing different sex determination mechanisms, even within species (Ogata et al. 2003; Lee et al. 2004).

In fish, amphibians and reptiles, key environmental cues cannot be predicted and may change between parents and offspring. This is the case for many teleost fish, particularly in the marine environment, where habitats ultimately occupied by juveniles often are related to the long-range dispersal associated to the planktonic larval stage (Mankiewicz et al. 2013). In this situation, adjusting sex ratios to environmental variation can be advantageous. For example, temperature fluctuations in the different habitats where fish dwell may alter biochemical pathways of sexual determination determining male or female development (Devlin and Nagahama, 2002). Furthermore, fish gonad development flexibility, which allows environmental factors to change gonadal fate, would offer a high number of opportunities along evolution for new sex determination mechanisms and environmental interactions to appear (Piferrer et al. 2012), which can help to explain the huge sex determination variation and the rapid transition between different systems in closely related fish species.

In this sense, sex determination in fish cannot be considered a cascade process but a network where different factors, both genetic and environmental, interact to determine sex. We have been studying sex determination from a mammalian point of view in every organism, looking for a sex "switch" in the top of a sex differentiation cascade. While most fish species present some sort of major genetic component in sex determination and finding this gene is still important, we should also aim to understand early gonad development and how the several genetic and environmental factors contribute to the fate of the gonad if we want to completely understand how sex is determined in fish, and so be able to control it.

3. Sex differentiation

Although it has been commonly assumed that downstream elements in the sex differentiation network are conserved among the different vertebrate lineages, this claim is based mainly in sequence homology and in a few studies on gene expression along gonad development. However, not only changes in the sex determination genes have been observed in fish, but also in other downstream elements of the sex differentiation network (Böhne et al. 2013; Herpin et al. 2013). Even genes with a very important sex-associated role in some species (male-like or female-like genes) have been found playing other relevant functions in

the opposite sex (Böhne et al. 2013). Mammalian sex differentiation is by far the most studied.

3.1. Sex differentiation in mammals

The process which leads to the development of a male or a female gonad can be divided in three steps: 1) Bipotential primordium formation, 2) sex determination, and 3) gonad differentiation (male or female pathways). The aim of this section is to summarize the role of the different genes involved in sex differentiation in mammals, which represent the best studied vertebrate group and a key reference for all other vertebrates.

3.1.1. Bipotential gonad formation

The genital ridge, which is composed of somatic cell lineages and germ cells, is the precursor of gonad primordium in both sexes. There are some key transcription factors involved in the formation and development of genital ridges (Tanaka and Nishinakamura, 2014). Among them, two genes are critical for the formation of the bipotential primordium. The first one is GATA-binding protein 4 (*gata4*), required for the initiation of genital ridge formation after its expression in the coelomic epithelium (Hu et al. 2013). *Gata4* is also responsible for the expression of the nuclear receptor subfamily 5, group A, member 1 (*nr5a1*) or steroidogenic factor 1 (*sf1*), the second critical gene in genital ridge formation, involved in the formation, development and proliferation of gonadal precursor cells, but also in the activation of a set of genes involved in steroidogenesis, such as *cyp17a1* or *3β-hsd* (Tanaka and Nishinakamura, 2014). *Nr5a1* or *sf1* has been proposed to act dose dependently and its expression depends on several other genes like Lim homeobox 9 (*Lhx9*; Birk et al. 2000) or the zinc finger transcription factor wilms tumor 1 (*wt1*; Kreidberg et al. 1993). Furthermore, *wnt* genes like *wnt4* or *wnt7*a are necessary for the formation of Müllerian ducts and its absence causes defects in both sexes (Parr and McMahon, 1998; Vainio et al. 1999).

After the formation of the undifferentiated gonad primordium, members of the insulin receptor family, *gata4*, *wt1* and *sf1*, promote the expression of *sry* in males (Park and Jameson, 2005).

3.1.2. Sex determination: *sry*

Sry is the sex determining gene in mammals. This gene is the founder of the *sox* family, which codifies for transcription factors with a DNA binding domain, similar to the chromatin high mobility group domain (HMG). It has only one exon which codifies a 204 amino acids protein. *Sry* is necessary and sufficient to initiate the differentiation of a male gonad. If *sry* is not active, a female gonad will develop. Nowadays, a single target is known for *sry*, *sox9*, which would be responsible for promoting the male gonad development cascade (Kashimada and Koopman, 2010).

3.1.3. Male gonad differentiation

Sry, together with *sf1*, activates directly *sox9* expression in the sertoli cells (Sekido and Lovell-badge, 2008), which is sufficient to induce testis formation and can replace *sry* if its expression is artificially induced. There are other genes regulating *sox9* expression. *Sry* starts a feedback loop between *sox9* and *fgf9*, increasing the expression of both genes which at the same time suppress the ovarian pathway through the inhibition of the *wnt/β-catenin* signaling pathway (Kim et al. 2006). *Sox9* is involved in another feedback loop with *ptgds*, which synthesizes prostaglandine D2, a signaling molecule which enhances *sox9* activity in Sertoli cells, inducing the expression of other genes like *amh* (Wilhelm et al. 2007a, Moniot et al. 2009). This gene belongs to the transforming growth factor β superfamily and is responsible for the regression of Müller ducts in the XY gonad (Shen et al. 1994), but also works as a negative regulator of aromatase, a key female enzyme responsible of conversion of androgens into estrogens (Le Page et al. 2010). Mice $amh^{-/-}$ present Müller ducts but develop normal testis, so *amh* does not seem to have an essential role in male gonad development in mammals (Behringer et al. 1994). *Sf1*, *gata4* or *wt1* are also involved in the regulation of *amh* (Wilhelm et al. 2007b). Sertoli cells also regulate the differentiation of Leydig cells, through the signaling activity of Desert Hedgehog gene (*dhh*; Yao et al. 2002) and the growth factor *pdgfa* (Brennan et al. 2003). Leydig cells produce testosterone, the male sex steroid hormone, which is synthesized by steroidogenic enzymes many of which are regulated by *sf1* as previously mentioned.

Other two *sox* genes are involved in male gonad development, *sox8* and *sox10*, which are structurally very similar to *sox9* and seem to reinforce and compensate its action if needed (Chaboissier et al. 2004). The DM gene family is also involved in sexual differentiation in a very wide range of species from corals or insects to fish and mammals (Miller et al. 2003, Raymond et al. 2000) and *dmrt1*, sex determining gene in birds and medaka, belongs to this family. In mammals, $dmrt1^{-/-}$ mutants presented gonad defects only after birth and so it is not critical for gonad development (Raymond et al. 2000).

3.1.4. Female gonad differentiation

Wnt/β-catenin signaling is the key female differentiation pathway which drives gonad differentiation when it is not repressed by *sox9*/*fgf9*. Mutations affecting this pathway lead to the masculinization of XX gonads and the overexpression of some of its genes can modify the fate of an XY gonad by promoting ovarian development (Maatouk et al. 2008). One of the tasks of the *wnt* pathway is to inhibit *sox9*, which has to be constantly repressed in an XX gonad. So, sex determination seems to be governed by a balance between two antagonistic pathways, *sox9*/*fgf9* for male development and *wnt/β-catenin* for female development (Kim et al. 2006).

Wnt4 is a member of the wingless family (*wnt*) which activates signaling in gonadal development (Maatouk et al. 2008) and so, it is important for sex determination (Kim et al. 2006) and female development (Vainio et al. 1999). *Wnt4* is also necessary for the initial

development of Müller ducts in both sexes (Vainio et al. 1999). It is also responsible for the inhibition of male specific processes in the XX gonad, working as an antagonist signal to *fgf9* and *sox9* (Kim et al. 2006). *Wnt4* works together with R-spondin 1 (*rspo1*) to regulate *ctnnb1*, which codifies for *β-catenin*, the final efector of the *wnt* pathway (Capel, 2006). *β-catenin* produces sex reversal in XY gonads, inhibiting the expression of male specific genes like *sox9* or *amh* and promoting the expression of specific female genes. On the contrary, if *β-catenin* is not expressed in XX gonads, ovary inducing genes are inhibited, but the expression of testis specific genes does not happen and so there is no sex reversal (Manuylov et al. 2008).

Another important gene in female differentiation is *foxl2*, which is a transcription factor exclusively expressed in the ovary. *Foxl2* acts directly, together with estrogen receptors α and β, to repress *sox9* during adulthood (Uhlenhaut et al. 2009). *Foxl2* is also implicated in estrogen synthesis regulation through the activation of aromatase (*cyp19a1*; Pannetier et al. 2006), which catalyzes the last steps of estrogen biosynthesis from androgens. The loss of *foxl2* does not impair sex determination or gonad differentiation until the perinatal stage (Ottolenghi et al. 2005).

3.1.5. Mammalian sex differentiation: conclusions

Despite mammalian sex determination relies upon a conserved mechanism with *sry* as a central element, the antagonism between *sox9*/*fgf9* and *wnt*/β-*catenin* pathways works as a threshold mechanism which can alter the fate of the gonad. However, in mammals the expression of these genes is so controlled, probably due to both tight genetic regulation and mammalian homeostasis, that when their expression is abnormal, in humans, we usually speak of disorders. Yet, the threshold mechanism is present.

3.2. Sex differentiation in other vertebrates

All described genes (*sox9*, *wt1*, *dmrt1*, *amh*, *sf1*, *foxl2*, …) have homologues in a wide spectrum of vertebrate species and they are apparently also involved in gonad differentiation, however, its function and importance might vary from one species to another.

For instance, *amh* expression precedes that of *sox9* both in chicken and alligator during early sex differentiation (Shoemaker and Crews, 2009; Smith and Sinclair, 2004), and in medaka *sox9* is not necessary for the formation of testis (Nakamura et al. 2008, 2012). Another example, despite *wnt4* antagonizes the expression of *fgf9* in mammals (Kim et al. 2006, Matson et al. 2011), this gene does not exist in fish (Forconi et al. 2013) and in birds it does not show a dimorphic expression pattern (Cutting et al. 2013). These observations question not only the importance of *fgf9* in sex determination in other vertebrates, but also the antagonism of *wnt4* and its signaling pathway. For example, *rspo2* is not expressed during medaka sex determination (Herpin et al. 2013) and *wnt4* is not expressed in a sexually dimorphic fashion in the early stages of gonad development in the same species (Oshima et al. 2005). An even more striking observation is the role of aromatase in East African cichlids. In

this group of fish, *cyp19a1* pattern is not consistent with its female role, since its expression was also detected in testis (Böhne et al. 2013). *Cyp19a1* expression has also been detected in rainbow trout testis, suggesting a role in male gonad (Kotula-Balak et al. 2008).

The examples of genes which belong to the sex differentiation cascade whose expression does not match that of mammals are numerous. Here, a few have been cited to illustrate that sex differentiation is not as conserved as traditionally assumed and so, its study in the different species can help us understand how gonad fate is determined.

4. Turbot

Turbot is the target species of this study; it is a flatfish with aquaculture importance.

4.1. Taxonomy

Turbot (*Scophthalmus maximus*, Linnaeus 1758) is a marine flatfish of the *Scophthalmidae* family (Order *Pleuronectiformes*). Nowadays, two classifications are accepted for turbot, *Scophthalmus maximus* and *Psetta maxima* (Froese and Pauly, 2006), so the use of one or other depends on the preferences of the author. Taking into account the small genetic distance based on allozymes (Bouza et al. 1997), the high karyotypic similarity (Pardo et al. 2001), and the hybridization observed between *S. maximus* and *S. rhombus*, we shall use the specific name *Scophthalmus maximus* as suggested by Hermida et al. (2013). Turbot complete taxonomic classification is as follows:

Superclass: *Gnathostomata*

Class: *Osteichthyes*

Subclass: *Actinopterygii*

Infraclass: *Actinopteri*

Superdivision: *Neopterygii*

Division: *Halecostomi*

Subdivision: *Teleostei*

Order: Pleuronectiformes

Suborder: *Pleuronectoidei*

Family: Scophthalmidae

Genus: *Scophthtalmus*

Species: *Scophthalmus maximus*

4.2. Biology

Turbot is a marine benthic species which inhabits sandy or rocky sea floors in a range of 20 to 100 meters depth. It is characterized by a flat body, almost circular, without bilateral symmetry. Both eyes are protruding on the left side of the fish. The skin does not present scales; instead it has rough bone bulges irregularly distributed on its dorsal part. The ventral part of the fish is white while the dorsal color is regulable, ranging from gray to brownish depending on the floor in order to provide camouflage. Turbot growth rate is one of the highest among flatfish, growing approximately 30 cm every three years. Turbot can live up to 25 years reaching one meter length and twelve kilograms weight (Froese and Pauly, 2006).

Figure 4. Turbot

Turbot distribution goes from the Baltic Sea to the Arctic Ocean all along the West coast of Europe (Blanquer et al. 1992). Turbot are carnivore, adult fish diet is based only in other teleost fish and cephalopods, however younger fish fed of small crustaceans and mollusks (Jones, 1970).

Reproductive season goes from May to July in the Atlantic coasts of the Iberia peninsula, in response to an increasing photoperiod and a raise of water temperatures. During this period turbot migrate from deep waters (50-100 m) to zones nearer to the coast (5-25 m). Females present very high fecundities, with up to a million eggs per kilogram of weight. Larvae hatch after an incubation period of five to seven days; they present bilateral symmetry and have a pelagic life style. Around 40 days later they go through metamorphosis and gain adult-like characteristics (Person-Le Ruyet, 1990).

4.3. Turbot aquaculture

Turbot is a very important commercial species in Europe and recently in China (FAO, 2014). Total turbot aquaculture production in the European Union in 2013 was of 7.721 tons, 14.5% lower than in 2012. The main European producer is Spain with a 88.3% of the production, and particularly Galicia with a 99.2% of Spanish production (APROMAR, 2014). In China turbot production started in 1992 and it is nowadays a very important aquaculture industry with 64000 tons in 2013 (FAO, 2014).

Turbot shows one of the most accentuated growth sex dimorphism among harvested fish species (Imsland et al. 1997). Females largely outgrow males and reach commercial size between four and six months earlier. Besides, males mature around 15-18 months old while females around 24 months (Purdom et al. 1972; Bye and Jones, 1981; Imsland et al. 1997). These data explain the interest of turbot industry in producing all female stocks, since they show higher growth rates, better energy conversion and a later sexual maturation (Penman and Piferrer, 2008).

4.4. Sex related studies in turbot

Sex determination and gonad differentiation define the number of males and females in a population and, so, sex ratios. Due to the mentioned growth sex dimorphism and its commercial interest, knowing the mechanisms of sex determination and differentiation are of the outmost importance in turbot, so an important effort have been devoted to understand sex determination mechanisms in this species.

4.4.1. Cytogenetic studies

Cytogenetic studies in turbot revealed that chromosome number is 2n=44, without any heteromorphic chromosome pair (Bouza et al. 1994; Pardo et al. 2001). Synaptonemal complexes were studied in spermatocytes and oocytes of diploid and triploid turbot (Cuñado et al. 2001), a phase of meiosis where chromosomes are more than 10 fold longer than mitotic chromosomes, and hence, being more resolute to detect unpaired regions associated with the putative sex differential region. No unpaired regions were observed in pachytene, which suggest that the sex determining region in this species is small as occurs in most marine species (Cuñado et al. 2001).

4.4.2. Sex ratio studies

Two gynogenetic families were obtained by UV irradiated sperm fertilization and cold shock, rendering a 1:3 male:female ratio in one of the families and 100% females in the other. Additionally, sex ratio of several triploid turbot families showed a female bias (3:1 female:male; Cal et al. 2006). These results moved these authors to suggest a XX/XY sex

determination system, since all female populations in gynogenetics and a female bias in triploid would be expected with such sytem (Cal et al. 2002, 2006).

Another analysis in 33 turbot families produced by crosses between regular females and genetic females hormonally reversed revealed that 26 families would adjust better to a ZZ/ZW system, although 15 families would fit both ZW and XY systems. In this study also a minor temperature effect on sex ratios was suggested (Haffray et al. 2009).

4.4.3. Sex-association marker studies

A first study using 2050 RAPD primers found four sex-associated markers in turbot. None of these markers allowed to correctly classifying 100% of the fish, but their combined use correctly assigned 90% of the males and 83% of the females (Casas et al. 2011).

Another study using cDNA-AFLPs in gonad, brain and liver was the first approximation to the study of comparative gene expression between sexes in this species. Several sex specific transcripts were found in the three organs. Significant expression differences were found for three genes, two in the gonads (meiosis-specific nuclear structural protein 1 and serie/threonine-protein kinase nek10) and another one in liver (complement component C9), the three being overexpressed in males (Taboada et al. 2012).

A final study using 540 RAPD primers detected a female specific marker. The sequencing and mapping of this marker revealed its linkage with *foxl2* and *wnt4* genes, related to sex development in females (Vale et al. 2014).

4.4.4. Genetic mapping and QTLs

The first turbot genetic map was obtained from 248 anonymous microsatellite markers (Pardo et al. 2007; Bouza et al. 2008). Later centromeres were located in most linkage groups (LG) using half-tetrad meiosis analysis with diploid gynogenetics (Martínez et al. 2008) and the exploitation of the first turbot expressed sequence tags (EST) database (Pardo et al. 2008) enabled to incorporate 31 microsatellite markers associated to ESTs. The integrated genetic map was used to identify chromosome regions associated with sex in several turbot families, a main sex determination region being located in the proximal end of LG5 (Martínez et al. 2009). The highest association was detected with Sma-USCE30 microsatellite and the distance with the sex determining locus was estimated in 1.4 million base pairs (Mb). This marker allowed correctly sexing 98.4% of the offspring of four out of the five analyzed families. Another three minor sex determination QTLs were also found in linkage groups 6, 8 and 21, suggesting that other minor genetic factors may be involved in sex determination. The number of markers in the genetic map was later increased to 496 (Bouza et al. 2012; Hermida et al. 2013) and the number of linkage groups reduced from 24 to 22, matching to the number of chromosomes (Taboada et al. 2014). Suppression of recombination was not found in the sex determination region, indicating that the sex determination system has a recent origin (Taboada et al. 2014).

4.4.5. Candidate genes

Linkage desequilibrium analyses were used to determine the position in the genetic map of several candidate genes related to sex differentiation in order to study their colocalization with sex-related QTLs. Among them, *sox9* and *sox17* were placed in LG21 and colocalize with a minor sex-related QTL. However, no candidate gene was found in the main sex determining region at LG5, despite two important sex differentiation genes, *amh* and *dmrta2*, are placed in LG5 (Viñas et al. 2012). Recently, six genes closely linked to SmaUSC-E30 sex associated marker were identified: *dnaj19*, *atp11b*, *sox2*, *ncbp2*, *dlg1* and *fxr1*. These genes were tested for sex association in a natural turbot population with negative results, suggesting that likely none of them is the sex determining gene (Taboada et al. 2014).

4.4.6. Genomic resources

A 454 sequencing run of tissues belonging to the brain-pituitary-gonad axis identified 1,410 genes related to reproduction, including for example *amh*, *cyp19a* or several *sox* genes. These sequences, together with those related to immune genes coming from Sanger (Pardo et al. 2008) and 454 (Pereiro et al. 2012) sequencing, were integrated in a turbot database and used for the design of a new version of the turbot microarray (Ribas et al. 2013).

Furthermore, turbot genome has been recently sequenced and assembled (Figueras et al, in preparation) and new transcriptome data from RNAseq (Robledo et al. 2014a) have been obtained. The genome is an essential tool for screening candidate regions and is currently being used to further study the sex determining region at LG5 and to refine the turbot transcriptome.

4.4.7. Environmental influences

The only environmental influence studied in turbot regarding sex determination was temperature. Haffray et al. (2009) analyzed six turbot families at three different temperatures (15, 18 and 23ºC) finding that two families presented a higher proportion of females than expected at 23ºC while another family presented more females at 15ºC. Despite the small number of families analyzed, temperature effects seem to be limited and family dependent in turbot and the study of these interactions might be advantageous for aquaculture.

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Aims

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The main objective of this work was to study sex determination and gonad differentiation through gene expression at the critical stages where sex differentiation begins in turbot. Special attention was paid to those genes involved in sex differentiation in other vertebrate species and to their role in turbot and fish in general, in order to assess their functional conservation. Furthermore, due to the great relevance of environmental factors and their interaction with genetic ones on sex determination in fish, another main objective was to study the effect of rearing temperature on the sex differentiation genes in relation to sex ratio. All data obtained in this work will also be framed in the practical application fo obtaining all-female populations by aquaculture industry.

The specific objectives were:

1) To setup the real-time PCR technique in turbot gonads, focusing on reference genes for the precise estimation of gene expression during sex differentiation and also for the validation of microarray experiments.

2) To evaluate the expression profile of several genes along turbot gonad differentiation and their degree of conservation when compared to other vertebrate species. Special attention will be paid to those genes present in the main sex determination region at LG5 or to those with a role in sex determination in other species.

3) To study the global differentiation process leading to the development of the undifferentiated gonad as testis or ovaries through the use of a turbot microarray. This will increase the available information on gonad differentiation in teleosts and also will help to understand the role of several genes during sex differentiation in turbot.

4) To investigate the effect of temperature on the expression of genes involved in sex differentiation. Our aim was to explore the sex ratio differences observed in some families when reared at different temperatures and to evaluate the influence of temperature on gene expression at relevant genes associated with the initial steps of gonad differentiation.

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Chapter 1

Chapter 1. Analysis of qPCR reference gene stability determination methods and a practical approach for efficiency calculation on a turbot (*Scophthalmus maximus*) gonad dataset.

Robledo D, Hernández-Urcera J, Cal RM, Pardo BG, Sánchez L, Martínez P, Viñas A. BMC Genomics 2014, 15: 648.

Analysis of qPCR reference gene stability determination methods and a practical approach for efficiency calculation on a turbot (*Scophthalmus maximus*) gonad dataset.

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Abstract

Gene expression analysis by reverse transcription quantitative PCR (qPCR) is the most widely used method for analyzing the expression of a moderate number of genes and also for the validation of microarray results. Several issues are crucial for a successful qPCR study, particularly the selection of internal reference genes for normalization and efficiency determination. There is no agreement on which method is the best to detect the most stable genes neither on how to perform efficiency determination. In this study we offer a comprehensive evaluation of the characteristics of reference gene selection methods and how to decide which one is more reliable when they show discordant outcomes. Also, we analyze the current efficiency calculation controversy. Our dataset is composed by gonad samples of turbot at different development times reared at different temperatures. Turbot (*Scophthalmus maximus*) is a relevant marine aquaculture European species with increasing production in the incoming years. Since females largely outgrow males, identification of genes related to sex determination, gonad development and reproductive behavior, and analysis of their expression profiles are of primary importance for turbot industry. We analyzed gene stability of six reference genes: *rps4*, *rpl17*, *gapdh*, *actb*, *ubq* and *b2m* using the comparative delta-CT method, Bestkeeper, NormFinder and GeNorm approaches in gonad samples of turbot. Supported by descriptive statistics, we found NormFinder to be the best method, while on the other side, GeNorm results proved to be unreliable. According to our analysis, *ubq* and *rps4* were the most stable genes, while *b2m* was the least stable gene. We also analyzed the efficiency calculation softwares LinRegPCR, LREanalyzer, DART and PCR-Miner and we recommend LinRegPCR for research purposes since it does not systematically overestimate efficiency. Our results indicate that NormFinder and LinRegPCR are the best approaches for reference gene selection and efficiency determination, respectively. We also recommend the use of *ubq* and *rps4* for normalization of gonad development samples in turbot.

1. Introduction

The main quantitative method for the study of gene expression is reverse transcription real-time quantitative PCR (qPCR), which is considered a highly sensitive technique. In qPCR, the amount of amplified product is monitored during the course of the reaction by measuring the fluorescence during the annealing phase of each amplification cycle. Fluorescence is produced by dyes or probes which bind to DNA, and so it is proportional to the amount of synthesized product. The DNA intercalating dye SYBR green I is one of the most widely applied systems, since the fluorescence readings can be obtained from any PCR amplicon, irrespective of its sequence (Spiess et al. 2008). Two types of qPCR can be performed: the expression levels of the genes can represent either an absolute quantification that relates the PCR signal to the initial copy number using a calibration curve or, as in our work, a relative quantification which measures the relative change in RNA expression level. A number of technical parameters such as RNA and cDNA quality, primer specificity, PCR efficiency and the genes used for normalization heavily condition the quality of qPCR results. Despite the widespread popularity of qPCR, there is a worrying lack of consensus on how it should be performed and how its results should be analyzed. The publication of the MIQE guidelines (Bustin et al. 2009) represented a landmark towards qPCR standardization, but not only are there many publications which still ignore the MIQE guidelines, but also new controversies have arisen which require further discussion.

Due to the quantitative nature of qPCR, an appropriate normalization method is critical to achieve reliable results. The purpose of normalization is to remove sampling noise (such as RNA differences in concentration and its quality) in order to estimate gene expression accurately (Vandesompele et al. 2002). Ideally, reference genes used for this purpose should show the same level of expression in all cells and tissues, and remain stable under different experimental conditions. As pointed out in several publications, there is no universal reference gene, and housekeeping gene expression can vary considerably (Glare et al. 2002), the best reference gene probably varying in the same species according to the tissue and the experimental conditions (Dang and Sun, 2011). So, as mentioned in the MIQE guidelines, normalization against a single reference gene is not recommended unless a clear evidence of its invariant expression is described for the specific experimental conditions of the study. The optimal number and choice of reference genes should be experimentally determined (Bustin et al. 2009), yet many publications employ a single normalization gene without appropriate validation. Several methods and software have been described to determine the optimum reference genes, however which method is the most suitable has still not been addressed.

Four reference gene determination methods are commonly used in qPCR studies: the comparative delta-Ct method (Silver et al. 2006), BestKeeper (Pfaffl et al. 2004), Genorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004). Gene expression stability is evaluated differently in each of the four methods. Briefly, the comparative delta-Ct method calculates the stability of each gene by obtaining the standard deviation of Cq differences (Cq or quantification cycle is the number of amplification cycles required to reach a selected fluorescence threshold) within each sample for each pairwise comparison with the other genes

and averaging them. NormFinder takes into account both intra-group and inter-group gene variation to evaluate its stability. BestKeeper ranks the genes according to the standard deviation (SD) of their Cqs, but the output includes more information, for example the coefficient of variation (CV), which was proposed as a validation method for the results offered by NormFinder and GeNorm (Caradec et al. 2010). GeNorm determines the pairwise standard deviation of Cq values of all genes, and then excludes the one with the lowest stability, repeating the process until only two genes remain, which are then considered the most stable ones.

Another topic, which has recently focused the attention of specialist on this ground, is the kinetics of qPCR and the efficiency determination associated to it. Traditionally, standard curves have been the gold standard to calculate qPCR efficiency. However, pipetting errors or poorly calibrated pipettes can greatly affect the accuracy of the standard curves due to the cumulative nature of error (Peirson et al. 2003; Rutledge and Côte, 2003). Also, cDNA may include PCR inhibitors which diminish the efficiency of the qPCR reaction. These inhibitors often remain in the samples from steps prior to qPCR amplification. The dilution steps involved in standard curve construction, which also dilute inhibitors, might lead to efficiency overestimation (Peirson et al. 2003). This can be easily confirmed by the existence of efficiencies above 100% and the usual practice of accepting a pair of primers as valid if its efficiency is between 90-110%. Theoretically, it is impossible to obtain qPCR efficiencies above 100%. More recently, several mathematical models have been published describing the kinetics of the qPCR reaction and trying to estimate qPCR efficiency from a single reaction. Many different models have been proposed, ranging from exponential (Peirson et al. 2003; Ramakers et al. 2003) to logistic ones employing up to five parameters (Spiess et al. 2008); even more complex models, which take into account the efficiency of each of the steps of the qPCR reaction, have been tackled (Booth et al. 2010). Here we analyzed four methods which allow an easy determination of efficiency for each reaction and amplicon: i) LinRegPCR (Ruijter et al. 2009), ii) LREanalyzer (Rutledge, 2011), iii) DART (Peirson et al. 2003) and iv) PCR-Miner (Zhao and Fernald, 2005), all publicly available and implemented in user-friendly software or online applications.

Marine flatfish represent a valuable group of teleosts because of their highly appreciated white flesh (Cerdà and Manchado, 2013). Turbot is a marine flatfish species with a notable aquaculture projection in Europe. It is predicted that by 2014 its production will duplicate that of 2009 (9142 t) (FEAP). Also, since turbot was introduced in China in 1992, the farming industry of this species has developed into one of the main mariculture industries with a production of 50000 tons per year (FAO, 2010). The main trait targets for genetic breeding programs in this species are growth rate, sex ratio and disease resistance (Bouza et al. 2012). Turbot shows one of the largest sex-dependent size dimorphism in marine aquaculture (Piferrer et al. 1995): females outgrow males by 50% when they reach commercial size. Some studies have demonstrated a ZZ/ZW system in turbot (Haffray et al. 2009; Martínez et al. 2009) and identified the main sex determining region in linkage group (LG) 5 (Martínez et al. 2009), but these authors also suggest the existence of other minor genetic and environmental

factors, for example temperature, which might affect sex determination. However, expression analyses have only been carried out in immune tissues so far (Millán et al. 2011; Pardo et al. 2012; Domínguez et al. 2013). Reference genes for qPCR have been characterized in different tissues of turbot (Dang and Sun, 2011) and in other flatfish (Øvergård et al. 2010; Infante et al. 2008), but gonads have not been included in these studies.

In this study, we evaluated the main factors which might compromise qPCR results, reference gene choice and qPCR efficiency determination, using gonads of turbot reared at different temperatures and along the development process. Our results suggest that for research purposes, NormFinder and LinRegPCR implement the best approaches for reference gene selection and efficiency determination, respectively, ant that *ubq* and *rps4* would be the best reference genes for the normalization of gonad development in turbot from 30 up to 135 days post fertilization. To our knowledge, this is the first qPCR evaluation in turbot gonads and no similar studies have been carried out in fish to date. Our approach, although applied in a particular tissue in turbot could be used as a guideline for qPCR development in other tissues or species.

2. Methods

2.1. Rearing conditions and sampling

Turbot fertilized eggs were obtained by crossing one female with two males and reared in tanks at the Instituto Oceanográfico de Vigo at three different temperatures (15°C, 18°C and 23°C). The samples were taken at the following stages: 30, 45, 60, 75, 90, 105, 120 and 135 days post fertilization (dpf). At each sampling point 10 individuals were taken per temperature (3x10) and their gonads excised as accurately as possible. The final number of samples tested was 240: eight different developmental stages, thirty gonad samples per stage (ten per each temperature). Samples were immediately embedded in RNAlater for preservation (Qiagen, Valencia, CA). Male and female gonads can be differentiated at 90dpf by histology (Cal R, Lluch N, Martínez P. Gonadal sex differentiation in turbot (*Scophthalmus maximus*). Also, *cyp19a1a* raw expression values by qPCR can perfectly distinguish females from males starting at 105 dpf (Supplementary Figure 1).

Animals were treated according to the Directive 2010/63/UE of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for experimentation and other scientific purposes. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain).

2.2. RNA isolation and cDNA synthesis.

Total RNA was extracted by homogenization in TRIZOL (Invitrogen, Paisley, UK) following the manufacturer's protocol. Total RNA was treated with RNase-free Recombinant DNase I(Roche Diagnostics, Mannheim, DE) and RNA concentration was assessed by spectrophotometry and its quality checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, US). Total RNA (1.2 µg) was reverse transcribed by random primers using AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) following the manufacturer's protocol and then diluted 1:2 with nuclease-free water.

2.3. Real-time PCR

Real-time PCR was performed on a Stratagene Mx3005P (Agilent Technologies) thermocycler using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix in a final volume of 12.5 µL following the manufacturer's protocol with 1µL of cDNA per reaction. Gene-specific primers for the reference genes *rpl17* (Ribosomal Protein L17), *b2m* (Beta-2-microglobulin) and *actb* (beta-actin) were obtained from (Dang and Sun, 2011) and primers for *ubq* (Ubiquitin), *rps4* (Ribosomal Protein S4) and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) were designed in our laboratory (Table 1). Specificity for each primer pair was first checked by melting curve profile and then confirmed by PCR product sequencing. *rpl17*, *b2m* and *actb* were chosen as putative reference genes because they were among the most stable genes in a previous study using different tissues in turbot (Dang and Sun, 2011), while *ubq*, *rps4* and gapdh were chosen because of their general use in many studies in other species and proved to be stably expressed in a microarray study carried out in our laboratory (unpublished data). Gene specific primers were also designed in our laboratory for six target genes involved in sex differentiation (*cyp19a1a, amh, sox9, sox19, sox17 and vasa*) (Table 1), and amplification was performed following the same procedure. Primer concentration was 300nM and each sample was run in duplicate. The cycling parameters were: 50°C for 2min, 95°C for 10min, followed by 40 cycles of amplification at 95°C for 15sec and 60°C for 1min. Finally, a dissociation step was performed after amplification to ensure the presence of a single amplification product. All the samples (240) were assayed for each gene. A sample maximization strategy was carried out, meaning that as many samples as possible were run in a single plate, and so, each gene was tested in the minimum amount of plates as possible. In every PCR plate, non-template controls were included to confirm the absence of contamination. In addition, three samples (interplate calibrators) were run in triplicate in every plate in order to correct inter-assay variation, each Cq value in a plate was corrected by adding or subtracting the difference between interplate calibrators mean value in the plate and their overall mean value for all the plates (Kubista et al. 2007). Real-time PCR data were obtained by the MxPro software (Agilent Technologies) and quantification cycle values (Cq) calculated for each replicate and then averaged to obtain the final Cq value. Cq determination

fluorescence threshold was the same for the six genes, a background-based threshold was determined for the six genes separately and the highest one applied for the six genes.

Table 1: Primer table

Gene name	Accession ID	Primer F (5' -> 3')	Primer R (5' -> 3')	Product Length (bp)
rps4	FE943956	CAACATCTTCGTCATCGGCAAGG	ATTGAACCAGCCTCAGTGTTTAGC	143
rpl17	DQ848879	ACCAGTGCGTCCCCTTCA	CTCATCTTCGGAGCCTTGTTC	214
gapdh	FE950888	CGCCCATAGCCCAGTCATAGC	TGGCAGAGGGAGGTGGAGAG	167
actb	EU686692	GTAGGTGATGAAGCCCAGAGCA	CTGGGTCATCTTCTCCCTGT	204
ubq	FE946708	GCGTGGTGGCATCATTGAGC	CTTCTTCTTGCGGCAGTTGACAG	124
b2m	DQ848854	CTCTGGCTGTTTTCGTCTGCT	TCCTTTCCGTTCTCTCCCG	86
cyp19a1a	JQ403643	CAGCGAGGAAGCTGGCAAACA	ACACGCAGACTCGGCTTTTTACATC	148
amh	JQ403642	CCAGGGCGGACCCCGATAAC	TGGCTGTGTTTGGACCCACGAG	99
sox9	JQ300535	ATCAGTACCCACACCTGCATAAC	TCAGCCTCCTCCACGAACG	103
sox19	JQ403639	ACCGAGCGGTTTGTGCCTTG	TCCTCTGGATGCAGTGCTGATTGT	122
sox17	JQ403638	TGTTCGGGAAGCAGGTGAAAGGT	CTTGTTGCCATTTTAGGGGACAGT	92
vasa	JX235364	CTTAGCTGTGGGCGTGGTGGG	ACGTTCTCCTGGCACATCAACG	190

Gene name, accession number, primer sequences and amplicon size of the reference genes (*rps4, rpl17, gapdh, actb, ubq, b2m*) and the target genes (*cyp19a1a, amh, sox9, sox19, sox17, vasa*) are shown.

2.4. Reference gene analysis

A total of six reference genes were selected for gene expression analysis in turbot gonad (Table 2). Their stability was analyzed with the comparative delta-Ct method (Silver et al. 2006), BestKeeper (Pfaffl et al. 2004), GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004), which use different approaches to establish gene stability, but in all of them, the lower the value the more stable the gene is. R program v. 3.0.2 (http://www.r-project.org) with the packages "psych", "gclus" and "fBasics" was used for other statistic operations and graphic generation. Comparisons between methods were performed with the whole data set and also with subsets of samples. We compare 25 subsets with 3 samples per

experimental group (72 samples in a total of 24 groups) and 25 subsets with 2 samples per experimental group (48 samples in a total of 24 groups) to assess robustness of each method. Furthermore, six target genes involved in sex differentiation were subjected to normalization by different reference gene combinations.

2.5. Efficiency analysis

Efficiency of each primer pair was checked for each reference gene by four different methods: LinRegPCR (Ruijter et al. 2009), LREanalyzer (Rutledge, 2011), DART (Peirson et al. 2003) and PCR-Miner (Zhao and Fernald, 2005). Each method calculates individual efficiency values for each qPCR reaction and then, these are averaged to obtained mean efficiency values for each gene. Raw fluorescence values (without baseline correction) were used as input for each efficiency determination method.

2.6. Normalization and efficiency correction on target genes

Efficiency corrected Cq values by LinRegPCR and PCR-Miner were obtained for the six target genes, following the formula "efficiency-corrected Cq = Cq * (log(E) / log(2))" (Kubista et al. 2007). These corrected Cqs were then normalized by *ubq+rps4* and *b2m* and then mean centered. This produced four datasets. Mean and standard deviation were obtained for temperature (high, normal and low) and sex (male and female) groups for each gene in each dataset.

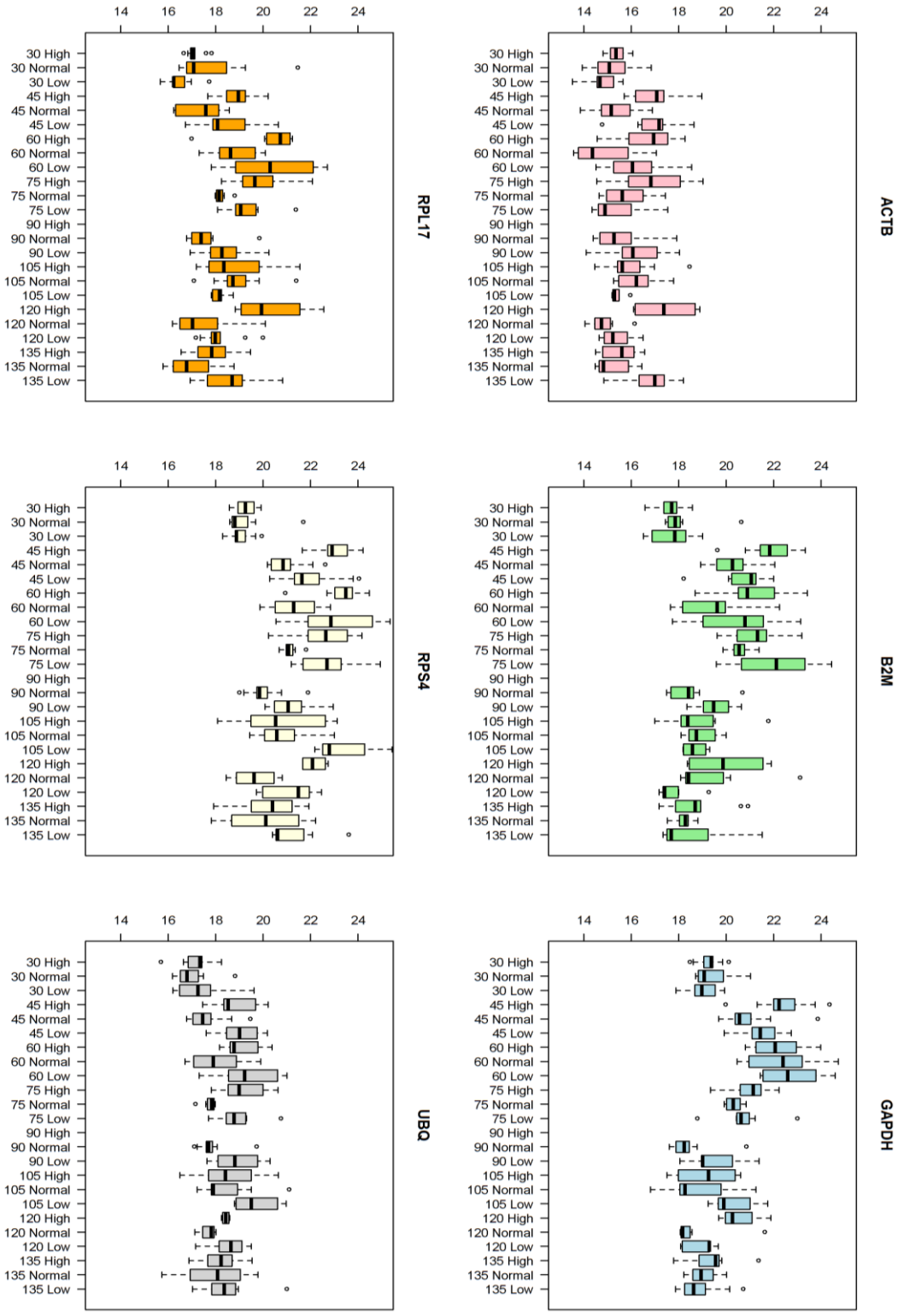
3. Results

3.1. Amplification

Amplification of each reference gene in 240 samples (two replicates per sample) produced a 480 Cq values dataset. Samples with missing Cq values or inconsistencies between replicates (Cq differences >1 cycle) in any of the reference genes were removed from the analysis. After averaging duplicates a total of 212 samples were kept (28 samples were removed) and we obtained descriptive statistics and Kolmogorov-Smirnov tests to check for normality for each of the assayed genes (Table 2). A single amplification product for each primer pair was confirmed by a single peak in the melting curve analysis and also by PCR product sequencing.

actb showed the highest expression (Cq mean = 15.87), amplification being more than two cycles earlier than any other gene. On the other side, *rps4* showed the lowest expression (Cq mean = 21.21). *ubq* standard deviation (SD) was the lowest (1.12) while *b2m* presented the largest variation between Cq values (SD=1.70). Also, reference gene Cq distributions were normal in every case but that of *b2m* (Kolmogov-Smirnov test p=0.009).

Figure 1. Reference gene Cq value distributions



Boxplots of the Cq values in each experimental group (fish age /temperature) for each of the six reference genes. Each group is named with a number, which indicates age in days post fertilization, and either "High", "Normal" or "Low" which indicates rearing temperature.

Table 2. Descriptive statistics of the reference genes Cq values

Gene	N	Mean	SD	Min Cq	Max Cq	KS-test p
actb	212	15.87	1.21	13.52	19.02	0.197
b2m	212	19.50	1.70	16.51	24.44	0.009
gapdh	212	20.18	1.65	16.81	24.72	0.477
rpl17	212	18.44	1.46	15.67	22.70	0.130
rps4	212	21.21	1.65	17.82	25.43	0.739
ubq	212	18.34	1.12	15.68	21.09	0.108

Number of samples (N), mean, standard deviation (SD), minimum Cq value (Min Cq), maximum Cq value (Max Cq) and p value of the Kolmogorov-Smirnov test (KS-test p) are shown for each candidate reference gene.

According to the experimental design, samples were divided in groups according to fish age in days post fertilization (dpf) and rearing temperature. This produced a total of 24 groups (8 age groups x 3 temperatures), with a minimum of six samples per group and a maximum of ten. A boxplot of all the groups for the six reference genes can be observed in figure 1. We also considered grouping our samples by degree-days, however, since groups remained basically the same (only two age-temperature groups would merge, so the number of groups would change from 24 to 23), which did not alter neither the results nor the discussion, we decided to name the groups by their age and rearing temperature since we considered it clearer (see Supplementary Tables 1 and 2).

A similar dataset of Cq values for six sex differentiation related genes was obtained and their descriptive statistics are presented in table 3. These genes are involved in gonad differentiation and were used to check normalization and efficiency correction effects.

Table 3. Descriptive statistics of the genes involved in gonad differentiation Cq values

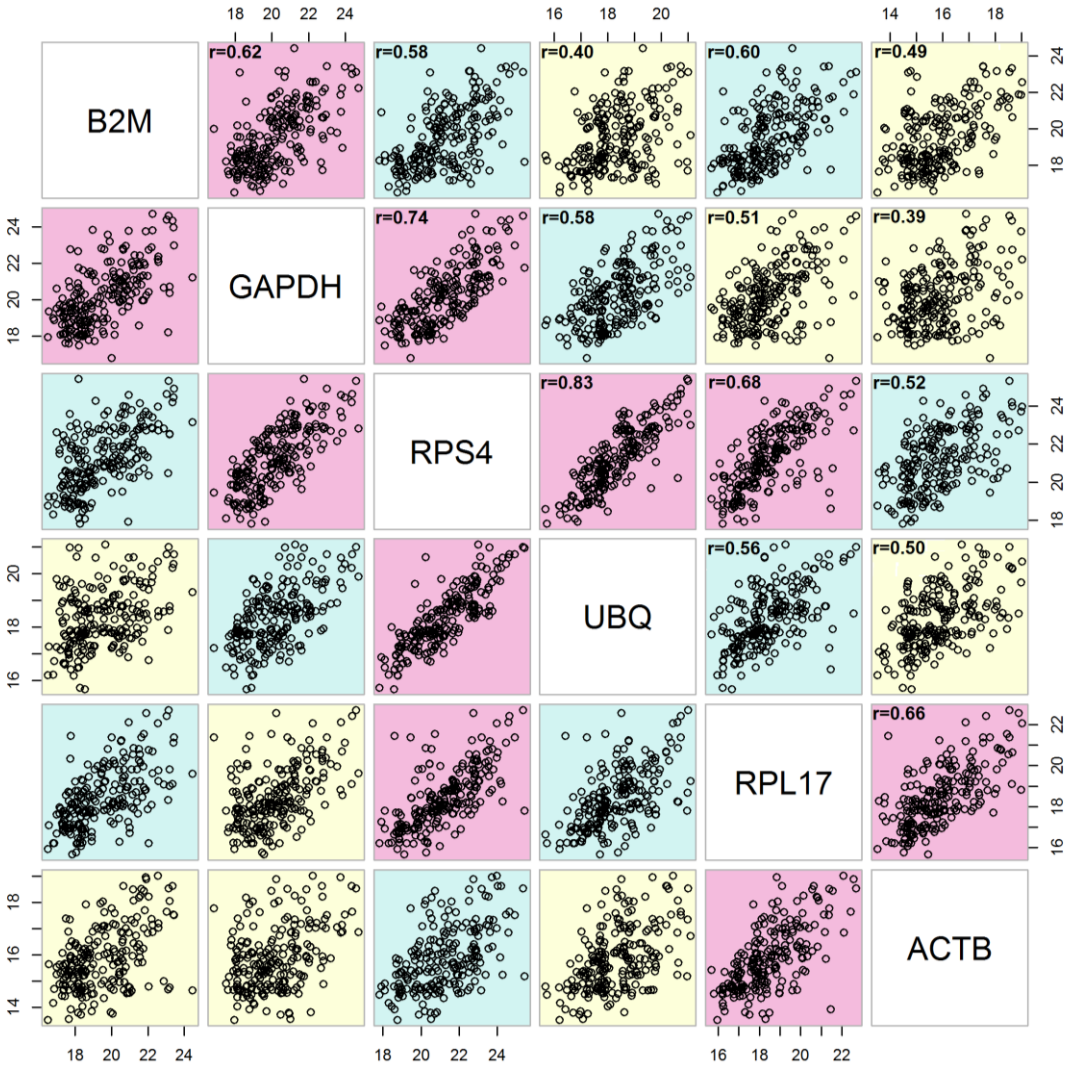
Gene	N	Mean	SD	Min Cq	Max Cq	KS-test p
cyp19a1a	224	31.87	5.35	20.44	40	0.003
amh	224	26.34	2.84	19.75	40	0.000
sox19	224	26.58	3.58	16.75	38.73	0.000
sox9	224	24.77	1.98	21.24	30.89	0.000
vasa	224	26.17	4.25	16.78	35.90	0.000
sox17	224	29.39	2.78	20.34	36.24	0.001

Number of samples (N), mean, standard deviation (SD), minimum Cq value (Min Cq), maximum Cq value (Max Cq) and p value of the Kolmogorov-Smirnov test (KS-test p) are shown for each candidate reference gene

3.2. Analysis of the reference genes

We analyzed the 212 Cq values obtained for each of the reference genes with comparative delta Ct method, Bestkeeper, NormFinder and GeNorm. For each method and gene a ranking of stability values is shown with the most stable gene at the top and the least stable at the bottom (Table 4). Due to the importance of gene-to-gene correlations for comparative delta-Ct method and GeNorm, correlations were graphically represented (Figure 2). Finally, the average intergroup and intragroup variation for each gene is shown in table 5 as reported by NormFinder. Groups were formed as specified above according to fish age and rearing temperature.

Figure 2. Correlation between reference genes



Correlation between reference genes Cq values. The highest correlations are colored in red, medium correlations in green and the lowest in yellow. Correlation coefficient (r) values are shown, p value < 0.001.

Table 4. Stability rankings obtained with the different reference gene determination methods

Rank	Comparative Delta-Ct	BestKeeper (SD)	BestKeeper (CV %)	NormFinder	GeNorm
1	*ubq* (1.267)	*ubq* (1.12)	*ubq* (4.96)	*rps4* (0.613)	*ubq* / *rps4*
2	*rps4* (1.278)	*actb* (1.21)	*actb* (6.16)	*ubq* (0.713)	
3	*rpl17* (1.323)	*rpl17* (1.46)	*rpl17* (6.34)	*rpl17* (0.721)	*rpl17* (1.154)
4	*actb* (1.381)	*rps4* (1.65)	*rps4* (6.43)	*actb* (0.785)	*actb* (1.202)
5	*gapdh* (1.431)	*gapdh* (1.66)	*gapdh* (6.66)	*gapdh* (0.85)	*gapdh* (1.290)
6	*b2m* (1.52)	*b2m* (1.70)	*b2m* (7.43)	*b2m* (0.851)	*b2m* (1.367)

Stability values obtained by each method are shown in parenthesis for each candidate reference gene. Both standard deviation (SD) and coefficient of variation (CV) rankings are shown for BestKeeper. The genes are ranked from most stable (1) to least stable (6).

In our analysis, *ubq* appears ranked first by BestKeeper, GeNorm and comparative delta-Ct method, and ranked second by NormFinder. *b2m*, a frequently used reference gene, is consistently ranked the last by all methods and does not show a strong correlation with any of the other genes (highest correlation 0.619 with *gapdh*) (Table 4, Fig. 2). This gene Cq distribution also deviated from normality. The inconsistency of *b2m* as reference gene has also been previously reported in human tissues (Silver et al. 2006; Sorby et al. 2010). The ranking between these two extremes varies depending on the method.

Table 5. Intra-group and inter-group variation estimates by NormFinder

Variation	*actb*	*b2m*	*gapdh*	*rpl17*	*rps4*	*ubq*
Intra-group	0.491	0.983	0.476	0.631	0.466	0.362
Inter-group	0.553	0.635	0.624	0.485	0.392	0.504

Average intra-group and inter-group Cq variation estimates obtained by NormFinder. Groups were constituted by fish of the same age and rearing temperature.

BestKeeper ranked *ubq* as the most stable gene with 1.12, a value above the recommended cutoff of 1 (Pfaffl et al. 2004). However, considering that our dataset includes samples coming from different tissues (ovary and testes), experimental conditions and development stages, a low standard deviation was not expected. *ubq* also shows the lowest CV and a high correlation with *rps4* (r=0.831). *Actb* is ranked as the second best reference gene by BestKeeper (SD=1.21), however, it does not show a high correlation with any of the other genes and it is ranked 4th by other methods (Table 4, Fig. 2). A possible explanation for this discrepancy might be the high expression shown by *actb* (mean Cq 15.87, more than two cycles higher than any other gene) (Table 2). This renders a lower error when measuring the fluorescence values and a lower copy number difference between samples, which does not imply higher stability between the different experimental conditions. This should be taken into account when choosing a reference gene since they are usually highly expressed genes. The most important characteristic of a reference gene is that its sample-to-sample variation must be representative of the technical error produced by the sampling, extraction and retrotranscription steps in order to reduce target gene Cq values error. Bestkeeper ranks *rpl17* third (SD=1.46), *rps4* fourth (SD=1.65) and *gapdh* fifth (1.66) (Table 4). An interesting fact is that only *rps4* shows high correlations with other genes (*ubq*: r=0.831, *gapdh*: r=0.741 and *rpl17*: r=0.677) (Fig. 2). Like *actb* but at the other extreme, *rps4* shows the lowest amplification cycle (Table 2), which can result in higher technical error. So, according to BestKeeper, *ubq* is the most stable gene, followed by *actb* and *rpl17*. As mentioned, probably *actb* is not a good reference gene and it seems that *rps4* might deserve a better ranking given its correlation with other genes.

NormFinder, which assesses inter-group variation (systematic differences due to age/temperature in our case) in order to discard regulated genes, ranked *rps4* as the most stable gene (0.613), *ubq* appears second (0.713) and *rpl17* third (0.721) (Table 4). *rps4* showed the lowest inter-group variation (0.392), which explains its ranking (Table 5). *gapdh* is clearly pointed as less stable than *rps4* and *rpl17* by NormFinder, with an overall score of 0.835 and an inter-group variation of 0.624. Surprisingly, NormFinder suggests *actb* and *rps4* as the most stable two gene combination. *actb* is ranked 4th by NormFinder (0.785) (Table 4, Fig. 2). The information provided by BestKeeper and NormFinder should be enough to decide among the most stable genes, to say, those with an acceptable low level of overall variation, low inter-group variation and high correlation between them.

The last two methods, comparative delta-Ct method and GeNorm, both follow pairwise approaches but with different procedures and outcomes; the first one ranks the genes following an average pairwise SD while the second follows a progressive exclusion of the least stable gene after pairwise comparison. Both methods agree with the results obtained by NormFinder. GeNorm recommends the couple of reference genes *ubq*/*rps4* with a value of 0.952 (the generally accepted cutoff value is 1.50), and points that adding another reference gene (*rpl17*) would not improve normalization (1.154 stability value for *ubq/rps4/rpl17*). Pairwise comparison methods tend to select those genes with the highest degree of similarity across the sample set, implying that the candidates with minimal expression variation do not

necessarily become top ranked (Andersen et al. 2004). While both approaches are based in pairwise comparisons, the progressive exclusion of genes by GeNorm increases the tendency to select the most correlated genes.

Since we have obtained inter-group variation estimates by NormFinder (Table 5) which points toward *rps4*, *ubq*, *rpl17* and possibly *actb* not being differentially expressed between groups, and also due to the unexpected presence of *actb* in the best normalization indexes calculated by NormFinder, we checked how the use of different normalization factors, geometric mean of different reference genes, affected six sex-differentiation related genes (*cyp19a1a*, *amh*, *sox19*, *sox9*, *vasa*, *sox17*) (Table 6). We normalized the samples by [*ubq]*, [*rps4*], [*ubq+rps4*], [*rps4+actb*], [*ubq+rps4+rpl17*], *[ubq+rps4+rpl17]* and [*ubq+rpl17+rps4+actb*]. We checked the intragroup and intergroup standard deviation for each of the six target genes and, since the samples were genetically sexed using the SmaUSC-E30 marker according to (Martínez et al. 2009), we also checked the standard deviation of male and female groups (Table 6). This way, we can have an independent measure out of the fish age / rearing temperature groups we have used to check the stability of the reference genes. Interestingly, *ubq* and *rps4* seem to behave differently. While *rps4* renders lower SD values for fish age / rearing temperature groups, *ubq* normalizes male and female groups better. However, the lowest SD estimates were obtained when both [*ubq+rps4*] were used for normalization, except for average intergroup SD where *rps4* alone performed better. The addition of *rpl17* or *actb* in the index for normalization did not yield lower SD estimates. The use of just [*ubq+rps4*] for normalization is in agreement with the results of GeNorm and also with the rankings produced by NormFinder and comparative delta-Ct method. The use of [*rps4+actb*] as suggested by NormFinder does not perform better.

Table 6. Standard deviation for target genes when normalized by different gene combinations

Gene combination for normalization	Average intragroup SD	Average intergroup SD	Average male group SD	Average female group SD
ubq	1.53	2.83	2.48	2.57
rps4	1.49	2.69	2.50	2.63
ubq+rps4	1.47	2.71	2.47	2.57
actb+rps4	1.53	2.79	2.63	2.8
ubq+rps4+rpl17	1.50	2.73	2.53	2.66
ubq+rps4+actb	1.48	2.78	2.54	2.68
ubq+rps4+rpl17+actb	1.50	2.74	2.61	2.78

Intragroup and intergroup normalized Cq standard deviations (SD) averaging the results for the six target genes are shown for Fish age + Rearing temperature groups when normalized by different candidate reference gene combinations. Standard deviations (SD) for males and females when normalized by the same combinations are also shown.

