Effects of environmental factors on the gonadal transcriptome of European sea bass (*Dicentrarchus labrax*), juvenile growth and sex ratios

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Ph.D. thesis 2014

Submitted in partial fulfillment of the requirements for the Ph.D. degree from the Universitat Pompeu Fabra (UPF). This work has been carried out at the Group of Biology of Reproduction (GBR), at the Department of Renewable Marine Resources of the Institute of Marine Sciences (ICM-CSIC).

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A mis padres

A Xavi

Acknowledgements

This thesis has been made possible by the support of many people who in one way or another, many times unknowingly, gave me the strength to overcome this "*long and winding road*".

First of all, I would like to thank my supervisor, Dr. Francesc Piferrer, for his patience, guidance and wise advice throughout all this Ph.D. experience. But above all, for the trust he placed on me almost seven years ago when he offered me the opportunity to be part of his team. Thanks also for teaching me how to question always everything, for sharing with me your enthusiasm for science and for giving me the opportunity of learning from you by participating in many projects, collaborations and scientific meetings.

I am also thankful to my colleagues (former and present Group of Biology of Reproduction members) for your support and encouragement throughout this journey. To the "exGBRs", thanks for helping me with my first steps into this world. Working as an undergrad with you Dr. Laia Navarro, Dr. Mercedes Blázquez, Dr. Jordi Viñas, Dr. Alejandro Mechaly and Dr. Natalia Ospina, sowed the spark of this thesis. But I also would like to thank my officemates during these years, Sílvia Joly, David Otero, Laia, Mecha and Dafni Anastasiadi; it has been a pleasure to share with you this journey through its highs and lows. Thanks are also due to Dr. Laia Ribas; I won't forget our long hours dissecting Zebrafish!! and your help on the final sprint of this thesis. Thanks also to Elvira Martínez, Gemma Fuster and Dr. Maxi Delgado for their help with fish maintenance, and to the undergrads I had the chance to teach and interact with: Kostantinos, Sergi, Rosa, David, Toni, Susana, Alejandro, Pablo…I have learned a lot from you.

During my thesis I also had the chance to be part of other research teams during short stages and collaborations. From them I treasure not only the knowledge received but the persons I have met that have broaden my visions of science and the world we are living in. I would like to express my gratitude to Prof. Francisco Pelegrí (University of Wisconsin-Madison) and his team, Dr. Sreelaja Nair and Dr. Robyn Lindeman for all the efforts they put on my project

and for being always willing to listen and advice. Thanks are also due to Prof. Lázló Orbán and Dr. Woei Chang (Temasek Life Sciences Laboratory, Singapur) who during a short but fruitful collaboration taught me a lot and made me feel part of their team.

I further extend my gratitude to the many people I had the chance to meet with at the Department of Renewable Marine Resources at the Institute of Marine Sciences (ICM-CSIC). For our coffee breaks conversations: Isabel, Jacopo, Balbina, Batis, Siscu, Laura, Toni, Amalia. To my Ph.D. colleagues at the ICM, past and present, that I will remember as friends: Vanessa, Cristina, Cinta, Àngel, Andrés, Samuele, Ainhoa, Sam, Giuliano, Valeria, Federico, Fernando, Marta, Anabel, Vanesa, Jose Antonio, Eva, Jordi, Raquel, Daniela, Paula, Giulia, Silvia, Dani, Carol, Marc, Alba, Sonia, and many more. Specially to Gorka, Elvira, David, Ari, Ulla and Dafni, for your unconditional friendship, for keeping my feet on the ground and cheer up my days.

Thanks are also due to my family and friends.

Gracias a mis amigos, los que os habéis mantenido a mi lado durante todos estos años, por entender mis ausencias, mis retrasos… simplemente por estar ahí aun cuando no entendierais muy bien el "per què de tot plegat". A mis "Nenis", Silvia, Lourdes, Laura y Ricard, por la felicidad de crecer juntos. A Marga y Robert por ser como de la familia. A Gema, que tuvo la osadía de seguir sus sueños hasta la Estación Biológica de Doñana y con quien he podido compartir aventuras y conversaciones de cómo salvar la ciencia, desde el ya lejano curso de aves en el que nos conocimos. A Maria y Alfonso por nuestras aventuras badaloninas (y las que nos quedan!), pero en especial a ti Maria, per estimar-me tant com per dir-me "jo! no vull que te'n vagis, però ves-te'n" i pels nostres "això ho podem fer nosaltres".

Pero este viaje empezó mucho antes. Gracias a mi familia por darme la mejor de las infancias, siempre rodeada de campo y playa. A todos ellos, a los que están y a los que nos han dejado, mil gracias, porque me habéis hecho la persona que soy hoy en día. A mis abuelos, por hacerme partícipe de su amor por los animales, la tierra y las viejas costumbres. A mis primas, por darme la mayor de las alegrías con esas criaturitas con las que se me cae la baba: Cesc,

Eduard, Valeria. A mi también familia, Vicente, Isabel y Jorge, por vuestro amor y apoyo. A ti Lorena, esa "roomie" inesperada y con retraso, porque cuando miro atrás siempre has estado ahí, gracias por acompañarme en esta recta final y ser mi "Floreta de Gràcia".

A mis padres, por su amor y apoyo incondicional. Por inculcarme la importancia de la educación, el esfuerzo y la bondad. Por darme lo que no tuvieron, posibilidades.

Y finalmente a ti, Xavi, por ser el mejor de los compañeros de viaje posible. Por estar siempre a mi lado animándome a ir "*Tras la parte más pequeña*".

Barcelona, 19 de mayo de 2014.

Abstract

In many gonochoristic fish, sex is plastic since it can be altered by the influence of environmental factors. In this thesis, using the European sea bass (*Dicentrarchus labrax*) model, a teleost fish with a polygenic sex determining system influenced by the environment, we have studied the effects of different environmental factors — including food supply, elevated temperatures and presence of estrogens— on growth, sex differentiation and gonadal development of juveniles. Global analysis of gene expression was carried out by a custom-made microarray. We found that, like in mammals, sex determines growth and that the first sex-related differences in growth are established before the appearance of the first molecular markers indicative of sex. Further, the juvenile testis transcriptome is influenced by poor growth during sex differentiation, while proper food supply during juvenile development is able to rescue the testis transcriptome of previously poor-growing individuals. We found that the previously observed masculinization as a result of elevated temperatures is caused by long-lasting effects at the transcriptomic level, by favoring the expression of male-related genes and decreasing that of female-related genes. In contrast, exposure to estrogen negatively affects both male- and female-related genes and pathways. Interestingly, the expression patterns of a suite of genes related to epigenetic regulatory mechanisms of gene expression showed different degrees of dependency to genetic background, developmental time and external influences according to their functional category.

Resum

A molts peixos gonocoristes, el sexe és plàstic donat que pot ésser alterat per la influència de factors ambientals. En aquesta tesi, utilitzant el llobarro (*Dicentrarchus labrax*) com a model, un peix teleosti amb un sistema poligènic de determinació del sexe influït per l'ambient, hem estudiat els efectes de diferents factors ambientals —incloent la disponibilitat d'aliment, temperatures elevades i presència d'estròg ens— en el creixement, la diferenciació sexual i el desenvolupament gonadal dels juvenils. L'anàlisi global

de l'expressió gènica s'ha realitzat mitjançant un xip d'ADN fet a mida. Hem trobat que, de la mateixa manera que ocorre en els mamífers, el sexe determina el creixement i que les primeres diferències en el creixement vinculades amb el sexe s'estableixen prèviament a l'aparició dels primers marcadors moleculars indicatius del sexe. A més, el transcriptoma de testicles juvenils està influït per un creixement pobre durant la diferenciació sexual, mentre que un subministrament adequat de menjar durant l'etapa juvenil és capaç de rescatar el transcriptoma testicular d'animals amb un pobre creixement previ. Hem trovat que la masculinització observada anteriorment com a resultat de les temperatures elevades està causada per efectes persistents a nivell transcriptòmic, afavorint l'expressió de gens relacionats amb el desenvolupament masculí i disminuint la dels gens relacionats amb el desenvolupament femení. Per contra, l'exposició a estrògens afecta negativament tant als gens relacionats amb el desenvolupament masculí com el femení. És destacable com els patrons d'expressió d'una sèrie de gens relacionats amb la regulació epigenètica de l'expressió gènica mostren graus diferents de dependència a factors genètics, període del desenvolupament i factors ambientals segons la seva categoria funcional.

Prologue

This thesis has been carried out at the Group of Biology of Reproduction (GBR), lead by Dr. Francesc Piferrer, at the Department of Renewable Marine Resources of the Institute of Marine Sciences (ICM-CSIC), Barcelona, under the International Ph.D. Programme in Biomedicine at the Experimental and Health Sciences Department of the Universitat Pompeu Fabra (UPF), during the period 2007-2014. The aim of this thesis was to study the relationship between growth and sex differentiation in the European sea bass (*Dicentrarchus labrax*) model, as well as to study some environmental effects, such as temperature or hormones, on its sex differentiation at a whole gonadal transcriptomic level.

The thesis is structured in two blocks with two chapters each. Note that each chapter is arranged in a self-repressentative way so repetitions in some of the common methods (i.e., statistical, qRT-PCR and microarray analyses) were inevitable.

Block A: Growth and sex differentiation in European sea bass

Chapter 1. The relationship between growth and sex differentiation in the European sea bass (*Dicentrarchus labrax*).

Chapter 2. Changes in food supply at the time of sex differentiation affect the testicular transcriptome of one-year-old juvenile European sea bass (*Dicentrarchus labrax*).

Block B: Environmental effects on fish phenotypic sex

Chapter 3. Exposure to elevated temperature during early development has lasting effects on the gonadal transcriptome of European sea bass (*Dicentrarchus labrax*) at the time of sex differentiation.

Chapter 4. Patterns of gene expression in European sea bass (*Dicentrarchus labrax*), a species with polygenic sex determination, at the time when gonads were experiencing opposite pathways of differentiation.

Each chapter corresponding to an article either published or in preparation for submission:

> Díaz, N., Ribas, L., Piferrer, F., 2013. The relationship between growth and sex differentiation in the European sea bass (*Dicentrarchus labrax*). Aquaculture, 408-409: 191-202.
>
> Díaz, N., Ribas, L., Piferrer, F. Changes in food supply at the time of sex differentiation affect the testicular transcriptome of one-year-old juvenile European sea bass (*Dicentrarchus labrax*). *In preparation*.
>
> Díaz, N., Piferrer, F. Exposure to elevated temperature during early development has lasting effects on the gonadal transcriptome of European sea bass (*Dicentrarchus labrax*) at the time of sex differentiation. *In preparation*.
>
> Díaz, N., Piferrer, F. Patterns of gene expression in European sea bass (*Dicentrarchus labrax*), a species with polygenic sex determination, at the time when gonads were experiencing opposite pathways of differentiation. *In preparation*.

During the realization of this thesis, I have participated in the following articles:

> Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., di Croce, L., Piferrer, F., 2011. DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PloS Genetics, 7(12): e1002447.
>
> Piferrer, F., Ribas, L., Díaz, N. 2012. Genomic approaches to study genetic and environmental influences on fish sex determination and differentiation. Marine Biotechnology, 14: 591-604.

And in a book chapter:

> Piferrer, F., Martínez, P., Ribas, L., Viñas, A., Díaz, N. 2012. Functional genomic analysis of sex determination and differentiation in teleost fish. In Functional Genomics in Aquaculture (eds. M. Saroglia and Z. Liu), Wiley-Blackwell, Oxford, UK. Pages 169-204.ISBN: 9781118350041.

I have also participated in other articles currently either under correction or in preparation:

> Ribas, L.- Liew, W.C., Díaz, N., Sreenivasan, R., Piferrer, F., Orban, L. Sex in hot water: Transcriptomic analysis of heat-induced masculinization in zebrafish. *Under correction*.
>
> Crespo, B., Ribas, L., Xavier, D., Kuhl, H., Rodríguez, J.M., Díaz, N., MacKenzie, S., Reinhardt, R., Moran, F., Zanuy, S., Gómez, A., Piferrer, F. Determination of the European sea bass (*Dicentrarchus labrax*) gonadal transcriptome during different stages of development by pyrosequencing, and construction of a reproduction-enriched custom oligo-microarray. *In preparation*.
>
> Anastasiadi, D., Díaz, N., Piferrer, F. Effects of temperature on global DNA methylation during early development in the European sea bass (*Dicentrarchus labrax*). *In preparation*.
>
> Ribas, L., Vanezis, K., Imúez, M.A., Sarrà, L., Ruiz, S., Genestar, M., Díaz, N., Anastasiadi, D., Piferrer, F. Development of an in vivo system in zebrafish to investigate the effects of altered DNA methylation during development. *In preparation*.

The results derived from this thesis have been presented at the following scientific meetings: I Simposi d'Aqüicultura de Catalunya. Barcelona (February 2008); Conference on Next Generation Sequencing: Challenges and Opportunities. Barcelona (October 2009); II Simposi d'Aqüicultura de Catalunya. Sant Carles de la Ràpita (October 2009); First Emerging technologies in Zebrafish meeting. Bilbao (November 2009); I Jornada de Cromatina i Epigenètica. Barcelona (March 2010); 9th International

Congress on the Biology of Fish: Sex Dimorphisms: Knowns and Unknowns. Barcelona (July 2010); II Jornada de Cromatina i Epigenètica. Barcelona (March 2011); Epigenetics and Developmental Programming Conference. Newcastle, UK (March 2011); XII Jornada de Biologia de la Reproducció. Barcelona (June 2011); Workshop on microarray analysis: Consolider Aquagenomics Project. Barcelona (June 2011); 9th International Symposium on Reproductive Physiology of Fish. Cochin, India (August 2011); 8º Congreso de la Asociación Ibérica de Endocrinología Comparada. Madrid (September 2011); Genomics in Aquaculture Symposium. Heraklion, Greece (September 2011); XIII Congreso Nacional de Acuicultura. Castelldefels (November 2011); 6th International Symposium on Vertebrate Sex Determination. Kona, USA (April 2012); Global Questions on Advanced Biology. Barcelona (July 2012); First Epiconcept Meeting. Antalya, Turkey (April 2013); XIII Jornada de Biologia de la Reproducció. Barcelona (June 2013); 9º Congreso de la Asociación de Endocrinología Comparada. Barcelona (July 2013); 17th International Congress of Comparative Endocrinology. Barcelona (July 2013); Genomics in Aquaculture. Bodo, Norway (September 2013); Epiconcept Workshop 2013. Sant Feliu de Guíxols (October 2013).

During the course of this thesis, I have done four short stays/collaborations in foreign research centers: 1) One short stay at Université Pierre et Marie Curie (Paris VI) at Villefranche sur Mer, France, under the supervision of Professor Muriel Umbhauer; 2) Two short stays at the University of Wisconsin-Madison, Madison, USA, under the supervison of Professor Francisco Pelegrí; 3) One collaboration at the National University of Singapore, Singapore, under the supervision of Professor László Orbán.

The different studies presented at this thesis were supported by the following research projects awarded to Francesc Piferrer:

SEXGENE (AGL2006-01359): Sex ratio determination in fishes: environmental regulation, molecular mechanisms involved in sexual differentiation and tests for its control in aquaculture (Sep. 2007 – Dec. 2009). From Ministerio de Educación y Ciencia.

AQUAGENOMICS (GSD2007-00002): Aquaculture production improvement through the use of biotechnology tools (Sep. 2007 – Dec. 2013). From Ministerio de Educación y Ciencia.

I-LINK (i-LINK0195): Combining thermal manipulation and transcriptomic analysis to decipher the environmental influences on sex ratio in fish populations (Jan. 2011 – Dec. 2012). From Consejo Superior de Investigaciones Científicas.

EPIGEN-AQUA (AGL2010-15939): External factors effects on the reproduction and fish sex ratio. Study of the epigenetic mechanisms involved in the response to the environment (Jan. 2011 – Sep. 2014). From Ministerio de Ciencia e Innovación.

Noelia Díaz Blanco was supported through this thesis by a scholarship (BES-2007-14273) from the Government of Spain (Sep. 2007 – Sep. 2011), by the INEM (Oct. 2011 - May 2012) and a contract linked to the project Epigen-Aqua (AGL2010-15939) (Jun. 2012 – Sep. 2014).

Table of contents

Introduction

1. The sex ratio

1.1. Definition

Sex ratio is defined as the number of males to females in a population and can be expressed as a percent of one sex versus the whole population or as the frequency of males versus females (males: females) (Hardy and Hardy, 2002). Furthermore, the sex ratio is referred to as primary, secondary, tertiary or quaternary depending upon whether it is calculated at fertilization, birth/hatch, the sexually active period or the post-reproductive period, respectively (Coney and Mackey, 1998).

1.2. Implications for natural populations

Due to the changing environment in which fish live, in some instances the sex ratio of the population can be altered (Baroiller et al., 2009). Thus, environmental changes can switch gonadal development, directing sex differentiation towards the other sex. Some of these sex ratio changes may be explained by the allocation theory of resources to male versus female reproduction in sexual species (Charnov, 1982; West, 2009) and by selection pressures (Fisher, 1930).

Many environmental factors are known to influence the final sex ratio, being temperature the most common environmental cue affecting sex, with 61 documented species as deduced in many instances from laboratory-based experiments (Baroiller et al., 2009) and causing an increase in male proportion with temperature, although the relevance in an actual ecological setting of these temperature influences has been questioned (Ospina-Álvarez and Piferrer, 2008). Temperature exerts its effects early in development, before the onset of histological differentiation of the gonads, not only in species with temperature-dependent sex determination (TSD), the most common form of environmental sex determination (ESD), but also in some species with genotypic sex determination (GSD) if the level of the thermoinsult is strong enough and/or prolonged for enough time (i.e., Strüssmann et al., 2008; Luckenbach et al., 2009; Magerhans et al., 2009; Baroiller et al.,

2009; Navarro-Martín et al., 2009b). Also, growth rate triggered or not by temperature, has been hypothesized to be governing sex differentiation in many fish, since temperature accelerates growth rate and females are found among the bigger fish in many species (Kraak and de Looze, 1993; Koumoundouros et al., 2002; Saillant et al., 2003; Vandeputte et al., 2007; Lawrence et al., 2008; Navarro-Martín et al., 2009b).

Other environmental factors can alter final sex ratios. In most ESD species where pH affects the sex ratio, acidic water during development produces an increase in percent males (Reddon and Hurd, 2013); although there are also species where acidic waters produce balanced populations (Römer and Beisenherz, 1996). Density in which larvae are raised in natural conditions may also be a factor affecting sex ratios. This is the case, for instance, of the sex-changing polygynous reef fish *Parapercis cylindrical*, where one sex is more fecund than the other, and where sex ratio bias toward the less fecund sex increases with density (Walker et al., 2010); or in eels (Anguillidae; Davey and Jellyman, 2005). Other minor factors such as size-specific predation (Britton and Moser, 1982), parental cannibalism of part of the offspring (Travers et al., 2000) or social status in some species (Francis and Barlow, 1993) can also affect the final sex ratio.

The anthropogenic-driven environmental effects on population sex ratio such as the ones caused by fishing pressure or contamination cannot be ignored. Geographic variations on the effects on sex ratios can be due to differential fishing pressures on males and females due to their growth sex dimorphism and can be even higher for sequential hermaphroditic species, being the length at the sex change age critical for the abundance of one of the genders (Adams et al., 2000; Sheperd et al., 2010; Chiba et al., 2013; Kendall and Quinn, 2013). On the other hand, many are the examples of fish species with a female bias due to their exposure to anthropogenic endocrine disrupting compounds (Piferrer, 2001; Devlin and Nagahama, 2002; Gross-Sorokin et al., 2006; Jeffries et al., 2010) as many are the examples of sex-changed fish by hormone administration (Saillant et al., 2001a; Gorshkov et al., 2004; Navarro-Martín et al., 2009a).

Among the factors known to affect sex ratios in natural populations, temperature, density and hypoxia are the most studied ones because these factors are of critical importance in fish culture. In many aquacultured species, temperatures used to raise fish are higher than the ones found in nature, hence masculinizing the batch (i.e., Strüssmann et al., 2008; Luckenbach et al., 2009; Magerhans et al., 2009; Baroiller et al., 2009; Navarro-Martín et al., 2009b).

2. Sex determination and sex differentiation

Sex ratio selection, the selection concerning the successful transmission of genetic factors through sperm or oocytes, shapes the evolution of sex-determining systems (Bull, 1983). This occurs when one genetic element is more transmitted through one reproductive cell than the other, and selection is favoring that particular element that is causing the sex ratio bias (Werren and Beukeboom, 1998). Thus, the evolution of the sex determining systems is influenced not only by the genes acting within an individual but also by the genes within the parents affecting either sex ratio or sex determination (Werren and Beukeboom, 1998). Primary sex ratio depends then on various types of factors, with different targets, modes of action and inheritances. These factors can be produced by the parents or the developing zygote and can also target either the parents or the offspring:

Parental sex ratio genes: those genes that are expressed and act within the parents to influence the offspring sex ratio. This can be achieved either by: a) *a sex-chromosome meiotic drive*, when parents alter the ratio of gametes containing sexual chromosomes but without directly affecting the zygotic sex determining mechanisms (Godfray, 1994), or by b) *a differential allocation of resources*, where parental phenotypes can allocate more resources to offspring of one sex and thus influence selection acting on zygotic sex determiners (Godfray, 1994). In ESD species, this can be achieved by selecting the oviposition sites (Janzen et al., 1997).

Parental-effect sex determiners: genes that are expressed in the parents but act on the zygote. Similar to zygotic sex determiners (see below), since they affect the developing zygote, although they

are submitted to the same selection pressures as sex ratio genes due to their expression in the parents. Their effects can be either a) *maternal effects*, including maternal products such as mRNAs or proteins deposited in the developing egg. These are extremely important in most organisms since zygotic genes are not expressed until the mid-blastula transition (MBT) and may have effects on sex determination (i.e., the *daughterless* gene in *Drosophila melanogaster*; Steinemann-Zwicky et al., 1990). Or b) *paternal effects*, i.e., in wasp a paternal sex ratio chromosome fails to condense and is lost, hence producing haploid males; while fertilized eggs develop as diploid female progeny (*Nasonia vitripennis*; Burton-Chellew et al., 2008).

Zygotic sex determiners: when major sex determiners or nascent sex chromosomes are involved. This is the case of dominant male determiners such as the Sex-determining Region on the Y chromosome (*SrY*) in mice and humans, or *sex-lethal* (*sxl*) in *Drosophila*.

The first two categories have been most studied in plants and in invertebrates, whereas research on vertebrates has concentrated perhaps more on the last type. Accordingly, the following sections will deal with factors (genes and environment) acting on the zygote.

As mentioned above, fish exhibit high phenotypic plasticity in response to environmental changes, hence also affecting the final sex ratio of a population. Parental effects aside, either of one type or the other as explained above, sex ratio in fish populations is then the result of the combination of two processes: sex determination and sex differentiation (Devlin and Nagahama, 2002).

2.1. Sex determination

The sex determination is the genetic or environmental process by which the gender of an individual is established (Devlin and Nagahama, 2002; Penman and Piferrer, 2008). In gonochoristic vertebrates, there are two major types of sex-determining systems: a) *genotypic sex determination* (GSD), where gender genetic differences are present and sex is determined at the moment of fertilization; b) *environmental sex determination* (ESD), where

there are no genetic differences between gender and sex is determined after fertilization (Bull, 1983; Sarre et al., 2004; Valenzuela, 2008; Penman and Piferrer, 2008). The inheritance of sex is then determined by major or minor sex factors, by environmental influences or by the combination of several effects (Bull, 1983; Penman and Piferrer, 2008) (Figure 1).

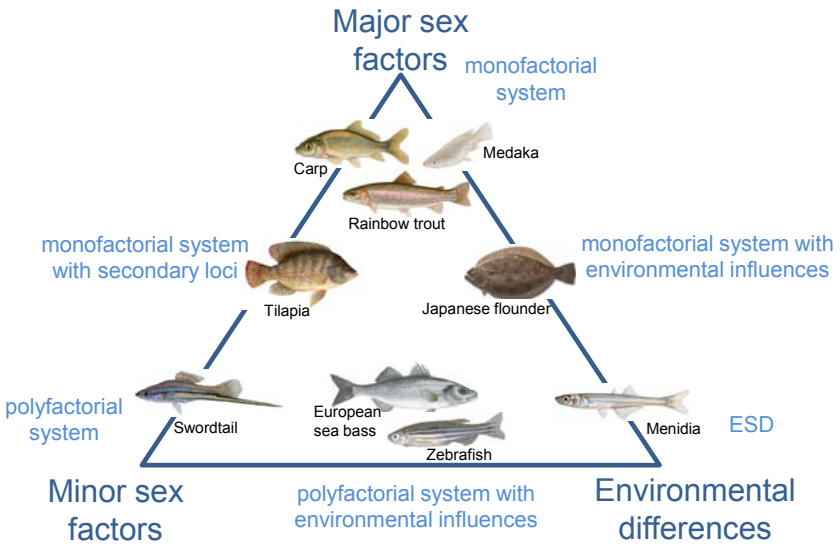


Figure 1. Representation of the three main types of zygotic factors or major types of sex determining mechanisms present in gonochoristic fish governing the final offspring sex ratio: major and minor sex factors and environmental influences. In light blue there are highlighted some sex determining systems with their correspondent species. From Penman and Piferrer, 2008 and Baroiller et al., 2009.

2.1.1. Genotypic sex determination (GSD)

As mentioned above, in GSD species sex is fixed at fertilization and depends essentially on the genetic content inherited from the parents. In mammals (XX/XY) and birds (ZZ/ZW) sex is determined at conception by sex chromosomes. Nevertheless, in fish the inheritance of sex can be based on:

Major sex factors or chromosomal sex determination: GSD including a monofactorial mechanism with a single or multiple pairs of sex chromosomes per individual or a multifactorial-single pair of

sex chromosomes system. In all of the monofactorial sex-determining species, fish can exhibit either female (ZW) or male (XY) heterogamety. In turn, sex chromosomes can be homomorphic (when no differences in size or shape are found but they can be distinguished using sex-specific or sex-linked traits or markers) or heteromorphic (when differences in size or shape are present under the microscope or if similar in size and shape, when distinguishable only by banding studies). For a more detailed description on this major sex factors classification and fish examples, see Penman and Piferrer (2008, Table 1). In multifactorial systems, there are three or more major sex-determining factors and in this system both sexes can be heterogametic.

Minor sex factors: there are no sex chromosomes and final sex ratio is the fruit of a polyfactorial combination of genetic factors found in autosomes, each with a minor additive effect. Thus, there are sex ratio fluctuations between families and maternal/paternal effects are found. Two examples of a polyfactorial sex determination system in GSD species with temperature influences on final sex ratios are the European sea bass (Vandeputte et al., 2007), and the model organism zebrafish (*Danio rerio*; Liew et al., 2012).

In most therian mammals, sex determination is triggered by just one gene. In humans (*Homo sapiens*), the master sex-deterring *SrY* (Sinclair et al., 1990), is part of the SOX family of genes. This family of genes is characterized by the presence of a HMG box DNA binding domain (Laudet et al., 1993) and known to bind and bend DNA (Pevny and Lovell-Badge, 1997) and also to act as an assembler of nucleoprotein complexes that allow correct gene expression (Grosschedl et al., 1994). Most importantly, *SrY* has been identified as the master inducer of the testis-determining pathway (Schafer, 1995) and to inactivate male development repressors (Jordan et al., 2001; Clarkson and Harley, 2002). Furthermore, it has been proved to be necessary and sufficient for the initiation and development of testis and its associated sexual characteristics in the majority of mammals (Wilhelm et al., 2007). Several studies in non-human mammals have proved the relationship between *Sry* and testis development although the temporal expression may vary. For instance, while in mouse (*Mus musculus*) its expression takes place during the period of sex

determination (Koopman et al., 1990) before the first signs of testis formation and then being related to the initiation of Sertoli precursors (Kim and Capel, 2006), in sheep (*Ovis aries*) and pigs (*Sus domesticus*) its expression persists after testis differentiation, suggesting other roles of *Sry* in these species (Parma et al., 1999).

Nevertheless, *Sry* has only been found in therian mammals (placental mammals and marsupials) (Schartl, 2004a) and it seems to be absent in the majority of non-mammalian vertebrates. Regarding invertebrates, *doublesex* (*dsx*; Erdman and Burtis, 1993) in Drosophila, and *male abnormal 3* (*mab*; Raymond et al., 1998) in *Caenorhabditis elegans* control their sex determination. These two genes encode for genes with a DM domain (zinc finger-like DNA-binding motif). In vertebrates, the DM domain gene *DMRT1* is implicated in sexual development. For instance, in birds, with a chromosomal sex determination system, two dosages of the Z-linked *DMRT1* gene (doublesex and mab-3-related transcription factor 1) are needed for male development (Smith et al., 2009). Also in turtles, *DMRT1* expression is connected to testis differentiation from undifferentiated gonads (Kettlewell et al., 2000). However, in *Xenopus laevis* sex determination is triggered by an ovary-determining gene, *DM*-W, a paralogue of *DMRT1* (Yoshimoto et al., 2008). Moreover, *DM-W* directs female sex as a sex-determining gene by antagonizing *DMRT1*, suggesting that DM-W diverged from *DMRT1* as a dominant-negative type gene (Yoshimoto et al., 2010). In medaka (*Oryzias latipes*), clones of *DMRT1, 2* and *3* genes are present and differentiate among a proto-Y and proto-X chromosomes (Schartl, 2004b). The Y-chromosomal copy *DMRT1bY* (*DMY*), a male master sex determining gene, is expressed before gonad differentiation and continuous from embryonic to adult stage (Kobayashi et al., 2004) and is only present in two of the five described *Oryzias sp.* (Matsuda, 2005).

Contrary to the situation of the *Sry* gene, *Sox3* (from which *Sry* is thought to derive) and *Sox9* genes are well conserved, widely distributed among all vertebrate types and required for testis development (Vidal et al., 2001; Graves, 2002; Clarkson and Harley, 2002; Galay-Burgos et al., 2004). Furthermore, recently some new sex determining gene candidates have been proposed (Kikuchi and Hamaguchi, 2013). Myosho et al., (2012) have proposed gonadal soma derived growth factor (*gsdf*) as the sex

determining (SD) gene in *O. curvinotus,* a closely related species of medaka. Its expression was previously related to primordial germ cell and spermatogonia proliferation in rainbow trout (Sawatari et al., 2007). Further, its spatial and temporal expression coincides with that of the *dmy* in medaka (Shibata et al., 2010). Additionally, its allele on the Y chromosome was sufficient to cause female-to-male sex-reversal in transgenic experiments and its promoter presents evolutionary changes in the upstream sequence of $gsdf^{Y}$, leading to male-specific higher expression of the Y allele associated to later testicular development (Myosho et al., 2012). Two candidate SD genes related to the anti-Müllerian hormone gene (*amh*) have been described in Patagonian pejerrey (*Odontesthes hatcheri*) and in the tiger pufferfish (*Takifugu rubripes*). The first, named *amhy*, is expressed in a sex-specific manner and detected before the first signs of morphological differentiation of ovaries and testes in genotypic male fish (Hattori et al., 2012). The latter is the anti-Müllerian hormone receptor type II (*amhr2*), where a single-nucleotide polymorphism (SNP) in its kinase domain is associated with the phenotypic sex of the fish (Kamiya et al., 2012). Interestingly, these three genes belong to the TGF-β superfamily (Imbeaud et al., 1995; Sawatari et al., 2007; Fan et al., 2011). Finally, *sdy* is the last SD proposed gene. Is a male-specific gene expressed in the somatic cells surrounding germ cells and shows a sequence homology with the interferon regulatory factor 9 (*irf9*) (Yano et al., 2012). Thus, so far a total of five different sex determining genes have been identified in fish: *dmy, gsdf, amhy, amhr2* and *sdy* (Table 1).

Table 1. Sex determining genes identified in fish.

Gene symbol	Gene description	Species	Common name	LG	Where	When	Mechanism	References
dmy/ dmrt1by	DM-domain gene on the Y chromosome	Expressed only in two of the five described *Oryzias sp. (*O. latipes *and* O. curvinotus*)*	Medaka and Malabar rice fish, respectively	LG1 (for both sp.)	B SD: at somatic cells surrounding germ cells; A SD: testis	From embryonic to adult stage	Segmental duplication of a small autosomal region containing precursor dmrt1 followed by an insertion of the duplicated region on the proto-Y chromosome. First: involved in germ cell proliferation. Second: induce development of pre-Sertoli cells into Sertoli cells	Matsuda et al., 2002; Nanda et al., 2002; Kondo et al., 2003; Kobayashi et al., 2004; Matsuda et al., 2005; Shibata et al., 2010
gsdf	gonadal soma derived growth factor	*Oryzias curvinotus*	Hynann ricefish	LG1	Somatic cells surrounding germ cells. Also in adult gonads at Sertoli and granulosa cells	Male-specific high expression during sex differentiation	Downstream of *Dmy* in the sex-determining cascade	Kobayashi et al., 2004; Shibata et al., 2010; Myosho et al., 2012

gsdf^{Y}	gonadal soma derived growth factor, chromosome Y	*Oryzias luzonensis*	Luzon rice fish	LG12	Somatic cells surrounding germ cells. Also in adult gonads at Sertoli and granulosa cells	Male-specific high expression during sex differentiation	Emergence of the *Dmy*-independent *Gsdf* allele	Kobayashi et al., 2004; Shibata et al., 2010; Myosho et al., 2012
amhy	Y chromosome-specific amh	*Odontesthes hatcheri*	Patagonian pejerrey	Y chromosome	Presumptive Sertoli cells of XY males	During the onset of and during gonadal differentiation	*amhy* has been inserted upstream of *amha* in the molecular cascade of sexual development. Leading to a subfunctionalization of amhs: *amhy* may have been encharged of sex determination and *amha* of testicular maturation and/or spermatogenesis	Hattori et al., 2012
amhr2	anti-Müllerian hormone receptor type II	*Takifugu rubripes*	Tiger pufferfish	Chromosome 19	Somatic cells surrounding germ cells.	Expressed in both sexes before the morphological differentiation of gonads	Single nucleotide polymorphism (SNP) in the kinase domain of *amhr2*. Sex is determined by a combination of the two alleles of amhr2 (heterozygous males and homozygous females)	Kamiya et al., 2012

sdy	sexually dimorphic on the Y-chromosome	*Oncorhynchus mykiss*	Rainbow trout	LG1 (RT01)	Domatic epithelial cells at the dorsal part of the gonad surrounding the somatic germ cell of differentiating testis	Predominant expression during testicular differentiation	Linked to the SEX locus on the Y chromosome, and it is both necessary and sufficient to induce testicular differentiation. Has evolved through neofunctionalization (from *irf9*) by losing its role in IFN signaling pathway	Yano et al., 2012
sdy	sexually dimorphic on the Y-chromosome	*Oncorhynchus mykiss*	Rainbow trout	LG1 (RT01)				
		Oncorhynchus masou	Masu salmon					
		Salmo salar	Atlantic salmon					
		Salmo trutta	Brown trout	LG18 (BT18)			*sdy* was strictly conserved as a male-specific	Yano et al., 2013
		Hucho hucho	Danube salmon					
		Parahucho perryi	Japanese huchen					
		Salvelinus alpinus	Artic charr					
		Salvelinus fontinalis	Brook trout					
		Salvelinus malma malma	Dolly varden					

Thymallus thymallus	Grayling	
Stenodus leucichthys	Sheefish	
Oncorhynchus tshawytscha	Chinook salmon	*sdy*-positive female
Salvelinus namaycush	Lake trout	*sdy*-negative male
Coregonus lavaretus	Lavaret	all males and all females were sdY positive
Coregonus clupeaformis	Lake whitefish	

Abbreviations: B SD, before sex differentiation; A SD, after sex differentiation.

2.1.2. Environmental sex determination (ESD)

In ESD species, phenotypic sex is determined by external factors and no consistent genetic differences are found between genders. The most common form of ESD is temperature-dependent sex determination (TSD), where the early ambient temperature is triggering the first ontogenetic differences between sexes and then fixing the final phenotypic sex. In reptiles, there are three patterns of response in front of temperature, ranging from all-female to all-male populations with higher male percent at extreme temperatures while balanced sex ratios are found at intermediate ones (Valenzuela and Lance, 2004).

Contrary to the situation in reptiles, in fish, there is only one pattern of response to temperature, with more males with increasing temperatures (Ospina-Álvarez and Piferrer, 2008). Many of the previously proposed fish species with TSD (~59 species) were recently reanalyzed following the "criteria" proposed by Valenzuela et al. (2003) and Conover (2004) based on the presence of sex chromosomes and/or sex ratios responses to temperatures that the species would not experience in the wild, and incorporating the concept of ecologically relevant temperatures precisely during the thermosensitive period. It was demonstrated that many of the TSD species were in fact GSD species with temperature effects (GSD+TE), since sex determination is normally under genotypic control and influenced by temperature only in rare circumstances (Ospina-Álvarez and Piferrer, 2008).

Furthermore, in the current scenario of global warming, an increase of just a few degrees in the average temperatures in many habitats of the planet, as predicted for the upcoming decades, could increase male percent in some TSD species if they could not relocate or adapt fast enough to the new situation (Ospina-Álvarez and Piferrer, 2008).

It could be argued that hypoxia is not a proper environmental factor influencing sex ratios but nevertheless it has been studied as a potential sex ratio distorter, since some studies where zebrafish were exposed to hypoxia yielded male-biased populations with slower growth rates (Shang et al., 2006). Hypoxia, as well as high densities (Jensen, 2003), triggers stress responses in fish, mainly by

increasing cortisol plasma levels. In fact, high cortisol levels have been associated with male differentiation in some fish species after stressful conditions (medaka: Hayashi et al., 2009; pejerrery: Fernardino et al., 2012; goldfish: Bernier et al., 2004; zebrafish: Shang and Wu 2004; Shang et al., 2006; Ribas et al., unpublished data; Egyptian mouth brooder (*Pseudocrenilabrus multicolor victoria*e): Friesen et al., 2012).

2.1.3. Continuous versus discrete dichotomy between GSD and TSD

Regarding the classification of sex determining mechanisms, some authors propose that GSD and TSD are two discrete processes that generate a continuous pattern of sex determination mechanisms (Valenzuela et al., 2003). Others see them as two ends of a continuum (Sarreet al., 2004). But, as already mentioned above (see Figure 1), the interactions among the three main factors that influence sex ratio inheritance can be explained by one factor or the combination of several effects, making any combination inside that triangle theoretically possible (Penman and Piferrer, 2008). Nowadays, the notion that "pure" GSD and TSD systems would be two ends of a continuum has gained more adepts.

2.2. Sex differentiation

Sex differentiation is the process by which a sexually undifferentiated gonad is transformed into testes or ovaries (Piferrer and Guiguen, 2008). This process gives rise to the phenotypic or gonadal sex. In gonochoristic species, once the gonad has developed as an ovary or as a testis it remains the same throughout life. The leading morphological and endocrinal aspects of this process are well understood and conserved among fish (Devlin and Nagahama, 2002). Nevertheless, in fish there are differences depending on the pattern of early gonadal development in gonochoristic species (Yamamoto, 1969). "Differentiated gonochoristic" species develop an ovary or testis from a previous undifferentiated gonad, while in "undifferentiated species" all individuals initially develop an ovarian tissue, and then

approximately while half of them continue with an ovarian differentiation, the remaining half experience a reorganization of the gonads with a tissue degeneration through apoptosis and a finally testis differentiation.

2.2.1. Morphological aspects

Gonadal development comprises: a) *gonadogenesis* that consists on a primordial gonad formation and a later differentiation into testes or ovaries; and b) *gametogenesis*, which is the formation of mature gametes (Piferrer and Guiguen, 2008). Contrary to what happens with the sex determination process, morphological development of gonads during early development is conserved not only among fish but to a certain extent even among vertebrates throughout evolution (Western and Sinclair, 2001).

Development of germ cells: Fish gonad structure is similar to that of other vertebrates, with germ cells (that have the potential to mitotically divide and enter meiosis) and associated supporting somatic cells (that will differentiate into associated structural and endocrine cell types) intermixed (Devlin and Nagahama, 2002). In zebrafish but also in many other fish, a DNA-helicase RNA (*vasa*) can be used as a marker of primordial germ cells (PGCs), the precursor of gamete-forming cells, since it is detectable since the two-cell stage at cleavage planes, remains associated to four cells (putative PGCs) through development until 1000-cell stage (Yoon et al., 1997) and after embryogenesis is solely detected in germ cells of the developing gonad (Yoon et al., 1997; Nagahama, 1999). Since germ line specification is not conserved as it is the specification of somatic tissue, two processes of specification are found: a) *preformation* where molecules that specify PGCs are inherited from the egg (germ plasm), and b) *epigenesis,*where extracellular signals trigger PGC specification from pluripotent precursors (Johnson et al., 2011).

Early gonadal development: In contrast to the rest of vertebrates, in fish all somatic cells appear to be derived from the cortex epithelial layer regardless of sex. PGCs are closely associated with endodermal tissues and migrate via the dorsal gut mesentery to the germinal ridge. Then, cell division occurs to form oogonia and

spermatogonia as well as somatic cell differentiation and migration. In ovarian development, somatic cells and PGCs differentiate to form follicles where oocytes are surrounded by an inner granulosa and an outer thecal layer (Nagahama et al., 1982). In testis, the somatic cells around the PGCs differentiate into seminiferous tubules and the supporting connective tissue, and into similar mammal-like Leydig and Sertoli cells (Van Vuren and Soley, 1990). Moreover, gonadal development takes place earlier in females than in males (Nakamura et al., 1998; Park et al., 2004).

Gametogenesis: Once the gonad is fully differentiated into either an ovary or a testis, gametogenesis or gamete formation can proceed. In females it starts earlier than in males but it is eventually stopped until the animal reaches puberty. In males, in contrast, does not start until puberty, when it proceeds until full completion. In fish, both oogenesis and spermatogenesis have already been well described elsewhere (Lubzens et al., 2010 and Schulz et al., 2010; respectively).

2.2.2. Endocrine control of gonadogenesis

Endocrine control of sex differentiation seems to involve a complex interaction between the brain, the pituitary and the gonads, and includes the production of pituitary-derived gonadotropins and gonad-derived steroids (Nagahama, 1994). Sex steroids not only have local direct effects on germ cell development, but also act as endocrine hormones that influence other cell types and organs involved in development (Devlin and Nagahama, 2002).