To assess the robustness of each method, we repeated the stability calculations in fifty subsets of the samples (Table 7), 25 subsets include 3 samples of each experimental group (fish age / rearing temperature) and another 25 include 2 samples of each experimental group (a total of 72 and 48 samples per subset respectively). We evaluated the 50 subsets together since the results show similar trends both with three and two samples per group. Since in many studies three genes are used for normalization, we compared not only the whole ranking but also the top3 genes. The most robust method is clearly BestKeeper SD, which renders an identical ranking as that obtained with the whole data set for a 44% of the subsets and, in 40% of the remaining subsets, it ranks the top three genes correctly (a total of 88%). NormFinder selected the same top3 genes also in 88% of the subsets, however the rank order was altered most of the times. On the contrary, the pairwise approaches showed a higher degree of variation, the top3 genes were different from those in the full dataset in 60% of the subsets for comparative delta Ct method and in 66% for GeNorm.

Table 7. Robustness of the gene stability determination method

	Full dataset ranking comparison	Comparative delta-Ct	Bestkeeper (SD)	NormFinder	GeNorm
3 samples per experimental group	Identical ranking	4	11	1	8
	Top 3 genes in different order	13	11	21	1
	Different ranking	8	3	2	16
2 samples per experimental group	identical ranking	2	11	4	4
	Top 3 genes in different order	11	9	18	4
	Different ranking	12	5	3	17
Total	Identical ranking	12%	44%	10%	24%
	Top 3 genes in different order	48%	40%	78%	10%
	Different ranking	60%	16%	12%	66%

Similarity of 50 subsets stability rankings by each method and the ranking obtained with the whole dataset. 25 subsets are formed by 3 samples per group (age/temperature) and another 25 subsets have 2 samples per group.

3.3. Efficiency determination analysis

We obtained mean gene efficiencies by LingRegPCR, LREanalyzer, Dart and PCR-Miner for each primer pair (Table 8) and correlations between mean efficiencies by each method for each gene (Table 9). There is around a 10% difference between the efficiencies

calculated by linear fit methods (LinRegPCR, DART) and non linear fit models (LREanalyzer, PCR-Miner), meaning that exponential methods might be underestimating efficiency or non linear methods overestimating it (or both). Two LREanalyzer efficiency estimates are over 100% (*rps4* and *ubq*), which is theoretically impossible for a PCR reaction, so LREanalyzer is likely overestimating qPCR efficiency. However, despite this 10% efficiency difference, mean efficiencies calculated by the four methods are correlated for each gene, indicating that although they are using different algorithms they are rendering similar relative results. Best correlation coefficient and p value are observed between LinRegPCR and PCR-Miner, which might be highlighting the importance of baseline correction since both methods use iterative approaches instead of relying on a fluorescence correction based on the average fluorescence of the first qPCR cycles. Also, LinRegPCR and PCR-Miner include several functions to remove outliers, so filtering the reactions before efficiency calculation might also be important to obtain more precise efficiency estimations.

Table 8. Efficiency values for each gene with each efficiency determination method

	actb	***b2m***	***gapdh***	***rpl17***	***rps4***	***ubq***
LREanalyzer	97.82%	98.00%	99.32%	94.46%	100.45%	101.78%
LinRegPCR	87.12%	90.27%	89.24	82.82%	88.61%	89.63%
DART	88.72%	92.62%	89.09%	86.04%	89.39%	90.84%
PCR-Miner	94.42%	99.72%	99.68%	92.23%	98.78%	99.69%

Mean efficiency values for each reference gene with LingRegPCR, LREanalyzer, DART and PCR-Miner.

Table 9. Correlation between efficiency determination methods

	LREanalyzer	**LinRegPCR**	**DART**
LinRegPCR	0.81 (0.052)		
DART	0.6 (0.205)	0.91 (0.013)	
PCR-Miner	0.82 (0.047)	0.94 (0.005)	0.82 (0.047)

Pearson correlation coefficients and p values (in parenthesis) for mean gene efficiencies with each of the four efficiency determination methods are shown.

Table 10. Efficiency-corrected delta Cqs by temperature group with each efficiency + reference gene combination

	High T Cq Mean	High T Cq SD	Normal T Cq Mean	Normal T Cq SD	Low T Cq Mean	Low T Cq SD
***cyp19a1a ubq+rps4* LinRegPCR**	1.11	4.33	-0.35	3.54	-0.74	5.41
***cyp19a1a ubq+rps4* PCR-Miner**	1.19	4.65	-0.38	3.81	-0.8	5.81
***cyp19a1a b2m* LinRegPCR**	0.78	5.26	-0.89	4	0.24	5.8
***cyp19a1a b2m* PCR-Miner**	0.83	5.66	-0.96	4.3	0.26	6.24
***amh ubq+rps4* LinRegPCR**	0.07	3.39	1.02	4.47	-1.27	1.48
***amh ubq+rps4* PCR-Miner**	0.07	3.65	1.09	4.8	-1.36	1.57
amh b2m LinRegPCR	-0.26	3.67	0.5	4.23	-0.32	2.16
***amh b2m* PCR-Miner**	-0.28	3.94	0.54	4.54	-0.34	2.32
***sox19 ubq+rps4* LinRegPCR**	-0.24	3.99	-0.92	3.83	1.33	2.39
***sox19 ubq+rps4* PCR-Miner**	-0.26	4.28	-0.98	4.11	1.42	2.56
***sox19 b2m* LinRegPCR**	-0.57	5.19	-1.46	3.97	2.31	3.08
***sox19 b2m* PCR-Miner**	-0.62	5.57	-1.56	4.26	2.48	3.31
***sox9 ubq+rps4* LinRegPCR**	0.38	1.82	0.17	1.44	-0.59	1.13
***sox9 ubq+rps4* PCR-Miner**	0.4	1.96	0.18	1.55	-0.64	1.21
***sox9 b2m* LinRegPCR**	0.04	2	-0.37	1.75	0.39	2.05
***sox9 b2m* PCR-Miner**	0.05	2.15	-0.4	1.88	0.42	2.21
***sox17 ubq+rps4* LinRegPCR**	0.58	2.12	-0.6	1.54	0.09	1.59
***sox17 ubq+rps4* PCR-Miner**	0.62	2.26	-0.63	1.65	0.09	1.7
***sox17 b2m* LinRegPCR**	0.25	3.47	-1.13	2.06	1.07	2.39
***sox17 b2m* PCR-Miner**	0.26	3.72	-1.21	2.21	1.15	2.56
***vasa ubq+rps4* LinRegPCR**	1.26	1.46	-0.96	4.04	-0.19	2.6
***vasa ubq+rps4* PCR-Miner**	1.35	1.57	-1.03	4.34	-0.21	2.79
***vasa b2m* LinRegPCR**	0.93	1.91	-1.5	3.8	0.79	2.46
***vasa b2m* PCR-Miner**	0.99	2.05	-1.61	4.08	0.85	2.64

Mean efficiency-corrected delta Cqs and SD values for the three rearing temperatures (T): high, normal and low; in the four datasets produced after efficiency correction with LinRegPCR or PCR-Miner and later normalization with *ubq+rps4* or *b2m*.

3.4. Normalization and efficiency correction on target genes

Six target genes (*cyp19a1a*, *amh*, *sox19*, *sox9*, *vasa*, *sox17*) involved in gonad differentiation were efficiency corrected and normalized by four different combinations of efficiency determination methods and reference gene combinations (LinRegPCR-*ubq+rps4*, LinRegPCR-*b2m*, PCR-Miner-*ubq+rps4* and PCR-Miner-*b2m*). For each combination, first, efficiency correction was performed on every Cq value of both reference and target genes. Afterwards, each target efficiency-corrected Cq value was normalized by the reference gene/s efficiency-corrected Cq values, obtaining efficiency-corrected delta Cq values. We computed mean efficiency-corrected delta Cq values and standard deviations for the three temperature groups (high, normal and low temperature) (Table 10) and also for males and females (Table 11). Two different patterns are shown in the tables, one caused by normalization and the other by efficiency correction. A higher standard deviation is obtained in most of the *b2m* normalized dataset compared to the *ubq+rps4* normalized ones, which is expected when a gene is not stable. However, this is not true for the *amh* normal temperature group neither for the gene *vasa*, suggesting some type of co-regulation. The other trend is observed when comparing the LinRegPCR efficiency corrected datasets with the PCR-Miner corrected ones. PCR-Miner produces higher mean Cqs (absolute value) increasing the difference between groups.

Furthermore, the use of a gene which presents systematic differences between groups for normalization can lead to changes in the mean Cq values of some genes. For example, *amh* gene expression in each temperature group is severely affected by normalization with *b2m*, varying from 0.07 to -0.26 at high temperature (when compared to normalization by *ubq+rps4*), from 1.02 to 0.5 at normal temperature and from -1.27 to -0.32 at low temperature (LinRegPCR values).

4. Discussion

4.1. Reference gene analysis

The four methods commonly used to check the stability of reference genes, comparative delta-Ct method, NormFinder, BestKeeper and GeNorm, represent viable strategies, although none of them is currently considered the best one and some problems can arise in certain experimental scenarios. The BestKeeper method is apparently the “common sense” solution to measure stability since standard deviation is a direct measure of variation. However, a gene might show a low standard deviation but still not be a good reference gene if its variation does not reflect the errors produced by sampling, RNA extraction and retrotranscription steps. This problem could be circumvented by analyzing the correlations between genes, assuming that the reference genes are not co-regulated. This means that sampling point differences (time and temperature in our experiment) affecting one of the genes should not affect the others, and so the correlations between them would reflect the inter-sample variation produced by the sample processing steps and not by co-regulation due to the experimental conditions.

Table 11. Efficiency-corrected delta Cqs by sex group with each efficiency + reference gene combination

	Female Cq Mean	Female Cq SD	Male Cq Mean	Male Cq SD
***cyp19a1a ubq+rps4* LinRegPCR**	-2.82	2.39	4.37	3.17
***cyp19a1a ubq+rps4* PCR-Miner**	-3.03	2.57	4.7	3.41
***cyp19a1a b2m* LinRegPCR**	-3.05	2.67	4.74	3.99
***cyp19a1a b2m* PCR-Miner**	-3.28	2.87	5.1	4.29
***amh ubq+rps4* LinRegPCR**	0.85	3.24	-1.32	3.54
***amh ubq+rps4* PCR-Miner**	0.92	3.49	-1.42	3.8
***amh b2m* LinRegPCR**	0.59	3.26	-0.91	3.69
***amh b2m* PCR-Miner**	0.63	3.51	-0.97	3.95
***sox19 ubq+rps4* LinRegPCR**	-2.31	2.54	3.58	1.25
***sox19 ubq+rps4* PCR-Miner**	-2.47	2.71	3.84	1.34
***sox19 b2m* LinRegPCR**	-2.54	3.73	3.95	1.59
***sox19 b2m* PCR-Miner**	-2.73	4	4.23	1.72
***sox9 ubq+rps4* LinRegPCR**	0.67	1.46	-1.04	0.95
***sox9 ubq+rps4* PCR-Miner**	0.72	1.57	-1.12	1.01
***sox9 b2m* LinRegPCR**	0.44	1.72	-0.68	2.07
***sox9 b2m* PCR-Miner**	0.47	1.85	-0.73	2.23
***sox17 ubq+rps4* LinRegPCR**	-0.98	1.1	1.53	1.62
***sox17 ubq+rps4* PCR-Miner**	-1.05	1.17	1.63	1.73
***sox17 b2m* LinRegPCR**	-1.22	2.35	1.89	2.39
***sox17 b2m* PCR-Miner**	-1.31	2.52	2.03	2.57
***vasa ubq+rps4* LinRegPCR**	-0.17	3.2	0.27	2.87
***vasa ubq+rps4* PCR-Miner**	-0.19	3.44	0.29	3.08
***vasa b2m* LinRegPCR**	-0.41	3.09	0.63	2.98
***vasa b2m* PCR-Miner**	-0.44	3.32	0.68	3.2

Mean efficiency-corrected delta Cqs and standard deviation (SD) values for males and females in the four datasets produced after efficiency correction with LinRegPCR or PCR-Miner and later normalization with *ubq+rps4* or *b2m*.

Nevertheless, it is risky to assume that genes are not co-regulated because this cannot be easily demonstrated. The GeNorm and the comparative delta-Ct method approaches present the same problem but in addition these methods rank genes mainly by their correlations, to say, GeNorm establishes the most stable genes by assuming "that the control reference genes are not co-regulated" (Vandesompele et al. 2002), and the same happens to the comparative delta-Ct method which follows a very similar approach. As a consequence, two co-regulated genes could fully spoil the analysis leading to wrong reference genes. Finally, NormFinder is not affected by the co-regulated gene drawback since it takes into account intergroup variation (finding genes which do not vary depending on time or temperature in our case), which should be as lower as possible for a good reference gene; however, similarly to BestKeeper, a low overall intergroup and intragroup variation does not necessarily mean that it is a good

reference gene. The advantages and disadvantages of each strategy should be taken into account when analyzing putative reference genes according to the experimental scenario.

NormFinder and GeNorm are the most extended methodologies to find the optimum reference genes. In many cases, NormFinder and GeNorm algorithms render very similar results, however, discrepancies between the output of NormFinder and GeNorm have been previously described (Caradec et al. 2010; Chen et al. 2011; Zhang et al. 2012). In these works, the CV has been used to decide which genes should be used for normalization, confirming NormFinder results in every case. While NormFinder results are non-biased, GeNorm stepwise exclusion can lead to awkward results by selecting reference genes which in fact are not the most stable. NormFinder, BestKeeper and comparative delta-Ct stability method results have also been reported to be more consistent among them than with those of GeNorm (Chen et al. 2011), although in other study BestKeeper was reported as the least consistent method (Zhang et al. 2012). Our results with the whole dataset support the high consistency between NormFinder and comparative delta-Ct method, while BestKeeper results seem to be the least consistent and only correlation values between reference genes seem to suggest a similar ranking.

However, when working with different subsets which include a lower number of samples, the pairwise approaches results vary significantly between subsets. This lack of robustness has been described previously: it was shown that the exclusion of a single sample could change the status of one gene from unstable to 2nd most stable gene by GeNorm (Silberberg et al. 2009). GeNorm lack of robustness can most likely be explained by the removal of the least correlated gene by pairwise comparison with all the others until only two genes are left, which can lead to stable genes being removed of the analysis early on. Robustness is a critical parameter. Since experiments are budget limited, it is important to be able to determine correct reference genes with a low number of qPCR reactions. BestKeeper and NormFinder appear to be more robust than comparative delta-Ct method and GeNorm in our study.

There is not a method to check how much normalization has improved our gene expression data. In principle, a reduction in the Cq variability of the gene of interest should be expected, however the highest reduction of this variability would also occur if the gene of interest and the reference gene(s) are co-regulated, so this is a risky strategy. An example of this is observed in *vasa* (and one *amh* group) standard deviation after normalization, obtaining a lower SD when normalized by a clearly not stable gene (*b2m*) than when normalized by *ubq+rps4*, suggesting co-regulation between *vasa* and *b2m*. The same applies to detecting significant/non-significant results depending on the reference(s) gene(s) used for normalization. This is only useful to stress the importance of choosing a good reference gene, not to choose between one or another since a co-regulated reference gene would lead to non-significant results even if there are expression differences between groups.

The fact that there is not a post-control which enables us to check if we have chosen the correct reference gene makes the choice even more critical. Every experiment and dataset is different, so the analysis has to be done carefully. Given the huge importance of

normalization and its great impact on the conclusions, it would be recommended to analyze each case separately, paying attention to details; using any method as a black box can lead up to low confident results. Several studies have solved the disagreement between the four methods by ranking them according to the geometric mean of the four ranking numbers for each gene, the lower the mean a gene gets the most stable it is (Chen et al. 2012). However, attributing the same weight to every method is arguable, especially because some of these methods include redundant information. This is a practical option without any biological meaning. If the four methods disagree, we recommend instead relying on the ranking provided by NormFinder, while ignoring its suggested combination, supported by descriptive statistics like mean, standard deviation and correlations, information offered by BestKeeper or any common statistical package. This approach would enable to assess the two most important and complementary issues: absence of inter-group variation and correlation between reference genes. This approach does not make any previous assumption and has proven to be robust when only a few samples are assayed.

To our knowledge this is the first experiment to analyze the stability of reference genes during the gonad development in fish. Even in mature organs, there is only one study carried out in zebrafish were testis and ovaries were analyzed separately (McCurley and Callard, 2008). However, studies have been carried out in other organs. The stability of several genes was studied in the liver, spleen, kidney, heart, brain, gill and muscle of turbot subjected to *Edwardsiella tarda* infection (Dang and Sun, 2011). Gene stability was checked before infection in all the organs together by NormFinder and GeNorm. In that study, out of eight genes, NormFinder ranked *RPSD* as the most stable one, followed by *actb*, *rpl17*, *b2m* and *gapdh* among those genes shared with our study, although primer pairs for gapdh were different. We tried to develop primers for *RPSD* but they were discarded due to late amplification cycle in gonad (>25). *ubq* and *rps4*, were not assayed in that work. *gapdh*, which has been classically used as a reference gene but recently classified as unstable in several studies (Vandesompele et al. 2002; Glare et al. 2002; Caradec et al. 2010; Sorby et al. 2010), performed badly in both our study and Dang and Sun (2011). However, *gapdh* is ranked as the most stable gene in heart and liver in Dang and Sun (2011), which emphasizes the importance of checking reference gene stability in each study separately, reference genes cannot be "exported". There are two *gapdh* isoforms in diploid teleost fish as a result of the fish-specific genome duplication event, however the same variant has been analyzed in both studies (*gapdh-2*).

There are two similar qPCR studies carried out in flatfish. The first one studied six reference genes during *Hippoglossus hippoglossus* development in sixteen different tissues using BestKeeper, NormFinder and GeNorm (Øvergård et al. 2010). Gonads were not included. They assayed *actb* which was found as one of the least stable genes. The most stable genes found were *EF1a1* and *RPL7*. The second study was carried out during larval development in *Solea senegalensis* and *Hippoglossus hippoglossus*. The stability of twelve genes, including *ubq*, *rps4*, *actb* and *gapdh-2*, was checked by GeNorm and NormFinder (Infante et al. 2008). The combined stability index of the two species ranked *ubq*, *rps4* and

eEF1a1 as the three best normalization genes by both methods, while *actb* appears in 5th and 6th place, respectively. Interestingly, in *Solea senegalensis*, *gapdh-2* appears ranked 3rd and 1st by GeNorm and NormFinder, respectively.

4.2. Efficiency determination analysis

Efficiency determination is an essential step in qPCR. Constant amplification efficiency in all compared samples is a very important criterion for reliable comparison between samples. It is also crucial for an accurate quantification of gene expression. Ideally, the efficiency of an assay should be 100%, which means that during the logarithmic phase of the reaction the PCR product is doubling each cycle.

Each of the four tested efficiency determination methods differ in their baseline fluorescence determination, type of fit to the log-linear phase of the qPCR reaction, and preprocessing steps to remove outliers. DART (Peirson et al. 2003) is based on a linear regression of the exponential phase of the qPCR reaction. Baseline subtraction is determined by fitting a saturation function to the first 2-10 cycles of the qPCR reaction. Then, a linear regression is performed in a 10-fold range around the middle point of the exponential phase, which is calculated using the maximum fluorescence and standard deviation of the fluorescence in the first 10 cycles. LinRegPCR (Ruijter et al. 2009) is also based on a linear regression fit to the log-linear phase of the amplification curve. LinRegPCR determines baseline fluorescence through an iterative algorithm to get the best fit of the linear regression to 4-6 points in the log-linear phase of the reaction. Then, after baseline subtraction, these points are used for efficiency determination. PCR-Miner (Zhao and Fernald, 2005) uses a non-linear regression fit. As LinRegPCR, baseline fluorescence is determined by an iterative fit to a four-parameter logistic model which also determines the exponential phase of the reaction. Then, a three-parameter exponential model is fitted to the exponential phase to determine efficiency. Finally, LREanalyzer (linear regression of efficiency) (Rutledge, 2011) uses a sigmoidal fit approach. Baseline subtraction is determined by averaging 6-12 cycles fluorescence values. Then, efficiency estimates are calculated for each cycle of the qPCR reaction. An LRE window is selected with those cycle efficiencies which fit to a linear regression. Finally, a derivative of the Boltzmann sigmoidal function is used for the calculation of the maximum efficiency of the reaction.

Logistic models are pure empirical models not designed to be kinetically realistic (Lievens et al. 2011) and rely purely in their good fit to the real-time PCR curve. As previously reported by other study, qPCR curves are not symmetric since they do not have the same curvature at both sides of the inflection point, implying the existence of two or more different mechanisms affecting the efficiency of the reaction (Spiess et al. 2008) and so, making this good-fit models hardly reliable. Furthermore, in some reactions SYBR green depletion might be the main mechanism leading to the plateau phase of the curve (Rutledge and Stewart, 2008). While SYBR green has an impact in the visualization of the real-time reaction, it does not have a connection with the kinetics of the PCR reaction.

A recent qPCR study has tried a new approach to assess qPCR efficiency, defining the global efficiency as the sum of denaturing efficiency, annealing efficiency, polymerase binding efficiency and elongation efficiency (Booth et al. 2010). The polymerase binding efficiency and the elongation efficiency can be constant provided that there is an excess of polymerase and a long elongation time. However, the denaturing efficiency is constantly decreasing each cycle at the same rate due to thermal damage in both the DNA and the polymerase. The annealing efficiency is also decreasing and depends on the proportion of ssDNA bound to the primers during this step and total ssDNA present. Some ssDNA chains might bind to its complementary strand instead of to the primers (Booth et al. 2010). This efficiency varies from cycle to cycle. This theoretical study was validated in (Louw et al. 2011). So, at first, a constant efficiency should not be assumed. Still, the qPCR curve shows a large exponential component, since, as confirmed by a previous study, in most cases the best fit to the log-linear region of the qPCR reaction is exponential (Spiess et al. 2008), suggesting that before and at the log-linear region the qPCR efficiency reduction is low.

A recent study analyzed all publicly available efficiency determination methods (Ruijter et al. 2013) in a large dataset, included four-point 10-fold dilution series, which allows calculation of the bias of each method. Similarly to our results, they report LinRegPCR and DART to produce an underestimation of efficiency and PCR-Miner and LRE analyzer an overestimation. They also analyze different parameters and find LinRegPCR and PCR-Miner amongst the best methods for most of the evaluated characteristics, for example precision and resolution, performing better than LREanalyzer and DART. The reader is encouraged to consult (Ruijter et al. 2013) to learn more about the different efficiency determination methods, their characteristics and performance differences. This study (Ruijter et al. 2013) is the most complete on qPCR efficiency determination methods done so far.

Both LinRegPCR and PCR-Miner performed similarly and produced highly correlated efficiency estimates in our study. The main difference was that while LinRegPCR underestimates efficiency, PCR-Miner overestimated it. Knowing this, LinRegPCR is probably the best choice for the average qPCR researcher since it will not produce erroneous significant differences between groups (false positives) or an overestimation of the fold change. However, LinRegPCR might not be the best option for clinical purposes, where the method of choice should be considered depending on the consequences of a false positive / overestimation or a false negative / underestimation. There are a good number of alternative efficiency estimation algorithms, however they are implemented as extensions to the open source statistical programming environment R (http://www.r-project.org) and probably not available for most researchers, so they have not been analyzed here. Still, LinRegPCR and PCR-Miner perform as well or better than all other methods, as shown in (Ruijter et al. 2013).

As a final remark, although (Ruijter et al. 2013) clearly improved our understanding of the different efficiency determination methods available, currently there is not a clear best method for estimating qPCR efficiencies. However, the publication of a theoretical study based on the PCR kinetics which defines the overall PCR efficiency as the product of the efficiency of each of the separate steps (Booth et al. 2010) and its experimental validation

(Louw et al. 2011) is a good step towards finding a biologically meaningful solution. Similar approaches will be likely applied in more studies in the near future provided they are implemented in appropriate user friendly softwares for the whole research community.

4.3. Normalization and efficiency correction on target genes

The effects of normalization with wrong genes are important; a high standard deviation will produce higher p values and, so, possibly lead to missing biologically relevant differences. Even worse, the use of a regulated gene (which shows systematical differences between experimental groups) for normalization, will lead to changes in gene values which can end in misguided results. The effect of efficiency correction, though not so dramatic, is also important since it can lead to overestimation (or underestimation) of differences between groups.

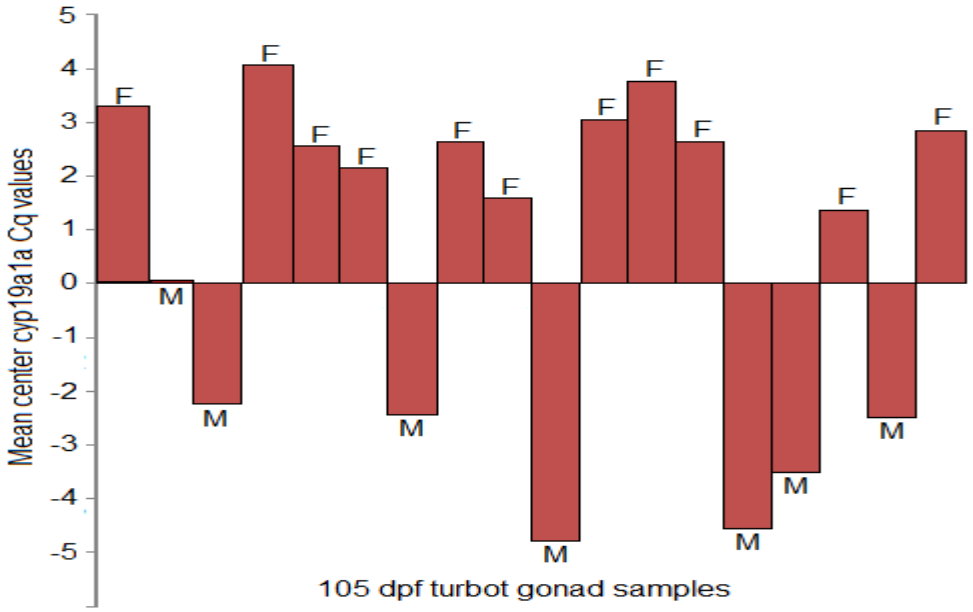
4.4 Concluding remarks

We found the ranking produced by NormFinder method as the most reliable one to choose reference genes for qPCR analysis when results differ between gene stability determination methods. NormFinder information should be complemented by the descriptive statistics offered by BestKeeper, especially the correlation coefficient. Accordingly, we found that *ubq* and *rps4* should be used as reference genes to study turbot gonad development from 30 up to 135 days post fertilization. We found pair-wise methods to be less robust than NormFinder and BestKeeper and also the suggested NormFinder two genes combination not reliable. We also recommend the use of LinRegPCR for efficiency determination for research purposes, however, efficiency determination is still a matter of discussion and probably new improved models will be published in the upcoming years.

Acknowledgements

This study was supported by a project from the Spanish Ministerio de Ciencia e Innovación (AFL2020-22326-C02-01). Diego Robledo was supported by a FPU fellowship from the Ministerio de Educación Cultura y Deporte of Spanish Governement. We are indebted to Lucía Insua, Susana Sánchez and Sonia Gómez for technical assistance.

Supplementary material



Supplementary Figure 1. *Cyp19a1a* expression levels at 105 dpf. *Cyp19a1a* mean centered Cq values in the gonads of turbot at 105 days post fertilization. High expression is observed in females (F) and low expression in males (M).

		Temperature		
		15	18	23
Days post fertilization	30	450	540	690
	45	675	810	1035
	60	900	1080	1380
	75	1125	1350	1725
	90	1350	1620	2070
	105	1575	1890	2415
	120	1800	2160	2760
	135	2025	2430	3105

Supplementary Table 1. The equivalence between Age+Temperature groups and degree-days is shown. Two groups (75dpf+18ºC and 90dpf+15ºC) would merge if we followed the "degree days" criterion, going from 24 to 23 groups.

Rank	NormFinder	NormFinder (Degree days)
1	*rps4*	*rps4*
	(0.613)	(0.619)
2	*ubq*	*ubq*
	(0.713)	(0.698)
3	*rpl17*	*rpl17*
	(0.721)	(0.707)
4	*actb*	*actb*
	(0.785)	(0.781)
5	*gapdh*	*gapdh*
	(0.85)	(0.844)
6	*b2m*	*b2m*
	(0.851)	(0.856)

Supplementary Table 2. Normfinder results for days post fertilization – temperature and degree-days groups are shown. We repeated NormFinder calculations for reference gene stability determination merging the two groups which would be grouped according to degree-days (75dpf+18ºC and 90dpf+15ºC) and compared them to the results shown in the manuscript for gene stability by NormFinder (the other methods for reference gene stability or efficiency determination are independent of grouping). The best suggested combination of two genes was actb and rps4 for both. As can be seen in the table, the ciphers vary but the classification remains the same and so the conclusions of the manuscript would be unaffected if we changed grouping.

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Chapter 2

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Gene expression analysis at the onset of sex differentiation in turbot (*Scophthalmus maximus*) at different rearing temperatures

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Abstract

Controlling sex ratios is essential for the aquaculture industry, especially in those species with sex dimorphism for relevant productive traits, hence the importance of knowing how the sexual phenotype is established in fish. Turbot, a very important fish for the aquaculture industry in Europe, shows one of the largest sexual growth dimorphisms amongst marine cultured species, being all-female stocks a desirable goal for the industry. Although an important knowledge has been achieved on the genetic basis of sex determination (SD) in this species, the master SD gene remains unknown. Recently, information has been gathered on gene expression profiles along gonad development by microarray analysis, but precise information on some key genes at the critical stage of sex differentiation is lacking. In the present work, we examined the expression profiles of 29 relevant genes related to sex differentiation, from the first larval stages up to 135 days post fertilization (dpf), when male and female gonads are differentiating. Also we considered the influence of three temperature regimes on the process of sex differentiation. The first sex-related differences in molecular markers could be observed at 90 dpf and so we have called that time the onset of sex differentiation. Three genes were the first to show differential expression between males and females and also allowed us to sex turbot accurately at the onset of sex differentiation (90 dpf) in 5-6 cm length fish: *cyp19a1a, amh* and *vasa.* The expression of genes related to primordial germ cell (*vasa, gsdf, tdrd1*) development starts to increase between 75–90 dpf and *vasa* and *tdrd1* later presented higher expression in females (105 dpf). Expression analysis on two genes placed on the SD region of turbot (*sox2, fxr1*) suggest that *sox2* could be discarded as sex determining gene and that *fxr1* does not show an expression pattern which clearly points towards a function as sex determinant, though it still cannot be ruled out. We also detected changes in the expression level of several genes (*ctnnb1, cyp11a, dmrt2 or sox6*) depending on culture temperature. Our results enabled us to identify the first sex-associated genetic cues (*cyp19a1a, vasa* and *amh*) at the initial stages of gonad development in turbot (90 dpf) and to

accurately sex turbot at this age, establishing the correspondence between gene expression profiles and histological sex. Furthermore, we profiled several genes involved in sex differentiation and found specific temperature effects on their expression.

1. Introduction

Sex is thought to have arisen in a single evolutive event in the last common ancestor of all eukaryotes, since sexual reproduction is almost universal and exclusive of this group (Javaux et al. 2001). Considering its consequences over the lifespan of an organism and its influence on population demography, it is thought that the sex-determination (SD) mechanism should be under strong selection forces (van Doorn, 2014). However, sex can be established by many different and fast-evolving mechanisms (Bull, 1983), indicating that SD triggers have emerged several times along evolution (Graves, 2008). Within vertebrates, different sex determining systems have been described. In therian mammals, with a XX/XY SD chromosome system, sex depends on the presence of the *Sry* gene, a paralogue of *sox3,* on the Y chromosome (Sinclair et al. 1990), while in birds with a ZZ/ZW SD system, the *dmrt1* gene with a double dosage is required for testis development (Smith et al, 2009). Also, in *Xenopus laevis* the *dm-w* gene, a paralogue of *dmrt1*, is responsible for SD (Yoshimoto et al. 2008). These SD genes encode for transcription factors belonging to DM and *Sox* families, thus suggesting a biased and recurrent recruitment of specific SD genes or families along evolution (Graves and Peichel, 2010).

Fish, with approximately 30.000 species (Nelson, 2006), is the most diverse group of vertebrates and its study has broaden our knowledge on SD. Fish diversity is also reflected by the variety of reproductive strategies: unisexuality, different types of hermaphroditism and gonochorism; and also by the diversity of SD systems (Devlin and Nagahama, 2002). In the last years, an important effort has been made in order to identify the SD gene in several model and aquaculture fish species. Different productive traits are sex-associated in farm fish such as growth rate, color, taste and flesh quality, hence, the interest of industry in producing mono-sex populations (Martínez et al. 2014). Nonetheless, detailed information at gene level is available for only a limited number of fish species. Five different master SD genes have been identified so far*: dmY/dmrt1by* in *Oryzias latipes* and in *O. curvinotus* (Matsuda et al. 2002), *gsdf* in *O. luzonensis* (Myosho et al. 2012), *amhy* in *Odontesthes hatchery* (Hattori et al. 2012), *amhr2* in *Takifugu rubripes*, *T. pardalis* and *T. poecilonotus* (Kamiya et al. 2012), and *sdY* in salmonid family (Yano et al. 2013). Recently, a distant cis-regulatory element of *sox3* necessary for male determination in *O. dancena*, a species with a XX/XY SD system, has also been identified (Takehana et al. 2014), and *dmrt1* has been suggested as the SD master gene in *Cynoglossus semilaevis* (Chen et al. 2014). However, little information is available, not only on the SD genes, but also on the initial molecular pathways related to sexual differentiation.

Traditionally, SD has been related to the switching mechanism of a hierarchical genetic network that causes the activation of downstream genes involved in gonad differentiation

(GD) leading to the differentiation of testes or ovaries (Schartl, 2004). Thus, concerning whether the first difference between future males and females is a difference in the expression of a gene or group of genes or the stength of an environmental factor, SD can be genetic (GSD) or environmental (ESD), although both ways can coexist (Penman and Piferrer 2008; Martínez et al., 2014).In the classical view of SD and GD, the downstream genetic cascade was assumed to be highly conserved, and only the genes at the top of the cascade would change by gene duplication (and by the recruitment of a downstream gene) or by allelic diversification, establishing a new SD mechanism (Schartl, 2004). Nowadays, the conservation of the downstream cascade has been questioned (Böhne et al. 2013; Herpin et al. 2013) and a new vision which considers sex as a threshold phenotype in which both genetic and environmental factors can act alone or in combination and, importantly, in different times during the period of GD is gaining support (Uller and Helantera, 2011; Heule et al., 2014). In this new view, different factors such as cell proliferation and hormone levels would be involved in determining a threshold which would give rise to a testis or an ovary, thus fitting to a threshold quantitative trait (Uller and Helanterä, 2011; Martínez et al., 2014).

Turbot is one of the most important species cultured in Europe, being Galicia (North-west of Spain) the main production region since the eighties. Production and quality of farmed fish rely on a deep knowledge of biological functions, especially those related to reproduction, growth and disease resistance. Being able to adjust the reproductive biology of cultured species allows exploiting sex-associated dimorphisms related to productive traits (Devlin and Nagahama, 2002). Turbot shows one of the strongest sexual growth dimorphisms amongst marine species and females can reach up to 50% bigger size than males (Imsland et al. 1997), thus industry is interested in the production of all-female populations. In the last years, an important effort has been devoted to understanding SD and GD in this species. Analysis on mitotic and meiotic chromosomes revealed the absence of an heteromorphic sex chromosome pair related to sex (Bouza et al. 1994; Cuñado et al. 2002). The major SD region was located on the LG5 at 2.6 cM of Sma-USCE30 marker (Hermida et al. 2013), but other minor sex-related QTLs were detected at LG6, LG8 and LG21 (Martínez et al. 2009). In that study a ZZ/ZW SD system was established in accordance with the sex ratios of progenies obtained from hormonally sex reversed parents (Haffray et al. 2009). Temperature also showed a minor influence on sex ratios in this species (Haffray et al. 2009). Close to the sex-associated marker several candidate genes were identified (*sox2*, *dnaj19*, *fxr1*), but apparently discarded because no association to sex was detected at the species level, so the SD gene remains unidentified in this species (Taboada et al. 2014). Considering the lack of information on the SD mechanism and the new scenario highlighting the relevance of downstream genes in gonad development, we decided to address an expression analysis on 29 relevant genes involved in GD at the initial critical stages of sex differentiation using a large amount of fish and sampling times in turbot. We also evaluated the effect of temperature along this period to ascertain its role on SD and its interaction with genetic factors. Our results enabled us to establish the correspondence between gene expression profiles and histological sex and to identify the first sex-associated genetic cues at the initial stages of gonad development in turbot.

2. Methods

2.1. Rearing conditions and sampling

Turbot fertilized eggs were obtained at the IEO (Instituto Oceanográfico de Vigo, Spain). Fish were reared in tanks at three different temperatures, (15ºC, 18ºC and 23ºC) with two replicates per temperature for a total of six tanks. Gonad samples were taken following a time series at the beginning of development embracing the critical period where the onset of GD takes place: 60, 75, 90, 105, 120 and 135 days post fertilization (dpf). This period was chosen based on a preliminary analysis on the expression GD key genes (*cyp19a1a*, *amh*, *sox9a*, *vasa*, *foxl2*) and on previous histological analyses of turbot gonads sampled every five days from 5 dpf, which did not show any change signals either in gene expression or GD before than 60 dpf. Ten fish per temperature and developmental stage were sampled and gonads dissected as accurately as possible considering the size of the fish. A total of 180 samples were used in this study: 6 stages x 3 temperatures/stage x 10 fish/stage-temperature. In fish of 105 dpf and above, gonads were split into two samples, one used for quantitative PCR (qPCR) and the other one for histological sexing. Samples for qPCR were immediately embedded in RNAlater for preservation (Qiagen, Valencia, CA).

Animals were treated according to the Directive 2010/63/UE of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for experimentation and other scientific purposes. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain).

2.2. Histology

Samples for histological analysis were kept in 4% paraformaldehyde buffer overnight. The next day, samples were cleaned with PBS and kept in 70% ethanol, and then gonads were dehydrated and embedded in paraffin, cut at 7 μm thick and stained with hematoxylin-eosin. These histological preparations allowed us to certify the sex of the dissected individuals from 105 dpf, where gonads were differentiated (see Results). Additionally, all samples were genetically sexed using the SmaUSC-E30 marker, which demonstrated a ~98% accuracy for sexing in turbot (Martínez et al. 2009). To establish the association between sex and alleles at this marker, parents and grandparents of each family were genotyped, and the expected genotypes of male and female offspring obtained following Taboada et al. (2014).

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted by homogenization in TRIzol (Invitrogen, Paisley, UK) following the manufacturer's protocol. Extracted RNA was treated with RNase-free Recombinant *DNaseI* (Roche Diagnostics, Mannheim, DE) and RNA concentration was assessed by spectrophotometry and its quality checked using an Agilent 2100 bionalyzer

(Agilent Technologies, Santa Clara, US). RNA (1.2 µg) was reverse transcribed by random primers using Affinity Script Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) following the manufacturer's protocol and then diluted 1:2 with nuclease-free water.

2.4. Quantitative PCR

qPCR was performed on a Stratagene Mx3005P thermocycler (Agilent Technologies) using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix in a final volume of 12.5 µL following the manufacturer's protocol with 1µL of cDNA per reaction. Specific primers for targeted genes were designed using Primer3 (Untergasser et al. 2012) from sequences obtained from the turbot EST database enriched with sex differentiation-related organs (gonad and brain; Ribas et al. 2013). When possible, primers were designed spanning different exons (Supplementary Table 1). Primer concentration was 300 nM and each sample was run in duplicate. The cycling parameters were: 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of amplification at 95ºC for 15 sec and 60ºC for 1 min. After amplification, a dissociation step was performed to ensure the presence of a single amplification product. Specificity for each primer pair was also confirmed by PCR product sequencing. In every PCR plate, non-template controls were included to confirm the absence of contamination. In addition, the same three samples were run in triplicate in every plate in order to correct inter-assay variation. qPCR data were obtained by the MxPro software (Agilent Technologies) and quantification cycle values (Cq) calculated for each replicate and then averaged to obtain the final Cq value. Three reference genes (*ubq*, *rps4*, *rpl17*) were used for normalization and LinRegPCR software (Ruijter et al. 2009) was used for efficiency determination following the recommendations by Robledo et al. (2014b) (Chapter 1). qPCR was performed in all the 180 samples for every gene. Samples with missing Cq values or inconsistencies between replicates (Cq difference > 1 cycle) were removed. Raw Cq values were transformed to the final fold difference values (FD) following the equations present in Kubista et al. (2007). Briefly, Cq values were normalized using the reference genes, efficiency corrected, log transformed and finally mean centered to obtain mean centered fold change values which were used for statistical analysis.

2.5. Statistical analysis

Statistical analyses were performed using R (version 3.0.2) (R Development Core Team, 2008). Pearson correlations for the heatmap were obtained using the "cor" function. Principal component analysis (PCA) was computed by the "prcomp" function. Length and gene expression differences between sexes and stages were checked by Mann-Whitney tests ($P < 0.05$) since our data mostly did not conform to a normal distribution. Discriminant analysis was performed using the "lda" function on the "MASS" package (Venables and Ripley, 2002). Multiple regression ($p<0.05$) was used to assess temperature effects on sex ratio,

introducing temperature and length in the model. The gvlma function of the gvlma R package was used to check if our dataset met the assumptions of the multiple regression. Furthermore, we performed two additional tests for every temperature significant effect on gene expression: i) a moderation analysis, to check if length was modulated by the temperature, a temperature-length interaction term was added to our model checking if the new model improved the previous one; and ii) a mediation analysis by Sobel test, to explore if the detected temperature effect on gene expression is partially or fully explained by size differences between individuals.

2.6. Co-localization of targeted genes with sex-related QTLs

Several SD related QTLs were previously reported in turbot (Martínez et al. 2009), and therefore, we considered relevant to establish the mapping position of the targeted genes regarding these QTLs in the last turbot map (Hermida et al. 2013). For this, we established the correspondence between the turbot linkage groups and the scaffolds of the recently sequenced turbot genome (Figueras et al. unpublished) using the mapped markers and their sequences. Target gene sequences were located in the turbot genome using local blast (Altschul et al. 1990) and then placed in the linkage map using the correspondence between linkage groups and scaffolds as far as accurately depending on the availability of markers in the vicinity.

2.7. Weighted correlation network analysis

Weighted correlation network analysis was performed in R (version 3.0.2) (R Development Core Team, 2008) using the WGCNA package (Langfelder and Horvath, 2008) following the author's tutorial. Co-expression networks were built for our genes and Cytoscape 3.0.2 was used to visualize the network (Shannon et al. 2003). This allowed us to obtain information about the functional relationships between the target genes.

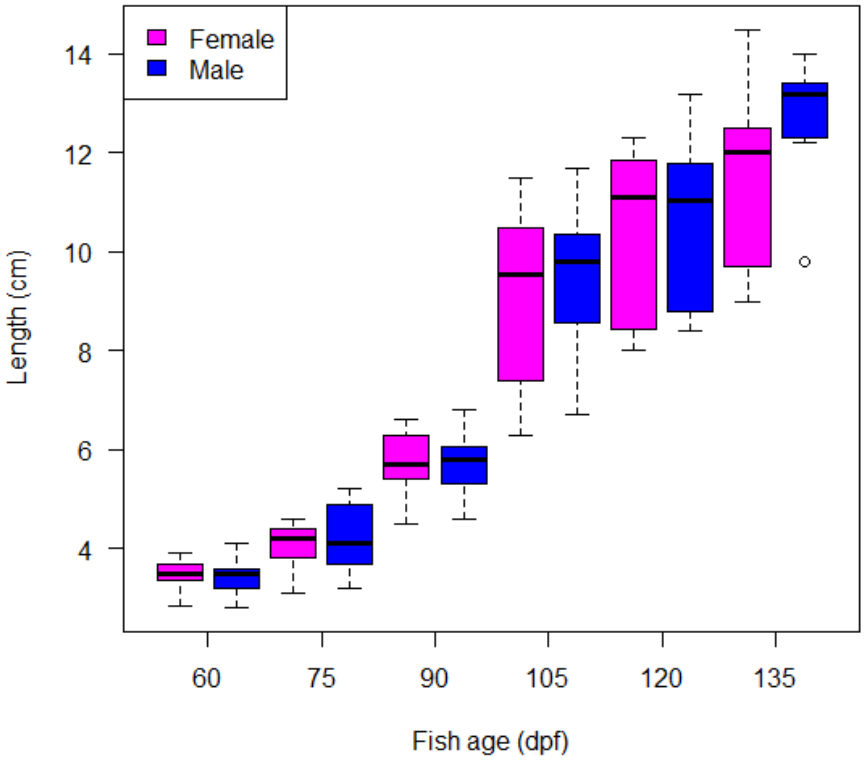
3. Results

3.1. Sampling and sexing

All the 180 turbot samples were genetically sexed using the Sma-USC30 marker and, additionally, the 105, 120 and 135 dpf samples were histologically sexed. Eighty-nine females and eighty-five males could be genetically sexed because the Sma-USC30 marker was informative, the remaining six samples being removed from this analysis since they could not be sexed. A 7% sexing discrepancy was observed between the genetic and histological information in the samples obtained at 105, 120 and 135 dpf, which provides an estimation of the error for sex-genotyping, a value close to that previously reported by Martínez et al. (2009). Given the reasonable accuracy of genetic sexing, the sex of samples below 105 dpf obtained through SmaUSC-E30 genotyping was considered for further analyses.

Males and females did not show length differences both in the whole dataset and at each development stage (Mann-Whitney test, $P < 0.05$) (Figure 1). However, significant differences were found at every stage between the three temperatures except at 90 and 120 dpf, where 18ºC reared animals did not differ in length from those reared at 23ºC (Table 1).

Figure 1. Male and female length by age



Mean length (centimeters) by age (days post fertilization) is shown in a boxplot for males and females separately. Females are represented in magenta and males in blue.

3.2. Gene selection and primer design

The 29 target genes were selected by: i) their importance for GD in other fish species; ii) previous data from our group in turbot (Viñas et al. 2012; Taboada et al. 2012); and iii) previous results from Ribas et al. (submitted) (Supplementary Table 2).

3.3. Co-localization of targeted genes with sex-related QTLs

A main SD QTL in linkage group 5 (LG 5) and three minor ones in LG6, LG8 and LG21 were previously reported in turbot (Martínez et al. 2009; Hermida et al. 2013). After establishing the relationship between the turbot map (linkage groups) and the turbot genome (scaffolds; Figueras et al. unpublished), 11 of our genes could be located in LGs harboring a

SD QTL (Figure 2). Five genes were found in LG5 and two of them, *sox2* and *fxr1*, co-localized with the main SD QTL; *ar1* co-localized with the sex QTL in LG8; four genes were placed in LG21 and two of them, *sox9* and *sox17*, within the confidence interval of the SD QTL.

Table 1. Length comparison between temperatures for each age group

Age (dpf)	Temperature (ºC)	Mean length ± SD (cm)	Percentage (%) / 18ºC	P value / 18ºC
60	15	3.14 ± 0.27	91.2	0.020
	18	3.44 ± 0.26	100	-
	23	3.72 ± 0.18	108.8	0.013
75	15	3.63 ± 0.43	87.8	0.044
	18	4.13 ± 0.45	100	-
	23	4.73 ± 0.38	114.6	0.006
90	15	4.95 ± 0.44	83.05	0.001
	18	5.90 ± 0.42	100	-
	23	6.02 ± 0.52	101.7	0.622
105	15	7.21 ± 0.68	73.5	0.000
	18	9.76 ± 0.51	100	-
	23	10.75 ± 0.47	109.2	0.000
120	15	8.33 ± 0.37	73.5	0.000
	18	11.26 ± 0.70	100	-
	23	11.85 ± 0.68	104.4	0.103
135	15	9.57 ± 0.33	78.1	0.000
	18	12.34 ± 0.36	100	-
	23	13.28 ± 0.71	108.1	0.003

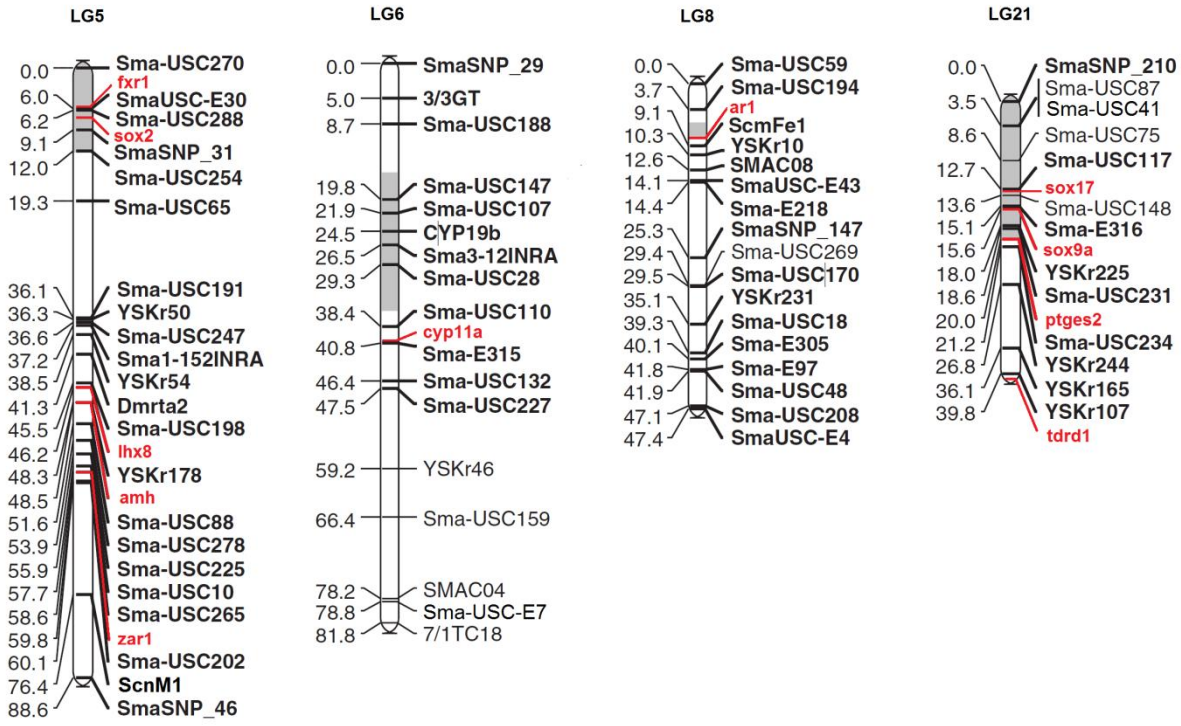
Mean length in cm and standard deviation (SD) for each turbot stage-temperature group. The percentage length difference for 15 and 23ºC groups referenced to the 18ºC group and the p value of 15 and 23ºC temperature lengths compared to18ºC are also shown.

3.4. Global expression patterns

Samples and genes were hierarchically clustered in a heatmap using the Pearson correlation coefficient as distance measure (Figure 3). For each sample, sex, age and rearing temperature are shown in the heatmap. Some samples are grouped according to sex or age and can be associated with particular groups of coexpressed genes. The samples of 60 and 75 dpf are clustered in two groups (labelled in grey) and they are characterized by the high

expression of *sox6*, *fxr1*, *wnt4*, *hsp27*, *ptges3*, *lhx8* and *dmrt2* (yellow circles on the right), but also by the nearly null expression of *tdrd1*, *vasa*, *cyp19a1a*, *foxl2* and *gsdf*,

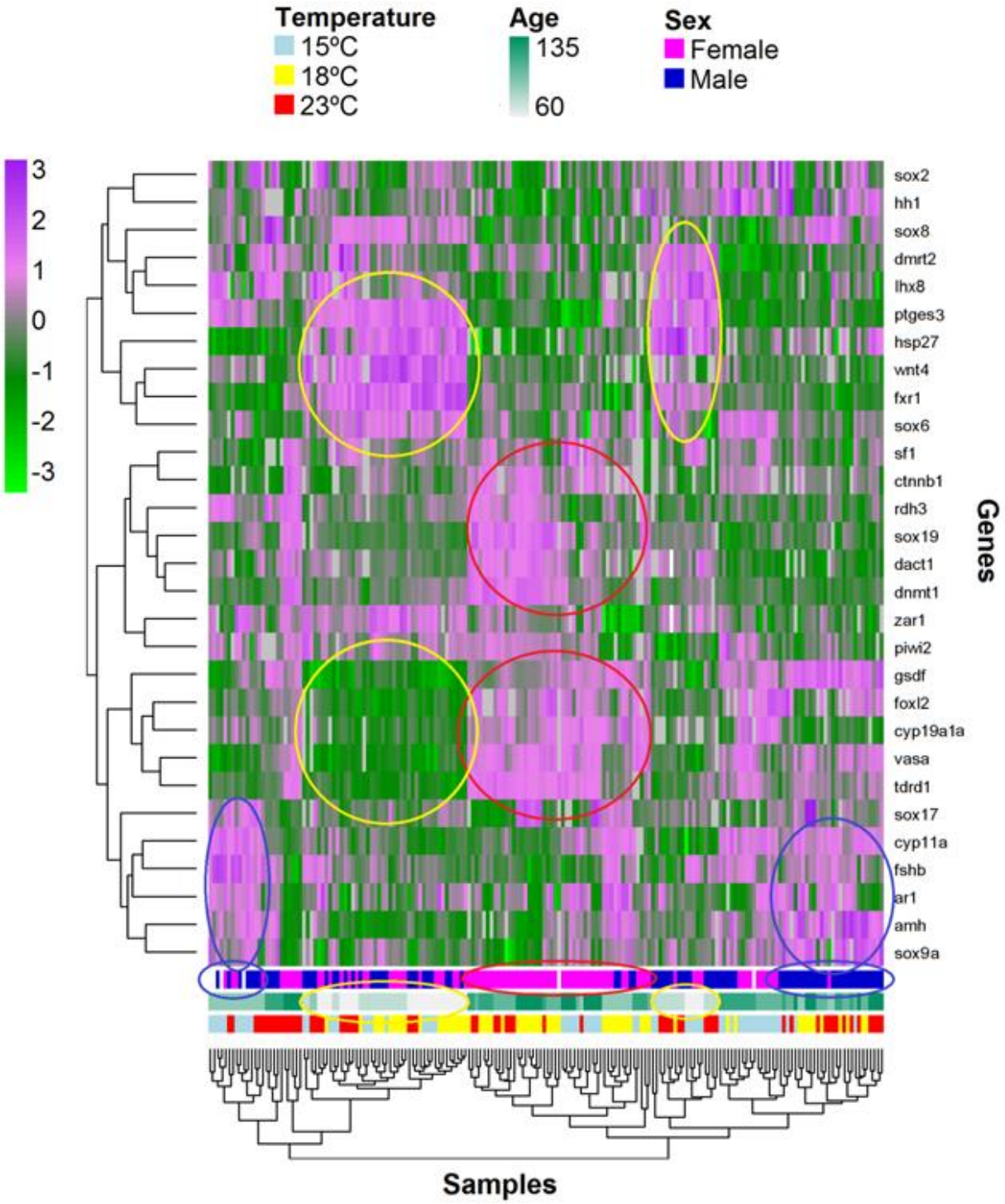
Figure 2. Turbot sex QTLs and target genes



Four turbot linkage groups are shown. Estimated location of the target genes is shown in red. Grey shaded LG areas represent the position of the SD QTLs.

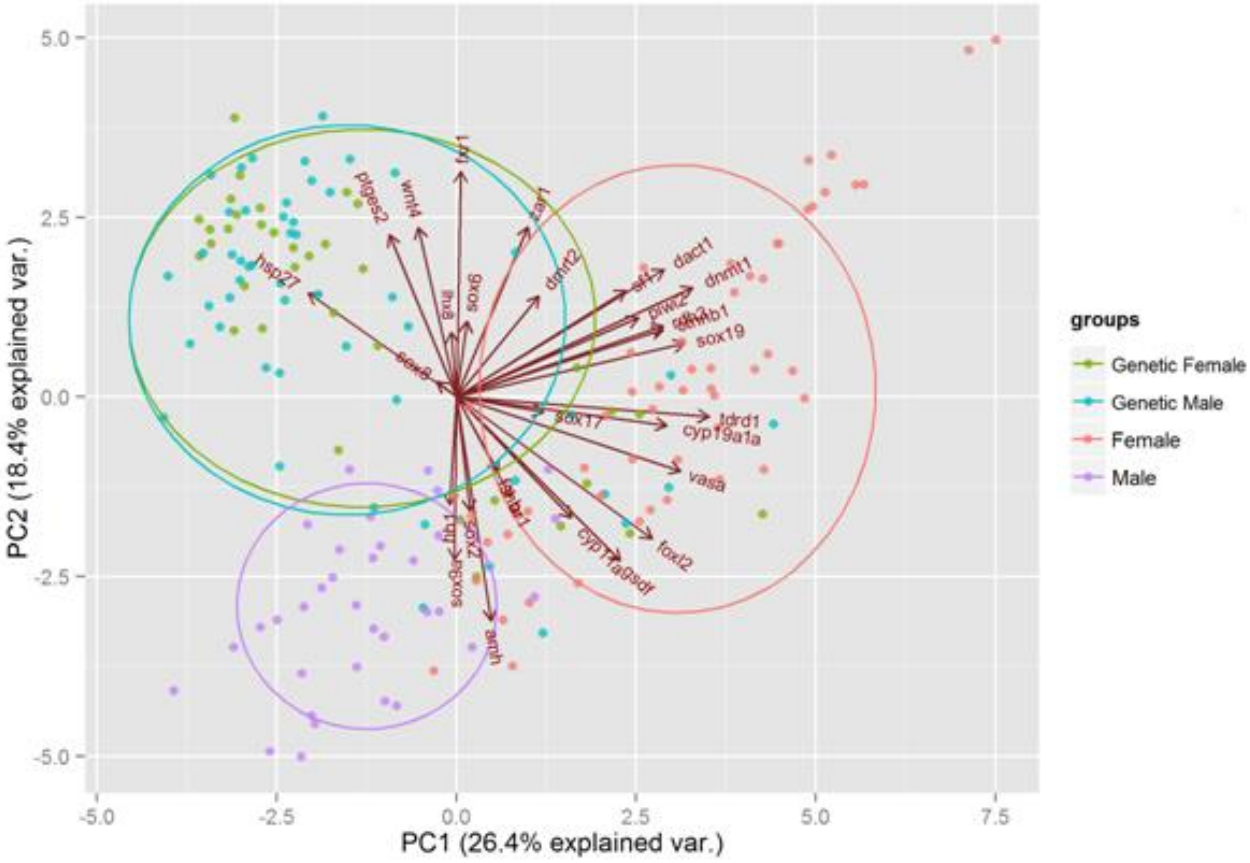
involved in gonad maturation and female differentiation (yellow circle on the left). These samples are grouped by age independently of temperature or sex, which do not seem to represent relevant factors on the diagnostic genes expressed at these stages. Two different groups of older fish, one related to females and another one with males, can be identified. The female group (red circle on the right) is mainly associated with the overexpression of two different clusters of genes, one containing *cyp19a1a*, *foxl2*, *vasa*, *tdrd1* and *gsdf*, a group of genes not expressed in undifferentiated individuals as outlined before, and another one containing *dnmt1*, *dact1*, *sox19*, *rdh3* and *ctnnb1* (red circle on the left). The male group (blue circles) is associated with the expression of *sox9*, *amh*, *ar1*, *fshb*, *cyp11a* (blue circle at the bottom). These "male" genes are also highly expressed in a mix of males and females of around 90 dpf and mostly reared at low temperatures (blue circle at the top). Some "female" genes (*foxl2*, *gsdf*, *vasa*, *tdrd1*) are also expressed to a lower extent in the male samples, suggesting a role in gonad development irrespective of sex (next to the blue circle at the bottom). As previously mentioned, some blocks connected to rearing temperature can also be seen, but, in general, it does not seem to be a determining factor for sample clustering.

Figure 3. Genes and samples heatmap



Heatmap of target genes and all gonad samples. Gene names are shown in the bottom of the figure while gene hierarchical cluster is shown in the top. Log fold change expression values representation ranges from purple (highest expression) to light green (lowest expression). Sample names are not shown, instead each sample is represented by the three colors at the left of the figure which indicate sex (magenta for females, and blue for males), age (ranging from 60 to 135 dpf corresponding to a scale going from grey to dark green) and temperature (light blue for 15ºC, yellow for 18ºC and red for 23ºC). Yellow, red or blue circles highlight expression patterns characteristic of undifferentiated, females or males individuals respectively.

Figure 4. Principal component analysis



Samples were grouped according to the fold change expression values of the target genes by a principal component analysis. Labeled as "Female" and "Male" and colored in red and purple respectively are gonad samples which have been histologically sexed, while labeler as "GenFemale" and "GenMale" and colored in olive green and light blue are gonad samples which have been genetically sexed. The circles for each group color represent a 66% probability that a sample belonging to that group will be placed inside the circle. The arrows with the name of the genes at the end represent how each gene contributes to the two principal analysis components represented in the figure.

PCA analysis on the 180 samples (Figure 4) revealed that histologically sexed males and females (≥105 dpf; males/females in the figure), can be mostly discriminated by their differential expression. However, younger individuals (only genetically sexed), appeared fully overlapped in the PCA, likely because they are still undifferentiated. A 66% prediction ellipse for each group is shown in Figure 4 indicating that if new individuals were added to our analysis from a certain group, 66% of them would expect to be placed inside the corresponding ellipse. Some of the genetically sexed individuals (60, 75 and 90 dpf) are also found in the ellipses for males or females which, also considering the previous heatmap results, indicates that turbot GD might start before 105 dpf. Interestingly, several genetic males are included in the female circle. This is very likely due to genotype sexing errors, as mentioned before (error rate around 7%), although any genetic male developing as female cannot be fully discarded. The arrows indicate the weight of each gene on the two first

principal components. Clearly, there is a large group of genes related to female differentiation (e.g., *cyp19a1a*, *sox19*, *tdrd1*, *dact1*), while the presumed male-related genes are fewer and not so markedly pointing towards male differentiation (*sox9*, *amh*, *sox2, hh1*). Apparently, the up-regulation of several female-like genes determines a female phenotype, while male development is characterized by the expression of very few specific genes. Also, as suggested in the heatmap, some genes are clearly related to undifferentiated individuals (*sox6*, *fxr1*, *wnt4*, *hsp27*, *ptges*).

3.5. Sex differences

Fold change expression values for those differentially expressed genes between males and females were analyzed gene by gene related to growth and age along development (Mann-whitney test; $P < 0.05$) (Figure 5).

Sex differences were first observed at 90 dpf when *cyp19a1a* ($FC_{F/M}$ = 2.0) and *vasa* ($FC_{F/M}$ = 1.2) are over expressed in females, and *amh* ($FC_{M/F}$ = 1.1) in males (Figure 5). *Amh* expression was already higher at 75 dpf in males ($FC_{M/F}$ = 0.5), although not significant (p = 0.11). These three genes presented an expression increase from 75 to 90 dpf in both sexes, also observed for *foxl2*, *tdrd1*, *gsdf* or even for *sox19* and *rdh3* (Figure 5). *Gsdf* expression increased very quickly from 60 to 75 dpf ($FC_{75/60}$ = 4.3). *Foxl2* showed a higher expression in females at 90 dpf ($FC_{F/M}$ = 1.2) although not significant (p = 0.12). *Foxl2* expression resembled that of *cyp19a1a*, although its expression increases in males at 105 dpf versus 90 dpf and also decreases in males from 105 dpf onwards (Figure 5). *Tdrd1* presented a pattern similar to *vasa* being more expressed in females at 105 dpf and onwards. Sex differences at 105 dpf and onwards

Other genes increased their expression at 105 dpf in females, while their expression remained at the undifferentiated stage level in males or even slowly decreased. FC values of females vs. males at 105 dpf were above 1 for *sox19* ($FC_{F/M}$ = 3.8), *dnmt1* ($FC_{F/M}$ = 3.1), *dact1* ($FC_{F/M}$ = 2.4), *rdh3* ($FC_{F/M}$ = 1.8), *ctnnb1* ($FC_{F/M}$ = 1.3), *sf1* ($FC_{F/M}$ = 1.1) and *piwi2* ($FC_{F/M}$ = 2.3) (Figure 5) and these differences increased between 35%-75% from 105 to 135 dpf.

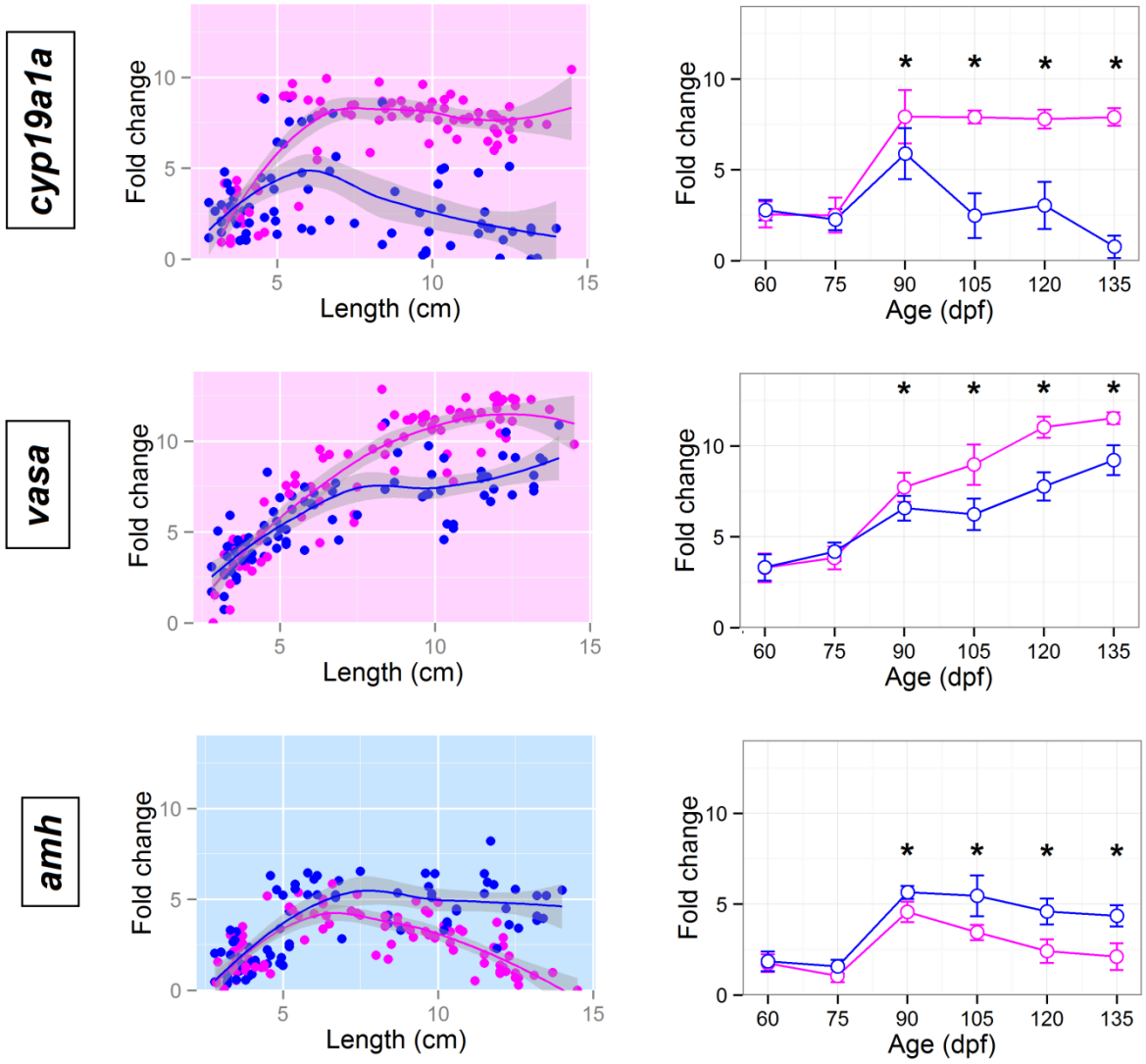
Fxr1 was also over expressed in females at 105 dpf ($FC_{F/M}$ = 1.2) and showed a 47% increase at 135 dpf (Figure 5), but presented higher expression levels in undifferentiated individuals, irrespective of sex. A similar pattern was also observed for *wnt4, dmrt2 and zar1*, genes which at some point during sex differentiation, 105-135 dpf, showed a higher expression in females, but its expression decreased from 75 to 90 dpf (Figure 5).

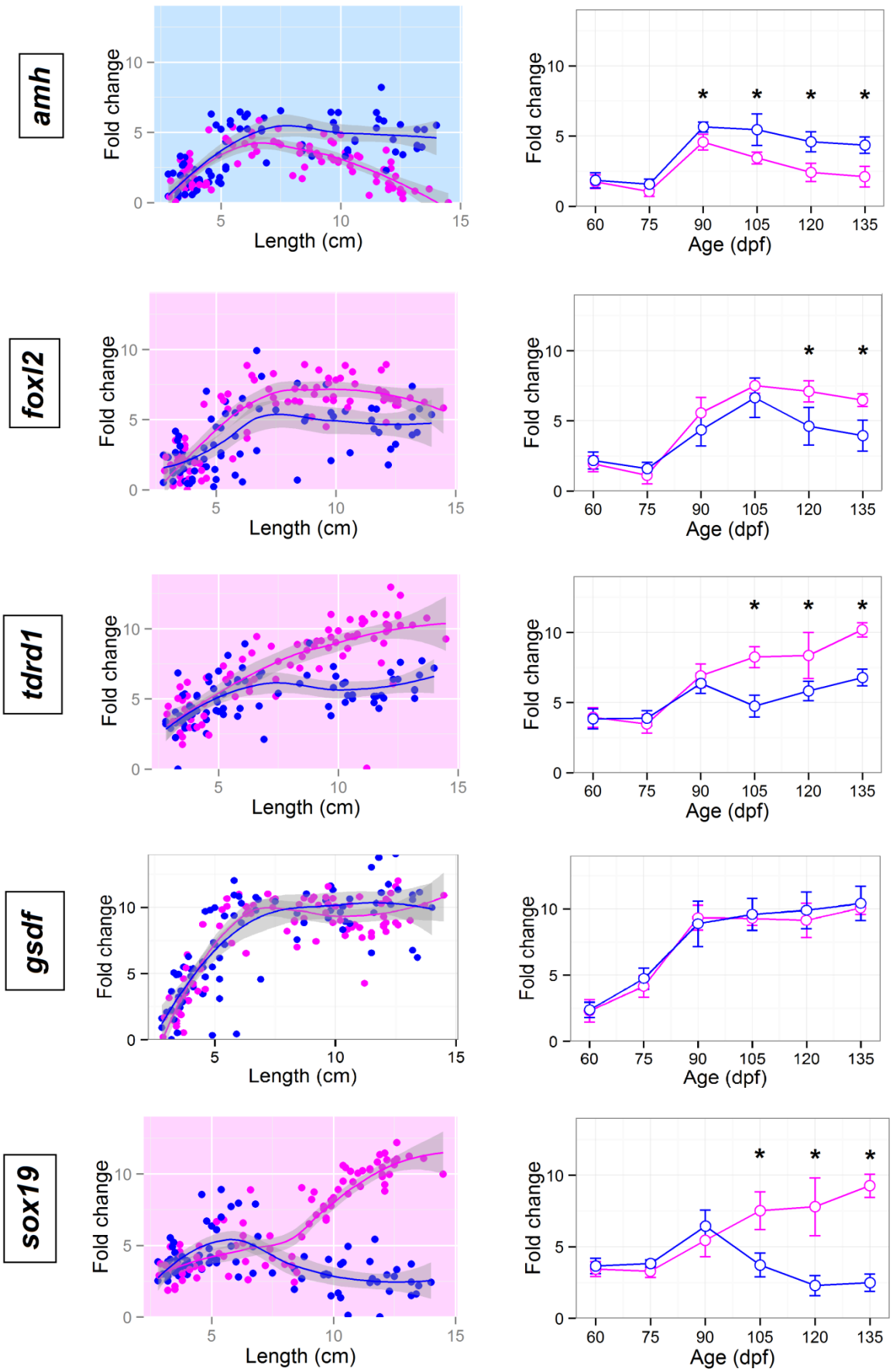
In contrast to that observed in females, in our study there were very few genes whose expression were higher in males and, even in these cases, the differences between males and females were low (Figure 5). One of them was *sox9a* for which sex differences increased from 105 dpf ($FC_{M/F}$ = 1.2) up to 135 dpf ($FC_{M/F}$=2.0). Another sox family gene, *sox8*, highly expressed in undifferentiated individuals in both sexes, was over expressed in males at 105 dpf ($FC_{M/F}$ = 0.8), but the difference remained constant at 135 dpf. Two additional genes, *fshb*

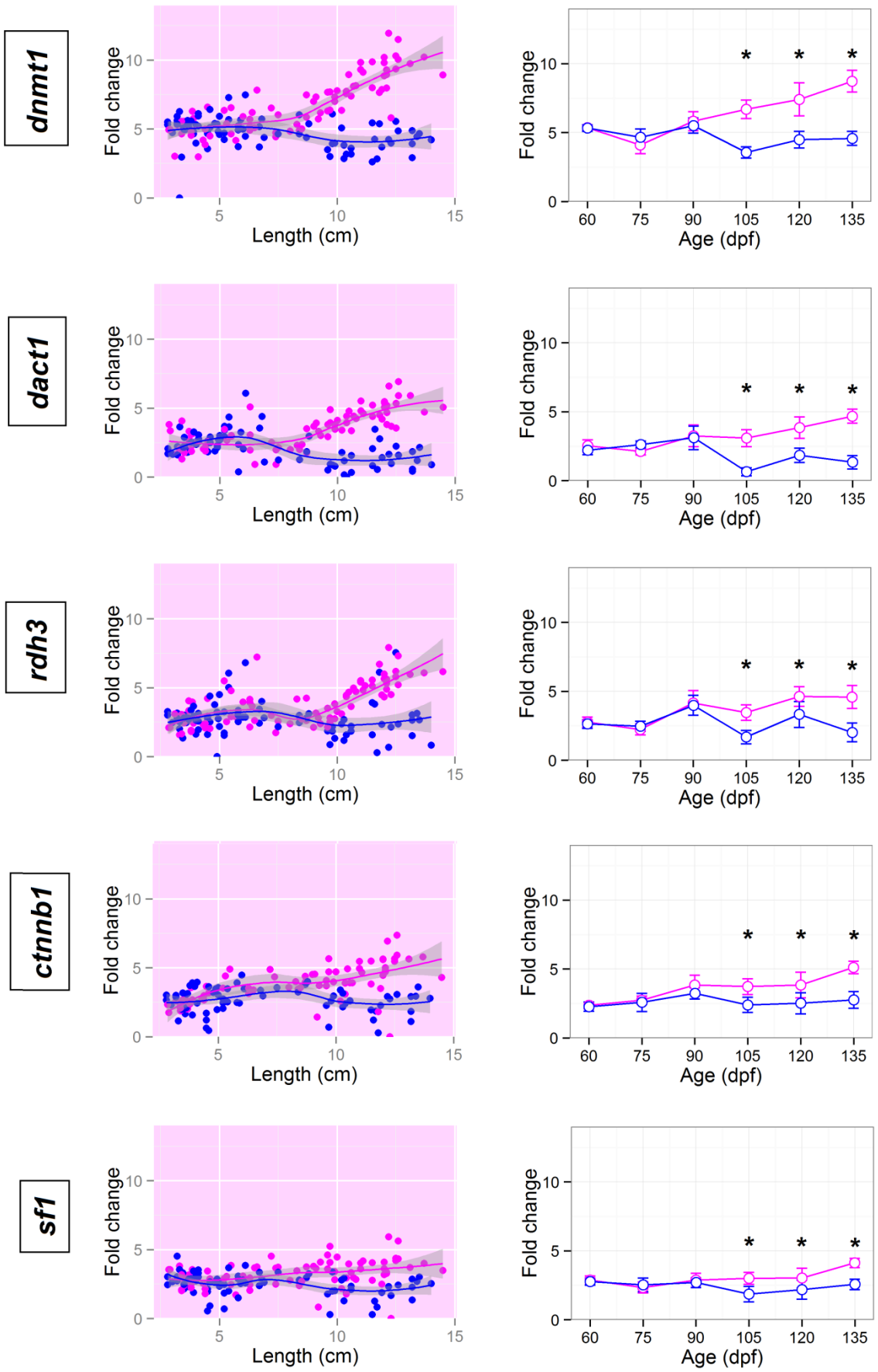
and *cyp11a*, showed mean expression values a little bit higher in males, but not significant (Supplementary figure 1).

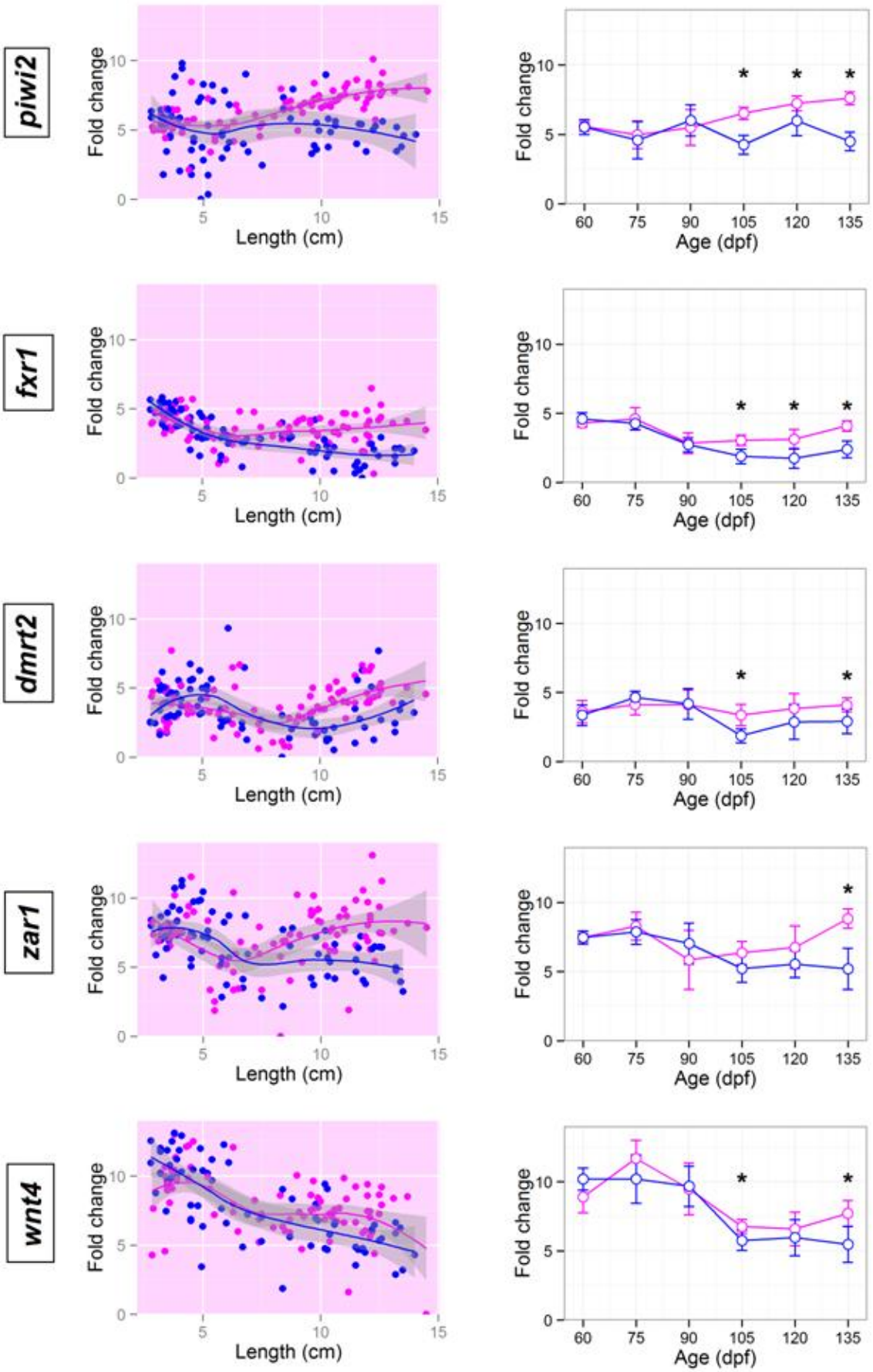
Some genes like *ptges3*, *hh1*, *hsp27* or *lhx8* did not show sex differences (Supplementary figure 1). Among these, a gene of the sox family, *sox17*, showed some groups of outliers whose expression was not explained either by sex or by length/age. Other two genes of this family, *sox2* and *sox6*, did not present any clear expression pattern along development or by sex, and androgen receptor 1, *ar1*, showed two differentially expressed groups of samples from 105 dpf onwards, but irrespective of sex, did not show dimorphic expression either. Finally, it is worth noting the high expression of *ptges3*, *zar1* and especially by *wnt4* at the beginning of gonad development.

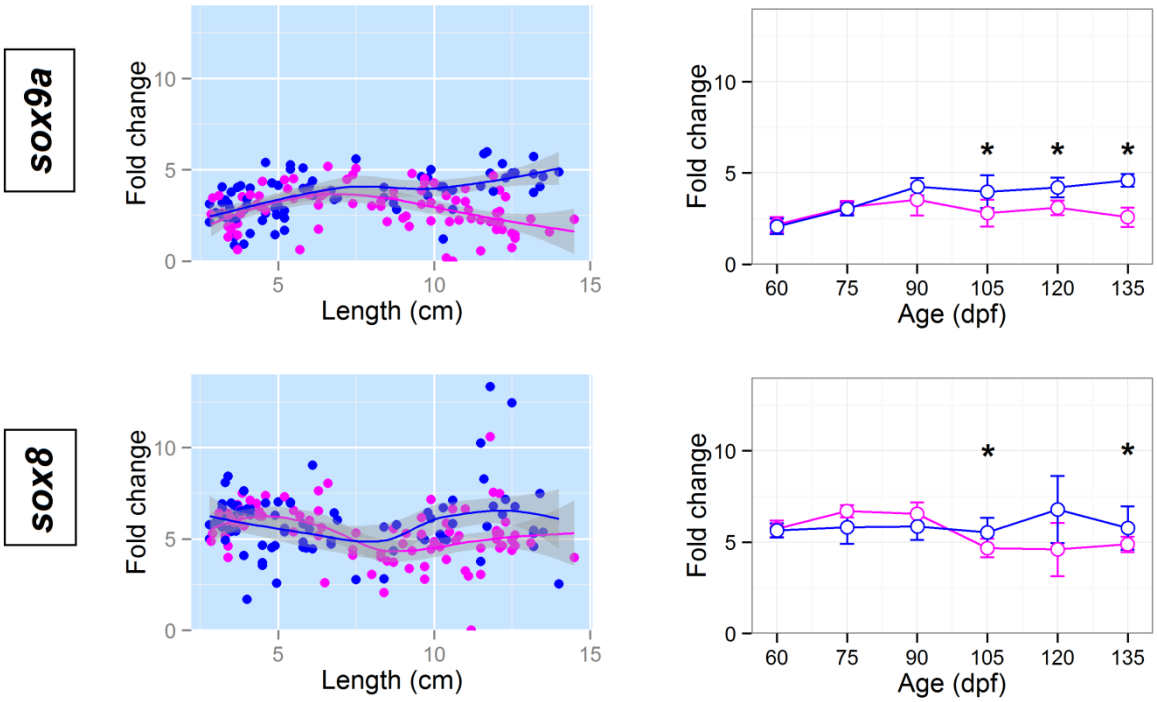
Figure 5. Gene expression along gonad development




amh
foxl2
tdrd1
gsdf
sox19
Fold change
Length (cm)
Age (dpf)
0
5
10
15
60
75
90
105
120
135
*


dnmt1
dact1
rdh3
ctnnb1
sf1
Fold change
Length (cm)
Age (dpf)
0
5
10
15
60
75
90
105
120
135
*


piwi2
fxr1
dmrt2
zar1
wnt4
Fold change
Length (cm)
Age (dpf)
0
5
10
15
60
75
90
105
120
135
*



Gene fold change values for each sample plotted according to both its length, in cm, and its age, in days post fertilization. Female samples are shown in magenta and male samples in blue. In the FC/length figure for each gene non-linear trend lines were calculated by loess regression and genes with significative differences between sexes at any age point present a pink background if the gene is overexpressed in females or a blue one if it is overexpressed in males. Genes without sex differences have a white background. In the FC/age figure, error bars represent the standard error of the mean, also an asterisk marks those age points were the differences in expression between males and females are significant.

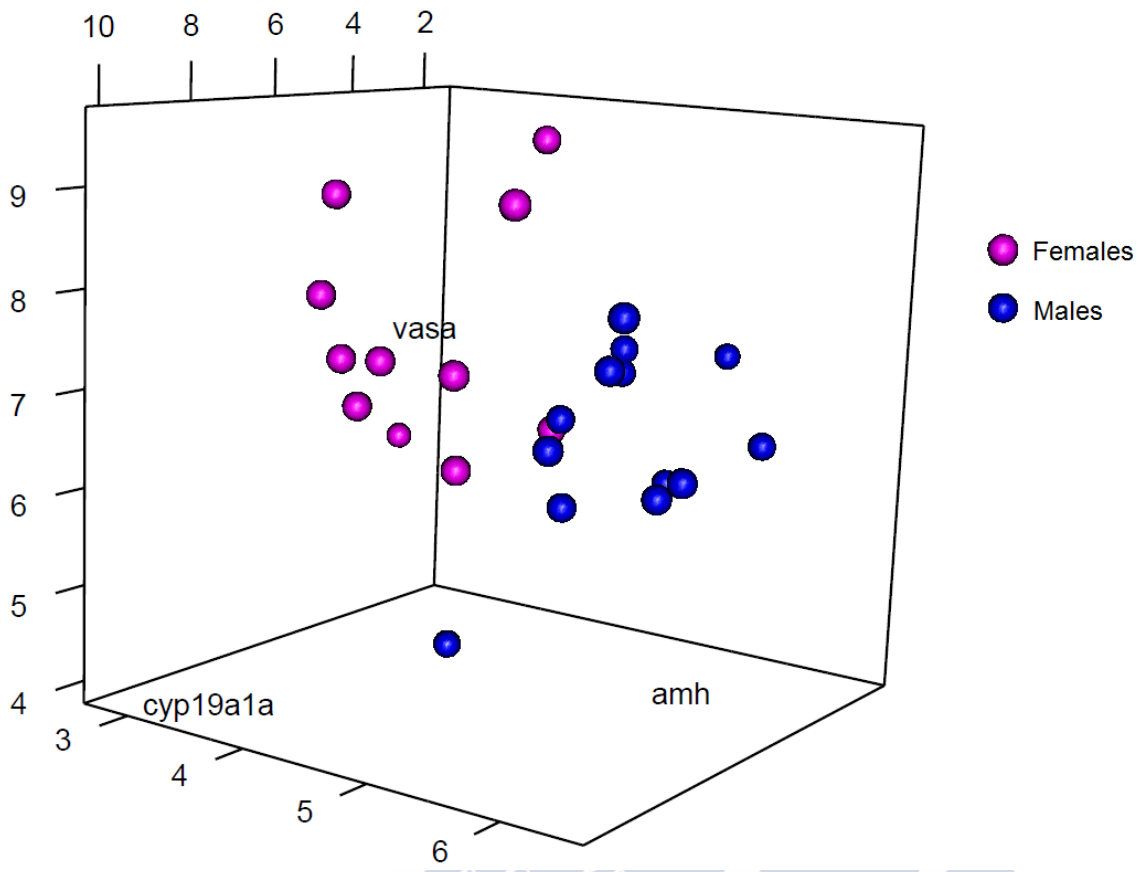
3.6. Discriminant analysis

A discriminant analysis considering the earlier dimorphic expressed genes (*cyp19a1a*, *amh* and *vasa*) enabled us to sex correctly 100% of the genetic males at 90 dpf and 82% of the genetic females, representing as a whole 91% of individuals correctly sexed (Figure 6). This difference was very close to the error observed for genetic sexing in our study, as outlined before. Furthermore, from 105 dpf onwards (sexed by histology), the expression of *cyp19a1a* alone is capable to perfectly discriminate males and females without error, additionally supporting the discrepancy due to sex genotyping error before this time.

3.7. Network analysis

To further understand the functional relationships between genes we performed a network analysis based on gene-to-gene correlations (Figure 7). A tight cluster with several female over-expressed genes (e.g., *cyp19a1a*, *foxl2*, *vasa*, *sox19*, *ctnnb1*) was found with all their genes inter-connected. Also, *sox9* and *amh* constituted a small male cluster together with *fshb*

Figure 6. Discriminant analysis 3D plot

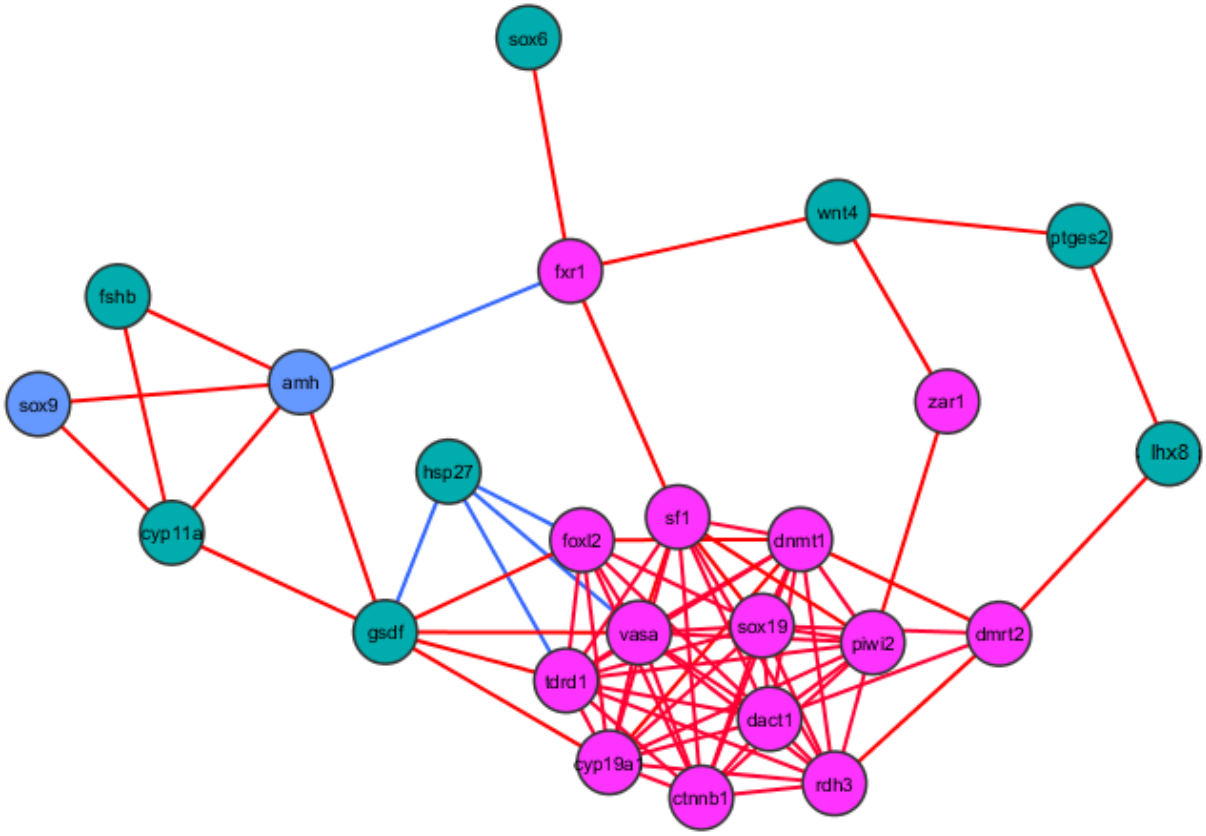


90 days post fertilization samples were plotted in a three dimensions graphaccordin to their fold change values for cyp19a1a, amh and vasa. Female samples are colored in magenta and males in blue.

and *cyp11a*. The two clusters are connected through two genes: *fxr1* and *gsdf*. *Fxr1* is located at the main sex determining region of turbot (Martínez et al. 2009), so its position connecting female and male genes may be relevant. Furthermore, *fxr1* is also related to *wnt4*, another gene with higher expression at undifferentiated stages.

The absence of some genes in the network (*sox2*, *sox8*, *sox17*, *ar1*) suggests that they do not show significant relationships with any other gene, at least in our analysis and for the chosen correlation threshold. This does not mean that they do not have any role in sex differentiation, since our study analyzed the expression of a limited number of genes (29). If more genes were added, it is possible that these genes showed connection to the network through them.

Figure 8. Network representation



Weighed correlation network performed with the fold change expression values of the genes is shown. Genes are represented as blue circles if they are overexpressed in males at any age, magenta if they are overexpressed in females, or dark green if no differences were found. Lines connecting genes indicate significant correlations, red lines are positive correlations and blue lines are negative correlations.

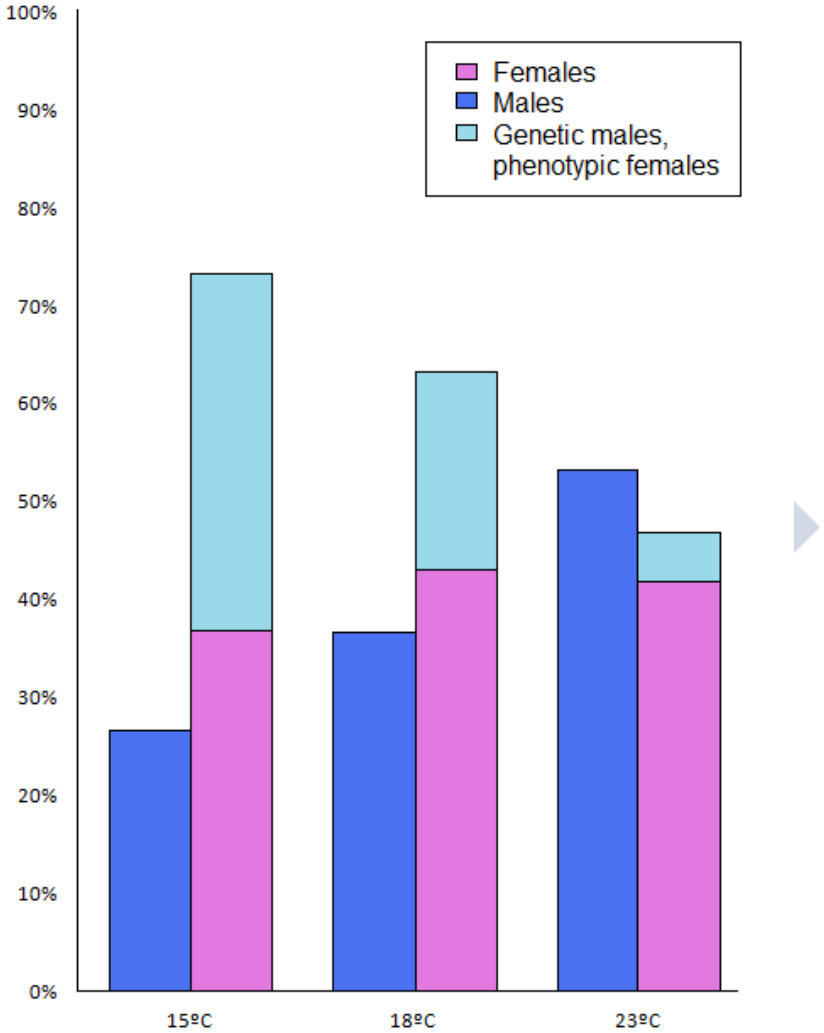
3.8. Temperature effects on gene expression

We found a higher proportion of females at both 15ºC and 18ºC than at 23ºC, where the male:female proportion is close to 1:1 (Figure 8). Genotyping of the SD marker strongly suggested that some genetic males developed as females, a total of 16 genetic males were classified as females by histology, so male-to-female sex reversal mechanism seems to be operating especially, at 15ºC. The detected 7% genetic sexing error might be the result of temperature effects on sex differentiation.

We analyzed the effects of temperature on gene expression in males and females separately and, since turbot length was different between temperatures in almost every development stage, we checked if the detected temperature differences were independent of length (Figure 9) or not (Supplementary Figure 2). Among those genes with length-independent temperature effects on expression (Figure 9), only *sox2* showed temperature effects which are not sex dependent. This gene showed higher expression at 15ºC and 23ºC,

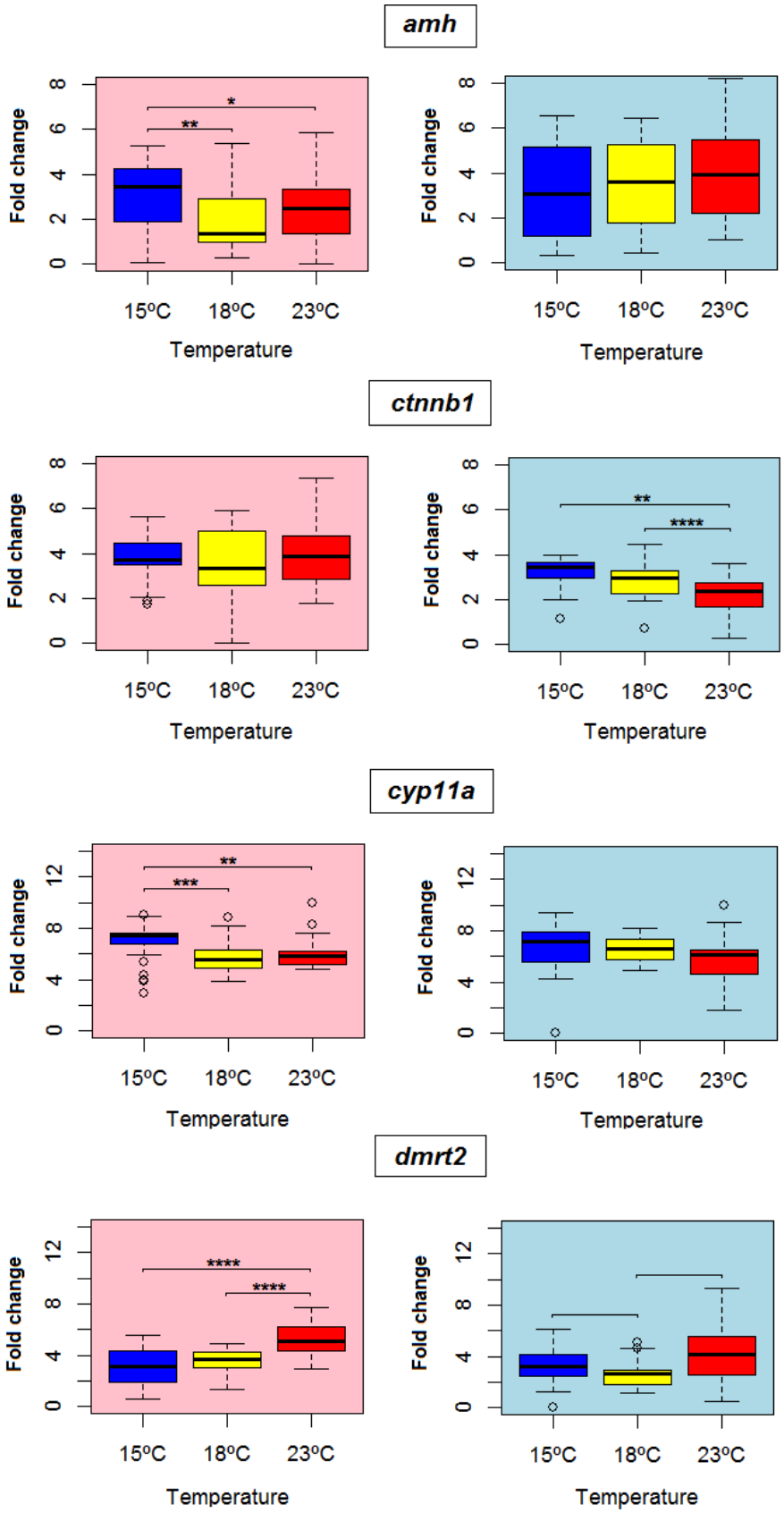
although the difference between 18 and 23ºC was not significant in females. Among those genes showing sex-specific temperature effects *amh*, *sox9a* and *cyp11a* were found to be more expressed at low temperatures in females, while *sox17* and *dmrt2* showed the opposite pattern with higher expression at 23ºC in females. It should be noted that these were previously identified as male-related genes. On the other side, *ctnnb1*, *piwi2*, *sf1* and *sox6* were overexpressed at low temperatures in males, and the four genes showed a very similar pattern.

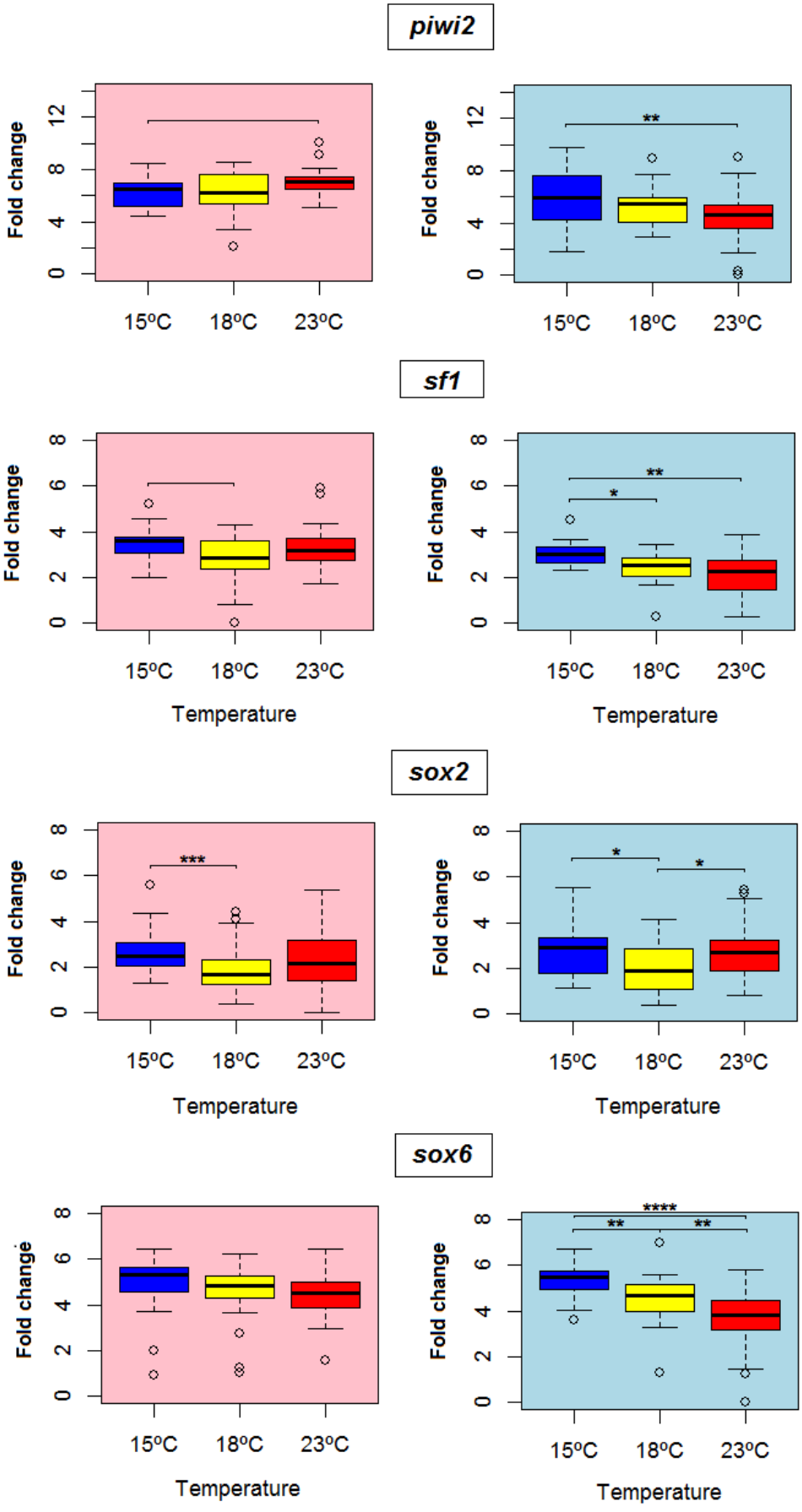
Figure 8. Sex proportions and temperature

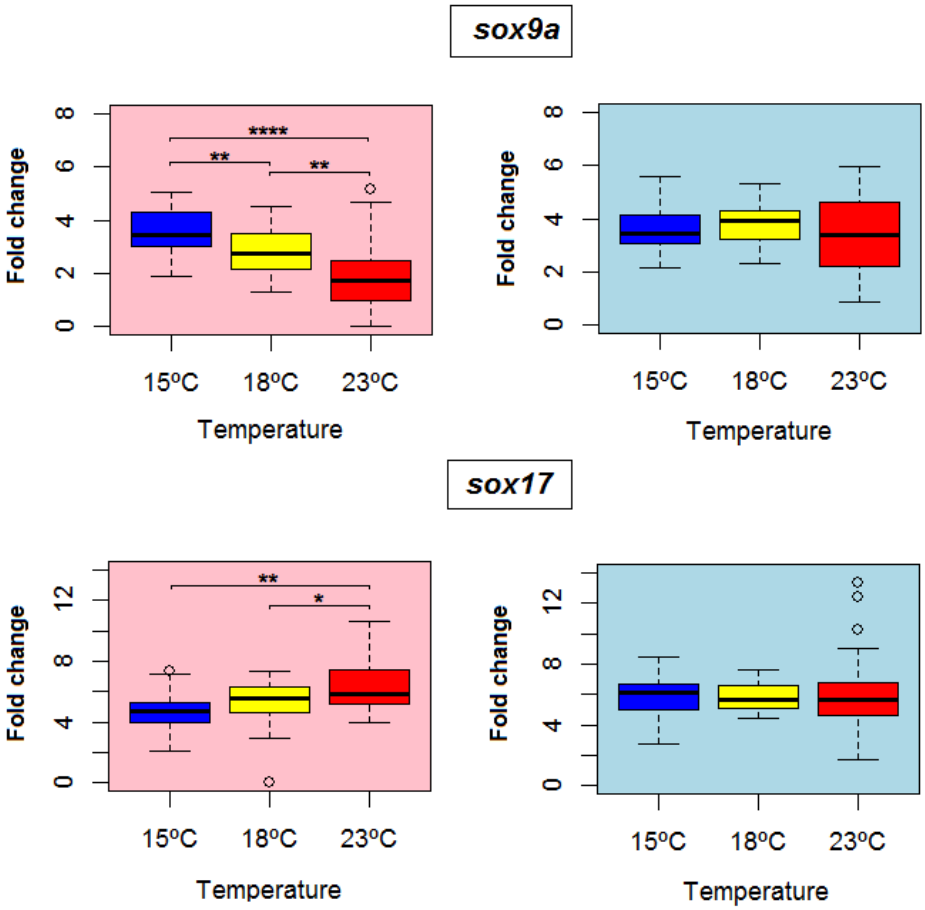


Percentage of male and female turbot, histologically sexed, at 15, 18 and 23ºC. Also, the percentage of phenotypic females which are genetic males is shown. No genetic females developed as males. Thirty fish per temperature.

Figure 9. Temperature effects on gene expression




piwi2
Fold change
0
4
8
12
15ºC
18ºC
23ºC
**
sf1
Fold change
0
2
4
6
8
15ºC
18ºC
23ºC
Temperature
*
**
sox2
Fold change
0
2
4
6
8
15ºC
18ºC
23ºC
Temperature
***
*
sox6
Fold change
0
2
4
6
8
15ºC
18ºC
23ºC
Temperature
****

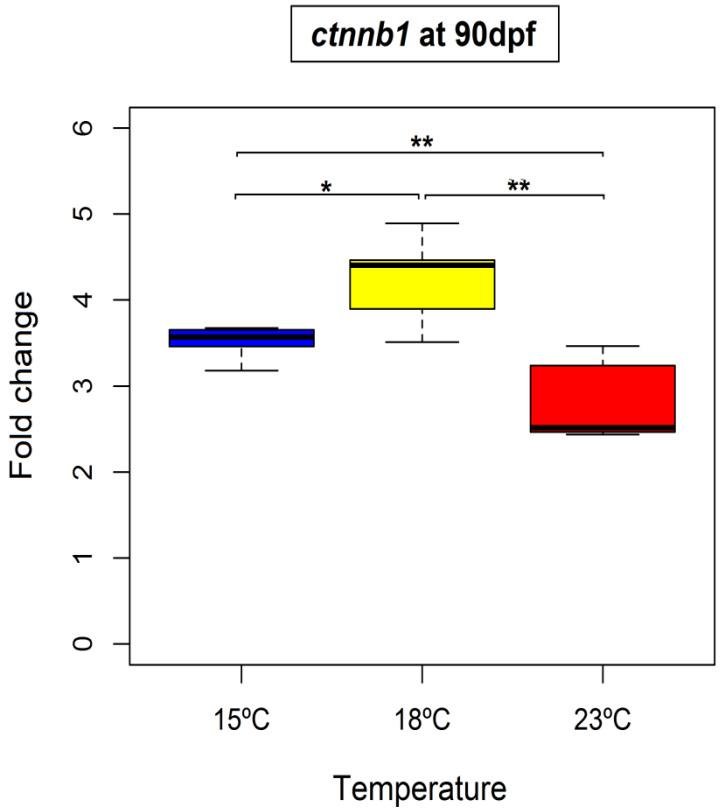


Mean fold change gene expression values at 15, 18 and 23ºC in the whole dataset are shown for males (light blue background) and females (pink background). Error bars represent standard deviation. Significate differences between temperatures are indicated by * ($0.01<p<0.05$), ** ($0.001<p<0.01$), *** ($0.0001<p<0.001$) or **** ($p<0.0001$). Black asterisks indicate that the detected difference is independent of fish length, while red asterisks indicate that fish length has an influence on the temperature differences.

Among the genes which showed expression differences between temperatures also influenced by growth (Supplementary figure 2), *dact, dnmt1, rdh3, sox8 and sox19* showed a similar pattern in females, presenting higher expression at higher temperatures; while in males the most interesting result was the higher expression of *fxr1* at lower temperatures. Expression analysis for genes related to chromatin remodelation showed differences depending on both size and temperature (*dnmt1, hh1, tdrd1*) (Supplementary figure 2), with the exception of *piwi2* which showed significant differences between 15 and 23ºC in males (Figure 9).

Finally, considering 90 dpf as the nearest stage to sex determination among the assayed stages, we decided to test for temperature differences at this stage which were independent of both length and sex, since most genes did not show dimorphic gene expression at this stage. Due to relative small sample size (only ten samples per temperature) a single gene showed significant different between temperatures at 90 dpf: *ctnnb1* (Figure 10). *Ctnnb1* highest expression was observed at 18ºC and at 23ºC showed the lowest expression.

Figure 10: Temperature effects on ctnnb1 expression at 90 dpf



Mean fold change gene expression values at 15, 18 and 23ºC at 90 dpf are shown for *ctnnb1*. Error bars represent standard deviation. Significate differences between temperatures are indicated by * ($0.01<p<0.05$) or ** ($0.001<p<0.01$).

4. Discussion

Recently, sex determination and differentiation has begun to be seen as a modular process rather than a cascade, where sex determination behaves as a threshold like character (Heule et al. 2014). In this model, gonadal fate depends on several factors acting coordinately, among them several genes and environmental variables. In this sense, and given the interest in obtaining single sex stocks in many aquaculture species, understanding the different gene patterns during early sex differentiation, supposedly the moment where gonad fate can be more easily controlled or altered, is of great importance to control sex ratios and manipulate sex determination. In this study we have analyzed the expression of 29 genes during turbot early sex differentiation. Several genes have been studied regarding sex differentiation in fish for the first time and a total of 21 genes were found to show dimorphic expression at some point during early sex differentiation in turbot. Furthermore, the influence of temperature was also assessed finding differences for 10 genes between temperatures. This study has

broadened our knowledge of gene expression patterns during early sex determination in turbot in particular and in fish in general.

4.1. Early sex differentiation

Here it has been shown that turbot could be accurately sexed by gene expression at 90 dpf and 5-6 cm length. Although morphological gonad differences between sexes were not detected at this developmental stage, the first molecular signals of sex differentiation were observed between 75 and 90 dpf. One of the first genetic signatures is the differential expression of *cyp19a1a*, *amh* and *vasa*, which allowed discriminating males (high *amh* expression) and females (high *cyp19a1a* and *vasa* expression) at this development stage. *Amh* and *cyp19a1a* are genes involved in sex differentiation across all vertebrate taxa (Cutting et al. 2013; Valenzuela et al. 2013). *Amh* is a member of the transforming growth factor β superfamily of glycoproteins and is expressed exclusively in gonads, and cyp19a1a is the enzyme responsible for the conversion of androgens into estrogens and is one of the most functionally conserved sex-related genes along evolution (Valenzuela et al. 2013). Although the Müllerian ducts are not present in modern teleosts (Miura et al. 2002), *amh* orthologs have been described in several species and characterized as key factors for gonad sex differentiation (Rodríguez-Marí et al. 2005, Klüver et al. 2007, Halm et al. 2007) and sex determination (Hattori et al. 2012). *Amh* and *cyp19a1a* have been reported as male and female-like genes, respectively, in several fish species (Callard et al. 2001, Poonlaphdecha et al. 2013; Li et al. 2013; Johnsen et al. 2013), and our results in turbot are in agreement with their reported roles.

4.2. Primordial germ cells

Vasa, *tdrd1* and *gsdf* are genes related to primordial germ cell development whose expression increased from 75 to 90 dpf, being among the first cues of GD in turbot. *Vasa* increased its expression in both sexes from 75 to 90 dpf, but 14-fold in females and 5-fold in males, while *tdrd1* and *gsdf* expression increased at 90 dpf, although *tdrd1* pro-female expression was not detected until 105 dpf and *gsdf* did not present any dimorphic pattern between sexes at all.