Since the brain is the integrator of all environmental and internal information, is actively involved in all the steps of the sexual cycle and along with the pituitary and the gonads forms the Brain-Pituitary-Gonad (BPG) axis (Colledge, 2008). Neurons in the preoptic regions of the brain, the neuroendocrine neurons, connect the central nervous system to the endocrine system. The common output in teleosts are up to three variants of the gonadotropic-releasing hormone (GnRH) (González-Martínez et al., 2001; Kim et al., 2014), corresponding to three GnRH populations (Kim et al., 2014) produced at different parts of the brain and with different phylogenetic relationships and functions (Andersson et al., 2001).

GnRH neurons directly innervate gonadotropin-releasing cells (gonadotropes) in the pituitary (Weltzien et al., 2004; Colledge et al., 2008; Kim et al., 2011). This pituitary stimulation produces two pituitary gonadotropins (GtHs): follicule-stimulating hormone (FSH) and luteinizing hormone (LH), which once released into the circulation, stimulate the gonads by binding to their specific receptors (Bogerd et al., 2005). Once in the gonads, gonadotropins stimulate gametogenesis and steroidogenesis (Levavi-Sivan et al., 2010). Other factors such as growth factors, other pituitary hormones and non-pituitary factors may also regulate gonadal function by exerting positive and negative feedback at any step of the BPG axis (Weltzien et al., 2004; Taranger et al., 2010). Although there is no direct evidence that in gonochoristic fish the central nervous system triggers the process, the hypothalamus-pituitary axis is active during the differentiation process and sex steroids (testosterone and estradiol) can have a feedback effect (positive or negative) on gonadotropin synthesis and secretion at different levels of the BPG axis (Moles et al., 2007; Colledge, 2008; Dufour et al., 2010). Furthermore, recently it has been shown that kisspeptin, a neuropeptide and its receptor (GPR54), play a major role in the HPG axis by regulating the onset of puberty (Roa et al., 2011; Chang et al., 2012).

In fish, another important factor involved in this cascade is the sex hormone binding globulin (*shbg*) that binds and transports sex steroids in blood, regulating also their access to target tissues (Siiteri et al., 1982). In European sea bass, it has been studied (Miguel-Queralt et al., 2007) and proved that its plasma levels were correlated to that of the sex steroids but there is still no information to its possible role in sex differentiation.

Sex steroids: Sex steroids are the product of all vertebrate gonads and are responsible of sex differentiation, the onset of puberty and the adult reproductive cycle (Piferrer and Guiguen, 2008). In fish, sex steroids have been since long known to be involved in fish gonadal sex differentiation (Yamamoto, 1969). The proper balance between androgens and estrogens is essential for normal sexual differentiation and reproductive function (Baroiller and D'Cotta, 2001). Furthermore, 11-ketotestosterone (11-KT), 11β-hydroxyandrostenedione and 11β-hydroxytestosterone are the sex steroids driving male sex differentiation instead of testosterone

(Piferrer et al., 1993; Borg, 1994; Baroiller et al., 1999), correlating androgen production with male sex differentiation (Nagahama, 2005). Besides, exogenous steroids administration early in development, mainly during the hormone sensitive period, strongly influences sex differentiation in both directions (Guiguen et al., 1999).

Steroidogenic enzymes: Sex steroids once formed cannot be stored. Thus, the enzymes that synthetize them play a key role controlling their abundance and availability. Cholesterol is the raw material in steroidogenesis (Figure 2), and hence, the first limiting step before its cleavage by the cytochrome P450 cholesterol side chain cleavage (*P450scc*), is its transport into the inner mitochondrial membrane. Steroidogenic acute regulatory protein (StAR) transports cholesterol through the aqueous intermembrane (Arakane et al., 1997) and its expression decreases when treating fish with exogenous estradiol (E_2) meaning that steroidogenesis is blocked at the first steps of the cascade. Other important enzymes in the pathway are: 17-hydroxylase/lyase (*P450c17*), 17β-hydroxysteroid-dehydrogenase (*17βhsd*), 3β-hydroxysteroid-dehydrogenase (*3βhsd*), 11β-hydroxylase (*P45011βhsd*) and P450 aromatase (*cyp19a1*). Aromatase is the enzyme responsible for the irreversible conversion of androgens into estrogens, and hence determines their balance in all vertebrates (Simpson et al., 2002). Also, *cyp19a1a* role during ontogenesis is well characterized (Baroiller et al., 1999; Guiguen et al., 1999; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002) and its expression reported before morphological sex differentiation (Baroiller et al., 1999), thus being able to play a crucial role in sex differentiation (Kwon et al., 2000). Additionally, high expression levels of aromatase are associated to ovarian development while the suppression of this enzyme triggers male development (Devlin and Nagahama, 2002).

In fish, aromatase activity and thus estradiol-17β (E_2) is essential for female development (Piferrer and Guiguen, 2008) and as well as the E_2 administration completely feminize fish populations (Saillant et al., 2001a; Navarro-Martín et al., 2009a), the administration of aromatase inhibitors such as Fadrozole (Fz) have an opposite masculinizing effect (Piferrer et al., 1994, Guiguen et al., 1999; Kitano et al., 2000; Navarro-Martín et al., 2009a). Since *cyp19a1a* is a key enzyme in fish sex differentiation and its expression has

been correlated with female differentiation in many teleost species (Guiguen et al., 1999; Kitano et al., 2000; Trant et al., 2001), *cyp19a1a* can be used as an early marker of ovarian differentiation in several species including rainbow trout (*Oncorhynchusmykiss;* Vizziano et al., 2007), the European sea bass (Blázquez et al., 2009), Southern flounder (*Paralichthyslethostigma*; Luckenbach et al., 2005) and halibut (*Hippoglossushippoglossus*; Matsuoka et al., 2006) to infer their gonadal sex before it can be identified histologically.

Cytochrome P450 11β-hydroxylase (*cyp11b2*) converts androstenedione and testosterone into 11β-hydroxyandrostenedione and 11β-hydroxytestosterone, being the latter a precursor of 11-ketotestosterone (11-KT), a major androgen in teleost fish (Piferrer et al., 1993: Baroiller et al., 1999; Liu et al., 2000; D'Cotta et al., 2001; Socorro et al., 2007) (Figure 2).

Sex steroid receptors: they are the mediators of androgen, estrogen and progesterone effects. Progesterone (*pgr*) and androgen receptors (*ar*) have evolved from an ancestral estrogen receptor (*er*) (Thornton, 2001). In fish, there are two subtypes of *ar*; *arα* and *arβ* that have been cloned in some teleosts (Takeo and Yamashita, 2000; Ikeuchi et al., 2001; Ogino et al., 2004; Blázquez and Piferrer, 2005). Moreover, *ar* changing levels during European sea bass sex differentiation suggested their involvement in sex differentiation and/or sexual maturation (Blázquez and Piferrer, 2005).

On the other hand, there are three *er*; *erα*, *erβ1* and *erβ2* that exhibit differences in tissue distribution, expression pattern, regulation, nucleotide sequence, different ligand-binding properties and they have also been identified in several teleost species (Menuet et al., 2002; Hawkins and Thomas, 2004; Filby and Tyler, 2005; Pinto et al., 2006; Nagler et al., 2007; Greytak and Callard, 2007). *erα* and *erβ* expression detectable before steroidogenic enzymes suggesting that estrogen (E_2) is already produced in gonads by the time of ovarian differentiation and may play an important role in sexual differentiation (Nagahama, 2005).

Furthermore, G-protein coupled membrane receptors *mER*, *mAR* and *mPR* have been described (Thomas et al., 2006), and they are

capable of eliciting biological responses much faster than their corresponding classical nuclear receptors, although their relation to sex differentiation remains still unclear.

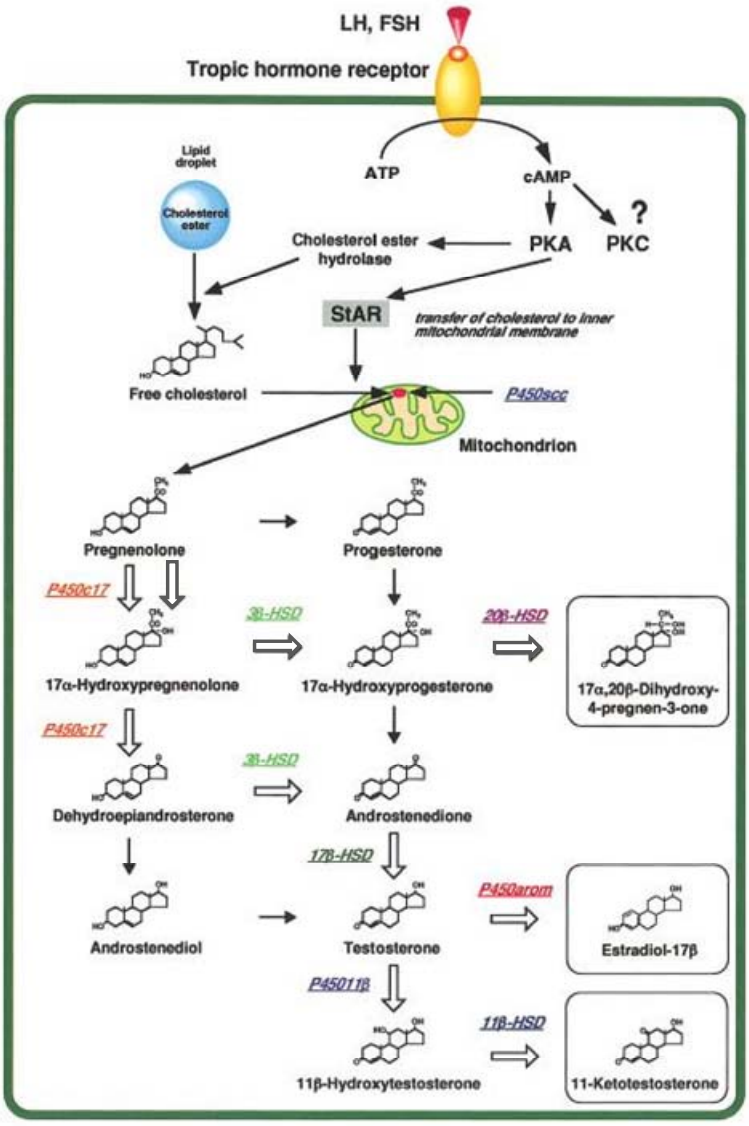


Figure 2. Steroidogenic pathway in the gonads of teleost fish from cholesterol. After the binding of LH/FSH to their receptors, StAR protein is synthesized in the cytoplasm and binds to cholesterol facilitating its transfer into the inner mitochondria where P450scc cleaves it and gives the raw material for steroidogenesis. From Young et al., 2005.

2.2.3. Other regulators of sex determination and differentiation

Transcription factors (TF) participate in regulating the expression of steroidogenic enzymes and other genes, although it is difficult to distinguish whether these transcription factors are related to sex determination, sex differentiation or gonadogenesis *per se* (Piferrer and Guiguen, 2008).

Doublesex and Mab-3 related transcription factor 1(dmrt1) is involved in vertebrate sex differentiation and has a similar role in fish (gonochoristic and hermaphroditic species). Its duplication in medaka derived into the sex determining gene *dmy* (Matsuda et al., 2002; Nanda et al., 2002) and in tilapia (*Oreochromis niloticus*) the two DM genes; *dmrt1* and *dmo* (*dmrt4*) are expressed in a sex-dependent manner (Guan et al., 2000). *Dmrt1* and its paralog genes are thought to be important in sex determination-differentiation, since variants or duplications of it contribute to this process in fish, amphibia, birds and mammals.

SRY-related high mobility group (HMG) containing box (SOX) genes constitute a family of genes involved in sex differentiation among other processes. Many sox genes have been identified in fish (Fukada et al., 1995; Kanda et al., 1998; Wang et al., 2003; Koopman et al., 2004; Galay-Burgos et al., 2004; Nakamoto et al., 2005). Among them: *sox9* (known to be involved in mammalian testis differentiation; Kent et al., 1996), *sox3* (suggested as a male development repressor), *sox24* (ovary expressed), *sox17* (involved in spermatogenesis: Wang et al., 2005, and with a role on ovarian development and function: Navarro-Martín et al., 2009c) and *sox19* (involvement in ovarian differentiation since it is strongly upregulated during this process Navarro-Martín et al., 2012) have been characterized in teleost species.

Steroidogenic factor-1 (sf1) / adrenal-4-binding protein (ad4bp) / fushitarazu factor-1 (ftz-f1). This nuclear receptor, also referred to as *nr5a1*, is involved in steroidogenic enzyme expression and is essential for mammalian gonadal development. The transcriptional activation of *cyp19a1a* driven by *sf1* has been reported in several teleosts (Yoshiura et al., 2003; Kobayashi et al.,

2005; Navarro-Martín et al., 2011; Díaz and Piferrer, zebrafish unpublished observations).

The dosage-sensitive sex-reversing adrenal hypoplasia congenital critical region on the X (Dax1 / Nr0b1). Nuclear receptor with a transcription repression function in mammalian female differentiation. In fish, its protein is highly conserved and the regulation of its function is a key step for normal male development and differentiation but without a clear sex specific role (Martins et al., 2007; Baron et al., 2008; Vizziano et al., 2008).

Helix/forkhead group (*foxl2*) is an early dimorphic marker of ovarian differentiation (Wang et al., 2004), and like the situation in many genes due to whole genome duplications, in fish it is present in duplicated copies (Baron et al., 2004). Furthermore, *in vitro* experiments showed activation of aromatase after treatment with foxl2 in Japanese flounder (*Paralichthys olivaceus;* Yamaguchi et al., 2007) and European sea bass (Navarro-Martín et al., 2011) but not in zebrafish, where *foxl2* binding sites at the gonadal aromatase promoter are missing (Díaz and Piferrer, unpublished observations).

Wilm's tumor suppressor-1 gene (Wt1) is a transcription factor with an essential role in the normal development of the urogenital system, hence in gonad formation. For instance, during murine gonadal ridge development, *Wt1* not only was expressed before *Dax-1* but also could activate it (Kim et al 1999; Western et al., 2000). Wt1 also forms a complex with SrY to regulate transcription during testis development. Thus, it is regarded as important for the identification and characterization of genes downstream of SrY (Matsuzawa-Watanabe et al., 2003).

Factor in the germline alpha (*figla*) is a germline specific transcription factor that hass a key regulatory role in the expression of several oocyte-specific genes, among them genes responsible of folliculogenesis initiation and zona pellucida genes (essential for fertilization and early embryonic survival). In humans, FIGLA is present in adult female ovaries, suggesting a role in normal ovarian development (Bayne et al., 2004). Furthermore, its expression has been identified in ovarian follicles, in mature oocytes and in pre-implantation embryos (Huntriss et al., 2002).

Growth factors: Naturally-occurring substances. From a chemical point of view, they are usually protein or steroid hormones with a regulating function in a huge variety of cellular processes. They can stimulate cellular growth and proliferation and are also involved in signaling processes between cells. Due to their importance in sex differentiation, steroid hormones have been dealt with above. Here we briefly discuss protein-based growth factors.

Anti-Müllerian hormone (amh) is a member of the TGF-β superfamily of growth and differentiation factors and displays a sexually dimorphic expression in teleosts during sex differentiation (Yoshinaga et al., 2004; Rodríguez-Marí et al., 2005; Halm et al., 2007). An expression pattern comparison between *amh* and *sox9* in zebrafish (Rodríguez-Marí et al., 2005) showed how *sox9* targets *amh* as previously described in mammals (Brennan and Capel, 2004).

Gonadal soma derived growth factor on the Y chromosome (Gsdf^{Y}) is a cytokine from the transforming growth factor (TGF)-β superfamily. Its expression in rainbow trout has been found in the genital ridge somatic cell that envelope the PGCs during embryogenesis and in granulosa and Sertoli cells afterwards. Its inhibition suppresses PGC proliferation and its addition is responsible of type-A spermatogonia proliferation (Sawatari et al., 2007). Furthermore, recently in medaka (*Oryzias luzonensis*) *Gsdf^{Y}* has been proposed as the new sex determining gene since its high expression is able to masculinize XX fish independently of the Dmy presence (Myosho et al., 2012).

Insulin-like growth factor 1 (igf1). This growth factor plays a role in fish growth, differentiation and reproduction. Also, besides its endocrine and paracrine/autocrine regulator function, its time-dependent production suggests that *igf1* is involved in Nile tilapia gonadal formation and differentiation (Fu et al., 2001; Berishvili et al., 2006). In the European sea bass, igf1 expression is limited to spermatogonia-containing cysts and it has been proposed to have effects during the proliferative stage (Viñas and Piferrer, 2008).

2.2.4. Transcriptomic studies on fish sex differentiation

Transcriptomic analysis during gonad formation and differentiation can provide an overall picture of the whole process and ultimately can lead to the discovery of new sex determining genes, regulatory factors and signaling pathways (Piferrer et al., 2013). Complications to this approach include the availability and correct annotation of piscine genomes and also the fact that analyzed fish, as we have seen, differ in its sex determination and differentiation system.

The gonadal transcriptome of zebrafish testis and ovaries (Santos et al., 2007) showed marked transcriptomic differences among sexes with 2,940 differentially expressed (DE) genes: 1,570 overexpressed in females and 1,370 overexpressed in males, an observation in agreement with previous experimental and computational studies on zebrafish testis and ovarian transcriptomes (Li et al., 2004; Wen et al., 2005). Moreover, sex-related differences for some *sox* genes, activins and insulin-like growth factors were found. *sox11b*, *sox21a* and *sox31* were overexpressed in ovaries while in testis the *sox9a* absence and *amh* overexpression induced not only testis development and differentiation but also sexual dimorphism maintenance (Santos et al., 2007). Also inhibins, inhibitors of FSH pituitary secretion, appeared overexpressed in testis in accordance with this inhibin pathway being activated in mature testes with a continuous gamete production. Forkhead box H1 (*FoxH1*), a transcription factor mediating TGF-β/activin signaling (Attisano et al., 2001) and a coexpressor of the *ar* (Chen et al., 2005), was overexpressed in ovaries, consistent with the synthesis and storage of maternal factors in mature oocytes and the suppression of androgen activity in this organ (Santos et al., 2007). As previously described (Wood et al., 2005), *igf1*, a gene related to growth and steroidogenesis, was overexpressed in testis and its receptors did not show any sex-related differences (Santos et al., 2007). Furthermore, transcriptomic variation was higher among females when compared to males. Since females presented two subgroups of growing follicles, early oocytes undergoing oogenesis and vitellogenic follicles, it was concluded that these different transcriptomic results may provide an explanation for relative fecundity reduction in aged females (Santos et al., 2007). Additionally, current gonadal microarray analysis of adult zebrafish gonads of both sexes submitted to different temperatures showed a

surprising pattern of response towards temperature and a considerable number of differentially expressed (DE) putative sex-related genes (Ribas et al., unpublished).

Gonadal transcriptomic analysis in Nile tilapia (Tao et al., 2013) showed sex-related expression of some XX-enhanced genes such as *snip1* (oocyte development), *wnt5a* (importance at the female reproductive system), *par-3* (involved in egg polarity) and *pen* (involved in primordial follicle formation and the newborn ovary homeobox); as well as some XY-enhanced genes such as *nanos2* (present in male germ cells), *arhgap42* (adherent junctions in testes), *dazap1* (mRNA transport during spermatogenesis), *hsp70* (potentially important in male testis and germ cells), and *m33* (its absence may cause sex-reversal towards a female).

Small-scale transcriptomic analysis of the Asian sea bass (*Lates calcarifer*) gonads from both sexes showed marked differences among differentiation stages (Ravi et al., 2014). Their analysis showed that by just analyzing 36 genes already known to have a role in reproduction, steroidogenesis or immunology-related pathways; it was possible to assess the sex and to discriminate among the different maturation stages in Asian sea bass, despite the presence of a constitutive high individual variability and complexity (Ravi et al., 2014).

Major efforts are now put in analyzing transcriptomes in response to environmental influences, both naturally-occurring (i.e., Gracey et al., 2004; Cossins et al., 2006) and of anthropogenic origin (i.e., Schiller et al., 2013a-b), or also after diet substitutions (i.e., Campos et al., 2010; Tacchi et al., 2011; Calduch-Giner et al., 2012). However, without a prior good knowledge of normal gonad development and differentiation, the understanding of the external effects at the transcriptomic level would be more difficult to appreciate.

We compiled transcriptomic data on piscine reproduction related genes and produced the table below.

Table 2. Fish reproduction-related genes from transcriptomic studies.

Symbol	Description	Male/ Female
Amh	Anti-Müllerian hormone	M>F
amhr2	Anti-Müllerian hormone receptor, type II	M>F
Amhy	Anti-Müllerian hormone Y-chromosome	M>F
aqp1o	Aquaporin 1o	M<F
bmp15	Bone morphogenetic protein 15	M<F
ctnnb1	Catenin beta-1	M<F
ctnnbip1	Beta-catenin-interacting protein 1	M<F
cyp11b2	Cytochrome P450 11β-hydroxylase	M>F
cyp19a1a	Cytochrome P450 aromatase	M<F
cyp26a1	Cytochrome P450 26A1	M<F
dax1	Dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome	M>F
dhh	Desert hedgehog	M>F
dmrt1	Doublesex- and mab-3-related transcription factor 1	M>F
dmrt1bY	Doublesex- and mab-3-related transcription factor 1	M>F
dmrt2	Doublesex- and mab-3-related transcription factor 2	M>F
dmrt3	Doublesex- and mab-3-related transcription factor 3	M>F
dmy	DM-domain gene on the Y chromosome	M>F
dvl2	Segment polarity protein dishevelled homolog DVL-2	M<F
erb1	Estrogen receptor beta1	M<F
ff1a	Ftz-F1 alpha	M<F
fgf2	Fibroblast growth factor 2	M<F
foxl2	Forkhead box L2	M<F
foxl2a	Forkhead box L2A	M<F
gsdf	Gonadal somatic cell derived factor	M>F
gsdf1	Gonadal soma derived factor 1	M>F
hsd11b2	Corticosteroid 11-beta-dehydrogenase isozyme 2	M>F
igf1	Insulin-like growth factor 1	M>F
nr0b1	Nuclear receptor subfamily 0 group B member 1	M>F
nr5a2	Nuclear receptor subfamily 5 group A member 2	M>F
pgr	Progesterone receptor	M<F
rspo1	R-spondin 1	M>F

rspo4	R-spondin 4	M<F
sdf1a	Stromal cell-derived factor 1	M>F
sdY	Sexually dimorphic on the Y-chromosome	M>F
sf1	Steroidogenic factor 1	M>F
sox11b	Transcription factor SOX-11b	M<F
sox17	Transcription factor SOX-17	M<F
sox19	Transcription factor SOX-19	M<F
sox19a	Transcription factor SOX-19a	M<F
sox21a	Transcription factor SOX-21a	M<F
sox24	Transcription factor SOX-24	M<F
sox3	Transcription factor SOX-3	M>F
sox31	Transcription factor SOX-31	M<F
sox9	Transcription factor SOX-9	M>F
sox9a	SRY-box containing gene 9a	M>F
sox9b	SRY-box containing gene 9b	M<F
srd5a2	Steroid-5-alpha-reductase, alpha polypeptide 2	M>F
sycp3l	Synaptonemal complex protein 3	M>F
vasa	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	M<F
wnt5a	Wingless-type MMTV integration site family, 5A	M<F
wt1	Wilm's tumor protein	M>F
wt1a	Wilm's tumor 1a	M>F
wt1b	Wilm's tumor 1b	M>F
zp2	Zonapellucida glycoprotein 2	M<F
zp3a	Zonapellucida glycoprotein 3a	M<F
zp3b	Zonapellucida glycoprotein 3b	M<F

Note: Summary based on fish transcriptomic results from Bobe et al., 2006; Kishi et al., 2006; Santos et al., 2007; Tao et al., 2013; Zheng et al., 2013; Ravi et al., 2014; Shao et al., 2014.

3. Epigenetics

The term epigenetics refers to "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in the DNA sequence" (Russo et al., 1996; Sasaki, 2005). This allows changes of the phenotype without changing the genotype. There are two important keywords in the above definition. The first one is the term *heritable*, which means that altered states of gene expression can be passed from one cell generation to the next through mitosis or even from parents to offspring through meiosis. The second is *without involving changes in the DNA sequence*.

Epigenetic modifications are involved in many normal cellular processes, switching on and off genes and determining which proteins are transcribed, thus contributing to cellular identity. For example, the human genome is able to give rise to more than 200 different cell types. Thus one genome produces more than 200 epigenomes. Furthermore, the distinct epigenome of a given cell type changes with time (i.e., Heyn et al., 2012).

Many important processes are regulated by epigenetic changes. For instance, epigenetic silencing is responsible for X-chromosome inactivation in female mammals (Egger et al., 2004) and also for the activation/inhibition of a set of genes in a tissue specific manner. Epigenetic regulatory mechanisms are also highly influenced by several factors such as environmental changes (Navarro-Martín et al., 2011; Feil and Fraga, 2012), age (Heyn et al., 2012), lifestyle (Heijmans et al., 2008) or disease state (Portela and Esteller, 2010), since organisms utilize these mechanisms to integrate both genomic and environmental information.

3.1. Operational epigenetics, mechanisms and integration of environmental cues

There are three categories of signals responsible of establishing epigenetic changes in the chromatin (Berger et al., 2009) (Figure 3):

Epigenators: signals from the environment (*sensu stricto* everything above the genome, and including the cytoplasmic, extracellular and external environment) such as temperature, differentiation signals or metabolites that trigger intracellular pathways. Everything occurring upstream of the first event on the chromosome would be part of the Epigenator signaling pathway, converted onto an intracellular Epigenator pathway and ending up activating the Epigenetic initiators (see below). The signal can be transient, but sufficient to trigger the response and be based on a protein-protein interaction or a modification event able to unleash the latent activity of the Initiator (Berger et al., 2009).

Epigenetic initiators: a signal that responds to an Epigenator and is necessary to mark the precise location of a chromosome where the epigenetic chromatin state is to be established. The Initiator could be a DNA-binding protein, a non-coding RNA or any factor which coordinates chromatin structure assembly, displays sequence recognition and/or is self-reinforced or self-renewed through positive feedback. In contrast to the Epigenator, the Initiator may persist with the Maintainer after triggering an epigenetic phenotype (Berger et al., 2009).

Epigenetic maintainers: a signal that sustains a given chromatin state in a given location in the first and subsequent generations, but is not sufficient to initiate it. Epigenetic maintainers involve different mechanisms and players such as DNA methylation, histone modifications, histone variants, nucleosome positioning and others. They do not present absolute DNA sequence specificity, thus conferring the ability of acting at any chromosomal location (Berger et al., 2009).

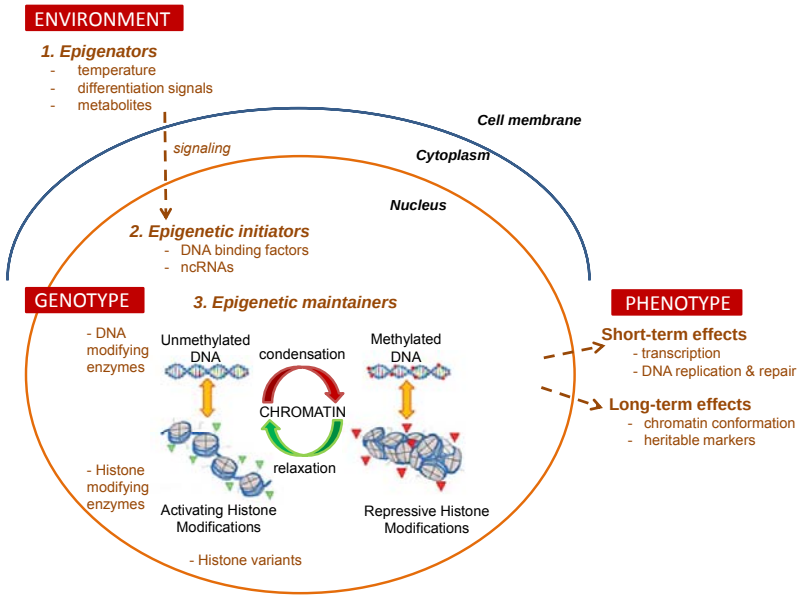


Figure 3. Schematic view of the three types of epigenetic mechanisms responsible of establishing the heritable characteristics. Modified from Piferrer, 2013 (which, in turn, was based on Berger et al., 2009) and Munro et al., 2010.

Epigenetic maintainers can be grouped into three main categories: DNA methylation, histone modifications and nucleosome positioning. However, the final phenotype would be the result of their interactions and the many positive and negative feedback mechanisms among them (Portela and Esteller, 2010) (Figure 4).

DNA methylation (Figure 4a): DNA methylation occurs almost exclusively in the context of CpG dinucleotides. These CpG dinucleotides tend to cluster in regions named CpG islands (regions with more than 200 bases with a G+C content of at least 50% and a ratio of observed *versus* statistically expected CpG frequencies of at least 0.6). This is important since about 60% of human promoters are associated with CpG islands (Straussman et al., 2009). In general, CpG island methylation is associated with gene silencing and plays a role in processes such as genomic imprinting (Kacem and Feil, 2009) or X-chromosome inactivation in females (Reik et al., 2005). DNA methylation exhibits different mechanisms of action since for instance it can recruit a methyl-CpG-binding (MBD) protein, that in turn recruits histone-modifying and

chromatin-remodeling complexes to methylated sites (Esteller, 2007) or by directly recruiting DNA binding proteins from their target sites (Kuroda et al., 2009). Furthermore, there is a new term known as CpG island shores (regions of lower CpG density in proximity of a CpG island) that not also can be methylated but also can be related to transcriptional inactivation too.

DNA methylation is mediated by DNA-methyltransferases (DNMTS) enzymes that catalyze the transfer of a methyl group from S-adenosyl methionine to DNA. In mammals, there are five reported DNMTs: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L, but only 1, 3a and 3b present a DNA methyltransferase activity. DNMT1 exhibits a maintenance activity while DNMT3a and DNMT3b have a *de novo* activity.

In zebrafish, eight different DNMTs have been described so far (Shimoda et al., 2005), but a recent update have shown that six of them evolved from a common proto-chromosome that suffered a duplication process generating, *dnmt3a* and *dnmt3b* that in turn, suffered a series of duplications. Then *dnmt3* (now *dnmt3b3*), *dnmt4* (now *dnmt3b1*), *dnmt5* (now *dnmt3b4*) and *dnmt7* (now *dnmt3b2*) originated from *dnmt3*b while *dnmt6* (now *dnmt3a1*) and *dnmt8* (now *dnmt3a2*) evolved from *dnmt3a* (Campos et al., 2012). Furthermore, *dnmt1* shows as in mammals a maintenance function, while *dnmt3b3* and *dnmt3b2* have a *de novo* function.

Histone modifications (Figure 4b): Histones can be divided into core histones, H2A, H2B, H3 and H4, which cluster into two H2A-H2B dimer and one H3-H4 tetramer to form the nucleosome, and linker histone H1, which binds to the linker DNA, i.e., the DNA between nucleosomes, and seals the nucleosome at the location where DNA enters and leaves. Core histones are globular structures with unstructured N-terminal tails where post-transcriptional modifications such as acetylation, methylation or phosphorylation occur. Moreover, chromatin can be in turn divided into heterochromatin (transcriptionally inactive) or euchromatin (actively transcribed). While euchromatin is characterized by high levels of acetylation and trimethylation of H3K4, H3K36 and H3K79; heterochromatin, in contrast, exhibits low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation (Li et al., 2007).

Nucleosome positioning (Figure 4c): is considered a transcription barrier since its positioning blocks the access of activators and transcription factors to their sites on DNA. They also inhibit transcript elongation by polymerases, hence regulating gene expression. Additionally, the position of nucleosomes around the transcription start sites (TSS) is also important for the initiation of transcription. Genes with 5' and 3' nucleosome-free regions are correlated with gene activation, since this provides space for the assembly of the transcription machinery (Cairns, 2009). In addition to the physical function of nucleosome positioning *per se*, other factors such as the incorporation of different histone variants, histone variant replacement by miRNAs or the presence of chromatin remodeling complexes (SWI/SNF, ISWI, CHD or INO80 family) can influence their position (Ho and Crabtree, 2010).

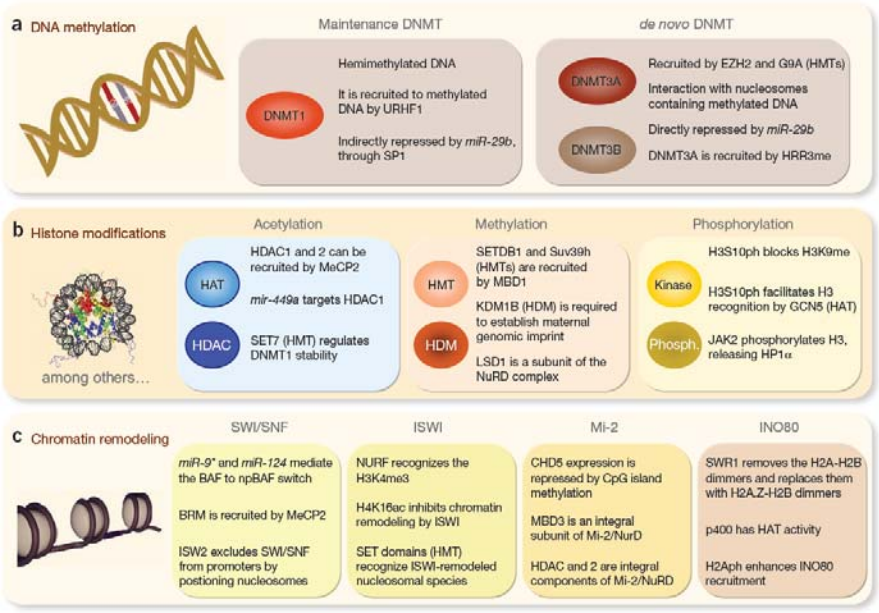


Figure 4. Epigenetic marks are catalyzed by the action of different epigenetic complexes (a-c). Final epigenetic regulation depends on the interaction among the different components: DNA methylation (a), histone modifications (b) and chromatin remodeling (c). From Portela and Esteller, 2010.

Epigenetic maintainer mechanisms can display short-term and long-term functional effects (Turner et al., 2007). When modifications occur rapidly and cyclically in response to external stimuli, maintainers such as histones, are normally linked to processes that are continuously occurring within the cell, hence can be considered as endpoints of signaling pathways. These changes are rapid responses of the genome towards environmental changes, thus these modifications are rapidly turned over. On the contrary, long-term effects, where the modification is maintained throughout the cell cycle or from one cell generation to the next, are also found. These constitute the true epigenetic modifications (Turner et al., 2007).

3.2. Epigenetics and sex determination/differentiation

Epigenetic mechanisms provide organisms with the ability to modify their gene expression patterns in response to external and internal environments, thus actively contributing to genotype x environment interactions (Turner, 2009).

It can be argued that sex determination and differentiation, key processes on perpetuation of species, may be highly sensitive to epigenetic influences. Undifferentiated gonads can develop into two mutually exclusive phenotypes and are subjected to the antagonistic signaling pathways and transcription networks (Kim and Capel, 2006). Final sex is then established by activating the testis or the ovarian pathway while repressing the alternative one in a sexually dimorphic manner which is then perpetuated across many cell divisions (Munger and Capel, 2006). The study of the contribution of the different epigenetic regulatory mechanisms to sex determination and differentiation is a quite recent subarea of research and as such has only been recently reviewed (Piferrer, 2013). The sections that follow are based on that review.

DNA methylation effects on sex determination: DNA methylation during development may lead to genomic imprinting. Differential methylation patterns at germ cell control regions may lead to maternal- or paternal-specific expression on a subset of genes in the genome, despite identical DNA sequence of the two parental chromosomes (Strogantsev et al., 2012). In plants,

epigenetic mechanisms involved in sex determination have been described to maintain repressed epigenetic states in maize (*Zea mays*; Parkinson et al., 2007). In the melon (*Cucumis melo*), the transition from male to female flowers is accomplished by the insertion of a transposon, which is required for the initiation and maintenance of the spreading of DNA methylation to the promoter of the CmWIP1 gene, involved in carpel abortion and hence in unisexual male flower development (Martin et al., 2009).

In medaka and European sea bass, gonadal aromatase promoter exhibits sex-related differences in its methylation pattern (Contractor et al., 2004; Navarro-Martín et al., 2011). Moreover, in European sea bass there are also differences in the methylation levels of the gonadal aromatase promoter at one year after early thermal treatment. Hence showing a direct effect of temperature on DNA methylation activity on the promoter, and therefore in aromatase expression levels. In addition, CpG in the gonadal aromatase promoter, also exhibits different sensitivities towards temperature (Navarro-Martín et al., 2011).

In the protandrous hermaphrodite black porgy (*Acanthopagrus schlegeli*), during the first two reproductive cycles the ovarian part remains inactive and irresponsive to E_2 treatment (Wu et al., 2010). Gonadal aromatase promoter methylation levels were higher in the inactive ovaries, suggesting an epigenetic control in addition to the transcriptional activation led by sf1 and foxl2 on the aromatase promoter (Wu et al., 2012a). In contrast, there were no differences in the methylation of the *dmrt1* promoter (a gene involved in testis differentiation, sexual fate determination and natural sex change in black porgy; Wu et al., 2012b) between inactive and active ovaries (Ching-Fong Chang, personal communication to Francesc Piferrer).

In the half-smooth tongue sole (*Cynoglossus semilaevis*), a gonochoristic species with a genotypic (ZZ/ZW) sex determining system with temperature influences, comparative analysis of the gonadal DNA methylomes of pseudo-males (ZW), females (ZW) and normal males (ZZ), showed that methylation modification in pseudo-males due to high temperature incubation were not only globally inherited by their ZW offspring, but that these offspring could develop as pseudo-males without a thermal treatment. Additionally, a transcriptome analysis revealed that dosage

compensation is present in a restricted, methylated cytosine enriched Z chromosomal region in pseudo-male testes, leading to an equal expression levels as in normal male testes, but not for W chromosomal genes that are suppressed by methylation regulation in pseudo-males (Shao et al., 2014).

In the red turtle (*Trachemys scripta*), a TSD species with an aromatase expression limited to the female-producing temperature during gonadogenesis, shifts from male- to female- producing temperature changed DNA methylation levels in the gonads. This is due to the demethylation of the aromatase promoter CpG sites leading to aromatase expression during gonad development in a temperature-specific manner (Matsumoto et al., 2013). Together, these examples illustrate how important is DNA methylation in ensuring proper gene expression/repression in relation to different stages of gonadal development across distantly related species.

Histone modification effects on sex determination: many of the enzymes involved in chromatin remodeling are sensitive to changes in the environment and metabolism and thus work as sensors that can alter gene expression (Turner, 2009). In some instances, epigenetic marks can be associated with a particular case as the imprinting found in the bug (*Planococcus citri*), where gametes originating from a given meiosis (same genome) differed in the levels of H3K9me (methylated lysine 9 of histone H3) and HPI (heterochromatin protein 1) (Buglia and Ferraro, 2004). In this sense, *EHMT2* is an important mammalian H3K9 methyltransferase in mouse embryogenesis since different methylation stages are regulated in a sex-differentially manner and its gene silencing function is crucial for proper meiotic prophase progression and gametogenesis regulation (Tachibana et al., 2007).

H3K9me2/1-specific demethylase JHDM2A directly binds and controls the expression of transition nuclear protein 1 (*tnp1*) and protamine 1 (*prm1*), necessary for packaging and condensating sperm chromatin (Okada et al., 2007). In mice, a loss of H3K9 demethylation leads to a *sry* downregulation during embriogenesis (Tachibana et al., 2012). Also CBX2 (chromobox homolog 2), the mouse homolog of polycomb, is involved in testis differentiation through regulation of *sry* gene expression, since the expression of some genes and transcription factors necessary for the gonadal

development were affected in CBX2 knock-out gonads (Katoh-Fukui et al., 2012).
The *Drosophila* PHD finger protein 7 (*phf7*) exhibits male specific expression from germ line stem cells through spermatogonia, promotes spermatogenesis in XX germ cells, binds to histone H3 N-terminal tails (to H3K4me2) and may be a conserved epigenetic mark that activates the male germ line (Yang et al., 2012).

Non-coding RNAs effects on sex determination: In *Drosophila*, *roX* and in mouse, *XIST* are two long non-coding RNAs implicated in dosage compensation, where specific gene expression inequality due to different number of sex chromosomes is inhibited through epigenetic chromatin modification of one of the two sex chromosomes (Angelopoulou et al., 2008). In cockroach (*Blattella germanica*), depletion of Dicer1, a key enzyme on miRNA formation, produced sterile females with huge alterations of oocyte development (Tanaka and Piulachs, 2012).

A recent study on the Atlantic halibut showed a sex-biased expression of miRNAs, with some of them altered after masculinization by androgen or aromatase inhibitor treatments, suggesting a miRNAs role as hormonal signaling (Bizuayehu et al., 2012).

Additionally, sex determination in chicken provides an interesting example of epigenetic mechanisms working together (Piferrer, 2013), since a ncRNA, *MHM* (male-hypermethylated) is transcriptionally silenced in males by methylation and regulates dosage compensation (Teranishi et al., 2001). In addition, the *MHM* locus is also strongly enriched for acetylation of histone H4 at lysine residue 16, a modification linked to female euchromatin and also with a role in Drosophila dosage compensation (Bisoni et al., 2005). Furthermore, *MHM* mRNA accumulates on the female Z chromosome next to *DMRT1* and influences its expression, then suggesting a possible direct role in chicken gonadal sex differentiation (Roeszler et al., 2012).

4. The European sea bass model

4.1 Biology

The European sea bass is a differentiated gonochoristic species belonging to the family of Moronidae (Nelson, 2006). European sea bass distribution includes the Eastern Atlantic, from Norway to Senegal, and from the Mediterranean to the Black sea, but it is absent from the White, Barents, Baltic and Caspian seas (Kottelat and Freyhof, 2007). Thus, the European sea bass is a eurythermal species with a broad range of temperature tolerance (8ºC-27ºC). Although primarily is a marine fish, its habitat also includes coastal lagoons, estuaries and coastal waters. It can even dwell in brackish and fresh waters since it enters river mouths in summer, but migrates offshore with cold weather. Therefore, the European sea bass is also a euryhaline species. Juveniles form schools in salty waters of a few meters depth (~10 m) and eat small crustaceans (shrimps and mollusks) and fish. Adults appear to be less gregarious and inhabit in coastal waters to a maximum of 100 m depth (Frimodt, 1995). Adults show a predator behavior, and capture their prey (small pelagic fish as sardines, sprats and sand smelts; crustaceans and squids) by a wide range of hunting techniques (Wheeler, 1975).