Trdrd are proteins which can associate with *piwil1* and *piwil2* both involved in the piRNA (piwi interacting small RNAs) pathway. This is a small RNA silencing system, which functions in germline specification, gametogenesis, transposon silencing, genome integrity, and stem cell maintenance across the animal phylogeny (Yi et al. 2014). *Tdrd1* has been found to bind to the piwi pathway proteins *ziwi* and *zili* (Huang et al. 2011) and also to interact with *vasa* (Kirino et al. 2010). *Tdrd1* proteins were detected in the primordial germ cells of zebrafish (*Danio rerio*) at 4 dpf and are involved in both oocyte and sperm development (Huang et al. 2011). *Vasa* gene is a highly specific marker of germ cells (Lasko and Ashburner, 1988; Hay et al. 1988; Komiya et al. 1994; Castrillon et al. 2000) required for

their development (Johnstone and Lasko, 2004) and conserved along several invertebrate and vertebrate taxa (Ephrussi and Lehman, 1992). It has been suggested that *vasa* promotes translation of target mRNAs involved in the development of the germ cell line (Johnstone and Lasko, 2004; Styhler et al. 2002; Liu et al. 2003) and it has also been connected with the piwi pathway (Tanaka et al. 2000), which, in turn, is essential for germline development (Aravin et al. 2007). Finally, *gsdf* is a highly conserved teleost-specific cytokine member of the TGF-β superfamily which has been reported to be expressed in the somatic cells surrounding the primordial germ cells in rainbow trout and promoting their proliferation (Sawatari et al. 2007). A copy of *gsdf*, named $gsdf^{Y}$, has been found to be the sex determinant gene in *Oryzias luzonensis* with a higher expression in males 10 days after hatching (Myosho et al. 2012). *Gsdf* has also shown higher expression in testis and co-localization with *dmy* expression during the early stages of GD in medaka (*Oryzias latipes*) (Shibata et al. 2010). In *Oryzias dancena sox3*, the SD gene, initiates testicular differentiation by upregulating *gsdf*, and it has been proposed that *sox3* is epistatic over *gsdf* in the SD pathway (Takehana et al. 2014). This gene has also shown higher levels of expression in testis in zebrafish, three-spot wrasse (*Halichoeres trimaculatus*) and coelacanth (*Latimeria menadoensis*) (Gautier et al. 2011, Horiguchi et al, 2013, Forconi et al. 2013). Also, recently, *gsdf* has been proposed as the sex determining gene in *Anoploma fimbria* (Rondeau et al. 2013). *Gsdf* does not seem to have such a male-like function in turbot, since it did not present a dimorphic expression at the critical period of GD, however it seems to be important for gonad development in both sexes since its expression greatly increased between 75 and 90 dpf. Even more, this is the only gene which shows a significant expression increase from 60 to 75 dpf in our study, which is consistent with a function as germ cell inductor, since its expression precedes that of *vasa* or *tdrd1*, germ cell genetic markers.

The expression pattern of *vasa*, *tdrd1* and *gsdf* in turbot suggests that primordial germ cells start proliferating between 75 and 90 dpf in both sexes, either faster in females or suffering a certain delay in males, as suggested by *vasa* and *tdrd1* expression levels. Another gene related to the *piwi* pathway and germ-line specific, *piwi2*, was investigated in our study. The expression pattern of this gene was slightly different from that of *vasa* or *tdrd1* since its activation was delayed until 105 dpf and only took place in females. In zebrafish, *piwi2* has been found to play a crucial role in meiosis (Houwing et al. 2008) and perhaps its different pattern of expression in turbot may be related to the start of meiosis in female germ cells.

The amount of primordial cells is recognized as one of the initial differences between male and female gonads in some fish species like zebrafish (Siegfried and Nüsslein-Volhard, 2008), medaka (Kurokawa et al. 2007) and stickleback (*Gasterosteus aculeatus*) (Lewis et al. 2008), although not in others like loach (*Misgurnus anguillicaudatus*) (Fujimoto et al. 2010) and goldfish (*Carassius auratus*) (Goto et al. 2012). Germ cell proliferation has been found to be associated with sex differentiation and even SD in several fish species. In medaka, germ cell proliferation is inhibited in males when the sex determining gene, *dmY*, is expressed at the SD stage before testis differentiation (Herpin et al. 2007). When *dmy* is not active in XY embryos, germ cells proliferate and enter meiosis like in XX embryos. Surprisingly, *amh* and

amhrII do not present a dimorphic expression in medaka during GD (Klüver et al. 2007), but *amh* has been found to control germ cell proliferation in this species, and mutations on its receptor (*amhrII*) lead to excessive proliferation of germ cells which caused male-to-female sex reversal (Morinaga et al. 2007), although female XY gonads still expressed *dmY*. Furthermore, if these *amhrII* mutants are depleted of germ cells, testis development takes place (Nakamura et al. 2012). So, *amh* seems to be a repressor of germ cell proliferation in medaka necessary for SD. This is also de case in fugu (*Fugu rubripes*), where a single SNP in the coding region of *amhrII* is likely responsible for SD (Kamiya et al. 2012). This SNP encodes a protein with a reduced function and is fixed in females, which are not sensitive to *amh*. On the other hand, fugu males are heterozygous and a fully functional *amhrII* allele mediates *amh* signaling, decreasing the number of germ cells (Kamiya et al. 2012). Also, in the Patagonian pejerrey, a copy of *amh*, *amhY*, has been found to be the sex determining gene and its action has been suggested to regulate germ cell proliferation and SD, being upstream to the autosomal *amh* and relegating the former to a function in testicular maturation and/or spermatogenesis (Hattori et al. 2012). If *amh* is indeed a germ cell inhibitor in turbot, its action could explain why *gsdf* expression is not dimorphic in turbot while germ cell markers like *vasa* and *tdrd1* show higher expression in females.

Interestingly, in the female gonad of zebrafish the maintenance of *cyp19a1a* expression, but not its activation, has been related to the presence of the primordial germ cells (Siegfried and Nüsslein-Volhard, 2008), pointing towards a model where *amh* is responsible of the control of germ cell proliferation while germ cells aid to maintain *cyp19a1a* expression. In our experiment, both *cyp19a1a* and *amh* showed higher expression in females and males, respectively, at 90 dpf. This pattern of *amh* expression was also reported in goldfish (Goto et al. 2012). According to information from other species, a threshold expression of *amh* could be controlling sexual fate. If *amh* does not reach the required expression level, primordial germ cells will proliferate and maintain *cyp19a1a* levels while *amh* levels decrease. On the contrary, if *amh* expression reaches a certain threshold, germ cells stop proliferating and *cyp19a1a* expression decreases.

Foxl2 pattern is similar to that previously described for *cyp19a1a*, *vasa*, *tdrd1* and *gsdf*. In fact, these five genes are clustered together in the heatmap. In this study *foxl2* increases its expression at 90 dpf, slightly more in females, though the difference is not significative; only after this stage it shows significant higher expression in females, although the severe down-regulation observed for cyp19a1a in males at the last stages was not observed for *foxl2*. *Foxl2* is a transcription factor that activates *cyp19a1a* transcription by binding to its promoter region (Wang et al. 2007) and both genes are strictly co-expressed in mammals (Pannetier et al. 2006). Its expression in turbot is consistent with an activation of *cyp19a1a*, however, the later decrease of *cyp19a1a* while *foxl2* expression is still high in males suggests other roles for *foxl2* not related to *cyp19a1a* activation at early stages of development. *Foxl2* expression has also been described in the male gonad of tilapia (*Oerochromis niloticus*), southern catfish (*Silurus meridionalis*) and goldfish (Wang et al. 2007; Liu et al. 2007; Goto et al. 2012). Also, cells with *cyp19a1a* expression without *foxl2* expression have been reported in medaka

(Herpin et al. 2013), so, apparently, *foxl2* is not essential for *cyp19a1a* expression maintenance in that species, although the authors did not exclude that those cells had earlier *foxl2* expression (Herpin et al. 2013). As previously suggested, *foxl2* might be a general regulator of steroidogenesis in gonads also involved in the initiation of the female gonad development cascade (Wang et al. 2007), however *foxl2* function seems to be more complex than in mammals and more factors appear to be involved in *cyp19a1a* regulation in fish.

Growth-related factors have also been associated to cell proliferation and gonad differentiation (Piferrer et al, 2012). In our study, we could not detect length differences between sexes at these initial stages of GD, which is consistent with previous reports which found the first signs of growth dimorphism at 240 dph in turbot (Imsland et al. 1997). So, the mechanism associated with SD and early GD does not produce sex dimorphic growth. However, we cannot rule out differences in gonad size between sexes that could be related to SD. Indeed, it would be interesting to explore the possible connections between differential growth and gonad size in species with growth dimorphism like turbot.

4.3. Female sex differentiation

Samples already started GD before 105- 135 dpf either towards males or females, and the sex is easily identified by *cyp19a1a* expression alone at these developmental stages. Several other turbot female-like genes were also detected when during this period of GD (*foxl2*, *vasa*, *tdrd1*, *sox19*, *dnmt1*, *dact1*, *rdh3*) and, overall, female differentiation involved a larger number of expressed genes than in males, where gene expression was very similar to undifferentiated fish, excluding a few classical male-like genes (*amh*, *sox9*, *sox8*). Female development seems to involve more complex genetic machinery. Preliminary results from turbot gonad microarray have led to the same conclusion (Ribas et al. submitted).

There are six genes, *dnmt1*, *rdh3*, *sox19*, *dact1*, *ctnnb1* and *sf1* which showed a similar increasing expression in females at 105 dpf. These genes showed high pair-wise correlation values within 105-135 dpf, and also the highest negative correlations with *amh* amongst all the assayed genes. So, *amh* down-regulation may be required for the activation of some genes important for female gonad development. *Dnmt1* is a DNA methylase which preferentially methylates hemimethylated DNA and is expressed in mouse non-proliferating growing oocytes where it plays a crucial role in maintaining imprinted genes during early embryogenesis (Howell et al. 2001). Conversely, *dnmt1* expression was found to be correlated with the proliferative state of male germ cells in mouse and down-regulated during arrest (Sakai et al. 2001). *Dnmt1* had been previously reported to be highly expressed in proliferating cells (Szyf et al. 1991). The expression of *dnmt1* in turbot started later than that of *vasa*, *tdrd1* or *gsdf*, so we do not expect this gene to be connected with the proliferation of germ cells. Instead, it might be related to the gonad somatic cells, which is in agreement with other results in mice, where *dnmt1* was found to be indispensable for the survival of these cells (Sakai et al. 2001). *Rdh3* is a retinol dehydrogenase involved in retinol metabolism and has been recently linked with meiosis in zebrafish, suggesting that retinoic acid may promote

the entrance in meiosis (Rodríguez-Marí et al. 2013). Retinol metabolism has also been associated with ovary differentiation in *Acanthopagrus schlegelii* (Lau et al. 2013). *Sox19*, although it has orthologous in other vertebrates, has undergone a rapid change and its function apparently differs between the different taxa. In fact, *sox15*, the *sox19* orthologous in mammals, is pseudogenized (Yamada et al. 2008). *Sox19* has not been studied in many fish species, but it has shown a conserved function in ovary development in fish (Navarro-Martín et al. 2012). *Dact1* is connected to the *wnt* signaling pathway and has been proposed to stabilize *ctnnb1*, and so, to modulate the transcriptional activation of target genes of this pathway (Waxman et al. 2004). *Ctnnb1* is the key downstream component of the canonical *wnt* signaling pathway, which antagonizes *sox9* and blocks testis development in mammals, thus promoting ovarian development (Maatouk et al. 2008). Our results are in agreement with a conserved female development function of this pathway, which has also been shown in zebrafish (Sreenivasan et al. 2014) and rainbow trout (Nicol et al. 2013). However, in turbot, the *wnt* pathway seems to be at least partially independent of *wnt4* since the expression of this gene was higher at undifferentiated stages and dropped at 90 dpf irrespective of sex, although later it showed higher expression in females. W*nt4* is highly expressed in the undifferentiated gonads of turbot at 60 and 75 dpf, which is consistent with a role in early gonad development also observed in mammals, where it is responsible for the development of Müller ducts in both sexes (Vainio et al. 1999), but needless to say its function has to be different in fish. *Wnt4* is also a key female gene in mammals which antagonizes *fgf9* and down-regulates *sox9* expression (Kim et al. 2006). *Wnt4* not so clear female-pattern during GD in turbot is likely related to the absence of *fgf9* in teleosts (Forconi et al. 2013). No dimorphic *wnt4* expression has been observed in rainbow trout (*Oncorhynchus mykiss*) (Nicol et al. 2011), zebrafish (Sreenivasan et al. 2014) or the more distant *Rana rugosa* (Oshima et al. 2005). *Wnt4* does not show a conserved function in female SD along evolution, and the results in our study suggest that it is not involved in the expression of *ctnnb1* in the female gonad development and so, other wnt proteins should be responsible for activating the *wnt* pathway, which seems to have a conserved female prominent function. *Sf1* or splicing factor 1 is involved in the assembly of the spliceosome and so, it is related to RNA splicing (Neubauer et al. 1998). There are several examples of differential splicing connected to sex development in insects (Nissen et al. 2012, Wang et al. 2014, Suzuki et al. 2014) and it is reiteratively found controlling the expression of key SD genes for example in *Drosophila* and other dipteran and hymenopteran insects (Salz, 2011). Alternative splicing of sex related genes has been found in fish too (von Schalburg et al. 2011; Miyake et al. 2012), but sex dimorphism has not been described for this gene. We found *sf1* overexpression in females, which suggests another layer of complexity to sex differentiation in fish involving dimorphic alternative splicing.

The coactivation of these six gene genes in female differentiation from 105 dpf, except for *ctnnb1* and *dact1*, does not seem to be related to a specific pathway considering their functional diversity, but with the activation of several concomitant pathways at the beginning of ovary development.

4.4. Male-like genes

Besides *amh*, we found two overexpressed genes in males from 105 dpf onwards: *sox9a* and *sox8*. Furthermore, the heatmap and network analyses suggested that *cyp11a* and *fshb* are related to male development as well. *Sox9a* is an essential player in sex differentiation and its male-like nature seems to be rather conserved along evolution, but its relevance seems to be variable (Smith and Sinclair, 2004; Shoemaker and Crews, 2009). In mammals, this gene is directly activated by *sry* and is responsible and sufficient for fating the male gonad (Ramkissoon and Goodfellow, 1996), also activating *amh* transcription which is responsible for Müllerian duct regression (Knower et al. 2003). In birds, *sox9a* is co-expressed with *amh* and induced by the SD gene *dmrt1* (Lambeth et al. 2014). However, in medaka, *sox9* is not required for testis development (Nakamura et al. 2008, 2012). In turbot, *sox9a* dimorphic expression is found later than that of *amh*, and its expression is more stable along the assayed stages, suggesting a less important role in GD in this species. The role of *sox8* is very similar to that of *sox9* in mammals. They belong to the *sox* family E group and *sox8* has been suggested to reinforce the action of *sox9* in male GD (Chaboissier et al. 2004). *Cyp11a* and *fshb* showed a positive correlation with the male genes in our study, but they did not display a dimorphic expression pattern at any stage. *Cyp11a* catalyzes the first step of steroidogenesis, the conversion of cholesterol to pregnenolone, a steroid hormone, precursor of androgens and estrogens (Miller and Auchus, 2011), while f*shb* is believed to control both vitellogenesis and spermatogenesis in adult fish (Swanson et al. 2003), but it has also been reported to be expressed before or during sex differentiation (Fan et al. 2003; Wu et al. 2009) in agreement with our results. However, we did not find any dimorphic expression of this gene, so it does not seem to drive gonad fating, which is in agreement with previous findings in tilapia (Yan et al. 2012).

4.5. Genes in the main SD region

Fxr1 is located in the turbot genome very close to Sma-USCE30 (Taboada et al. 2014), the highest sex-associated marker in this species, within the main SD region at linkage group 5 (LG5) (Martínez et al. 2009), thus representing a potential SD candidate gene. In this experiment, *fxr1* is highly expressed before the first GD signs and precedes the expression of *cyp19a1a* and *amh*, although at this time the expression is not sexually dimorphic. Also, *fxr1* position in the functional network is quite intriguing, connecting male and female clusters through its negative correlation with *amh* and positive with *sf1*. *Fxr1* is a RNA-binding protein and together with *Fxr2* is an autosomal paralogue of *fmrp* (fragil X mental retardation 1), important for normal female reproductive function and cognition development in humans. It should be noted at this point that turbot shows a ZZ/ZW SD system (Martínez et al. 2009), so the SD gene should be likely related to the activation of the GD pathway. *Fxr1* is involved in miRNA-mediated post-transcriptional gene regulation, and has been related to female gametogenesis in pigs (Yang et al. 2012) and *Xenopus laevis* (Mortensen et al. 2011). This gene regulates intracellular transport and local translation of certain mRNAs in mouse

(Mientjes et al. 2004), and presents several mRNA variants and protein isoforms in mammals (Huot et al. 2001). The function of this gene as a post-transcriptional regulator, its expression pattern at the very beginning of GD in turbot, and its co-localization with the main SD region support *fxr1* as a SD candidate in turbot. Also *fxr1*, *fxr22* and *fmrp* have Tudor domains (Adams-Cioaba et al. 2010) which recently have been associated with effector proteins involved in histone methylation. Several tudor domain containing proteins were found to interact with methylated lysine in histones and also with methylated arginine residues in non-histone proteins involved in the regulation of RNA metabolism, small RNA pathways or germ cell development. It is also intriguing the higher expression level of *fxr1* at low temperature which is associated with an increase of females proportion (see next subsection). However, further analysis will be required for examining expression of this gene in younger individuals looking for a putative dimorphic pattern between sexes. Finally *sox2*, also located in the main SD region of turbot (Viñas et al. 2012), did not present a dimorphic expression pattern and also showed a relatively steady expression along all the assayed stages, suggesting no role of this gene in turbot SD.

4.6. Temperature effects

Cold temperatures (15ºC and 18ºC) rendered a higher proportion of phenotypic females in the studied fish. This effect was due to the influence of the temperature itself and not to genetic segregation distortion, since genotyping of the SD marker strongly suggested that some genetic males developed as females. This is very interesting for aquaculture, especially if turbot were to show compensatory growth when changed to warmer temperatures for faster growth after being reared at 15ºC for higher female proportion during early sexual differentiation. Similar results showing low-temperature-induced feminization have been reported in a tilapia species (*Oreochromis mossambicus*) (Wang and Tsai, 2000). Haffray et al. (2009) also reported the influence of temperature on sex ratio in turbot, but it was family-dependent and not always in the same way: two families presented a higher proportion of females than expected at 23ºC while another family presented more females at 15ºC. Despite the small number of families analyzed, temperature effects seem to be limited and family dependent in turbot, but further work should be done to evaluate this issue.

We were able to validate temperature effects which were not influenced by length for some of the genes assayed: *amh*, *ctnnb1*, *cyp11a*, *dmrt2*, *piwi2*, *sf1*, *sox2*, *sox6*, *sox9a* and *sox17*. Among these, only *sox2* effects seem to be sex independent. This gene showed higher expression both at 15 and 23ºC, which may indicate some kind of stress response. *Sox2* is a transcription factor regulating several genes and it is also involved in the maintenance of stem-cell identity (Andreu-Agullo et al. 2012). Its expression pattern during sex differentiation process does not suggest a sex-dependent function in GD of turbot despite being located in the main SD region (Taboada et al. 2014).

Among the genes which presented sex dependent temperature effects, *ctnnb1*, *piwi2*, *sf1* and *sox6* showed higher expression at low temperatures in males. This group is particularly

interesting since we found genetic males developing as females at both 15 and 18ºC. Among these, *ctnnb1* is remarkable because it shows a downstream position in the *wnt* signaling pathway (Capel, 2006) and it also showed expression differences between temperatures at 90 dpf, the earliest stage assayed where SD has already started. *Wnt* signalling pathway needs to be upregulated for developing an ovary in zebrafish (Sreenivasan et al. 2014). So, it is likely that genes involved in the *wnt* signalling pathway machinery are regulated by temperature and responsible for this increase in *ctnnb1* expression and, possibly, responsible for the higher proportion of females. Consistently with our results, elevated *ctnnb1* expression has been reported connected to low temperatures in rats (Zhang et al. 2008) and tilapia (Tsai et al. 2007) although in other tissues. A recent study in oyster also found a biased sex ratio towards females related to higher *ctnnb1* expression at lower rearing temperatures (Santerre et al. 2013). Therefore, gene or genes related to the *wnt* pathway and to *ctnnb1* are candidates for the detected temperature effect producing a higher proportion of females at cold temperatures and this pathway is interesting for future studies on sex-temperature interaction.

For females, some of the genes with temperature influences are not easy to explain. We found *amh*, *sox9a* and *cyp11a*, male-related genes, more expressed at 15ºC where a higher proportion of females was observed. A possible explanation is that the mechanism causing male-to-female sex reversion at this temperature produces an over-expression of certain female genes (i.e. *ctnnb1*) which drive GD towards an ovary, while male genes are not affected until later in development. So, the observed higher expression at 15ºC would be due to genetic males expressing male related genes (*amh*, *sox9a, cyp11a*), even though they were differentiating as females.

Other two genes showed the opposite pattern in females, with higher expression at higher temperatures: *dmrt2* and *sox17*. *Dmrt2* has been shown to be upregulated during gonad development and also expressed in germ cells in the swamp eel (*Monopterus albus*) (Sheng et al. 2014), while in *Rana rugosa* it was found expressed in the developing gonad during SD without any dimorphic pattern, suggesting a function both in testicular and ovarian differentiation (Matsushita et al. 2007). S*ox17* has been associated with ovarian development in *Dicentrarchus labrax* (Navarro-Martín et al. 2009), although it did not present dimorphic expression in turbot and so, apparently, it is not related to female differentiation in this species. Yet, this gene seemed to have a peak of expression at 90 dpf in both sexes, so it could have some function in early gonad development in turbot for both males and females. In the swamp eel, *sox17* was also expressed both in testis and ovary (Wang et al. 2003).

Finally, among those significant temperature effects which we could not separate from length effects, we detected a decrease of *fxr1* expression in males at low temperature, which may be related to the role of this gene on SD in this species; however this has yet to be proved. Furhtermore, *cyp19a1a*, showed higher expression levels at low temperatures as occurred in sea bass (Navarro-Martín et al. 2009).

Temperature effects are gene and sex specific. Given the labile nature of SD in fish related to specific morphogenetic thresholds, several genes could be responsible for sex ratio

shifts. As seen in this study, several genes involved in sex show expression differences due to temperature, and so these genes are potential candidates for sex ratio alterations.

4.7. Concluding remarks

Turbot sex differentiation is ongoing at 90 dpf and sex can be distinguished by the expression levels of three genes when fish are 5-6 cm length: *cyp19a1a, amh* and *vasa*; while later females are easily discriminated by the expression of *cyp19a1a*. The first molecular signs of sex differentiation are the dimorphic expression of these three genes and an increase in the expression of *vasa*, *gsdf* and *tdrd1*, connected with primordial germ cells, suggesting their proliferation from 75 to 90 dpf and an important role in sex differentiation Expression analysis on genes placed on the SD region of turbot suggest that *sox2* could be discarded as sex determining gene and that *fxr1* has an expression pattern consistent with a candidate sex determinant. Our data suggest that female development has more complex machinery and is strongly regulated, suggesting the involvement of both methylation and splicing mechanisms. Furthermore, we have observed that temperature affects the expression of several genes and suggest that the *Wnt/β-catenin* pathway is a likely candidate for feminization at low temperatures, since *ctnnb1* expression is higher at low temperatures and we also observed a higher proportion of females at 15 and 18ºC.

Turbot sex differentiation seems to involve several genes and mechanisms, leading to a complex process with many factors involved. These results are more compatible with a view of sex determination as a network where the activation or repression of several genes can affect gonad fate. This view of sex determination as a threshold character could help us to understand temperature effects during sex differentiation.

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Supplementary material

Supplementary Table 1: Primer table

Gene	Primer F (5' -> 3')	Primer R (5' -> 3')	Product length (bp)
amh	CCAGGGCGGACCCCGATAAC	TGGCTGTGTTTGGACCCACGAG	99
ar1	TCGGATGCACGTCTCCACCA	GGAGGGAGTCCAGGAGTCGGG	275
ctnnb1	AGCTGTGTCGGTCGCGGTTT	GCCAGCCTCTGGACGTTGGTG	404
cyp11a	TCTGGGTTTGATGCTGGACT	ACCTTGGTTGAAGATCCCGTC	178
cyp19a1a	CAGCGAGGAAGCTGGCAAACA	ACACGCAGACTCGGCTTTTTACATC	148
dact	TCAGAGGGCAAAAATGGGCT	ACTTCAGTGGGCTTCCTGTG	144
dmrt2	GACTTTCTGTCCAAGCCCCT	GGGCGTGGGTCTTTTCAGTA	91
dnmt1	GGAGTACGCGCCCATCTTT	GTCCTCCGTGAAGCAGTTGA	169
foxl2	GGCGGGGAGAGGAAGGGGAAT	ATCCGGCGGCGTCTCCTGTA	89
fshb	TGCAAACTGGCCAACATCAC	CCGTTAATGTGCTTCGCCTC	179
fxr1	AGGTGCCCTTCAGTGATGTC	TCTCGTTGTAGGTGGCATCA	200
gsdf	CTGGGCTGGAACAACTGGAT	GGCACCATTTCCTGGGAGTT	173
hh1	AGAGAGCCAAGTATCGGAGG	ATCCTTCAGCCTTCAGAGCC	132
hsp27	AGGAGAGGAAGGATGAGCACGGC	TTGTTGTCGGCGGTGACGGG	191
lhx8	TTCACCAGCGTTCATTCGTC	CACCGAACTACACAAGCAGA	280
piwi2	ACAACACAGCGAACCTCACA	GGCATACTTGCATGGTGCTG	113
ptges3	TCTACGACCGCACCATCAAC	TCATGCTCCCAGTCTCTCCA	133
rdh3	CTGACGACCACACACCTTGA	GCGACTCCAGCATTGTTCAC	119
rpl17	ACCAGTGCGTCCCCTTCA	CTCATCTTCGGAGCCTTGTTC	214
rps4	CAACATCTTCGTCATCGGCAAGG	ATTGAACCAGCCTCAGTGTTTAGC	143
sf1	TCACCAACACCACCCTCTGT	CCATGAGGGACAGGTACTCC	155
sox2	ACAGACAAATGTCGGGGTTGGGGA	CCGGCTCCTCTCGAGCTTCCT	147
sox6	CCCATTTCTCCCTCCTCTCT	CCTTTCCGAGGAGACTGTTG	193
sox8	AAGACGCTGGGGAAACTGT	CGGGGCTGGTACTTGTAGTC	138
sox9a	ATCAGTACCCACACCTGCATAAC	TCAGCCTCCTCCACGAACG	103
sox17	TGTTCGGGAAGCAGGTGAAAGGT	CTTGTTGCCATTTTAGGGGACAGT	92
sox19	ACCGAGCGGTTTGTGCCTTG	TCCTCTGGATGCAGTGCTGATTGT	122
tdrd1	TGAGCCTTTGGTGTGGTCTT	ACCATAGCCCGATACCATGC	254
ubq	GCGTGGTGGCATCATTGAGC	CTTCTTCTTGCGGCAGTTGACAG	124
vasa	CTTAGCTGTGGGCGTGGTGGG	ACGTTCTCCTGGCACATCAACG	190
wnt4	TTGGCAAGGTGGTCACGCAGG	AACGCCACACTGGCTGCTGAG	76
zar1	ACGCCTTCAACAACCAGCAG	GATGTCCTCCCACGCGATATG	118

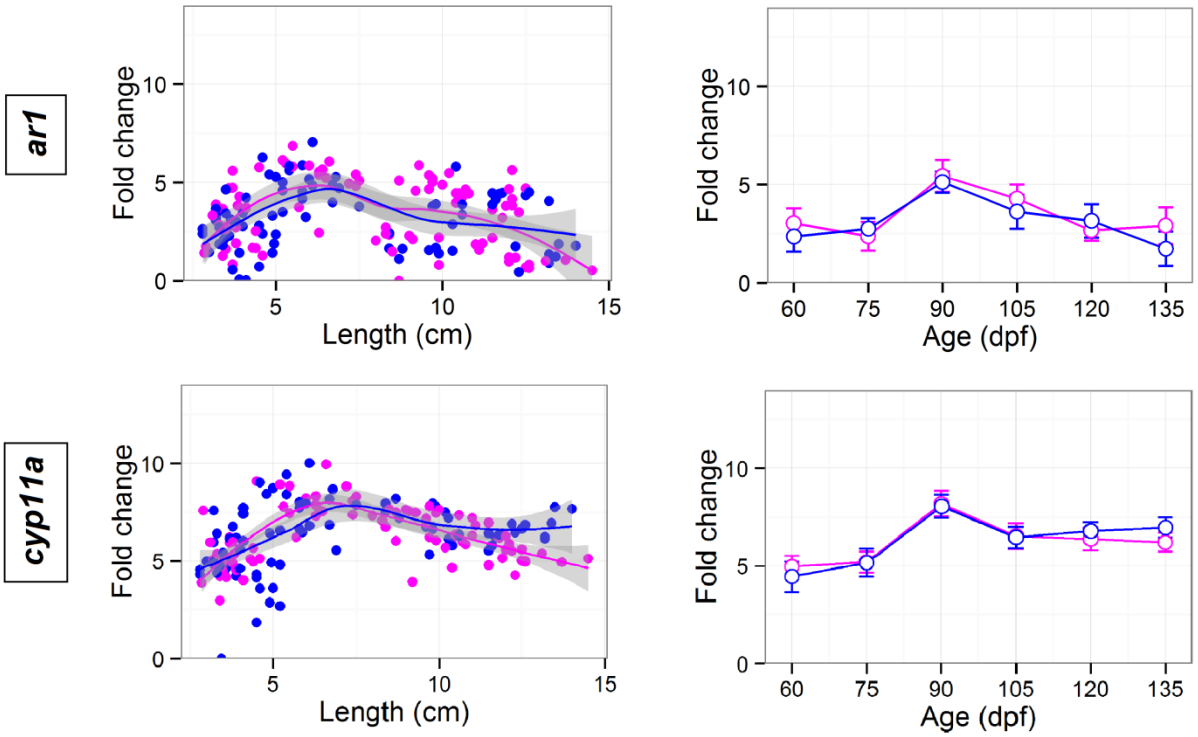
Forward and reverse primers for each gene and the length of the amplified fragment are shown.

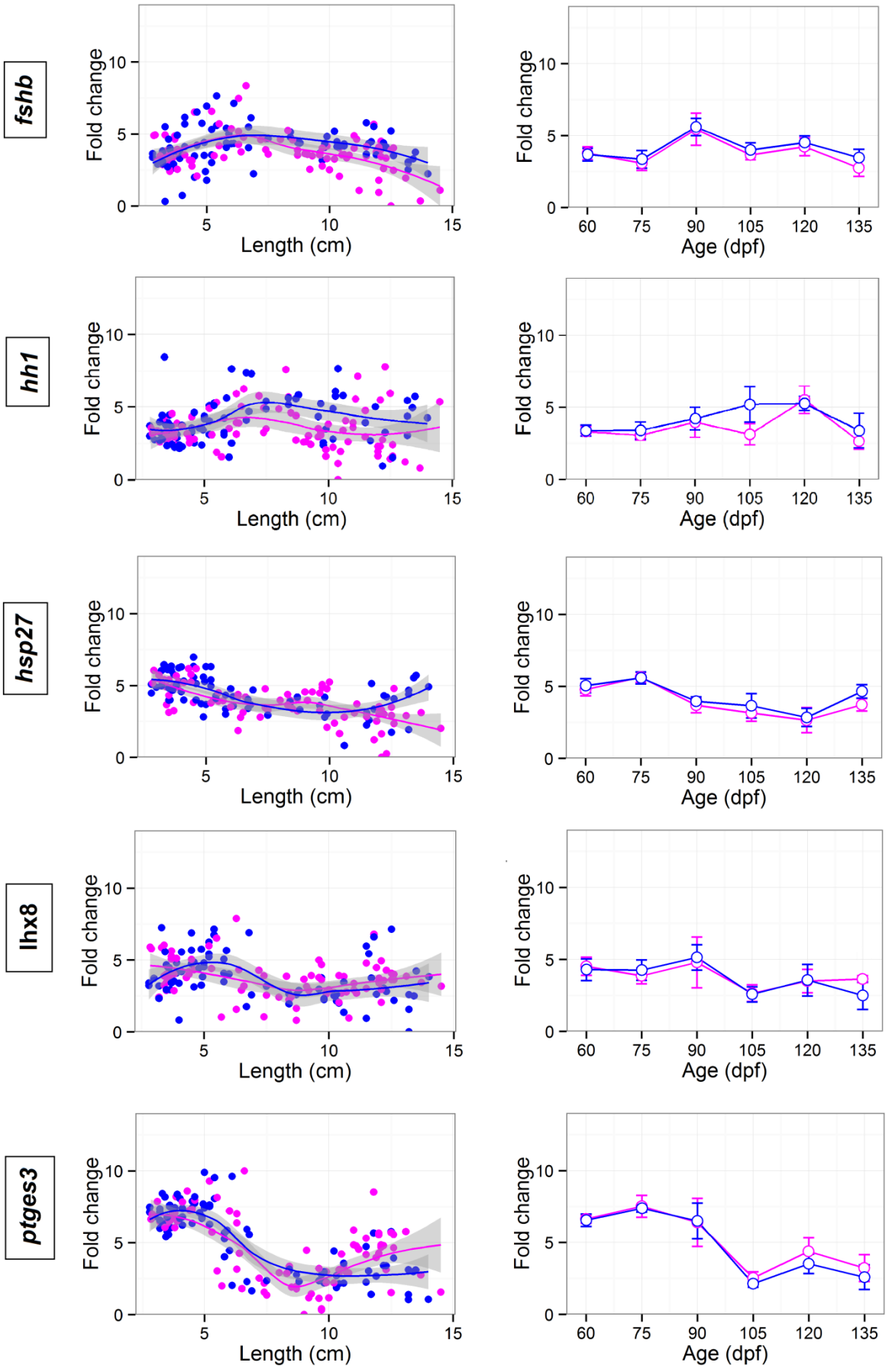
Supplementary Table 2: Brief description of the studied genes

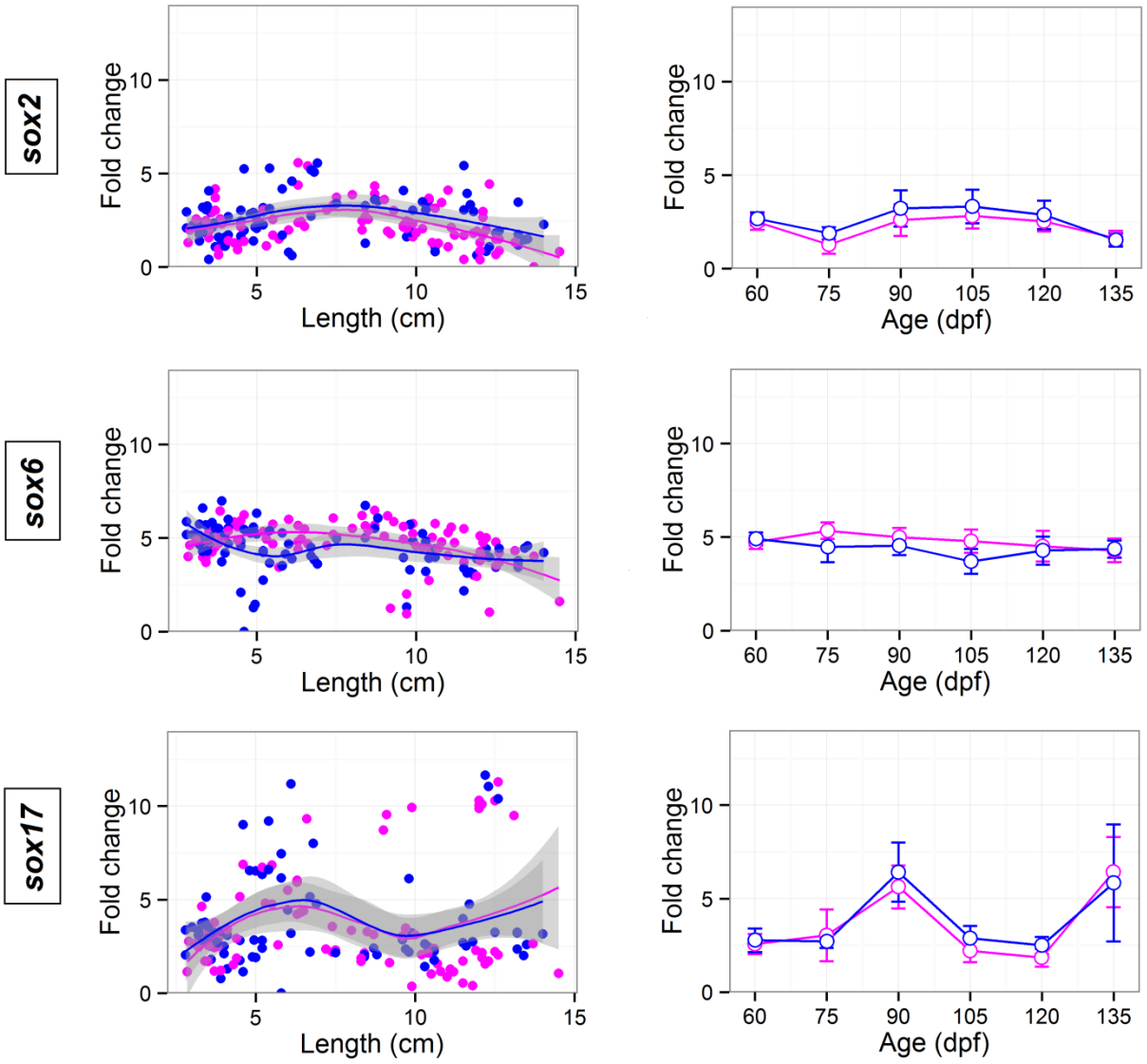
Gene Symbol	**Annotation**	**Function/Relevance**
amh	Müllerian-inhibiting factor	Belongs to the TGF (transforming growth factor) super family. Regression of the Müllerian ducts in male mammals (Cate et al. 1986)
ar1	Androgen receptor 1	Male differentiation (Walters et al. 2010)
ctnnb1	Catenin β-1	Downstream effector of the canonical *Wnt* signaling pathway (Kühl and Wedlich, 1997)
cyp11a1	Cytochrome P450 11A1	Testosteron biosynthetic process (Strushkevich et al. 2011)
cyp19a1a	Aromatase	Catalyzes the formation of estrogens from androgens (Corbin et al. 1988), in fish is essential for female development (Piferrer and Guiguen, 2008).
dact1	Dapper homolog 1	Function in stabilizing CTNNB1 (catenin □-1). Promotes the membrane localization of CTNNB1 (Sensiate et al. 2014)
dmrt2	Doublesex- and mab-3-related transcription factor 2	Expressed in testis. Not required for sex differentiation in mice (Kim et al. 2003)
dnmt1	DNA (cytosine-5)-methyltransferase 1	Methylates CpG residues. Preferentially methylates hemimethylated DNA (Pradhan et al. 2008)
foxl2	Forkhead box protein L2	Critical factor essential for ovary differentiation and maintenance (Ottolenghi et al. 2005)
fshb	Follitropin subunit β	Stimulates development of follicle and spermatogenesis in the reproductive organs (Wreford et al. 2001)
fxr1	Fragile X mental retardation syndrome-related protein 1	Located at the main SD turbot QTL (Martinez et al. 2009)
gsdf	Gonadal soma derived factor	Belongs to the (transforming growth factor) TGF–β super family, gonad specific expression (Gautier et al. 2011), in medaka has been proposed as the sex determining gene (Myosho et al. 2012)
hh1	Histone H1	Chromatin structure protein (Th'ng et al. 2005)
hsp27	Heat shock 27 kDa protein	Regulates androgen receptor levels (Stope et al. 2012)
lhx8	LIM homeobox 8	Involved in ovarian formation and folliculogenesis in mouse (Choi et al. 2008; Jagarlamudi and Rajkovic 2012)
piwi2	Piwi-like protein 2	Germ cell differentiation. Essential for the germline integrity, repressing transposable elements (Aravin et al. 2007)
ptges3	Prostaglandin E synthase 2	Prostaglandin biosynthesis (Murakami et al. 2003)
rdh3	Retinol dehydrogenase 3	Acts on retinol bound on cellular retinol-binding protein (CRBP) (Chai et al. 1995). Retinoic acid is involved in germ cell meiosis entry (Bowles and Koopman, 2010)
sf1	S factor 1	Necessary for spliceosome assembly (Wang et al. 1999)
sox2	Transcription factor *SOX-2*	Negative regulation of canonical Wnt signaling pathway (Mansukhani et al. 2005)
sox6	Transcription factor SOX-6	Cellular response to transforming growth factor beta stimulus (Kim and Im, 2011)

sox8	Transcription factor SOX-8	Involved in male SD, reinforcement of *sox9* action (Barrionuevo et al. 2009)
sox9a	Transcription factor SOX-9	Male sex differentiation (Cameron and Sinclair, 1997)
sox17	Transcription factor SOX-17	Involved in spermatogenesis (Wang et al. 2003) also is related to ovarian development (Navarro-Martín et al., 2009). Inhibits Wnt signaling, promotes degradation of activated CTNNB1 (Liu et al. 2010)
sox19	Transcription factor *Sox-19*	Transcriptional activator belonging to the sox B1 group (Okuda et al. 2006), involved in ovarian differentiation (Navarro-Martín et al. 2012)
tdrd1	Tudor domain-containing protein 1	Participating in the repression transposable elements preventing their mobilization in humans has a central role in spermatogenesis (Reuter et al. 2009)
vasa	Probable ATP-dependent RNA helicase DDX4	Germ cell marker (Castrillon et al. 2000)
wnt4	Protein Wnt-4	Gonad development and female SD (Jordan et al. 2001).
zar1	Zygote arrest protein 1	Essential for female fertility (Wu et al. 2003)

Supplementary Figure 1: Expression of non-dimorphic genes along gonad development

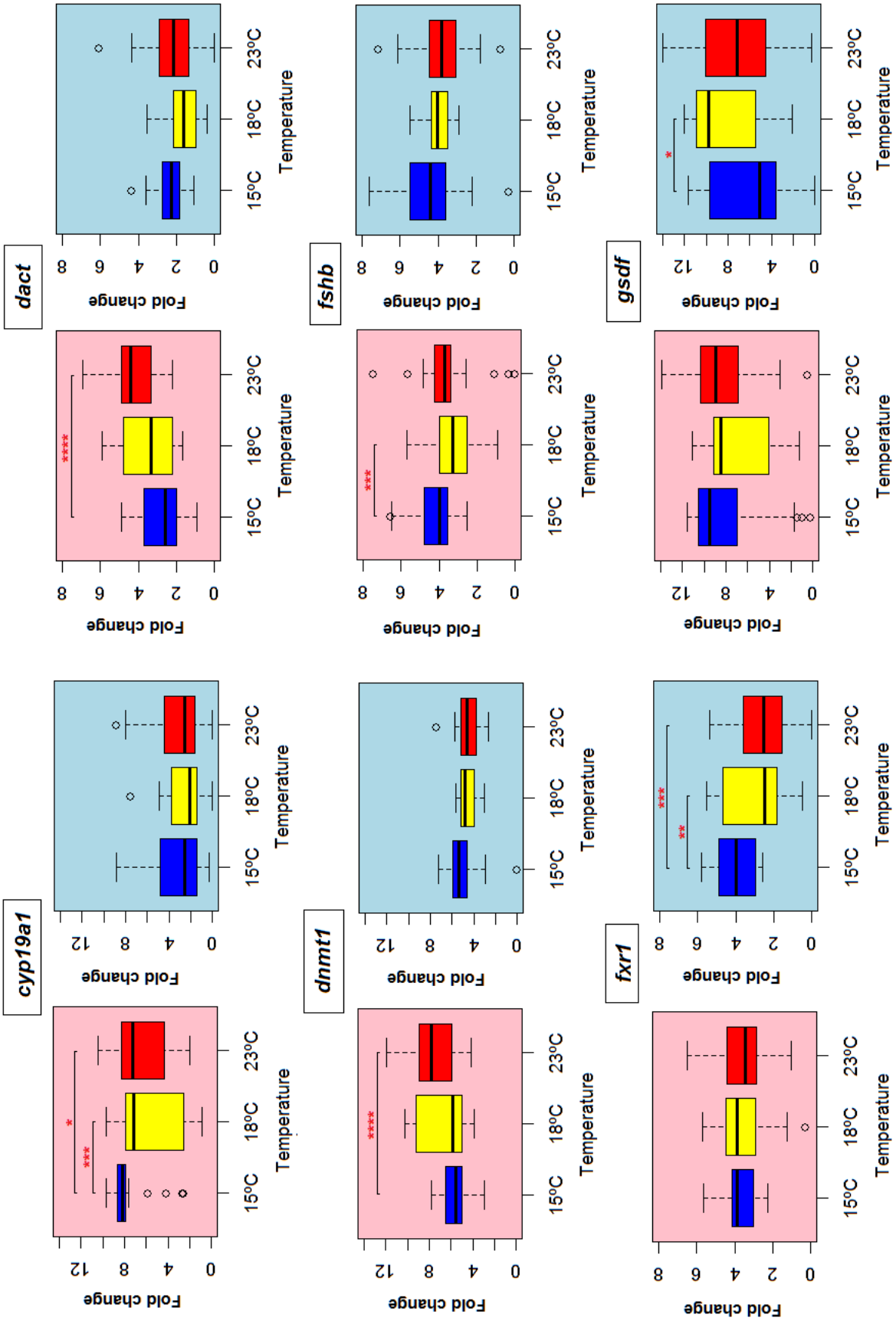


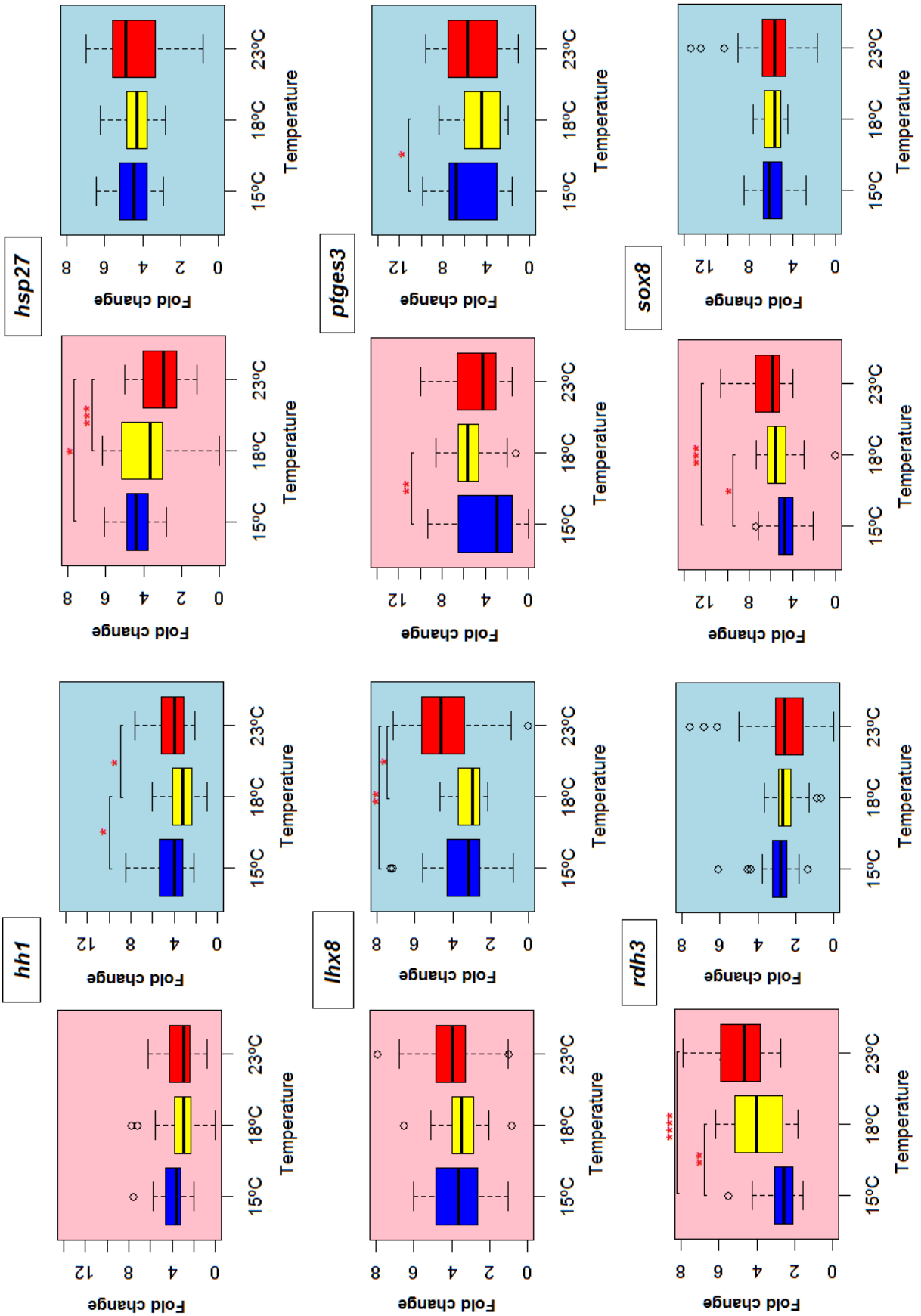

fshb
hh1
hsp27
lhx8
ptges3
Fold change
Length (cm)
Age (dpf)
0
5
10
15
60
75
90
105
120
135

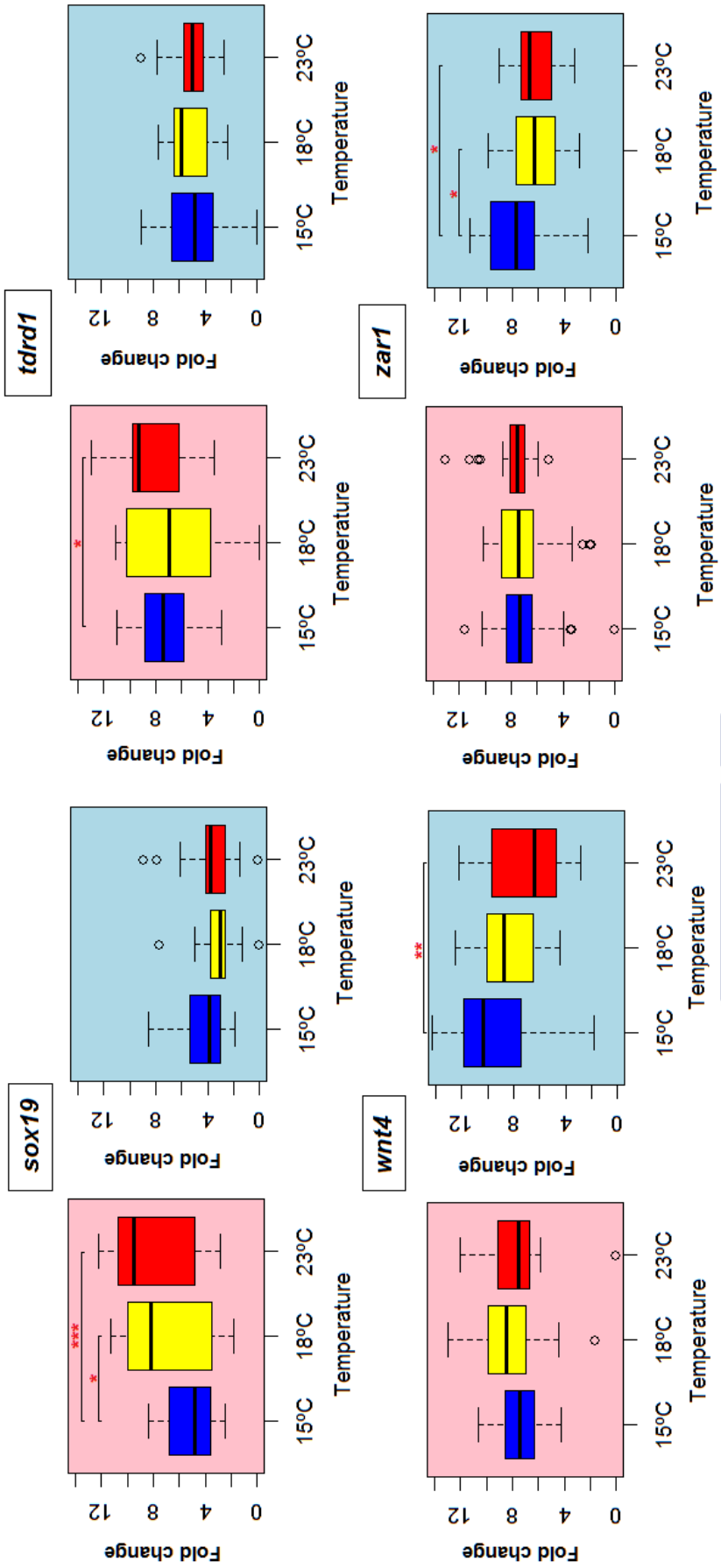


Gene fold change values for each sample plotted according to both its length, in cm, and its age, in days post fertilization. Female samples are shown in magenta and male samples in blue. In the FC/length figure for each gene non-linear trend lines were calculated by loess regression. In the FC/age figure, error bars represent the standard error of the mean

Supplementary Figure 2: Temperature+length effects on gene expression

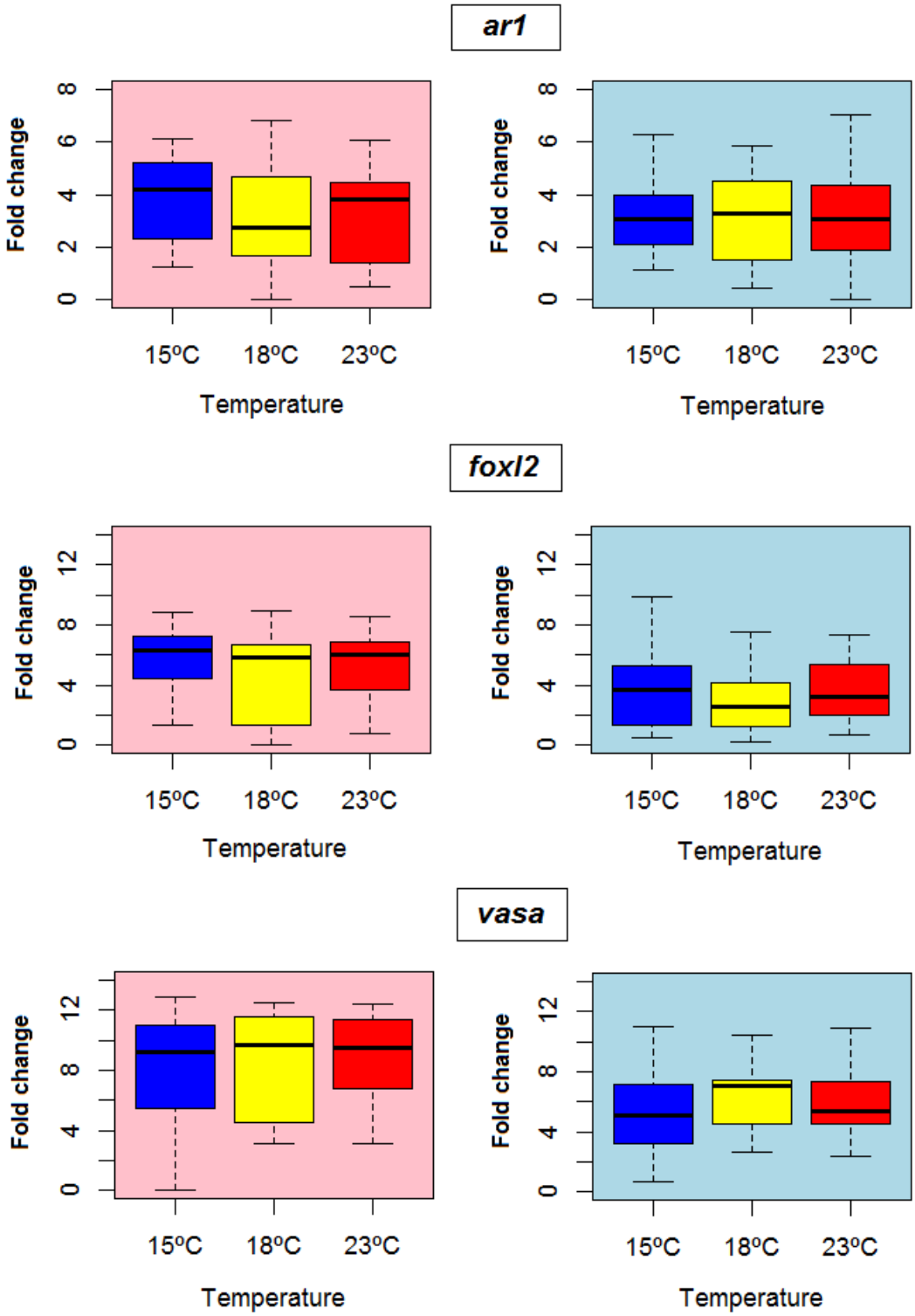



hsp27
pfges3
sox8
hh1
lhx8
rdh3
Fold change
Temperature
15ºC
18ºC
23ºC



Mean fold change gene expression values at 15, 18 and 23ºC in the whole dataset are shown for males (light blue background) and females (pink background). Error bars represent standard deviation. Significate differences between temperatures are indicated by * (0.01<p<0.05), ** (0.001<p<0.01), *** (0.0001<p<0.001) or **** (p<0.0001), however red asterisks indicate that fish length has an influence on the temperature differences.

Supplementary Figure 3: Gene expression insensitive to temperatures



Mean fold change gene expression values at 15, 18 and 23ºC in the whole dataset are shown for males (light blue background) and females (pink background). Error bars represent standard deviation.

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Chapter 3

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Transcriptomic analysis of the process of gonadal sex differentiation in the turbot (*Scophthalmus maximus*)

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Abstract

Turbot (*Scophthalmus maximus*) is a flatfish species with one of the most remarkable sexual size dimorphism among cultured fish. The interest of turbot industry in getting all-female populations has promoted a big research effort to understand the processes of sex determination and gonad differentiation. Gonad primordium is a bipotential tissue where complex genetic and environmental processes determine its fate either as a testis or as an ovary. To better understand the complexity of gonad plasticity throughout development in turbot we explored the gonadal transcriptomic landscape from undifferentiated to juvenile fish using a custom microarray enriched in reproduction-related genes. Gene expression profiles revealed that females started sex differentiation sooner than males (at ~5 cm total length (TL) compared to ~10 cm TL), which suggested that gonads developed slower in males. Some genes proved to be female (*cyp19a1a*) and male (*dmrt3*) early sex markers; *cyp19a1a* expression starting at 90 dpf and *dmrt3* at 140 dpf. The expression profiles of 18 canonic reproduction-related genes were studied along gonad development, verifying the key role of *cyp19a1a* in female development. Furthermore, 45 differentially expressed genes (DEG) were identified as pro-female genes during ovarian differentiation (140 dpf onwards) and 12 pro-male DEG were related for the first time to testis development. The global transcriptome analysis revealed that a larger number of DEG were found in ovaries than in testes when compared to differentiating gonads. However, when juvenile testis and ovaries were compared, there were more DEG upregulated in males than in females. To identify sex determining candidate genes, ~4.000 DEG between male and female juvenile gonads were located in the turbot genetic map and its position compared with that of previously identified sex- and growth-related quantitative trait loci (QTL). Only two (*foxl2* and *17βhsd*) of the canonical reproduction-related genes mapped to growth-QTLs in linkage group (LG) 15 and

LG6, respectively, but none to sex-related QTLs. Sex-related QTLs showed a larger (but not significant) amount of male-biased DEG in LG8, while transcripts mapping near growth-related QTLs were only found in females but not in males. Overall results can aid to better understand sex differentiation specifically in this cultured fish species, but also contribute towards understanding the high sex determination and sex differentiation diversity observed in teleosts.

1. Introduction

Reproduction is one of the major concerns for fish aquaculture industry. Understanding the genetic basis of reproduction is essential to control the onset of puberty, sex ratios or egg spawning cycles (Piferrer et al. 2005; Taranger et al. 2010). Fish show diverse reproduction strategies likely as a consequence of the high diversity of environments where they live (Penman and Piferrer, 2009). One of the most important aspects of fish reproduction is gonad development. Gonad development relies on two basic interacting processes: sex determination (SD) and gonad differentiation (GD). SD is the process that establishes the sex of individuals, and it can be switched by the action of a single master gene, the interaction of several minor loci and/or by the actions of environmental factors (such as temperature). On the other hand, GD is the process leading to the formation of an ovary or a testis once the fate of the undifferentiated gonad has been determined (Piferrer, 2009). SD and GD have been traditionally seen as two independent consecutive processes, but new data on cold-blooded vertebrates, especially fish, has opened a debate and both processes have been proposed to be part of a single continuous development network where genetic factors and environmental cues interact to determine sex (Heule et al. 2014). This new model would aid to better understand the high evolutionary turnover of SD in fish (Uller and Helanterä 2011). The undifferentiated gonad is a bipotential tissue with high plasticity which makes it unique for studying the transcription architecture responsible of its fate (Munger and Capel, 2012). Recently, major advances on the description of genes involved in SD and GD in fish and their interactions have been reported (Bachtrog et al. 2014; Shen and Wang, 2014), however there is yet much to be discovered in this field due to the complexity and diversity of reproduction systems in fish.

Turbot (*Scophthalmus maximus*) is a marine flatfish species with a highly appreciated white flesh that has been traditionally farmed in Europe and particularly in Spain with a production over 10,000 tones (T) in 2012 (FAO, 2014). Its increasing worldwide demand has determined its introduction in the Chinese market (Wang et al. 2010) and a production exceeding 60,000 T has been reported in China (FAO, 2014). Turbot shows one of the largest sex size dimorphisms in marine aquaculture species, females largely outgrowing males and thus reaching commercial size several months earlier (Piferrer et al. 1995; Imslad et al. 1997). Obtaining all-female progenies is one of the main interests for turbot industry. Turbot shows a ZZ/ZW sex determination system (Haffray et al. 2009; Martínez et al. 2009) and the main sex determining region was identified in linkage group (LG) 5, although three other minor sex-

related QTLs were located in LG6, LG8, and LG21 (Martínez et al. 2009; Taboada et al. 2014). Although the main sex determining gene(s) still remain(s) unknown, several sex-associated markers have been developed so far (Casas et al. 2011; Viñas et al. 2012; Taboada et al. 2014), which are being applied by turbot industry for precocious sexing. Recently, two female-associated random amplified polymorphism DNA (RAPD) marker were identified and placed on the turbot genetic map close to the transcription factor forkhead box L2 (*foxl2*), an important gene related to initial steps of sex differentiation, and close to the wingless-type MMTV integration site family member (*wnt*) 1, a gene involved in ovarian development (Vale et al. 2014). Other genes related to sex differentiation have also been localized in the turbot genetic map: the anti-Müllerian hormone (*amh*) and doublesex and mab-3 related transcription factor 2 (*dmrt2*) in LG5; the SRY (sex determining region Y)-box 9a (*sox9a*) and *sox17* in LG21; and the cytochrome P450, family 19, subfamily B (*cyp19b*) in LG6 (Viñas et al. 2012).

Little is known about gene expression during sex differentiation in turbot. A first transcriptomic analysis using cDNA-AFLPs was performed in gonad, brain and liver of male and female turbot (Taboada et al. 2011), enabling the identification of some sex-related expressed genes in adult tissues. With the development of new sequencing technologies, flatfish genomic resources have increased significantly (Cerdà et al. 2010; Cerdà and Manchado, 2013). Specifically in turbot, two 454 pirosequencing runs, one related to immune (Pereiro et al. 2012) and another to reproduction related-organs (Ribas et al. 2013) have greatly increase the number of publicly available turbot expressed sequence tags (EST). The reproduction run was based on brain-hypophysis-gonadal axis mRNA obtained at different development stages. A total of 34,400 novel turbot sequences, including 1,410 related to reproduction, were identified for the first time in turbot. With all these genomic resources it was possible to generate a reproduction-related enriched microarray including a total of 43,803 turbot-specific probes.

The objective of this study was to identify genes driving testis or ovary development. The turbot reproduction-enriched microarray was used to evaluate gene expression profiles in undifferentiated, differentiating and juvenile turbot. Results obtained led to the identification of sex-specific marker genes and novel genes related to testis or ovary development, which will help to understand GD processes in both turbot and other teleost species and might be helpful for sex control strategies in turbot.

2. Methods

2.1. Turbot gonad sampling

Fish were obtained at the facilities of Centro Tecnológico Gallego de Acuicultura (CETGA; Ribeira, NW Spain). Fish were treated in agreement with the European convention for the protection of animals used for experimental and scientific purposes (ETS Nu 123, 01/01/91). A total of 30 turbot gonad samples were collected from a mixture of unrelated genetic families. In order to obtain the widest representation of expressed transcripts, fish with

gonads at different stages of development were used: i) early sexually undifferentiated gonads (PU), at 75 days post-fertilization (dpf); ii) late sexually undifferentiated gonads (U; 90 dpf); iii) sexually differentiating gonads (D; at 135, 137 and 151 dpf; for simplicity, an average value of 140 dpf will be used from now on); and iv) gonads from female (FJ) and male (MJ) juveniles, sexually differentiated, collected at 309, 315 and 485 dpf (average used value of 400 dpf). PU and U stage gonads were very small and their complete isolation was very difficult likely including a small portion of the surrounding tissues. Similarly, D stage gonads may contain a bit of attached epithelium, sometimes difficult to remove. FJ and MJ samples contained exclusively gonadal tissue. Weight and length of each fish was recorded for each sample and sex was visually assessed and classified as undifferentiated, males or females. In some fish, gonads were divided into two parts. One was rapidly flash frozen in liquid nitrogen for RNA extraction and molecular analysis and the other was used for histological sex verification (see below).

2.2. Sex verification

For histological analysis, samples were fixed in 4% paraformaldehyde buffer overnight, rinsed in phosphate buffer saline the next day and stored in 70% ethanol. Samples were then dehydrated in a series of alcohols and embedded in paraffin, sectioned at 7 μm thick and stained with hematoxylin-eosin and observed under the microscope. Some samples at the beginning of gonadal development (75–140 dpf) were genetically sexed using the SmaUSC-E30 marker which demonstrated a high accuracy for offspring sexing in turbot families (Martínez et al. 2009).

2.3. RNA isolation, cDNA synthesis and library construction

RNA was individually extracted by RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Quantity was determined using a Nanodrop spectrophotometer (Nanodrop Technologies, USA) and quality (RNA integrity number, RIN) using a Bioanalizer (Agilent Technologies, USA). RNA samples with RIN > 8.1 were further processed for microarray analysis. Briefly, 50 ng of total RNA were labeled using the Low Input Quick Amp Labeling Kit, One-Color (Cy3; Agilent Technologies).

2.4. Microarray hybridization and scanning

Samples were hybridized individually in a custom 4x44 K Agilent platform at the Universidad of Santiago de Compostela (USC) Genomics Platform. This custom-microarray was based on the last version of the turbot database, particularly enriched in reproduction and immune organs, and previously validated (Pereiro et al. 2013; Ribas et al. 2013). The microarray platform consists on a total of 45,200 spots, 43,803 corresponding to gene transcripts and 1,417 to Agilent controls. Copy RNA (cRNA) was prepared by overnight hybridization with the corresponding buffers during 17 h at 65°C and washed on the

following day. Hybridized slides were scanned using an Agilent G2565B microarray scanner (Agilent Technologies). The scanner images were segmented with the Agilent Feature and the Agilent software was applied to avoid saturation in the highest intensity range. Agilent Feature Extraction produced the raw data for further pre-processing. The processed signal (gProcessed-Signal) value was the chosen parameter for the absolute hybridization signal as recommended (Millán et al. 2010). Microarray data was submitted to Gene Expression Omnibus (GEO) with accession number GSE64280.

2.5. Data analysis

Normalization using all microarray data was done by the Aquantile method implemented in the Limma R package (R version 3.0.2). Samples were normalized together for all the microarray analysis. We used the microarray analysis software Multiple Experiment Viewer (MeV) version 4.8.1 to statistically analyze the data previous $\log_2$ transformation of the fluorescence values. Samples were *a priori* assigned to specific groups by two different ways, one based on their transcriptomic profiles considering both sampling time and the results of Principal Component Analysis (PCA), and the other using sex-marker gene profiles on considering gonadal aromatase (*cy19a1a*) as a female marker and doublesex and mab-3 related transcription factor 3 (*dmrt3*) as a male marker along gonadal development.

Differentially expressed genes (DEG) between the groups were discovered by Significance Analysis of Microarrays test (SAM) (Tusher et al. 2001) with a False Discovery Rate (FDR) adjusted q value < 0.01. Scatter plots showed the microarray $\log_{10}$ transformed intensity distribution for all genes relating two different gonad development stages. Heatmaps were constructed by hierarchical clustering of samples and selected genes using Pearson correlation. Venn diagrams were built by Venny online software (http://bioinfogp.cnb.csic.es/tools/venny/). The SOTA program (Self Organizing Tree Algorithm; Dopazo and Carazo, 1997; Yin et al. 2006) was used to identify groups of genes with similar expression patterns. A linear model for microarray data (Limma; p value < 0.05; Wettenhall and Smyth, 2004) was applied to identify genes with similar expression patterns, specifically to gonadal aromatase (*cyp19a1a*). Putative transcription factor binding sites in the promotor of sequences of genes of interest were analyzed by MatInspector (Genomatix Software).

2.6. GO terms and Kegg pathway analysis

We used the software Blast2GO (Conesa et al. 2005) to analyze the enriched GO terms in the DEG between groups. A Fisher exact test (P < 0.05) FDR corrected for multiple testing was performed using all genes in our microarray as background and the DEG of each comparison as query. KEGG pathway enrichment was assessed by DAVID Bioinformatic Database (Huang and Lempicki, 2009) using *Danio rerio* as background (P < 0.05).

2.7. Gene network analysis

Weighted correlation network analysis (WGCNA) was performed in R (version 3.0.2) (R Development Core Team, 2008) using the WGCNA package following the tutorial written by Langfelder and Horvath (2008). Briefly, the microarray expression file was first pre-processed by removing those genes or samples with excessive missing data as recommended. Co-expression networks were then built and clusters of genes were grouped into different color modules, allowing a minimum of 30 genes per module. These modules were checked for association with male or female phenotypes ($r > 0.5$, $P < 0.01$). Gene lists for the modules significantly associated with these traits were obtained, keeping only those module genes that showed a correlation with the trait of interest ($r > 0.6$). Thereafter, GO and KEGG enrichment analyses were performed for these gene lists. At the same time, co-expression networks were built for a selection of relevant genes (a total of 54) using a high threshold power to keep only the most relevant connections. To visualize the network, Cytoscape 3.0.2 software was used (Shannon et al. 2003).

2.8. Mapping genes to QTLs

The identified DEG between FJ and MJ in the microarray analysis were mapped to the turbot genome (Figueras et al. in preparation) using local BLAST in order to assess DEG co-localized with sex- and growth-QTL markers. Sequences corresponding to each DEG were blasted and the best hit for each sequence selected. Blast results showed the reliability of the analysis since different probes for the same DEG were localized in exactly the same region of the genome. Turbot QTL positions were obtained from Hermida et al. (2013) and a range of ± 1 Mb around the highest associated QTL marker was checked for over or under-representation of male and female genes by chi-square tests with Yates correction ($P < 0.05$). Microarray transcript distribution along the genome was used as reference to study the male and female representation. Visual representations of the linkage groups (LG) and the genes localized on them were produced by the script genetic-mapper.pl (http://code.google.com/p/genetic-mapper/).

2.9. Microarray validation by real-time PCR

RNA was reverse transcribed by random primers using AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) following the manufacturer's protocol. Real-time PCR (qPCR) was performed on a Stratagene Mx3005P using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies) in a final volume of 20 µL following the manufacturer's protocol. Gene-specific primers for 20 DEG were designed in spanning exons when possible. Primer concentration was 300 nM and each sample was run in duplicate. The cycling parameters were: 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of amplification at 95ºC for 15 sec and 60ºC for 1 min. Finally, a dissociation step was performed after amplification to ensure the presence of a single amplification product.

Specificity for each primer pair was also confirmed by PCR product sequencing. qPCR data were obtained by the MxPro software (Agilent Technologies) and quantification cycle values (Cq) calculated for each replicate and then averaged to obtain the final Cq value. Raw Cq values were transformed to the final fold change difference (FC) values following the guidelines present in Kubista et al. (2007). Three reference genes (ubiquitin*;* *ubq*, ribosomal protein S4; *rps4* and glyceraldehyde-3-phosphate dehydrogenase; *gapdh*) were used for normalization and LinRegPCR software (Ruijter et al. 2009) was used for efficiency determination. References genes and efficiency determination were previously validated for turbot gonad samples by Robledo et al. (2014b) (Chapter 1). Primer sequences used for qPCR validation are presented in Supplementary Table 1.

3. Results

3.1. Microarray validation

Microarray results were validated by qPCR using 16 differentially expressed genes (see below) in different comparisons, totaling 34 gene expression differences. Results obtained with the two methods were highly correlated (r = 0.914, P = 4.53e-14; Supplementary Figure 1). Furthermore, a two-tail Mann-Whitney test was performed to ensure that microarray and qPCR mean values were not significantly different (P = 0.103). Thus, results obtained with the microarray were fully validated.

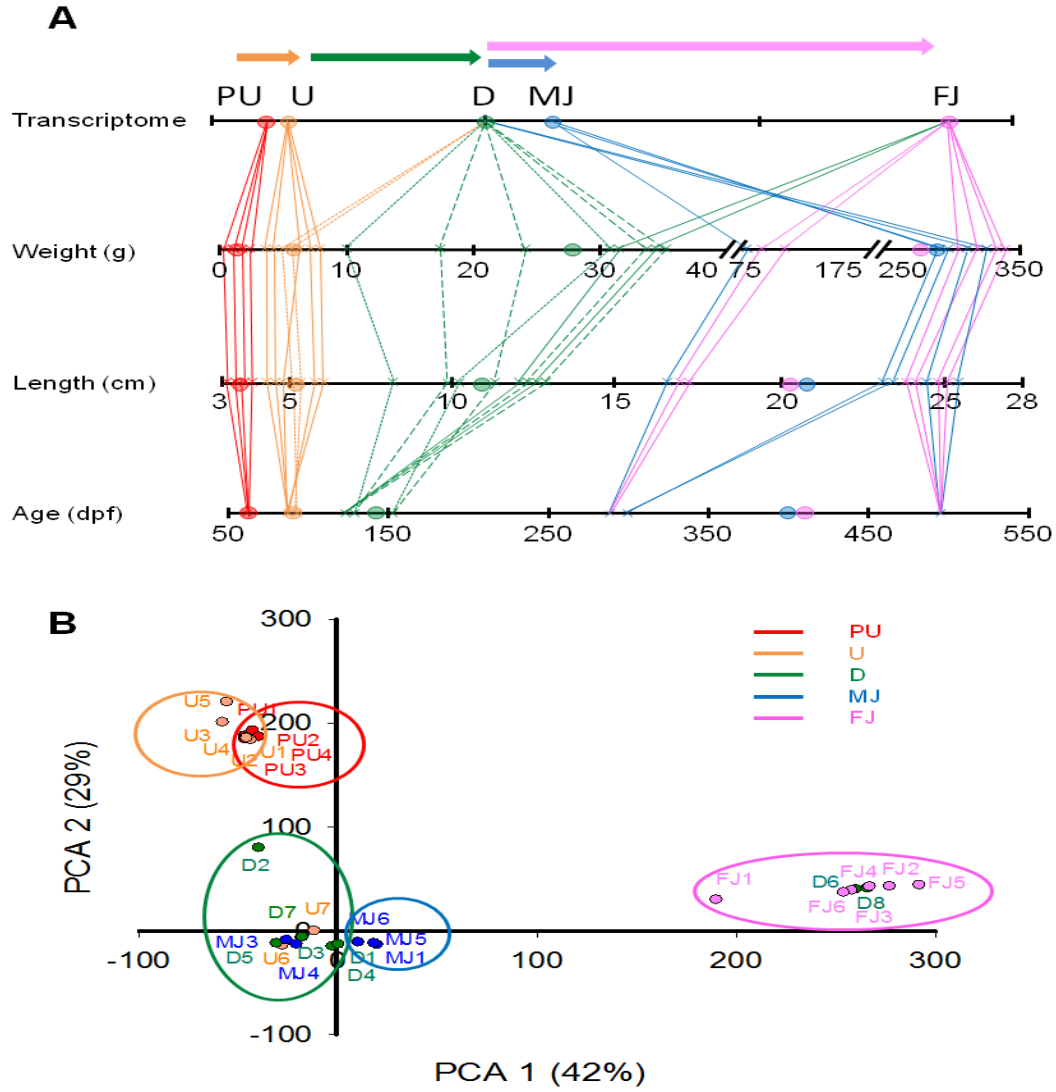
3.2. Morphometric and transcriptomic overview

As illustrated in Figure 1A (and in more detail in Supplementary Figure 2) total length (TL) and body weight (BW) of the 30 fish used in this study ranged from 3.8 ± 0.05 cm and 0.828 ± 0.02 g (mean ± SEM; average of n = 5 fish) in the PU group to 23.4 ± 1.81 cm and 264.100 ± 46.70 g (mean ± SEM; average of n = 6 fish) in the FJ group. Based on length and weight, significant differences ($P < 0.05$) existed among PU+U, D and MJ+FJ (Supplementary Figure 2A and 2B, respectively). The relationship between age and length, a better proxy than weight for assessing gonadal development in fish, and also between length and weight, is shown in Supplementary Figures 2C and 2D, respectively.

The genetic relationship among samples was represented in a Euclidean space based on a Principal Component Analysis, which aims to explain a large proportion of the total variance in a few non-correlated variables by re-dimensioning the expression values of all the probes. The component 1 alone already explained 42.2% of the variation and the first three components reached up to 82.5% of the total genetic variation. PCA classified samples into three main clusters (Figure 1B). Cluster I included all U+PU fish (TL ~5 cm), which had a uniform gonadal transcriptome regardless of size. Cluster II included most D stage fish (TL ~10 cm) as well as all MJ fish (~21 cm TL). Cluster III comprised all FJ fish (TL ~20 cm) and two D fish (~12 cm TL), which also had a uniform gonadal transcriptome. To better classify the samples, we also performed a complementary hierarchical clustering on gene expression profiles (Supplementary Figure 3A). Based on both grouping methods (PCA and hierarchical

clustering), we decided to aggregate the samples in five groups and the final adscription of the sampled fish was as follows: 1) PU: PU1, PU2, PU3, PU4; 2) U: U1, U2, U3,U4, U5; 3) D: U6, U7, D1, D2, D3, D4, D5, D7, MJ3, MJ4; 4) MJ: MJ1, MJ5, MJ6; 5) FJ: D6, D8, FJ1, FJ2, FJ3, FJ4, FJ5, FJ6.

Figure 1. Overview of transcriptomic groups during sex differentiation of turbot



A) Information of turbot fish used in this study with gonads at different stages of development: prior appearing as undifferentiated gonads (PU) at 75 days post-fertilization (dpf), undifferentiated gonads (U; 90 dpf), differentiating gonads (D; at 135, 137 and 151 dpf; for simplicity an average value of 140 dpf is plotted), and gonads from female (FJ) and male (MJ) juveniles collected at 309, 315 and 485 dpf (for simplicity an average value of 400 dpf is plotted. Male and female ages are represented by a blue and pink dots respectively). Each fish is represented by one line that relates its age, length and weight with its transcriptomic group. Circles show the mean value for a given variable. Short dashed lines show pro-female fish, long dashed lines show pro-male fish based on *cyp19a1a* expression levels. In the line above ("Transcriptome") distances among circles are proportional to the Euclidean distance in the PCA (see below) using D as a centroid. B) Clustering turbot fish based on their gonadal transcriptomic profiles by PCA analysis. Percent refer to total variance. Circles correspond to each transcriptomic group which individuals were classified.

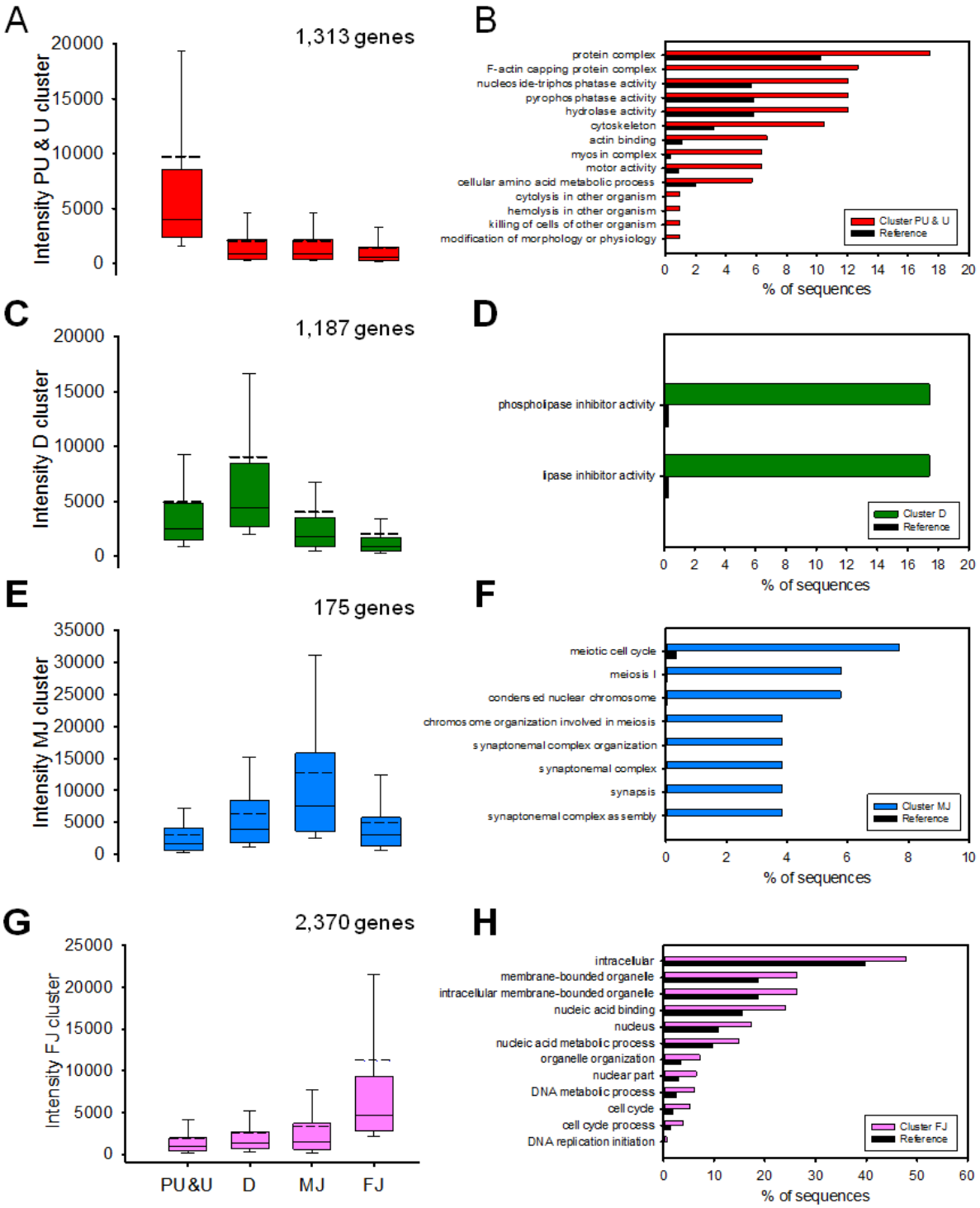
To additionally verify that our groups were consistent for studying sex differentiation in turbot we took a total of 39 genes with well-established functions in reproduction and, using the average expression values for each of the five groups, a heatmap was constructed hierarchically clustering both genes and groups (Supplementary Figure 3B). These genes were steroidogenic enzymes (e.g. *cyp19a1a*, 17-beta hydroxy steroid dehydrogenase, *17βhsd*, 11-beta-hydroxylase, *cyp11β*), transcription factors (e.g. *sox9b*, *sox19*, *foxl2*, *dmrt3*, folliculogenesis specific basic helix-loop-helix, *figα*), sex steroid receptors (e.g. androgen receptor, *ar*), growth factors (e.g. gonadal somatic cell derived factor, *gsdf*, insulin-like growth factor 2, *ifg2*) and other relevant genes related to gonadal development (e.g. steroidogenic acute regulatory protein; *star*, *wnt1*, fanconi anemia (*fancl*), follicle stimulating hormone receptor; *fshr*, progesterone receptor, *pgr*). This subset of genes produced a similar result to that observed in the PCA, PU and U groups were found tightly clustered, same as D and MJ groups, and FJ grouped far apart from the other groups.

3.3. Comparative analysis between different gonadal developmental stages

A SOTA analysis (Herrero and Dopazo, 2002; Yin et al. 2006) was performed, grouping genes by their expression values, in order to identify genes with similar expression patterns along turbot gonad development. PU and U groups could not be discriminated since we did not find gene clusters specific for any of them. Thus, using SOTA we identified four clusters of genes with higher expression in PU+U (1,313 genes), D (1,187), MJ (175) or FJ (2,370) groups (Figure 2 A, C, E, G). As remarkable examples, PU+U over-expressed prostaglandin 3 (*ptge3*), a gene which, together with other prostaglandins, is required for ovary development in fish (Pradhan and Olsson, 2014) and spermatogenesis associated 1 (*spata1*), a gene involved in spermatogenesis (Giesecke et al. 2009); the D cluster showed specific expression of *star* and the luteinizing hormone receptor (*lhr*), both genes are involved in gonadal development in fish (Maugars and Schmitz, 2008; Levavi-Sivan et al. 2010; Yu et al. 2014); MJ over-expressed *spata22* and sperm associated antigen (*spag*), both involved in spermatogenesis (Schroter et al. 1999; Buchold, 2012); and finally the FJ cluster presented genes involved in ovarogenesis like vitellogenin (*vtg*) and choriogenin (*chg*) genes (Lee et al. 2002; Wheeler et al. 2005).

The four SOTA clusters were subjected to GO enrichment analysis using all the genes represented in the microarray as background. We identified a total of 14, 2, 8 and 12 significantly overrepresented GO terms for the PU+U, D, MJ and FJ, respectively (Figures 2 B, D, F and H). For the PU+U cluster, several GO terms were related to protein complex, nucleoside-triphosphatase activity, pyphosphatase and hydrolase activity as well as GO terms related to cytoskeleton (acting binding and myosin complex). The D cluster only showed two enriched GO terms, related to phospholipase and lipase inhibitor activity. The MJ cluster GO terms were related to meiosis, synapsis and chromosomal organization (condensed nuclear chromosome, synaptonemal complex) while those in the FJ cluster were associated with

Figure 2. SOTA analysis for different gonadal developmental stages in turbot



A) Boxplot of the PU+U cluster, showing a total of 1,313 genes. B) GO enriched terms of the PU+U cluster. C) Boxplot of the D cluster, showing a total of 1,187 genes. D) GO enriched terms of the D cluster. E) Boxplot of the MJ cluster, showing a total of 175 genes. F) GO enriched terms of the MJ cluster. G) Boxplot of the FJ cluster, showing a total of 2,370 genes. H) GO enriched terms of the FJ cluster. Abbreviations as in Figure 1. In the boxplot, the solid and dashed lines indicate the median and mean, respectively; the lower and upper edges indicate the 25th and 75th percentiles, respectively; and the lower and upper whiskers indicate the 5th and 95th percentiles, respectively.

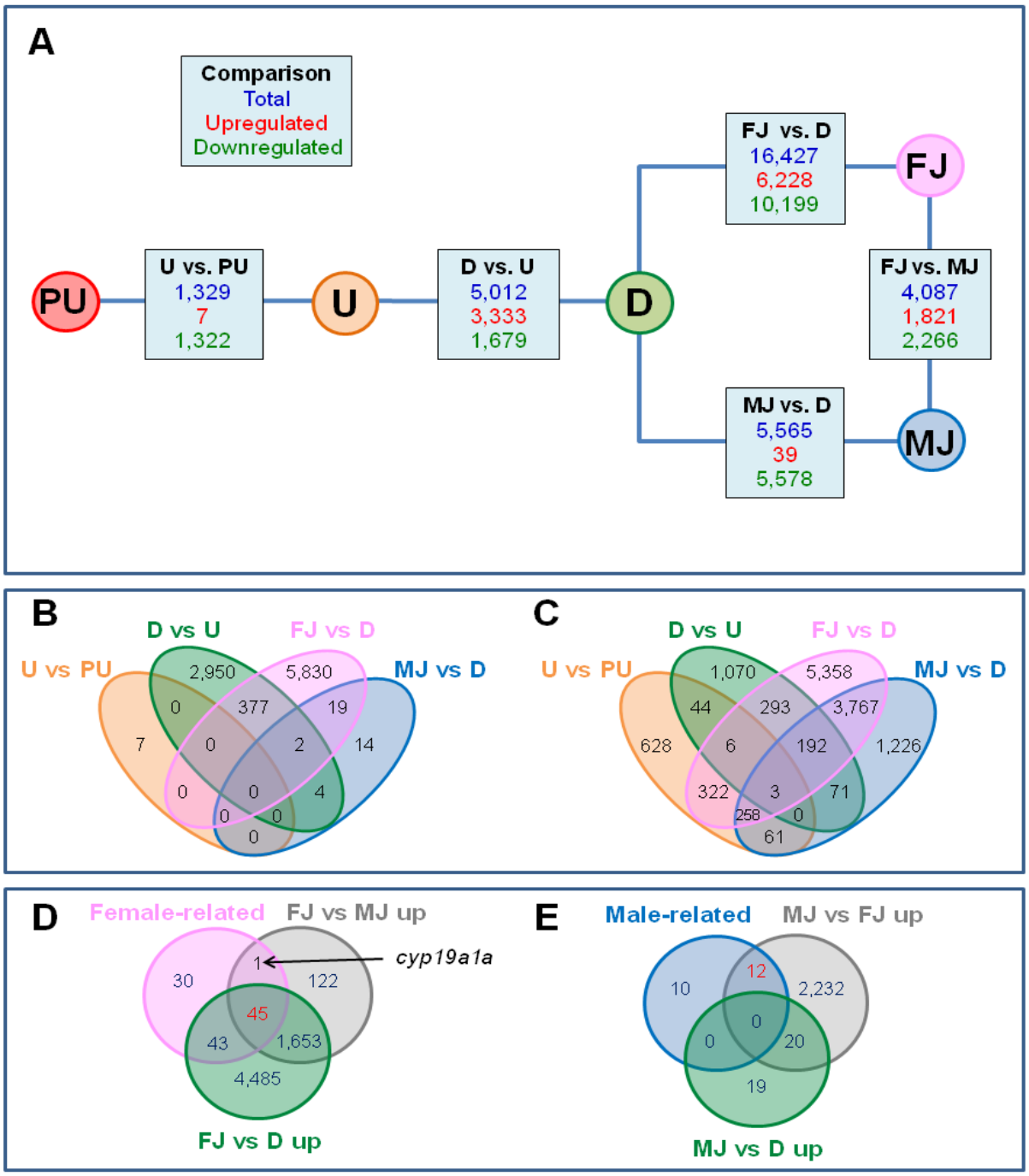
intracellular, membrane-bounded organelle, nucleic acid binding and organelle organization among others.

To further analyze the process of gonad development in turbot, we identified statistically significant gene expression differences between the four groups (five comparisons) by SAM (FDR corrected p value < 0.01) (Figure 3A). The 20 most up-regulated and down-regulated DEG observed in each pair-wise comparison are listed in Supplementary Table 2. In all but the D *vs.* U comparison the downregulated genes outnumbered the upregulated ones and this was particularly evident in the MJ *vs.* D and U *vs.* PU. Despite the large number of DEG in the PU *vs.* U and D *vs.* MJ comparisons, these groups were previously found to be clustered very close in the PCA analysis, so either these DEG are not so relevant for the transcriptomic classification of the gonad samples or the FC differences are not relevant enough to cluster them in different groups.

The number of DEG exclusive of a given comparison and those shared by two or more comparisons is shown using Venn diagrams (Figures 3B and 3C for up- and downregulated genes, respectively). Three common genes were found to be downregulated in all comparisons (plectin domain containing protein, *plec*, tetratricopeptide repeat protein 36, *ttc36*, and uncharacterized protein), while there were no upregulated genes common to all of them. A group of 377 upregulated genes common to the D *vs.* U and FJ *vs.* D comparisons was identified, constituting a group of female-related genes whose expression might start at undifferentiating stage and keep increasing until ovary formation. Among them, there were several genes related to female development such as *fancl* or nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*Nf-kβ*), but also genes related to immune system such as caspase 8 (*casp8*), to methylation such as histone-lysine N-methyltransferase (*ehmt*) or to cell division such as cell division cycle 2 (*cdc2*) were found. At the same time, a set of 293 genes were down-regulated during ovarian development. These included heat shock protein 70 (*hsp70*) and also genes related to immune system (tumor necrosis factor alpha, *tnfα*, major histocompatibility complex I, *mhci*). In contrast, during testis development (D *vs.* U and MJ *vs.* D) we found very few common upregulated genes (4) and also not many downregulated (71). An important group of 3,767 genes was downregulated in both FJ and MJ when compared to D group, genes expressed in the differentiating gonads but repressed when gonads were differentiated. This large number of genes contrasted with the few number of common upregulated genes upregulated (19).

We performed a GO term enrichment analysis in the DEG lists, using the whole microarray data as background, to obtain a global view of the gonad differentiation processes. The overrepresented GO terms for the three GO categories and their fold enrichment for each of the comparisons are detailed in Supplementary Table 3. We also identified, when possible, significantly KEGG pathways involved in each set of DEG comparisons using DAVID (FDR $P < 0.05$; Supplementary Table 3). The largest number of enriched GO terms appeared in the FJ *vs.* D comparison, among the GO terms overrepresented in the upregulated

Figure 3. Description of the number of DEG in different comparisons based on transcriptomic groups



A) Number of DEG in different comparisons throughout gonadal development. B) Venn diagram showing the number of upregulated genes in different comparisons. C) Venn diagram showing the number of downregulated genes in different comparisons. D) Venn diagram showing DEG with expression patterns similar to genes with a well-established role in ovary differentiation (Female-related genes). E) Venn diagram showing DEG with expression patterns similar to genes with a well-established role in testis differentiation (Male-related genes). Abbreviations as in Figure 1.

DEG there were several connected to transcription regulation (chromatin, protein-DNA complex or transcription from RNA polymerase II promoter) and also to histone modifications, while in the downregulated DEG calcium ion binding and metabolic-related GO terms (glycolysis/gluconeogenesis) were overrepresented. We also identified an enrichment of pathways related to spliceosome in the FJ group and oxidative phosphorylation and fructose and mannose metabolism in the D group.

3.4. Gene expression differences at early stages of gonadal development

We were interested in exploring genes involved in early turbot sex differentiation. For this purpose, we used two complementary strategies. First, we used the transcriptomic D group according to the PCA and, based on *cyp19a1a* expression levels, a key gene responsible for estrogen synthesis and conserved in nearly all teleost fish (Guiguen et al. 2009), we compared D-pro-females, expressing *cyp19a1a* (U6, U7, D1 and D4) *vs.* D-pro-males (D5, D7, D2, D3, MJ3 and MJ4) by Limma statistical analysis. We found 16 genes upregulated in the *cyp19a1a* expressing samples, while another group of 8 genes was upregulated in pro-males which did not show *cyp19a1a* expression (Table 1). The gene with the largest expression difference between D-pro-females and D-pro-males was, as expected, *cyp19a1a*, which was 8.2 times higher in D-pro-females. Other genes greatly upregulated in D-pro-females were *foxl2*, extracellular matrix protein 1 (*ecm1*), collagen alpha-1(XVIII) chain (*col18*) and gap junction Cx32.7 protein (*cx32.7*), but we also identified other genes related to female development as *sox11* and genes related to methylation (spalt-like transcription factor 1, *sall1*), immune system (interferon regulatory factor 5, *irf5*), cell division (structural maintenance of chromosomes 2, *smc2*) and cell matrix (sushi nidogen and EGF-like domains 1, *sned1*). Among the genes upregulated in D-pro-males, collagen type VI alpha 3 (*col6a3)* showed the largest difference, followed by *dmrt3* (FC = -3.02), which belongs to the gene family (doublesex/mab-3 related) of the well-known male determining gene *dmrt1*. Also some immune-related genes (complement c1q tumor necrosis factor-related protein 4, *clqtnf4* and ccr4-not transcription complex subunit 6, *cnot6*) together with actin binding (tropomyosin 4, *tpm4*) or cell catalytic activity (cwf19-like 1 cell cycle control, *cwf19l1*) genes that were more expressed in the D-pro-male group.

The relevance of the doublesex/mab-3 related family in sex differentiation is well-known and widespread through the vertebrate phylogeny, hence we analysed in more detail the expression pattern of *dmrt3* regarding *cyp19a1a*. *Cyp19a1a* expression was first detected at 90 dpf samples, whereas *dmrt3* was only detected at 140 dpf. The expression of these two genes was negatively correlated ($r = -82.74$, $P < 0.0001$; Supplementary Figure 4A), strongly suggesting opposite roles of these two sex genes in turbot sex differentiation. Therefore these two genes could be potential sex markers for femaleness and maleness at early stages of gonad development in turbot. For additional confirmation, we sexed each of the 30 fish used in this study, representing different stages of gonad development, based on the expression profiles of these two markers (Supplementary Figures 5A and 5B). The sex of a subsample of

21 of these fish was further verified either genetically (using the SmaUSC-E30 marker) or histologically (Supplementary Table 4). A perfect match between assigned sex based on *cyp191a1* and *dmrt3* levels and actual sex was observed in all cases, indicating that the expression levels of these two genes are reliable sex markers in turbot.

Table 1. Early development pro-females vs pro-males

Gene	Annotation	Fold change
cyp19a1a	Cytochrome P450 aromatase A	8.2
foxl2	Forkhead box protein L2	5.5
ecm1	Extracellular matrix protein 1	5.0
col18a1	Collagen alpha-1(XVIII) chain	4.6
cx32.7	Gap junction Cx32.7 protein	4.3
scg5	Neuroendocrine protein 7B2	3.4
aadacl4	Arylacetamide deacetylase-like 4	3.4
smc2	Structural maintenance of chromosomes 2	3.3
hs3st1l2	Heparan sulfate 3-O-sulfotransferase 5	3.2
sned1	Sushi, nidogen and EGF-like domain-containing protein 1	3.0
sall1	Sal-like protein 1	3.0
irf5	Interferon regulatory factor 5	3.0
ube2w	Ubiquitin-conjugating enzyme E2 W	2.76
slc26a6	Solute carrier family 26 member 6	2.6
sox11	Transcription factor SOX-11	2.4
fam213a	Redox-regulatory protein FAM213A	1.9
syndig1	Synapse differentiation-inducing gene protein 1	-1.6
cwf19l1	CWF19-like protein 1	-1.7
mkl1	Myocardin-like protein 1	-2.0
tpm4	Tropomyosin alpha-4	-2.2
cnot6	CCR4-NOT transcription complex subunit 6	-2.2
c1qtnf4	Complement C1q tumor necrosis factor-related protein 4	-2.5
dmrt3	Doublesex- and mab-3-related transcription factor 3	-3.02
col6a3	Collagen alpha-3(VI) chain	-5.2

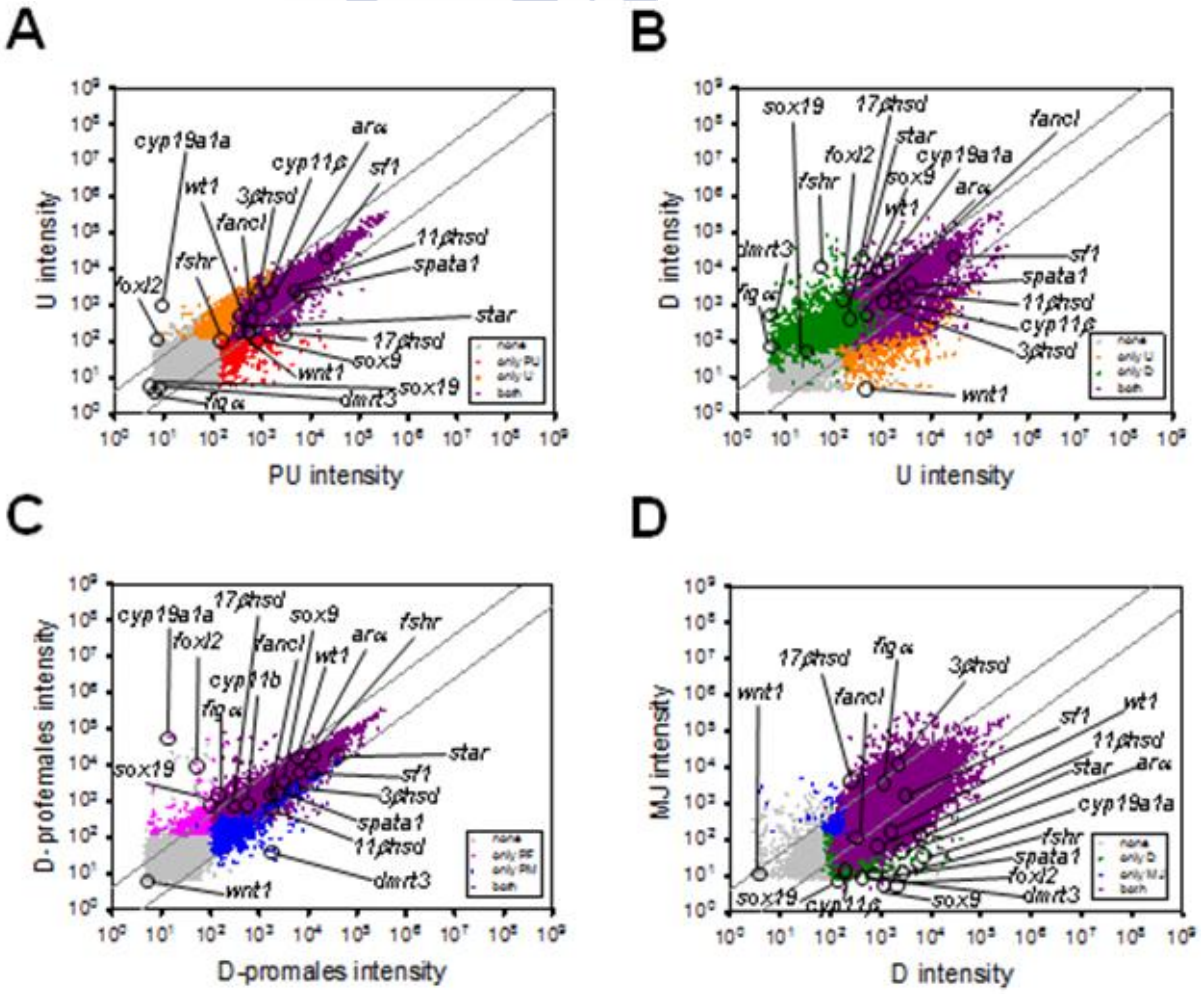
List of the DEG ($P < 0.01$) between pro-female fish (U6, U7, D1 and D4) and pro-male fish (D5, D7, D2, D3, MJ3 and MJ4) belonging to the transcriptomic group D.

3.5. Expression of canonical reproduction-related genes

Scatter plots in Figure 4 show the intensity of all the expressed genes for the different comparisons in the transcriptomic groups, with particular attention to a total of 18 canonical reproduction-related genes selected for their importance for reproduction and sex differentiation in fish (Baroiller et al. 1999; Gardner et al. 2005; Arukwe, 2008; Piferrer and Guiguen 2008; Hsu et al. 2009; Guiguen et al. 2010; Rodríguez-Marí and Postlethwait, 2011; Navarro-Martín et al. 2012). Eight of these genes belong to the steroidogenic pathway (*cyp19a1a, 17βhsd, 3βhsd, 11βhsd, cyp11β, star, arα, fshr*), six are transcription factors

involved in sex differentiation (*foxl2*, *sox19*, *figα; dmrt3*, *sox9b* and splicing factor, *sf1*) and four present different reproduction-related functions (wilms tumor 1b, *wt1b*, *fancl*, *wnt1* and *spata1*). In the first comparison U *vs.* PU (Figure 4A), two key genes in the female pathway (*cyp19a1a* and *foxl2*) were already expressed in the U group (although not in all individuals) and their activity continued along development as observed in D group (D *vs.* U, Figure 4B), in the D pro-female group (D pro-female *vs.* D pro-male comparison, Figure 4C) and in the FJ group (FJ *vs.* MJ comparison, Figure 4F). In the FJ *vs.* D comparison, although both *cyp19a1a* and *foxl2* were expressed in both groups, their expression was higher in the D group. A high positive correlation between these two key female sex differentiation related genes was found ($r = 0.9837$, $P < 0.0001$; Supplementary Figure 4B), indicating that both genes are co-expressed during turbot sex differentiation process.

Figure 4. Expression scatterplots between different development stages and canonical reproduction-related genes



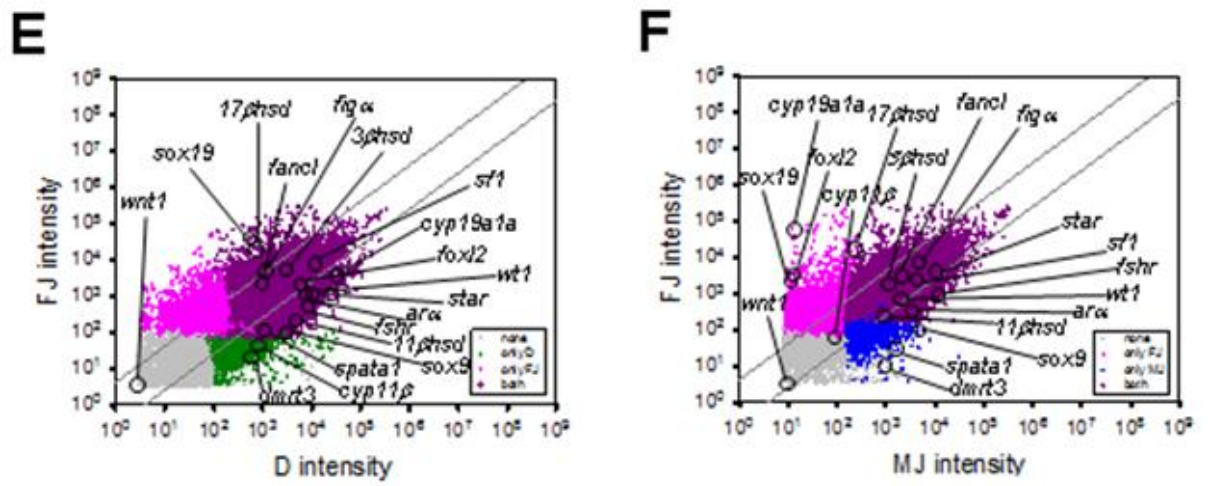


Figure 4. Variation in the expression of all genes in the turbot microarray based on their $\log_{10}$ transformed intensity in different comparisons, with 18 canonical reproduction-related genes highlighted. Genes were considered to be expressed in a given sample if they had raw fluorescence values over 200 and a gene was considered to be expressed in a certain group if it was detected in at least two individuals of that group. Comparisons included are between the U *vs.* PU (A), D *vs.* U (B), D pro-females *vs.* D pro-males (C), MJ *vs.* D (D), FJ *vs.* D (E), and FJ *vs.* MJ (F) groups. Intensity values were $\log_{10}$-transformed. Grey dots represent genes not expressed in any of the two groups, and deep purple dots represent genes expressed in both groups. Genes exclusively expressed in a given group are also color coded (red color belongs to PU group; orange color belongs to U group; green color belongs to D group; pink color to D pro-females or FJ groups; blue color to D pro-males or MJ groups). Grey lines indicate 2 and -2 fold differences between groups. Abbreviations as in Figure 1. Full gene names are shown in Supplementary Table 7.

We also studied if these 18 canonical genes presented significant differences (FDR corrected *P* value < 0.01) in the studied comparisons (Table 2). The steroidogenic gene *3βhsd* was over-expressed in PU than in U (FC = 1.1); *wnt1* and *cyp11b* over-expressed in U *vs.* D, but *sox9b*, *star* and *fshr* were over-expressed in D *vs.* U. As expected, the largest number of DEG among these 18 canonical was found between FJ *vs.* MJ comparison when the gonads were already differentiated.

We finally studied the expression pattern of these 18 canonical reproduction-related genes along gonad development based on the expression of the two identified sex markers (*cyp19a1a* and *dmrt3*; Supplementary Figure 5). The intensity observed for each of the seven studied genes of the steroidogenic pathway, except for *cyp19a1a*, did not show clear differences between sexes, confirming this gene as a prominent female marker among steroidogenic genes. Only *17βhsd* showed an association with sex but in more advanced stages of development (Figure 5A). Of the six transcription factors studied, three showed a clear female-like pattern (Figure 5B): *foxl2*, *sox19* and *figα*. *Foxl2* expression, clearly female-specific, began at 90 dpf in females. The transcription factors *dmrt3* and *sox9b* showed a male bias although only at 140 dpf and 400 dpf respectively, and *sf1* was expressed at early stages in both sexes and later higher in males. Finally, the expression pattern of the four genes with a reproduction-related function was studied (Supplementary Figure 6); *wt1β* did not show any

sex bias, *fancl* presented higher expression in juvenile females, *wnt1* did not show clear differences between sexes and *spata1*, as previously observed by SOTA analysis, showed higher expression at early stages of development and was later more downregulated in females than in males during gonad development.

Table 2. Canonical reproduction-relates genes along gonad development

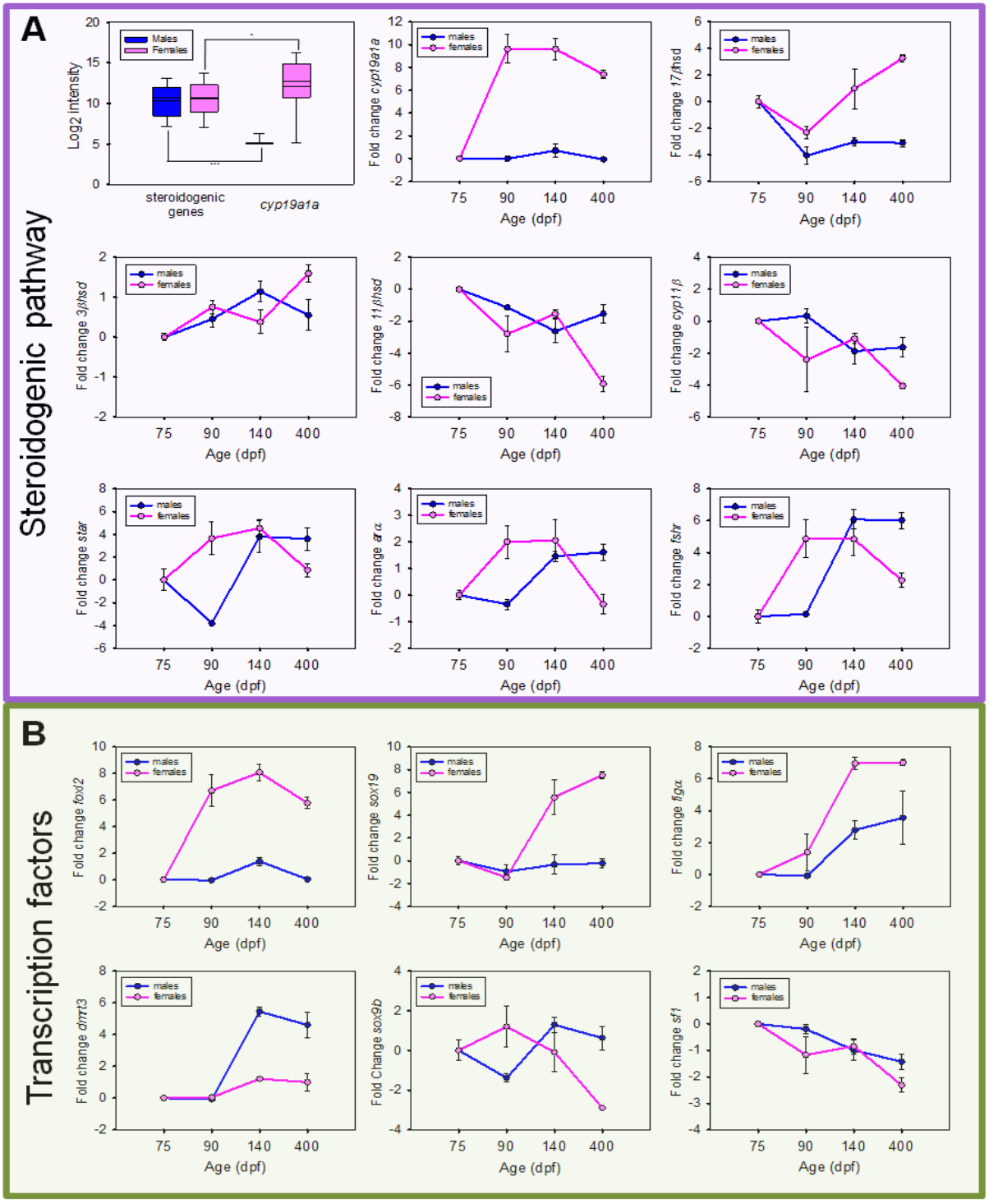
Sex-related	Gene name	U *vs.* PU	D *vs.* U	Df *vs.* Dm	MJ *vs.* D	FJ *vs.* D	FJ *vs.* MJ
Female	*cyp191a1*	-	-	8.2	-	-	5.2
	foxl2	-	-	5.5	-	-	
	sox19	-	-	-	-	-	6.9
	sf1	-	-	-	-	-	-
	wnt1	-	-1.1	-	-	-	-
	fancl	-	-	-	-	-	-
	figα	-	-	-	-	-	1.13
	sox9b	-	2.4	-	-	-	-3.3
	dmrt3	-	-	-3.0	-	-	-
Male	*wt1*	-	-	-	-	-	-
	spata1	-	-	-	-	-	-2.2
	arα	-	-	-	-	-	-
	cyp11β	-	-1.6	-	-	-	-
	star	-	5.1	-	-	-	-3.4
	17βhsd	-	-	-	-	-	-
	fshr	-	4.8	-	-	-	-3.2
Both	*11βhsd*	-	-	-	-	1.0	-
	3βhsd	-1.1	-	-	-2.3	-3.5	-

Differential expression of 18 canonical reproduction-related genes ($P < 0.01$) during gonadal development across the different comparisons. Numbers indicate fold change. Abbreviation as in Figure 1 and in Supplementary Table 7.