European sea bass becomes sexually mature between 2-7 years of age depending on the gender and the latitude (in the Mediterranean < 4 years). However, under artificial rearing conditions, males mature earlier than females (Bruslé and Roblin, 1984) and earlier than in the wild (male mature around the second year, while females mature during the third year; precocious males and females can mature even one year earlier). Spawning takes place during the spring near the British Islands and earlier in its southern range. For instance, in the Northwestern Mediterranean, spawning takes place in winter (January-March). Female European sea bass spawn in batches (Murua and Saborido-Rey, 2003), releasing between a quarter to half million eggs per kilogram. Reproduction, spawning and hatching occur in sea water and while eggs and pre-larvae drift towards coastal zones, larvae actively search nursery sites in more low-salinity environments (Barnabé, 1989).

Sex ratios data from semi-natural enclosures showed a female-biased population (Arias, 1980). A recent studies on 13 wild European sea bass populations showed that as a whole, sex ratio is skewed towards females (59.4% females, $P<0.001$). Sex ratio at young ages is balanced (52.0% females, $P=0.15$). However, these results could be also explained by differential longevity, by a sampling bias (Vandeputte et al., 2012) or by differential fishing pressure at some critical moments.

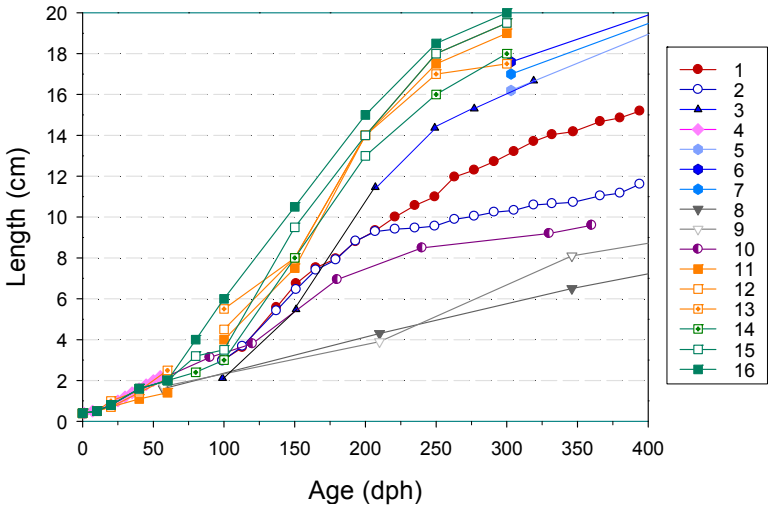
4.2. Growth

In fish, individual variability in weight is often high. Thus, a group of fish from the same paternal origin, the same batch (same age) and homogeneous in their initial weight may progressively differ in growth and yield a non homogenous population (Gardeur et al., 2001a; Campeas et al., 2009). Heterogeneity of growth is a current issue in aquaculture, since it facilitates cannibalism (Baras and Jobling, 2002) or imposes the need of grading practices to optimize food utilization. Several studies have shown differential growth performance between individuals of the same batch subjected to the same environmental conditions due to factors such as feeding protocol (Gélineau et al., 1998), fight for food resource (Carter et al., 1993), hierarchical structure (Martins et al., 2006) or food utilization (Toguyeni et al., 1997). In this context, it is not surprising that individual analysis of European sea bass growth from the same batch showed differences in specific growth rate (SGR), indicating that homogenous initial growth does not lead to similar SGR and neither to similar final body weight (BW) (Gardeur et al., 2001a).

There is also an important gender effect on growth since a higher SGR correlated with a low male ratio (Gardeur et al., 2001a), hence confirming the occurrence of sexual growth dimorphism prior to the age of 10 months (Bruslé and Roblin, 1984; Blázquez et al., 1999; Gardeur et al., 2001; Saillant et al., 2001; Navarro-Martín et al., 2009b; Díaz et al., 2013), with bigger females at the commercial size (Gardeur et al., 2001a; Navarro-Martín et al., 2009b). Furthermore, Vandeputte et al. (2007) also found a strong

correlation between sex and growth and that even after correcting for this sex dimorphism, families with high female percent still showed a size advantage. Length at maturation was also affected in a sex-related manner, meaning that sexual growth dimorphism in European sea bass appears in a population of mature males and immature females (Blázquez et al., 1999; Gardeur et al., 2001a; Saillant et al., 2001a; Díaz et al., 2013). Nevertheless, this sexual growth dimorphism (Carrillo et al., 1995) is still present at 2 years when males have 20-40% less BW than females (Felip et al., 2006).

Opposite to what has just been mentioned, in some cases growth variability in European sea bass cannot be explained by sexual growth dimorphism, social hierarchies within the group or the initial weight. Differences may be due the individual genetic growth potential inherited from the parents (Carter et al., 1993; Gardeur et al., 2001a). Furthermore, studies on the genetic influence on sex in European sea bass showed how a size advantage in families with high proportions of females was present, even when correcting for sex dimorphism (Vandeputte et al., 2007). This parental component of growth performance can also be seen when comparing fish from different parental origins submitted to changes in the same environmental factor, temperature (Figure 5). Although differences in growth performance can be observed due to the thermal treatment, a common pattern is also visible, fish from the same genetic origin cluster together when compared to fish from other paternal origins.



Legend code	Treatment	Period	Reference
1	High temperature (21°C)	From 20 dpf and onwards	Chapter 3
2	Low temperature (15°C)	From 20 dpf and onwards	Chapter 3
3	Low temperature (15°C)	Until 60 dpf, then at 21°C	Navarro-Martín et al., 2009b
4	Natural temperature	Always	Skretting tables
5	13°C	Half-epiboly to mid metamorphosis	Pavlidis et al., 2000
6	15°C	Half-epiboly to mid metamorphosis	Pavlidis et al., 2000
7	20°C	Half-epiboly to mid metamorphosis	Pavlidis et al., 2000
8	High temperature	>19°C from 19 dpf and onwards	Saillant et al., 2002
9	Low temperature	13°C always	Saillant et al., 2002
10	Natural temperature	Always	Roblin, 1983
11	13°C	Larval period	Mylonas et al., 2005
12	17°C	Larval period	Mylonas et al., 2005
13	21°C	Larval period	Mylonas et al., 2005
14	13°C	Nursery period	Mylonas et al., 2005
15	17°C	Nursery period	Mylonas et al., 2005
16	21°C	Nursery period	Mylonas et al., 2005

Figure 5. Growth performance in different European sea bass batches submitted to different thermal treatments. Modified from Navarro-Martín et al., 2009b; Skretting's Gemma Wean performance guidelines; Pavlidis et al., 2000; Saillant et al., 2002; Roblin, 1983 and Mylonas et al., 2005.

Additionally, individual feeding behavior studies have also pointed out the inter-individual differences that are even more important than physiological variables (Millot et al., 2008). These differences in feeding behavior that are normally present after size-grading of European sea bass populations are however, transient changes and do not improve the growth of the graded fish in self-feeding conditions (Benhaïm et al., 2011).

Food availability also influences individual growth. In nature, after periods of food deprivation and with a consequent decrease on growth rates, fish can experience an accelerated growth converging in size with those fish that did not suffer from the food restriction (Ali et al., 2003). In fact, the presence of compensatory growth in

animals indicates that growth rates are usually below the normal growth trajectory (Jobling, 2010). In addition, the degree of growth compensation may differ depending upon the magnitude of the growth deviation from the normal growth trajectory. Accordingly, we can speak of full compensation (full convergence in growth relative to control), partial compensation (recovery in growth, shortening weight differences relative to control) or no compensation (recovery in growth after food deprivation without convergence). Because of this, the definition of growth compensation can be misleading since size-recovery attribute in fact includes three different types of growth recovery (Jobling, 2010) (Figure 6):

Compensatory growth: Animals restore body composition and energy reserves by increasing growth rates. However, their final size may be lower and the composition of the gain may differ when compared to individuals that grew under normal environmental conditions.

Catch-up growth: animals exhibit full convergence in growth, without necessarily a recovery of body composition after showing different growth histories.

Recovery growth: a combination of compensatory and catch-up growth.

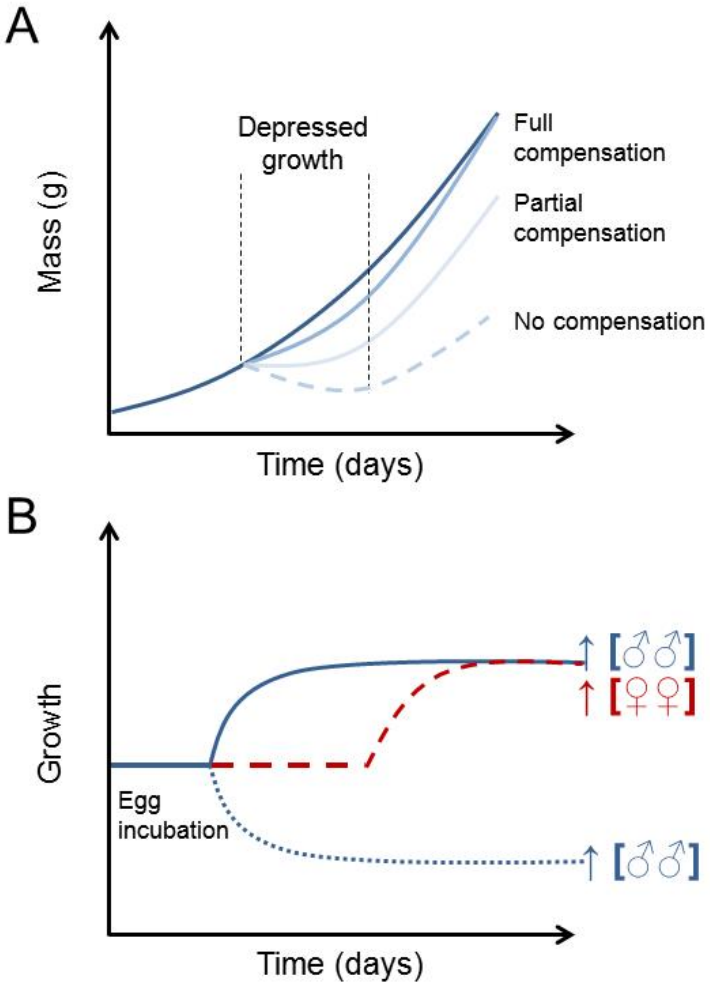


Figure 6. Growth responses to environmental conditions. **A)** Full, partial or non-compensated growth general models (modified from Jobling, 2010) and **B)** the European sea bass growth performance with three scenarios: accelerated growth rates induced by applying high temperatures consistently results on a highly male-biased population (blue solid line); maintaining slow growth rates by reducing temperature until the end of thermosensitive period and then increasing temperature (and thus food consumption) to take advantage of catch-up growth yields the highest possible female percent, the actual number depending on the particular genotype (red long dashed line); and maintaining low growth rates with sustained low temperatures for a long period also produces a consistently high male percent since fish do not attain the needed standard length on time to trigger sex differentiation (blue dotted line).

The endocrine system is behind the control of compensatory growth through the GH/IGF axis. Fish go from an anabolic normal stage to a catabolic phase where endogenous energy reserves are mobilized (food depression) and a final hyperanabolic stage (refeeding), when the actual accelerated growth occurs (Won and Borski, 2013).

During the catabolic phase, endogenous energy reserves are depleted and ghrelin is both enhancing appetite and stimulating growth hormone (GH) synthesis. This increase on GH levels has a decreasing effect on the lipostatic signaling and exploits energy reserves. Moreover, insulin-like growth factors (IGFs) are suppressed due to liver GH resistance (Figure 7). Afterwards, when normal feeding is re-established, hyperanabolism is characterized by a resumption of hepatic GH sensitivity to IGF-I levels which promotes accelerated growth. The negative feedback produced by high levels of IGF-I and the increase in energy reserves returns GH and appetite to the normal levels and normal growth rates are observed again.

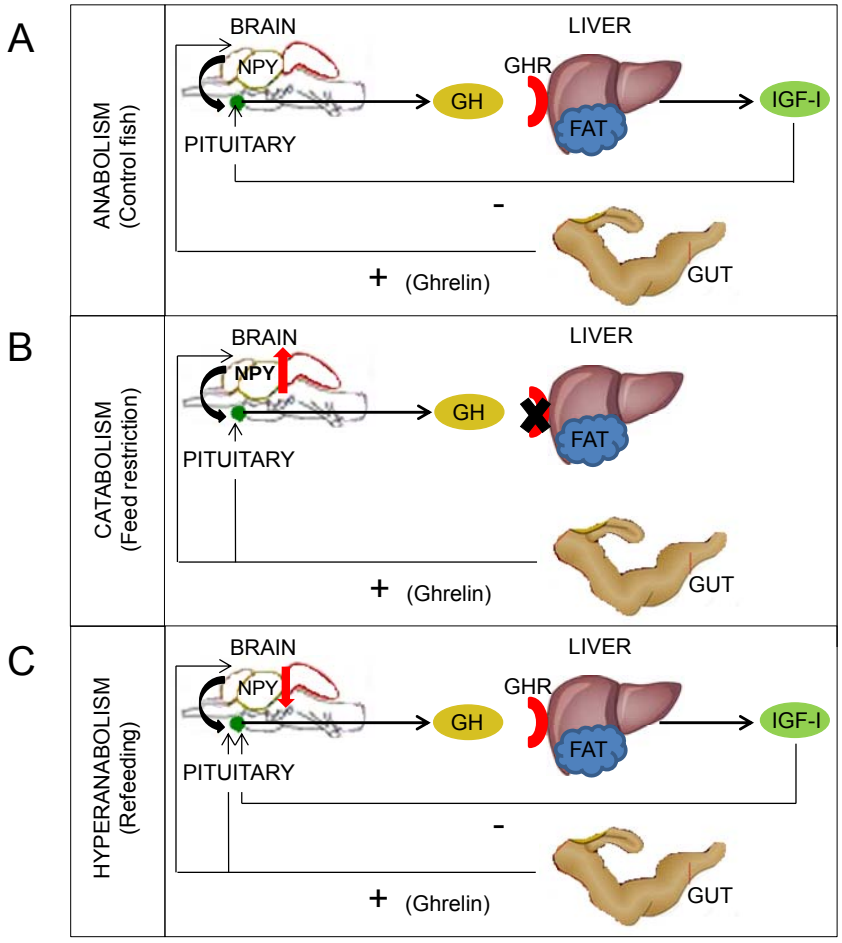


Figure 7. Endocrine control of compensatory growth: A) Anabolism: in a normal situation, the brain stimulates the pituitary to produce GH that will bind its receptor in the liver, produce IGF-I that will exert a negative feedback at the brain; B) Catabolism: during the feed restriction, the produced GH cannot bind its receptor (liver insensitivity) and IGF-I is not synthetized. Ghrelin then stimulates the brain and the pituitary; and C) Hyperanabolism: the refeeding sets everything but Ghrelin to its normal levels. Ghrelin continues to positively stimulate the brain and the pituitary, causing an increase in appetite and GH production what causes growth acceleration (Modified from Won and Borski, 2013).

4.3. Reproductive biology

The European sea bass, as many teleosts (Devlin and Nagahama, 2002), is a gonochoristic species where individuals develop only as males or females, and maintain the same sex throughout their life span. Among the different strategies of early gonadal development (differentiated or undifferentiated gonochoristic species), European sea bass is an example of differentiated gonochorism, where early gonad development proceeds from an undifferentiated gonad directly to ovary or testis (Blázquez et al., 1998).

The European sea bass karyotype consists of 24 subtelocentric-acrocentric chromosome pairs (Volckaert et al., 2008). The smallest acrocentric pair is heteromorphic for C-heterochromatin in males (Cano et al., 1996), suggesting an early differentiation stage of sex chromosomes in European sea bass, although no recognizable proper heterochromosomes (sex chromosomes) are present.

4.4. Sex determination and sex differentiation

The European sea bass is thought to present a polygenic sex determining system (Vandeputte et al., 2007) influenced by temperature (Piferrer et al., 2005). It should be noted that although a polygenic sex determination system is thought to be the ancestral type of sex determination in fish (Kirpichnikov, 1981), due to their intrinsic unstable nature (Bull, 1983) it has been accepted just in a few organisms (Vandeputte et al., 2007).

From a genetic point of view, in European sea bass female homogamety can be ruled out since sex ratios of normal diploid and gynogenetic offspring are equivalent (Felip et al., 2002; Peruzzi et al., 2004). Furthermore, since the offspring from the masculinized females is not female-biased, female homogamety (XX-XY) and male homogamety (ZW-ZZ) would not be possible (Blázquez et al., 1999). However, other interpretations of sex ratio data of hormone-treated fish suggest that male homogamety with environmentally male-biased sex ratio would still be a possibility (Vandeputte et al., 2007). Nevertheless, the actual accepted model fits with the definition of a polygenic sex determining system, where there is an

underlying sex tendency (determined both by polygenes and environmental effects) greater or less than a threshold value (Vandeputte et al., 2007). In addition, just one masculinizing environmental condition, temperature (Piferrer et al., 2005), can influence European sea bass sex ratios, and the genetic component seems to be due to differential sensitivity to the masculinizing power of temperature (Saillant et al., 2002).

Moreover, the statistical model analysis performed by Vandeputte et al. (2007) proved that assuming environmental variance, a two-factor system can be excluded but that a four-factor system (two biallelic loci) would also explain sex ratios results found in wild European sea bass populations. The variations found on female percent due to thermal treatments also show that genetic and environmental components are of comparable magnitude, supporting the notion of a continuum between GSD and ESD components of sex determination (see section 2.1.3. above) in the European sea bass.

Recent research performed in our laboratory (Navarro-Martín et al., 2011) showed how temperature during early development is linked to the production of male-biased populations through differences in the methylation levels of the gonadal aromatase promoter at one year (juvenile males double the DNA methylation levels of females). Thus, elevated (>17°C) temperatures at early stages (0–60 days post fertilization) masculinize about half of the fish that with lower temperature would have developed as females by hypermethylation of, at least, the gonadal aromatase promoter, hence inhibiting aromatase gene expression and preventing female differentiation due to the lack of estrogen.

The above effect is not a generalized thermal effect on gene promoters since the housekeeping gene beta-actin was not affected by temperature neither in brain or gonads. Also the temperature methylating effect was restricted to the gonads since no methylation differences were found for gonadal aromatase promoter at the brain (where gonadal aromatase mRNA is also detected, at a much lower levels than in the gonads). Different CpGs loci within the *cyp19a1a* promoter also showed different sensitivities to temperature. The analysis of *cyp19a1a* and beta-actin in the gonads and brain of undifferentiated fish (separated as high or low aromatase expressors

to reflect putative future females and males, respectively) showed the same effects. Thus, methylation of gonadal aromatase promoter is the cause of the lower expression of aromatase in the temperature-masculinized fish.

Further, *in vitro* experiments inducing the methylation of the aromatase promoter, suppressed the ability of *sf1* and *foxl2* transcription factors to stimulate gonadal aromatase synthesis. Since in normal situations these two transcription factors not only stimulate the production but also present and additive effect (Navarro-Martín et al., 2011).

In the European sea bass, gonadal differentiation (Figure 8) takes place in a caudo-craneal direction (Bruslé and Roblin, 1984). Gonads remain undifferentiated during post-larval stages until fish attain about 8 cm standard length, when sex differentiation, a process more related to length than to age (Blázquez et al., 1999) starts. This takes place when farmed European sea bass are usually about 5–6 months of age in females (Bruslé and Roblin, 1984; Saillant et al., 2003; Papadaki et al., 2005; Piferrer et al., 2005; Navarro-Martín et al., 2009b; Díaz et al., 2013). European sea bass females differentiate earlier than males by entering into meiosis and initiating somatic cell proliferation to form the ovarian cavity (Bruslé and Roblin, 1984), are bigger than males and mature later (Blázquez et al., 1999; Navarro-Martín et al., 2009b; Díaz et al., 2013). While still sexually undifferentiated, European sea bass gonads can be influenced by environmental abiotic factors (see section 4.5. below) or external factors such as sex steroids (Saillant et al., 2003b; Navarro-Martín et al., 2009a); but once sex is determined remains the same throughout life (Gorshkov et al., 1999; Zanuy et al., 2001).

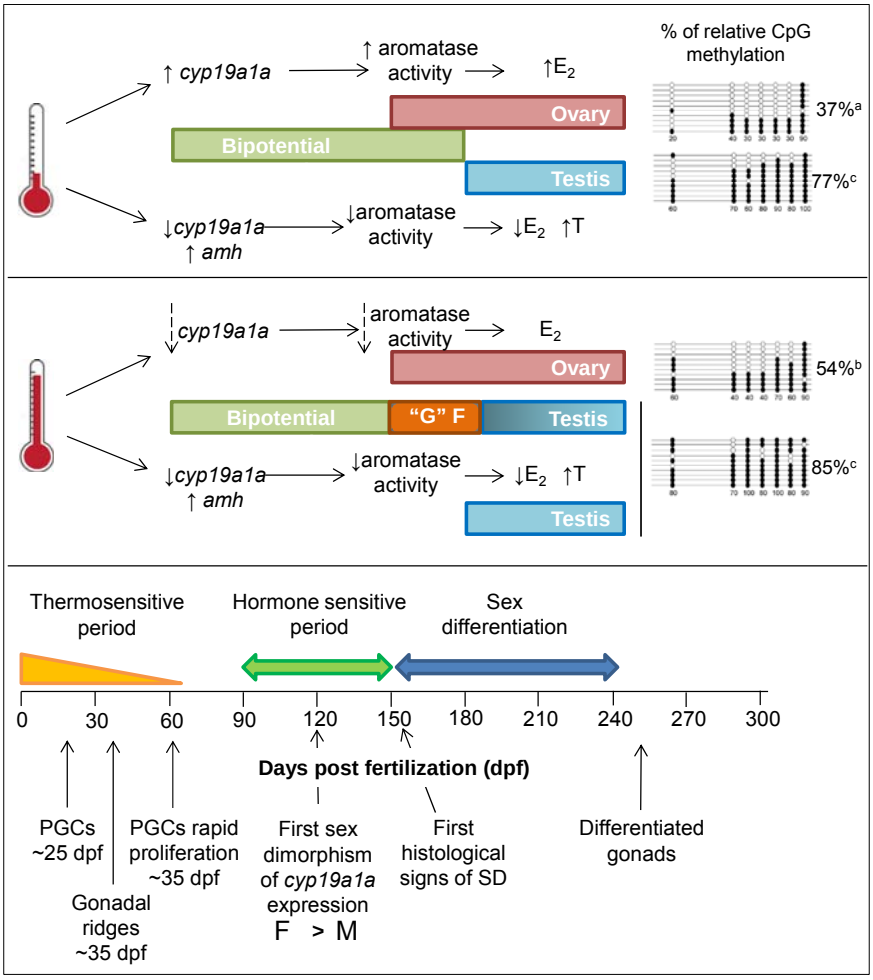


Figure 8. A schematic view of the European sea bass gonadal differentiation. At the bottom of the figure the morphological aspects of European sea bass gonadal differentiation are summarized, from the primordial germ cells (PGC) appearance to fully differentiated gonads. Also the thermosensitive and hormonal sensitive period are highlighted. At the upper panel, "normal" situation, i.e., temperatures below 17ºC during the thermosensitive period do not affect sex differentiation neither for females or males, is shown. However, at the middle panel, unusually high temperatures (around 21ºC, as the ones used in European sea bass hatcheries to speed up larval growth) affect the bipotential gonad by blocking aromatase expression, hence inhibiting enzyme activity. High levels of testosterone are observed since it is not converted to estrogens by aromatase, which implies the masculinization of part of the "G" F, genotypic females. The CpG relative percent methylation (solid circles) of the gonadal aromatase promoter is also shown at the right part of the figure with different letters indicating statistical differences. Based on Piferrer et al., 2005; Navarro-Martín et al., 2009b and Navarro-Martín et al., 2011.

Among the several genes involved in fish sex differentiation, some of them having two copies due to the genome duplication in the European sea bass; aromatase (cytochrome P450, *cyp19a1a*) is predominantly expressed in the ovary (Dalla-Valle et al., 2002) while *cyp19a1b* is mostly expressed in the brain (Blázquez and Piferrer, 2004). *cyp19a1a* promoter presents some interesting conserved binding sites such as sf1, sox, foxl2 or androgen receptor elements. Among them, sf1 transcription factor have shown to specifically bound to the promoter to directly regulate *cyp19a1a* transcription (Galay-Burgos et al., 2006). This observation is in accordance with an *in vitro* analysis where the addition of sf1 not only activated *cyp19a1a* expression but also showed an additive effect when added together with foxl2 (Navarro-Martín et al., 2011). *cyp19a1a* expression in adult European sea bass is higher in ovaries than in testis and also higher in gonads when compared to brain levels (Dalla-Valle et al., 2002). In addition, significant differences among gender are visible at 120 days post hatch (dph), just before the first histologically observable signs of sex differentiation and are maximal when sex differentiation is completed. Thus, *cyp19a1a* can be considered as an earlier marker of ovarian differentiation (Blázquez et al., 2008).

A total of 13 different sox genes have been identified in European sea bass (Galay-Burgos et al., 2004) belonging to four different Sox gene families (B, C, E and F), with some of them showing sexual dimorphic expression such as *sox9.2* at the end of the first year (Galay-Burgos et al., 2004) and suggesting a role in late testis differentiation. *sox19* and *sox17* are upregulated during ovarian differentiation (Navarro-Martín et al., 2009c and 2012). The expression of the latter increasing at around 150 dph, more or less coinciding with the onset of ovarian differentiation and then showing a higher expression in the ovaries (at 250 dph), hence suggesting also a role during ovarian development (Navarro-Martín et al., 2009c).

dmrt1 has two transcripts in European sea bass (*dmrt1a* and *dmrt1b*) with the same pattern of expression in gonads (Deloffre et al., 2009), with a sex dimorphic expression (more expression in males than in females) and with a high expression during gonad development. Altogether, these observations suggest that *dmrt1* in

European sea bass is not involved in early sex differentiation but, it is in gonad development and spermatogenesis. In contrast, another male-related gene, *dax1*, does not show any evidence of sexual dimorphism during sex differentiation (Martins et al., 2007). Besides, the 11beta-hydroxylase (*cyp11b*), an enzyme involved in the synthesis of 11-oxygenated androgens, shows a high dimorphic expression during European sea bass gonadogenesis with higher levels in testis and is not affected by temperature changes (Socorro et al., 2007), suggesting an involvement in testis differentiation.

Sex steroids effects on European sea bass sex differentiation have already been well studied (Zanuy et al., 2001), and in European sea bass, exogenous estrogens are able to induce ovarian differentiation (Blázquez et al., 1998). Endogenous estrogens are present even before the first primary oocytes appear (Papadaki et al., 2005). On the other hand, androgens do not appear to be involved in testis differentiation since their expression (testosterone) is posterior to the onset of sex differentiation and thus they can be considered just as a product of male sex differentiation (Rodríguez et al., 2004; Papadaki et al., 2005). Moreover, 11-KT levels are also related to spermatogenesis but not to male differentiation (Papadaki et al., 2005).

In European sea bass, there are three estrogen receptors (*erα, erβ1* and *erβ2;* Halm et al., 2004). e*rα* shows high expression levels at around 200 dph while *erβ1* and *erβ2* highest expression takes place around 250 dph (Halm et al., 2004), showing a higher expression in males (Blázquez et al., 2008), mainly in spermatogonia and spermatocytes (Viñas and Piferrer, 2008), and suggesting a role in testis maturation and spermatogenesis (Blázquez et al., 2008).

4.5. Influences of the environment

In the European sea bass, it is clear that sex determination is labile and affected by environmental factors, mainly temperature (Piferrer et al., 2005). Other factors have also been studied:

Density has no direct effect on the male-bias found in aquaculture facilities since sex ratios where similar between high and low

density reared fish, and no differences in growth were present after 200 dpf (Saillant et al., 2003b). Moreover, repeated size-grading plus density treatment do not affect sex ratios (Saillant et al., 2003b). However, if high density depresses growth for a long period during a critical developmental time it can lead to an increase in male percent (Piferrer et al., 2005).

Exogenous administration of steroids during the hormone sensitive period can alter the process of sex differentiation, as in many other fish. In that sense, treatments with androgens or estrogen inhibitors would lead to male differentiation, while the opposite is true for estrogen administration (Zanuy et al., 2001; Saillant et al., 2001a; Navarro-Martín et al., 2009a; Díaz et al., 2013).

Photoperiod is considered one of the most important environmental stimuli affecting the onset of puberty in fish (Rodríguez et al., 2012). Hence, photoperiod control may avoid early maturing during the first year. However, since continuous light regimes, known to reduce precocious males in European sea bass (Begtashi et al., 2004; Felip et al., 2008; Carrillo et al., 2009), may be causing permanent stress to fish and are difficult to apply at the outdoor facilities (Carrillo et al., 2009; Taranger et al., 2010). Instead, a short-term continuous light exposure during September has been proposed to reduce precocious gametogenesis in sea bass (Rodríguez et al., 2012). However, there is no evidence of photoperiod effects on European sea bass sex differentiation.

4.6. Importance for aquaculture

The European sea bass is one of the most important species in European aquaculture. Thus, while captures are stable at around 10 KTsince 2002, in contrast, its aquaculture production has increased from ~4 KT to 144 KT in just 20 years (www.fao.org).

Zootechnical aspects of European sea bass culture are well established, allowing all year production through the manipulation of culture conditions. Photoperiod and temperature are among the most important parameters. Regarding the photoperiod, for larvae and post-larvae a French method is applied (darkness from

fertilization to 5-7 days post fertilization (dpf) and then a gradual increase of light exposure) and the broodstock is submitted to natural photoperiod at least at the gametogenesis period to allow proper gonad maturation (Moretti et al., 1999). On the other hand, aquaculture protocols suggest raising European sea bass at 13ºC-15ºC from fertilization/spawning to swim bladder inflation and then gradually increase temperature at a ratio of 0.5ºC/day until 20ºC (Moretti et al., 1999). Furthermore, Dülger and collaborators (2012) have suggested raising European sea bass at 25ºC all year round. Although this protocol is widely applied at commercial hatcheries, it does not take into account the effect it may have on sex ratio bias in favor of males (~75-100%) and neither its related problems such as precocious male maturity (Carrillo et al., 1995), increase in aggressiveness, etc.

From a commercial point of view, this implies a loss in potential produced biomass since not only female European sea bass grow bigger than males (Carrillo et al., 1995; Blázquez et al., 1999; Pavlidis et al., 2000; Saillant et al., 2001a; Saillant et al., 2003b; Navarro-Martín et al., 2009b; Díaz et al., 2013) but also immature males grow less than mature males during the first year and they also grow smaller than immature females either at one (Gorshkov et al., 1999; Saillant et al., 2003b; Begtashi et al., 2004; Papadaki et al., 2005) or at two years old (Felip et al., 2006). Furthermore, there are no growth differences between females and feminized fish by E_2 treatment and in turn these females are bigger than males (Saillant et al., 2001a). Altogether these results are of great interest to the private sector and that is why all-female European sea bass stocks can be of advantage. In this sense, several approaches have been proposed from hormonal treatments (Zanuy et al., 2001) to environmental abiotic factors control (Zanuy et al., 2001; Piferrer et al., 2005; Navarro-Martín et al., 2009b).

Among the abiotic environmental factors, temperature is the most important factor affecting European sea bass sex ratios. While 15ºC has been proposed as the optimal temperature for larval stages (Koumoundouros et al., 2001), optimal growth in juveniles is found at 26ºC and 13ºC is considered as detrimental (Person-Le Ruyet et al., 2004). Hatcheries apply high temperatures to speed up growth rates to be able to sell fish as fast as possible. However, on-growers

face the problem of male-bias and the consequent loss of the biomass mentioned above.

Many studies of the thermal effects on sex ratios in European sea bass have been performed. Initial studies showed that in the European sea bass the thermolabile period includes from half-epiboly to mid-metamorphosis (17-18 mm total length; ~70 dph) (Pavlidis et al., 2000; Koumoundouros et al., 2002). Treating fish with high temperatures, as the aquaculture enterprises do, masculinize a high proportion of the population (Carrillo et al., 1995). Furthermore, treating fish with temperatures never surpassing 17°C until metamorphosis yielded the maximum female percent found to date (Pavlidis et al., 2000). However, raising fish at low temperatures (15ºC) for a long period also masculinize the population even more than a high temperature thermal treatment (Saillant et al., 2002). In that sense, our research group developed (Navarro-Martín et al., 2009b) and patented a thermal protocol to maximize the number of females (patent noN200802927 "Método para el control térmico de la proporción de sexos en la lubina"). Such protocol consisted on maintaining 17ºC water temperature during the thermosensitive period, and then increasing the temperature as a ratio of 0.5ºC/day until 21ºC to allow high growth rates. This allows the highest proportion of females in the population by avoiding the masculinizing effects of the high and sustained low temperatures (Navarro-Martín et al., 2009b) without affecting growth. These results have been independently confirmed by similar experiments in other labs (Sfakianakis et al., 2013) and furthermore, they have suggested that growing fish at low temperatures during early life not only also increases female proportion and hence growth performance, but also decreases skeletal abnormalities.

4.7. Pending issues in regards to its sex determination and sex differentiation

The European sea bass is a species with a proposed four-factor polygenic sex determination system. Thus, it follows that, knowing the true nature of the four-factor system governing European sea bass sex and how external factors, particularly temperature,

influence them would be undoubtedly very useful not only because factors in polygenic systems are unknown, but also for the development of knowledge-based sex control techniques to improve aquaculture production.

Future analysis using Next Generation Sequencing (NGS) such as RNA-sequencing or custom-made microarrays would allow a better understanding of the genes that are not only involved in the sex determination/differentiation process at normal conditions but also to analyze disruptions due to changes in environmental conditions. This will be facilitated by the publication of the European sea bass genome.

Furthermore, since the European sea bass is a eurythermal and euryhaline species which can adapt to a wide range of environments, a broader analysis of the epigenetic mechanisms that govern sex determination and differentiation are needed in order to determine the relevance of these mechanisms in such species.

Aims

The overall objective of this thesis was to contribute to our understanding of the effects of environmental factors on the juvenile growth, gonadal transcriptome and resulting sex ratios of fish, a group of vertebrates known for having extraordinary diversity and a very plastic sex. For this, we used the European sea bass (*Dicentrarchus labrax*), a marine teleost with a polygenic system of sex determination where genetics and environment contribute to sex ratios.

The research involved carrying out experiments with captive sea bass under controlled environmental conditions and subjected to specific treatments. In addition to general biometric analysis, the work focuses on analyses of sex ratios, gonadal morphology and, particularly, an in-depth analysis of global gene expression including signaling pathways and estimation of protein-protein interactions.

The specific objectives were:

1. To investigate the relationship between growth and sex ratios in the European sea bass by altering growth during the period of sex differentiation, using size-grading and hormonal therapy and then examining the resulting sex ratios of different populations.

2. To analyze the consequences of growth on the molecular endocrinology of sexually undifferentiated and juvenile gonads. This is achieved by using both fish with different natural growth rates and fish where growth has been manipulated through changes in food intake, and then using a custom-made microarray to study differential gene expression, affected pathways and protein-protein interactions.

3. To understand how the early environment sets permanent changes that result in long-lasting consequences in the sexual phenotype. This is achieved by studying the effects of elevated temperature at the whole gonadal transcriptomic level, not after, but at the time of sex differentiation, in order to gain a better

understanding of the genes and the involved pathways that are directly affected by temperature.

4. To determine whether the same patterns of gene expression involved in sex differentiation that operate in species with chromosomal systems of sex determination also operate in species with a polygenic system of sex determination. This is achieved by comparing patterns of gene expression at the time when gonads are experiencing environmentally- and hormonally-induced opposite pathways of differentiation.

Block A: Growth and sex differentiation

Chapter 1

Block A: Growth and sex differentiation

Chapter 1. The relationship between growth and sex differentiation in the European sea bass (*Dicentrarchus labrax*).

Díaz N., Ribas L., Piferrer F. The relationship between growth and sex differentiation in the European sea bass (Dicentrarchus labrax). Aquaculture (2013). 408-409: 191-202. doi:10.1016/j.aquaculture.2013.05.032

Chapter 2

CHANGES IN FOOD SUPPLY AT THE TIME OF SEX DIFFERENTIATION AFFECT THE TESTICULAR TRANSCRIPTOME OF ONE-YEAR-OLD JUVENILE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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Abstract

Proper food supply is one of the main factors influencing growth rates in both natural and farmed fish populations since food restriction results in reduced growth and has deleterious effects on reproduction. In the European sea bass, sex-related differences in growth are established just before fish attain ~4 cm, i.e., before histological sex differentiation. At that point, females are more abundant among the larger size ranges, although they are still present among the lower size ranges, where males predominate. The objective of this study was to analyze the consequences of growth on the molecular endocrinology of sexually undifferentiated and juvenile gonads. We analyzed the gonadal transcriptome of European sea bass at two developmental times utilizing a custom-made microarray: 1) 4-month-old sexually undifferentiated fish, comparing the gonads of the fish with the highest growth rates with those with the slowest growth rates when food supply was not limited, and 2) testis from 11-month-old juveniles were growth was manipulated during ~7 months through changes in food intake. The four groups used were: a) sustained fast growth, b) sustained slow growth, c) accelerated growth, or d) decelerated growth. Results showed that the transcriptomes of undifferentiated gonads were not drastically affected by initial natural differences in growth rates, and that regardless whether the gonad was sexually undifferentiated or differentiated, significant changes in the expression of genes

associated with protein turnover were seen, favoring catabolism in slow-growing fish and anabolism in fast-growing fish. Moreover, while fast-growing fish took energy from glucose, as deduced from the pathways affected and the analysis of protein-protein interactions examined, in slow-growing fish lipid metabolism and gluconeogenesis was favored. The highest transcriptomal differences were found when forcing initially fast-growing fish to decelerate their growth, while accelerating growth of initially slow-growing fish resulted in transcriptomal convergence with sustained fast-growing fish. Taken together, these results indicate that food availability during sex differentiation shapes the juvenile testis transcriptome, as evidenced by physiological adaptations to different energy balances. Remarkably, this occurs in absence of major histological changes in the cellular composition of the testis. Thus, fish are able to transcriptionally recover their testes if they are provided with enough food supply during sex differentiation; however, an initial fast growth does not represent any advantage in terms of transcriptional fitness if later food becomes scarce.

1. Introduction

Food availability and energetic demands fluctuate in most habitats. Animals are capable of sensing their inner energy levels and the external energy availability and thus act accordingly by long-term investments in processes such as growth, immune functions or reproduction when food availability is not a problem, or by ensuring survival when food is scarce (Schneider, 2004). Moreover, there is a tight relationship between food availability and reproduction (Schneider, 2004; Castellano et al., 2009) since it can alter the timing and duration of spawning, fecundity and egg size (Volkoff et al., 2005; Morgan et al., 2013), or the timing of the reproductive cycles (Yoneda and Wright, 2005). Favorable feeding conditions produce early maturation of individuals (Kjesbu, 1994) while a decrease in food availability causes a decrease in energy transfer to the gonads (Marshall et al., 1999), but this relationship may present important differences between species since fish is a vast phylogenetic group with different behaviors and reproduction types (Devlin and Nagahama, 2002).

The European sea bass (*Dicentrarchus labrax*) is a gonochoristic species with a polygenic sex determining system (Vandeputte et al., 2007) presenting a long sexually undifferentiated process with sexual dimorphism at the time of sex differentiation (SD) onset (~150 days post hatch, dph, for females and ~180 dph for males) (Roblin and Bruslé, 1983; Blázquez et al., 1995; Mylonas et al., 2005; Papadaki et al., 2005). However, this dimorphism is more related to the attained length than to age (Blázquez et al., 1999). The relationship between growth and SD has been previously studied in sea bass (Blázquez et al., 1999; Koumoundouros et al., 2002; Mylonas et al., 2005; Papadaki et al., 2005; Saillant et al., 2003a, 2003b; Vandeputte et al., 2007; Navarro-Martín et al., 2009b; Díaz et al., 2013). There is a relationship between body weight and sex since not only sea bass females are larger than males, but also both males and females in batches with higher percent females were bigger than males and females of batches with a lower female percent (Vandeputte et al., 2007). Further, early size-gradings of the population (at 66 and 123-143 dph, Papadaki et al., 2005; at 70 dph, Koumoundouros et al., 2002; at 82 dph, Saillant et al., 2003b) selecting for the largest fish resulted in ~90% of females, but the opposite, i.e., selecting for the smallest fish produces only ~65% males at one year of age, meaning that while the largest fish are essentially all females, among the smallest fish there are both males and females (Papadaki et al., 2005).

Recently, two experiments on growth rate alteration by manipulating food supply during the SD period were conducted on European sea bass in our laboratory (Díaz et al., 2013). The first experiment showed that transiently but severely reducing food supply starting towards the end, middle or even at the beginning of the SD period, and thus negatively affecting growth, did not affect the course of sex differentiation, indicating that gender was already fixed before the SD period started (Díaz et al, 2013). The second experiment involved four groups of fish which, through controlling food supply, were made to experience different growth rates during the SD period. Two groups, one fast-growing and the other slow-growing, originated from the fast-growing fish at 127 dph. The other two groups, also one fast-growing and the other slow-growing, originated from the slow-growing fish at 127 dph. In this case, there were differences in the final sex ratio of the population as fast-growing derived groups presented more number of females (~40%) than the

slow-growing groups (~10%). Thus, the differences in the final sex ratio were not related to the growth rate during the SD but to whether fish derived from the fast- or slow-growing fish at 127 dph. These results confirmed the results of the first experiment and indicated that before the first signs of SD appear, the relationship between growth and sex is already established and confirmed that in the European sea bass larger sizes are associated with female development (Papadaki et al., 2005; Díaz et al., 2013).

Partition of consumed energy into growth, energy storage, and gonads according to temporal food availability, metabolic demands, and reproductive needs have been studied since a long time ago (Adams et al., 1982). Recently, with the advent of new technologies, the underlying mechanisms including associated changes in global gene expression can be investigated. However, transcriptomic analyses in fish have traditionally addressed nutrition and reproduction topics separately. Hence, while efforts on growth studies have been put towards the effects of diet substitutions (Geay et al., 2011; Calduch-Giner et al., 2012; Campos et al., 2010; Tacchi et al., 2011), stocking density and food ration (Salas-Leiton et al., 2010; Martin et al., 2010; Yi et al., 2013), or comparing domesticated vs. transgenic fish (Overtuf et al., 2010); reproduction transcriptome analyses have focused on describing gonad maturation and gonadal differences between sexes (Sun et al., 2013; Ravi et al., 2014; Tao et al., 2013; Rolland et al., 2009), environmental effects (Bozinovic and Oleksiak, 2011) or hormonal treatment effects (Schiller et al., 2013a and 2013b).