3.6. Identification of novel genes related to turbot sex differentiation

Another objective was to identify novel genes with expression patterns correlated to genes with a well-established role in sex differentiation. First, we looked in the scatter plot lists for female-related and male-related genes. We selected those genes with a FC > 2 between D-pro-female *vs.* FJ (244 *vs.* 4,452), on one hand, and between D-pro-male *vs.* MJ (131 *vs.* 712), on the other. A total of 119 female-related and 22 male-related genes were identified, although not DEG. Next, we looked in these gene lists for common DEG either upregulated in FJ *vs.* D and *vs.* MJ for female-related genes (Figure 3C), and between MJ *vs.* D and *vs.* FJ to find male related genes (Figure 3D). As a result, a total of 12 and 45 DEG related to testis and ovary differentiation, respectively, were obtained. The former were DEG in females at 140 dpf (Figure 6A, Supplementary Figure 7 and Supplementary Table 5).

Figure 5. Sex-specific gene expression patterns during gonadal development of turbot



Expression levels of: A) genes involved in the steroidogenic pathway (*cyp191a1*, *17βhsd*, *3βhsd*, *11βhsd*, *cyp11β*, *star*), gonadotrophin and androgen action (*fshr*, *arα*), and B) transcription factors known to be involved in GD process (*foxl2*, *sox19*, *fishα*, *dmrt3*, *sox9b*, *sf1*). Data shown as mean ± SEM of fold change (log_2) using the PU mean as a reference. * = $P < 0.05$, ** = $P < 0.01$ based on the Kruskal-Wallis non-parametric test. Age in days post fertilization (dpf) refers to average age values of fish sampled at each group. Full gene names are shown in Supplementary Table 7.

These included 14 genes with known reproduction-related function such as alveolin (*alv*), zona pelucida sperm binding proteins (*zp*), growth derived factor (*gdf*) 9 and LIM homeobox (*lhx*) 8. The remaining genes included eight cell component genes (e.g. transmembrane protein 144-like, *tmem144a*, peroxisomal membrane protein, *pex*, component of oligomeric golgi complex 3, *cog3*), five genes with immune-related functions (e.g. *cd98*, V-set and immunoglobulin domain-containing protein, *vsig*, 10 cell death-inducing DFFA-like effector, *ccidec*), four metabolic-related genes (e.g. glycerol-3-phosphate dehydrogenase 1, *gpd1*, insulin receptor substrate 2-B-like, *isr2*, or glutathione S-transferase, *gstal*), three related to DNA machinery (e.g. general transcription factor IIIC), two related to cell-cell adhesion (e.g. protocadherin 15a, *pcdh15a*) one related to circadian cycle (i.e. cryptochrome, *cry*2), and nine non-annotated which had not previously been connected to gonad development or reproduction.

Regarding the 12 DEG related to testis development, we found that only one gene was previously known to be involved in reproduction (angiotensin converting enzyme, *ace*), while two were cell component members (i.e. calpain, *capn8* and *type4-ice*), one had a role in cell-cell adhesion (neurexophilin, *nxph*) and eight genes were not annotated.

While the 45 genes involved in ovary development presented similar expression patterns, the 12 testis-related ones could be classified in three different groups based on their expression pattern along gonad development (Figure 6, Supplementary Figure 8 and Supplementary Table 5). Pattern 1 (Figure 6B) consisted of genes which exhibited either no major expresion change or downregulation as gonad development progresses, attaining lower expression values in females than in males (*ace* and R4_8750). Pattern 2 (Figure 6C) consisted of genes with no expression changes in females along gonad development but variable levels of upregulation in males from 140 dpf onwards (*capn8*, R4_6635, R4_27229, R4_12716r and R4_1679r). Finally, pattern 3 (Figure 6D) consisted of genes upregulated in males from 140 dpf onwards and likewise downregulated in females (*nxph*, type4-ice, R4_18369, R4_66731r and R4_68497).

To further explore the role of these genes during gonad development and their interaction with other genes in the reproduction cascade, the putative transcription factor binding sites in the promotor of some of these genes were explored. Three genes, *alv*, *zp* and *gdf9*, out of the 45 female genes identified as involved in ovary development and three, *type- ice*, *ace*, *nxph1*, out of the twelve in testis development were studied. Results showed that a total of 15 and 22 putative transcription factor binding sites where overrepresented with a Z-score value above 1.5 (Z-score values below -2 or above 2 can be considered statistically significant, corresponding to a *P* value of about 0.05, Sui et al. 2005) among the female- and male-related gene promoters, respectively. Among them, in *zp* and *gdf9* two putative binding sites related to reproduction related genes were identified: the estrogen response elements (ERE) binding site ($P > 0.05$) and heterodimer binding site retinoid receptor (RXR, $P < 0.05$); in the *type-ice*, *ace*, and *nxph1* genes also two interesting binding sites were predicted: steroidogenic factor (SF) 1 binding site ($P > 0.05$) and specificity protein (SP) 1 binding site ($P < 0.05$; Supplementary Table 6).

Figure 6. Expression of new sex-related genes

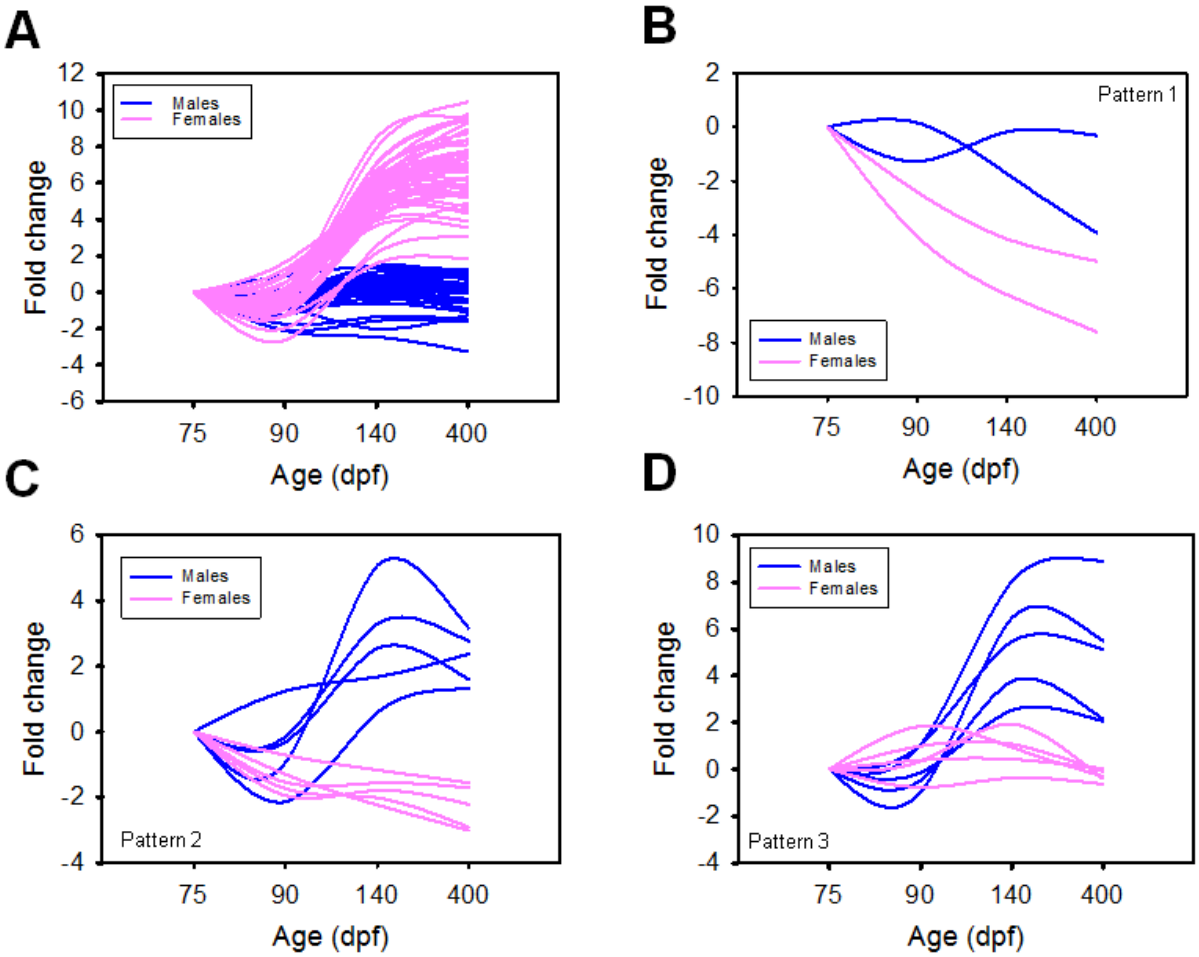
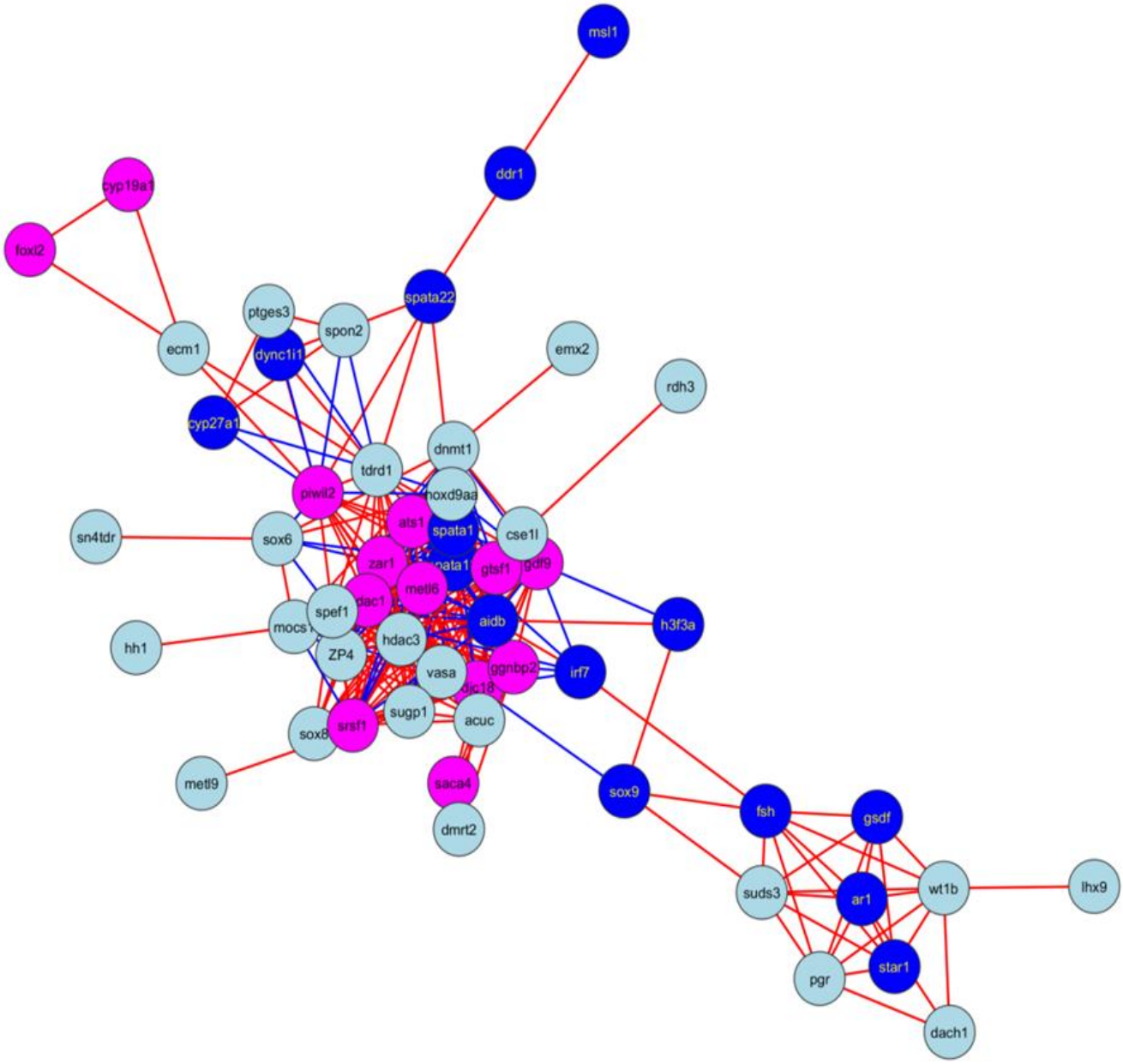


Figure 6. Identified genes with similar behaviour to female-and male-related genes with a well-established role in sex differentiation in turbot. A) Expression of 45 genes associated with ovarian development. B) Pattern 1: expression of genes that exhibit either no major change or ion with development, attaining lower expression values in females than in males. C) Pattern 2: expression of genes that exhibit no major change in females but different levels of upregulation in males. D) Pattern 3: expression of genes that are upregulated in males and downregulated in females during gonadal development. Data is shown as fold change (log_2) using the PU group values as a reference. Age in days post fertilization (dpf) refers to average age values of fish sampled at each group. Full gene names are shown in Supplementary Table 7.

3.7. Sexual differences in juvenile fish

We also studied the expression differences between ovary and testis in juvenile fish. A total of 4,087 DEG where found in the FJ *vs.* MJ comparison (Figure 3A), being larger the number of genes found upregulated in testes (2,266) than in ovaries (1,821). When looking specifically at the previously studied 18 canonical reproduction-related genes (Table 2) we found significant ($P < 0.01$) sex differences between FJ and MJ for some genes, either upregulated in the ovary (i.e. *cyp19a1a* FC = 5.2, *sox19* FC = 6.9, *figα* FC = 1.13) or in testis (i.e. *sox9b* FC = -3.3, *spata1* FC = -2.2, *star* FC = -3.4 and *fshr* FC = -3.2).

Figure 7. MJ vs FJ Network analysis

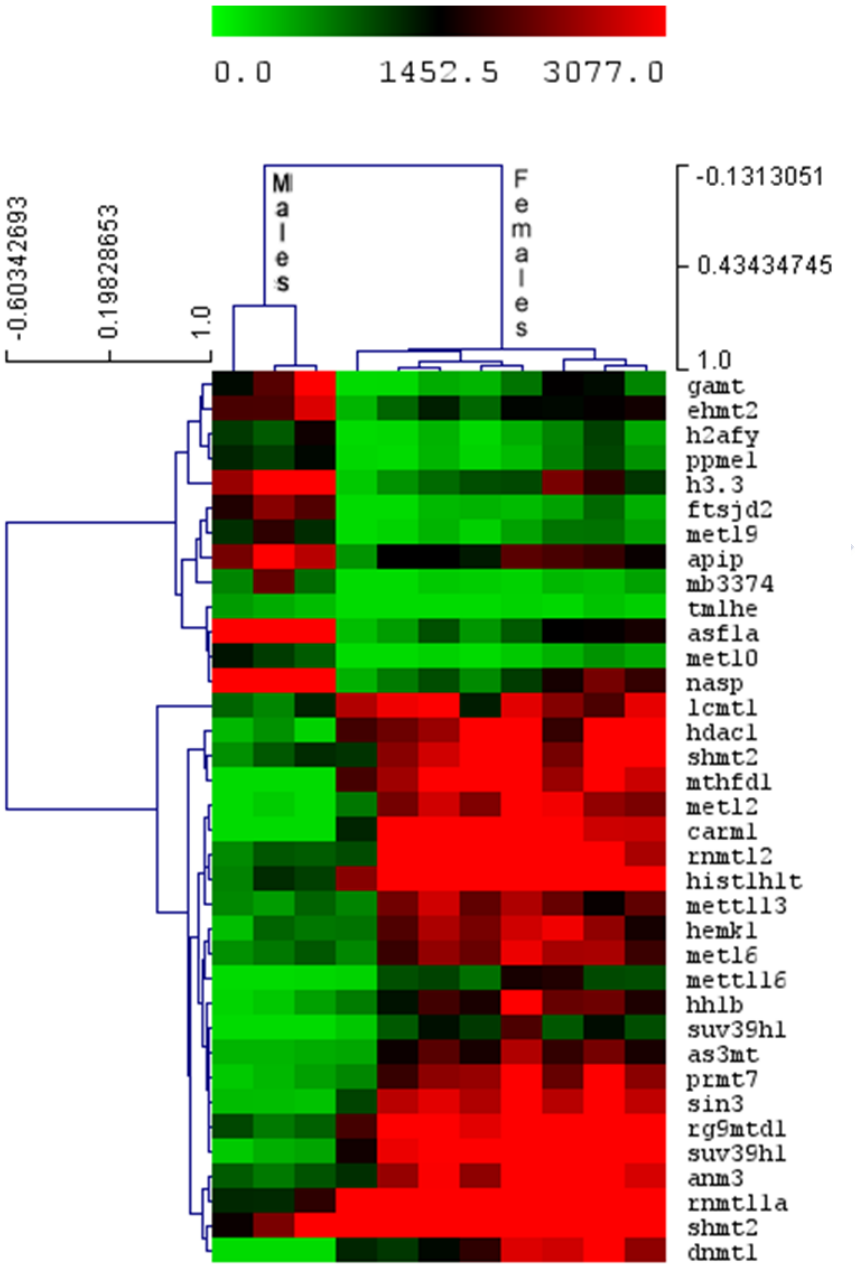


Network analysis with genes involved in sex differentiation and reproduction. The genes found upregulated in the FJ *vs.* MJ comparison are represented in magenta in females and in blue in males. Lines connecting gene symbols indicate a positive correlation of their expression if depicted in red and a negative correlation if depicted in blue. Full gene names are shown in Supplementary Table 7.

The total number of DEG (5,565 genes) between MJ and FJ found by SAM analysis (Figure 3A) were selected for a visual network representation (Figure 7). Results showed two main gene clusters, one related to females and another to males. A larger group of genes mainly connected to female phenotype in the centre of the network was observed. These genes were involved in ovarian development (*gsd9*, *zar1*), germ cell line maintenance (*piwil2*) and, as already observed in the heatmap and GO analysis, methylation (*metl6*, *metl9*), histone

deacetylation (*hdca3*, *hdac1*) and splicing (SURP and G patch domain containing, *sugp1*, and serine/arginine-rich splicing factor 1, *srsf1*). In agreement with previous analysis we found *cyp19a1a* connected with *foxl2* and *ecm1*. The female and male-related gene groups were connected mainly by three genes: *sox9b*, *irf7* and histone H3.3 (*h3f3a*). These three genes showed a positive correlation with other male-related genes (*ar1*, *fsh*, *gsdf*, *star1*, *pgr*, *wt1β*), histone desacethylation (suppressor of defective silencing 3 homolog, *suds3*) and transcription factors (*lhx9*, dachshund family transcription factor 1, *dach1*).

Figure 8. Epigenetic genes



Heatmap analysis of epigenetic regulatory mechanisms-related genes during gonadal development. Full gene names are shown in Supplementary Table 7.

Among the DEG in the FJ *vs.* MJ comparison, we found several genes involved in epigenetic regulatory mechanisms (Figure 8). There were 13 upregulated genes in the MJ group, e.g. methyltransferase-like protein 9 (*metl9), metl10*, euchromatic histone-lysine N-methyltransferase 2 (*ehmt2*) or anti-silencing function 1A *histone chaperone (asf1a)*; and 23 upregulated genes in the FJ group, e.g. *dnmt1*, *metl6*, histone H1-beta (*H1b*) or ribosomal RNA methyltransferase 2-like (*rnmtl2*).

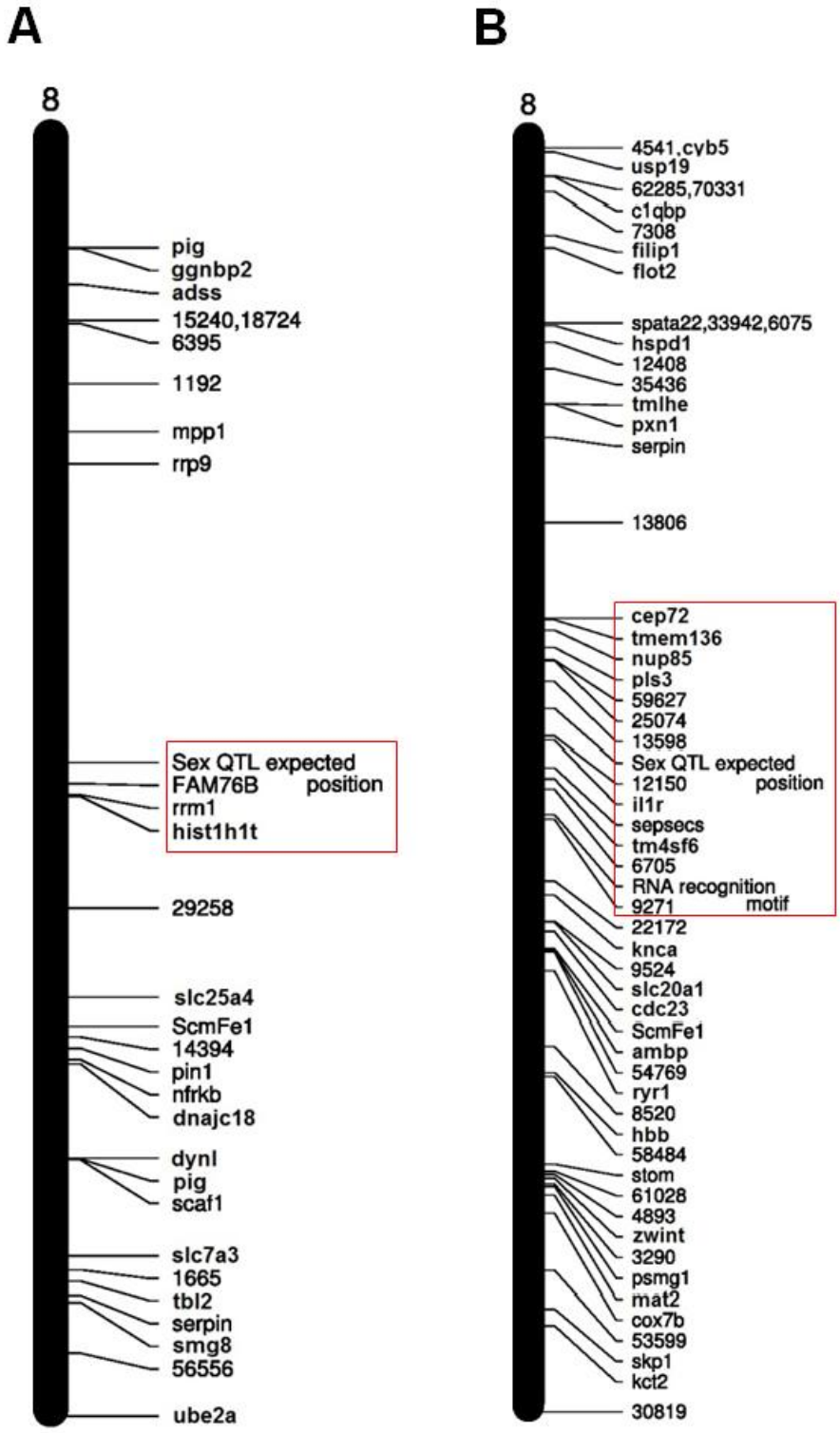
3.8. Localization of reproduction-related genes in the sex- and growth-QTL markers

With the purpose of identifying putative candidate sex determining genes in turbot, we localized in the turbot genome the 18 studied canonical reproduction-related genes and those DEG in MJ (2,266) and FJ (1,821). None of the 18 canonical genes mapped to the reported sex- or growth-related QTLs with the exception of *foxl2* and *17βhsd*, which mapped close to the growth-related QTLs in LG15 and LG6, respectively (Sánchez-Molano et al. 2011).

The DEG between FJ and MJ were located in the turbot genetic map to assess their co-localization with the previously reported sex- and growth-related QTLs (Martínez et al. 2009; Sánchez-Molano et al. 2011) considering ±1Mb around the most significant sex- and growth-related QTL markers (Figure 7C and 7D; Supplementary Figures 9 and 10). Microarray IDs were used to represent unannotated genes. LG5 holds the main sex determination QTL in turbot (Martínez et al. 2009), unfortunately the markers associated to the sex determining region are located in a small scaffold of the turbot genome (scaffold 83) and very few DEG were placed on it, only two for each sex. The two female genes were non-annotated and the two male genes were the fragile X mental retardation syndrome-related protein 1 (*fxr1*) and the RNA-binding protein 8A (*rbm8a*). In the region surrounding a minor sex-QTL in LG6 (scaffold 15), a total of 14 female upregulated genes were localized, while 21 upregulated male genes were found, although none with a remarkable sex-related function. Yet, in males, some interesting genes were found just above the sex-QTL expected position and inside the +-1Mb interval: two genes related to the immune system (*mhci*, immunoglobulin superfamily containing leucine-rich repeat 2, *isrl2*), one involved in histone acetylation (mortality factor 4 like 1 gene, *morf4l1*) and two transcription factors (DNA-j-like, leucine rich repeat containing, *lrrc6*). In LG8 (scaffold 14) we also found more genes upregulated in MJ (14 genes) than in FJ (4 genes). We also found a larger number of male DEG mapped to LG21 (12 *vs.* 9), again some genes related to immune system (Fc receptor, *fcr*) or histone modification (*H3* histone family, *h3.3*).

Differences were also found for upregulated genes in MJ and FJ around growth-QTL markers (Supplementary Figure 10) in LG5 (scaffold 34) and LG16 (scaffold 40). No MJ upregulated genes were placed near LG5 and LG16 growth-QTLs, while two FJ genes were found next to the LG5 growth-QTL marker (aldehyde dehydrogenase 9, *aldh9a1*, and glutamine synthetase, *glul*) and three more next to the LG16 growth-QTL marker (R4_48196, tRNA 5-methylaminomethyl-2-thiouridylate and transmembrane protein 209, *tmem209*). However none of them showed an obvious relationship with growth processes.

Figure 9. MJ vs FJ genes mapped to LG8



Genes upregulated (A) or downregulated (B) in FJ *vs.* MJ mapped to the LG8, indicating the position of the turbot sex-associated QTL marker. A region of ±1 Mb around the highest associated genetic marker to the sex–related QTL is shown in square. Full gene names are shown in Supplementary Table 7.

Finally, we performed an enrichment analysis for each QTL to check if the region was particularly enriched in either FJ or MJ DEG, using the genome position of all the microarray genes as background. Only an almost significant enrichment (P = 0.056) in male DE genes was detected in the sex-QTL region of LG8 (Figures 9A and 9B).

4. Discussion

To our knowledge, this is the second study, after that previously reported in tilapia (Tao et al. 2013), where a deep transcriptomic analysis from undifferentiated to differentiated gonad development has been performed in fish. The work presented here aimed to identify sex markers and novel genes that may help to improve turbot production through sex control and maturation, but also to gain knowledge about the gonad differentiation process in turbot.

4.1. Transcriptomic overview during gonadal development

We classified individuals into different transcriptomic groups showing that, for example, females started sex differentiation at ~5 cm of total length (TL), earlier than males (~10 cm of TL). Once the groups were defined, we used SOTA analysis to find groups of genes with expression patterns specific of each development period. At early steps of gonadal development (PU+U group) genes related to both female and male pathways were already expressed (e.g. *ptge3, spata1*) as well as in D group (e.g. *star* and *lhr*). MJ group over-expressed genes involved in spertamotegenesis like *spata22* and *spag*, while FJ group genes involved in ovarogenesis like *vtg* and *chg*.

A differential expression analysis was performed between the different developmental groups, finding the highest differentiation between FJ and D. Similar observations were reported in tilapia, where a higher number DEG was found at 30 days post hatching (Tao et al. 2013) coinciding with the formation of the future ovarian cavity and the first meiotic divisions (D'Cotta et al. 2001). With the exception of the U *vs.* D comparison, more genes were found to be downregulated than upregulated in a certain stage when compared to the previous developmental stage, showing that gene repression seems to be critical for gonad development. A higher number of genes was found upregulated in testis when compared to ovaries (2,266 in MJ *vs.* 1,821 in FJ), as previously documented in other fish species like zebrafish (Small et al. 2009) or tilapia (Tao et al. 2013) but also in mammals (i.e. mice; Munger et al. 2009) and invertebrates (i.e. *Drosophila*; Brown et al. 2014). In contrast, a much higher number of genes (6,228) were required for ovary development than for testis development (39) from D stage. In fact, D was the most heterogeneous group, formed by ~90 and ~135 dpf individuals but also by ~400 dpf males, and therefore the influence of these samples on the expression of pro-male genes should not be underestimated. Some D individuals (pro-male) and MJ expressed male-related genes such as *sox9b*, involved in sex determination and testicular development (Nakamoto et al. 2005), the gonadotropin *fsh*, required for spermatogenesis (Zohar et al. 2010), or *ar*, required for male sex differentiation (Blázquez and Piferrer, 2005). These genes together with other male-related genes like

spata1, *wt1* and *star* involved in testis maturation (Huffman et al. 2012) were also positively correlated in the gene network analysis.

Due to the emerging importance of epigenetics in fish gonad development (Piferrer, 2013; Shao et al. 2014), we identified a group of epigenetic-related DEG that showed a dimorphic gene pattern between testes and ovaries. Since the first epigenetic mechanism described in fish, which showed the influence of temperature during early stages of development in the methylation levels of the *cyp19a1a* promoter in the European sea bass gonads (Navarro-Martín et al. 2011), several studies have revealed the importance of this cellular mechanisms for controlling sex differentiation in fish. For example, hypermethylation of *cyp19a1a* promoter was observed in the ovotestis and testis of the hermaphroditic ricefield eel (*Monopterus albus*) when compared to the ovary (Zhang et al. 2013), and also higher methylation levels of this promoter during sex differentiation in carps (*Gobiocypris rarus*) subjected to bisphenols during fish development have been reported (Liu et al. 2014). Turbot microarray results showed genes involved in epigenetic regulation throughout gonad development (13 and 23 genes upregulated in males and females, respectively). Females showed up-regulation of *dnmt1*, which plays a crucial role in the maintenance of methylation patterns (Pradhan et al. 1999); some methyltransferase proteins such as *metl6*, which also presented a positive correlation in the gene network analysis with other female-related genes; and histones like *h1b*, responsible for chromatin condensation with other histones (Ohe et al. 1989). In MJ, methyltransferase proteins such as *metl9*, *metl10* and *ehmt2* or the histone chaperone *asf1* were upregulated regarding FJ. However, it is clear that further efforts are required to understand the epigenetic mechanisms involved during sex differentiation in turbot.

4.2. Identification of *cyp19a1a* and *dmrt3* as good early sex markers

Cyp19a1a is a key enzyme responsible for converting androgen to estrogen in the fish ovary during sex differentiation (Devlin and Nagahama, 2002; Guiguen et al. 2010). This process requires high expression of *cyp19a1a* in the developing ovary (Guiguen et al. 1999) and, therefore, *cyp19a1a* is considered an early marker of ovarian differentiation in several fish species such as sea bass (Blázquez et al. 2008), rainbow trout (Guiguen et al. 1999), half-smooth tongue-sole (*Cynoglossus semilaevis*; Deng et al. 2009), Southern flounder (*Paralichthys lethostigma*; Luckenbach et al. 2005) and Atlantic halibut (*Hippoglossus hippoglossus*; Matsuoka et al. 2006). During early gonad development in turbot, at 90 dpf when fish were ~5 cm of TL, *cyp19a1a* over-expression was already observed in some individuals and it continued during ovary differentiation and maturation. In contrast, fish with low *cyp191a1a* levels exhibited high levels of *dmrt3* at ~140 dpf (~10 cm TL). *Dmrt3* belongs to the doublesex/mab-3 related family of transcription factors, involved in sex-specific differentiation in all animals studied so far (Kopp, 2012). The most notorious member of this family is *dmrt1*, sex determining gene in *Oryzias latipes* (Nanda et al. 2002), *Xenopus laevis* (Yoshimoto et al. 2008), birds (Smith et al. 2009) and probably also in *Cyanoglossus*

semilaevis (Chen et al. 2014). *Dmrt1*was not present in the microarray, however similar expression patterns between *dmrt1* and *dmrt3* have been observed in some fish species, for example in Japanese pufferfish (*Takifugu rubripes*; Yamaguchi et al. 2006). In swamp eel (*Monopterus albus*), *dmrt3,* together with *dmrt2* and *dmrt2b,* were also detected in testis but not in ovaries (Sheng et al. 2014). In zebrafish, *dmrt3* expression was detected in the developing germ cells of both gonads, mainly in spermatogonia and spermatocytes but also in developing oocytes (Li et al. 2008). So far, data suggest that *dmrt3* is expressed in the developing testis of most fish species, as in turbot, and therefore *dmrt3* can be considered a male marker as its well-known paralog *dmrt1*. Genetic or histological sexed fish always matched with the previously assigned sex based on *cyp19a1a* and *dmrt3* expression levels. The observation that the expression of these two genes was negatively correlated supports their opposite roles in turbot sex differentiation. Together, these results show that *cyp19a1a* and *dmrt3* are reliable markers of ovarian and testicular development, respectively, for sex assignment in the turbot.

To further explore the genetic basis of gonad differentiation, the behavior of 18 canonical genes, including *cyp19a1a* and *dmrt3*, selected by their importance during sex differentiation in fish, was studied. First, we analysed the expression pattern of eight genes related to the steroidogenic pathway for their important role during gonad differentiation: *cyp191a1, 17βhsd, 3βhsd, 11βhsd, cyp11β, star, arα* and *fshr*. None of these genes, excluding *cyp19a1a*, showed a clear sex dimorphic expression, which reinforced the relevance of *cyp191a1* as an ovarian development sex marker in turbot. Secondly, we studied the expression pattern of six canonical transcription factors along gonadal development. As expected, *foxl2*, *figα* and *sox19* were expressed in females, while *dmrt3* and *sox9b* were expressed in males. *Foxl2* is expressed in the somatic cells of the ovary and is critical for ovarian determination, its deletion in mice increased the expression of testis differentiation markers such as *sox9* leading to testis development (Uhlenhaut et al. 2009; García-Ortiz et al. 2009). *Figα* is expressed at the primordial follicle stage and its expression persists during oocyte growth. *Figα* also suppresses male specific genes, being crucial for ovarian fate (Joshi et al. 2007). *Sox19* is a fish specific expressed gene since its orthologue in other vertebrate lineages, termed *sox15*, is pseudogenized (Okuda et al. 2006). *Sox19* is expressed in many adult tissues in European sea bass (*Dicentrarchus labrax*) but mostly in gonads and brains suggesting a role in ovarian differentiation (Navarro-Martín et al. 2012). So far, *sox19* has been found in rice field eel (Liu and Zhou, 2001), fugu (Koopman et al. 2004), sturgeon (Hett and Ludwig 2005), zebrafish (Okuda et al. 2006) and European sea bass (Navarro-Martín et al. 2012). In turbot *sox19* was previously mapped to LG2 (Viñas et al. 2012), but its female-specific expression pattern is reported here for the first time. *Sox9* is involved in sex determination and testicular development in all vertebrates (Nakamoto et al. 2005; Bagheri-Fam et al. 2010). This transcription factor is expressed in Sertoli cells sharing functions with *sox8* during testis differentiation in mammals (Chaboissier et al. 2004). The last studied transcription factor, s*f1* (*nr5a1*), exerts several functions in the reproduction system by regulating the expression of several genes such as *star, cyp19a1a* or *amh* (Brennan and Capel, 2004; Kuo et al. 2005). In

teleosts, members of the *nr5a* family have been identified in a number of species, like zebrafish (von Hofsten and Olsson, 2005) or European sea bass (Crespo et al. 2013). The highest *sf1* expression in turbot was found at early stages of gonadal development when several factors are required for the growth of the early bipotential gonad (Brennan and Capel 2004). Among the other canonical reproduction-related genes analysed, *fancl* and *spata1* showed different patterns between the two sexes. *Fancl*, which guarantees the survival of female germ cells (Rodriguez-Marí and Postlethwait, 2011), was more expressed in juvenile ovaries although the difference was not statistically significant; and *spata1* showed higher expression at early stages of development and it was more downregulated in females than in males during gonad development. *Spata1* has been reported to play a role in testis maturation (Huffman et al. 2012), so it is unclear why higher expression of this gene was found at early stages of development in turbot when the gonads are still undifferentiated.

4.3. Identification of novel genes associated with ovarian or testis development

Forty-five DEG were identified as pro-female genes involved in ovarian differentiation and all of them were differentially expressed in females at 140 dpf onwards. Among them we found a total of twelve genes with known reproduction-related functions. Two of them, *alv* and *zps*, are expressed in egg envelop. *Alv* is a protease that helps to trigger the egg hardening after fertilization (Shibata et al. 2012). *Alv* has also been identified in other fish species like medaka (Shibata et al. 2000), coho salmon (*Oncorhynchus kisutch*; Luckenbach et al. 2008) or Atlantic cod (*Gadus morhua*; Kleppe et al. 2014). *Zps* are glycoproteins that are accumulated in oocytes during oogenesis and play an important role during fertilization (Ringuette et al. 1988). They have also been identified in a large number of fish species like Atlantic bluefin tuna (*Thunnus thynnus*; Gardner et al. 2012) or sturgeon (*Acipenser transmontanus*; Murata et al. 2014). *Lhx8* is a transcriptional regulator involved in ovarian formation and folliculogenesis in mouse (Choi et al. 2008; Jagarlamudi and Rajkovic 2012) and its deficient transcription in *lhx8 -/-* ovaries downregulated the expression of germ cell specific genes like *gdf9*, bone morphogenic protein (*bmp15*) or *figα*, generating infertile mice (Pangas et al. 2006). *Gdf9* is required during folliculogenesis for granulosa cell proliferation (Wu et al. 2004) and in fish it has been identified in ovaries in several fish species like European sea bass (Halm et al. 2008), rainbow trout (Bobe et al. 2008), eel (Lokman et al. 2010), zebrafish (Clelland and Kelly, 2011) and ricefield eel (*Monopterus albus*; He et al. 2012).

Among pro-female genes, we also identified some not previously related to sex differentiation. Eight were cell components, for example membrane proteins (*tmem144a*, *pex*) or Golgi complex components (*cog3*). Five genes were related to the immune system: *cd98*, *vs.ig10*, *ldlr*, *cidec* and cell-surface antigen heavy chain-like. The activation of the immune system during gonadal development has been described in several fish species like in carp (Xu et al. 2011), Atlantic cod (Sundaram et al. 2012), eel (Sower et al. 2009) or zebrafish, where the immune system is required to initiate female cell apoptosis during male sex

differentiation, leaving space for male cells to form the developing testes (Uchida et al. 2002; Maack and Segner, 2003). We also identified four metabolism-related genes, like *gpd1*, *irs2* or *gstal*, which were upregulated in ovaries. Finally, three DEG related to transcription machinery (*rex1*and *gtf3c1*) and histone DNA binding (*h1f5*), two genes involved in cell adhesion (*pcdh15a* and *pcdh15*) and one in circadian rhytm (*cry2*) were also preferably expressed in developing ovaries. The influence of the circadian system in reproduction has been described in mice, where knockout *cry2* males showed increased expression of several cytochrome P450 enzymes in the liver reaching levels typically found in females (Bur et al. 2009). In zebrafish, alterations of the circadian clock at early stages of development resulted in female bias in the final population (Villamizar et al. 2012).

On the other hand, a group of 12 novel genes were identified as pro-male genes involved in testis development and they were classified in three different expression patterns. Pattern 1, characterized by a lower expression in females than in males, included genes such as *ace* and one non annotated (R4_8750). *Ace* is an enzyme with higher activity in testes than ovaries in mice (Lim et al. 2002) and plays an important role in the regulation of male fertility (Hagaman et al. 1998). Although its function in male gonads has been studied in other vertebrates, like frogs (Bramucci et al. 2004), no data was available in fish on this regard. Pattern 2, characterized by up-regulation in males from 140 dpf onwards, included four non annotated genes and *capn8*. Calpain system is formed by various proteins involved in various physiological functions like remodeling the cytoskeletal/membrane attachments, different signal transduction pathways or apoptosis (Goll et al. 2003). In mice, c*alp8* has been related to muscle (Sorimachi et al. 1993), while *calp5* and *11* were detected in testis (Dear et al. 1999; Dear and Boehm, 1999). This system is believed to be not only conserved, but even expanded in the teleost lineage. However, more effort is required to understand the role of each member of this family. Pattern 3, composed by genes downregulated in females during gonadal development, includes *nxph*, a member of the neurexophilin family which promotes the adhesion between dendrites and axons (Missler and Sudhof, 1998). Studies of *nxph1* during zebrafish embryonic development indicate that this gene is expressed in the central nervous system (Thomas-Jinu and Houart, 2013), but its role in gonadal development is described here for the first time. The study of the transcription binding sites on the promoters of some of these pro-male and pro-female genes suggests that some of these genes could be regulated by transcription factors involved in gonad differentiation, like estrogens or *sf1*, which reinforces our hypothesis that these genes might have a role in sex differentiation.

4.4. Relationship between reproduction-related genes and sex- and growth-QTL markers

We localized in the turbot genetic map (Hermida et al. 2013) the 18 canonical reproduction-related genes and the DEG between MJ (2,266) and FJ (1,821). None of the 18 canonical genes mapped near sex- or growth-related QTL markers with the exception of *foxl2* and *17βhsd*, close to growth-QTL markers in LG15 and LG6 respectively. The mapping of FJ *vs.* MJ DEG revealed an almost significant enrichment in male transcripts in the sex-QTL

region of LG8. However, the main sex determination QTL is in LG5 (Martinez et al. 2009) where we found *fxr1*, a gene already described as relevant for sex determination in turbot (Taboada et al. 2014).

4.5. Concluding remarks

Our results revealed the complexity of the gene expression patterns underlying gonad development in turbot. The study of gene expression along gonadal development provided new insights on the importance of specific sets of genes for each gonad developmental stage from undifferentiated gonads up to the formation of testes or ovaries. Gonadal transcriptome showed that ovarian differentiation in turbot started at ~5 cm of TL while testis differentiation started later and male gonads remained transcriptomically more similar to undifferentiated gonads. Two early sex markers were identified: *cyp191a1* in females from 90 dpf and *dmrt3* in males from 140 dpf. Furthermore, a group of 45 pro-female and 12 pro-male genes, with clear sex biased patterns, were identified during ovary or testis development for the first time. The functions of these genes were not exclusively related to reproduction but also to metabolism, immune system or circadian clock processes, and it will be interesting to refine their role in sex differentiation in the future. Finally, DEG between male and female juveniles were mapped to sex- and growth-QTLs, although no sex determination candidates were found and no QTL showed a significantly bias in male or female genes.

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Supplementary material

Supplementary Table 1. Genes for microarray validation

Gene name	Gene symbol	Forward primer	Reverse primer
Follitropin subunit beta	*fshb*	TGCAAACTGGCCAACATCAC	CCGTTAATGTGCTTCGCCTC
Tudor domain-containing protein 1	*tdrd1*	TGAGCCTTTGGTGTGGTCTT	ACCATAGCCCGATACCATGC
Retinol dehydrogenase 3	*rdh3*	CTGACGACCACACACCTTGA	GCGACTCCAGCATTGTTCAC
Flg-hepta	*flg-h*	TGGTCTTTGGGAGTGGGGA	TGAATCGAGCAGTGTCCCAAA
Piwi-like protein 2	*piwil2*	ACAACACAGCGAACCTCACA	GGCATACTTGCATGGTGCTG
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	GGAGTACGCGCCCATCTTT	GTCCTCCGTGAAGCAGTTGA
SRY-box containing transcription factor 6	*sox6*	CCCATTTCTCCCTCCTCTCT	CCTTTCCGAGGAGACTGTTG
Gonadal soma derived factor	*gsdf*	CTGGGCTGGAACAACTGGAT	GGCACCATTTCCTGGGAGTT
Prostaglandin synthase E3	*ptges3*	TCTACGACCGCACCATCAAC	TCATGCTCCCAGTCTCTCCA
Lim homeobox 8	*lhx8*	TTCACCAGCGTTCATTCGTC	CACCGAACTACACAAGCAGA
SRY-box containing transcription factor 19	*sox19*	ACCGAGCGGTTTGTGCCTTG	TCCTCTGGATGCAGTGCTGATTGT
Aromatase	*cyp19a1a*	CAGCGAGGAAGCTGGCAAACA	ACACGCAGACTCGGCTTTTTACAT
Catenin beta-1	*ctnnb1*	AGCTGTGTCGGTCGCGGTTT	GCCAGCCTCTGGACGTTGGTG
Doublesex- and mab-3-related transcription factor 2	*dmrt2*	GACTTTCTGTCCAAGCCCCT	GGGCGTGGGTCTTTTCAGTA
Alcohol dehydrogenase Class VI	*adh6*	GCTTATCGCTGGACGCACTTG	TGGCTTCACTGACAACAACGC
UTP--glucose-1-phosphate uridylyltransferase	*ugp1*	CAGGAGTTTCTGTCCAGGTTTGAG	ATTGGCGATGATGATGACGGTTC
c-X-C motif chemokine 14	*cxcl14*	CACACGCACCCTCCAAATA	CCGAGTGTTTACAGCAGCAC
Similar to WAF-1/CIP1 stabilizing protein	*fkbpl*	CGACAGCACAGATGATGG	CTTCGTAGTAACCGTTCTCC
Interferon-related developmental regulator 1	*ifrd1*	CGCTGAGAAGAAGAACATC	CAAGTCACGATGGGTAAAG
S-phase kinase-associated protein 1	*skp1*	TCACAGAGGAGGAGGAAGC	CAACCAGTTAGCAGAGACAATC

Gene names, gene symbols and primer sequences of the genes used for microarray validation by qPCR

Supplementary Table 2. DEG in the different comparisons

U vs PU			
Up-regulated		**Down-regulated**	
Anotation	**Fold change**	**Anotation**	**Fold change**
Period 4	3.07	Annexin A1	-3.14
Cytochrome oxidase subunit Via	2.08	Trypsin domain	-3.15
Calsequestrin	0.96	Sperm acrosome membrane-associated protein 4	-3.15
Zgc:103752	0.82	Periplakin	-3.17
Enolase	0.61	Envoplakin	-3.26
		Envoplakin	-3.29
		Nucleolar protein Nop52	-3.32
		Herpes_gp2	-3.35
		RNA_capsid	-3.41
		Herpes_gp2	-3.46
		ATP-binding cassette, subfamily A	-3.54
		Guanine nucleotide binding protein-like 3	-3.73
		TT_ORF1 domain	-3.78
		Keratin 1	-3.81
		Trypsin domain	-4.63
		Secreted trypsin-like serine protease	-4.95
		Elongation factor 1-alpha, oocyte form	-5.22
		Gastric chitinase	-5.90
		Metalloproteinase	-6.10
		Chitinase 1	-7.02

D vs U			
Up-regulated		**Down-regulated**	
Anotation	**Fold change**	**Anotation**	**Fold change**
Steroidogenic acute regulatory protein	5.08	DNA-directed RNA polymerase II subunit A	-7.77
Gonadal soma derived factor	4.88	Myhz2 protein	-7.81
Follicle stimulating hormone receptor	4.79	I-set multi-domain protein	-7.91
Inhibin	4.70	Desmin	-7.98
Isoform 3 of Cell division protein kinase 14	4.65	Titin a	-7.99
solute carrier family 43, member 3	4.58	Nucleoside diphosphate kinase B	-7.99
Cardiac myosin light chain1	4.49	eEF1A2 binding protein	-8.05
Nipsnap homolog 3A	4.38	SET and MYND domain containing 1a	-8.08
Polyprotein	4.30	Kelch repeat and BTB (POZ) domain containing 10	-8.24
WD repeat domain 20	4.28	Myosin, heavy polypeptide 1, skeletal muscle	-8.25
Serine hydrolaselike protein	4.22	Myosin light chain 3 (Fragment)	-8.25
7tm_7 domain	4.15	Myosin heavy chain, striated muscle	-8.29
Transcription termination factor, RNA polymerase I	4.11	Myosin heavy chain	-8.33

Creatine kinase, brain a	3.86	Adenosine monophosphate deaminase	-8.33
Carbonyl reductaselike	3.77	Myozenin 1b	-8.48
20betahydroxysteroid dehydrogenase			
Tropomyosin1 alpha chain	3.75	Myosin binding protein C	-8.66
HydroxyacylCoenzyme A dehydrogenase	3.66	Myhz2 protein	-8.70
Zgc:154009	3.65	FYDLN_acid	-8.73
Smoothelinlike	3.64	Aspartic acid-rich protein aspolin2	-8.81
Vascular cell adhesion molecule 1	3.55	Calsequestrin	-9.29

FJ vs D

Up-regulated		Down-regulated	
Anotation	**Fold change**	**Anotation**	**Fold change**
Zona pellucida spermbinding protein 2	7.99	7tm_7 domain	-4.61
Zona pellucida protein X	7.92	Zonadhesin	-4.64
Zygote arrest 1like	7.87	Novel protein	-4.66
RNA binding motif protein 4.1	7.86	Similar to 3(2), 5-bisphosphate nucleotidase 1	-4.67
ATPbinding cassette, subfamily A (ABC1)	7.74	Fast muscle troponin I	-4.69
Creatine kinase, muscle	7.70	Myo-inositol monophosphatase	-4.75
Zona pellucida protein X	7.62	Es1 protein	-4.75
Mucin 1	7.56	Fumarylacetoacetase	-4.78
Lge1 domain containing protein	7.42	mucin-5B	-4.82
Alveolin	7.26	nipsnap homolog 3A	-4.88
Kinesin family member 20/23	7.09	HRAS-like suppressor 2	-4.97
Egg envelope component ZPAX	6.98	Replication factor C (Activator 1) 3	-5.01
Cyclin A2	6.92	C1orf123 homolog	-5.05
ELOVL family member 6	6.82	Trypsin	-5.05
CTH1 protein	6.80	Isoform 3 of Cell division protein kinase 14	-5.08
Rho GTPase activating protein 11A	6.70	DNA mismatch repair protein MSH6	-5.09
Cathepsin Z	6.68	Aquaporin 1a	-5.25
Lowdensity lipoprotein receptors domain class A	6.65	Solute carrier family 12 member 3	-5.44
Choriogenin L	6.65	Collagen, type VI, alpha 3	-5.53
Protooncogene tyrosineprotein kinase Yes	6.60	SSU rRNA	-5.63

MJ vs D

Up-regulated		Down-regulated	
Anotation	**Fold change**	**Anotation**	**Fold change**
UPF0575 protein	2.67	Zonadhesin	-3.41
Hyaluronoglucosaminidase	2.62	ATP-synt_B	-3.41
Stromal antigen 1like	2.61	TRAM_LAG1_CLN8 domain	-3.41
U6 snRNAassociated Smlike protein LSm6	2.44	Adducin 3	-3.43
Binding protein 2 (liprin beta 2)	2.38	Fibrinogen-like protein 1	-3.45
Novel protein (Zgc:92501)	2.14	SelP_N domain	-3.46
Exonuc_XT domain	2.08	UDP-glucuronosyltransferase 2A1	-3.52
Small nuclear Ribonucleoproteinassociated protein B	2.00	Collagen, type IV, alpha	-3.52

TMEM9 domain family, member B	1.98	AFG3 ATPase family gene 3-like 2	-3.54
UracilDNA glycosylase	1.94	Neurofascin isoform 7 precursor	-3.54
U6 snRNAassociated Smlike protein LSm6	1.89	Transglutaminase 2	-3.58
ZPC domain containing protein 5	1.89	Zinc finger protein 560	-3.59
Serine dehydrataselike	1.87	Cytoglobin-1	-3.62
Solute carrier family 25 member 19	1.86	Phosphatidylinositol-4-phosphate 5-kinase-like protein 1	-3.64
LEDGF/p75 transcription factor	1.84	Ras-related protein Rab-25	-3.69
Ribonuclease H2 subunit C	1.77	Protein kinase C and casein kinase substrate in neurons protein 1	-3.73
eIF_4EBP domain	1.73	Leucine rich repeat containing 32	-3.80
Histone H2A	1.70	Adducin 3 (gamma) b	-3.81
Checkpoint protein HUS1	1.66	Histone-lysine N-methyltransferase SETD7	-3.83
Mortality factor 4 like 1	1.64	6-pyruvoyl tetrahydrobiopterin synthase	-4.21

FJ vs MJ

Up-regulated		**Down-regulated**	
Anotation	**Fold change**	**Anotation**	**Fold change**
Riboflavinbinding protein	7.23	wu:fi30e01	-7.53
Proenkephalin	6.67	CTH1 protein	-7.58
Collagen, type VI, alpha 3	5.29	Alveolin	-7.81
Ribosomal protein S27	5.08	Creatine kinase, muscle	-7.88
Adaptorrelated protein complex 3, beta 1 subunit	4.52	Glutathione S-transferas	-8.08
UPF0575 protein	4.48	Early nodulin-75	-8.14
Dynein light chain Tctex type 1	4.46	Zgc:92083	-8.25
cell adhesion molecule 1a	4.41	Zona pellucida sperm-binding protein 2	-8.26
Luteinizing hormone receptor	4.29	Mucin 1	-8.39
Si:ch211199g17.1	4.27	Kinesin family member 20/23	-8.57
Prostaglandin E synthase 3	4.26	Rho GTPase activating protein 11A	-8.58
Brainspecific polypeptide PEP19	4.26	Cathepsin Z	-8.58
Primaryamine oxidase	4.10	low-density lipoprotein receptor	-8.65
Sideroflexin 2	4.07	ATP-binding cassette, subfamily A, member 5	-8.77
Nonclathrin coat protein zeta1COP	4.06	ELOVL family member 6	-8.95
leucyltRNA synthetase	4.01	GH05993p	-9.16
Acyl carrier protein	3.97	Quinone reductase	-9.28
Binding protein 15	3.96	Choriogenin L	-9.34
SEC23 interacting protein	3.94	Choriogenin L	-9.48
NADHubiquinone oxidoreductase chain 3	3.83	NAD(P)H dehydrogenase quinone 1	-9.57

Top 20 up-regulated and down-regulated DEG obtained by SAM for each comparison during turbot gonad development.

Supplementary Table 3. Enriched GO terms

	GO term	*FDR P* value	Enrichment
U vs PU			
Downregulated			
Biological Process	Pore complex assembly	1.30E-02	31.01
	Hemolysis in other organism involved in symbiotic interaction	3.10E-02	4.33
Molecular Function	Actin binding	3.10E-02	28.95
D vs U			
Downregulated			
Cellular component	Myosin filament	1.81E-06	12.67
	F-actin capping protein complex	1.23E-02	17.99
Biological Process	Actin cytoskeleton organization	3.25E-02	3.74
	Glycolysis	3.25E-02	22.44
	Substrate adhesion-dependent cell spreading	6.07E-06	4.89
Molecular Function	Motor activity	5.97E-05	4.21
	Actin binding	3.25E-02	4.1
Upregulated			
Molecular Function	Double-stranded RNA binding	1.34E-04	4.95
FJ vs D			
Downregulated			
Molecular Function	Calcium ion binding	1.24E-03	1.56
Kegg pathways	Glycolysis / Gluconeogenesis	2.39E-03	2.69
	Oxidative phosphorylation	3.59E-03	2.11
	Fructose and mannose metabolism	4.04E-03	2.73
Upregulated			
Cellular component	Nucleoplasm part	6.51E-03	2.06
Biological Process	Chromatin	9.30E-03	2.25
	Protein-DNA complex	3.79E-02	2.53
	Cell division	1.09E-03	2.39
	DNA repair	1.08E-02	2.09
	Transcription from RNA Polymerase II promoter	1.67E-02	2.03
	Nucleosome assembly	2.07E-02	2.53
	Mitosis	2.27E-02	2.36
	tRNA processing	2.58E-02	3.78
	Histone modification	3.36E-02	2.94
Molecular Function	DNA binding	9.40E-05	1.56
	Zinc ion binding	3.25E-04	1.45
	ATP-dependent helicase activity	4.70E-03	2.23
Kegg pathways	Spliceosome	1.18E-02	2.82
MJ vs D			
Downregulated			
Biological Process	Phosphorylation	1.29E-02	1.77
	Protein phosphorylation	2.10E-02	1.77
FJ vs MJ			
Upregulated			
Kegg pathways	Aminoacyl-tRNA biosynthesis	4.46E-02	5.52

Supplementary Table 7. List of enriched GO terms among the differentially expressed genes between different gonad development stages.

Supplementary Table 4. Samples sexed by *cyp19a1a* and *dmrt3*

Age (dpf)	Fish ID	*cyp191a1a* levels	*dmrt3* levels	Assigned sex	Verified sex
75	PU1	low	low	none	male (G)
75	PU2	low	low	none	male (G)
75	PU3	low	low	none	female (G)
75	PU4	low	low	none	female (G)
90	U1	low	low	male	-
90	U2	high	low	female	-
90	U3	low	low	male	-
90	U4	low	low	male	-
90	U5	low	low	male	-
90	U6	high	low	female	female (G)
90	U7	high	low	female	female (G)
140	D1	high	low	female	-
140	D2	low	high	male	-
140	D3	low	high	male	-
140	D4	high	low	female	-
140	D5	low	high	male	male (G)
140	D6	high	low	female	female (G)
140	D7	low	medium	male	male (G)
140	D8	high	low	female	female (G)
400	MJ1	low	medium	male	male (P)
400	MJ3	low	high	male	male (P)
400	MJ4	low	high	male	male (P)
400	MJ5	low	medium	male	male (P)
400	MJ6	low	medium	male	male (P)
400	FJ1	high	low	female	female (P)
400	FJ2	high	low	female	female (P)
400	FJ3	high	low	female	female (P)
400	FJ4	high	low	female	female (P)
400	FJ5	high	low	female	female (P)
400	FJ6	high	low	female	female (P)

For each one of the 30 fish gonadal sample used for microarray analysis of gene expression, fish age and ID, and the assigned sex based solely on the *cyp19a1a* and *dmrt3* expression levels. In a subsample of 21 fish sex was verified either by genotyping, using the turbot sex probe (G), or by histological analysis based on their phenotype (P). In all cases, there was coincidence between assigned sex and actual sex. Age in days post fertilization (dpf) refers to average age values of fish sampled at each group.

Supplementary Table 5. Genes with similar expression to well-known reproduction genes

Sex-related	Function	Gene description	Gene symbol
Female	Reproduction	Alveolin	*gstal*
Female	Reproduction	zona pellucida protein Y1	*zpy1*
Female	Reproduction	kelch domain containing 1	*kelch*
Female	Reproduction	3-oxo-5-beta-steroid 4-dehydrogenase	*ake1d1*
Female	Reproduction	LIM homeobox 8a	*lhx8*
Female	Reproduction	Adrenodoxin-like protein	*fdx1l*
Female	Reproduction	zona pellucida protein Y1	*zpy1*
Female	Reproduction	zona pellucida sperm-binding protein	*zp*
Female	Reproduction	rho guanine nucleotide exchange factor 19	*gtf3c1*
Female	Reproduction	similar to egg envelope component	*zpax*
Female	Reproduction	maternal B9.10 protein	-
Female	Reproduction	Growth differentiation factor 9	*gdf9*
Female	Cell component	transmembrane protein 144	*tmem144a*
Female	Cell component	neurabin-1-like isoform X6	*nrb1*
Female	Cell component	kinesin family member 16B	*pcsk6*
Female	Cell component	component of oligomeric golgi complex 3	*cog3*
Female	Cell component	proprotein convertase subtilisin/kexin type 6	*pcsk6*
Female	Cell component	WASH complex subunit FAM21	*wash1*
Female	Cell component	peroxisomal membrane protein	*pex*
Female	Cell component	bolA family member 1	*bola1*
Female	Immunology	V-set and immunoglobulin domain-containing protein 10	*vsig10*
Female	Immunology	CD98 solute carrier family 3 member 2	*cd98*
Female	Immunology	LDL receptor-related protein	*ldlr*
Female	Immunology	cell death-inducing DFFA-like effector c	*cidec*
Female	Immunology	cell-surface antigen heavy chain	-
Female	Metabolism	glycerol-3-phosphate dehydrogenase 1	*gpd1*
Female	Metabolism	insulin receptor substrate 2-B	*irs2*
Female	Metabolism	acyl-Coenzyme A dehydrogenase family member 11	*acd11*
Female	Metabolism	Glutathione S-transferase	*gstal*
Female	DNA machinery	RNA exonuclease 1 homolog	*rex1*
Female	DNA machinery	general transcription factor IIIC	*gtf3c1*
Female	DNA machinery	Spy1 domain containing protein	*h1f5*
Female	Cell-cell adhesion	Protocadherin 15a	*pcdh15a*
Female	Cell-cell adhesion	similar to protocadherin 15	*pcdh15*
Female	Circadian	cryptochrome-2	*cry2*
Female	Unknown	Unknown	-
Female	Unknown	Unknown	-
Female	Unknown	Unknown	-
Female	Unknown	Unknown	-
Female	Unknown	Unknown	-
Female	Unknown	Unknown	-

Female	Unknown	Unknown	-
Female	Unknown	Unknown	-
Female	Unknown	Unknown	-
Male	Reproduction	angiotensin-converting enzyme	*ace*
Male	Cell component	calpain-8 catalytic subunit	*capn8*
Male	Cell component	Type-4 ice-structuring protein	*type4-ice*
Male	Cell-cell adhesion	neurexophilin 1	*nxph1*
Male	Unknown	Unknown	-
Male	Unknown	Unknown	-
Male	Unknown	Unknown	-
Male	Unknown	Unknown	-
Male	Unknown	Unknown	-
Male	Unknown	Unknown	-
Male	Unknown	Unknown	-
Male	Unknown	Unknown	-

List of DEG with similar behaviour to genes with a well-established role in sex differentiation expressed in differentiating to differentiated ovary (a total of 45 genes) or testis (a total of 12 genes).

Supplementary Table 6. Transcription factor binding sites in male- and female-related genes

Sex-related	TF Families	Nr. of Matches in Input	Expected (promoters)	Std.dev.	Over representation (promoters)	Z-Score (promoters)
female	V$PLAG	19	3.33	1.82	5.71	8.32
female	V$CARE	3	0.99	1	3.02	1.51
female	V$MEF3	6	2.26	1.5	2.65	2.15
female	V$CTCF	8	3.13	1.77	2.56	2.47
female	V$CP2F	8	3.21	1.79	2.49	2.39
female	V$ZF02	10	4.04	2.01	2.47	2.72
female	V$ZFHX	18	7.51	2.74	2.4	3.65
female	**V$EREF**	**9**	**4.6**	**2.14**	**1.96**	**1.82**
female	**V$RXRF**	**24**	**12.81**	**3.58**	**1.87**	**2.99**
female	V$GRHL	20	11	3.31	1.82	2.56
female	V$NEUR	11	6.69	2.59	1.64	1.47
female	V$HESF	12	7.43	2.72	1.62	1.5
female	V$IKRS	5	3.1	1.76	1.61	0.8
female	V$CHRF	10	6.36	2.52	1.57	1.25
female	V$KLFS	16	10.37	3.22	1.54	1.59
male	V$LTFM	7	1.34	1.16	5.23	4.46
male	V$ZF04	4	0.88	0.94	4.53	2.79
male	V$INSM	6	1.37	1.17	4.38	3.53
male	V$ZF57	6	1.48	1.22	4.05	3.3
male	V$MOKF	10	3.28	1.81	3.05	3.44
male	V$MITF	7	2.58	1.61	2.71	2.44
male	**V$SF1F**	**5**	**1.95**	**1.4**	**2.57**	**1.83**
male	V$SP1F	13	5.28	2.3	2.46	3.14
male	V$MYOD	20	8.71	2.95	2.3	3.66
male	V$NF1F	8	3.59	1.9	2.23	2.06
male	V$PRDF	17	7.85	2.8	2.16	3.09
male	V$EGRF	11	5.62	2.37	1.96	2.06
male	V$SIXF	7	3.8	1.95	1.84	1.39
male	V$AP1R	33	18.06	4.24	1.83	3.4
male	V$MEF3	4	2.26	1.5	1.77	0.82
male	V$NFAT	20	11.42	3.38	1.75	2.4
male	V$NEUR	11	6.69	2.59	1.64	1.47
male	V$RORA	12	7.38	2.72	1.63	1.52
male	V$CTCF	5	3.13	1.77	1.6	0.78
male	V$CHRF	10	6.36	2.52	1.57	1.25
male	V$IRXF	17	11.09	3.33	1.53	1.63
male	V$TALE	15	9.82	3.13	1.53	1.49

Putative promoter binding sites of three female- (*alv, zp* and *gdf9*) and three male- (*type4 ice, ace, nxph1*) related genes.

Supplementary Table 7. Figure abbreviations

Gene symbol	Gene description	Gene symbol	Gene description
11bhsd	11-beta hydroxysteroid dehydrogenase 1	*lyrm5*	LYR motif-containing protein 5
17bhsd	17-beta hydroxysteroid dehydrogenase 1	*malt1*	Mucosa associated lymphoid tissue lymphoma translocation gene 1
3bhsd	3-beta hydroxysteroid	*mapk14a*	Mitogen-activated protein kinase 14
ace	Angiotensin-converting enzyme	*mapk4*	Mitogen-activated protein kinase kinase 4
acuc	Acetoin utilization protein AcuC	*mat2*	Methionine adenosyltransferase II
adprm	Manganese-dependent ADP-ribose/CDP-alcohol diphosphatase	*maz*	MYC-associated zinc finger protein
adss	Adenylosuccinate synthetase	*mb3374*	Methyltransferase Mb3374
aidb	Acyl-CoA dehydrogenase	*met10*	Methyltransferase 10 domain containing
aldh9a1	Aldehyde dehydrogenase 9 family member A1a	*metl2*	Methyltransferase 2 domain containing
ambp	Alpha-1-microglobulin	*metl6*	Methyltransferase-like protein 6
anks1b	Ankyrin repeat and sterile alpha motif domain-containing protein 1B	*metl9*	Methyltransferase-like protein 9
anm3	Arginine N-methyltransferase 3	*mettl13*	Methyltransferase-like protein 13
apip	APAF1 interacting protein	*mettl16*	Methyltransf_16 domain containing protein
ar1	Androgen receptor beta	*mlst8*	Target of rapamycin complex subunit lst8
ar2	Androgen receptor alpha	*mocs1*	Molybdenum cofactor biosynthesis enzyme 1
arl5	ADP-ribosylation factor 5	*morf4l1*	Mortality factor 4 like 1
armc2	Armadillo repeat containing 2	*mpp1*	Membrane protein, palmitoylated 1, 55kDa
arpp	Cyclic AMP phosphoprotein	*msl1*	Male-specific lethal 1 homolog
as3mt	Arsenite methyltransferase	*mthfd1*	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial precursor
asf1a	Anti-silencing function 1A histone chaperone	*mtu1*	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase 1
ats1	N-acetyltransferase ats1	*mustn1*	Musculoskeletal embryonic nuclear protein 1
B4GALNT3	Beta-1,4-N-acetyl-galactosaminyl transferase 3	*myof*	Myoferlin
birc5a	Baculoviral IAP repeat containing 5	*nap1*	Nucleosome assembly protein 1
blm	Bloom syndrome helicase	*nap1l4a*	Nucleosome assembly protein 1-like 4
C17orf89	Chromosome 17 open reading frame 89	*nasp*	Nuclear autoantigenic sperm protein (histone-binding)
c1qbp	Complement component 1, q subcomponent binding protein	*ndufa4*	NADH dehydrogenase 1 alpha subcomplex subunit 4
caprin1b	Cell cycle associated protein 1	*neil*	Nei endonuclease VIII
carm1	Coactivator-associated arginine methyltransferase 1	*nfrkb*	Nuclear factor related to kappaB binding protein
cby1	Chibby homolog 1	*nme*	Nucleoside diphosphate kinase
CDC23	Cell division cycle 23	*nt5c*	5'-nucleotidase
cenp	Inner centromere protein	*nup85*	Nucleoporin NUP85
cep55l	Centrosomal protein 55kDa	*nxnl2*	Nucleoredoxin-like protein 2
cep72	Centrosomal protein of 72 kDa	*nxph1*	Neurexophilin 1
cnbp	Cellular nucleic acid-binding protein	*patl2*	Protein associated with topoisomerase II

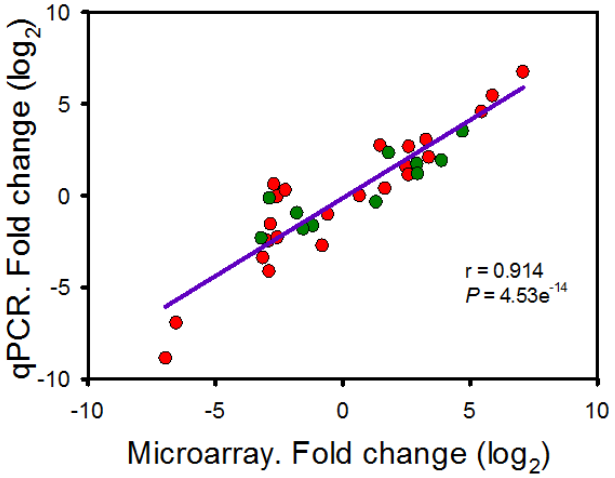
commd5	COMM domain containing 5
cox5a	Cytochrome c oxidase subunit 5A
cox6b1	Cytochrome c oxidase subunit VIb
cox7b	Cytochrome c oxidase subunit VIIb
cpt1a	Carnitine palmitoyltransferase 1A (liver)
creld2	Cysteine-rich with EGT-like domain 2
cry2	Probable DNA photolyase
cse1l	Exportin-2
ctdspl2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like 2
ctnnb1	Catenin (cadherin-associated protein), beta 1
ctsh	Cathepsin H
cyb5	Cytochrome b5
cyp11b	Steroid 11-beta-hydroxylase
cyp19a1a	Gonadal aromatase
cyp27a1	Cytochrome P450 family 27 subfamily A polypeptide 1
dach1	Dachshund homolog 1
dagl	Diacylglycerol lipase
ddb2	DNA damage-binding protein 2
ddr1	Discoidin domain receptor family member 1
dhtkd1	Dehydrogenase E1 and transketolase domain containing 1
dio	Iodothyronine deiodinase
djc18	DnaJ homolog subfamily C member 18
dlgap5	Discs, large (Drosophila) homolog-associated protein 5
dmc1	Dosage suppressor of mck1 homolog
dmrt2	DM-related transcriptional factor Dmrt2
dmrt3	Doublesex and mab-3 related transcription factor 3
dnaja4	DnaJ-like subfamily A member 4
dnajb14	DnaJ (Hsp40) homolog, subfamily B, member 14
dnajc18	DnaJ (Hsp40) homolog, subfamily C, member 18
dnmt1	DNA (cytosine-5-)-methyltransferase 1
dtx1	Deltex1
duf1421	DUF1421 multi-domain protein
dync1i1	Cytoplasmic dynein 1 intermediate chain homolog 2
pdap1	28 kDa heat-and acid-stable phosphoprotein
pdp2	Pyruvate dehyrogenase phosphatase catalytic subunit 2
pgd2	Prostaglandin D2
pgr	Progesterone receptor
phf6	PHD finger protein 6
phldb2	Pleckstrin homology-like domain, family B, member 2
pig	Phosphatidylinositol glycan
pin1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1
piwil2	Piwi-like protein 2
plekhb1	Pleckstrin homology domain containing, family B (evectins) member 1
pls3	Plastin 3 isoform 5
pnpla2	Patatin-like phospholipase domain containing 2
ppef1	Serine/threonine-protein phosphatase
ppme1	Protein phosphatase methylesterase 1
ppp1cb	Serine/threonine-protein phosphatase PP1-beta
prc1	Protein regulator of cytokinesis 1
prkar1a	cAMP-dependent protein kinase type I-alpha regulatory subunit
prmt7	Protein arginine methyltransferase 7
prss8	Prostasin
psmd7	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 7
psmg1	Proteasome (prosome, macropain) assembly chaperone 1
PTB domain	PTB domain containing protein
ptges3	Prostaglandin E synthase 3
pts	6-pyruvoyl tetrahydrobiopterin synthase
pxn1	Jeltraxin
Ras domain	Ras domain containing protein
rassf7	Ras association (RalGDS/AF-6) domain family (N-terminal) member 7
rbm8a	Rna-binding protein 8a
rcn2	Reticulocalbin 2, EF-hand calcium binding domain
rdh3	Retinol dehydrogenase 3
rg9mtd1	Mitochondrial ribonuclease P protein 1 precursor
rhot2	Mitochondrial Rho GTPase 2
rnmtl1a	RNA methyltransferase like 1

	1
dynl	Dynein light chain
ecm1	Extracellular matrix protein 1
ehmt2	Euchromatic histone-lysine N-methyltransferase 2
eif2ak1	Heme-regulated initiation factor 2 alpha kinase
elof1	Translation elongation factor EF-1alpha
eme1	Wu:fc30c07 isoform 2
emx2	Homeobox protein EMX2
eps8l1	EPS8-like protein 1
ergic2	ERGIC and golgi 2
etfb	Electron-transfer-flavoprotein, beta polypeptide
fam117	Family with sequence similarity 117
FAM76B	Family with sequence similarity 76, member B
fancl	Fanconi anemia, complementation group G-like
fbxl	F-box and leucine-rich repeat
fbxl14b	F-box and leucine-rich repeat protein 14
fcr	Fc receptor
fdxr	Ferredoxin reductase
fgf7	Fibroblast growth factor 7
fgl2	Fibrinogen 2
fig7	FIg-Hepta
figa	Factor in the germline alpha
filip1	Filamin A interacting protein 1
flnc	Filamin C
flot2	Flotillin 2
foxl2	Forkhead box protein L2
fshb	Follicle-stimulating hormone beta subunit
fshr	Follicle stimulating hormone receptor
ftsjd2	S-adenosyl-L-methionine-dependent methyltransferase ftsjd2
fxr1	Fragile X mental retardation, autosomal homolog 1
gamt	Guanidinoacetate N-methyltransferase
gdf9	Growth differentiation factor 9
gfm2	Ribosome-releasing factor 2
ggnbp2	Gametogenetin-binding protein 2
glul	Glutamine synthetase
gorasp1	Golgi reassembly stacking protein 1, 65kDa
gpi	Glucose-6-phosphate isomerase
rnmtl2	Ribosomal RNA methyltransferase 2
rps27	Ribosomal protein S27
rrm1	Ribonucleotide reductase M1
rrp9	Ribosomal RNA processing 9, small subunit (SSU) processome component, homolog
ryr1	Ryanodine receptor RyR1
saca4	Sperm acrosome membrane-associated protein 4
scaf1	SR-related CTD-associated factor 1
scaper	S-phase cyclin A-associated protein in the ER
sec23ip	SEC23 interacting protein
sepsecs	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase
serpin	Serpin peptidase inhibitor
sf1	zf-C4 multi-domain protein
sf1	Splicing factor 1
shmt2	Serine hydroxymethyltransferase
sin3	Histone deacetylase complex, SIN3 component
sirt	Sirtuin
skp1	S-phase kinase-associated protein 1
slc20a1	Solute carrier family 20 member 1b
slc25a22	Mitochondrial glutamate carrier 1
slc25a4	ADP-ATP translocase
slc35b1	Solute carrier family 35 member B1
slc5	Solute carrier family 5
slc7a3	Solute carrier family 7
smg8	*SMG8* nonsense mediated mRNA decay factor
sn4tdr	4SNc-Tudor domain protein
snapc1	Small nuclear RNA activating complex, polypeptide 1, 43kDa
sox11	Probable ubiquitin-conjugating enzyme E2 W
sox19	SRY-box containing gene 19
sox6	SRY-box containing gene 6
sox8	SRY-box containing gene 8
sox9	SRY-box containing gene 9
spata1	Spermatogenesis associated 1
spata13	Spermatogenesis associated 13
spata22	Spermatogenesis-associated protein 22
spef1	Sperm flagellar protein 1
spon2	Spondin-2

gsdf	Gonadal soma derived factor	*srd5a3*	Steroid 5 alpha-reductase 3
gsta	Glutathione S-transferase	*srsf1*	Splicing factor. arginine/serine-rich 1
gtsf1	Gametocyte-specific factor 1	*star*	Steroidogenic acute regulatory protein
h1f0	Histone H1.0-like	*star1*	Steroidogenic acute regulatory protein 1
h2afy	H2A histone family, member Y	*stom*	Stomatin
h3.3	Replacement histone H3.3	*suds3*	Sin3 histone deacetylase corepressor complex component SDS3
h3f3a	Replacement histone H3.3	*sugp1*	Splicing factor 4
hbb	Beta-type globin	*suv39h1*	Histone-lysine N-methyltransferase SUV39H1
hdac1	Histone deacetylase 1	*suv39h1*	Suppressor of variegation 3-9 homolog 1
hdac3	Histone deacetylase 3	*taldo1*	Transaldolase
hemk1	HemK methyltransferase family member 1	*tbc1d24*	TBC1 domain family member 24
hh1	Histone H1	*tbl2*	Transducin (Beta)-like 2
hh1b	Histone H1-beta, late embryonic	*tdrd1*	Tudor domain-containing protein 1
hist1h1t	Histone H1t	*tm4sf6*	Transmembrane 4 L6
hla-a	MHC class IA antigen	*tmem136*	Transmembrane protein 136
hoxd9aa	Homeobox protein Hox-D9a	*tmem138*	Transmembrane protein 138
hsd17b7	Hydroxysteroid (17-beta) dehydrogenase 7	*tmem209*	Transmembrane protein 209
hspd1	Heat shock protein 60	*tmem88*	Transmembrane protein 88
igf2	Insulin growth factor 2	*tmlhe*	Trimethyllysine dioxygenase
il17re	Interleukin-17 receptor E	*tob1*	Transducer of ERBB2, 1
il1r	Interleukin 1 receptor	*tomm70a*	Translocase of outer mitochondrial membrane 70 homolog A
irf7	Interferon regulatory factor 7	*trim14*	Tripartite motif-containing 14
islr2	immunoglobulin superfamily containing leucine-rich repeat 2	*tspan18b*	Tetraspanin 18
itfg3	Integrin alpha FG-GAP repeat containing 3	*tspan3*	Tetraspanin-3
itga3b	Integrin, alpha 3b	*ttll12*	Tubulin tyrosine ligase-like family member 12
jade3	Jade family PHD finger 3	*txnrd1*	Thioredoxin reductase TrxR1
kct2	KCT2 protein	*ube2a*	Ubiquitin-conjugating HR6A
kif22	Kinesin family member 22	*usp19*	Ubiquitin specific peptidase 19
kif23	Kinesin family member 23	*vasa*	Vasa rna helicase
kiss1	Vertebrate breast cancer metastasis-suppressor 1	*wdr24*	WD repeat-containing protein 24
klhdc10	Kelch domain containing 10	*wnt1*	Wingless-type MMTV integration site family, member 1
knca	Potassium voltage-gated channel	*wt1*	Wilms tumor 1
lact	Lactamase	*wt1b*	Wilms' tumor suppressor 1b
lcmt1	Leucine carboxyl methyltransferase 1	*zar1*	Zygote arrest protein 1
ldha	L-lactate dehydrogenase A	*zp3*	Zona pellucida sperm-binding protein 3
lhx9	LIM/homeobox protein Lhx9	*zp4*	Zona pellucida sperm-binding protein 4
lmf2a	Lipase maturation factor 2	*zwint*	ZW10 kinetochore associated

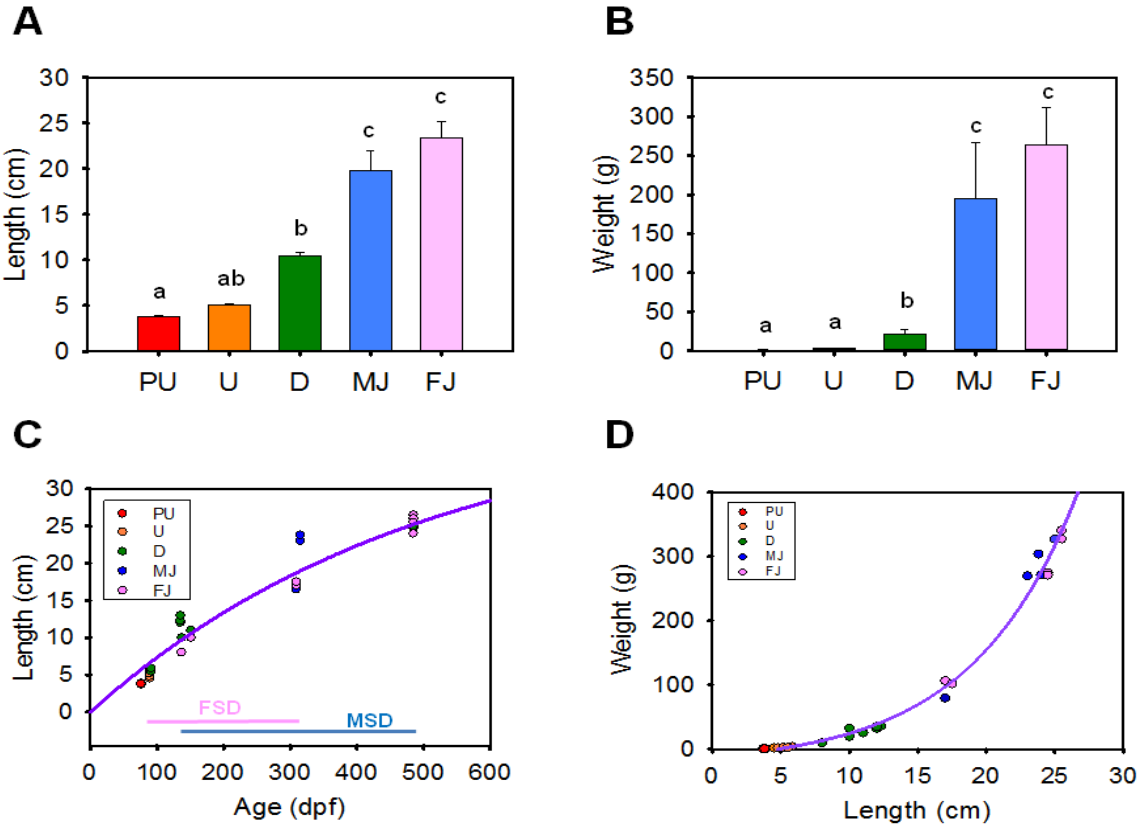
Gene description for each gene abbreviation used in the figures of this chapter.

Supplementary Figure 1. Microarray validation



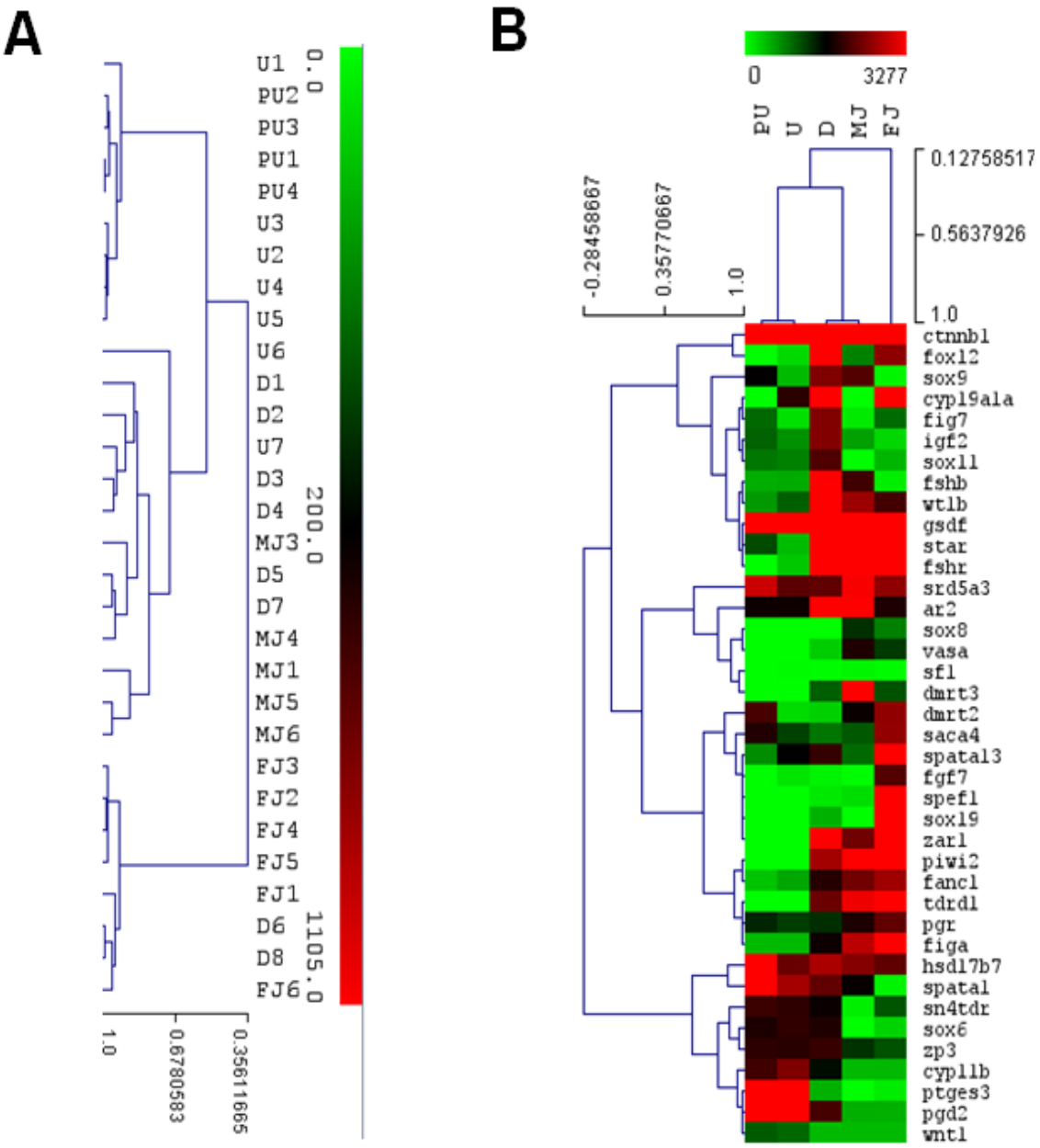
Validation of the microarray results was performed by qPCR in a total of 16 pairs of primers corresponding to 16 different genes and across the five comparisons (PU *vs.* U, D *vs.* U, MJ *vs.* D, FJ *vs.* D). A total of 34 microarray-qPCR pairs of expression values were compared. Data points in red indicate genes whose expression is presented with detail in this study.

Supplementary Figure 2. Turbot growth at different stages of gonadal development



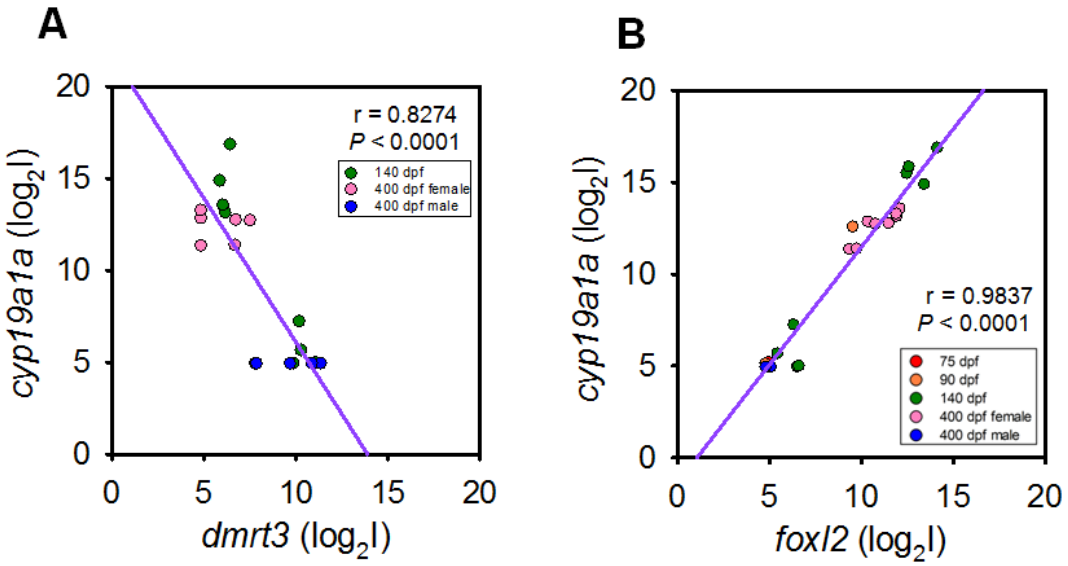
A) Total length (TL). B) Body weight (n = 30 fish; range 4–7 fish/group). Groups with different letters have statistically significant differences ($P < 0.05$). C) Relationship between age (days post fertilization, dpf) and TL. D) Relationship between TL and body. Data point symbols are color-coded according to the transcriptomic group. Abbreviations regarding gonadal development: prior appearing as undifferentiated gonads (PU), undifferentiated gonads (U), differentiating gonads (D), and gonads from female (FJ) and male (MJ) juveniles. The lines below MSD and FSD in C indicate the duration of male and female sex differentiation, respectively.

Supplementary Figure 3. Transcriptomic grouping assesment



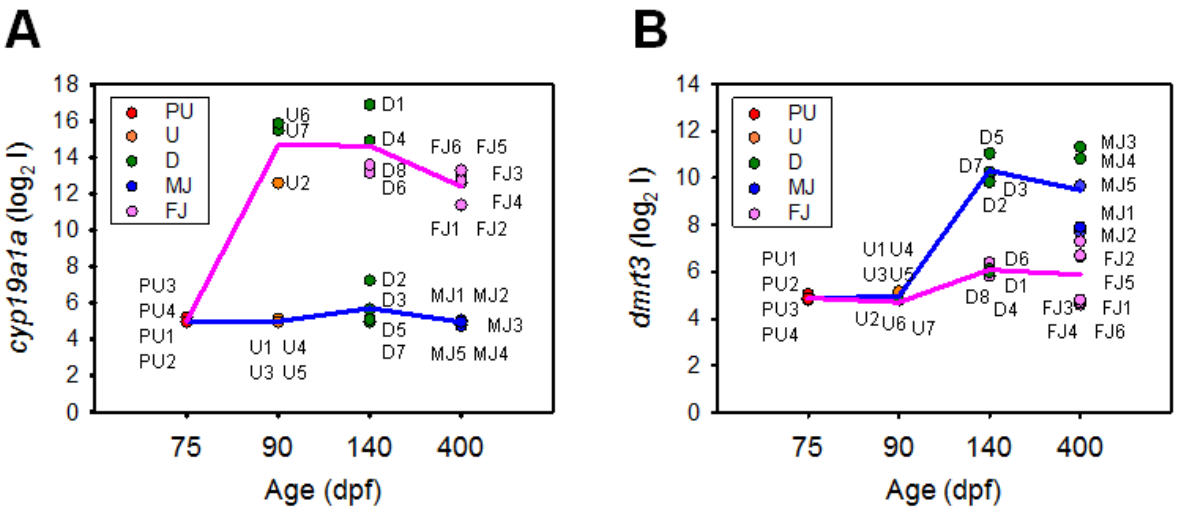
A) Hierarchical clustering analysis of all the samples hybridized in the microarray. B) Heatmap clustering of reproduction-related genes during gonadal development. Clustering was performed using mean gene expression for each group. Full gene names are shown in Supplementary Table 7. Abbreviations as in Supplementary Figure 2.

Supplementary Figure 4. *Cyp19a1a* correlation with *dmrt3* and foxl2



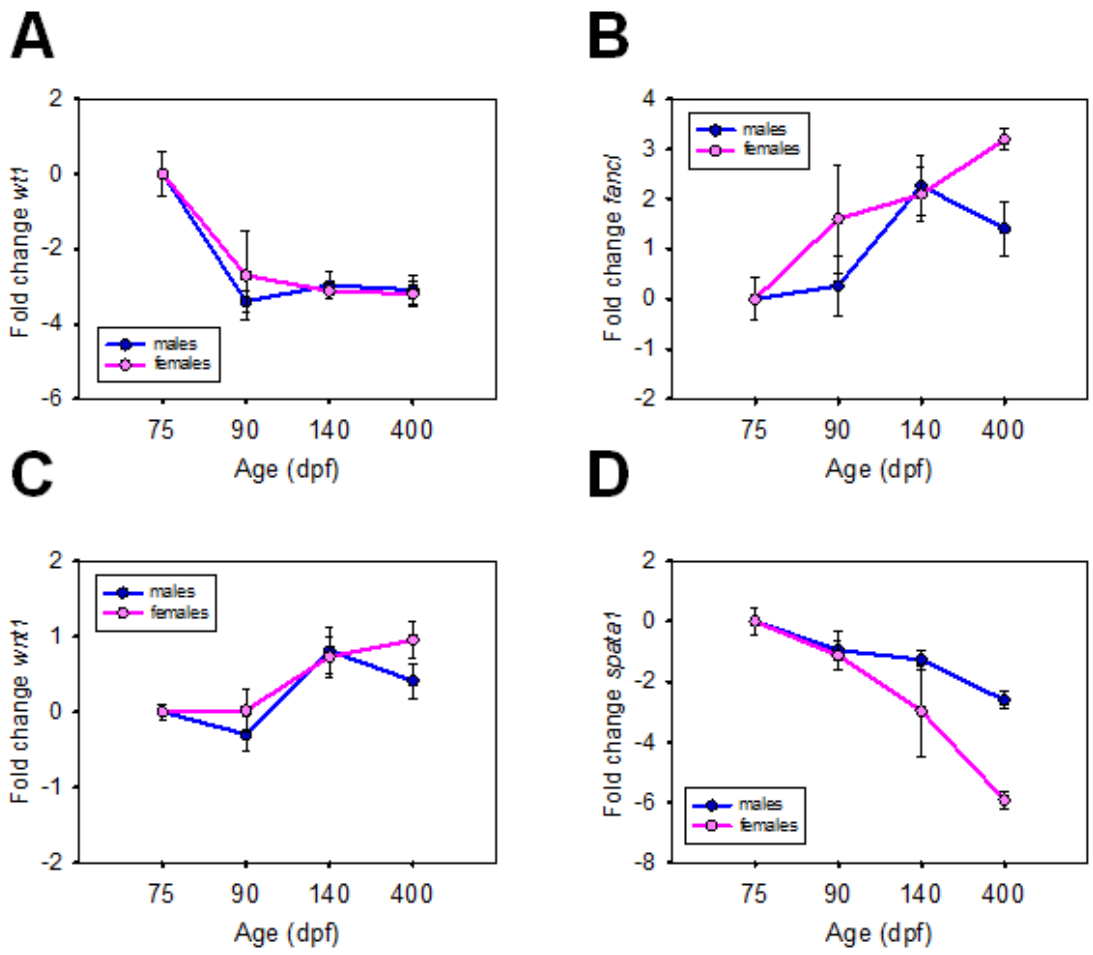
Pearson correlation between two antagonistic, *cyp19a1a-dmrt3* (A), and two agonistic, *cyp19a1a-foxl2* (B), gene pairs. Abbreviations as in Supplementary Figure 2.

Supplementary Figure 5. *Cyp19a1a* and *dmrt3* expression for each sample



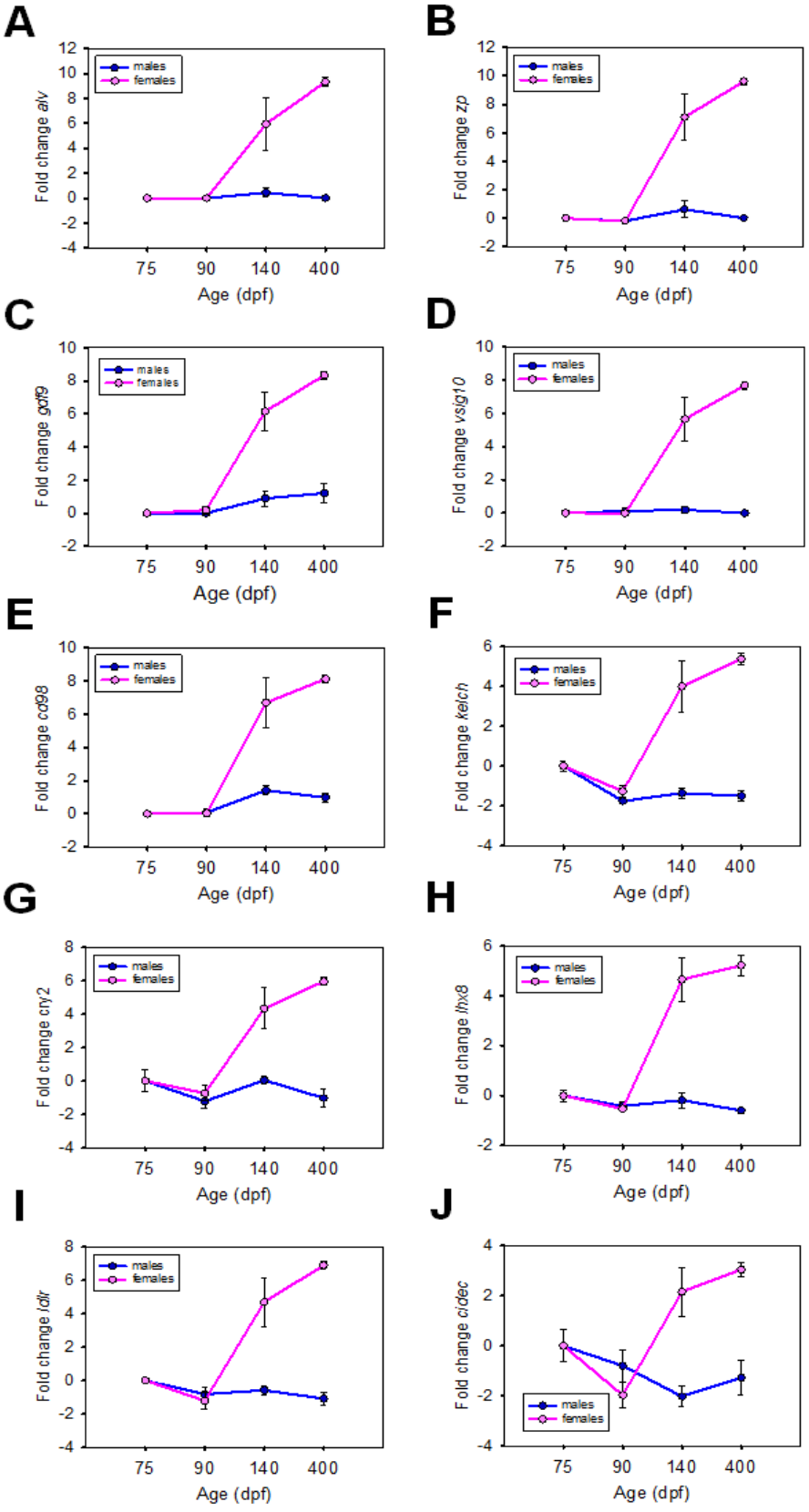
Gene expression patterns of two early markers of phenotypic sex in turbot in developing males (blue line) and females (pink line). A) *Cyp191a1* mRNA expression levels. B) *Dmrt3* mRNA expression levels. Each individual is colored based on their assigned transcriptomic group. Abbreviations as in Supplementary Figure 2.

Supplementary Figure 6. Sex-specific expression patterns of reproduction-related genes during gonadal development in turbot



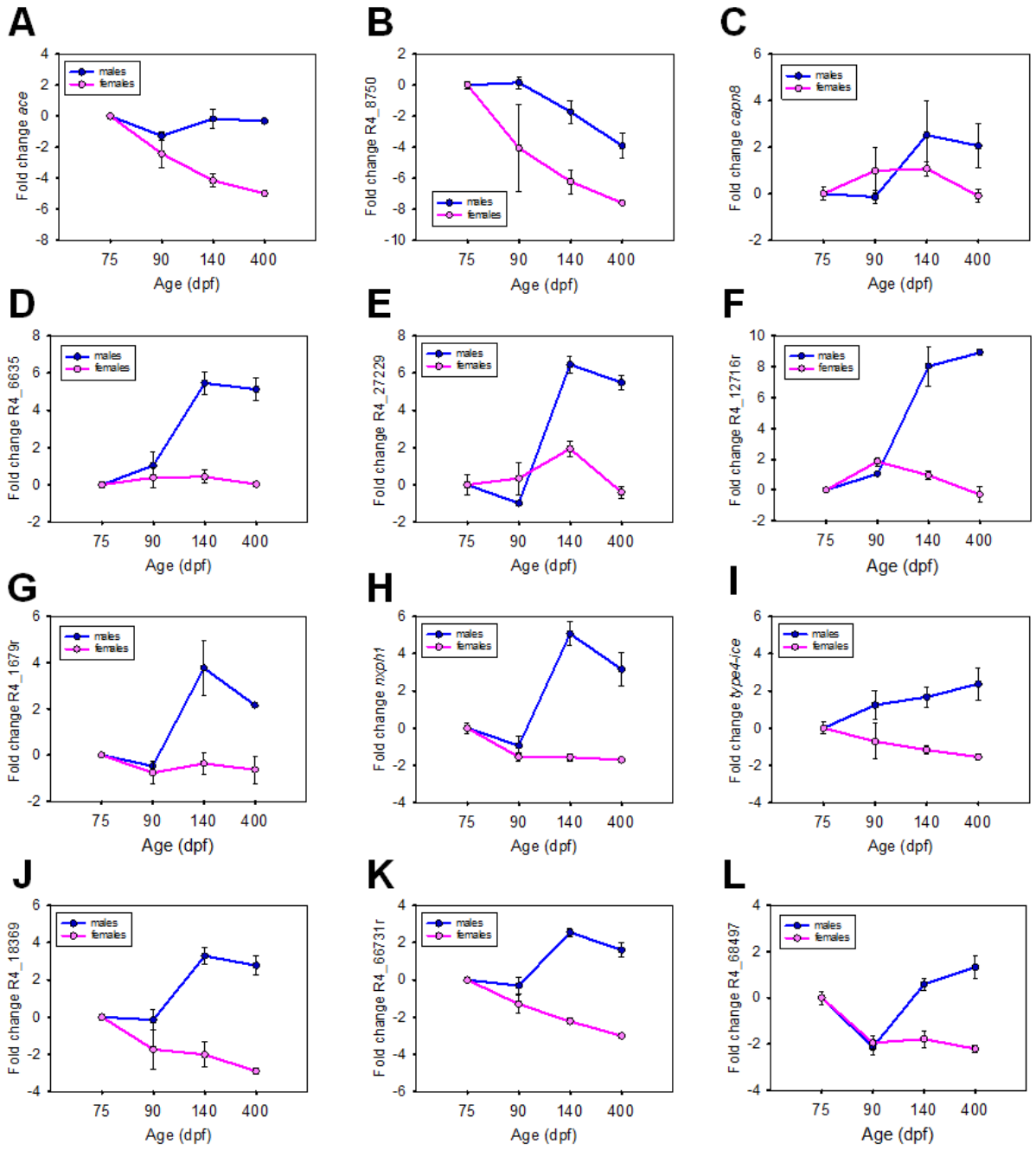
Expression of A) *wt1*, B) *fancl*, D) *wnt1* and D) *spata1*. Age in days post fertilization (dpf) refers to average age values of fish sampled at each group. Full gene names are shown in Supplementary Table 7.

Supplementary Figure 7. Pro-female genes



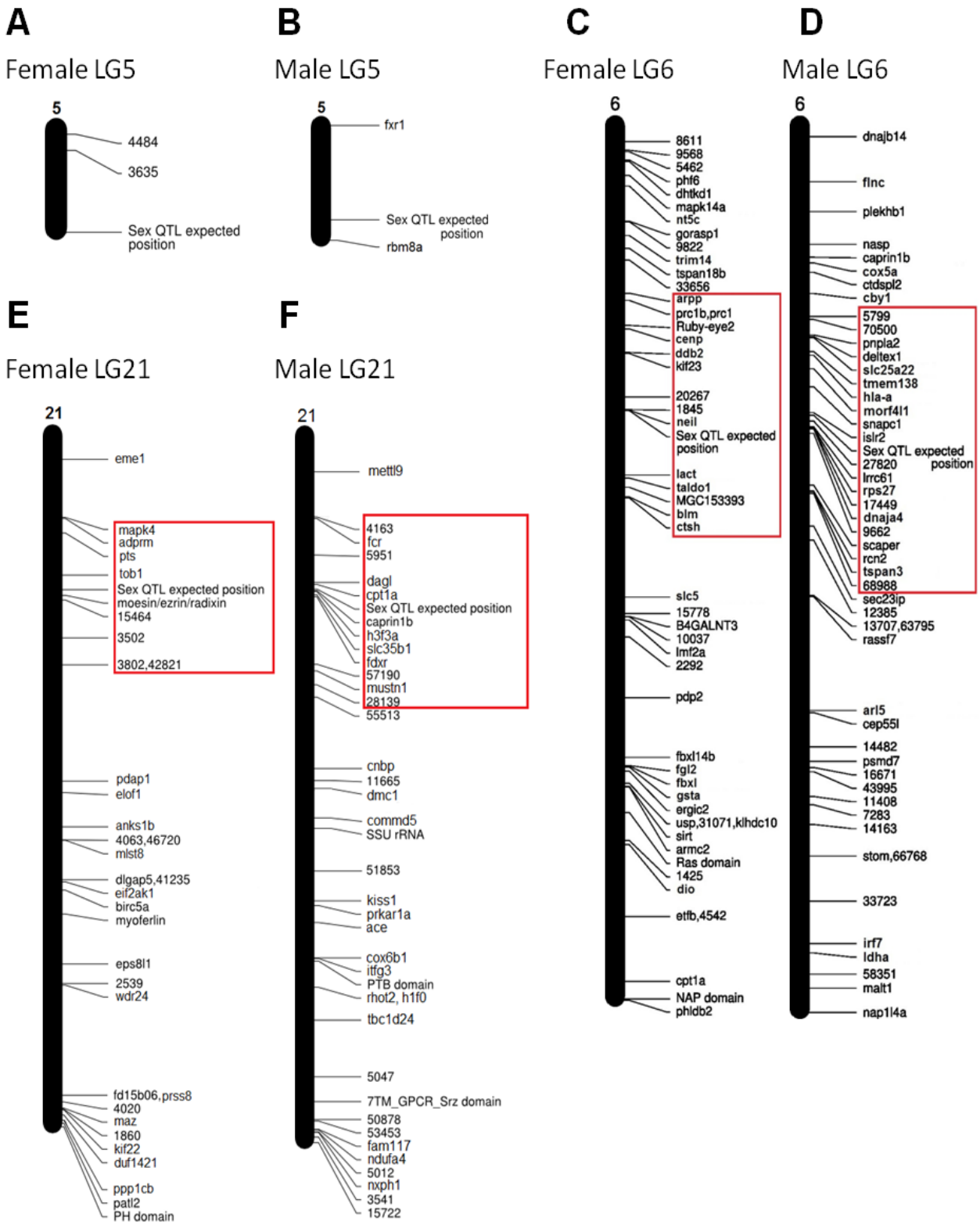
Sex-specific expression patterns of ten out of 45 pro-female genes found during gonadal development in turbot. A) *alv*, B) *zp*, C)*gdf9*, D) *vs.ig10*, E) *cd98*, F) *kelch*, G) *cry2*, H) *lhx8*, I) *ldlr* and J) *cidec*. Age in days post fertilization (dpf) refers to average age values of fish sampled at each group. Full gene names are shown in Supplementary Table 7.

Supplementary Figure 8. Pro-male genes



Sex-specific expression patterns of 12 pro-male genes found during gonadal development in turbot. Three different patterns can be observed. Pattern 1, no changes or downregulated in males, always downregulated in females: A) *ace* and B) R4_8750. Pattern 2, no changes in females, upregulated in males: C) *capn8*, D) R4_6635, E) R4_27229, F) R4_12716r and G) R4_1679r). Pattern 3, downregulated in females, upregulated in males: I) *nxph*, J) *type4-ice*, K) R4_18369, L) R4_66731r and M) R4_68497. Age in days post fertilization (dpf) refers to average age values of fish sampled at each group. Full gene names are shown in Supplementary Table 7.

Supplementary Figure 9. MJ vs FJ DEG mapped to sex determination QTLs



Mapping in different linkage groups of genes upregulated in female juveniles *vs.* male juveniles (A, C, E) or genes upregulated in males juveniles when compared to female juveniles (B, D, F) indicating the position of the turbot sex-associated QTL marker. Genes that map within a region of ±1 Mb around the highest associated genetic marker to the sex–related QTL are shown inside a square.

Supplementary Figure 10. MJ vs FJ DEG mapped to growth QTLs

Mapping in different linkage groups (LG5 and LG16) upregulated genes in female juveniles when compared to male juveniles (A, C) or genes upregulated in male juveniles when compared to female juveniles (B, D) indicating the position of the turbot growth-QTL marker. Genes that map within a region of ±1 Mb around the highest associated genetic marker to the growth–related QTL are shown inside a square.

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Discussion

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One of the objectives of studying sex is to understand the evolution of sex determination and its implications. However, the study of sex in fish is also related to practical issues for those aquaculture species with sex dimorphism. Several flatfish species are harvested worldwide and growth differences between males and females are usual, however, very few studies on sex differentiation have been carried out on this group. Turbot is a relevant flatfish aquaculture species with a notable growth sex dimorphism. The main objective of this thesis was to study gene expression during sex differentiation in turbot to gain knowledge about this process in general and about sex determination in turbot in particular. Each study has been detailed in the previous chapters. However, below all the work is discussed in an integrative way aiming to obtain a deeper understanding of sex differentiation in turbot and fish.

1. qPCR setup in turbot gonads

The real-time PCR (qPCR) is considered a highly sensitive technique to quantify gene expression; however, several parameters deeply condition the quality of the results. One of the aims of this thesis, showed in chapter 1, was to evaluate those parameters for real-time experiments along gonad development in turbot.

1.1. Primer validation

Though not strictly a component of the qPCR setup, we sequenced the qPCR product for every pair of primers used to ensure that they were amplifying the target gene. Researchers usually rely on the presence of a single peak in the melting curve of the qPCR reaction, or, at most, use a gel electrophoresis of the PCR product to confirm the expected size, but these approaches do not fully confirm that the target gene is being amplified. In our study, we found one pair of primers, originally designed for *fxr1*, which presented a single melting curve peak and a single electrophoresis band but it was amplifying a different transcript. This might be especially problematic for species which have suffered recent whole genome duplications, as is the case of teleosts (Glasauer and Neuhauss, 2014).

1.2. Efficiency determination

Efficiency can alter the final fold change (FC), the estimator commonly used to evaluate differential expression regarding controls. Efficientcy may increase or decrease FC depending on the chosen method, and so, over or underestimate differences between groups. The efficiency of the qPCR reaction has been traditionally determined by calibration curves, and efficiencies between 90 and 110% have been considered appropriate. However, it is a non-sense to speak about efficiencies above 100% in a PCR reaction, so we looked for alternatives to the standard curve. We tested several available methods for efficiency determination, finding LinRegPCR (Ruijter et al. 2009) and PCR-Miner (Zhao and Fernald, 2005) to be the most accurate ones. LingRegPCR results underestimate efficiency, while PCR-Miner

overestimates. Finally, we decided to use LinRegPCR efficiency estimations for our qPCR experiments since PCR-Miner would lead to FC overestimation, while LinRegPCR results are more conservative, as we mentioned before. Similar results investigating a larger number of efficiency determination methods were found by Ruijter et al. (2013). These new methods of efficiency determination have not yet been applied into fish expression studies. Among 25 qPCR studies perfomed in fish in 2013 and 2014, efficiency calculation by standard curve was performed in 7 of them (Salmerón et al. 2013; Li et al. 2013; Luo et al. 2013, 2014; Gomes et al. 2014; Tian et al. 2014;; Walock et al. 2014;), while no method for efficiency determination is mentioned in the remaining ones (Jones et al. 2013; Wang TT et al. 2013; Wang Y et al. 2013; Jang et al. 2013; Takeuchi and Okubo, 2013; Chen et al. 2013; Lin et al. 2013; Gao et al. 2013; Mu et al. 2013; Doyle et al. 2013; Liu et al. 2013; Bellaïche et al. 2014; Umasuthan et al. 2014a, 2014b; Tomaszkiewicz et al. 2014; Ravi et al. 2014; Malandrakis et al. 2014). This does not mean that efficiencies were not calculated in these studies, since several of them mention them or use relative quantification methods which require efficiencies. The only noteworthy exception is the work by Sundström et al. (2013) which used LinRegPCR (Ruijter et al. 2009).

1.3. Reference genes

One of the most critical issues of a qPCR is the normalization of the results. Genes of interest quantitation cycle (Cq) values are normalized by reference genes in order to remove technical sample-specific variation. So, it is important to use reference genes that are relatively stable through the experimental conditions tested. We tested six different candidate reference genes in our whole dataset and found ribosomal protein s4 (*rps4*) and ubiquitin (*ubq*) to be the most stable ones. The 60S ribosomal protein L17 (*rpl17*) was found to be the third most stable gene. It is also worth highlighting that β-actin (*actb*) and glyceraldehydes 3-phosphate dehydrogenase (*gapdh)* genes, two of the most commonly used genes for normalization, were found to be unstable, along with beta-2-microglobulin (*b2m*). Appropiate reference genes for gonad development studies were recently studied in another teleost fish, *Monopterus albus* (Hu et al. 2014). Five genes were assayed, among them *gapdh* and *actb*, which were found not stable, but also *rpl17*, which was found to be stable along with *EF1α*. These results are in accordance with our findings in turbot gonad. Gene stability is clearly tissue- and condition-dependent, and *gapdh* and *actb* were found to be the most stable genes in turbot kidney infected with *Edwardsiella tarda* (Dang and Sun, 2011). So, it would probably be more consistent to use reference genes obtained in other species but in the same experimental conditions, than those from other tissues in the same species.