However, a study directly analyzing the effects of food supply on reproduction and particularly on the development of juvenile gonads has never been described in fish. In mitten crab (*Eriocheir sinensis*) during early development, when crabs store significant amounts of energy in the hepatopancreas, Jiang and collaborators (2009) found four genes in the hepatopancreas and 13 genes in testis related to nutritional control, and three genes in the hepatopancreas and eight in the testes related to regulation of reproduction. Among the former, arginine kinase, zinc-finger proteins or leptin were upregulated in the hepatopancreas transcriptome as a sign of energy storage for further energy-demand of the reproductive processes. Genes involved in the regulation of reproduction such as cyclins, kinetochore spindle formation or the heat shock protein 70 were upregulated in testis and

promote an increase in cell division during spermatogenesis. In rats, dietary energy intake changes (restrictions and excesses) but also food availability have profound effects in gonads from both sex at different levels (biochemical, endocrine, behavioral and genetic; Martin et al., 2007). Moreover, a transcriptomic analysis of these gonads facing diet restriction or excess, showed how females were more affected by ration changes than males. Males were also better adapted to an intermittent fasting by increasing the probability of an eventual fertilization, while females were able to sense the food restriction and behaved as sub-fertile females (Martin et al., 2009).

The present study is based on samples collected in experiment 2 of Díaz et al. (2013) described above and had two objectives: 1) to analyze the transcriptional differences in sexually differentiating European sea bass gonads from the fastest growing vs. the slowest growing fish at 127 dph, i.e., before the first histological signs of SD at 150 dph (Papadaki et al., 2005) but after the first signs of molecular SD at 120 dph (Blázquez et al. 2009) to explore a possible link of transcriptomic signatures with future sex, and 2) the consequences of food availability between 133-337 dph (juvenile growth) on the subsequent testes transcriptome by analyzing the effects of growth acceleration and deceleration.

2. Materials and methods

2.1 Animals and rearing conditions

The fish that were transcriptomically analyzed in this study are the same fish described in Experiment 2 of Díaz et al. (2013). Briefly, European sea bass eggs obtained from a commercial hatchery were collected at one day post fertilization (dpf) on March 2009, transported to our experimental aquarium facilities and hatched following established procedures for this species (Moretti et al., 1999) with minor changes, as previously described (Navarro-Martín et al. 2009b; Díaz et al., 2013). Fish were reared under natural conditions of photoperiod, pH (~7.9), salinity (~37.8 ppt), oxygen saturation (85–100%) and with a water renewal rate of 30% vol·h^{-1}. In order to avoid temperature influences on the sex ratio, the thermal regime used and previously described (Díaz et al., 2013) included

egg spawning at 13–14°C and larval rearing at 16 ± 1°C until 60 dpf. Then, temperature was increased to 21°C at a rate of 0.5-1°C·day^{-1} and maintained until the first fall, when it was let to follow the natural temperature. The rearing density was kept low to avoid any possible distorting effect on sex ratios (details in Díaz et al., 2013). Fish were manually fed three times a day with artemia AF, then artemia EG enriched with aminoacid (INVE Aquaculture, Belgium) and dry food (ProAqua, S.A., Spain) of the appropriate pellet size as fish grew. Unless otherwise stated, juveniles and adults were fed *ad libitum* two times a day. Fish were treated in agreement with the European Convention for the Protection of Animals used for Experimental and Scientific Purpouses (ETS Nu 123, 01/01/91).

2.2 Experimental design

Details of the experimental design can also be found in Díaz et al. (2013). Briefly, fish were individually size-graded at 127 dph, i.e., at ~4 cm standard length (SL), before the histological process of SD started (~8 cm SL), and separated into two groups according to the SL they had attained and comprising the two extremes of the normal curve distribution: a fast growth group (group F), with a mean size of 5.0 cm SL (range 4.2–6.4 cm), and a slow growth group (group S), with a mean size of 3.5 cm SL (range 2.6–3.7 cm). After checking that fish of each group was of the desired size, then at 133 dph (time 1, T1, i.e., when fish were 4 months old) each group was further subdivided into two tanks (n = 79 fish per tank). On one hand, the F group was subdivided into two groups with initial similar mean SL and BW: the fast-fast group (FF), in which growth rates from that moment onwards were as before, and the fast-slow group (FS), in which the growth rate was reduced to match what had been the growth rate of group S until then. On the other hand, the S group was also subdivided into two groups with initial similar mean SL and BW: the slow-fast group (SF), in which the growth rate was increased to match what had been the growth rate of group F until then, and the slow-slow group (SS), in which the growth rates from that moment onwards were as before (see Fig. 1A for a diagram of the experimental design). Food supply changed as follows: prior to T1, all fish were fed *ad libitum* three times a day. Then, between T1 and time 2 (T2), groups FF and SF (accelerated growth) were fed *ad*

libitum four times a day, with an amount of food per day equivalent of 3–6% of the mean BW. On the other hand, groups FS and SS (decelerated growth) were feed only two times a day with an amount of food per day equivalent of 1.5–3% of the mean BW. The growth rate of all groups was carefully monitored by periodic samplings and the amount of food adjusted if necessary. Animals were sacrificed with an overdose of 2-phenoxyethanol (2PE) when juveniles were 337 dph (T2, i.e., when fish were 11 months old) (range of fish per tank at that moment: 52-70). There was no mortality associated to treatments.

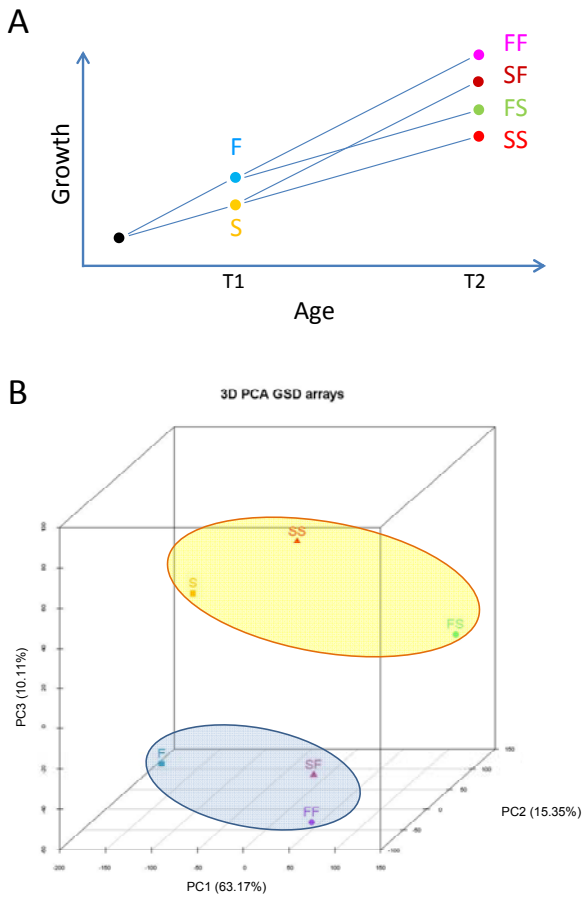


Figure 1. A) Experimental design of the European sea bass subjected to food restriction at different times during the sex differentiation period. B) Principal component analysis representation of the six groups. Time 1 fast- (F) and slow- (S) growing groups, and time 2 F- (FF and FS) and S- (SF and SS) derived groups.

2.3 Samplings

Details on the follow-up of growth, including sexual growth dimorphism, gonadosomatic, hepatosomatic and carcass indices, as well as sex ratio and the degree of gonadal development of these fish have been previously described (Díaz et al., 2013). When possible, sex was visually determined and confirmed histologically if necessary (Díaz et al., 2013). Only males were considered for the present study and histological results indicated that testis contained no spermatozoa. The number of fish used for each group and the biometry is shown in Supplementary Table 1. Here, we focus only on the RNA extraction for transcriptomic analysis of gene expression and for microarray validation by qRT-PCR.

2.4 RNA extraction and cDNA synthesis

Total RNA was extracted from sexually undifferentiated gonads (mean SL ~4 cm) at 133 dph (T1) and sexually differentiated juvenile testis at 337 dph (T2).

A classical chloroform-isopropanol-ethanol RNA extraction protocol after a Trizol (Live Technologies, Scotland, UK) homogenization was used. RNA quality and concentration were measured by a ND-1000 spectrophotometer (NanoDrop Technologies) and checked on a 1% agarose/formaldehyde gel. RNA integrity was measured by a Bioanalyzer 2100 (RNA 6000 Nano LabChip kit Agilent, Spain). Samples with a 100-200 ng/µl RNA concentration and RIN>7 were used for microarray hybridizations.

In parallel, 200 ng of total RNA were treated by *E.coli* DNAse H and retrotranscribed (100 ng) to cDNA using SuperScript III RNase Transcriptase (Invitrogen, Spain) and Random hexamer (Invitrogen, Spain) following manufacturer's instructions.

2.5 Microarray

2.5.1. Experimental design

Hybridizations were performed at the Universitat Autònoma de Barcelona (UAB, Spain). The experiment included, on one hand, the comparison of 15 undifferentiated gonads from two groups (seven individual gonads from the F group against eight individual gonads from the S group) at 133 dph (T1), to explore transcriptomic differences among two groups with different growth rates since they were selected from the opposite extremes of the normal distribution curve. On the other hand, individual testes from each one of the four groups (groups FF, SF, FS and SS) differentially fed and sampled at 337 dph (T2), were analyzed to investigate the growth acceleration or growth deceleration effects on their transcriptomes. Thus, a total of 35 microarrays (one per fish) were used to analyze the gonadal transcriptome of the six groups considered in this study. To avoid batch effects samples were evenly distributed among the slides.

2.5.2 RNA sample preparation and array hybridization

RNA labelling, hybridizations, and scanning were performed according to manufacturer's instructions. Briefly, total RNA (100 ng) was amplified and Cy3-labeled with Agilent's One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labelling kit) along with Agilent's One-Color RNA SpikeIn Kit. Then cRNA was purified with RNeasy mini spin columns (Qiagen), quantified with the Nanodrop ND–1000 and verified using the Bioanalyzer 2100. Each sample (1.65 μg) was hybridized to *Dicentrarchus labrax* array (Agilent ID 023790) at 65°C for 17 h using Agilent's GE Hybridization Kit. Washes were conducted as recommended by the manufacturer using Agilent's Gene Expression Wash Pack with stabilization and drying solution. Arrays were scanned with Agilent Technologies Scanner, model G2505B. Spot intensities and other quality control features were extracted with Agilent's Feature Extraction software version 10.4.0.0. The experiment has been submitted to Gene Expression Omnibus (GEO)-NCBI database (GSE54362) and the platform that validates the microarray has the accession number (GPL13443).

2.6 Quantitative real time PCR (qRT-PCR)

Microarray validations were carried out by qRT-PCR analysis. Two genes from each one of the six possible microarray comparisons (see Supplementary Table 2) were used for qRT-PCR validation, including one up- and another downregulated. Primers were designed using Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) against the annotated gene sequences directly from the European sea bass genome (Tine et al., 2014 unpublished), always trying to design the primers between exons to avoid DNA contamination (Supplementary Table 2). Primer amplification efficiencies were tested by linear regression analysis from a cDNA dilution series and by running a melting curve (95ºC for 15 s, 60ºC for 15 s and 95ºC for 15 s). Efficiency (E: $E=10^{(-1/slope)}$, with values between 1.80 and 2.20), standard curves ranging from –2.9 to –3.9 and linear correlations (R^2) higher than 0.92 were measured (Supplementary Table 2). cDNA was diluted 1:10 for all the target genes and 1:500 for the endogenous control (the housekeeping gene *r18S*).

qRT-PCR was analyzed by an ABI 7900HT (Applied Biosystems) under standard cycling conditions. Briefly, an initial UDG decontamination cycle (50ºC for 2 min), an activation step (95ºC for 10 min) was followed by 40 cycles of denaturation (95ºC for 15 s) and one annealing/extension step (60ºC for 1 min). A final dissociation step was also added (95ºC for 15 s and 60ºC for another 15 s). Each sample was run in triplicate in 384-well plates in a final 10 µl volume (2 µl of 5x PyroTaq EvaGreen qPCR Mix Plus (ROX) from Cultek Molecular Bioline, 4 µl distilled water, 2 µl primer mix at a 10 µM concentration and 2 µl of cDNA). Negative controls were run per duplicate and *r18S* was used to calculate intra- and inter-assay variations. SDS 2.3 software (Applied Biosystems) was used to collect raw data and RQ Manager 1.2 (Applied Biosystems) was used to calculate gene expression. qRT-PCR data was analyzed by adjusting for E and normalizing to the *r18S* reference gene (Schmittgen and Livak, 2008).

2.7 Statistical analysis of data

2.7.1 Microarray raw data normalization

Feature Extraction output data was corrected for background using normexp method (Ritchie et al., 2007) and also was quantile normalized (Bolstad, 2001). Reliable probes showed raw foreground intensity at least two times higher than the respective background intensity and were not saturated nor flagged by the Feature Extraction software. Our sea bass custom-made microarray presents most of the probes (64.7%) in duplicate but also with more than three identical probes for some genes. Median intensities per gene were calculated and a probe was considered reliable when at least half of its replicates were reliable as defined above. An empirical Bayes approach on linear models (Limma) (Smyth, 2004) was used to perform a differential expression analysis. A False Discovery Rate (FDR) method was used to correct for multiple testing. Differentially expressed (DE) genes were filtered by fixing an absolute fold change (FC) of 1.5 and an adjusted *P*-value<0.01. MA data analyses were performed with the Bioconductor project (http://www.bioconductor.org/) in the R statistical environment (http://cran.rproject.org/) (Gentleman et al., 2004).

2.7.2 qRT-PCR statistics

Quantitative qRT-PCR statistical analysis was performed using 2DCt from the processed data (Schmittgen and Livak, 2008). 2DCt results were then checked for normality, homoscedasticity of variance and a one-way ANOVA test was used to assess differences between treatments using SPSS v.19 software.

2.8 Gene annotation and enrichment analysis

Gene data (names, abbreviations, synonyms and functions) were determined using Genecards (http://www.genecards.org/) and Uniprot (http://www.uniprot.org/). The web based tool AMIGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) (Carbon et al., 2009) was used to look for the sequences of the DE genes found at the MA. After obtaining these sequences, Blast2GO software

(http://www.blast2go.com) (Conesa et al., 2005) was used to enrich GO term annotation and to analyze the subsequent altered KEGG pathways (http://www.genome.jp/kegg/), which were also further explored by DAVID (http://david.abcc.ncifcrf.gov/; Huang et al., 2009a, 2009b). Completing the analysis, Blast2GO with Fisher's Exact Test with Multiple Testing Correction of the False Discovery Rate (Benjamini and Hochberg, 1995) was used to analyze our DE genes using the custom-made microarray as background.

Protein names from the DE genes were then uploaded to the web-based tool STRING v9.1 (http://string-db.org/) (Franceschini et al., 2013) to analyze physical and functional protein interactions. Furthermore, an FDR test was applied to determine if the protein list was enriched (higher values meaning higher significances). A Mean Linkage Clustering (MLC or UPGMA), a simple agglomerative hierarchical clustering included in STRING was performed to group proteins. This method clusters proteins based on pairwise similarities in relevant descriptor variables.

3. Results

3.1. Overall view of transcriptomic results

Visualization of the spatial distribution of the microarray data of the six studied groups along the three major axis of the PCA is shown in Figure 1B. Component 1 contributed to 63.17% of the variation while the first three components together explained 88.63% of the variation. Two clusters could be observed, one containing group F and the F-derived groups with an accelerated growth (groups FF and SF), and the other formed by group S and the S-derived groups with growth deceleration (groups SS and FS).

The number of DE genes found in the only possible comparison at T1 as well as in the six possible comparisons between the four groups at T2 is shown in Table 1. The comparison with larger number of genes was FS vs. SS, while the FF vs. SF comparison gave no DE genes. From each one of the comparisons with DE genes, the most upregulated and the most downregulated genes (a total of twelve) were selected for a qRT-PCR validation (see details

and quality control data of the designed primers in Supplementary Table 2). All genes tested showed the same fold change tendency, thus validating the microarray results (Table 2). Among the tested genes four of them (*cct6a*, *rps15*, *fabp3* and *rpl9*) showed statistical differences ($P<0.05$) when analyzed by qRT-PCR.

Table 1. Differentially expressed genes at the different comparisons

Group comparisons	Total # of genes	# Upregulated genes			# Downregulated genes		
		Total	Real	NA	Total	Real	NA
F vs. S	76	41	20	11	35	20	6
FF vs. SS	155	71	43	9	114	70	30
FS vs. FF	1092	662	316	47	431	153	111
SF vs. SS	94	42	26	1	53	37	9
					6		
					0		
SF vs. FS	938	507	184	162	4	303	40
FF vs. SF	0	0	0	0	0	0	0
				10			
FS vs. SS	2014	1452	717	8	562	261	202

Table 2. Microarray validations by qRT-PCR

Comparison	Gene	Microarray FC	Microarray adj. *P*-value	qRT-PCR FC	qRT-PCR SEM	qRT-PCR Student *t*-test
F vs. S	*flna*	2.85	0.004	1.801	0.509	0.332
	tspan1	-5.90	0.007	-2.47	0.185	0.356
FF vs. SS	*cct6a*	2.33	0.001	2.98	0.551	0.009
	rps15	-13.28	0.000	-1.46	0.407	0.001
FS vs. FF	*ggps1*	11.58	0.006	22.63	16.673	0.284
	fabp3	-15.34	0.007	-7.51	0.125	0.001
SF vs. SS	*rpl9*	2.61	0.001	18.79	7.023	0.047
	pcca	-14.03	0.000	-42.13	0.009	0.970
SF vs. FS	*lpl*	13.93	0.006	2.23	0.440	0.364
	tspan13	-10.27	0.006	-240.52	0.002	0.204
FS vs. SS	*ca1*	36.70	0.004	2.49	0.591	0.631
	agpat5	-13.38	0.000	-2.38	0.292	0.849

In the comparisons containing DE genes, analysis of the associated GO terms related to biological processes (BP), molecular function (MF) and cell component (CC) provided further information on the molecular signatures of each treatment (Supplementary Table 3). Seven selected BP subcategories based on prior knowledge that they take place in the gonads are shown in Figure 2. Metabolic process, response to stimulus and signaling were, in that order, the most represented subcategories. Regarding MF and CC subcategories, no clear differences were seen among the different comparisons. The most represented MF subcategories among the comparisons were binding and catalytic activities.

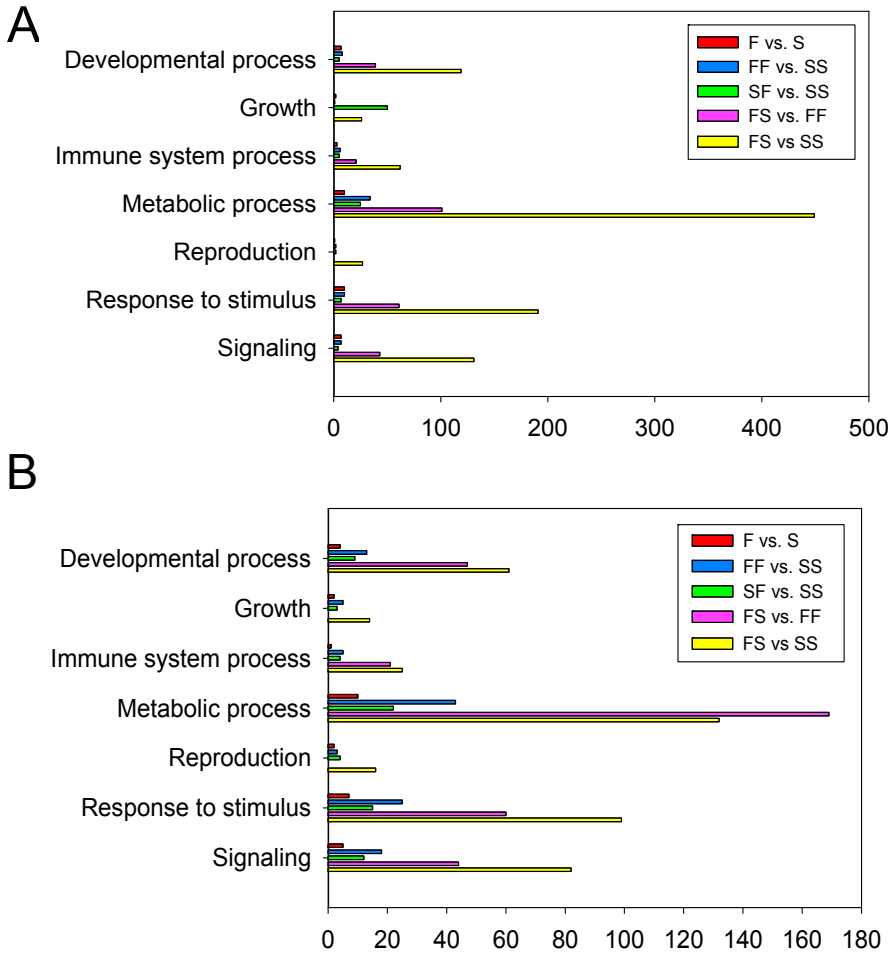


Figure 2. Seven selected GO subcategories for the Biological process (BP) category. A) Upregulated GO-terms and B) downregulated GO terms, for all of the studied comparisons.

3.2. Transcriptome of sexually undifferentiated gonads of initial fast-growing vs. initial slow-growing fish (group F vs. group S comparison)

All fish from the F group clustered together and all but one fish from the S group did the same as shown in the heatmap (Figure 3A). Of the total 40 DE genes, the 20 upregulated included genes were related to transcription, immune response or cytoskeleton structure, whereas among the 20 downregulated genes there were genes mainly related to mitochondrial functions (Supplementary Table 4).

Further analyzing the BP subcategories for the up- and downregulated GO terms (Figure 2A and 2B, respectively) showed how the number of GO terms for all the subcategories was always low when compared with T2 group comparisons. Tyrosine-protein kinase gene, a gene involved in male gonad development, was upregulated in the F group. In general, genes related to the response to stimulus and metabolic processes were downregulated. This applied also to genes related to growth such as growth hormone (GH) and adrenomedullin which is related to male gonad development and response to stimulus.

DAVID analysis of DE genes showed two gene clusters within the upregulated genes related to cytoskeleton organization and lumen (enrichment scores 2.6 and 1.14, respectively), and five clusters within the downregulated genes mainly related to mitochondrion, binding, membrane structure and ion binding (enrichment scores 3.36, 1.56, 1.47, 0.31 and 0.22, respectively). KEGG pathway analysis of DE genes showed nine affected pathways: three were upregulated and included T-cell receptor signaling and linoleic acid metabolism, and six were downregulated and mainly related to the metabolism of xenobiotics and amino acid degradation (Supplementary Table 5).

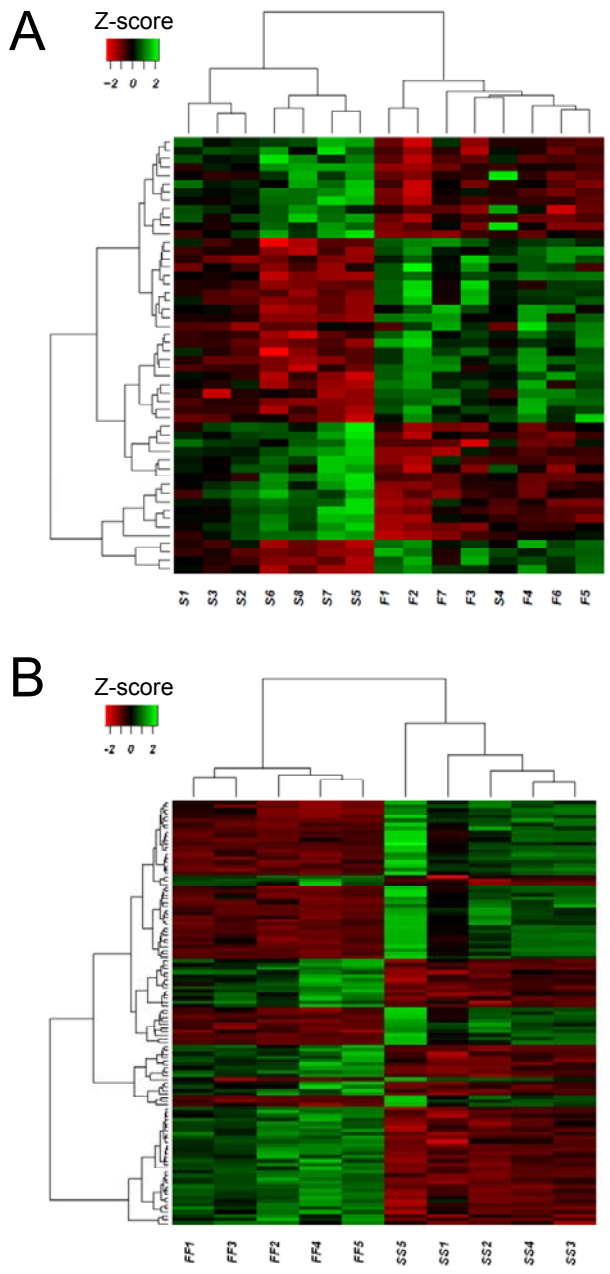


Figure 3. Individual heatmap representation of the transcriptome analysis of (A) undifferentiated gonads of F versus S growing fish, and (B) differentiated testis of F and S growing controls (FF and SS, respectively). Only DE genes are represented at the figure. High to low expression is shown by a degradation color from green to red, respectively.

Only seven interactions were found among the proteins coded by these DE genes; however, they were enriched in interactions (P<0.001) (data not shown). Proteins from the upregulated DE genes were required for the 60S ribosomal subunit biogenesis and mRNA stability such as Ilf3, Nop56, Nop58 and Noc2l; with combined scores of protein-protein interactions ranging from 0.573 to 0.924 (a value of 1 represents the highest possible relationship). Nevertheless, when analyzing the proteins from the downregulated DE genes, three clusters of relationships were observed and related to: 1) respiratory electron transport (Uqcrc2 and Etfa; combined score: 0.969), 2) amino acid degradation (Bckdha and Ivd; combined score: 0.915), and 3) glutathione-mediated detoxification pathway (Gstk1 and Ggh; combined score: 0.899).

3.3. Transcriptome of juvenile testes of sustained fast-growing vs. sustained slow-growing fish (group FF vs. group SS comparison)

There were 113 DE genes when comparing the testis of FF and SS groups (43 up- and 70 downregulated genes; see Supplementary Table 6 for a detailed list). A heatmap visualization of the data (Figure 3B), clearly separated individuals according to their group of origin.

The three most regulated GO terms in the BP category were related to metabolic processes and response to stimulus, followed by developmental process in the upregulated GO terms, and to signaling for the downregulated GO terms (Figure 2A and 2B, respectively).

DAVID analysis of the DE genes yielded seven up- and 20 downregulated gene clusters mainly related to Rps and Rpl ribosomal protein families. KEGG analysis showed twelve altered pathways: three that were upregulated in the FF fish and that showed an opposite behavior to what had been observed for the F vs. S comparison (for example, the drug metabolism and the xenobiotics and glutathione metabolisms). There were also nine downregulated pathways related to the accelerated growth and metabolism (see Supplementary Table 7 for a detailed list of the pathways). Although

low representation of sequences was found for each pathway, after a FDR test, ribosome was the most enriched pathway among both up- and downregulated pathways, while proteasome was also highly enriched among the downregulated DE genes.

A Fisher's Exact Test with Multiple Testing Correction of FDR analysis of the most specific terms showed that eight biological processes, three molecular functions and three cell components GO terms were over-represented when using the whole microarray as a background (see Supplementary Table 8 for further details). Most of the GO terms were related to the ribosome structure and the translation process.

Protein-protein interaction analysis showed that upregulated proteins clustered in three different groups, where the largest one was related to the Rps (six different Rps) and Rpl (seven different Rpl) ribosomal protein families. These groups of proteins are respectively found at the small and large ribosomal subunits (combined scores ranging from 0.401 to 0.999; Figure 4A). The other two clusters were conformed by Iars2 and Cct6a proteins, which are related to translation and folding, as well as the 60S ribosomal subunit biogenesis-related proteins (Ube2a, Nop58, Sf3b1 and Cpsf1). On the other hand, downregulated protein interactions clustered in four groups, being the largest formed by the Rpl protein family (nine different Rpl proteins), but also forming part of the small ribosomal subunit and of the proteasome accessory complex (Figure 4B). The other three clusters were conformed by: 1) Agpat2 and Agpat5, which are involved in phospholipid metabolism, 2) Psmd13, Psmd8 and Psmc1, which are involved in ubiquitinated protein degradation, and 3) Prl and Ren, which are mainly involved in growth regulation and apoptosis.

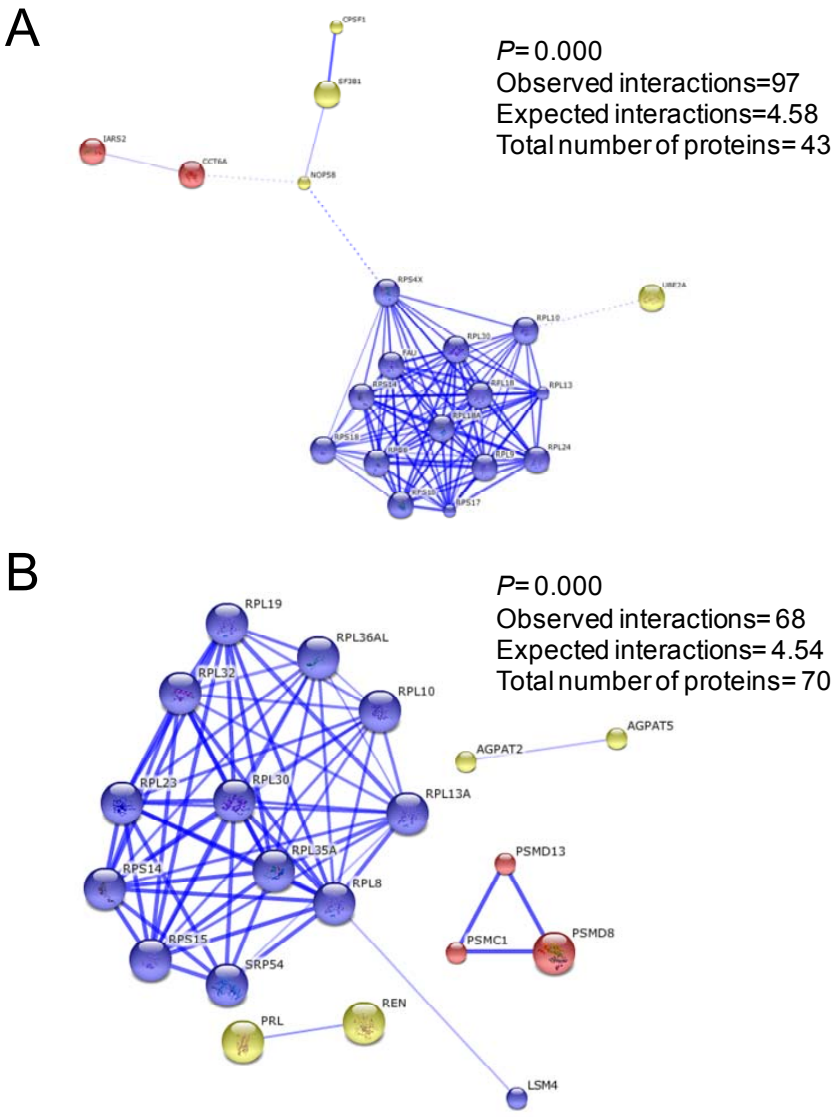


Figure 4. Protein-protein predicted confidence interactions for the time 2 FFvs. SS comparison. (A) For the 43 proteins from the upregulated DE genes and (B) for the 70 proteins from the downregulated DE genes.

3.4. The effects of accelerating growth

3.4.1. Transcriptome of juvenile testes of growth-accelerated fish vs. sustained slow-growing fish (group SF vs. group SS comparison)

Despite significant differences ($P<0.01$) in SL and BW in favor of fish from group SF when compared to fish of the SS group (Suppl. Table1), the two groups had a similar sex ratio with a clear male bias (90.57 and 92.19% males, respectively; reported in Díaz et al., 2013). However, the transcriptional comparison of the SF vs. the SS group had a low or moderate number of DE genes in the testis transcriptome. A heatmap analysis (Figure 5A) visually representing the 63 DE genes, 26 up- and 37 downregulated genes (Supplementary Table 9 for further details), showed that these two groups clustered separately.

BP subcategories were analyzed for the up- and downregulated GO terms (Figure 2A and 2B, respectively). Metabolic process GO terms were the most upregulated and contained five genes that were mainly related to amino acid metabolism (*ren, psme1, psmc1, trdmt1* and *agpat5*). Renin and prolactin (*prl*), genes involved in positive regulation of growth, male gonad development, response to hormone stimulus and signaling (hormone-mediated or through G-protein coupled receptors) were downregulated.

DAVID analysis of the data with the highest stringency showed seven clusters for the upregulated genes (enrichment score of 23.68 to 0.42), being protein biosynthesis and translational elongation the most enriched ones. Among the downregulated genes, four clusters (enrichment scores from 1.48 to 0.06) were present and mostly related to peptidase activity. KEGG analysis showed 16 pathways altered due to the growth acceleration (three up- and twelve downregulated KEGG pathways; Supplementary Table 10) that were mostly related to amino acids, glutathione and lipid metabolisms. Fisher's Exact Test with Multiple Testing Correction of FDR of the most specific terms showed eight biological processes, three molecular functions and two cellular components that were over-represented when compared against our microarray background and were mainly related to ribosome structure and translational elongation and termination (Supplementary Table 11).

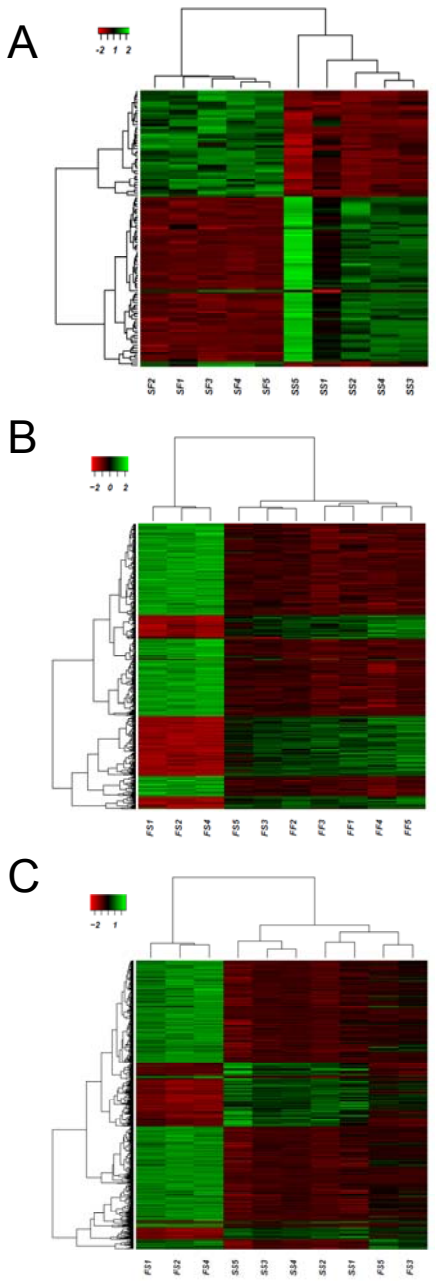


Figure 5. Individual heatmap representation of the transcriptome analysis of European sea bass one-year old testis. A) SF versus SS comparison, B) FS versus FF comparison, C) FS versus SS comparison. Only DE genes are represented at the figure. High to low expression is shown by a degradation color from green to red, respectively. The scale bar shows Z-score values for the heatmap.

Protein-protein interaction analysis with an MLC clustering method, showed proteins from the upregulated DE genes clustering together and being enriched in interactions (P<0.001). These proteins were either ribosomal (Rpl and Rps ribosomal protein families) or ribosome associated proteins (e.g., Ef1a1, Ubc or Fau). On the contrary, proteins from the downregulated DE genes were not enriched in interactions (P=0.067) and there was just one interaction between Prl and Cort proteins, which are known to be involved in growth control and signaling (data not shown).

3.4.2. Transcriptome of juvenile testes of growth-accelerated fish vs. sustained fast-growing fish (group SF vs. group FF comparison)

Transcriptional analysis of the SF vs. FF group returned zero DE (Table 1) even when we looked for genes with a lower P-value (0.05) and lower FC (1.2). Although the two groups had different sex ratios (90.57% and 67.65% males, respectively) due to their initially different growth rates before size-grading, and FF fish were bigger in BW but not in SL at the time of sampling. However, from a transcriptional point of view, they had no differences, indicating a full recovery from the early naturally slow growth rates.

3.5. The effects of decelerating growth

3.5.1. Transcriptome of juvenile testes of growth-decelerated fish vs. sustained fast-growing fish (group FS vs. group FF comparison)

Fish that experienced the same initial fast-growing rate also had a similar sex ratio (67.65% and 61.43% males, respectively) when compared to the S-derived groups (P<0.001) that were highly male-biased (>90%). However, when comparing growth between decelerated fish (FS) vs. sustained fast-growing fish (FF) there were differences in the final growth due to the different feeding regimes (for SL and BW; FF>FS) during the SD period.

Differences at the transcriptomic level were found (469 DE genes: 316 up- and 153 downregulated genes; Supplementary Table 12). A

heatmap visualization of the data (Figure 5B), showed that two FS individuals (FS3 and FS5) shared a transcriptomic pattern with those of the FF group.

The three most regulated GO terms in the BP category were related to metabolic processes, response to stimulus and developmental process in the upregulated GO terms as signaling was for the downregulated subcategory (Figure 2A and 2B, respectively).

DAVID analysis showed 37 clusters from the upregulated genes (enrichment scores from 3.66 to 0.07) and functions were mainly related to proteolysis, regulation of ubiquitin, proteasome and protein modifications processes. On the contrary, downregulated genes (37 clusters; enrichment score from 1.82 to 0.0) had functions mostly related to biosynthesis of phospho- and glycerolipids, anabolic processes and RNA processing and splicing. These DE genes were part of 56 affected pathways (41 upregulated and 15 downregulated; Supplementary Table 13). Upregulated pathways were the most altered ones after filtering for high stringency and were related to pyrimidine metabolism ($P<0.001$), RNA polymerase ($P<0.05$), oxidative phosphorylation ($P<0.05$), terpenoid backbone biosynthesis ($P<0.05$), epithelial cell signaling ($P<0.05$), purine metabolism ($P<0.05$), glutathione metabolism ($P<0.05$), glycosylphophatidylinositol (GPI)-anchor biosynthesis ($P<0.05$). With this high stringency filtering criteria, only proteasome ($P<0.001$) and ubiquitin mediated proteolysis ($P<0.05$) were affected among the downregulated pathways.

The Fisher's Exact Test with Multiple Testing Correction of FDR of the most specific terms showed twelve biological processes, eight molecular functions and three cellular components that were over-represented when compared against our microarray as a background and were related to mitochondria and transport activity, while receptor activity was found under-represented (Supplementary Table 14).

The protein-protein interaction analysis showed that proteins corresponding to both DE up- (four different clusters; Supplementary Figure 1) and downregulated (ten different clusters; Supplementary Figure 2) genes were enriched in interactions ($P<0.001$). Upregulated protein clusters were conformed by: a)

proteasome-related proteins (e.g., Psma, Cct6a, Skp1 or Ube2v2), b) signaling and cholesterol storage-proteins (e.g., Dmd, Mtor or Lpl), c) transcription regulator proteins (e.g., Max, Pdcd10 or Itgb4), and d) mitochondrial membrane respiratory chain (e.g., Mt-co1, Mt-nd1 or Mt-nd4). Downregulated proteins clustered in ten different groups but containing a few proteins, being the most enriched ones playing a role in: a) signaling and protein degradation (e.g., Mapk14, Htra2), b) translation initiation and protein folding (e.g., Eif4g1, Pdfn1) or c) transcription (e.g., Polr2h, Gtf3a).

3.5.2. Transcriptome of juvenile testes of growth-decelerated fish vs. sustained slow-growing fish (group FS vs. group SS comparison)

The analysis of testes from fish that suffered from a growth deceleration during the sex differentiating period (FS) compared to fish with a sustained slow growth (SS group) showed differences (P<0.001) in the final sex ratio (61.43% and 92.19%, respectively) and in the final growth (FS>SS for both SL and BW). These results were further corroborated by the large transcriptomic differences found (978 DE genes: 717 up and 261 downregulated genes; Supplementary Table 15). Heatmap visualization of data (Figure 5C) showed that the two individuals from the FS group (FS3 and FS5), that previously clustered with the FF group, were also clustering with the SS individuals.

Analysis of the BP subcategories from the up- and downregulated GO terms (Figure 2A and 2B, respectively) showed how decelerating growth causes the highest changes in all subcategories. None of the genes from these GO terms of the FS vs. SS comparison were coincident with the other decelerating comparison (FS vs. FF).

Further analysis of the data showed how these 978 DE genes were classified in 71 altered KEGG pathways (62 up- and nine downregulated), and were mostly related to RNA translation and elongation (Supplementary Table 16). Moreover, clustering analysis of these genes with the highest stringency yielded 82 clusters for the up- and 48 clusters for the downregulated genes. The most enriched upregulated gene clusters were mainly related to the peroxisome, RNA splicing or nucleotide biosynthetic process, while the most enriched downregulated clusters were mostly related to protein

catabolism processes, DNA modifications such as methylation, and response to nutrients.

A Fisher's Exact Test with multiple corrections for FDR of the most specific terms gave two BP, one MF and three CC GO terms that were over-represented when comparing to our custom microarray and these GO terms were mainly related to the ribosome structure and translation (Supplementary Table 17). On the other hand, there was one GO term under-represented and it was related to the regulation of the immune system.

The highest representation of protein-protein interactions for this comparison (FS vs. SS) showed after a MLC clustering an enrichment in interactions (P<0.001), and presented six clusters for the upregulated proteins (Supplementary Figure 3) related to: a) ribosomal protein families (Rpl and Rps), b) post-replication repair of damaged DNA and proteasome (e.g., Rad18 and Psm6, respectively), c) response to stress (e.g., Tp53, Apex1, Ing1), d) 60S ribosomal biogenesis and mRNA synthesis (e.g., Nop58, Nop16, Polr2f) and e) respiratory chain (e.g., Ndufa1, Nduf53, Atp5g1). The twelve clusters for the downregulated proteins (Supplementary Figure 4) were mainly related to: a) regulation of metabolic pathways and chromosome stability (e.g., Csnk2b, Mapre1, Tubgcp2), b) translation initiation (e.g., Eif4a2, Eif4e, Eif3d) and c) regulation of cell responses (e.g., Prl, Irf1, Wipi1).