We also tested how the use of "wrong" reference genes would affect our results, and found higher standard deviations. Furthermore, if the reference genes used for normalization are regulated in the tested conditions it would lead to misguided results due to changes in gene expression estimates. However, even though the great impact of normalization has been posed in the literature for several years, many studies disregarded reference gene validation

and instead used a house-keeping gene, assuming its stability. Over 25 articles published in teleost fish in 2013 and 2014 using real-time PCR, normalization was performed using a single gene with no validation in 14 of them (Tian et al. 2013; Li et al. 2013; Jones et al. 2013; Wang TT et al. 2013; Wang Y et al. 2013; Jang et al. 2013; Takeuchi and Okubo, 2013; Chen et al. 2013; Lin et al. 2013; Gao et al. 2013; Mu et al. 2013; Bellaïchi et al. 2014; Umasuthan et al. 2014a, 2014b), while other two more used a single gene validated in different species and experimental conditions (Luo et al. 2013; Tomaszkiewicz et al. 2014). Only three of the 25 studies used more than one gene for normalization (Doyle et al. 2013; Ravi et al. 2014; Luo et al. 2014).

Finally, we also assessed the current methods available for reference gene stability determination, concluding that NormFinder method is the best one for this task and GeNorm the least suitable one, while Bestkeeper and comparative delta-ct method ranked in-between offering some useful information.

As outlined before, there is a worrying lack of consensus when performing real-time PCR but also a lack of minimum validation in many studies, which can seriously compromise the reliability of expression estimates. This is an important problem in the scientific community which should be addressed as soon as possible, however, it does not have an straightforward solution.

2. Expression studies in turbot gonads

Gene expression along turbot gonad development has been assayed both by real-time PCR and microarrays in this study. In this section we discuss the results of both studies in an integrated manner.

2.1. Searching for sex determination genes

Several genes located at the major sex determining region in the linkage group 5 (LG5) of the turbot genetic map were included in the turbot microarray and their expression evaluated in gonads of both males and females at different developmental stages. However, most of them either did not show significant expression over the background (*atp11b*, *dlg1*, *pik3ca*, *cp*) or did not show dimorphic expression (*dnajc19*, *fam102b*, *ttc14*). *Tmem69* (Transmembrane protein 69) showed a higher expression in female juveniles, but this dimorphic pattern was not observed in differentiating females. Overall, none of the sex determination candidates assayed in the microarray study presented an expression pattern consistent with a sex determining role.

Two of the more suggestive candidates were also assayed by qPCR*: sox2* and *fxr1*. The presence of a gene belonging to the *sox* family in the major sex determining region of turbot was an exciting discovery (Taboada et al. 2014). However, *sox2* expression analysis both by microarray and qPCR did not show any significant difference between sexes along gonad

development. *Sox2* is associated with pluripotency in mammals (Wang et al. 2012) and it is required for germline specification in mouse (Yabuta et al. 2006). However, that is not the case of humans, where *sox2* is not expressed in the gonad (Perrett et al. 2008) and, moreover, its repression is necessary for germline differentiation. Additionally, when overexpressed, it changes the cell fate from the germline to the neural lineage (Lin et al. 2014). *Sox2* has also been detected in chicken primordial germ cells of both sexes (Motono et al. 2008). Gonadal expression of *sox2* has not been studied in other fish, but the results in turbot suggest that *sox2* is not involved in gonad differentiation.

Fxr1, in the qPCR study, was found to be highly expressed in undifferentiated individuals (60-75 days post fertilization (dpf)) irrespective of sex, dropping to low levels at the onset of gonad differentiation (90 dpf) and later showing female-specific expression (105-135 dpf). However, in the microarray *fxr1* was found to be over-expressed in male juvenile gonads (~400 dpf). *Fxr1* has been involved in female gametogenesis in pigs (Yang et al. 2012) and *Xenopus laevis* (Mortensen et al. 2011). In fish, it has only been partially studied in the gonad of adult zebrafish, where it is expressed in immature spermatogenic cells (Engels et al. 2004) in agreement with its pattern in mouse (Huot et al. 2001) and human (Tamanini et al. 1997). *Fxr1* has been poorly studied in gonads and its function is not well understood. Our results point to different functions of this gene depending on the developmental stage and sex of the fish.

According to these results, *fxr1* would be the most promising candidate sex determining gene in turbot. *Fxr1* had also been previously proposed as a possible sex determination gene given its location close to Sma-USC30 (Taboada et al. 2014), the marker with the highest sex association in the main turbot sex determination region (Martínez et al. 2009). None of the other studied genes presented expression patterns which suggested they could be the sex determining gene in turbot.

2.2. The start of sex differentiation: length, age and genetic components

In the qPCR study, turbot gonad differentiation started at 90 dpf and dimorphic sex expression of some genes was found at this stage (~5.5 cm length), however, our microarray results show that for the same age and size there are differences between individuals: some of the 90 dpf and ~5.5 cm samples used in the microarray were undifferentiated while others were differentiating; and the same is true for 135 dpf samples (12.5 cm length) which can be either differentiating fish or female juveniles. These results suggest a genetic component related to sexual differentiation timing and, perhaps, also to sexual maturation, although environmental factors could also be involved. If so, this could have interesting implications for aquaculture industry since sexual maturation delays growth, disperse sizes, and determines higher susceptibility to diseases (Martinez et al. 2014). In Atlantic salmon (*Salmo salar*), sexual maturation variability is enormous, especially in males, which might reach maturity from 1 up to 7 years (Simpson, 1992), with an underlying genetic component associated at least to one QTL (Gutierrez et al. 2014). Sex maturation QTLs have also been described in

rainbow trout (*Oncorhynchus mykiss*; Easton et al. 2011) and Arctic charr (*Salvelinus alpines*; Küttner et al. 2011). Turbot male and female size differences begin at one year age (Imsland et al. 1997), reaching commercial size at ~2 years. Finding a genetic component for sexual maturation which allowed delaying the onset of sex size dimorphism would be really appealing for the aquaculture industry.

2.3 Aromatase

The enzyme cytochrome P450 aromatase, encoded by the gene *cyp19a1,* is responsible for the synthesis of estrogens (Ryan, 1982). In fish, there are two different genes encoding the aromatase: *cyp19a1a*, encoding a gonad aromatase, and *cyp19a1b*, encoding a brain aromatase. Here we have assayed *cyp19a1a*. Aromatase seems to have one of the most conserved roles on gonad differentiation along vertebrate evolution and, consequently, in its sex dimorphic expression pattern, among those genes involved in sex differentiation. Aromatase inhibition has been shown to cause sex reversal in fish (medaka), reptiles (American alligator and red-eared slider turtle), amphibians (*Bufo bufo* and *Xenopus laevis*), birds (chicken) and mammals (mouse) (Ditewig and Yao, 2005). Turbot *cyp19a1a* expression pattern is very similar to that of *cyp19a1/cyp19a1a* in other vertebrates, with a female-specific expression. Specific over-expression of *cyp19a1a* during ovarian differentiation has been observed in many fish species including southern flounder (*Paralichthys olivaceus*; Luckenbach et al. 2005), Atlantic halibut (*Hippoglossus hippoglossus*; van Nes et al. 2005), zebrafish (*Danio rerio*; Sawyer et al. 2006), Nile tilapia (*Oreochromis niloticus*; Esterhuyse et al. 2008), rainbow trout (*Oncorhynchus mykiss;* Vizziano et al. 2007), European seabass (*Dicentrarchus labrax*; Blázquez et al. 2008) and Japanese medaka (*Oryzias latipes*; Patil and Gunasekera, 2008).

However, a transient peak of *cyp19a1a* expression was also observed in males at the start of gonad differentiation in turbot. In zebrafish, gonads develop initially as "juvenile ovaries" and, so, *cyp19a1a* is expressed, but this gene is then down-regulated in animals undergoing testicular differentiation (Wang and Orban, 2007). A peak in *cyp19a1a* levels has also been described in European sea bass (Blázquez et al. 2008), which suggests a role of *cyp19a1a* for the development of the undifferentiated gonad, and perhaps also required for later testis development. In *Rana rugosa cyp19a1* is also expressed in the undifferentiated gonads prior to sex determination (Nakamura, 2013), and in mammals both testicular somatic cells and germ cells are a source of estrogens in immature and mature males (Carreau et al. 2012).

Cyp19a1a gene expression was proposed as an early marker of sex differentiation in the Southern flounder (Luckenbach et al. 2005), Atlantic halibut (Matsuoka et al. 2006) and rainbow trout (Vizziano et al. 2007). That is also the case in turbot, where *cyp19a1a* expression can reliably discriminate females at 105 dpf onwards with an accuracy of the 100%, which allowed us to sex some of the undifferentiated and differentiating individuals for microarray analysis.

2.4. *Cyp19a1a-amh* relationship

Amh is a member of the Transforming Growth Factor β family and its function in mammals, birds, reptiles and amphibians is to repress the Müllerian ducts (Josso et al. 2001). Among fish, only early evolved ray-finned species present Müllerian ducts (Wrobel, 2003), so, *amh* role in sexual differentiation is not well understood in teleosts, though it has been linked with the inhibition of germ cell proliferation in several fish species (Nakamura et al. 2012; Kamiya et al. 2012; Hattori et al. 2012). *Amh* is initially expressed in the undifferentiated gonad of both sexes and then at higher levels in males during gonad differentiation in japanese flounder (Yoshinaga et al. 2004), zebrafih (Rodríguez-Mari et al. 2005, Wang and Orban, 2007), Nile tilapia (Ijiri et al. 2008; Poonlaphdecha et al. 2013) and rainbow trout (Baron et al. 2005). However, in medaka, *amh* does not show dimorphic gene expression and seems to contribute to both male and female gonad development (Klüver et al. 2007). In this species, *dmY*, the sex determination gene, is expressed before gonad differentiation and inhibits the proliferation of germ cells in males. It seems that *dmY* has superseded *amh*'s role in medaka as regulator of germ cell proliferation, and so, *amh* has lost its dimorphic expression in this species. This germ cell inhibition seems to be critical for male development, since not only *dmY* in medaka but also *gsdf* in *Oryzias luzonensis* (Myosho et al. 2012), *amhrII* in *Fugu rubripes* (Kamiya et al. 2012) and *amhY* in the Patagonian pejerrey (*Odontestes hatcheri*; Hattori et al. 2012), four sex determining genes out of the six described in fish, are directly connected with germ cell proliferation control.

In mammals, *amh* has been reported to directly down-regulate the expression of *cyp19a1a* (di Clemente et al. 1992; Josso et al. 1998). However, such direct regulation has not been observed in our study, where *amh* and *cyp19a1a* are co-expressed at the onset of gonad sex differentiation. Instead, *amh* and *cyp19a1a* seem to be regulated by the same mechanism but causing opposite effects according to sex (up-regulation or down-regulation), which leads to the observed opposite pattern of expression of these two genes in later stages in turbot and in other fish species like zebrafish (Rodríguez-Mari et al. 2005; Wang and Orban, 2007), pejerrey (Fernandino et al. 2008) or rainbow trout (Vizziano et al. 2007). The one-way regulation of *cyp19a1a* by *amh* might be a mammal-specific mechanism since that observation has not been reported in birds (Vaillant et al. 2001) and other fish species like zebrafish (Schulz et al. 2007) or pejerrey (Fernandino et al. 2008). In Nile tilapia *cyp19a1a* expression was found to precede that of *amh* by at least four days (Poonlaphdecha et al. 2013). These authors proposed that *amh* would be a target of aromatase/estrogen rather than the opposite.

Amh dimorphic expression is among the first genetic cues related to gonad differentiation in turbot. *Amh* early expression, its relation with *cyp19a1a* and its documented connection with germ cells, along with its direct involvement in sex determination in two fish species, make *amh* a very good candidate to understand the mechanisms of sex determination and the onset of gonad differentiation.

2.5. Genes involved in early sex differentiation

Aromatase expression allowed us to discriminate putative females and males in the microarray for undifferentiated and differentiating individuals, which in turn led to the discovery of sets of genes differentially expressed in the two groups: 15 genes up-regulated in aromatase-expressing fish and 8 in non-aromatase-expressing ones. Among the "early female genes", co-expressed with aromatase, we found *foxl2*, which is expected to act as *cyp19a1a* activator (Pannetier et al. 2006); structural maintenance of chromosomes protein 2 (*scg5*), required for the formation of mitotic-like chromosomes (Mbikay et al. 2001); *Sushi*, nidogen and EGF-like domain-containing protein 1 (*sned1*), which shows an epidermal growth factor domain (Leimeister et al. 2004); sal-like protein 1 (*sall1*), a transcriptional repressor involved in organogenesis of ureteric tissues (Sweetman and Münsterberg, 2006); interferon regulatory factor 5 (*irf5*), transcription factor involved in the response to virus infection (Barnes et al. 2001); and *sox11*, important in the developing nervous system (Haslinger et al. 2009). Among the "early male genes" the most interesting gene found was doublesex- and mab-3-related transcription factor 3 (*dmrt3*), which has been hypothesized to regulate transcription during sexual development in mouse and with higher expression in males (Kim et al. 2003). Furthermore, *dmrt3* was found to be expressed in zebrafish undifferentiated gonad at 17 dpf, and in adults it was expressed in germ cells of both sexes (Li et al. 2008). All these genes are candidates to try to unravel the mysteries behind *cyp19a1a*/*amh* regulation in turbot and perhaps in other fish species, but especially interesting are *sox11* and *dmrt3* since they belong to gene families previously involved in sex determination and gonad differentiation along the whole animal world.

2.6. *Sox* genes

The *sox* family of transcription factors takes its name from the mammalian sex determination gene (Sry-related HMG box) and the presence of both *sry* and *sox9* makes this family an interesting target for sex determination and differentiation studies. Besides *sox2*, whose role has been previously discussed, several other *sox* genes were assayed by qPCR in our study: *sox6*, *sox8*, *sox9a*, *sox17* and *sox19*; and some others using microarrays: *sox3*, *sox9b*, *sox11* and *sox14*.

In turbot *sox9a/sox9b*, *sox11* and *sox19* showed the most interesting results. *Sox9* is expressed directly downstream of *sry* in mammals (Kim et al. 2006) triggering the male development cascade. In our studies, *sox9a* male-biased dimorphic expression was only observed after the first signs of sex differentiation and after the dimorphic expression of *amh*. However, *sox9b*, studied in the microarray, showed a higher expression in females at 90dpf, but later its expression became male specific at 140 and 400 dpf. The differential expression pattern of these paralogs is a classic example of neo-functionalization due to gene duplication: since both genes originally accomplished the same task, one of them evolved independently to gain a new function. Consistent with the mammalian *sox9* expression, *sox9a* showed a male specific pattern in turbot, but also in cod (*Gadus morrhua*; Yokoi et al. 2002) and zebrafish

(*Danio rerio*; Klüver et al. 2005). Neo-functionalization of the *sox9b* paralog was also supported in these species, although *sox9b* expression pattern was different in each case (Klüver et al. 2005; Nakamura et al. 2012). *Sox9* is critical for male sex determination in mammals but, for example in chicken and alligator, *amh* expression precedes that of *sox9* during early sex differentiation (Smith and Sinclair, 2004; Shoemaker and Crews, 2009). Furthermore, in medaka *sox9* is not necessary for male gonad formation (Nakamura et al. 2008, 2012). The importance of *sox9* in male sex differentiation varies from one species to another, but it does not seem to be critical in fish as in mammals or even as important as in birds, where it is expressed in both sexes but higher in males, consistent with a function in testis development (Caetano et al. 2014).

Sox19 is orthologous to the mammalian *sox15* and *Xenopus laevis soxD*, however they show very divergent sequences, even belonging to different *sox* groups (Okuda et al. 2006; Ito, 2010). *Sox19* expression is strongly female biased in turbot. These results are consistent with those in sea bass (*Dicentrarchus labrax*), the only other species where the gonad expression of this gene has been studied to date (Navarro-Martín et al. 2012). *Sox11*, despite only being assayed in the microarray, shows one of the most interesting patterns. As previously mentioned, *sox11* is expressed in aromatase-expressing undifferentiated and differentiating individuals and, unlike *foxl2* and *cyp19a1*, *sox11* is not strongly expressed in any other development stage, even neither in female juveniles. *Sox11* has been proposed to be involved in oogenesis and sex change in the orange-spotted grouper, showing higher expression in the ovary (Zhang et al. 2008). This gene has not been assayed in any other vertebrate species related to gonad differentiation. Both *sox11* and *sox19* are interesting gonad differentiation-female related genes which have not been extensively studied so far and might play important roles in sex differentiation in fish.

Sox6 did not show any dimorphic expression nor by qPCR neither by microarray in turbot, but in the latter, its expression decreases in juvenile fish, which may indicate a role in undifferentiated and differentiating gonads. However, in mouse the pattern is completely different, being expressed in adult testis and also slightly in the ovary (Narahara et al. 2002). *Sox6* has not been studied in the gonad of other species, so it is risky to make assumptions about its function or conservation. *Sox3*, *sox8*, *sox14* and *sox17* hardly showed expression in the microarray in all samples, however *sox8* was found to be over-expressed at 105 dpf onwards in males (FC = ~1) by qPCR and *sox17* expression was sex-independent at the onset of sex differentiation. *Sox8* was also found to be over-expressed in testis in *Epinephelus coioides* (Liu et al. 2012), but it does not present dimorphic expression in chicken (Takada et al. 2005) or turtles (Takada et al. 2004). In mammals *sox8* is thought to reinforce *sox9* action in the gonad differentiation process (Chaboissier et al. 2004), so, if *sox9* is less important in other vertebrates, it makes sense that *sox8* expression is not conserved. *Sox17* was found to be a transcriptional activator in the premeiotic germ cells of mouse during germ cell maturation (Kanai et al. 1996), while in the rice field eel (*Monopterus albus*) it is expressed in both ovary and testis during sex reversal (Wang et al. 2003), and in sea bass its expression pattern during spermatogenesis was consistent with a role in the proliferative events of premeiotic germ cells

(Viñas and Piferrer, 2008). *Sox17* expression in turbot turned up at the onset of sex differentiation in both sexes, coincident with a raise in the expression of germ cell markers like *vasa* and *tdrd1*, so a role for *sox17* in germ cell proliferation would be consistent with the results of our study.

2.7. Wnt/β-catenin pathway

The *wnt*/β-catenin signaling pathway controls various steps in mammalian organogenesis (Niehrs, 2012; de Lau et al. 2014) and is key for female differentiation. The final effector protein of this pathway is the β-catenin (encoded by the *ctnnb1* gene), which activates target gene expression in the nucleus and promotes female determination (Maatouk et al. 2008). β-catenin is synthesized constitutively in the cytoplasm, however, in the absence of activators of the *wnt*/β-catenin pathway it is degraded before reaching the nucleus (MacDonald et al. 2009). In XX mammals, *wnt4* and *rspo1* are the genes responsible for activating the *wnt*/β-catenin pathway, although there could be other positive regulators (Chassot et al. 2012).

Of these three genes (*ctnnb1*, *wnt4*, *rspo1*), we assayed *ctnnb1* both by qPCR and microarray and *wnt4* by qPCR. *Ctnnb1* expression was higher in females with both methods, consistent with its role in female development which seems to be conserved over the whole vertebrate taxa. However, we found that *wnt4* expression was higher at undifferentiated stages but rapidly decreased at the onset of sex differentiation, showing after 105 dpf a small overexpression in females. *Wnt4* is predominantly expressed in the ovary in mammals (Vainio et al. 1999) and the same expression pattern has been described in chicken (Smith et al. 2008). In the red-eared slider turtle (*Trachemys scripta elegans*), *wnt4* expression only becomes dimorphic at late ovarian differentiation (Shoemaker et al. 2007), while in *Rana rugosa wnt4* is not expressed in a sexually dimorphic fashion; instead it is transcribed in the embryos at the late gastrula stage and its expression maintained until the undifferentiated gonad develops into a testis or an ovary (Oshima et al. 2005). In the teleost fish black porgy (*Acanthopagrus schlegelii*), *wnt4* expression remains unchanged during ovarian differentiation (Wu and Chang, 2009) and in rainbow trout it is not expressed in the ovary during early gonadal differentiation, but other *wnt* genes have shown sex-specific expression (Nicol and Guiguen, 2011). Besides *wnt4*, other *wnt* genes are expressed in a sex-specific manner in mice: *wnt5a*, *wnt6* and *wnt9a* in the ovary (Bouma et al. 2004; Cederroth et al. 2007) and *wnt1*, *wnt3* and *wnt7a* in the testis (Bouma et al. 2004, Visel et al. 2004). *Wnt5a*, *wnt7b* and *wnt10a* were assayed in our microarray. *Wnt5a* showed very low expression in differentiated fish of both sexes; *wnt7b* also showed low expression but in undifferentiated individuals; and *wnt10a* was not expressed at all. Turbot *wnt4* pattern is similar to that found in *Rana rugosa*, which suggests *wnt4* is not important for sex differentiation in fish and amphibians but plays a role in undifferentiated gonad development. *Wnt4* seems to have gained importance in sex differentiation along evolution as suggested by these results and those in reptiles, birds and mammals. It will be very interesting to test *rspo1* expression and other possible inductors of

the *wnt* / β-catenin pathway along gonad development. *Ctnnb1* turbot expression is in agreement with a conserved role in female gonad differentiation along vertebrate evolution.

2.8. Germ line

We assayed the expression of *vasa*, *tdrd1*, *gsdf*, *dmrt2* and *piwil2* by qPCR, a set of genes connected in one way or another to germ cells. All of them, except *gsdf*, presented dimorphic female biased expression. *Vasa*, *tdrd1* and *gsdf* levels increased at the onset of gonad differentiation; however *dmrt2* and *piwil2* expression rose later in gonad development. *Vasa* sex dimorphic expression was one of the first detected, along with *cyp19a1a* and *amh*. This suggests that germ cell number may be directly involved in sex determination in turbot, as is the case for example of medaka, where the absence of germ cells determines the formation of a male gonad, and overproliferation of germ cells leads to female development (Nakamura et al. 2012). Similarly, in zebrafish the germ line is essential for ovarian differentiation (Siegfried and Nüsslein-Volhard, 2008). However, in mammals, germ cells are not essential for testicular differentiation although they participate in several aspects of ovarian differentiation (Choi and Rajkovic, 2006). *Vasa* was the first molecular marker discovered for germ cells in teleost, and in the European sea bass *vasa* levels increased between 45 and 72 dpf, concomitant with the start of PGC divisions and proliferation (Blázquez et al. 2011). Dimorphic expression pattern of this gene has been found in tilapia (Kobayashi et al. 2000), gibel carp (Xu et al. 2005) and catfish (Raghuveer and Senthilkumaran, 2010). *Tdrd1* expression pattern is very similar to that of *vasa*, and so it seems a promising marker for germ cells as well. The expression of these two germ cell markers becomes higher in females at 90 to 105 dpf and onwards, meaning that probably there is a higher number of germ cells than in males. Interestingly, *gsdf*, which regulates the proliferation of the primordial germ cells (Gautier et al. 2011), does not show any dimorphic pattern, suggesting that its effects are different depending on sex or that there are other factors in play promoting germ cell proliferation.

Dmrt2 and *piwil2* expression is different to those of *vasa*, *tdrd1* and *gsdf*. The expression pattern of another gene, retinol dehydrogenase 3 (*rdh3*), is also similar to that of *dmrt2* and *piwil2*. One of the first recognizable differences between male and female gonad development is the onset of meiosis. In mice, retinol acid promotes germ cells in the ovary to enter meiosis, while retinol acid is degradated in male gonads by the action of *cyp26* (Bowles et al. 2006; Koubova et al. 2006). Perhaps these three genes are indicating the entry in meiosis of germ cells, which would explain the that they present a different pattern than *vasa* and *tdrd1*, markers of germ cell number, or even than *gsdf*, a germ cell proliferation factor, which seems to be somehow ineffective in turbot males at the onset of sex differentiation.

The onset of gonad differentiation seems to coincide with germ cell proliferation in turbot and the involvement of these cells in sex determination has been reported in other fish species (Herpin et al. 2007; Siegfried and Nüsslein-Volhard, 2008; Kamiya et al. 2012). The study of these cells during early sex differentiation might help understanding the sex determination

mechanism and is indeed a very interesting topic of research, in connection with *cyp19a1a* and *amh* gene expression.

2.9. Epigenetic mechanisms

Epigenetic mechanisms are involved in gene expression and they are fundamental for the execution of developmental transcriptional programs leading to the different cells, tissues, organs (Morgan et al. 2005). Undifferentiated gonad can develop as an ovary or testis and differential epigenetic regulation is expected to be critical in this process.

We found a gene involved in methylation, DNA (cytosine-5)-methyltransferase 1 (*dnmt1*), to be up-regulated in females in our qPCR study, but also several other genes involved with epigenetic mechanisms connected with female or male differentiation in the microarray study; for example methyltransferase-like protein 13 (*mettl13*), histone deacetylase 1 (*hdac1*) or arginine N-methyltransferase 3 (*prmt7*). We identified more epigenetic-related genes involved in female than in male development, approximately in a 2:1 relationship. This suggests that ovary development is subjected to extensive chromatin modifications of the undifferentiated gonad, while male fate may be closer to the default gonad development in turbot. In zebrafish, the knock-down of *dnmt1* caused defects in the differentiation of the intestine, pancreas and retina (Rai et al. 2006), so this gene is indeed involved in organogenesis in fish. Methylation has been linked to sex differentiation in the European sea bass, so fish reared at masculinization temperatures showed hypermethylation of the *cyp19a1a* promoter, determining a higher proportion of males (Navarro-Martín et al. 2011). Haffray et al. (2009) have shown the influence of temperature in turbot sex determination in some families, so these genes involved in epigenetic mechanisms might help to understand the link between temperature and sex in turbot.

2.10. Splicing

Several genes related to alternative splicing which are more expressed in female gonads were identified: splicing factor 1 (*sf1*), serine-arginine-rich splicing factor 1 (*srsf1*), splicing factor proline/glutamine rich (*sfpq*), pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16 (*dhx38*), splicing factor 3a, subunit 3 (*sf3a3*), pre-mRNA-splicing factor 38A (*prpf38a*), splicing factor, arginine/serine-rich 3 (*sfrs3b*), splicing factor, arginine/serine-rich 4 (*sfrs4*) and splicing factor, arginine/serine-rich 1 (*sfas1*). Splicing has been characterized as a key sex differentiation factor in insects like drosophilids (Salz, 2011), lepidopterans (Wang et al. 2014) or silkworms (Suzuki et al. 2014), but there are also studies in vertebrates, for example on *dmrt1* in a crocodile species (Anand et al. 2008) and in chicken (Zhao et al. 2007), or about *sox17* in *Dicentrarchus labrax* (Navarro-Martín et al. 2009) suggesting a similar implication. Splicing is poorly characterized during sex differentiation, but the raise of the new deep sequencing technologies will surely contribute to this matter. Nonetheless, it is interesting that so many splicing related genes are over expressed in turbot female gonads.

2.11. Temperature effects on gene expression

Temperature affects sex ratios in turbot in a family-dependent manner (Haffray et al. 2009). The qPCR study was performed in a single family showing a higher proportion of females at low temperatures. Several genes demonstrated expression profiles related to rearing temperatures, sexes and their interaction.

A decrease of *cyp19a1a* expression at high temperatures has been described in several fish species like zebrafish (Uchida et al. 2004), tilapia (D'Cotta et al. 2001) and Japanese flounder (Kitano et al. 2007), and usually has been associated with higher male proportions at high temperatures. This is similar to what happens in most crocodile species where high temperatures produce male offspring (Western et al. 1999). We did not detect a *cyp19a1a* expression decrease at high temperatures in turbot, however no sex ratio bias was observed at 23ºC, the male:female proportion being close to 1:1. *Foxl2* expression at high temperatures was also found to be suppressed in Japanese flounder (Kitano et al. 2007) but not in turbot where *foxl2* did not show any temperature effects at all. So, we could not find clear temperature effects on the expression of *cyp19a1a* or *foxl2* in turbot.

Still, we did find another female related gene, *ctnnb1*, more expressed at low temperatures in the male gonad of developing turbot, which might help to explain why genetic males develop as phenotypic females. Elevated *ctnnb1* levels have been reported connected to low temperatures in rats (Zhang et al. 2008) and tilapia (Tsai et al. 2007) in other tissues. A recent study in oyster also found a biased sex ratio towards females associated with higher *ctnnb1* expression at lower rearing temperatures (Santerre et al. 2013). *Ctnnb1* and the *wnt*/β-catenin pathway are good candidates to explain temperature effects in those species with sex ratios shifted towards females at cold temperatures. So, the untangling of the gene or genes controlling this signaling pathway in fish gonad appears to be relevant regarding this aim.

Furthermore, *fxr1*, a female biased gene located in the sex determining region of turbot, also presented temperature effects on gene expression. This gene has been outlined before as a possible sex determinant gene in turbot; if that were the case, an over-expression of this gene at cold temperatures, as observed, could directly explain a higher proportion of females. There are no previous studies testing the effect of temperature on *fxr1* expression in other organisms.

Effects on two genes related to germ cells have also been observed; *piwil2* and *dmrt2* show lower expression at high temperatures. This might mean that germ cells are not proliferating, that germ cells are dying, or that the entry in meiosis is being altered. There are only two *dmrt2* studies in fish. In medaka, *dmrt2* expression was not found in the developing gonad but it was expressed on adult testis (Winkler et al. 2004). On the contrary, in the swamp eel (*Monopterus albus*) *dmrt2* expression was found in developing germ cells and suggested its involvement in gonad differentiation (Sheng et al. 2014). *Dmrt2* expression is widespread at gonad development in mammals, being expressed both in ovary and testis, although seemingly higher in males (Bratus and Slota, 2009); however, it has been usually linked more to somite than to sexual development (Seo et al. 2006). In *Rana rugosa*, *dmrt2* was found expressing in the developing gonad during sex determination with no dimorphism,

suggesting it has a general function in gonad differentiation (Matsushita et al. 2007). *Piwil2* role as an important regulator of germ cell division seems to be more clear and conserved, with studies in mouse, platypus and chicken (Lim et al. 2013) or the half-smooth tongue sole (*Cynoglossus semilaevis*; Zhang et al. 2014). The effects of temperature on these genes have not been previously studied in any species. Further clarification of the function of these genes regarding germ cells in turbot and in fish is required before drawing any conclusions.

3. Concluding remarks

In our study, we have deepened on turbot sex differentiation, a complex process with many genes involved. Thousands of genes are implicated in the development of testis or ovaries following a rather hierarchical network, however the sex determination switching gene remains still elusive, although *fxr1* is still an interesting candidate. We have found temperature effects on gene expression, which offer a molecular explanation for the sex ratio shifts observed in some families. The expression profile of some genes, like *ctnnb1*, might help to explain why some families show sex ratio shifts and others do not.

From the data reported here and those in other fish species, we think that sex differentiation is organized following a network pathway. In turbot, we observed dimorphic expression of many genes during early sex differentiation (90-105 dpf) and also the effects of temperature on gene expression, which even led to biased sex ratios. Our results fit to a model where several factors affect the fate of the gonad. In fact, the artificial alteration of important genes, like *cyp19a1a*, is a common way to produce all-female or all-male stocks, proving that changes in gene expression during early sex differentiation can avoid the effect of a possible sex-master gene. The huge variation in sex determination systems among fish can also be more easily explained under this network model. There are several genes increasing or decreasing their expression by mutations that can drive the gonad development towards testis or ovaries, and so there are plenty of options for the arousal of new sex determination genes. In our opinion, this network model does not invalidate the concept of a master sex determination gene, since most fish species still present a major locus responsible for establishing sex in most individuals. However, we think that the definition of sex determination as a process which establishes sex by a single gene can be misleading, since the process controlling gonad fate overlaps with the early gonad differentiation, and so, other genes and environmental factors can influence the expression of key morphogenetic facts approaching sex to a threshold-like complex trait. Phenotypic sex is mainly determined by gonad development and the outcome depends on the variation at several loci in the genome and their interaction with the environment.

Both mammalian and avian sex determination are special cases, where the environment does not vary, not only due to endothermia but also internal fecundation, and so there is no need to adjust sex ratios to environmental variation, which allowed the fixation of strong genetic sex determination systems. Reptiles also present higher conservation of their sex determination systems than amphibians or fish, which might be related to internal fecundation

and/or better environmental regulation. It would be interesting to check if iguanas or snakes live in more stable environments or have better regulatory mechanisms than other reptiles with more variable sex determination systems.

In short, environment appears to be relevant in the evolution of the sex determination systems. The rapid change between different systems is facilitated by the threshold-like architecture of sex determination where the expression of many genes, which depends both on several genes and environmental variables, determines gonad fate.

4. Future prospects

There are new ongoing approaches in order to gain more information about sex determination and differentiation in turbot.

The first experiment consists in a RAD sequencing analysis in a large number of turbots belonging to a high number of families (>50) involving a factorial mating design. Offspring will be sexed and genotyped for a high number of markers (10,000 SNPs) which will allow us to refine the main sex determining region at LG5, narrowing the region to mine for candidate sex determination genes. Furthermore, the factorial mating design and the large number of families will also make possible to obtain information about the three minor sex-related QTLs, their interaction with the main LG5 QTL and how they affect to sex ratios. Finally, the factorial design will also provide information about maternal and paternal effects, and their interaction.

The second experiment consists in sequencing the main sex determinining region at LG5 both in turbot males and females to find sequence differences between sexes. For this experiment, we have available ZZ males and WW superfemales for a more efficient comparison on the differential sex determining region. Two different approaches have been considered. A first option would be to isolate the Z and the W chromosomes and fully sequence them apart from the rest of the genome. However, the isolation of single chromosomes is not an easy task, especially considering the small fish chromosomes and the low morpholical differences existing between chromosomes which make chromosome identification difficult. The other possibility is to sequence the whole genome but enriching the sex determination region in LG5 through specific probes such as in the protocol of Sure-select (Agilent Technologies).

The third and final approach consists in analyzing female and male skin microarrays in order to find genes differentially expressed between sexes. Those genes will be analyzed by real-time PCR in a larger number of individuals and at different development stages. With this strategy we aim to obtain a sex marker for precociously sexing of turbot. Though turbot sexing would be almost definitive if we finally discover the main sex determination gene, a phenotypic skin marker would still be useful for those cases where other genetic or environmental factors are influencing gonad fate.

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Conclusions

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1. There is a worrying lack of consensus on real-time PCR studies in fish. We setup the real-time PCR for expression studies along gonad development in turbot, validating three suitable reference genes and several pairs of primers for genes of interest involved in sex differentiation. We also studied different real-time PCR parameters in the process, recommending LinRegPCR and NormFinder methods for efficiency and stability calculation respectively.

2. We could not identify the sex determination gene of turbot. The expression profiles of the assayed genes located in the main sex determination region did not bring any clue about this issue. With the current information at hand, our best candidate is *fxr1*, but more studies are still need to find that gene.

3. Sex differentiation in turbot is first detected at 90 dpf and a discriminant analysis with three genes related to sex differentiation, *cyp19a1*, *amh* and *vasa*, is capable of correctly assigning sex to each fish.

4. Sex differentiation in turbot is a complex process which involves classical sex differentiation genes, germ cell related signals, new genes previously not related to sex, and epigenetic and splicing mechanisms, among others. We found genes clearly behaving as expected by studies in other organisms, i.e. *cyp19a1a*, *amh* or *sox9*, but also others like the *wnt* genes whose role in sex differentiation does not appear to be conserved. Less studied genes like *sox19*, *sox11*, *dmrt3* or *tdrd1* seem to be relevant for gonad development and further studies in turbot and other species should be addressed. Germ cells seem also to have a very important role in the process of gonad differentiation in turbot and possibly in sex determination.

5. Temperature effects on gene expression during gonad development point toward the wnt/β-catenin pathway, and to some degree also to germ cells, as the mechanisms responsible of a higher proportion of females at cold temperatures, which could be interesting for turbot farms interested in obtaining female-biased sex ratios.

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Anexo: Resumen

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El sexo es un proceso biológico característico de los organismos eucariotas. Este proceso consiste en la fusión de dos células especializadas que combinan su material genético para dar lugar a un único individuo. El sexo habitualmente implica la fusión de gametos haploides de dos individuos con roles reproductivos diferenciados, conocidos como machos y hembras. La característica más importante del sexo es la aparición de nuevas combinaciones génicas, debido tanto al proceso de formación de los gametos mediante meiosis en cada progenitor como a la unión del material genético de dos progenitores. Estas nuevas combinaciones génicas representan el sustrato sobre el que opera la evolución. Sin embargo, otras características del sexo producen efectos, a priori, evolutivamente desfavorables, por lo cual el origen del sexo es un asunto controvertido y es pertinente preguntarse cuál es su origen y por qué se ha mantenido y extendido en los organismos eucariotas.

El origen del sexo tuvo lugar en los primeros estadios de la vida en la Tierra y probablemente apareció por primera vez en el último antecesor común de los eucariotas. Existen distintas hipótesis para explicar el origen del sexo y, dada la dificultad para diseñar experimentos que reproduzcan las condiciones de tiempos evolutivamente tan lejanos, actualmente no hay ninguna que cuente con un mayor número de pruebas a favor. Parece que está mucho más claro el por qué, o más bien en qué circunstancias, ha sido mantenido el sexo a lo largo de la evolución de los eucariotas. Las nuevas combinaciones génicas producidas por la reproducción sexual pueden ser favorables en un ambiente cambiante, en el que los organismos deben adaptarse a condiciones ambientales impredecibles. En estas circunstancias, las ventajas del sexo superan a sus desventajas, seleccionándose y fijándose así la reproducción sexual.

El sexo implica la existencia de machos, habitualmente individuos con gametos pequeños y móviles, y hembras, con gametos más grandes y carentes de movilidad. Dos conceptos importantes en el estudio del sexo son la determinación sexual, que es el proceso que establece el sexo de un individuo, y la diferenciación sexual, que es el proceso por el que se desarrolla el fenotipo masculino o femenino. Tradicionalmente se ha considerado que los mecanismos de determinación sexual varían entre especies y grupos, mientras que la diferenciación sexual estaría mucho más conservada. Sin embargo, esta visión clásica separando ambos procesos está siendo cuestionada y últimamente se ha propuesto que la determinación y la diferenciación constituyen un único proceso en las primeras etapas de la diferenciación gonadal que conforma una red de desarrollo sexual en la que distintos factores interactúan entre si.

El sexo de un individuo puede ser determinado por los genes, el ambiente o una combinación de ambos; en este sentido, normalmente se habla de determinación sexual genética (GSD) o determinación sexual ambiental (ESD) como los extremos de un modelo en el que intervienen ambos factores. Los estudios sobre la determinación sexual se han centrado habitualmente en encontrar el gen que actuaría como interruptor, desencadenando el desarrollo de un macho o una hembra a partir de una gónada inicialmente indiferenciada. Ese sería el gen determinante del sexo, que puede funcionar mediante un mecanismo de presencia / ausencia o mediante un mecanismo de dosis génica. Los cromosomas en los que están

situados los genes determinantes del sexo se conocen como cromosomas sexuales. El par sexual presenta dos cromosomas sexuales distintos, uno contiene el gen determinante del sexo y el otro no, y habitualmente uno de los dos sólo aparece en heterozigosis. Según cuál sea el sexo heterogamético se habla de un sistema XX/XY, en el que el sexo heterogamético es el masculino; o ZZ/ZW, en el que el sexo heterogamético es el femenino. Existen otros tipos de sistemas de cromosomas sexuales, por ejemplo sistemas de cromosomas múltiples con más de dos cromosomas sexuales o sistemas XX/X0 en el que el sexo masculino sólo tiene un cromosoma sexual. Habitualmente el origen del gen determinante del sexo se asocia con alelos que proporcionan una ventaja a uno de los sexos pero son desfavorables para el otro, un fenómeno conocido como antagonismo sexual. En estos casos, la selección favorecerá la presencia de un gen determinante en la proximidad y suprimirá la recombinación provocando que los cromosomas sexuales se diferencien en contenido génico y, eventualmente, morfológicamente. La supresión de la recombinación es un paso crítico para la evolución de los cromosomas sexuales y afecta principalmente al cromosoma específico del sexo heterogamético (Y o W), ya que el otro cromosoma puede mantener la recombinación en el sexo homogamético (XX o ZZ). Sin embargo, el cromosoma heterogamético sufrirá un proceso de degeneración debido a la ausencia de recombinación, acumulándose mutaciones y secuencias repetidas.

Los sistemas de determinación sexual son muy variables en vertebrados. Sin embargo, dos de los grandes grupos, mamíferos y aves, presentan sistemas de determinación muy conservados en la mayoría de las especies analizadas. Los mamíferos, el grupo más estudiado, presentan un sistema de determinación sexual XX/XY en el que el gen determinante del sexo es el denominado sry (sex-determining region Y), localizado en el cromosoma Y, el cual determina el desarrollo de una gónada masculina. Las aves tienen un sistema de cromosomas sexuales ZZ/ZW y en este grupo el gen determinante del sexo es dmrt1 (Doublesex and mab-3 related transcription factor 1) que está presente en el cromosoma Z, determinando el sexo aparentemente por un sistema de dosis génica.

Estos cromosomas tienen un origen diferente a los cromosomas sexuales de los reptiles, que exhiben una gran variedad de sistemas de determinación sexual. Mayoritariamente presentan sistemas de determinación sexual ambiental por temperatura; es el caso de cocodrilos y tortugas. Sin embargo, las serpientes presentan determinación sexual genética con un sistema ZZ/ZW. Los anfibios también tienen diversos sistemas de determinación sexual, aunque generalmente presentan determinación sexual genética.

Los peces teleósteos muestran una gran variedad de mecanismos de determinación sexual y todos los tipos de estrategias reproductivas. Constituyen el grupo más numeroso de vertebrados con 27.000 especies que ocupan prácticamente todos los ambientes acuáticos del planeta. El control de la determinación y diferenciación sexual en este grupo es muy importante para la industria de la acuicultura (hoy en día se cultivan más de 350 especies de peces), ya que algunas especies presentan dimorfismo sexuales que convierten a uno de los sexos, machos o hembras, en más deseables para el cultivo. Por ejemplo, el caso más evidente es que muchas de las especies de acuicultura presentan mayores tasas de crecimiento en uno

de los sexos, pero el sexo también puede estar asociado a características como color o forma, relacionadas con el valor comercial. También existen casos particulares como el del esturión, en el que sólo las hembras producen caviar.

Debido a la importancia del sexo en este grupo, se han realizado diversos estudios destinados a identificar el gen determinante del sexo en varias especies de peces, con éxito en medaka (dmY), Oryzias luzonensis (gsdfY), Oryzias dancena (sox3), Takifugu rubripes (amhr2), trucha arcoíris (sdY) y pejerrey patagónico (amhy), mientras que en otras dos especies existen claros genes candidatos (Gsdf en Anoplopoma fimbria y dmrt1 en Cynoglossus semilaevis). Los peces son un grupo especial que exhibe rápidas transiciones entre diferentes sistemas de determinación sexual, lo cual se refleja en especies muy próximas con sistemas sexuales diferentes. Por ejemplo, en el género Oryzias se han detectado siete pares de cromosomas sexuales distintos en ocho especies en las que se ha encontrado par sexual. También se han detectado efectos ambientales en la determinación sexual. Aunque la determinación sexual ambiental pura es rara, la mayoría de las especies presentan efectos de la temperatura sobre la determinación sexual genética. En general, la determinación y diferenciación sexual en peces son procesos muy plásticos, lo cual puede representar un mecanismo para ajustar las proporciones sexuales a un hábitat particular.

Tradicionalmente, en oposición a lo observado con la determinación sexual, se ha considerado que la diferenciación sexual es un proceso conservado y que los genes que participan en la diferenciación sexual en mamíferos actúan de manera similar en el resto de vertebrados. Sin embargo, en los últimos años se ha visto que no siempre es así. En mamíferos sry, el gen determinante del sexo, está presente sólo en el cromosoma Y funcionando como interruptor. La expresión de sry activa la expresión de sox9, el cual desencadena el desarrollo masculino. Durante la diferenciación sexual y tras la expresión de sox9 se expresan distintos genes importantes para la correcta formación de la gónada masculina, por ejemplo amh, sf1, fgf9, otros genes de la familia sox o genes de la familia dmrt. Por el contrario, en los individuos XX, sry no está presente y por lo tanto sox9 no se expresa, produciéndose el desarrollo de una gónada femenina mediante la expresión de genes característicos como cyp19a, foxl2 o genes de la ruta wnt / β-catenina. La diferenciación sexual en mamíferos es la más estudiada y el modelo que se ha utilizado para estudiar el resto de especies. Aunque los genes implicados en la diferenciación sexual en mamíferos están presentes, por lo general, también en el resto de vertebrados, se han encontrado diferencias importantes lo cual parece indicar que la función de algunos de estos genes no está tan conservada como inicialmente se creía.

El rodaballo (Scophthalmus maximus) es un pez plano de gran importancia para la acuicultura, especialmente en Galicia (con una producción de casi 7000 toneladas en 2013), y un marcado dimorfismo sexual. Las hembras maduran sexualmente más tarde y crecen más rápido que los machos, alcanzan la talla comercial antes y presentan menor susceptibilidad a las enfermedades. Por ello, la industria está interesada en obtener stocks solo hembras, de ahí la importancia del estudio de la determinación y diferenciación sexual en esta especie. En este sentido, se han realizado distintos estudios previos, por ejemplo a nivel citogenético, que han

evidenciado la ausencia de heteromorfismos cromosómicos asociados con el sexo. Estudios sobre las proporciones sexuales en distintas familias de rodaballos llegaron a la conclusión de que debía presentar un sistema de determinación sexual genética ZZ/ZW, aunque con cierto efecto de la temperatura en algunas familias. Este sistema ZZ/ZW fue posteriormente confirmado por el desarrollo de un mapa genético del rodaballo, en el que se localizó la principal región determinante del sexo en el grupo de ligamiento 5 de esta especie y tres QTLs secundarios en otros grupos de ligamiento (LG6, LG8, LG21). Asimismo, se realizaron distintos análisis con marcadores y genes candidatos (implicados en determinación y diferenciación sexual en otras especies o identificados en la región determinante del sexo en el rodaballo) para intentar localizar el gen determinante del sexo, sin éxito hasta ahora. Sin embargo, hasta la fecha no se ha realizado ningún estudio de expresión en relación con la diferenciación sexual.

Los objetivos de esta tesis son cuatro: 1) Establecer y validar los parámetros técnicos para el estudio de la expresión génica en gónada de rodaballo mediante PCR en tiempo real (qPCR); 2) evaluar los niveles de expresión de genes relevantes durante la diferenciación sexual del rodaballo en comparación con otras especies; 3) estudiar el proceso global de diferenciación gonadal en el rodaballo; y 4) investigar el efecto de la temperatura en la diferenciación sexual del rodaballo.

La técnica que hemos empleado para analizar con precisión la expresión de diferentes genes en el desarrollo gonadal del rodaballo es la qPCR. Antes de realizar este análisis es necesario validar la estabilidad de la expresión de los genes de referencia que se van a utilizar para normalizar los resultados de los genes de interés. Nuestro primer trabajo consistió en valorar la estabilidad de seis posibles genes de referencia durante el desarrollo gonadal del rodaballo: rpl17 (Ribosomal Protein L17), b2m (Beta-2-microglobulin), actb (beta-actin), ubq (Ubiquitin), rps4 (Ribosomal Protein S4) y gapdh (glyceraldehyde-3-phosphate dehydrogenase). Revisando la bibliografía detectamos la falta de consenso existente en varios aspectos técnicos relacionados con la qPCR, los cuales afectaban directamente a la determinación de los genes de referencia para nuestro estudio. Es por ello que, previamente y utilizando los datos de nuestros genes de referencia, estudiamos dos de los aspectos en los que mayor discusión ha habido en la literatura sobre qPCR durante los últimos años: 1) los distintos métodos para determinar la estabilidad de los genes de referencia; y 2) los métodos para calcular la eficiencia de amplificación de cada pareja de cebadores.

Se han publicado cuatro métodos distintos para establecer la estabilidad de los genes de referencia: 1) comparative delta-Ct method, 2) BestKeeper, 3) GeNorm y 4) NormFinder. Encontramos que el método NormFinder, que valora la estabilidad mediante las desviaciones inter- e intra-grupales, proporcionó los resultados más fiables. NormFinder mostró los resultados más robustos cuando se redujo el número de muestras utilizadas para determinar la estabilidad de un gen de referencia. Además, comprobamos los efectos producidos por distintas combinaciones de genes de referencia al normalizar seis genes objetivo, encontrando que con la combinación ubq+rsp4, los dos genes más estables según NormFinder, obteníamos los mejores resultados.

Por otro lado, utilizamos también cuatro métodos distintos para calcular la eficiencia de cada par de cebadores: 1) LinRegPCR, 2) LREanalyzer, 3) Dart y 4) PCR-Miner. De los cuatro métodos analizados, encontramos que tanto LinRegPCR como PCR-Miner proporcionan estimas de eficiencia de fiabilidad similar. De hecho, las estimas de eficiencia de ambos métodos están correlacionadas. La principal diferencia radica en que las estimas de eficiencia de LinRegPCR son más bajas que las de PCR-Miner, que en algunos casos superan el 100%. Por ello, decidimos utilizar LinRegPCR para evitar sobreestimar las diferencias de expresión.

Nuestro segundo trabajo consistió en estudiar la expresión de 29 genes relacionados con la diferenciación sexual durante los primeros estadios del desarrollo gonadal a tres temperaturas distintas. Los objetivos de este trabajo eran: 1) intentar identificar el gen y mecanismos implicados en la determinación del sexo en rodaballo; 2) estrechamente relacionado con lo anterior, estudiar la diferenciación gonadal desde el punto de vista de la expresión génica especialmente durante el período crítico de diferenciación gonadal; y 3) valorar los efectos de la temperatura en la expresión génica en relación con las proporciones sexuales como factor ambiental clave en los procesos de determinación sexual. La expresión de los 29 genes fue estudiada en seis estadios distintos (60, 75, 90, 105, 120 y 135 días post fertilización) y a tres temperaturas (15, 18 y 23ºC) con 10 peces por cada estadio y temperatura, para un total de 180 peces. Todos los peces fueron sexados genéticamente (mediante una herramienta desarrollada y patentada por nuestro grupo) y, además, los peces de 105, 120 y 135 días post fertilización fueron también sexados histológicamente.

El análisis conjunto de los datos mediante dendrogramas a partir de las correlaciones, y mediante el análisis de componentes principales mostró una clara separación de las muestras sexadas por histología (105 a 135 días post fertilización) en dos grupos: machos y hembras; grupos en los que se incluyeron algunos individuos de estadios anteriores, básicamente de 90 días. A mayores, subconjuntos de genes también se asociaron según su pefil de expresión a uno de los grupos: individuos indiferenciados, machos o hembras.

El análisis individual de la expresión de cada gen confirmó lo apuntado por los análisis globales. A 90 días post fertilización se detectan las primeras diferencias entre machos y hembras: cyp19a1a y vasa muestran mayor expresión en hembras y amh mayor en machos. La expresión de estos tres genes permitió sexar correctamente casi un 90% de los individuos en este estadio. En estos individuos de 90 días también se observó un incremento en la expresión de genes relacionados con las células germinales con respecto a los individuos de 75 días, lo cual sugiere su proliferación entre 75 y 90 días post fertilización. A partir de 105 días la expresión de la aromatasa discriminó completamente los machos y las hembras, y a partir de este estadio varios genes mostraron sobreexpresión en hembras (dact1, ctnbb1, dnmt1, …) y, en menor número, también en machos (sox9a, sox8).

En relación con la determinación sexual, se estudiaron dos genes mediante qPCR situados en la región determinante del sexo del grupo de ligamiento 5: fxr1 y sox2. El patrón de expresión mostrado por fxr1 podría ser consistente con este rol, ya que presentó alta expresión

en estadios aún indiferenciados, descendiendo su expresión en el momento en el que se detectan los primeros signos de diferenciación a 90 días post fertilización para aumentar su expresión específicamente en hembras en estadios posteriores. Por el contrario, la expresión de sox2 no varió en el periodo estudiado en machos y hembras, lo cual no parece compatible con el gen determinante del sexo en el rodaballo ni que tenga un papel relevante en la diferenciación gonadal.

El estudio de los efectos de la temperatura reveló un aumento de la proporción de hembras con el descenso de la temperatura y también efectos sobre la expresión génica. El resultado más interesante fue la sobreexpresión de la β-catenina en machos a temperaturas frías, coincidente con una mayor proporción de hembras. Esto sugiere que la ruta wnt / β-catenina podría ser responsable o al menos estar relacionada con los efectos de la temperatura sobre las proporciones sexuales en esta especie.

El último estudio consistió en un análisis del transcriptoma de la gónada de rodaballo mediante un oligo-microarray a distintos estadios de desarrollo gonadal entre 75 y 485 días. Según la edad de los rodaballos muestreados, su sexo y su agrupamiento en función de su perfil de expresión mediante análisis de componentes principales se establecieron cinco grupos: preindiferenciados (PU), indiferenciados (U) y diferenciándose (D), en los que a priori se desconocía el sexo, y machos juveniles (MJ) y hembras juveniles (FJ). Análisis globales mediante componentes principales y agrupamiento jerárquico mostraron que las gónadas FJtienen un perfil transcriptómico más diferenciado que MJ respecto a D. Es más, estos dos grupos, D y MJ, así como PU y U, se agruparon prácticamente juntos en ambos análisis. Los distintos análisis de expresión diferencial entre grupos confirmaron la similitud entre estos grupos y las grandes diferencias de FJ con el grupo D y MJ.

Con la intención de estudiar la diferenciación sexual temprana y utilizando los resultados del anterior capítulo, utilizamos el gen cyp19a1a para sexar los individuos del grupo D y separarlo en dos subgrupos: individuos con y sin expresión de aromatasa, supuestas hembras y machos, respectivamente. El análisis de expresión diferencial entre estos dos grupos reveló 16 genes relacionados con estas hembras en desarrollo y 8 relacionados con los machos que, por tanto, podrían estar implicados en la diferenciación sexual temprana. Entre los genes más destacados, además de foxl2, que se expresa simultáneamente con cyp19a1a, encontramos sox11, sobre-expresado en hembras, y dmrt3, sobre-expresado en machos. Al estudiar la expresión de dmrt3 en el resto de muestras encontramos que es un gen específico de machos el cual puede ser utilizado, como cyp19a1a, para distinguir ambos sexos durante este período.

También se comprobó el comportamiento a lo largo del desarrollo de 18 genes considerados canónicos relacionados con la reproducción. Entre ellos, se estudió la expresión de siete genes relacionados con la esteroidogénesis, de los cuales solo cyp19a1a y 17βhsd mostraron diferencias entre sexos. También se estudiaron siete factores de transcripción, de los cuales tres (foxl2, sox19 and figα) mostraron mayor expresión en hembras y otros tres (dmrt3, sox9b, sf1) en machos.

Además, identificamos un total de 45 y 12 genes relacionados con el desarrollo gonadal de hembras y machos, respectivamente, que no estaban relacionados a priori con ninguno de los dos sexos. Entre los genes relacionados con hembras detectamos gdf9 o lhx8 por ejemplo. También encontramos numerosos genes diferencialmente expresados entre machos y hembras juveniles relacionados con la metilación, mayoritariamente sobre-expresados en hembras.

En resumen, en este trabajo se ha puesto a punto la técnica de qPCR para el estudio del desarrollo gonadal en el rodaballo y se ha estudiado la expresión génica mediante esta técnica y mediante microarrays durante la diferenciación sexual. La utilización de ambas aproximaciones ha permitido obtener resultados similares y a la vez complementarios sobre la expresión génica durante el desarrollo gonadal en rodaballo, obteniendo una visión global del proceso y a la vez permitiéndonos conocer en detalle qué ocurre en el inicio de la diferenciación sexual. Uno de los objetivos principales era encontrar el gen determinante del sexo en esta especie, lo cual no se ha conseguido mediante ninguna de las dos aproximaciones. Sin embargo si se ha obtenido mucha información sobre la expresión génica a lo largo del desarrollo, tanto de genes implicados en la diferenciación sexual de los vertebrados como de nuevos genes previamente no relacionados con el sexo que parecen estar implicados en el desarrollo gonadal, por ejemplo genes implicados en mecanismos de metilación y splicing. Toda esta información es importante si consideramos que la determinación y la diferenciación sexual son procesos que siguen un modelo de red en el que varios factores, genéticos y ambientales, interactúan para definir el sexo. En este sentido, hemos podido relacionar efectos de la temperatura con efectos en la expresión de varios genes y, además, hemos encontrado que la ruta wnt / β-catenina podría estar directamente implicada en las desviaciones de las proporciones sexuales causadas por la temperatura.

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