The comparison between fast- and slow-growing groups vs. the FS group, a group that shows high transcriptomic activity but still some transcriptomical similarities with both groups (FF and SS), showed 253 DE genes in common (Figure 6). These genes were mainly grouped by five main functions: positive regulation of ubiquitination, RNA splicing and mRNA processing, regulation of apoptosis, glycerolipid and phospholipid metabolic process and regulation of phosphorylation. Among these common 253 DE genes, there were just five genes that showed an opposite pattern of expression: *atf4, prelid1, rps17, psma6* and *dmd*, which were more expressed in the F-derived groups (FF>FS>SS) and mainly related to the proteasome complex and ribosomal structure. Renin (*ren*) and prolactin (*prl*), two genes involved in the positive regulation of growth, growth hormone and G-protein coupled receptor signaling pathways and in male gonad development were downregulated in both comparisons

(FS vs. SS and FF; Figure 6) with a low expression of these genes in the initial fast-growing groups. This inhibition was also observed in fish with forced accelerated growth when compared to the slow-growing fish (SF vs. SS). These results indicated that these pathways are inhibited when the food availability is altered. Regarding the signaling function, apart from *ren* and *prl,* there were 4 coincident genes with the same pattern of expression in both comparisons (FS vs. SS and FF). Two of them, *atf4* and *ppkp2*, were upregulated in the FS group and involved in unfolded protein response and cell-cell signaling, while the other two genes (*errb* and *fkbp14*) were downregulated in FS group and involved in steroid hormone mediated signaling pathway and also in unfolded protein response.

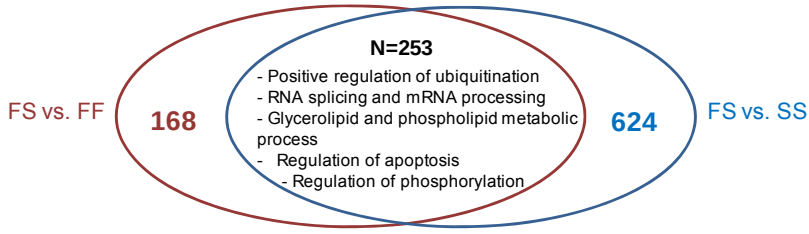


Figure 6. Venn diagram analysis of the DE genes by comparing (FS vs. FF) vs. (FS vs. SS). N represents the total number of common genes per comparison and the main categories which genes are clustered.

4. Discussion

The relationship between growth and SD has long been known for the European sea bass, where the largest fish are essentially all females whereas both sexes are found among the smallest fish, although males predominate. Early size-grading experiments (between 66-143 dph) have confirmed this (Koumoundouros et al., 2002; Saillant et al., 2003b; Papadaki et al., 2005) by obtaining ~90% females among the largest selected fish. Moreover, in a previous study we found that altering growth rates during the SD period in size-graded and non-size-graded populations did not alter the sex ratios (Díaz et al., 2013). Here, it is presented for the first time a microarray analysis of undifferentiated gonads from 4-month-

old sea bass with opposite growing rates just after the size-grading (T1), and on differentiated testis (11-month-old juveniles, T2). The study of whether naturally occurring differences in somatic growth are somehow translated in observed trasnscriptomic differences in the gonads during SD has never been explored in fish. Nevertheless, a similar work was performed in mitten crabs (Jiang et al., 2009), where it was separately analyzed the relationship between nutrition, on the hepatopancreas transcriptome, and reproduction, on testes transcriptome. Regardless the differences among experimental designs and the model organisms used, some traits found for crab as the differential expression of some heat shock proteins, cell death suppressors, RNA-dependent DNA polymerases or controllers of splicing, were also found in our study. Similarly to what has been previously reported in fish liver and muscle transcriptomes (Drew et al., 2008; Martin et al., 2010; Tacchi et al., 2011; Calduch-Giner et al., 2014), it seems that juvenile testis are also affected by food supply. Interestingly, thus there are common transcriptomic responses with the above mentioned tissues, but not with the brain transcriptomic responses (Drew et al., 2008).

Fast growing vs. slow growing fish before the onset of SD

Differences between naturally fast- vs. slow-growing (F vs. S) European sea bass of the same family early in development (T1) were not reflected in major transcriptomic changes in their undifferentiated gonads since only 40 DE genes were found. Translation was an active process in F fish gonads since genes coding for ribosomal structure, protein translation and folding were highly expressed. Immune response (positive regulation of apoptosis) and reproduction-related pathways were upregulated, although the only gene directly related to reproduction, the tyrosine-protein kinase-like (*ptk*), which is associated with male gonad development function, is also present in other biological functions such as cytoskeleton reorganization or cell proliferation. In contrast, gonads from fish that showed the slowest growth before size-grading (group S) were undergoing catabolism processes and protein recycling, since pathways related to negative regulation of growth, protein and amino acid catabolism, protein modification and fatty acid biosynthesis were highly expressed. The lack of abundance in reproduction-related genes among the DE genes present in the F vs.

the S comparison may be due to the fact that the custom-made microarray used in this study was enriched for immunology and growth-related terms rather than reproduction related terms.

These results indicate that large intrafamily differences in body growth within a group of 4-month-old sea bass due to natural variation are not necessarily translated in a large number of DE genes in their sexually undifferentiated gonads. This occurs despite the fact that groups made selecting the largest fish contain more future females than the group made selecting the smallest fish, as shown before (Koumoundouros et al., 2002; Saillant et al., 2003b; Papadaki et al., 2005), and as evidenced by actual subsequent differences in the sex ratios of these groups (Díaz et al., 2013).

Effects of unrestricted growth on the testis transcriptome

Food intake is one of the main factors influencing growth rates in aquatic production (Salas-Leiton et al., 2010) and food restriction is directly associated with reduced growth rates in fish including the European sea bass (Díaz et al., 2013). The lack of transcriptomic differences between the SF and FF groups highlights the balance between protein synthesis and degradation, i.e., protein turnover, as one of the most important active processes in the gonads. Protein turnover relies on proteins mainly obtained from the diet, since high protein contributions from diet or low protein turnover (catabolism) are translated into higher growth rates (Houlihan et al., 1995). In fact, we found lower expression of genes related to catabolism in the accelerated growth group (SF) when compared to the group with sustained growth (FF). The genes related to protein turnover, together with genes involved in the immune system, were also downregulated in the juvenile testis, as it had been found before in the liver of Atlantic salmon (*Salmon salar*) fed with a supplemental diet (Martin et al., 2010), and it was suggested that this is so because they are involved in the regulation of the decrease in whole body metabolic demands, result in less energy wastage and an enhancement in growth performance (Tacchi et al., 2011).

Groups with unrestricted growth during the SD period (FF and SF) showed in common an increased expression of genes related to high protein translation and folding, mainly of proteins related to

ribosome structure. This, together with the lack of histological differences between groups, shows how gonads from slow-growing fish can still recover after a period of slow growth if food supply is not a limiting factor thereafter. This is remarkable since it shows the plasticity of the gonads during the SD period to environmental effects, since fish present a capacity of exploiting a situation of food abundance and recover from a slow growth period.

Effects of restricted food supply on the juvenile testis transcriptome

As also found in the Atlantic salmon liver (Martin et al., 2010) and white muscle transcriptomes (Martin et al., 2002; Tacchi et al., 2011) after food deprivation, protein synthesis and degradation decreased in European sea bass juvenile testes, since both processes are highly demanding in terms of energy (Houlihan et al., 1995). It is known that defective or damaged proteins (proteolysis process) are constantly degraded by the proteasome following two main pathways: lysosome or ubiquitin-proteasome pathways (Tanaka and Chiba, 1998; Craiu et al., 1997). Our results show that in sea bass gonads proteolysis was mainly achieved by the ubiquitin-proteasome pathway rather than the lysosome pathway, as described before in rainbow trout and gilthead sea bream (Martin et al., 2002; Palstra et al., 2013; Calduch-Giner et al., 2014), since we observed a larger representation of genes involved in the ubiquitin-proteasome pathway. The ubiquitin-proteasome pathway, mainly responsible for protein degradation, was downregulated in the group FS when compared to the group FF and contrasting with the SF and SS groups. Also, food supply restriction was accompanied by a downregulation of genes related to protein synthesis and degradation, and with the immune system, in agreement with previous observations made in the Atlantic salmon liver transcriptome (Martin et al., 2010) and in white muscle (Tacchi et al., 2011) after food deprivation; although some genes of the complement system (Boshra et al., 2004) were still upregulated. Moreover, a decrease in the lipoprotein levels, in transcription, in tRNA synthesis, and in protein synthesis and elongation in group FS as a consequence of food deprivation, was coincident with previous results in fasted cod as an energy-conserving mechanism (Kjaer et al., 2009), and is a common and strong downregulated response of the energy-generating processes in the adipose tissue (Higami et al.,

2004). This may be due to the fact that during food deprivation hormonal signals such as growth hormone or insulin levels are translating food restriction signals into a protein turnover change by decreasing the protein synthesis and increasing catabolism (Gabillard et al., 2006) to save energy (Dobly et al., 2004; Schneider, 2004).

Several studies in muscle and liver in fish have transcriptomically analyzed the responses to starvation by studying catch-up growth (Rescan et al., 2007) or the effects on the muscle (Salem et al., 2006; Calduch-Giner et al., 2014), but to our knowledge this is the first time that these studies have been performed in fish gonads. Two individuals from group FS (FS3 and FS5) clustered with individuals from the FF and SS groups, showing transcriptome similarities. This suggest that these FS fish still conserved some traits of the pre-T1 period but also traits related to the T1–T2 period when food supply was reduced, reflecting the existence of an adaptation to sudden feeding changes (Schneider, 2004). Most of these changes were related to protein turnover. This may be due to the adaptive capacity of fish to sense environmental changes that in turn drive changes in their metabolism, since protein turnover is a highly energy-demanding process (Houlihan et al., 1995; Tacchi et al., 2011).

The comparison of groups FS vs. FF or SS evidenced common features of expression where processes such as apoptosis, ubiquitin catabolism, peroxisome, kinase activity or regulation of cellular growth were increased. On the other hand, processes such as proteolysis, regulation of protein modifications, RNA processing, regulation of transcription factors, chromatin assembly, response to nutrients or gamete generation and reproduction were decreased, opposite to what has been found for sea bream heart transcriptome, where transcription was enhanced and transcription inhibitors downregulated (Calduch-Giner et al., 2014). These observations indicate that FS fish had to cope with the dramatic reduction of food intake by saving energy at different levels (Schenider, 2004; Martin et al., 2010; Tacchi et al., 20112011; Díaz et al., 2013). This is also supported by the fact that pathways related to metabolism such as lipid mobilization, or purine and pyrimidine metabolism were upregulated, as well as the pathways related to stress response such as the mTOR signaling pathway, which is involved in DNA damage and nutrient deprivation. In contrast, amino acid metabolism, xenobiotic removal or glucose metabolism were downregulated

when comparing the FS to the FF and SS groups, showing how the naturally fast-growing fed with a non-restrictive diet and later subjected to food restriction did not obtain enough dietary energy and therefore had to start mobilizing lipids and activating gluconeogenesis. Moreover, in agreement with what has been found for sea bream for both white and red muscle under food restriction (Calduch-Giner et al., 2014), mitochondria and ATP transport GO terms were enriched in juvenile testes, a fact that has been proposed as a link between food restriction and stress response mediated by cortisol (Calduch-Giner et al., 2014).

No matter to which of the extreme groups the FS group was compared, processes related to translation and protein regulation such as unfolded protein response, proteasome and postregulation of damaged DNA were highly active. Processes such as translation initiation, protein folding, transcription and cell-cell signaling were also taking place. Together, these results indicated that although food was scarce and growth was decelerated the transcriptional and the translational machineries in testis were still active in the FS group. Furthermore, the steroid biosynthesis pathway was downregulated in the FS group when compared to the SF and SS groups, suggesting that the adaptation to the growth decrease could be affecting the energy dedicated to future gonad maturation, although without apparent major consequences, since there were no differences at a histological level at the time when fish were sampled. Reproduction-related processes such as steroid biosynthesis, steroid hormone-mediated signaling and cholesterol storage were affected by growth deceleration, since they were downregulated in the FS group when compared to FF group. However, the FS group showed an increase in GO terms related to spermatogenesis/male gamete generation, indicating that the FS fish, although being the most different group from a transcriptomic point of view due to food restriction during the SD period, were still allocating some of the energy in preparation for gonad maturation during the second year.

Conclusions

To the best of our knowledge, this is the first study evaluating the effects of different growth rates on gonadal development in fish with a transcriptomic approach.

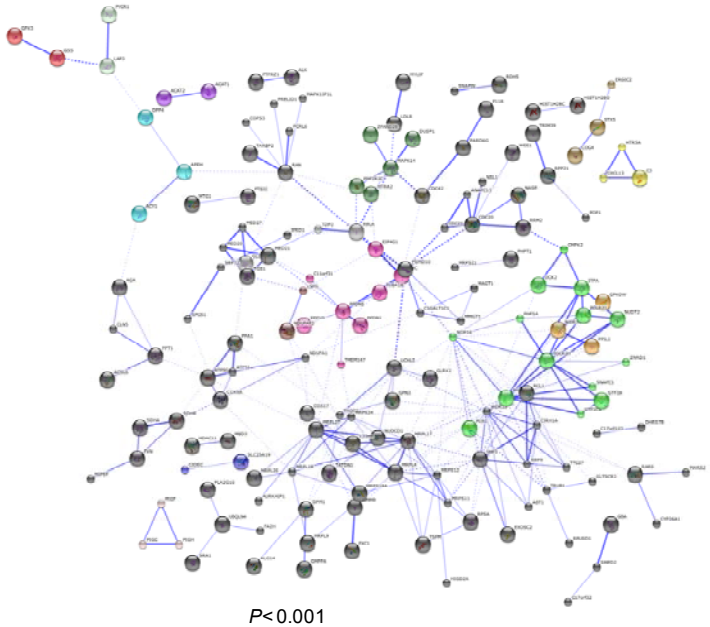
The transcriptome of undifferentiated gonads were not drastically affected by initial natural differences in growth rates (fish from the opposite sides of the normal distribution curve). In addition, regardless the maturation status of the gonad (T1 and T2), as it has also been shown previously for liver and muscle, slow-growing fish transcriptomes had an altered protein turnover with a higher catabolism, represented by a reduction in transcription and translation, a decreased immunological response, and a metabolism based on lipids and gluconeogenesis. On the other hand, the transcriptome of fast-growing fish reflects an enhancement of anabolic processes such as transcription, translation, protein synthesis and elongation and a metabolism based on glucose.

In differentiated juvenile gonads, the highest effects on the testis transcriptome were found when forcing a naturally fast-growing fish to decelerate its growth through food restriction, since those fish showed high transcriptomic differences when compared to the sustained fast-growing fish and even more differences when compared to the fish with sustained slow-grow. These results suggest that food availability during the SD period is indeed able to modulate the testis transcriptome.

Interestingly, individuals with an initial slow grow but later with accelerated growth due to increased food supply during SD showed a full recovered transcriptome. These results suggest that fish are able to transcriptionally recover their testes from a “bad start” if they are provided with enough food supply during the SD period. Nevertheless, the opposite is not true, since in naturally fast-growing fish a “good start” does not ensure any advantage in terms of transcriptional fitness if later, food becomes scarce. These results have implications for natural fish populations subjected to fluctuating food supply as well as for populations or a part thereof of farmed fish under suboptimal feeding regimes.

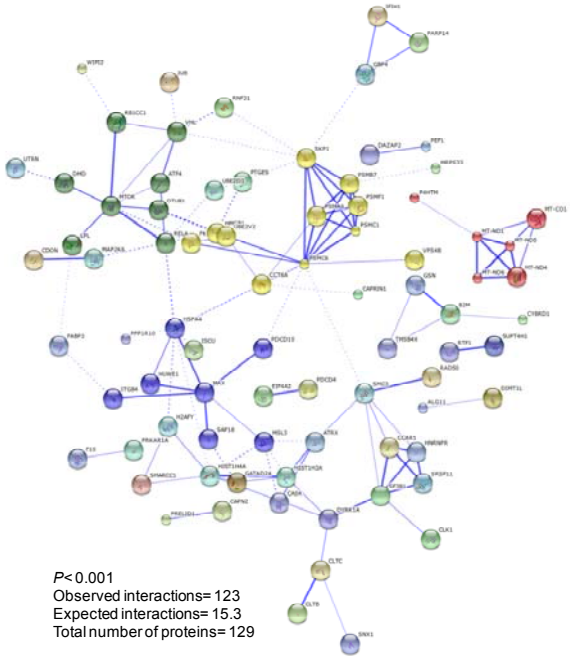
Acknowledgements

Research was supported by the Spanish Ministry of Science projects "Epigen-Aqua" (AGL2010-15939) and "Aquagenomics" (CDS2007-0002) to FP. ND was supported by a scholarship from the Government of Spain (BES-2007-14273) and then by a contract by the Epigen-Aqua project. LR was supported by an Aquagenomics postdoctoral contract and Epigen-Aqua project.
Thanks to S. Joly for technical assistance.

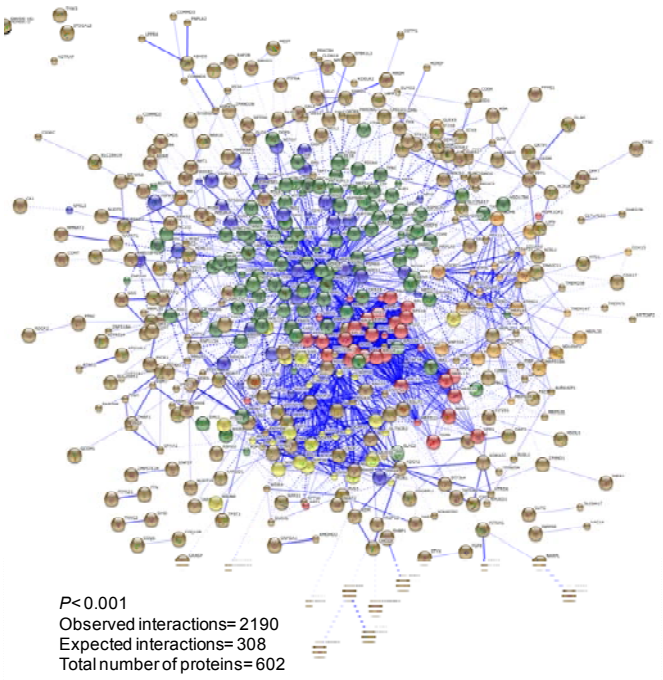


Supplementary figures

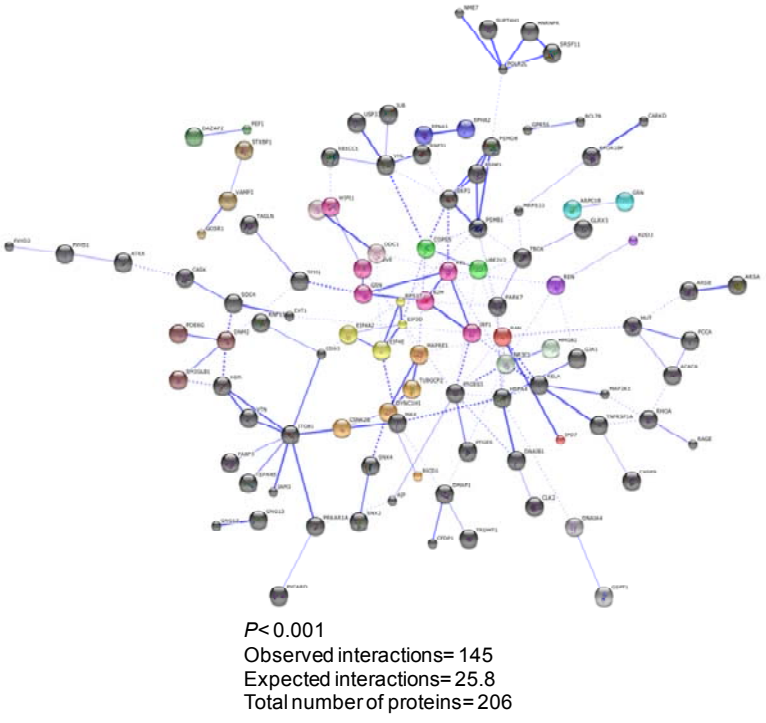
Supplementary Figure 1. Protein-protein predicted confidence interactions for the FS vs. FS comparison. The interactions of 266 proteins from the upregulated DE genes are shown.



Supplementary Figure 2. Protein-protein predicted confidence interactions for the FS vs. FF comparison. The interactions of 129proteins from the downregulated DE genes are shown.



Supplementary Figure 3. Protein-protein predicted confidence interactions for the FSvs. SS comparison. The interactions of 602proteins from the upregulated DE genes are shown.



Supplementary Figure 4. Protein-protein predicted confidence interactions for the FSvs. SS comparison. The interactions of 206 proteins from the downregulated DE genes are shown.

Supplementary tables

Supplementary Table 1. Biometric data of the individuals used for the transcriptomic analysis

	N	SL (cm)	BW (g)
Time 1			
Group F	7	4.9±0.218**	1.93±0.279
Group S	8	3.6±0.043	0.70±0.029
Time 2			
Group FF	5	15.06±0.730[a]	60.20±4.609[a]
Group SF	5	13.56±0.256[ab]	45.07±2.134[b]
Group FS	5	14.14±0.564[a]	40.24±2.370[b]
Group SS	5	12.26±0.244[b]	28.07±1.105[c]

*Statistical differences ($P<0.01$) between Time 1 groups after runing a t-test.

Different letters mark statistical differences among Time 2 groups by an ANOVA analysis. Note that Group S BW did not follow a normal distribution and had to be log transformed prior to statistical analysis.

Supplementary Table 2. Quantitative RT-PCR primer characteristics

Comparison	Genes	Gene abbrev.	Primer name	Primer sequence (5'→3')	Efficiency (E)	Slope	R^2	Fold change	adjusted p-value
F vs. S	filamin-A	*Flna*	flna-Fw	GTGCTCCAGGTCTGTGTCCT	1.96	-3.42	0.96	2.849	0.004
			flna-Rev	TTGGGGTCAACGATCTCTTC					
	tetraspanin 1	*tspan1*	tspan1-Fw	CAAATCCTGGAGACCCTCAA	1.8	-3.89	0.96	-5.899	0.007
			tspan1-Rev	GTCCAGGTGGCAGTACAGGT					
FF vs. SS	T-complex protein 1 subunit zeta	*cct6a*	cct6a-Fw	TTACTTCGCTGGTTGTGCTG	2.14	-3.01	0.97	2.335	0.001
			cct6a-Rev	CGGTCCCAGGTTACTTTTCA					
	40S ribosomal protein S15	*rps15*	rps15-Fw	TGGCGGATACCGAGATTAAG	2,00	-3.32	0.99	-13.276	0.000
			rps15-Rev	CTGCATCAGCTGCTCATAGG					
FSvs. FF	fatty acid-binding protein, heart	*fabp3*	fabp3-Fw	CCACCACCATCATCTCAGTG	2.03	-3.26	0.97	15.341	0.007
			fabp3-Rev	CGTCAAACTCCTCTCCAAGC					
	geranylgeranyl pyrophosphate synthase	*ggps1*	ggps1-Fw	TCAAACAGCTCAGCCAAATG	2.03	-3.25	0.97	-11.577	0.006
			ggps1-Rev	TGTTTTAGCGGAGTGTGCAG					
SF vs. SS	ribosomal protein L9	*rpl9*	rpl19-Fw	CGTCCTTCCTTCCTCCTTTC	2.2	-2.92	0.92	2.606	0.001

			rpl19-Rev	CACAATACTGTCCGCACCTG					
	propionyl-CoA carboxylase alpha chain, mitochondrial	*Pcca*	pcca-Fw	CACACAGGCTCTCTCCATCA	1.96	-3.41	0.98	-14.035	2.452
			pcca-Rev	TGGTGTTGTAGACGGTGGAA					
FS vs. SS	carbonic anhydrase	*ca1*	ca1-Fw	TGCCATAGTTGCTAACGCAC	1.97	-3.4	0.98	36.701	0.004
			ca1-Rev	CTCATGGGACAGCCCTAACA					
	1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	*agpat5*	agpat5-Fw	GCTGACTGGATCATTGCTGA	1.95	-3.44	0.97	-13.376	7.796
			agpat5-Rev	TCCTCCGTGCTGAGAGAAAT					

Supplementary Table 3. List of number of GO terms found for each category for all the comparisons studied.

	F vs. S	FF vs. SS	SF vs. SS	FS vs. FF	FS vs. SS
Upregulated GO terms					
Biological process					
Single-organism process	12	27	22	76	367
Signaling	7	7	4	43	131
Rhythmic process	0	0	0	0	0
Response to stimulus	10	10	7	61	191
Reproduction	1	2	2	0	27
Multi-organism process	2	9	14	19	67
Multicellular organismal process	6	9	6	45	143
Metabolic process	10	34	25	101	449
Locomotion	3	3	2	0	25
Localization	7	15	15	32	178
Immune system process	3	6	5	21	62
Growth	2	1	50	0	26
Developmental process	7	8	5	39	119
Cellular process	16	35	26	103	507
Cellular component organization or biogenesis	9	15	17	40	202
Cell killing	0	0	0	0	0
Biological regulation	12	19	14	70	287
Biological adhesion	1	2	1	0	15
Cellular component	0	0	0	0	0
Molecular funcion					
Transporter activity	1	2	1	0	32
Translation regulator activity	0	2	2	0	0
Structural molecule activity	2	14	17	0	55
Receptor activity	0	0	1	0	14
Protein binding transcription factor activity	1	0	0	0	20
Nucleic acid binding transcription factor activity	0	3	1	11	15
Molecular transducer activity	2	1	1	0	15
Enzyme regulator activity	0	2	1	0	23
Electron carrier activity	0	0	0	0	0
Chemoattractant activity	0	0	0	0	0
Channel regulator activity	0	1	1	0	0

Catalytic activity	4	11	8	60	283
Binding	15	25	18	107	453
Antioxidant activity	0	0	0	0	0
Cellular component					
Virion	0	0	0	0	0
Synapse	1	0	0	0	0
Organelle	15	29	25	86	450
Nucleoid	0	1	0	0	0
Membrane-enclosed lumen	5	12	6	40	173
Membrane	8	8	7	42	211
Macromolecular complex	10	24	23	55	253
Extracellular region	3	0	1	16	33
Extracellular matrix	0	0	1	0	0
Cell junction	0	2	1	0	20
Cell	16	34	26	103	518
Downregulated GO terms					
Biological process					
Single-organism process	8	42	18	136	140
Signaling	5	18	12	44	82
Rhythmic process	0	2	2	0	0
Response to stimulus	7	25	15	60	99
Reproduction	2	3	4	0	16
Multi-organism process	2	12	5	20	21
Multicellular organismal process	4	20	10	53	81
Metabolic process	10	43	22	169	132
Locomotion	1	4	2	0	19
Localization	6	21	6	54	75
Immune system process	1	5	4	21	25
Growth	2	5	3	0	14
Developmental process	4	13	9	47	61
Cellular process	10	52	26	194	168
Cellular component organization or biogenesis	3	21	11	80	73
Cell killing	0	1	1	0	0
Biological regulation	6	29	17	101	124
Biological adhesion	1	7	4	0	21
Molecular function					
Transporter activity	2	8	2	14	19
Structural molecule activity	1	9	0	15	11
Receptor regulator activity	0	0	0	0	0

Receptor activity	0	4	1	0	19
Protein binding transcription factor activity	0	3	1	11	0
Nucleic acid binding transcription factor activity	1	4	2	0	12
Molecular transducer activity	1	4	0	0	20
Metallochaperone activity	0	0	0	0	0
Enzyme regulator activity	0	2	1	0	12
Electron carrier activity	1	1	0	0	0
Chemoattractant activity	0	0	0	0	0
Channel activity	0	0	0	0	0
Catalytic activity	5	26	15	110	85
Binding	12	45	26	170	158
Antioxidant activity	0	0	0	0	0
Cellular component					
Synapse	0	0	1	0	0
Organelle	10	37	20	169	126
Membrane-enclosed lumen	3	12	10	76	46
Membrane	9	17	11	93	99
Macromolecular complex	3	24	9	100	87
Extracellular region	2	7	5	15	25
Extracellular matrix	0	2	1	0	0
Cell junction	0	2	0	0	13
Cell	14	50	25	198	166

Supplementary Table 4. Differentially expressed gene list. Fast versus Slow comparison

Description	Gene symbol	Fold change	Adjusted *P*-value
protein kinase ORF73	*orf73*	2.859	0.007
filamin-A	*flna*	2.849	0.004
arachidonate 12-lipoxygenase	*alox12*	2.407	0.002
Gelsolin	*gsn*	2.192	0.010
hypothetical YFW family protein 1	*yfw1*	2.106	0.003
clathrin heavy chain	*chcA*	2.051	0.006
mesoderm development candidate 1	*mesdc1*	1.874	0.002
nucleolar protein 56	*nop56*	1.830	0.009
interleukin enhancer-binding factor 3	*ilf3*	1.798	0.002
nucleolar protein 58	*nop58*	1.788	0.006
chemokine CXC-like protein	*cxc*	1.784	0.006
tyrosine-protein kinase Blk	*blk*	1.756	0.002
pre-mRNA processing factor 4 homolog	*prpf4*	1.716	0.009
protein-tyrosine sulfotransferase 1	*tpst1*	1.656	0.002
hypoxia-inducible factor	*hif*	1.640	0.007
band 4.1-like protein 1	*epb41l1*	1.614	0.007
nucleolar complex associated 2 homolog	*noc2l*	1.597	0.008
apoptosis-stimulating protein of p53 protein 2	*tp53bp2*	1.594	0.008
transcription initiation factor TFIID subunit 3	*taf3*	1.519	0.008
formin-like protein 1	*fmnl1*	1.518	0.008
high affinity copper uptake protein 1	*slc31a1*	-1.512	0.007
transmembrane protein 150A	*tmem150a*	-1.555	0.008
unknown protein		-1.588	0.008
isoavaleryl-CoA dehydrogenase, mitochondrial	*ivd*	-1.589	0.007
oxysterol-binding protein 1	*osbp*	-1.627	0.007
unknown protein		-1.806	0.008
iron-sulfur cluster assembly enzyme ISCU, mit.	*iscu*	-1.816	0.008
golgi pH regulator B	*gpr89b*	-1.829	0.008
2-oxoisovalerate dehydrogenase subunit alpha, mit.	*bckdha*	-1.873	0.008
adrenomedullin-1	*adm1*	-1.891	0.008

Somatotropin	*gh*	-1.965	0.007
CD151 antigen	*cd151*	-1.982	0.004
calcipressin-1	*rcan1*	-1.986	0.006
glutathione S-transferase kappa 1	*gstk1*	-2.002	0.007
cytochrome b-c1 complex subunit 2, mitochondrial	*uqcrc2*	-2.042	0.008
electron transfer flavoprotein subunit alpha, mit.	*etfa*	-2.076	0.008
3-hydroxybutyrate dehydrogenase type 2	*bdh2*	-2.097	0.008
unknown protein		-2.148	0.008
RNA, 28S Ribosomal 1	*rna28s1*	-2.165	0.004
tetraspanin 1	*tspan1*	-5.899	0.007

Supplementary Table 5. Affected KEGG pathways for F vs. S comparison

Pathways	# Sequences	# Enzymes	up/down
Alpha-Linolenic acid metabolism	1	1	down
Butanoate metabolism	1	1	up
Drug metabolism-cytochrome P450	1	1	up
Glutathione metabolism	1	2	up
Linoleic acid metabolism	1	1	down
Metabolism of xenobiotics by cytochrome P450	1	1	up
Synthesis and degradation of ketone bodies	1	1	up
T cell receptor signaling pathway	1	1	down
Valine, leucine and isoleucine degradation	1	1	up

Supplementary Table 6. A detailed list of the DE genes for the FF versus SS comparison

Description	Gene symbol	Fold change	Adjusted *P*-value
T-complex protein 1 subunit zeta	*cct6a*	2.335	0.001
basic leucine zipper and W2 domain-containing protein 2	*bzw2*	2.291	0.000
60S ribosomal protein L9	*rpl9*	2.275	0.006
myelin-associated neurite-outgrowth inhibitor	*fam168b*	2.262	0.003
cyclic AMP-dependent transcription factor ATF4	*atf4*	2.170	0.006
60S ribosomal protein L18	*rpl18*	2.165	0.000
splicing factor 3B subunit 1	*sf3b1*	2.143	0.005
ubiquitin-conjugating enzyme E2 A	*ube2a*	2.138	0.005
voltage-dependent anion-selective channel protein 2	*vdac2*	2.121	0.001
type II inositol 1,4,5-trisphosphate 5-phosphatase	*inpp5b*	2.115	0.010
60S ribosomal protein L13	*rpl13*	2.104	0.000
ATP synthase F(0) complex subunit C1, mitochondrial	*atp5g1*	2.006	0.003
Glutathione S-transferase Mu 1	*gstm1*	1.995	0.002
60S ribosomal protein L10	*rpl10*	1.984	0.003
40S ribosomal protein S17	*rps17*	1.967	0.000
40S ribosomal protein S30	*rps30*	1.941	0.002
chaperonin containing TCP1, subunit *6A* (Zeta 1)	*cct6a*	1.924	0.001
nucleolar protein 58	*nop58*	1.917	0.009
cleavage and polyadenylation specificity factor subunit 1	*cpsf1*	1.906	0.005
thioredoxin, mitochondrial	*txn2*	1.876	0.006
60S ribosomal protein L30	*rpl30*	1.874	0.000
reverse transcriptase-like protein	*rtl*	1.867	0.000
40S ribosomal protein S18	*rps18*	1.809	0.000
40S ribosomal protein S30	*rps30*	1.797	0.007
polycomb protein SCMH1	*scmh1*	1.760	0.010
cold-inducible RNA-binding protein	*cirbp*	1.714	0.004
60S ribosomal protein L24	*rpl24*	1.695	0.004
proteasome activator complex subunit 1	*psme1*	1.685	0.004

presequence protease, mitochondrial	*pitrm1*	1.682	0.006
40S ribosomal protein S14	*rps14*	1.682	0.000
40S ribosomal protein S8	*rps8*	1.679	0.000
thyroid receptor-interacting protein 1	*trip6*	1.646	0.009
transcription termination factor, mitochondrial	*mterf*	1.637	0.001
40S ribosomal protein S4	*rps4*	1.624	0.001
CG13731	*cg13731 -PA*	1.618	0.009
lanC-like protein 1	*lancl1*	1.607	0.010
40S ribosomal protein S10	*rps10*	1.591	0.001
plakophilin-2	*ppkp2*	1.590	0.006
phosphatidylserine synthase 1	*ptdss1*	1.565	0.003
grb2-associated-binding protein 1	*gab1*	1.559	0.007
golgin subfamily A member 5	*golga5*	1.545	0.009
60S ribosomal protein L18a	*rpl18a*	1.524	0.004
VHSV-induced protein	*ftr53*	1.509	0.006
isoleucine-tRNA ligase	*iars2*	1.505	0.006
40S ribosomal protein S15	*rps15*	-13.276	0.000
1-acyl-sn-glycerol-3-phosphate acyltransferase beta	*agpat2*	-12.126	0.000
7,8-dihydro-8-oxoguanine triphosphatase	*nudt1*	-9.391	0.000
excitatory amino acid transporter 3	*slc1a1*	-6.884	0.000
carboxypeptidase N catalytic chain	*cpn1*	-6.731	0.000
ubiquitin carboxyl-terminal hydrolase 33	*usp33*	-6.600	0.003
26S proteasome non-ATPase regulatory subunit 13	*psmd13*	-6.101	0.000
platelet glycoprotein IX	*gp9*	-5.319	0.000
histone chaperone ASF1A - Protein HIRA	*asf1a-hira*	-4.966	0.001
E3 ubiquitin-protein ligase Midline-1	*mid1*	-4.922	0.000
26S proteasome non-ATPase regulatory subunit 8	*psmd8*	-4.654	0.000
cystatin-A	*csta*	-4.464	0.000
gamma-aminobutyric acid receptor subunit rho-2	*gabrr2*	-4.359	0.000
aquaporin-8	*aqp8*	-3.972	0.000
leucine-rich repeat-containing protein 40	*lrrc40*	-3.884	0.008
60S ribosomal protein L35A	*rpl35a*	-3.684	0.000
tetratricopeptide repeat protein 39C	*ttc39c*	-3.064	0.010

protease serine 1	*prss1*	-2.929	0.000
protein FAM49B	*fam49b*	-2.843	0.001
eukaryotic peptide chain release factor GTP-binding subunit ERF3A	*gspt1*	-2.838	0.000
60S ribosomal protein L32	*rpl32*	-2.795	0.003
propionyl-CoA carboxylase alpha chain, mitochondrial	*pcca*	-2.787	0.003
peptidyl-prolyl cis-trans isomerase FKBP14	*fkbp14*	-2.786	0.004
60S ribosomal protein L10	*rpl10*	-2.751	0.007
plexin-C1	*plxnc1*	-2.632	0.000
glutamate receptor 3	*gria3*	-2.527	0.000
CTP synthase 1	*ctps1*	-2.492	0.008
unhealthy ribosome biogenesis protein 2 homolog	*urb2*	-2.428	0.000
26S protease regulatory subunit 4	*psmc1*	-2.332	0.003
V-type proton ATPase 116 kDa subunit a isoform 1	*atp6v*	-2.304	0.000
armadillo repeat-containing protein 1	*armc1*	-2.245	0.001
40S ribosomal protein S14	*rps14*	-2.236	0.005
U6 snRNA-associated Sm-like protein LSm4	*lsm4*	-2.176	0.001
60S ribosomal protein L8	*rpl8*	-2.155	0.000
coatomer subunit epsilon	*cope*	-2.122	0.002
E3 ubiquitin-protein ligase TRIM39	*trim39*	-2.081	0.007
ephrin-A1	*efna1*	-2.063	0.007
protein EURL homolog	*eurl*	-2.033	0.000
type II inositol 1,4,5-trisphosphate 5-phosphatase	*inpp5a*	-2.031	0.000
upstream-binding protein 1	*ubp1*	-2.028	0.001
steroid hormone receptor ERR2	*esrrb*	-1.956	0.001
60S ribosomal protein L36A-like protein	*rpl36al*	-1.901	0.000
1-phosphatidylinositol 3-phosphate 5-kinase	*pip5k3*	-1.767	0.004
60S ribosomal protein L13A	*rpl13a*	-1.730	0.002
bestrophin-2	*best2*	-1.717	0.000
ubiquitin-like modifier-activating enzyme ATG7	*atg7*	-1.699	0.003
peptide BmKa1	*ka1*	-1.684	0.005
GPI-linked NAD(P)(+)-arginine ADP-ribosyltransferase 1	*art1*	-1.633	0.006
butyrophilin-like protein 1	*btnl1*	-1.612	0.003
60S ribosomal protein L30	*rpl30*	-1.487	0.004

protein-arginine deiminase type-2	*padi2*	-1.450	0.005
cyclic AMP-dependent transcription factor ATF-3	*atf3*	-1.431	0.003
asparagine--tRNA ligase, cytoplasmic	*nars*	-1.412	0.010
tRNA (cytosine(38)-C(5))-methyltransferase	*trdmt1*	-1.389	0.007
reverse transcriptase	*reverse transcriptase*	1.360	0.008
short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	*acadsb*	1.363	0.004
craniofacial development protein 1	*cfdp1*	1.366	0.010
sister chromatid cohesion protein DCC1	*dscc1*	1.367	0.002
signal recognition particle 54 kDa protein	*srp54*	1.384	0.008
SET and MYND domain-containing protein 5	*smyd5*	1.400	0.001
Renin	*ren*	1.456	0.001
60S ribosomal protein L19	*rpl19*	1.462	0.006
serine/threonine-protein kinase B-raf	*braf*	1.478	0.007
1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	*agpat5*	1.480	0.003
shaw type potassium channel Kv3.3	*kcnc3*	1.484	0.002
transposable element Tc3 transposase	*tc3a*	1.485	0.003
Prolactin	*prl*	1.491	0.006
60S ribosomal protein L23	*rpl23*	1.492	0.006
syntaxin interacting protein 1	*sip1*	1.497	0.003

Supplementary Table 7. Affected KEGG pathways for FF vs. SS comparison

Pathways	# Sequences	# Enzymes	up/down
Cysteine and methionin metabolism	1	1	down
Drug metabolism-cytochrom P450	1	1	up
Fatty acid biosynthesis	1	1	down
Glutathione metabolism	1	1	up
Glyoxylate and dicarboxylate metabolism	1	1	down
Inositol phosphate metabolism	1	1	down
Metabolism of xenobiotics by cytochrome P450	1	1	up
Phosphatidylinositol signaling system	1	1	down
Propanoate metabolism	1	1	down
Purine metabolism	1	1	down
Pyrimidine metabolism	1	1	down
Valine, leucine and isoleucine degradation	1	1	down

Supplementary Table 8. Two-tails Fisher's exact test with multiple testing corrections of FDR results for the FF versus SS comparison

GO Term	Name	Type	FDR	single test *P*-value	# in test group	# in reference group	# non annot test	# non annot reference group	over/ under
GO:0003735	structural constituent of ribosome	MF	6,40E-13	5,80E-17	22	154	70	7394	over
GO:0006415	translational termination	BP	1,80E-09	1,30E-12	12	43	80	7505	over
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	BP	1,90E-08	2,20E-11	12	57	80	7491	over
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	BP	2,30E-08	3,20E-11	12	59	80	7489	over
GO:0006414	translational elongation	BP	2,10E-07	5,00E-10	12	77	80	7471	over
GO:0019083	viral transcription	BP	2,10E-07	5,10E-10	11	59	81	7489	over
GO:0022627	cytosolic small ribosomal subunit	CC	1,70E-06	4,60E-09	8	26	84	7522	over
GO:0022625	cytosolic large ribosomal subunit	CC	1,10E-05	3,30E-08	8	35	84	7513	over
GO:0006413	translational initiation	BP	1,60E-04	6,00E-07	11	125	81	7423	over
GO:0000028	ribosomal small subunit assembly	BP	5,80E-03	3,30E-05	3	3	89	7545	over
GO:0030490	maturation of SSU-rRNA	BP	1,40E-02	9,10E-05	3	5	89	7543	over
GO:0005234	extracellular-glutamate-gated ion channel activity	MF	2,20E-02	1,40E-04	2	0	90	7548	over
GO:0022624	proteasome accessory complex	CC	2,80E-02	1,90E-04	3	7	89	7541	over
GO:0003723	RNA binding	MF	4,80E-02	3,40E-04	16	494	76	7054	over

Supplementary Table 9. DE genes from SF versus SS comparison

Description	Gene symbol	Fold change	Adjusted *P*-value
ribosomal protein L9	*rpl9*	2.606	0.001
ribosomal protein L10	*rpl10*	2.362	0.000
cold-inducible RNA-binding protein	*cirbp*	2.283	0.005
glutathione S-transferase Mu 1	*gstm1*	2.188	0.000
ribosomal protein L13	*rpl13*	2.075	0.000
ATP synthase F(0) complex subunit B1, mitochondrial	*atp5f1*	2.063	0.003
ribosomal protein S3	*rps3*	2.031	0.001
ribosomal protein L18	*rpl18*	1.975	0.001
ribosomal protein L24	*rpl24*	1.904	0.001
ribosomal protein S17	*rps17*	1.867	0.000
Polyubiquitin-C	*ubc*	1.842	0.004
T-complex protein 1 subunit eta	*cct7*	1.772	0.003
ribosomal protein S18	*rps18*	1.756	0.001
ribosomal protein S8	*rps8*	1.728	0.000
ribosomal protein S30	*rps30*	1.692	0.010
ribosomal protein S14	*rps14*	1.690	0.001
40S ribosomal proteinS16	*rps16*	1.675	0.000
plakophilin-2	*pkp2*	1.668	0.003
proteasome activator complex subunit 1	*psme1*	1.663	0.008
LanC-like protein 1	*lancl1*	1.651	0.009
ribosomal protein L30	*rpl30*	1.650	0.007
ribosomal protein S4	*rps4*	1.598	0.001
elongation factor 1-alpha 1	*eef1a1*	1.559	0.007
ribosomal protein L19	*rpl19*	1.545	0.003
ribosomal protein L13a	*rpl13a*	1.528	0.000
phosphatidylserine synthase 1	*ptdss1*	1.519	0.008
Propionyl-CoA carboxylase alpha chain, mitochondrial	*pcca*	14.035	0.000
1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	*agpat5*	13.439	0.000
histone chaperone ASF1A/Protein HIRA	*asf1a-hira*	-6.974	0.000
Prolactin	*prl*	-6.604	0.000

1-phosphatidylinositol 3-phosphate 5-kinase	*pip5k3*	-6.406	0.000
short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	*acadsb*	-6.317	0.000
peptide BmKa1	*ka1*	-6.247	0.000
potassium voltage-gated channel subfamily C member 3	*kcnc3*	-5.400	0.000
E3 ubiquitin-protein ligase Midline-1	*mid1*	-5.066	0.001
MKIAA*0133 protein*	*urb2*	-4.649	0.003
GPI-linked NAD(P)(+)-arginine ADP-ribosyltransferase 1	*art1*	-4.232	0.000
peptidyl-prolyl cis-trans isomerase FKBP14	*fkbp14*	-4.127	0.000
butyrophilin-like protein 1	*btnl1*	-3.557	0.000
dual specificity protein phosphatase 7	*dusp7*	-3.455	0.010
Renin	*ren*	-2.829	0.002
craniofacial development protein 1	*cfdp1*	-2.805	0.004
ionotropic glutamate recetor subunit *3 alpha*	*fglur3a*	-2.775	0.000
leucine-rich repeat-containing protein 40	*lrrc40*	-2.773	0.000
26S proteasome non-ATPase regulatory subunit 13	*psmd13*	-2.535	0.002
cyclic AMP-dependent transcription factor ATF-3	*atf3*	-2.533	0.001
carboxypeptidase N, polypeptide 1	*cpn1*	-2.428	0.000
plexin-C1	*plxnc1*	-2.426	0.000
tRNA (cytosine(38)-C(5))-methyltransferase	*trdmt1*	-2.374	0.000
V-type proton ATPase catalytic subunit A	*atp6v*	-2.288	0.000
tetratricopeptide repeat protein 39C	*ttc39c*	-2.249	0.001
chromosome 21 open reading frame 7	*c21orf7*	-2.111	0.000
chromodomain-helicase-DNA-binding protein 1-like	*chd1l*	-2.105	0.008
delta-1-pyrroline-5-carboxylate synthase	*aldh18a*	-2.087	0.003
syntaxin-binding protein 1	*stxbp1*	-2.084	0.000
protein-argi*nine deiminasae type II*-like	*?*	-1.963	0.001
serine protease HTRA1	*htra1*	-1.913	0.001
dTDP-D-glucose 4,6-dehydratase	*tgds*	-1.842	0.003
Cystatin	*cst*	-1.750	0.000
armadillo repeat-containing protein 1	*armc1*	-1.639	0.008
sister chromatid cohesion protein DCC1	*dscc1*	-1.619	0.003

Supplementary Table 10. KEGG pathways of SF versus SS comparison

Pathways	# Sequences	# Enzymes	up/down
Arginine and proline metabolism	1	2	down
Biosynthesis of vancomycin group antibiotics	1	1	down
Cysteine and methionine metabolism	1	1	down
Drug metabolism-cytochrome P450	1	1	up
Fatty acid biosynthesis	1	1	down
Glutathione metabolism	1	1	up
Glycerolipid metabolism	1	1	down
Glycerophospholipid metabolism	1	1	down
Glyoxylate and dicarboxylate metabolism	1	1	down
Metabolism of xenobiotics by cytochrome P450	1	1	up
Polyketide sugar unit biosynthesis	1	1	down
Propanoate metabolism	1	1	down
Sphingolipid metabolism	1	1	down
Streptomycin biosynthesis	1	1	down
Valine, leucine and isoleucine degradation	1	1	down

Supplementary Table 11. Fisher's Exact Test with Multiple Corrections for FDR for SF vs. SS comparison

GO Term	Name	Type	FDR	single test p-value	# in test group	# in reference group	Over/ Under
GO:0003735	structural constituent of ribosome	MF	3,40E-08	6,20E-12	14	162	over
GO:0022627	cytosolic small ribosomal subunit	CC	3,40E-06	2,10E-09	7	27	over
GO:0006415	translational termination	BP	6,70E-05	7,30E-08	7	48	over
GO:0006414	translational elongation	BP	9,90E-05	1,30E-07	8	81	over
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	BP	2,00E-04	3,60E-07	7	62	over
GO:0019083	viral transcription	BP	2,00E-04	4,00E-07	7	63	over
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	BP	2,00E-04	4,40E-07	7	64	over
GO:0000028	ribosomal small subunit assembly	BP	2,20E-03	5,90E-06	3	3	over
GO:0030490	maturation of SSU-rRNA	BP	4,70E-03	1,60E-05	3	5	over
GO:0006413	translational initiation	BP	9,00E-03	3,40E-05	7	129	over
GO:0005234	extracellular-glutamate-gated ion channel activity	MF	1,10E-02	4,60E-05	2	0	over
GO:0004970	ionotropic glutamate receptor activity	MF	2,70E-02	1,40E-04	2	1	over
GO:0022625	cytosolic large ribosomal subunit	CC	3,80E-02	2,00E-04	4	39	over

Supplementary Table 12. DE genes for comparison FS versus FF

Description	Gene symbol	Fold change	Adjusted *P*-value
Fatty acid-binding protein, heart	*fabp3*	-15.341	0.007
Lipoprotein lipase	*lpl*	-14.392	0.006
Mullerian-inhibiting factor	*amh*	-12.314	0.007
Integrin beta-4	*itgb4*	-6.390	0.006
Actin-related protein 2/3 complex subunit 1	*arpc1*	-5.203	0.008
26S protease regulatory subunit 10B	*psmc6*	-4.905	0.007
V-type proton ATPase subunit B1	*vha-b1*	-4.861	0.007
Keratin, type II cytoskeletal 1	*krt1*	-4.822	0.006
Collagen alpha-1(XVIII) chain	*col18a*	-4.333	0.008
NADH-ubiquinone oxidoreductase chain 1	*mt-nd1*	-4.319	0.006
NADH-ubiquinone oxidoreductase chain 4	*mt-nd4*	-4.187	0.006
Cytochrome c oxidase subunit 1	*mt-co1*	-4.009	0.007
Lipopolysaccharide-induced tumor necrosis factor-alpha factor	*litaf*	-3.663	0.009
Ornithine decarboxylase	*odc1*	-3.547	0.002
T-lymphoma invasion and metastasis-inducing protein 1	*tiam1*	-3.502	0.006
Unknown	?	-3.452	0.006
S-phase kinase-associated protein 1	*skp1*	-3.441	0.005
Retinol-binding protein 1	*rbp1*	-3.439	0.007
Thymosin beta-4	*tmsb4x*	-3.171	0.008
Proteasome inhibitor PI31 subunit	*psmf1*	-3.162	0.006
NADH-ubiquinone oxidoreductase chain 5	*mt-nd5*	-3.161	0.008
Core histone macro-H2A.1	*h2afy*	-3.159	0.005
DAZ-associated protein 2	*dazap2*	-3.157	0.006
Keratin, type II cytoskeletal 2 epidermal	*krt2*	-3.155	0.007
Serine incorporator 2	*serinc2*	-3.081	0.003
Structural maintenance of chromosomes protein 3	*smc3*	-3.018	0.005
Beta-2-microglobulin	*b2m*	-3.007	0.007
Cyclic AMP-dependent transcription factor ATF-4	*atf4*	-3.006	0.005
Clathrin heavy chain 1	*cltc*	-2.995	0.008

PRELI domain-containing protein 1, mitochondrial	*prelid1*	-2.958	0.006
DNA-directed RNA polymerases I, II, and III subunit RPABC4	*polr2k*	-2.899	0.006
CUGBP Elav-like family member 1	*celf1*	-2.889	0.004
Cytochrome b reductase 1	*cybrd1*	-2.874	0.004
Fatty aldehyde dehydrogenase	*aldh3a2*	-2.870	0.009
Prostaglandin E synthase	*ptges*	-2.869	0.006
40S ribosomal protein S17	*rps17*	-2.807	0.006
Poly [ADP-ribose] polymerase 14	*parp14*	-2.770	0.007
Gelsolin	*gsn*	-2.768	0.009
Calpain-2 catalytic subunit	*capn2*	-2.730	0.006
Transmembrane protein 206	*tmem206*	-2.725	0.007
Negative elongation factor E	*nelfe*	-2.723	0.005
Unknown	?	-2.718	0.007
T-complex protein 1 subunit zeta	*cct6a*	-2.660	0.002
cAMP-dependent protein kinase type I-alpha regulatory subunit	*prkar1a*	-2.631	0.006
Large proline-rich protein BAG6	*bag6*	-2.625	0.004
Splicing factor 3B subunit 1	*sf3b1*	-2.615	0.003
Heat shock 70 kDa protein 4	*hspa4*	-2.570	0.008
Peflin	*pef1*	-2.570	0.006
Sorting nexin-1	*snx1*	-2.555	0.007
Unknown	?	-2.539	0.006
Peripheral plasma membrane protein CASK	*cask*	-2.531	0.005
Myelin-associated neurite-outgrowth inhibitor	*fam168b*	-2.496	0.003
Unknown	?	-2.481	0.006
Iron-sulfur cluster assembly enzyme ISCU, mitochondrial	*iscu*	-2.471	0.007
NADH-ubiquinone oxidoreductase chain 6	*mt-nd6*	-2.467	0.008
RB1-inducible coiled-coil protein 1	*rb1cc1*	-2.463	0.007
Interferon-induced helicase C domain-containing protein 1	*ifih1*	-2.452	0.007
NF-kappa-B inhibitor-like protein 1	*nfkbil1*	-2.445	0.006
Caprin-1	*caprin1*	-2.432	0.009
Programmed cell death protein 4	*pdcd4*	-2.432	0.005
Histone H4	*hist1h4a*	-2.428	0.005
Zinc finger protein 1	*znf1*	-2.413	0.006

Dual specificity tyrosine-phosphorylationregulated kinase 1A	*dyrk1a*	-2.403	0.008
Calmodulin-sensitive adenylate cyclase	*cya*	-2.394	0.006
DNA repair protein RAD50	*rad50*	-2.384	0.005
Histone H3.1	*hist1h3a*	-2.378	0.007
GTP-Binding Protein 4	*gbp4*	-2.378	0.008
Ubiquitin-conjugating enzyme E2 D3	*ube2d3*	-2.356	0.007
Protein tyrosine phosphatase type IVA 2	*ptp4a2*	-2.355	0.005
E3 ubiquitin-protein ligase RNF13	*rnf13*	-2.321	0.006
Prostaglandin reductase 1	*ptgr1*	-2.316	0.006
E3 ubiquitin-protein ligase UBR1	*ubr1*	-2.294	0.009
Clathrin light chain B	*cltb*	-2.235	0.006
Unknown	unknown	-2.234	0.008
LIM domain-containing protein ajuba	*ajuba*	-2.224	0.006
Unknown	?	-2.220	0.007
Vesicle transport protein SEC20	*bnip1*	-2.211	0.007
Peroxiredoxin-2	*prdx2*	-2.197	0.010
Von Hippel-Lindau disease tumor suppressor	*vhl*	-2.193	0.010
Basic leucine zipper and W2 domain-containing protein 2	*bzw2*	-2.184	0.000
Serine/threonine-protein phosphatase 1 regulatory subunit 10	*ppp1r10*	-2.136	0.009
Unknown	?	-2.124	0.006
Eukaryotic initiation factor 4A-II	*eif4a2*	-2.119	0.006
UPF0600 protein C5orf51 homolog	*wu:fd42g01*	-2.092	0.006
MOB kinase activator 3A	*mob3a*	-2.062	0.006
Ketosamine-3-kinase	*fn3krp*	-2.049	0.008
Programmed cell death protein 10	*pdcd10*	-2.044	0.006
Utrophin	*utrn*	-2.036	0.007
Serine/arginine-rich splicing factor 11	*srsf11*	-2.029	0.009
Histone deacetylase complex subunit SAP18	*sap18*	-2.016	0.006
Proteasome subunit alpha type-6	*psma6*	-2.015	0.008
Leucine-rich repeat, immunoglobulin-like domain and transmembrane domain-containing protein 1	*lrit1*	-2.003	0.006
Mt-myomegalin	*myomegalin*	-2.002	0.009
Putative uncharacterized protein	*b230110c*	-1.993	0.007
Proteasome subunit beta type-7	*psmb7*	-1.970	0.007

Heterogeneous nuclear ribonucleoprotein R	*hnrnpr*	-1.969	0.010
B-cell CLL/lymphoma 7 protein family member B	*bcl7b*	-1.966	0.010
Y-box-binding protein 3	*ybx3*	-1.961	0.006
Vacuolar protein sorting-associated protein 4B	*vps4b*	-1.959	0.007
Transcriptional regulator ATRX	*atrx*	-1.957	0.010
Porimin	*porimin*	-1.951	0.006
Dystrophin	*dmd*	-1.947	0.009
26S protease regulatory subunit 4	*psmc1*	-1.945	0.007
Serine/threonine-protein kinase mTOR	*mtor*	-1.944	0.006
ATP synthase subunit alpha, mitochondrial	*atp5a*	-1.943	0.006
SWI/SNF complex subunit SMARCC1	*smarcc1*	-1.929	0.007
Dual specificity protein kinase CLK1	*clk1*	-1.929	0.006
Transmembrane prolyl 4-hydroxylase	*p4htm*	-1.927	0.006
Probable dimethyladenosine transferase	*dimt1*	-1.922	0.008
High mobility group protein B1	*hmgb1*	-1.914	0.005
Transcriptional repressor p66-alpha	*gatad2a*	-1.912	0.007
Coagulation factor X	*f10*	-1.911	0.007
FBP32	*fbp32*	-1.907	0.010
Male-specific lethal 3 homolog	*msl3*	-1.898	0.009
Peptidyl-prolyl cis-trans isomerase FKBP3	*fkbp3*	-1.897	0.007
Splicing factor, arginine/serine-rich 19	*scaf1*	-1.895	0.002
Protein yippee-like 5	*ypel5*	-1.891	0.008
ETS translocation variant 1	*etv1*	-1.878	0.008
39S ribosomal protein L53, mitochondrial	*mrpl53*	-1.872	0.007
Protein Red	*ik*	-1.863	0.007
DCN1-like protein 1	*dcun1d1*	-1.849	0.008
Protein max	*max*	-1.831	0.006
Tetraspanin-13	*tspan13*	-1.830	0.010
Ubiquitin-conjugating enzyme E2 variant 2	*ube2v2*	-1.821	0.008
Unknown	?	-1.820	0.007
E3 ubiquitin-protein ligase RNF31	*rnf31*	-1.797	0.009
Dual specificity mitogen-activated protein kinase kinase 6	*map2k6*	-1.790	0.008
Forkhead box protein J2	*foxj2*	-1.781	0.007
NEDD4-like E3 ubiquitin-protein ligase WWP1	*wwp1*	-1.780	0.007

Charged multivesicular body protein 3	*chmp3*	-1.757	0.007
Forkhead box protein K1	*foxk1*	-1.751	0.006
cAMP-dependent protein kinase type I-alpha regulatory subunit	*prkar1a*	-1.744	0.008
Tax1-binding protein 1	*tax1bp1*	-1.734	0.007
Inositol monophosphatase 1	*impa1*	-1.733	0.009
Apoptosis-stimulating of p53 protein 1	*ppp1r13b*	-1.733	0.009
Zinc finger CCCH domain-containing protein 7B	*zc3h7b*	-1.726	0.009
Serine/threonine-protein kinase ICK	*ick*	-1.716	0.007
RNA polymerase-associated protein RTF1 homolog	*rtf1*	-1.682	0.008
EF-hand calcium-binding domain-containing protein 6	*efcab6*	-1.680	0.009
GDP-Man:Man(3)GlcNAc(2)-PP-Dol alpha-1,2-mannosyltransferase	*alg11*	-1.679	0.006
Apoptosis-resistant E3 ubiquitin protein ligase 1	*arel1*	-1.676	0.007
Unknown	?	-1.669	0.010
Transcription factor p65	*rela*	-1.659	0.007
28S ribosomal protein S33, mitochondrial	*mrps33*	-1.655	0.006
Serine/threonine-protein kinase tousled-like 2	*tlk2*	-1.634	0.006
Unknown	?	-1.623	0.010
WD repeat domain phosphoinositide-interacting protein 2	*wipi2*	-1.623	0.006
Unknown	?	-1.621	0.009
E3 ubiquitin-protein ligase HUWE1	*huwe1*	-1.615	0.010
Cell division cycle and apoptosis regulator protein 1	*ccar1*	-1.577	0.007
UBX domain-containing protein 4	*ubxn4*	-1.559	0.008
Ubiquitin thioesterase OTUB1	*otub1*	-1.537	0.006
Complement C3	*c3*	18.227	0.007
Cystatin-S	*cst4*	16.203	0.007
Geranylgeranyl pyrophosphate synthase	*ggps1*	11.577	0.006
Cytochrome c oxidase copper chaperone	*cox17*	11.010	0.006
Histone H2AX	*?*	9.738	0.005
Tetraspanin-13	*tspan13*	9.442	0.007
Sorting nexin-10	*snx10*	7.389	0.007
Cell division cycle protein 20 homolog	*cdc20*	7.227	0.010
Unknown	*?*	6.897	0.007

Cell death activator CIDE-3	*cidec*	6.714	0.010
Cytochrome P450 26A1	*cyp26a1*	6.336	0.009
Histone H2B type 1-C/E/F/G/I	*hist1h2bc*	6.082	0.006
Periphilin-1	*pphln1*	5.272	0.009
Uridine-cytidine kinase 2	*uck2*	5.177	0.007
Ribonucleoside-diphosphate reductase subunit M2	*rrm2*	4.848	0.009
Tetraspanin-13	*tspan13*	4.796	0.009
Nuclear autoantigenic sperm protein	*nasp*	4.793	0.010
Zona pellucida sperm-binding protein 5	*zpc5*	4.683	0.007
Cytochrome c oxidase subunit 5A	*cox5a*	4.612	0.006
ER membrane protein complex subunit 8	*emc8*	4.594	0.006
Selenoprotein H	*selh*	4.463	0.006
Complex III assembly factor LYRM7	*lyrm7*	4.411	0.008
DNA-directed RNA polymerases I, II, and III subunit RPABC3	*polr2h*	4.337	0.007
Glutaredoxin-2, mitochondrial	*glrx2*	4.189	0.007
Elongation factor Ts, mitochondrial	*tsfm*	4.110	0.007
Protein FAM60A	*fam60a*	4.008	0.006
Acetyl-CoA acetyltransferase, mitochondrial	*acat1*	3.978	0.006
Transcription factor SOX-3	*sox3*	3.865	0.006
Prefoldin subunit 1	*pfdn1*	3.742	0.006
Hepcidin	*hamp*	3.714	0.007
THAP domain-containing protein 4	*thap4*	3.679	0.007
Ceroid-lipofuscinosis neuronal protein 5	*cln5*	3.645	0.006
Unknown	?	3.593	0.007
Glutathione peroxidase 3	*gpx3*	3.561	0.010
39S ribosomal protein L17, mitochondrial	*mrpl17*	3.512	0.005
Inactive hydroxysteroid dehydrogenase-like protein 1	*hsdl1*	3.495	0.009
Frataxin, mitochondrial	*fxn*	3.422	0.007
Wee1-like protein kinase	*wee1*	3.377	0.003
RNA-binding protein 7	*rbm7*	3.377	0.006
Epididymal secretory protein E1	*npc2*	3.353	0.006
DNA-directed RNA polymerase II subunit RPB11-a	*polr2j*	3.316	0.008
Si:busm1-211o13.10	*si:busm1-211o13.10*	3.302	0.006

Peptidyl-prolyl cis-trans isomerase-like 1	*ppil1*	3.286	0.007
Probable aminopeptidase NPEPL1	*npepl1*	3.239	0.006
Lutropin-choriogonadotropic hormone receptor	*lhcgr*	3.235	0.007
Histone H2B type 1-C/E/F/G/I	*hist1h2bc*	3.165	0.006
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1	*ndufa1*	3.123	0.008
Dual specificity protein phosphatase 1	*dusp1*	3.087	0.007
Coenzyme Q-binding protein COQ10 homolog B, mitochondrial	*coq10b*	3.087	0.008
Eukaryotic translation initiation factor 4 gamma 1	*eif4g1*	3.055	0.009
Probable cytosolic iron-sulfur protein assembly protein CIAO1	*ciao1*	3.050	0.005
Probable tRNA pseudouridine synthase 1	*trub1*	3.046	0.007
Histone H2B type 1-O	*hist1h2bo*	3.045	0.008
Ribonuclease P protein subunit p21	*rpp21*	3.027	0.006
Unknown	*?*	3.024	0.006
U6 snRNA-associated Sm-like protein LSm1	*lsm1*	3.018	0.007
Unknown	?	3.012	0.007
Junctional adhesion molecule A	*f11r*	3.005	0.008
AKT-interacting protein	*aktip*	2.981	0.007
Unknown	*?*	2.923	0.010
Cyclin-dependent kinase 5 activator 1	*cdk5r1*	2.901	0.006
MAD2L1-binding protein	*mad2l1bp*	2.890	0.007
Lactoylglutathione lyase	*glo1*	2.884	0.008
Mitochondrial translocator assembly and maintenance protein 41 homolog	*tamm41*	2.884	0.006
DTW domain-containing protein 2	*dtwd2*	2.869	0.009
Inactive L-threonine 3-dehydrogenase, mitochondrial	*tdh*	2.856	0.006
GTP-binding nuclear protein Ran	*ran*	2.830	0.006
28S ribosomal protein S24, mitochondrial	*mrps24*	2.828	0.008
39S ribosomal protein L35, mitochondrial	*mrpl35*	2.827	0.007
Trans-L-3-hydroxyproline dehydratase	*l3hypdh*	2.825	0.010
Hnrp1	*hnrp*	2.794	0.006
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	*sdh1*	2.744	0.007
Embryonic stem cell-specific 5-hydroxymethylcytosine-binding protein	*hmces*	2.734	0.006

BTB/POZ domain-containing protein KCTD14	*kctd14*	2.730	0.007
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	*sdhb*	2.704	0.010
DNA-directed RNA polymerase I subunit RPA12	*znrd1*	2.689	0.008
Anaphase-promoting complex subunit 13	*anapc13*	2.683	0.007
Fibroblast growth factor 8	*fgf8*	2.683	0.007
Unknown	*?*	2.668	0.007
Putative ribosomal RNA methyltransferase 2	*ftsj2*	2.658	0.008
MIT domain-containing protein 1	*mitd1*	2.630	0.005
RNA 3'-terminal phosphate cyclase-like protein	*rcl1*	2.605	0.007
Nonstructural protein P125-2	*p125*	2.601	0.005
Transcription factor BTF3 homolog 4	*btf3l4*	2.577	0.009
N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	*aga*	2.569	0.007
Protein Dr1	*dr1*	2.555	0.009
SNARE-associated protein Snapin	*snapin*	2.549	0.005
Glioma tumor suppressor candidate region gene 2 protein	*gltscr2*	2.548	0.007
Activator of basal transcription 1	*abt1*	2.526	0.006
Methionine--tRNA ligase, mitochondrial	*mars2*	2.514	0.008
S-acyl fatty acid synthase thioesterase, medium chain	*olah*	2.513	0.007
ER membrane protein complex subunit 6	*emc6*	2.503	0.006
Kinetochore-associated protein NSL1 homolog	*nsl1*	2.500	0.006
ALK tyrosine kinase receptor	*alk*	2.491	0.006
39S ribosomal protein L33, mitochondrial	*mrpl33*	2.487	0.007
Transmembrane protein 53	*tmem53*	2.481	0.007
Transmembrane protein 70, mitochondrial	*tmem70*	2.476	0.006
Mitogen-activated protein kinase 14	*mapk14*	2.454	0.006
Vitamin K epoxide reductase complex subunit 1	*vkorc1*	2.453	0.006
Mitochondrial import inner membrane translocase subunit Tim10 B	*timm10b*	2.448	0.010
39S ribosomal protein L18, mitochondrial	*mrpl18*	2.445	0.007
Mitochondrial import inner membrane translocase subunit Tim9	*timm9*	2.444	0.009
Histone-lysine N-methyltransferase SETDB1	*setdb1*	2.433	0.005

MARVEL domain-containing protein 3	*marveld3*	2.423	0.007
Myc proto-oncogene protein	*myc*	2.417	0.007
28S ribosomal protein S12, mitochondrial	*mrps12*	2.416	0.005
AN1-type zinc finger protein 2B	*zfand2b*	2.411	0.006
AP-2 complex subunit alpha-1	*ap2a*	2.408	0.009
Acylpyruvase FAHD1, mitochondrial	*fahd1*	2.401	0.008
Arginine--tRNA ligase, cytoplasmic	*rars*	2.392	0.008
39S ribosomal protein L4, mitochondrial	*mrpl4*	2.391	0.007
Tetratricopeptide repeat protein 27	*ttc27*	2.390	0.008
Sideroflexin-4	*sfxn4*	2.390	0.006
LYR motif-containing protein 1	*lyrm1*	2.388	0.006
28S ribosomal protein S11, mitochondrial	*mrps11*	2.386	0.007
Catechol O-methyltransferase	*comt*	2.376	0.007
Acidic leucine-rich nuclear phosphoprotein 32 family member A	*anp32a*	2.368	0.006
Fc receptor-like protein 6	*fcrl6*	2.367	0.006
Zgc:194819 protein	*faf2*	2.360	0.009
Transmembrane protein 199	*tmem199*	2.359	0.006
Cytosol aminopeptidase	*lap3*	2.337	0.006
Membrane magnesium transporter 1	*mmgt1*	2.310	0.007
Mediator of RNA polymerase II transcription subunit 27	*med27*	2.295	0.008
Probable inactive tRNA-specific adenosine deaminase-like protein 3	*adat3*	2.293	0.007
MARVEL domain-containing protein 1	*marveld1*	2.287	0.007
39S ribosomal protein L27, mitochondrial	*mrpl27*	2.286	0.007
Aurora kinase A-interacting protein	*aurkaip1*	2.277	0.009
Unknown	?	2.274	0.007
PHD finger-like domain-containing protein 5A	*phf5a*	2.273	0.010
Sphingomyelin phosphodiesterase 2	*smpd2*	2.261	0.009
Peroxisomal membrane protein 4	*pxmp4*	2.255	0.007
HIG1 domain family member 2A	*higd2a*	2.253	0.005
General transcription factor 3C polypeptide 5	*gtf3c5*	2.252	0.006
RNA pseudouridylate synthase domain-containing protein 1	*rpusd1*	2.244	0.003
IP05929p	*cg14903*	2.225	0.006
COP9 signalosome complex subunit 3	*cops3*	2.216	0.006

Glycine receptor subunit alpha-3	*glra3*	2.212	0.006
28S ribosomal protein S21, mitochondrial	*mrps21*	2.205	0.007
Protein THEM6	*them6*	2.204	0.006
AP-1 complex subunit sigma-3	*ap1s3*	2.196	0.009
HRAS-like suppressor 3	*pla2g16*	2.187	0.005
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 3	*ndufaf3*	2.183	0.005
Transmembrane protein 134	*tmem134*	2.183	0.006
Anaphase-promoting complex subunit CDC26	*cdc26*	2.181	0.007
Ubiquitin carboxyl-terminal hydrolase isozyme L5	*uchl5*	2.173	0.007
Putative deoxyribonuclease TATDN1	*tatdn1*	2.173	0.007
N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	*aga*	2.169	0.008
Cysteine-rich with EGF-like domain protein 2	*creld2*	2.163	0.007
Protein LZIC	*lzic*	2.158	0.007
Rab5 GDP/GTP exchange factor	*rabgef1*	2.151	0.009
Autophagy-related protein 16-1	*atg16l1*	2.141	0.006
Mitochondrial ribosome-associated GTPase 1	*mtg1*	2.139	0.007
Protein disulfide-isomerase-like protein of the testis	*pdilt*	2.133	0.009
Ribosome biogenesis protein WDR12	*wdr12*	2.129	0.007
Acetyl-CoA acetyltransferase, cytosolic	*acat2*	2.126	0.007
SET and MYND domain-containing protein 4	*smyd4*	2.120	0.007
Transmembrane protein 147	*tmem147*	2.115	0.008
N-alpha-acetyltransferase 20	*?*	2.113	0.007
Methyl-CpG-binding domain protein 3	*mbd3*	2.108	0.006
Cell cycle control protein 50A	*tmem30a*	2.108	0.007
11-cis retinol dehydrogenase	*rdh5*	2.108	0.006
Signal peptidase complex subunit 1	*spcs1*	2.106	0.008
Unknown	?	2.105	0.009
Zinc finger CCHC domain-containing protein 4	*zcchc4*	2.103	0.007
5-hydroxytryptamine receptor 5A	*htr5a*	2.099	0.006
tRNA pseudouridine synthase A, mitochondrial	*pus1*	2.097	0.009
Transcription factor p65	*rela*	2.093	0.009

Lipase maturation factor 2	*lmf2*	2.086	0.009
NudC domain-containing protein 1	*nudcd1*	2.083	0.007
U3 small nucleolar RNA-interacting protein 2	*rrp9*	2.082	0.007
28S ribosomal protein S18a, mitochondrial	*mrps18a*	2.081	0.009
Mediator of RNA polymerase II transcription subunit 20	*med20*	2.080	0.010
Methylosome protein 50	*wdr77*	2.080	0.010
Mitochondrial import inner membrane translocase subunit Tim21	*timm21*	2.071	0.009
V-type proton ATPase subunit F	*atp6v*	2.071	0.006
Serine protease HTRA2, mitochondrial	*htra2*	2.068	0.007
OX-2 membrane glycoprotein	*cd200*	2.060	0.007
Endoplasmic reticulum-Golgi intermediate compartment protein 2	*ergic2*	2.052	0.006
ATP synthase subunit e, mitochondrial	*atp5i*	2.045	0.010
Pyrroline-5-carboxylate reductase 1, mitochondrial	*pycr1*	2.033	0.006
RWD domain-containing protein 2B	*rwdd2b*	2.031	0.008
UMP-CMP kinase 2, mitochondrial	*cmpk2*	2.030	0.007
3-oxoacyl-[acyl-carrier-protein] reductase FabG	*fabg*	2.028	0.008
Phosphatidylinositol N-acetylglucosaminyltransferase subunit H	*pigh*	2.027	0.006
Tyrosyl-DNA phosphodiesterase 2	*tdp2*	2.026	0.008
Zinc finger CCHC domain-containing protein 10	*zcchc10*	2.021	0.006
39S ribosomal protein L9, mitochondrial	*mrpl9*	2.018	0.007
Ubiquilin-4	*ubqln4*	2.011	0.007
Mediator of RNA polymerase II transcription subunit 21	*med21*	2.008	0.009
Vasopressin-neurophysin 2-copeptin	*avp*	1.995	0.006
Ribonucleases P/MRP protein subunit POP1	*pop1*	1.994	0.007
N-acetylglucosamine-1-phosphotransferase subunit gamma	*gnptg*	1.993	0.006
14 kDa phosphohistidine phosphatase	*phpt1*	1.993	0.010
ATP synthase F(0) complex subunit C1, mitochondrial	*atp5g1*	1.988	0.010
Inorganic pyrophosphatase	*ppa1*	1.985	0.006
Carboxypeptidase Z	*cpz*	1.983	0.006
UDP-N-acetylglucosamine transferase subunit ALG14 homolog	*alg14*	1.982	0.006

Bis(5'-nucleosyl)-tetraphosphatase	*nudt2*	1.973	0.006
Putative N-acetylglucosamine-6-phosphate deacetylase	*amdhd2*	1.969	0.006
Phosphatidylinositol transfer protein beta isoform	*pitpnb*	1.968	0.009
26S proteasome non-ATPase regulatory subunit 10	*psmd10*	1.965	0.006
Glucosylceramidase	*gba*	1.960	0.010
Palmitoyl-protein thioesterase 1	*ppt1*	1.957	0.007
Low-density lipoprotein receptor	*ldlr*	1.955	0.008
Ribonucleases P/MRP protein subunit POP1	*pop1*	1.937	0.010
Dehydrogenase/reductase SDR family member 7B	*dhrs7b*	1.936	0.006
Rab5 GDP/GTP exchange factor	*rabgef1*	1.935	0.006
ER membrane protein complex subunit 10	*emc10*	1.933	0.006
Sodium bicarbonate cotransporter 3	*slc4a7*	1.929	0.005
Peroxisomal membrane protein 11A	*pex11a*	1.926	0.007
Receptor-type tyrosine-protein phosphatase zeta	*ptprz1*	1.915	0.009
Acylamino-acid-releasing enzyme	*apeh*	1.900	0.010
Inosine triphosphate pyrophosphatase	*itpa*	1.894	0.007
Unknown	?	1.891	0.008
Elongation factor 1-delta	*eef1d*	1.890	0.007
Long-chain-fatty-acid--CoA ligase 6	*acsl6*	1.884	0.008
Glucosylceramidase	*gba*	1.883	0.006
Nitrogen permease regulator 3-like protein	*nprl3*	1.880	0.006
Phosphatidylinositol-glycan biosynthesis class F protein	*pigf*	1.869	0.006
Solute carrier family 35 member F2	*slc35f2*	1.867	0.007
Mitochondrial inner membrane protein OXA1L	*oxa1l*	1.864	0.009
G patch domain and KOW motifs-containing protein	*gpkow*	1.854	0.009
Calponin-2	*cnn2*	1.851	0.009
Nucleolar protein 16	*nop16*	1.850	0.007
Protein KTI12 homolog	*kti12*	1.847	0.009
histone deacetylase 11	*hdac11*	1.839	0.007
WD repeat-containing protein 41	*wdr41*	1.832	0.006
39S ribosomal protein L52, mitochondrial	*mrpl52*	1.814	0.010
Steroid receptor RNA activator 1	*sra1*	1.810	0.009

Cirhin	*cirh1a*	1.808	0.009
Conserved oligomeric Golgi complex subunit 4	*cog4*	1.807	0.008
Exosome complex component RRP4	*exosc2*	1.795	0.007
Arylamine N-acetyltransferase 1	*nat1*	1.794	0.008
V-type proton ATPase subunit G 1	*atp6v*	1.794	0.007
Pseudouridine-5'-monophosphatase	*hdhd1*	1.792	0.007
MKI67 FHA domain-interacting nucleolar phosphoprotein	*nifk*	1.790	0.006
Unknown	?	1.790	0.010
Unknown	*?*	1.784	0.007
CD82 antigen	*cd82*	1.780	0.006
Mitochondrial intermediate peptidase	*mipep*	1.780	0.008
THO complex subunit 7 homolog	*thoc7*	1.775	0.008
Beta-catenin-interacting protein 1	*ctnnbip1*	1.774	0.008
Partitioning defective 6 homolog gamma	*pard6g*	1.772	0.008
E3 ubiquitin-protein ligase MYLIP	*mylip*	1.771	0.009
Solute carrier family 25 member 40	*slc25a40*	1.771	0.008
Signal recognition particle subunit SRP72	*srp72*	1.769	0.008
Histidine triad nucleotide-binding protein 3	*hint3*	1.768	0.008
Unknown	?	1.764	0.008
Tail-anchored protein insertion receptor WRB	*wrb*	1.761	0.009
U3 small nucleolar ribonucleoprotein protein IMP3	*imp3*	1.759	0.009
E3 ubiquitin-protein ligase TRIM39	*trim39*	1.743	0.005
SET and MYND domain-containing protein 5	*smyd5*	1.734	0.009
DNA-directed RNA polymerases I and III subunit RPAC1	*polr1c*	1.734	0.009
UPF0511 protein C2orf56 homolog	?	1.733	0.005
Protein FAM193B	*fam193b*	1.731	0.006
Protein TBRG4	*tbrg4*	1.727	0.010
Protein FAM46A	*fam46a*	1.724	0.007
Glycerol-3-phosphate acyltransferase 3	*agpat9*	1.721	0.006
C-X-C motif chemokine 13	*cxcl13*	1.715	0.007
Protein GTLF3B	*gtlf3b*	1.712	0.009
Tubulin alpha-1B chain	*tuba1b*	1.710	0.007
ATPase family AAA domain-containing protein 1	*atad1*	1.709	0.006

Inositol monophosphatase 1	*impa1*	1.707	0.007
Probable RNA-binding protein EIF1AD	*eif1ad*	1.699	0.009
COMM domain-containing protein 3	*commd3*	1.699	0.007
UPF0402 protein	*egm_09475*	1.692	0.007
Trimethylguanosine synthase	*tgs1*	1.691	0.008
Syntaxin-5	*stx5*	1.691	0.007
E3 ubiquitin-protein ligase RNF126	*rnf126*	1.687	0.009
Glutathione synthetase	*gss*	1.685	0.008
Alpha/beta hydrolase domain-containing protein 14A	*abhd14a*	1.683	0.009
Ankyrin repeat domain-containing protein 16	*ankrd16*	1.678	0.010
Acidic fibroblast growth factor intracellular-binding protein	*fibp*	1.676	0.007
C1GALT1-specific chaperone 1	*c1galt1c1*	1.669	0.008
Spermatogenesis-associated protein 31A1	*spata31a1*	1.669	0.006
Interferon-related developmental regulator 1	*ifrd1*	1.667	0.010
E3 ubiquitin-protein ligase RAD18	?	1.666	0.009
Unknown	?	1.666	0.009
Unknown	?	1.665	0.009
FBP32	*fbp32*	1.661	0.008
Unknown	?	1.659	0.007
Molybdopterin synthase sulfur carrier subunit	*mocs2*	1.656	0.010
Osteopetrosis-associated transmembrane protein 1	*ostm1*	1.644	0.009
Transcription factor Sp9	*sp9*	1.639	0.007
14 kDa phosphohistidine phosphatase	*phpt1*	1.634	0.004
snRNA-activating protein complex subunit 1	*snapc1*	1.630	0.008
E3 ubiquitin-protein ligase RNF146	*rnf146*	1.628	0.006
SH2 domain-containing protein 5	*sh2d5*	1.622	0.006
Mitochondrial thiamine pyrophosphate carrier	*slc25a19*	1.615	0.007
Serine incorporator 3	*serinc3*	1.611	0.008
Serine/threonine-protein phosphatase 2A [≈ High power LED current, peak 2.7 A] 55 kDa regulatory subunit B gamma isoform	*ppp2r2c*	1.608	0.009
Cell division control protein 42 homolog	*cdc42*	1.606	0.007
Prefoldin subunit 5	*pfdn5*	1.604	0.007
Lariat debranching enzyme	*dbr1*	1.602	0.007

Dipeptidyl peptidase 4	*dpp4*	1.600	0.007
PRELI domain-containing protein 1, mitochondrial	*prelid1*	1.597	0.006
Thioredoxin-related transmembrane protein 2	*tmx2*	1.593	0.009
RISC-loading complex subunit TARBP2	*tarbp2*	1.590	0.007
Dolichol-phosphate mannosyltransferase	*dpm1*	1.568	0.009
Unknown	*?*	1.560	0.006
40S ribosomal protein SA	*rpsa*	1.557	0.007
Transcription termination factor, mitochondrial	*mterf*	1.554	0.007
MAPK-interacting and spindle-stabilizing protein-like	*mapk1ip1l*	1.549	0.006
Mitochondrial dynamics protein MID51	*miefl*	1.545	0.009
Ecto-NOX disulfide-thiol exchanger 1	*enox1*	1.541	0.007
Integral membrane protein 2C	*itm2c*	1.541	0.010
Cell cycle control protein 50A	*tmem30a*	1.528	0.007
Antizyme inhibitor 1	*azin1*	1.526	0.010
Transcription factor IIIA	*gtf3a*	1.523	0.008
Grainyhead-like protein 2 homolog	*grhl2*	1.521	0.010
Magnesium transporter protein 1	*magt1*	1.514	0.008
GPN-loop GTPase 3	*gpn3*	1.512	0.009
Probable N-acetyltransferase 8B	*nat8b*	1.504	0.006
Phosphatidylinositol N-acetylglucosaminyltransferase subunit C	*pigc*	1.503	0.009
Mannose-1-phosphate guanyltransferase alpha	*gmppa*	1.502	0.006

Supplementary Table 13. KEGG pathways from FS versus FFcomparison

Pathways	# Sequences	# Enzymes	up/ down
Amino sugar and nucleotide sugar metabolism	1	1	up
Aminoacyl-tRNA biosynthesis	2	2	up
Arachidonic acid metabolism	2	2	down
Arginine and proline metabolism	1	1	down
Arginine and proline metabolism	2	2	up
Benzoate degradation	1	1	up
Beta-Alanine metabolism	1	1	down
Betalain biosynthesis	1	1	up
Biosynthesis of unsaturated fatty acids	1	1	up
Biotin metabolism	1	1	up
Butanoate metabolism	1	1	up
Chloroalkane and chloroalkene degradation	1	1	down
Citrate cycle (TCA cycle)	2	1	up
Drug metabolism-cytochrome P450	2	2	down
Drug-metabolism-other enzymes	1	1	up
Ether lipid metabolism	1	1	up
Fatty acid biosynthesis	2	2	up
Fatty acid metabolism	1	1	up
Fructose and mannose metabolism	1	1	up
Glutathione metabolism	3	3	up
Glycerolipid metabolism	1	1	up
Glycerophospholipid metabolism	2	2	up
Glycine, serine and threonine metabolism	2	2	down
Glycolysis/Gluconeogenesis	2	2	down
Glycosylphophatidylinositol (GPI)-anchor	2	2	up
Glycerolipid metabolism	1	1	down
Glyoxylate and dicarboxylate metabolism	1	1	up
Histidine metabolism	1	1	down
Lysine degradation	2	2	up
Metabolism of xenobiotics by cytochrome P450	2	2	down
mTOR signaling pathway	1	1	up
Naphthalene degradation	1	1	down

N-Glycan biosynthesis	1	1	up
One carbon pool by folate	1	1	down
Other glycan degradation	3	2	up
Other types of O-glycan biosynthesis	1	1	up
Phenylalanine metabolism	2	2	down
Phenylpropanoid biosynthesis	1	1	down
Phosphonate and phosphinate metabolism	1	1	up
Porphyrin and chlorophyll metabolism	1	1	up
Propanoate metabolism	1	1	up
Purine metabolism	11	7	up
Pyrimidine metabolism	11	6	up
Pyruvate metabolism	2	2	up
Selenocompound metabolism	1	1	up
Sphingolipid metabolism	3	2	up
Steroid biosynthesis	1	1	up
Steroid hormone biosynthesis	1	1	up
Synthesis and degradation of ketone bodies	1	1	up
T cell receptor signaling pathway	2	2	up
Terpenoid backbone biosynthesis	1	1	up
Thiamine metabolism	1	1	up
Tryptophan metabolism	1	1	up
Ubiquinone and other terpenoid-quinone biosynthesis	1	1	up
Valine, leucine and isoleucine degradation	1	1	up

Supplementary Table 14. Fisher's Exact Test with Multiple Corrections for FDR for FS vs. FF comparison

GO Term	Name	Type	FDR	single test p-Value	# in test group	# in reference group	Over/ Under
GO:0005739	Mitochondrion	CC	1,60E-02	2,40E-05	79	852	over
GO:0000786	Nucleosome	CC	2,20E-02	4,30E-05	8	18	over
GO:0005730	Nucleolus	CC	2,80E-02	6,90E-05	47	442	over
GO:0004872	receptor activity	MF	2,80E-02	7,40E-05	3	300	under
GO:0010467	gene expression	BP	2,80E-02	1,10E-04	115	1435	over
GO:0010604	positive regulation of macromolecule metabolic process	BP	2,80E-02	1,50E-04	50	494	over
GO:0051724	NAD transporter activity	MF	2,80E-02	1,50E-04	3	0	over
GO:0005347	ATP transmembrane transporter activity	MF	2,80E-02	1,50E-04	3	0	over
GO:0051018	protein kinase A binding	MF	2,80E-02	1,50E-04	3	0	over
GO:0043132	NAD transport	BP	2,80E-02	1,50E-04	3	0	over
GO:0015867	ATP transport	BP	2,80E-02	1,50E-04	3	0	over
GO:0015866	ADP transport	BP	2,80E-02	1,50E-04	3	0	over
GO:0035350	FAD transmembrane transport	BP	2,80E-02	1,50E-04	3	0	over
GO:0035349	coenzyme A transmembrane transport	BP	2,80E-02	1,50E-04	3	0	over
GO:0014911	positive regulation of smooth muscle cell migration	BP	2,80E-02	1,50E-04	3	0	over

GO:0015230	FAD transmembrane transporter activity	MF	2,80E-02	1,50E-04	3	0	over
GO:0015228	coenzyme A transmembrane transporter activity	MF	2,80E-02	1,50E-04	3	0	over
GO:0015217	ADP transmembrane transporter activity	MF	2,80E-02	1,50E-04	3	0	over
GO:0080122	AMP transmembrane transporter activity	MF	2,80E-02	1,50E-04	3	0	over
GO:0080121	AMP transport	BP	2,80E-02	1,50E-04	3	0	over
GO:0031325	positive regulation of cellular metabolic process	BP	2,90E-02	1,60E-04	50	498	over
GO:0034645	cellular macromolecule biosynthetic process	BP	3,60E-02	2,10E-04	108	1348	over
GO:0006334	nucleosome assembly	BP	4,80E-02	3,10E-04	10	41	over

Supplementary Table 15. DE genes from the comparison FS vs. SS

Description	Gene symbol	Fold change	Adjusted *P*-value
Carbonic anhydrase 1	*ca1*	36.701	0.004
Complement receptor-like	*?*	18.232	0.004
Tetraspanin-13	*tspan13*	13.125	0.002
Gamma-interferon-inducible lysosomal thiol reductase	*ifi30*	12.332	0.004
Cytochrome c oxidase copper chaperone	*cox17*	12.064	0.002
Mid1-interacting protein 1	*mid1ip1*	11.400	0.001
Histone H2AX	*h2afx*	10.117	0.001
50S ribosomal protein L4	*rpld*	9.307	0.004
Geranylgeranyl pyrophosphate synthase	*ggps1*	9.157	0.003
Apo-Eif4aiii	*aurandraft_58937*	8.632	0.002
Cell death activator CIDE-3	*cidec*	8.194	0.003
Sorting nexin-10	*snx10*	7.612	0.003
Cytochrome P450 26A1	*cyp26a1*	7.406	0.003
Tetraspanin-13	*tspan13*	6.542	0.002
Metallothionein-2	*mt2a*	6.233	0.009
Histone H2A	*h2a.zl1*	6.168	0.001
Cytoplasmic dynein 1 heavy chain 1	*dync1h1*	6.034	0.004
Periphilin-1	*pphln1*	5.963	0.004
Histone H2B 1/2/3/4/6	*h2b-i*	5.540	0.003
Nuclear autoantigenic sperm protein	*nasp*	5.534	0.004
HAUS augmin-like complex subunit 6	*haus6*	5.472	0.002
Multifunctional protein ADE2	*paics*	5.434	0.002
Translation initiation factor eIF-2B subunit alpha	*eif2b1*	5.305	0.001
Erythropoietin	*epo*	5.042	0.005
Uridine-cytidine kinase 2	*uck2*	5.042	0.004
Glutathione peroxidase 3	*gpx3*	4.996	0.002
Cytochrome c oxidase subunit 5A	*cox5a*	4.666	0.002
Transcription factor SOX-3	*sox3*	4.507	0.001
Coenzyme Q-binding protein	*coq10b*	4.503	0.001

COQ10 homolog B, mitochondrial			
60S acidic ribosomal protein P0	*rplp0*	4.410	0.000
Selenoprotein H	*selh*	4.383	0.003
DNA-directed RNA polymerases I, II, and III subunit RPABC3	*polr2h*	4.240	0.005
Junctional adhesion molecule A	*f11r*	4.237	0.001
ATP-dependent RNA helicase DDX39	*ddx39*	4.157	0.006
Glutaredoxin-2, mitochondrial	*glrx2*	4.107	0.004
ER membrane protein complex subunit 8	*emc8*	4.102	0.003
Inactive hydroxysteroid dehydrogenase-like protein 1	*hsdl1*	4.097	0.001
Fructose-bisphosphate aldolase B	*aldob*	4.068	0.009
Profilin-2	*pfn2*	4.053	0.010
28S ribosomal protein S24, mitochondrial	*mrps24*	3.957	0.001
Mitochondrial translocator assembly and maintenance protein 41 homolog	*tamm41*	3.950	0.000
Ceroid-lipofuscinosis neuronal protein 5	*cln5*	3.944	0.001
Voltage-gated hydrogen channel 1	*hvcn1*	3.851	0.005
28S ribosomal protein S17, mitochondrial	*mrpl17*	3.826	0.000
Complex III assembly factor LYRM7	*lyrm7*	3.760	0.009
Prefoldin subunit 1	*pfdn1*	3.724	0.002
RNA, 5.8S Ribosomal 1	*rn5-8s1*	3.671	0.003
Unknown	*?*	3.670	0.002
Betaine--homocysteine S-methyltransferase 1	*bhmt*	3.612	0.007
Ribonuclease P protein subunit p21	*rpp21*	3.594	0.001
Cyclin-dependent kinase 9	*cdk9*	3.578	0.002
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1	*ndufa1*	3.570	0.001
Putative surface protein	*mgas10750_spy1694*	3.561	0.001
Lactoylglutathione lyase	*glo1*	3.529	0.001
Protein FAM60A	*fam60a*	3.521	0.002
Nonstructural protein P125-2	*p125*	3.461	0.000
Sodium/potassium-transporting	*atp1a1*	3.443	0.002

ATPase subunit alpha-1			
RNA-binding protein 7	*rbm7*	3.422	0.002
Dual specificity protein phosphatase 2	*dusp2*	3.355	0.002
zona pellucida complex 5	*zpc5*	3.354	0.009
THAP domain-containing protein 4	*thap4*	3.350	0.005
Si:busm1-211o13.10	*si:busm1-211o13.10*	3.343	0.002
DTW domain-containing protein 2	*dtwd2*	3.335	0.002
Type II inositol 1,4,5-trisphosphate 5-phosphatase	*inpp5b*	3.329	0.000
Lamina-associated polypeptide 2, isoform alpha	*tmpo*	3.303	0.000
Elongation factor 1-delta	*eef1d*	3.293	0.000
Ribonuclease UK114	*hrsp12*	3.292	0.007
MARVEL domain-containing protein 1	*marveld1*	3.285	0.000
Transaldolase	*taldo1*	3.281	0.002
THAP domain-containing protein 1	*thap1*	3.269	0.000
Lutropin-choriogonadotropic hormone receptor	*lhcgr*	3.269	0.003
ATP synthase subunit gamma, mitochondrial	*atp5c1*	3.264	0.001
Cob(I)yrinic acid a,c-diamide adenosyltransferase, mitochondrial	*mmab*	3.260	0.003
Epididymal secretory protein E1	*npc2*	3.245	0.002
Endothelial lipase	*lipg*	3.229	0.004
Cyclin-dependent kinase 5	*cdk5*	3.218	0.001
Putative deoxyribonuclease TATDN1	*tatdn1*	3.203	0.000
Vitamin K epoxide reductase complex subunit 1	*vkorc1*	3.200	0.000
Glioma tumor suppressor candidate region gene 2 protein	*gltscr2*	3.178	0.001
Vinculin	*deb-1*	3.174	0.006
Frataxin, mitochondrial	*fxn*	3.172	0.003
Lactotransferrin	*ltf*	3.157	0.002
Synaptonemal complex protein SC65	*leprel4*	3.130	0.003
Sarcolemmal membrane-associated protein	*slmap*	3.116	0.004
Hnrp1	*hnrp*	3.097	0.001

AN1-type zinc finger protein 2B	*zfand2b*	3.094	0.000
Isocitrate dehydrogenase [NADP], mitochondrial	*idh2*	3.081	0.001
Probable aminopeptidase NPEPL1	*npepl1*	3.041	0.002
39S ribosomal protein L22	*rpl22*	3.037	0.000
histone cluster 1	*hist1h2bo*	3.027	0.005
DNA primase small subunit	*prim1*	3.022	0.003
DNA-directed RNA polymerase II subunit RPB11-a	*polr2j*	3.005	0.008
Acidic leucine-rich nuclear phosphoprotein 32 family member A	*anp32a*	2.993	0.001
39S ribosomal protein L27, mitochondrial	*mrpl27*	2.987	0.001
Cyclic AMP-dependent transcription factor ATF-4	*atf4*	2.981	0.000
Serine/threonine-protein phosphatase PGAM5, mitochondrial	*pgam5*	2.967	0.001
Interferon-induced GTP-binding protein Mx1	*mx1*	2.958	0.006
Acylamino-acid-releasing enzyme	*apeh*	2.953	0.000
Nucleoside diphosphate kinase A	*nme1*	2.947	0.000
Aurora kinase A-interacting protein	*aurkaip1*	2.940	0.001
Methionine--tRNA ligase, cytoplasmic	*mars*	2.919	0.002
ER membrane protein complex subunit 6	*emc6*	2.917	0.001
Interactor protein for cytohesin exchange factors 1	*ipcef1*	2.914	0.002
Kinesin-like protein KIF22	*kif22*	2.913	0.002
Sorting nexin-24	*snx24*	2.896	0.010
Myc proto-oncogene protein	*myc*	2.888	0.001
Transmembrane protein 208	*tmem208*	2.874	0.000
MIT domain-containing protein 1	*mitd1*	2.871	0.000
Aspartyl aminopeptidase	*dnpep*	2.868	0.001
ER membrane protein complex subunit 10	*emc10*	2.858	0.006
BTB/POZ domain-containing protein KCTD14	*kctd14*	2.857	0.003
MARVEL domain-containing protein 3	*marveld3*	2.855	0.001
Signal recognition particle 9 kDa	*srp9*	2.844	0.002

protein			
Succinate dehydrogenase assembly factor 2, mitochondrial	*emi5*	2.831	0.003
5-methylcytosine rRNA methyltransferase NSUN4	*nsun4*	2.827	0.001
Wee1-like protein kinase	*wee1*	2.825	0.001
Catechol O-methyltransferase	*comt*	2.816	0.001
Transcription factor BTF3 homolog 4	*btf3l4*	2.786	0.007
Transmembrane protein 70, mitochondrial	*tmem70*	2.782	0.001
N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	*aga*	2.782	0.001
Protein Tob1	*tob1*	2.779	0.005
Oxysterol-binding protein-related protein 3	*osbpl3*	2.770	0.002
Trans-L-3-hydroxyproline dehydratase	*l3hypdh*	2.769	0.007
Cytochrome P450 27C1	*cyp27c*	2.768	0.004
THO complex subunit 4	*alyref*	2.763	0.004
Activator of basal transcription 1	*abt1*	2.757	0.001
Methylosome protein 50	*wdr77*	2.747	0.005
Embryonic stem cell-specific 5-hydroxymethylcytosine-binding protein	*hmces*	2.740	0.001
Cell cycle control protein 50A	*tmem30a*	2.740	0.000
Aktip protein	*aktip*	2.738	0.002
RNA polymerase II subunit A C-terminal domain phosphatase SSU72	*ssu72*	2.738	0.002
28S ribosomal protein S18a, mitochondrial	*mrps18a*	2.733	0.001
39S ribosomal protein L18, mitochondrial	*mrpl18*	2.731	0.002
Mitotic spindle assembly checkpoint protein MAD2B	*mad2l2*	2.730	0.004
Ras-related protein Rab-8A	*rab8a*	2.728	0.004
Chromobox protein homolog 5	*cbx5*	2.728	0.005
Argininosuccinate synthase	*ass1*	2.711	0.006
S-acyl fatty acid synthase thioesterase, medium chain	*olah*	2.706	0.002
Histone-lysine N-methyltransferase SETDB1	*setdb1*	2.706	0.000
Zinc finger CCHC domain-	*zcchc4*	2.704	0.002

containing protein 4			
LYR motif-containing protein 1	*lyrm1*	2.700	0.001
Mitochondrial import inner membrane translocase subunit Tim10 B	*timm10b*	2.696	0.003
Biogenesis of lysosome-related organelles complex 1 subunit 1	*bloc1s1*	2.694	0.001
Tetratricopeptide repeat protein 27	*ttc27*	2.694	0.002
Palmitoyl-protein thioesterase 1	*ppt1*	2.689	0.000
SUMO-activating enzyme subunit 2	*uba2*	2.682	0.007
Unknown	*?*	2.673	0.001
Mitogen-activated protein kinase 14	*mapk14*	2.659	0.001
28S ribosomal protein S2, mitochondrial	*mrps2*	2.658	0.001
Transmembrane protein 134	*tmem134*	2.651	0.000
Peroxisomal membrane protein 4	*pxmp4*	2.651	0.001
Fibroblast growth factor 8	*fgf8*	2.651	0.004
Mitochondrial import inner membrane translocase subunit Tim21	*timm21*	2.649	0.001
RNA 3'-terminal phosphate cyclase-like protein	*rcl1*	2.648	0.002
AP-1 complex subunit sigma-3	*ap1s3*	2.647	0.002
Transcription initiation protein SPT3 homolog	*supt3h*	2.640	0.002
G patch domain and KOW motifs-containing protein	*gpkow*	2.639	0.001
Protein KTI12 homolog	*kti12*	2.626	0.001
Sideroflexin-4	*sfxn4*	2.624	0.001
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	*sdhb*	2.614	0.008
Transmembrane protein 147	*tmem147*	2.607	0.001
Acyl-CoA dehydrogenase family member 11	*acad11*	2.607	0.001
Autophagy-related protein 16-1	*atg16l1*	2.597	0.000
Rab5 GDP/GTP exchange factor	*rabgef1*	2.597	0.001
Protein disulfide-isomerase-like protein of the testis	*pdilt*	2.593	0.001
Lipid phosphate phosphatase-related protein type 4	*lppr4*	2.583	0.008

39S ribosomal protein L35, mitochondrial	*mrpl35*	2.570	0.008
Very-long-chain (3R)-3-hydroxyacyl-[acyl-carrier protein] dehydratase 3	*ptplad1*	2.560	0.004
U3 small nucleolar RNA-interacting protein 2	*rrp9*	2.559	0.002
HMG domain-containing protein 4	*hmgxb4*	2.558	0.003
Putative ribosomal RNA methyltransferase 2	*ftsj2*	2.549	0.002
Fc receptor-like protein 6	*fcrl6*	2.545	0.001
Putative N-acetylglucosamine-6-phosphate deacetylase	*amdhd2*	2.544	0.000
Coiled-coil domain-containing protein R3HCC1L	*r3hcc1l*	2.530	0.002
Homeobox protein VOX2	*vox2*	2.530	0.005
General transcription factor 3C polypeptide 5	*gtf3c5*	2.525	0.001
EH domain-binding protein 1	*ehbp1*	2.511	0.002
Probable cytosolic iron-sulfur protein assembly protein CIAO1	*ciao1*	2.502	0.003
39S ribosomal protein L15, mitochondrial	*mrpl15*	2.501	0.001
Trafficking protein particle complex subunit 5	*trappc5*	2.497	0.001
Serine protease HTRA2, mitochondrial	*htra2*	2.495	0.001
Cytosol aminopeptidase	*lap3*	2.494	0.001
Tyrosyl-DNA phosphodiesterase 2	*tdp2*	2.489	0.003
39S ribosomal protein L10	*rpl10*	2.484	0.000
Zinc finger protein 518A	*znf518a*	2.482	0.002
Transcription factor p65	*rela*	2.481	0.002
Cytochrome b-c1 complex subunit 1, mitochondrial	*uqcrc1*	2.457	0.003
Cellular tumor antigen p53	*tp53*	2.433	0.006
Arginine--tRNA ligase, cytoplasmic	*rars*	2.432	0.002
Ubiquitin carboxyl-terminal hydrolase isozyme L5	*uchl5*	2.425	0.002
Transmembrane protein 199	*tmem199*	2.415	0.002
DnaJ-like subfamily B member 11	*hsp40b11*	2.404	0.003
U6 snRNA-associated Sm-like protein LSm1	*lsm1*	2.401	0.010

Nucleophosmin	*npm1*	2.398	0.002
39S ribosomal protein L4, mitochondrial	*mrpl4*	2.390	0.004
28S ribosomal protein S11, mitochondrial	*mrps11*	2.378	0.004
Sphingomyelin phosphodiesterase	*smpd1*	2.367	0.003
U3 small nucleolar ribonucleoprotein protein IMP3	*imp3*	2.361	0.002
Acetyl-CoA acetyltransferase, cytosolic	*acat2*	2.358	0.001
39S ribosomal protein L9, mitochondrial	*mrpl9*	2.347	0.002
Ancient ubiquitous protein 1	*aup1*	2.339	0.003
15 kDa selenoprotein	*42248*	2.339	0.002
Interleukin enhancer-binding factor 2	*ilf2*	2.329	0.009
Probable tRNA pseudouridine synthase 1	*trub1*	2.327	0.008
Retinol dehydrogenase 1 (All trans)	*rdh1*	2.324	0.002
snRNA-activating protein complex subunit 1	*snapc1*	2.319	0.000
40S ribosomal protein S12, mitochondrial	*tko*	2.311	0.004
40S ribosomal protein SA	*rpsa*	2.309	0.008
Solute carrier family 35 member F2	*slc35f2*	2.309	0.000
N-acetylglucosamine-1-phosphotransferase subunit gamma	*gnptg*	2.307	0.001
RING finger protein 113A	*rnf113a*	2.306	0.002
Mitochondrial import inner membrane translocase subunit Tim9	*timm9*	2.304	0.008
Ubiquitin-like protein ATG12	*atg12*	2.302	0.002
Ras-related protein Rab-32	*rab32*	2.301	0.001
Long-chain-fatty-acid--CoA ligase 1	*acsl1*	2.298	0.004
Double-stranded RNA-specific adenosine deaminase	*adar*	2.296	0.002
Factor in the germline alpha	*figla*	2.292	0.008
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	*ndufa9*	2.283	0.006
Glycine receptor subunit alpha-3	*glra3*	2.280	0.002
HMG domain-containing protein 4	*hmgxb4*	2.276	0.001

Protein disulfide-isomerase A4	*pdia4*	2.276	0.007
Acyl-coenzyme A thioesterase 8	*acot8*	2.274	0.002
Schwannomin-interacting protein 1	*schip1*	2.271	0.000
Pyrroline-5-carboxylate reductase 1, mitochondrial	*pycr1*	2.268	0.001
Eukaryotic translation initiation factor 4E transporter	*eif4enif1*	2.267	0.006
Glucosidase 2 subunit beta	*prkcsh*	2.266	0.004
Proteasome subunit alpha type-3	*psma3*	2.249	0.004
Long-chain-fatty-acid--CoA ligase 6	*acsl6*	2.247	0.001
Protein THEM6	*them6*	2.247	0.002
Fatty acyl-CoA reductase 2	*far2*	2.243	0.002
Pseudouridine-5'-monophosphatase	*hdhd1*	2.242	0.000
AGAP012577-PA	*agap012577-pa*	2.241	0.000
COP9 signalosome complex subunit 3	*cops3*	2.240	0.002
Protein MTO1 homolog, mitochondrial	*mto1*	2.236	0.002
Alanyl-tRNA editing protein Aarsd1	*aarsd1*	2.236	0.001
ATP synthase subunit e, mitochondrial	*atp5i*	2.235	0.003
CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, mitochondrial	*pgs1*	2.233	0.001
RNA pseudouridylate synthase domain-containing protein 1	*rpusd1*	2.228	0.001
Methyl-CpG-binding domain protein 3	*mbd3*	2.225	0.001
Glucosylceramidase	*gba*	2.225	0.001
claudin 18	*cldn18*	2.225	0.002
OTTMUSG00000010694 protein	*zfp600*	2.222	0.004
Membrane magnesium transporter 1	*mmgt1*	2.220	0.005
Cyclin-dependent kinase 10	*cdk10*	2.218	0.007
Cysteine-rich with EGF-like domain protein 2	*creld2*	2.216	0.003
SET and MYND domain-containing protein 4	*smyd4*	2.215	0.002
UDP-N-acetylglucosamine transferase subunit ALG14 homolog	*alg14*	2.213	0.001

Thioredoxin-2	*trx2*	2.198	0.000
Ectonucleoside triphosphate diphosphohydrolase 8	*entpd8*	2.192	0.000
DNA primase large subunit	*prim2*	2.191	0.005
Metaxin-2	*mtx2*	2.190	0.004
FGFR1 oncogene partner 2	*fgfr1op2*	2.182	0.001
Mediator of RNA polymerase II transcription subunit 17	*med17*	2.182	0.002
Glucosamine-6-phosphate isomerase 1	*gnpda1*	2.180	0.005
Translation initiation factor eIF-2B subunit alpha	*eif2b1*	2.179	0.004
39S ribosomal protein L45, mitochondrial	*mrpl45*	2.179	0.001
UBX domain-containing protein 2A	*ubxn2a*	2.167	0.001
Ufm1-specific protease 2	*ufsp2*	2.167	0.001
E3 ubiquitin-protein ligase MYLIP	*mylip*	2.166	0.001
Partitioning defective 6 homolog gamma	*pard6g*	2.166	0.002
ATP-dependent RNA helicase DDX55	*ddx55*	2.165	0.001
Mediator of RNA polymerase II transcription subunit 27	*med27*	2.162	0.008
Calcium release-activated calcium channel protein 1	*orai1*	2.160	0.003
Densityregulated protein	*denr*	2.159	0.001
Im:7145112 protein	*elac2*	2.158	0.002
28S ribosomal protein S26, mitochondrial	*mrps26*	2.149	0.002
ADP-ribosylation factor-like protein 6-interacting protein 1	*arl6ip1*	2.149	0.002
IP05929p	*cg14903*	2.145	0.002
Inositol monophosphatase 1	*impa1*	2.145	0.000
Transcription factor p65	*rela*	2.143	0.005
Cryptochrome-1	*cry1*	2.140	0.001
tRNA pseudouridine synthase A, mitochondrial	*pus1*	2.134	0.005
Carboxypeptidase Z	*cpz*	2.133	0.001
CAAX prenyl protease 1 homolog	*zmpste24*	2.132	0.001
Mitochondrial Rho GTPase 1	*rhot1*	2.132	0.002
Propionyl-CoA carboxylase beta	*pccb*	2.132	0.002

chain, mitochondrial			
Endonuclease 8-like 1	*neil1*	2.131	0.001
Target of rapamycin complex 2 subunit MAPKAP1	*mapkap1*	2.113	0.000
DNA polymerase beta	*polb*	2.113	0.002
Zinc finger CCHC domain-containing protein 10	*zcchc10*	2.109	0.002
Glycerol-3-phosphate acyltransferase 3	*agpat9*	2.108	0.000
Nucleolar complex protein 4 homolog	*noc4l*	2.107	0.002
Interferon-related developmental regulator 1	*ifrd1*	2.107	0.002
Mediator of RNA polymerase II transcription subunit 20	*med20*	2.105	0.006
Histone-lysine N-methyltransferase SMYD3	*smyd3*	2.105	0.002
Putative protein MSS51 homolog, mitochondrial	*mss51*	2.105	0.003
Peroxisomal membrane protein 11A	*pex11a*	2.104	0.002
Unknown	*?*	2.102	0.002
Ras GTPase-activating-like protein IQGAP2	*iqgap2*	2.101	0.004
Pre-mRNA-splicing factor RBM22	*rbm22*	2.089	0.008
Ubiquitin-like protein 4A	*ubl4a*	2.089	0.002
Ribonuclease P protein subunit p38	*rpp38*	2.088	0.008
V-type proton ATPase subunit G 1	*atp6v*	2.087	0.001
3-oxoacyl-[acyl-carrier-protein] synthase, mitochondrial	*oxsm*	2.085	0.003
tRNA wybutosine-synthesizing protein 3 homolog	*tyw3*	2.084	0.004
Heat shock 70 kDa protein 14	*hspa14*	2.082	0.000
39S ribosomal protein L18	*rpl18*	2.081	0.000
DNA-(apurinic or apyrimidinic site) lyase	*apex1*	2.077	0.007
Ribonuclease P protein subunit p40	*rpp40*	2.077	0.002
UMP-CMP kinase 2, mitochondrial	*cmpk2*	2.074	0.004
Histone-lysine N-methyltransferase SETD7	*setd7*	2.071	0.005
Tight junction protein ZO-2	*tjp2*	2.070	0.002
Zinc finger protein 277	*znf277*	2.067	0.001

2',3'-cyclic-nucleotide 3'-phosphodiesterase	*cnp*	2.064	0.006
Transcription factor BTF3	*btf3*	2.061	0.002
5-hydroxytryptamine receptor 5A	*htr5a*	2.061	0.002
HRAS-like suppressor 3	*pla2g16*	2.052	0.002
Anoctamin-10	*ano10*	2.051	0.003
Zinc transporter ZIP12	*slc39a*	2.051	0.002
39S ribosomal protein L9, mitochondrial	*rpl9*	2.049	0.004
Type-1 angiotensin II receptor-associated protein	*agtrap*	2.045	0.007
Cell differentiation protein RCD1 homolog	*rcd1*	2.038	0.002
DNA polymerase kappa	*polk*	2.038	0.003
RNA-binding protein NOB1	*nob1*	2.037	0.007
26S proteasome non-ATPase regulatory subunit 10	*psmd10*	2.037	0.002
Unknown	*?*	2.036	0.009
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 3	*ndufaf3*	2.034	0.003
Selenoprotein T	*selt*	2.034	0.002
Pleiotrophin	*ptn*	2.029	0.002
Zgc:162641	*slc25a17*	2.029	0.001
Cathepsin C	*ctsc*	2.029	0.007
HIG1 domain family member 2A	*higd2a*	2.029	0.002
Transmembrane protein 147	*tmem147*	2.025	0.006
Inorganic pyrophosphatase	*ppa1*	2.024	0.001
39S ribosomal protein L22, mitochondrial	*mrpl22*	2.024	0.002
39S ribosomal protein L4	*rpl4*	2.017	0.003
DNA damage-binding protein 2	*ddb2*	2.010	0.004
ATP synthase F(0) complex subunit C1, mitochondrial	*atp5g1*	2.007	0.001
OX-2 membrane glycoprotein	*cd200*	2.007	0.004
Tether containing UBX domain for GLUT4	*aspscr1*	2.006	0.000
Methyltransferase-like protein 4	*mettl4*	2.004	0.005
Ubiquitin-like protein 7	*ubl7*	2.004	0.000
Mitochondrial thiamine pyrophosphate carrier	*slc25a19*	2.004	0.000

NADH dehydrogenase (Ubiquinone) Fe-S protein 3 (Predicted), isoform CRA_c	*ndufs3*	2.004	0.005
TIP41-like protein	*tiprl*	2.003	0.002
Methionine aminopeptidase 1D, mitochondrial	*metap1d*	2.002	0.001
Mitochondrial inner membrane protein OXA1L	*oxa1l*	1.999	0.003
NTF2-related export protein 1	*nxt1*	1.996	0.006
Cathepsin B	*ctsb*	1.996	0.008
Ankyrin repeat and SOCS box protein 13	*asb13*	1.995	0.008
Nucleolar protein 16	*nop16*	1.992	0.002
Cytochrome b-c1 complex subunit 8	*uqcrq*	1.992	0.006
60S ribosomal protein L24	*rpl24*	1.990	0.000
60 kDa heat shock protein, mitochondrial	*hspd1*	1.988	0.003
EH domain-containing protein 1	*ehd1*	1.987	0.008
DDB1- and CUL4-associated factor 13	*dcaf13*	1.986	0.005
SET and MYND domain-containing protein 5	*smyd5*	1.983	0.001
BAG family molecular chaperone regulator 1	*bag1*	1.983	0.002
Acidic fibroblast growth factor intracellular-binding protein	*fibp*	1.981	0.002
39S ribosomal protein L52, mitochondrial	*mrpl52*	1.981	0.002
RNA-binding protein 39	*rbm39*	1.976	0.008
U5 small nuclear ribonucleoprotein 40 kDa protein	*snrnp40*	1.967	0.002
Nicotinate phosphoribosyltransferase	*naprt1*	1.965	0.001
Solute carrier family 25 member 40	*slc25a40*	1.965	0.004
39S ribosomal protein L5	*rpl5*	1.962	0.007
Betaine aldehyde dehydrogenase	*aldh9a*	1.962	0.006
Rab5 GDP/GTP exchange factor	*rabgef1*	1.961	0.002
Proteasome activator complex subunit 1	*psme1*	1.958	0.000
Mitochondrial intermediate peptidase	*mipep*	1.958	0.002
Serine/threonine-protein phosphatase 2A	*ppp2r2c*	1.958	0.002

ES1 protein homolog, mitochondrial	*c21orf33*	1.957	0.005
Cirhin	*cirh1a*	1.956	0.002
ATP synthase subunit g, mitochondrial	*atp5l*	1.954	0.006
Protein phosphatase methylesterase 1	*ppme1*	1.954	0.010
Mortality factor 4-like protein 1	*morf4l1*	1.953	0.001
m7GpppX diphosphatase	*dcps*	1.952	0.009
Myocardial zonula adherens protein	*gcom1*	1.949	0.003
39S ribosomal protein L49, mitochondrial	*mrpl49*	1.949	0.008
Receptor-type tyrosine-protein phosphatase zeta	*ptprz1*	1.949	0.005
Low-density lipoprotein receptor	*ldlr*	1.948	0.005
Unknown	*?*	1.945	0.003
Ephrin type-A receptor 2	*epha2*	1.945	0.006
39S ribosomal protein L30	*rpl30*	1.944	0.000
ADP-sugar pyrophosphatase	*nudt5*	1.943	0.002
Glutaredoxin-related protein 5, mitochondrial	*glrx5*	1.942	0.003
Probable ATP-dependent RNA helicase DHX37	*dhx37*	1.939	0.003
Unknown	*?*	1.934	0.002
Histone H2A	*h2afv*	1.931	0.008
TATA box-binding protein-associated factor RNA polymerase I subunit A	*taf1a*	1.927	0.001
Unknown	*?*	1.925	0.001
Inosine triphosphate pyrophosphatase	*itpa*	1.919	0.003
39S ribosomal protein L13	*rpl13*	1.919	0.001
Glycosylphosphatidylinositol anchor attachment 1 protein	*gpaa1*	1.918	0.003
Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]	*nudt2*	1.917	0.003
Cleavage and polyadenylation specificity factor subunit 5	*nudt21*	1.914	0.004
Heat shock cognate 71 kDa protein	*hspa8*	1.914	0.003
Lysophospholipase-like protein 1	*lyplal1*	1.913	0.003
SH3 domain-binding glutamic acid-rich-like protein	*sh3bgrl*	1.913	0.004

tRNA-dihydrouridine(47) synthase [NAD(P)(+)]-like	*dus3l*	1.908	0.007
Geranylgeranyl transferase type-2 subunit beta	*rabggtb*	1.903	0.002
40S ribosomal protein S17	*rps17*	1.903	0.000
Caspase-1	*casp1*	1.903	0.005
Histidine triad nucleotide-binding protein 3	*hint3*	1.898	0.002
Mitochondrial ribonuclease P protein 3	*kiaa0391*	1.898	0.001
WD repeat-containing protein 41	*wdr41*	1.896	0.001
Splicing factor 45	*rbm17*	1.888	0.004
Protein VAC14 homolog	*vac14*	1.888	0.002
Reverse transcriptase-like protein	*rtl*	1.885	0.000
Glutathione S-transferase Mu 1	*gstm1*	1.884	0.001
GTPase Era, mitochondrial	*eral1*	1.883	0.003
Large neutral amino acids transporter small subunit 2	*slc7a8*	1.880	0.002
Riboflavin kinase	*rfk*	1.880	0.006
Alpha/beta hydrolase domain-containing protein 14A	*abhd14a*	1.880	0.002
Probable tRNA N6-adenosine threonylcarbamoyltransferase, mitochondrial	*osgepl1*	1.876	0.005
Pericentrin	*pcnt*	1.875	0.006
Ribosome production factor 1	*rpf1*	1.875	0.005
ATP-dependent RNA helicase DDX56	*ddx56*	1.862	0.002
Cytoplasmic dynein 1 intermediate chain 2	*dync1i2*	1.862	0.001
cAMP-dependent protein kinase inhibitor alpha	*pkia*	1.860	0.004
Cytoskeleton-associated protein 2	*ckap2*	1.859	0.007
Leukocyte immune-type receptor TS32.15 L1.1a	*?*	1.858	0.009
Protein FAM46A	*fam46a*	1.854	0.002
Hydroxyacylglutathione hydrolase, mitochondrial	*hagh*	1.853	0.002
STON1-GTF2A1L protein	*ston1-gtf2a1l*	1.852	0.005
Glycine cleavage system H protein, mitochondrial	*gcsh*	1.850	0.001
Unknown	*?*	1.850	0.007
Steroid receptor RNA activator 1	*sra1*	1.847	0.005

Anaphase-promoting complex subunit 7	*anapc7*	1.846	0.003
Rho-associated protein kinase 2	*rock2*	1.844	0.007
Translation initiation factor IF-3, mitochondrial	*mtif3*	1.844	0.000
Probable E3 ubiquitin-protein ligase HERC3	*herc3*	1.844	0.001
NEDD8-activating enzyme E1 catalytic subunit	*uba3*	1.844	0.001
Endoplasmin	*hsp90b1*	1.843	0.004
Poly(rC)-binding protein 2	*pcbp2*	1.842	0.002
Protein-tyrosine sulfotransferase 1	*tpst1*	1.842	0.000
Glycogen phosphorylase, muscle form	*pygm*	1.841	0.009
ADM	*adm*	1.839	0.004
Endoplasmic reticulum-Golgi intermediate compartment protein 2	*ergic2*	1.838	0.001
Tripeptidyl-peptidase 1	*tpp1*	1.835	0.004
CD82 antigen	*cd82*	1.834	0.002
Signal recognition particle subunit SRP72	*srp72*	1.833	0.003
39S ribosomal protein L28, mitochondrial	*mrpl28*	1.833	0.002
Nitrogen permease regulator 3-like protein	*nprl3*	1.826	0.003
40S ribosomal protein S18	*rps18*	1.826	0.000
Eukaryotic initiation factor 4A-I	*eif4a1*	1.825	0.003
TNFAIP3-interacting protein 1	*tnip1*	1.824	0.002
Polycomb group RING finger protein 2	*pcgf2*	1.822	0.001
Pre-mRNA-splicing factor SPF27	*bcas2*	1.822	0.009
Exosome complex exonuclease rrp4	*exos2*	1.821	0.010
Arylamine N-acetyltransferase 1	*nat1*	1.819	0.001
Ankyrin repeat domain-containing protein 16	*ankrd16*	1.817	0.002
Phosphatidylinositol-glycan biosynthesis class F protein	*pigf*	1.815	0.002
Homeodomain-interacting protein kinase 1	*hipk1*	1.814	0.004
Transferrin receptor protein 1	*tfrc*	1.813	0.001
Phosphatidylinositol N-acetylglucosaminyltransferase	*pigh*	1.810	0.009

subunit H			
Ribonucleases P/MRP protein subunit POP1	*pop1*	1.810	0.009
Phosphatidylinositol N-acetylglucosaminyltransferase subunit A	*piga*	1.809	0.002
tRNA methyltransferase 112 homolog	*trmt112*	1.809	0.000
Nucleolar protein 14	*nop14*	1.809	0.001
Conserved oligomeric Golgi complex subunit 4	*cog4*	1.806	0.004
Eukaryotic translation initiation factor 6	*eif6*	1.803	0.001
Protein GTLF3B	*gtlf3b*	1.802	0.010
Golgi SNAP receptor complex member 2	*gosr2*	1.799	0.001
Inhibitor of growth protein 1	*ing1*	1.798	0.010
Nuclear receptor coactivator 1	*ncoa1*	1.798	0.000
Hypoxanthine-guanine phosphoribosyltransferase	*hprt1*	1.796	0.008
E3 ubiquitin-protein ligase RAD18	*rad18*	1.795	0.002
Sodium bicarbonate cotransporter	*nbc*	1.794	0.002
C-X-C chemokine receptor type 2	*cxcr2*	1.793	0.002
Protein arginine N-methyltransferase 5	*prmt5*	1.790	0.004
Histone acetyltransferase KAT8	*kat8*	1.790	0.002
Dipeptidyl peptidase 2	*dpp2*	1.789	0.003
WD40 repeat-containing protein SMU1	*smu1*	1.788	0.006
Band 4.1-like protein 3	*epb41l3*	1.787	0.004
Probable ATP-dependent RNA helicase DDX49	*ddx49*	1.786	0.002
Serine incorporator 3	*serinc3*	1.785	0.002
Coiled-coil-helix-coiled-coil-helix domain-containing protein 2, mitochondrial	*chchd2*	1.782	0.008
Adenylosuccinate synthetase isozyme 2	*adss*	1.782	0.006
mRNA-capping enzyme	*rngtt*	1.781	0.002
28S ribosomal protein S29, mitochondrial	*dap3*	1.778	0.002
Sodium-dependent neutral amino acid transporter SLC6A17	*slc6a17*	1.778	0.004
Putative uncharacterized protein	*dkfzp686d02116*	1.777	0.001

DKFZp686D02116			
SPARC	*sparc*	1.776	0.002
T-complex protein 1 subunit beta	*cct2*	1.776	0.005
FAD synthase	*flad1*	1.771	0.008
V-type proton ATPase subunit F	*atp6v*	1.768	0.002
UPF0511 protein C2orf56-like protein, mitochondrial	*eag_10011*	1.767	0.001
Hyaluronidase-1	*hyal1*	1.766	0.001
Methyltransferase-like protein 2B	*mettl2b*	1.762	0.004
Ribonucleoside-diphosphate reductase subunit M2 B	*rrm2b*	1.758	0.001
Elongation factor Ts, mitochondrial	*tsfm*	1.758	0.002
tRNA pseudouridine synthase-like 1	*pusl1*	1.758	0.001
MAD2L1-binding protein	*mad2l1bp*	1.756	0.001
40S ribosomal protein S15	*rps15*	1.754	0.000
40S ribosomal protein S3	*rps3*	1.754	0.002
LanC-like protein 1	*lancl1*	1.753	0.009
Ras-related protein Rap-2b	*rap2b*	1.751	0.009
Derlin-2	*derl2*	1.751	0.004
Dehydrogenase/reductase SDR family member 7B	*dhrs7b*	1.749	0.008
SH2 domain-containing protein 5	*sh2d5*	1.748	0.001
Transmembrane protein 60	*tmem60*	1.747	0.003
Molybdopterin synthase sulfur carrier subunit	*mocs2*	1.747	0.003
Dystrophin	*dmd*	1.745	0.005
THO complex subunit 4	*alyref*	1.744	0.008
C-X-C motif chemokine	*cxcl*	1.739	0.003
Protein FAM76B	*fam76b*	1.739	0.002
Tail-anchored protein insertion receptor WRB	*wrb*	1.739	0.006
Zinc finger protein 330	*znf330*	1.738	0.005
RISC-loading complex subunit TARBP2	*tarbp2*	1.734	0.004
Peroxisomal biogenesis factor 19	*pex19*	1.733	0.000
Nucleolar protein 58	*nop58*	1.732	0.007
PRELI domain-containing protein 1, mitochondrial	*prelid1*	1.732	0.001
WD repeat-containing protein 13	*wdr13*	1.730	0.002

EKC/KEOPS complex subunit TPRKB	*tprkb*	1.730	0.001
Transcription termination factor, mitochondrial	*mterf*	1.729	0.004
cAMPregulated phosphoprotein 21	*arpp21*	1.727	0.002
Osteopetrosis-associated transmembrane protein 1	*ostm1*	1.727	0.004
Protein TEX261	*tex261*	1.724	0.002
39S ribosomal protein L19	*rpl19*	1.724	0.000
Alpha-soluble NSF attachment protein	*napa*	1.723	0.003
Adenylate cyclase type 2	*adcy2*	1.723	0.002
Transcription factor Sp9	*sp9*	1.722	0.002
THO complex subunit 7 homolog	*thoc7*	1.721	0.008
Ras-related protein Rab-9A	*rab9a*	1.720	0.006
V-type proton ATPase 16 kDa proteolipid subunit	*atp6v*	1.717	0.008
Radical S-adenosyl methionine domain-containing protein 2	*rsad2*	1.717	0.000
Tetratricopeptide repeat protein 27	*ttc27*	1.717	0.002
3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	*hibch*	1.717	0.002
Ras-related protein Rap-1A	*rap1a*	1.715	0.006
Transcription elongation factor SPT4-B	*supt4h1b*	1.710	0.002
Transmembrane protein 69	*tmem69*	1.710	0.007
Estradiol 17-beta-dehydrogenase 12	*hsd17b12*	1.709	0.010
ATP-dependent RNA helicase DDX18	*ddx18*	1.709	0.009
N-lysine methyltransferase SETD6	*setd6*	1.708	0.005
Enteropeptidase	*tmprss15*	1.705	0.009
Lactosylceramide alpha-2,3-sialyltransferase	*st3gal5*	1.705	0.004
Legumain	*lgmn*	1.704	0.003
DNA-directed RNA polymerase III subunit RPC9	*crcp*	1.703	0.002
Junction plakoglobin	*jup*	1.703	0.001
AP-3 complex subunit sigma-2	*ap3s2*	1.702	0.004
E3 ubiquitin-protein ligase TRIM13	*trim13*	1.702	0.002
DnaJ homolog subfamily C member 11	*dnajc11*	1.702	0.004

RING finger protein 121	*rnf121*	1.702	0.004
AN1-type zinc finger protein 1	*zfand1*	1.701	0.009
Zinc finger protein 706	*znf706*	1.699	0.008
Histone deacetylase 11	*hdac11*	1.699	0.009
Purine nucleoside phosphorylase	*pnp*	1.697	0.000
Platelet-activating factor acetylhydrolase IB subunit gamma	*pafah1b3*	1.695	0.006
28S ribosomal protein S17, mitochondrial	*mrps17*	1.693	0.010
Protein LZIC	*lzic*	1.693	0.006
Ubiquinone biosynthesis monooxygenase COQ6	*coq6*	1.692	0.008
Growth hormoneregulated TBC protein 1	*grtp1*	1.692	0.004
Receptor-type tyrosine-protein phosphatase alpha	*ptpra*	1.688	0.009
Glutamine-rich protein 1	*qrich1*	1.688	0.008
Keratinocyte-associated protein 2	*krtcap2*	1.687	0.010
Homeobox and leucine zipper protein Homez	*homez*	1.687	0.006
Tubulin alpha-1B chain	*tuba1b*	1.686	0.006
Mucin-2	*muc2*	1.686	0.000
Trimethylguanosine synthase	*tgs1*	1.686	0.010
Galactocerebrosidase	*galc*	1.686	0.005
Cat eye syndrome critical region protein 5	*cecr5*	1.686	0.005
MKI67 FHA domain-interacting nucleolar phosphoprotein	*nifk*	1.686	0.004
E3 ubiquitin-protein ligase RNF126	*rnf126*	1.685	0.006
Golgin subfamily A member 5	*golga5*	1.684	0.001
Palmitoyltransferase ZDHHC23	*zdhhc23*	1.683	0.002
Probable ATP-dependent RNA helicase DHX8	*dhx8*	1.682	0.008
Coatomer subunit epsilon	*cope*	1.681	0.008
Wu:fj80h11 protein	*si:dkey-256h2.1*	1.678	0.005
Kynurenine--oxoglutarate transaminase 1	*ccbl1*	1.676	0.007
Transmembrane channel-like protein	*tmc6*	1.674	0.003
Ras-related protein R-Ras2	*rras2*	1.674	0.001

Elongation factor G, mitochondrial	*gfm1*	1.672	0.003
Patatin-like phospholipase domain-containing protein 2	*pnpla2*	1.671	0.002
Alpha-2C adrenergic receptor	*adra2c*	1.671	0.002
UPF0402 protein	*egm_09475*	1.670	0.005
Protein BANP	*banp*	1.669	0.001
Eukaryotic translation initiation factor 3 subunit A	*eif3a*	1.666	0.003
Tyrosine--tRNA ligase, mitochondrial	*yars2*	1.659	0.006
UNC119-binding protein C5orf30 homolog	*d1ertd622e*	1.659	0.004
C1GALT1-specific chaperone 1	*c1galt1c1*	1.657	0.005
Nucleoside diphosphate kinase 6	*nme6*	1.657	0.009
Threonine--tRNA ligase, cytoplasmic	*tars*	1.657	0.006
Polymerase delta-interacting protein 2	*poldip2*	1.656	0.004
Serine/threonine-protein kinase A-Raf	*araf*	1.654	0.004
Calcium uptake protein 2, mitochondrial	*micu2*	1.652	0.010
COMM domain-containing protein 3	*commd3*	1.652	0.002
Cytoplasmic dynein 1 intermediate chain 2	*dync1i2*	1.651	0.002
WD repeat-containing protein 6	*wdr6*	1.647	0.002
E3 ubiquitin-protein ligase RNF146	*rnf146*	1.647	0.002
39S ribosomal protein L20, mitochondrial	*mrpl20*	1.643	0.009
Septin-2	*sept2*	1.643	0.009
Fanconi anemia group F protein	*fancf*	1.641	0.006
Serine/threonine/tyrosine-interacting protein	*styx*	1.641	0.005
Mesoderm-specific transcript homolog protein	*mest*	1.639	0.004
Tensin-4	*tns4*	1.638	0.002
HCLS1-associated protein X-1	*hax1*	1.634	0.004
Dynamin-1-like protein	*dnm1l*	1.634	0.004
Nuclear transcription factor Y subunit alpha	*nfya*	1.633	0.003
Golgin subfamily B member 1	*golgb1*	1.633	0.009
Tetratricopeptide repeat protein 17	*ttc17*	1.632	0.005

40S ribosomal protein S8	*rps8*	1.631	0.000
Transcription factor 25	*tcf25*	1.631	0.001
UDP-glucose 4-epimerase	*gale*	1.626	0.007
40S ribosomal protein S30	*rps30*	1.626	0.003
Pre-rRNA-processing protein TSR1 homolog	*tsr1*	1.625	0.008
Phosphoglucomutase-2	*pgm2*	1.623	0.002
Eukaryotic translation initiation factor 4E-binding protein 1	*eif4ebp1*	1.622	0.009
Translationally-controlled tumor protein	*tpt1*	1.622	0.008
Nicotinamide mononucleotide adenylyltransferase 3	*nmnat3*	1.622	0.001
ATPase family AAA domain-containing protein 1-B	*atad1b*	1.621	0.003
Protein arginine N-methyltransferase 7	*prmt7*	1.619	0.008
Presequence protease, mitochondrial	*pitrm1*	1.618	0.003
Ribosomal protein S6 kinase alpha-1	*rps6ka1*	1.615	0.004
Plakophilin-2	*pkp2*	1.615	0.001
tRNA (guanine-N(7)-)-methyltransferase non-catalytic subunit WDR4	*wdr4*	1.614	0.005
Cell cycle control protein 50A	*tmem30a*	1.613	0.001
Syntaxin-5	*stx5*	1.612	0.005
Glutathione synthetase	*gss*	1.611	0.008
CG13731	*dmel_cg13731*	1.610	0.002
Cytochrome b561 domain-containing protein 2	*cyb561d2*	1.610	0.003
Ecto-NOX disulfide-thiol exchanger 1	*enox1*	1.607	0.002
COMM domain-containing protein 2	*commd2*	1.606	0.002
Tetratricopeptide repeat protein 39C	*ttc39c*	1.606	0.008
Mannose-1-phosphate guanyltransferase alpha	*gmppa*	1.604	0.001
Proteasome subunit alpha type-6	*psma6*	1.599	0.006
SRSF protein kinase 1	*srpk1*	1.599	0.002
ER lumen protein retaining receptor 2	*kdelr2*	1.598	0.005
Cytochrome c oxidase assembly protein COX15 homolog	*cox15*	1.596	0.009

General transcription factor IIH subunit 3	*gtf2h3*	1.595	0.010
Troponin C, skeletal muscle	*tnnc2*	1.594	0.003
Protein slowmo homolog 2	*slmo2*	1.594	0.003
E3 ubiquitin-protein ligase DTX3L	*dtx3l*	1.594	0.001
Protein NEDD1	*nedd1*	1.593	0.002
Arrestin domain-containing protein 3	*arrdc3*	1.593	0.006
GTPase Era, mitochondrial	*eral1*	1.592	0.002
Putative humanin peptide	*mt-rnr2*	1.590	0.002
UPF0105 protein C14orf124 homolog	*cn124*	1.590	0.003
GDP-mannose 4,6 dehydratase	*gmds*	1.588	0.001
39S ribosomal protein L18a	*rpl18a*	1.584	0.001
Cytochrome c oxidase protein 20 homolog	*cox20*	1.584	0.005
40S ribosomal protein S14	*rps14*	1.583	0.000
Copper homeostasis protein cutC homolog	*cutc*	1.583	0.002
Decapping and exoribonuclease protein	*dxo*	1.580	0.003
Protein FAM193B	*fam193b*	1.579	0.009
Cyclin-D1-binding protein 1 homolog	*ccndbp1*	1.579	0.010
Serine/threonine-protein kinase RIO2	*riok2*	1.578	0.005
High mobility group protein 20A	*hmg20a*	1.575	0.001
TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor	*taf7*	1.574	0.003
DNA-directed RNA polymerases I, II, and III subunit RPABC2	*polr2f*	1.572	0.002
Polyubiquitin-C	*ubc*	1.571	0.008
Guanine nucleotide exchange factor MSS4	*rabif*	1.569	0.001
Nucleolar complex protein 2 homolog	*noc2l*	1.567	0.008
GRB2-associated-binding protein 1	*?*	1.566	0.002
Putative methyltransferase UPF0383	*eag_01969*	1.565	0.002
Fatty-acid amide hydrolase 2-B	*faah2b*	1.563	0.005
Hyperosmotic glycine rich protein	*?*	1.561	0.005

Vacuolar ATP synthase 16 kDa proteolipid subunit-like protein	*?*	1.559	0.008
N-alpha-acetyltransferase 10	*naa10*	1.558	0.009
General receptor for phosphoinositides 1-associated scaffold protein	*grasp*	1.553	0.006
Chromodomain-helicase-DNA-binding protein 1	*chd1*	1.553	0.003
Cytosolic Fe-S cluster assembly factor NARFL	*narfl*	1.551	0.009
Serine/threonine-protein kinase B-raf	*braf*	1.550	0.001
1-acylglycerol-3-phosphate O-acyltransferase ABHD5	*abhd5*	1.550	0.005
Nucleolysin TIA-1 isoform p40	*tia1*	1.546	0.009
AP-5 complex subunit mu-1	*ap5m1*	1.546	0.004
Metaxin-3	*mtx3*	1.545	0.002
Solute carrier family 25 member 43	*slc25a43*	1.543	0.003
Vesicle transport through interaction with t-SNAREs homolog 1B	*vti1b*	1.540	0.003
E3 ubiquitin-protein ligase BRE1A	*rnf20*	1.539	0.004
Folliculin	*flcn*	1.538	0.009
Syntaxin-12	*stx12*	1.537	0.009
Cyclin-dependent kinases regulatory subunit 1	*cks1b*	1.537	0.002
39S ribosomal protein L12	*rpl12*	1.537	0.004
Charged multivesicular body protein 4c	*chmp4c*	1.536	0.006
Sorting and assembly machinery component 50 homolog	*samm50*	1.535	0.010
39S ribosomal protein L13a	*rpl13a*	1.534	0.000
Serine/threonine-protein kinase Nek4	*nek4*	1.533	0.001
Diphthamide biosynthesis protein 1	*dph1*	1.533	0.001
U8 snoRNA-decapping enzyme	*nudt16*	1.531	0.005
Mitochondrial inner membrane protease ATP23 homolog	*xrcc6bp1*	1.527	0.007
COMM domain-containing protein 5	*commd5*	1.523	0.004
Peroxisomal multifunctional enzyme type 2	*hsd17b4*	1.520	0.007
Cytochrome P450 2C33-like	*?*	1.519	0.007

Grainyhead-like protein 2 homolog	*grhl2*	1.519	0.007
GPN-loop GTPase 3	*gpn3*	1.517	0.005
Lysosome-associated membrane glycoprotein 1	*lamp1*	1.516	0.004
40S ribosomal protein S15a	*rps15a*	1.509	0.003
39S ribosomal protein L23	*rpl23*	1.508	0.000
Protein TBRG4	*tbrg4*	1.508	0.009
Ribosome biogenesis protein BMS1 homolog	*bms1*	1.507	0.008
Ribosome maturation protein SBDS	*sbds*	1.507	0.007
DNA-directed RNA polymerases I, II, and III subunit RPABC5	*polr2l*	1.506	0.001
ATP-dependent helicase	*?*	1.504	0.004
Band 4.1-like protein 3	*epb41l3*	1.502	0.003
Ras-related protein Rab-11B	*rab11b*	1.501	0.002
1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	*agpat5*	-13.376	0.000
Fatty acid-binding protein, heart	*fabp3*	-11.365	0.005
Propionyl-CoA carboxylase alpha chain, mitochondrial	*pcca*	-10.257	0.000
Histone chaperone asf1-b	*as1lb*	-7.635	0.000
1-phosphatidylinositol 3-phosphate 5-kinase	*pikfyve*	-7.044	0.000
Glutamate receptor ionotropic, kainate 4	*grik4*	-6.743	0.000
MORN repeat-containing protein 3	*morn3*	-6.441	0.008
Prolactin	*prl*	-6.355	0.000
Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	*acadsb*	-6.107	0.000
Unhealthy ribosome biogenesis protein 2 homolog	*urb2*	-5.794	0.001
Unknown	*?*	-5.716	0.006
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	*ndufa3*	-5.610	0.006
Meiotic recombination protein DMC1/LIM15 homolog	*dmc1*	-5.456	0.010
Exostosin-1	*ext1*	-5.396	0.001
E3 ubiquitin-protein ligase Midline-1	*mid1*	-5.327	0.000
Unknown	*?*	-5.228	0.003

Transgelin	*tagln*	-5.224	0.009
Ras-related protein O-RAL	*?*	-5.208	0.003
Transposable element Tc3 transposase	*tc3a*	-5.009	0.003
Unknown	*?*	-4.984	0.000
Nucleoside diphosphate kinase	*nme7*	-4.871	0.002
Actin-related protein 2/3 complex subunit 1A	*arpc1a*	-4.853	0.010
Potassium voltage-gated channel subfamily C member 3	*kcnc3*	-4.820	0.000
Integrin beta-1	*itgb1*	-4.773	0.008
WD repeat domain phosphoinositide-interacting protein 1	*wipi1*	-4.687	0.005
Butyrophilin Like Protein 4	*btnl4*	-4.669	0.002
GPI-linked NAD(P)(+)--arginine ADP-ribosyltransferase 1	*art1*	-4.494	0.000
Mesothelin	*msln*	-4.428	0.002
Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	*gspt1*	-4.427	0.000
Tetratricopeptide repeat protein 25	*ttc25*	-4.294	0.003
Unknown	*?*	-4.252	0.002
Ephrin type-A receptor 2	*epha2*	-4.248	0.002
Lipopolysaccharide-induced tumor necrosis factor-alpha factor	*litaf*	-4.207	0.004
PDZ and LIM domain protein 3	*pdlim3*	-4.152	0.001
JunDLa	*jundla*	-4.144	0.003
Junctional adhesion molecule C	*jam3*	-4.068	0.002
Cytochrome P450 aromatase brain isoform	*cyp19a2*	-4.027	0.001
Unknown	*?*	-3.907	0.004
Sodium-coupled neutral amino acid transporter 4	*slc38a*	-3.804	0.009
Probable G-protein coupled receptor 139	*gpr139*	-3.736	0.002
Tropomyosin alpha-1 chain	*tpm1*	-3.691	0.002
cAMP-dependent protein kinase type I-alpha regulatory subunit	*prkar1a*	-3.685	0.000
Unknown	*?*	-3.641	0.000
Methionine-R-sulfoxide reductase B3	*msrb3*	-3.598	0.008
Dual specificity protein	*dusp7*	-3.561	0.002

phosphatase 7			
Sodium/potassium-transporting ATPase subunit gamma	*fxyd2*	-3.529	0.006
Gap junction alpha-1 protein	*gja1*	-3.504	0.002
A-kinase anchor protein 14	*akap14*	-3.452	0.009
Vacuolar protein sorting-associated protein 4B	*vps4b*	-3.423	0.004
Peptidyl-prolyl cis-trans isomerase FKBP14	*fkbp14*	-3.402	0.001
DNA methyltransferase 1-associated protein 1	*dmap1*	-3.366	0.004
Phospholemman	*fxyd1*	-3.293	0.009
Vacuolar protein sorting-associated protein 37A	*vps37a*	-3.288	0.008
Spon2b protein	*spon2b*	-3.226	0.006
Importin-7	*ipo7*	-3.208	0.001
Prostaglandin E synthase	*ptges*	-3.200	0.001
Ionotropic glutamate recetor subunit 3 alpha	*fglur3a*	-3.175	0.000
Uncharacterized protein	*psmd8*	-3.165	0.000
S-phase kinase-associated protein 1	*skp1*	-3.120	0.001
Serine incorporator 2	*serinc2*	-3.077	0.006
Tetraspanin-5	*tspan5*	-3.064	0.002
Peptidyl-prolyl cis-trans isomerase FKBP1B	*fkbp1b*	-3.050	0.001
Tropomyoshin1-1	*tpm1-1*	-2.973	0.009
NAD(P)H-hydrate epimerase	*apoa1bp*	-2.968	0.009
Cytosolic Fe-S cluster assembly factor NUBP1	*nubp1*	-2.966	0.002
Renin	*ren*	-2.945	0.000
Potassium voltage-gated channel subfamily E member 1-like protein	*kcne1l*	-2.900	0.001
Embigin	*emb*	-2.894	0.003
Dynamin-2	*dnm2*	-2.883	0.010
tRNA (cytosine(38)-C(5))-methyltransferase	*trdmt1*	-2.879	0.000
Butyrophilin subfamily 1 member A1	*btn1a1*	-2.852	0.000
Transaldolase	*taldo1*	-2.850	0.003
CD63 antigen	*cd63*	-2.837	0.010
E3 ubiquitin-protein ligase TRIP12	*trip12*	-2.835	0.003

Vitronectin	*vtn*	-2.777	0.001
Craniofacial development protein 1	*cfdp1*	-2.774	0.001
Transcriptionalregulating factor 1	*trerf1*	-2.749	0.004
Heat shock 70 kDa protein 4	*hspa4*	-2.732	0.002
Four and a half LIM domains protein 1	*fhl1*	-2.708	0.007
Voltage-dependent anion-selective channel protein 2	*vdac2*	-2.706	0.007
Cytoplasmic dynein 1 heavy chain 1	*dync1h1*	-2.697	0.004
Steroid hormone receptor ERR2	*esrrb*	-2.685	0.005
Unknown	*?*	-2.646	0.004
Doublesex- and mab-3-related transcription factor 1	*dmrt1*	-2.643	0.003
Lysophosphatidic acid receptor 6	*lpar6*	-2.641	0.002
Unknown	*?*	-2.598	0.003
Beta-2 microglobuli	*b2m*	-2.585	0.002
Phosphoglucomutase-like protein 5	*pgm5*	-2.547	0.005
Prostaglandin reductase 1	*ptgr1*	-2.543	0.001
Syndecan 4	*sdc4*	-2.537	0.008
Hydroxycarboxylic acid receptor 2	*hcar2*	-2.527	0.005
Myelin proteolipid protein	*plp1*	-2.512	0.001
26S proteasome non-ATPase regulatory subunit 8	*psmd8*	-2.493	0.001
DAZ-associated protein 2	*dazap2*	-2.446	0.007
Peripheral plasma membrane protein CASK	*cask*	-2.445	0.001
Unknown	*?*	-2.422	0.004
Proteasome inhibitor PI31 subunit	*psmf1*	-2.421	0.004
Intraflagellar transport protein 52 homolog	*ift52*	-2.417	0.003
EF-hand calcium-binding domain-containing protein 7	*efcab7*	-2.408	0.002
Prostaglandin E synthase	*ptges*	-2.390	0.007
Plexin-C1	*plxnc1*	-2.379	0.000
Negative elongation factor E	*nelfe*	-2.365	0.002
Uncharacterized protein C21orf59-like protein	*cgi_10004163*	-2.348	0.006
Tumor necrosis factor receptor superfamily member 1A	*tnfrsf1a*	-2.337	0.007
Plasma membrane calcium-	*atp2b1*	-2.325	0.003

transporting ATPase 1			
Unknown	*?*	-2.317	0.009
NFKBIL1	*nfkbil1*	-2.306	0.007
Transcription factor p65	*rela*	-2.298	0.001
Clathrin light chain A	*clta*	-2.293	0.001
Ligand-dependent nuclear receptor-interacting factor 1	*lrif1*	-2.289	0.007
Protein-arginine deiminasae type II-like	*?*	-2.283	0.000
Collagen type IV alpha-3-binding protein	*col4a3bp*	-2.262	0.003
Eukaryotic initiation factor 4A-II	*eif4a2*	-2.257	0.001
DnaJ homolog subfamily A member 4	*dnaja4*	-2.254	0.008
Granulins	*grn*	-2.241	0.004
Carboxypeptidase N catalytic chain	*?*	-2.237	0.000
Caspase-9	*casp9*	-2.231	0.002
DnaJ homolog subfamily B member 1	*dnajb1*	-2.228	0.009
?	*?*	-2.206	0.005
Peflin	*pef1*	-2.198	0.002
?	*?*	-2.193	0.008
Leucine-rich repeat-containing protein 40	*lrrc40*	-2.183	0.000
Sugar phosphate exchanger 2	*slc37a*	-2.181	0.010
Cytochrome P450 aromatase brain isoform	*cyp19a2*	-2.170	0.006
Protein RER1	*rer1*	-2.169	0.006
?	*?*	-2.167	0.003
sorting nexin variation 2	*snx2*	-2.165	0.005
Vesicle-associated membrane protein 3	*vamp3*	-2.160	0.006
Gelsolin	*gsn*	-2.155	0.003
?	*?*	-2.153	0.001
Ornithine decarboxylase	*odc1*	-2.150	0.005
Suppressor of cytokine signaling 4	*socs4*	-2.147	0.003
EGF-containing fibulin-like extracellular matrix protein 1	*efemp1*	-2.144	0.001
Golgi SNAP receptor complex member 1	*gosr1*	-2.137	0.003
Heterogeneous nuclear	*hnrnpr*	-2.134	0.003

ribonucleoprotein R			
LYR motif-containing protein 5	*lyrm5*	-2.126	0.009
RB1-inducible coiled-coil protein 1	*rb1cc1*	-2.124	0.007
Glucocorticoid receptor	*nr3c1*	-2.098	0.003
?	*?*	-2.097	0.006
Tetraspanin-18	*tspan18*	-2.081	0.001
Krueppel-like factor 13	*klf13*	-2.066	0.000
Calcium/calmodulin-dependent protein kinase type 1	*camk1*	-2.064	0.007
Prostaglandin E synthase 3	*ptges3*	-2.061	0.005
N-acetyl lactosaminide beta-1,3-N-acetyl glucosaminyl transferase	*cpipj_cpij017866*	-2.049	0.007
Chromodomain-helicase-DNA-binding protein 1-like	*chd1l*	-2.041	0.002
FXYD5b	*?*	-2.035	0.008
Ubiquitin carboxyl-terminal hydrolase 33	*usp33*	-2.035	0.002
Long-chain-fatty-acid--CoA ligase 1	*acsl1*	-2.033	0.002
Transmembrane protein 131	*tmem131*	-2.028	0.005
B-cell CLL/lymphoma 7 protein family member B	*bcl7b*	-2.014	0.004
RING finger protein 24	*rnf24*	-2.011	0.010
LIM domain-containing protein ajuba	*ajuba*	-2.011	0.003
FBP32	*?*	-2.007	0.004
Syntaxin-binding protein 1	*stxbp1*	-2.002	0.000
Ras-specific guanine nucleotide-releasing factor RalGPS2	*ralgps2*	-1.985	0.003
Platelet glycoprotein IX	*gp9*	-1.984	0.003
?	*?*	-1.982	0.000
Uncharacterized protein C10orf12	*c10orf12*	-1.975	0.006
Aldehyde dehydrogenase ALDH2b	*aldh2b*	-1.964	0.004
Von Hippel-Lindau disease tumor suppressor	*vhl*	-1.961	0.008
MAPK/MAK/MRK overlapping kinase	*mok*	-1.957	0.004
Proteasome subunit beta type-1	*psmb1*	-1.956	0.009
GA20768	*dpse\ga20768*	-1.956	0.002
Serine/arginine-rich splicing factor 11	*srsf11*	-1.931	0.009

orphan nuclear receptor DAX2	*nr0b1b*	-1.921	0.008
Ubiquitin-conjugating enzyme E2 variant 2	*ube2v2*	-1.920	0.002
Ubqln4-prov protein	*ubqln4-prov*	-1.919	0.003
Bifunctional apoptosis regulator	*bfar*	-1.918	0.004
High mobility group protein B2	*hmgb2*	-1.917	0.003
Interleukin-1 receptor-like 1	*il1rl1*	-1.911	0.002
Reverse transcriptase-like protein	*rtl*	-1.910	0.006
E3 ubiquitin-protein ligase RNF13	*rnf13*	-1.910	0.007
Eukaryotic translation initiation factor 4E	*eif4e*	-1.909	0.004
40S ribosomal protein S17	*rps17*	-1.905	0.005
GTP-binding nuclear protein Ran	*ran*	-1.902	0.006
FYVE, RhoGEF and PH domain-containing protein 6	*fgd6*	-1.900	0.004
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-13	*gng13*	-1.899	0.007
Ret finger protein-like 1	*rfpl1*	-1.883	0.007
11-beta-hydroxysteroid dehydrogenase-like protein	*hsd11bl*	-1.882	0.003
Receptor-type tyrosine-protein phosphatase S	*ptprs*	-1.875	0.004
Glycine-gated ion channel alpha3 subunit	*?*	-1.873	0.007
Transmembrane protein 107	*tmem107*	-1.861	0.005
?	*?*	-1.860	0.004
Serine/threonine-protein phosphatase 2A [≈ High power LED current, peak 2.7 A] regulatory subunit B" subunit alpha	*ppp2r3a*	-1.857	0.002
Dual specificity protein kinase CLK2	*clk2*	-1.849	0.003
G-protein coupled receptor 56	*gpr56*	-1.847	0.007
Gamma-tubulin complex component 2	*tubgcp2*	-1.845	0.010
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12	*gng12*	-1.843	0.009
V-type proton ATPase subunit a, Golgi isoform	*stv1*	-1.835	0.000
Transforming protein RhoA	*rhoa*	-1.820	0.008

Ephrin-A1	*efna1*	-1.819	0.003
Retinal rod rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit gamma	*pde6g*	-1.817	0.006
Sulfotransferase family 1, cytosolic sulfotransferase 6	*sult1st6*	-1.817	0.004
Serine protease HTRA1	*htra1*	-1.812	0.001
Sister chromatid cohesion protein PDS5 homolog A	*pds5a*	-1.810	0.000
Transcriptional regulator ATRX	*atrx*	-1.809	0.009
LON peptidase N-terminal domain and RING finger protein 1	*lonrf1*	-1.806	0.003
Microtubule-associated protein RP/EB family member 1	*mapre1*	-1.797	0.007
COP9 signalosome complex subunit 5	*cops5*	-1.791	0.005
Aldehyde dehydrogenase, mitochondrial	*aldh2*	-1.790	0.009
Protein max	*max*	-1.784	0.003
Isovaleryl-CoA dehydrogenase, mitochondrial	*ivd*	-1.780	0.004
?	*?*	-1.778	0.001
Fos-related antigen 2	*fosl2*	-1.778	0.007
UV excision repair protein RAD23 homolog B	*rad23b*	-1.777	0.004
Neurobeachin-like protein 2	*nbeal2*	-1.772	0.002
AH receptor-interacting protein	*aip*	-1.768	0.000
Tubulin-specific chaperone A	*tbca*	-1.768	0.003
Methylmalonyl-CoA mutase, mitochondrial	*mut*	-1.751	0.003
ATP-dependent (S)-NAD(P)H-hydrate dehydratase	*carkd*	-1.745	0.001
Cytosolic carboxypeptidase 6	*agbl4*	-1.743	0.003
Apoptosis-associated speck-like protein containing a CARD	*pycard*	-1.740	0.002
Tetratricopeptide repeat protein 9C	*ttc9c*	-1.736	0.004
Casein kinase II subunit beta	*csnk2b*	-1.729	0.002
Xylosyltransferase 2	*xylt2*	-1.721	0.003
E3 ubiquitin-protein ligase RNF31	*rnf31*	-1.711	0.008
Arylsulfatase A	*arsa*	-1.710	0.003
28S ribosomal protein S33, mitochondrial	*mrps33*	-1.709	0.001
Si:ch211-125c5.1 protein	*spen*	-1.705	0.001

Structural glycoprotein p40	*p40*	-1.691	0.010
Vascular endothelial growth factor receptor 2	*kdr*	-1.690	0.006
Gamma-adducin	*add3*	-1.688	0.007
Sorting nexin-4	*snx4*	-1.684	0.010
Arylsulfatase B	*arsb*	-1.681	0.005
RNA, 18S Ribosomal	*rna18s1*	-1.676	0.008
Inositol 1,4,5-trisphosphate receptor-interacting protein	*itprip*	-1.670	0.003
Syntaphilin	*snph*	-1.666	0.005
Probable E3 ubiquitin-protein ligase HERC6	*herc6*	-1.666	0.008
BTB (POZ) domain containing 1	*btbd1*	-1.665	0.005
Cell cycle progression protein 1	*ccpg1*	-1.663	0.009
Glutaredoxin-3	*glrx3*	-1.661	0.003
KCNAB1 protein	*kcnab1*	-1.659	0.008
Cystatin-SN	*cst1*	-1.652	0.000
Pre-mRNA-splicing factor 18	*prpf18*	-1.647	0.000
Porimin	*tmem123*	-1.643	0.004
Acetyl-CoA carboxylase 1	*acaca*	-1.643	0.008
ATP synthase subunit alpha, mitochondrial	*atp5a*	-1.628	0.004
Protein DJ-1	*park7*	-1.628	0.008
Zinc finger protein-like 1	*zfpl1*	-1.625	0.006
Glycosyltransferase-like domain-containing protein 1	*gtdc1*	-1.624	0.005
Eukaryotic translation initiation factor 3 subunit D	*eif3d*	-1.619	0.010
Endophilin-B1	*sh3glb1*	-1.617	0.004
DNA-directed RNA polymerases I, II, and III subunit RPABC5	*polr2l*	-1.604	0.005
LIM/homeobox protein Lhx1	*lhx1*	-1.596	0.007
Interferon regulatory factor 1	*irf1*	-1.595	0.009
Peptide YY-like	*pyy*	-1.594	0.002
Protein archease	*zbtb8os*	-1.585	0.002
Spermidine synthase	*srm*	-1.575	0.002
?	*?*	-1.571	0.002
Ribosomal protein 63, mitochondrial	*mrp63*	-1.564	0.008
Protein BEX2	*bex2*	-1.558	0.003

Mitogen-activated protein kinase kinase kinase 1	*map3k1*	-1.552	0.003
Gamma-aminobutyric-acid receptor rho-2B subunit	*?*	-1.549	0.004
?	*?*	-1.540	0.007
Torsin-1A-interacting protein 1	*tor1aip1*	-1.539	0.008
MKIAA4125 protein	*bicd1*	-1.532	0.001
?	*?*	-1.524	0.007
39S ribosomal protein L34, mitochondrial	*mrpl34*	-1.524	0.001
Kelch-like protein 26	*klhl26*	-1.518	0.009
Histone-lysine N-methyltransferase SETDB2	*setdb2*	-1.512	0.008
?	*?*	-1.507	0.005

Supplementary Table 16. KEGG pathways for the FS versus SS comparison

Pathways	# Sequences	# Enzymes	up/ down
Alanine, aspartate and glutamate metabolism	3	4	up
Alpha-linolenic acid metabolism	3	3	up
Amino sugar and nucleotide sugar metabolism	5	5	up
Aminoacyl-tRNA biosynthesis	5	5	up
Aminobenzoate degradation	1	1	up
Arachidonic acid metabolism	2	2	down
Arginine and proline metabolism	5	5	up
Benzoate degradation	1	1	up
Betalain biosynthesis	1	1	up
Biosynthesis of unsaturated fatty acids	2	2	up
Butanoate metabolism	2	2	up
Caprolactam degradation	1	1	up
Chloroalkane and chloroalkene degradation	1	1	down
Citrate cycle (TCA cycle)	2	3	up
Cutin, suberine and wax biosynthesis	1	1	up
Drug metabolism-other enzymes	5	4	up
Ether lipid metabolism	5	4	up
Fatty acid biosynthesis	2	2	down
Fatty acid degradation	4	4	up
Fatty acid elongation	3	2	up
Fructose and mannose metabolism	4	4	up
Galactose metabolism	3	3	up
Geraniol degradation	1	1	up
Glucerophospholipid metabolism	8	7	up
Glutathione metabolism	5	5	up
Glycerolipid metabolism	6	5	up
Glycerophospholipid metabolism	1	1	down
Glycine, serine and threonine metabolism	2	2	up
Glycolysis/Gluconeogenesis	2	3	up
Glycosaminoglycan biosynthesis-chondro	1	1	down
Glycosaminoglycan biosynthesis-heparan	2	3	down
Glycosaminoglycan degradation	1	1	up
Glycosphingolipid biosynthesis-ganglio	1	1	up
Glycosphingolipid biosynthesis-lacto	1	2	up

Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	2	2	up
Glyoxylate and dicarboxylate metabolism	2	2	down
Linoleic acid metabolism	2	1	up
Lysine degradation	6	4	up
Methane metabolism	1	1	up
Naphthalene degradation	1	1	down
Nicotinate and nicotinamide metabolism	3	3	up
Nitrogen metabolism	1	1	up
One carbon pool by folate	1	1	up
Other glycan degradation	2	2	up
Oxidative phosphorylation	7	5	up
Pentose phosphate pathway	4	4	up
Phenylalanine metabolism	3	4	up
Phenylalanine, tyrosine and tryptophan biosynthesis	1	1	up
Phenylpropanoid biosynthesis	2	1	up
Phosphatidylinositol signaling system	2	2	up
Phosphonate and phosphinate metabolism	1	1	up
Porphyrin and chlorophyll metabolism	2	4	up
Primary bile acid biosynthesis	2	2	up
Propanoate metabolism	3	2	down
Purine metabolism	24	19	up
Pyrimidine metabolism	16	12	up
Pyruvate metabolism	4	3	up
Riboflavin metabolism	2	1	up
Selenocompound metabolism	2	1	up
Sphingolipid metabolism	5	5	up
Starch and sucrose metabolism	2	2	up
Steroid biosynthesis	1	1	up
Streptomycin biosynthesis	2	2	up
Synthesis and degradation of ketone bodies	1	1	up
T cell receptor signaling pathway	2	2	up
Terpenoid backbone biosynthesis	2	2	up
Toluene degradation	1	1	up
Tropane, piperidine and pyridine alkaloid biosynthesis	1	1	up
Tryptophan metabolism	3	3	up
Tyrosine metabolism	2	1	up
Ubiquinone and other terpenoid-quinone	1	1	up

Supplementary Table 17. Fisher's Exact Test with Multiple Correction for FDR for FS versus SS comparison

GO Term	Name	Type	FDR	single test p-Value	# in test group	# in reference group	# non annot test	# non annot reference group	Over/ Under
GO:0005840	Ribosome	CC	6,50E-06	2,60E-09	73	245	799	6489	over
GO:0003735	structural constituent of ribosome	MF	4,90E-05	4,90E-08	46	130	826	6604	over
GO:0006412	Translation	BP	1,00E-03	1,70E-06	78	324	794	6410	over
GO:0005739	Mitochondrion	CC	1,20E-03	2,10E-06	152	779	720	5955	over
GO:0000028	ribosomal small subunit assembly	BP	1,20E-03	2,20E-06	6	0	866	6734	over
GO:0002682	regulation of immune system process	BP	1,60E-02	4,50E-05	6	184	866	6550	under
GO:0005730	Nucleolus	CC	5,00E-02	1,70E-04	83	406	789	6328	over

Block B: Environmental effects on phenotypic sex

Chapter 3

EXPOSURE TO ELEVATED TEMPERATURE DURING EARLY DEVELOPMENT HAS LASTING EFFECTS ON THE GONADAL TRANSCRIPTOME OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) AT THE TIME OF SEX DIFFERENTIATION

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Abstract

Background. Sex in fish is plastic and in several species can be influenced by environmental factors. In sensitive species, elevated temperatures have a masculinizing effect. Previous studies on the effects of temperature on gene expression have been restricted to a few cognate genes, mostly related to testis or ovarian development and analyzed in gonads once they had completed the process of sex differentiation. However, studies on the effect of temperature at the whole gonadal transcriptomic level are scarce in fish and, in addition, temperature effects at the time of sex differentiation and also at the transcriptomic level are unknown. Here, we used the European sea bass, a gonochoristic teleost with a polygenic sex determination system influenced by temperature, and exposed larvae to elevated temperature during the period of early gonad ridge formation. Transcriptomic analysis of the gonads was carried out about three months after the end of temperature exposure, shortly after the beginning of the process of sex differentiation.

Results. Elevated temperature doubled the number of males with respect to untreated controls. Transcriptomic analysis of early differentiating female gonads showed that 27 genes were differentially expressed (18 up- and 9 downregulated) in the treated group. Heath caused: 1) an up-regulation of genes related to

cholesterol transport (*star*), the stress response (*nr3c1*) and testis differentiation (*amh*, *dmrt,* etc.), 2) a decrease in the expression of genes related to ovarian differentiation such as *cyp19a1a*, and 3) an increase in the expression of several genes related to epigenetic regulatory mechanisms (*hdac11, dicer1, ehmt2, jarid2a, pcgf2, suz12, mettl22*). Further analysis showed an upregulation of GO terms related to catalytic activity and a downregulation of processes such as reproduction, growth, immune response or signaling. Functional pathway analysis yielded four KEGG pathways that were related to oocyte maturation, tight junction, chemokine and hormone-mediated signaling. Protein-protein interaction analysis confirmed the above mentioned pathway results.

Conclusions. Taken together, the results of this study contribute to the understanding of how the early environment sets permanent changes that result in long-lasting consequences, in this case in the sexual phenotype. Results also show the usefulness of comparing the effects of heat on the behavior of cognate genes related to sex differentiation as well as that of genes involved in establishing and maintaining cell identity through epigenetic mechanisms.

1. Introduction

Identifying environmental cues and their perception and transduction mechanisms is a central focus of research in developmental biology within an ecological context (eco-devo research) (Sultan, 2007). Changes in environmental variables can have profound influences on differentiation, growth and reproduction in many organisms (Somero, 2011). Temperature is the main abiotic factor that affects many biological functions at different levels of organization by changing the rates of chemical reactions and physiological processes, or by changing the three-dimensional shapes of biomolecules (Beitinger and Lutterschmidt, 2011; Schulte et al., 2011).

Fishes exhibit enormous diversity in their morphology, in habitat occupancy, and in their biology (Nelson, 2006). This diversity is also remarkable as regards to their reproductive strategies (Breder and Rosen, 1966) including sex determination and differentiation

(Devlin and Nagahama, 2002), two processes that contribute to the establishment of the sex ratio, a crucial demographic parameter important for population viability and for the continuation of all species with sexual reproduction.

Sex determination is the process by which the sex (gender, male or female) of an individual is established in a simple binary fate decision (Penman and Piferrer, 2008). There are two major sex-determining systems in vertebrates: genetic (GSD) and environmental sex determination (ESD), with temperature-dependent sex determination (TSD) being the most common type of ESD (Bull, 1983; Penman and Piferrer, 2008). Master sex determining genes are not conserved among vertebrates with GSD (Kikuchi and Hamaguchi, 2013) and eight genes with such a function have been identified so far: *Sry* in mammals (Sinclair et al., 1990), *DMRT-1* in birds (Smith et al., 2009), *DM-W* in *Xenopus laevis* (Yoshimoto et al., 2008), and *sdy*, *amhr2*, *amhy*, *dmy* and *gsdf* in fish (Kikuchi and Hamaguchi, 2013; Heule et al., 2014).On the other hand, sex differentiation (SD) includes the various molecular, genetic and physiological processes responsible for the transformation of a sexually undifferentiated gonad into testes or ovaries in an individual of a given genotype and parents in a given environment, and thus is responsible for the final gonadal sex (Bull, 1983; Piferrer and Guiguen, 2008). Some of the genes involved in this process are fairly conserved from fish to mammals, at least regarding their structure and dimorphic expression (Place and Lance, 2004). Genes involved in testis differentiation include *dmrt1, dax1, sox9*, *arb, amh, cyp11b* (Liu et al., 2000; Wang and Orban, 2007; Socorro et al., 2007; Sreenivasan et al., 2008; Blázquez et al., 2009), *sox9a2*, *tbx1a* and *tbx1b* (Yano et al., 2012); whereas genes involved in ovarian differentiation include *cyp19a1a, foxl2, er, fst* (Luckenbach et al., 2005; Matsuoka et al., 2006; Vizziano et al., 2007; Blázquez et al., 2009), *hsd3b* and *star* (Yano et al., 2012). However, the order of expression and interactions among these genes may change between groups (Cutting et al., 2013) or depending on environmental conditions (Volff et al., 2007). Estrogens are essential for proper ovarian differentiation. From the above mentioned genes, a major player in vertebrate SD is *cyp19a1,* the gene that encodes for aromatase, an enzyme that catalyzes the conversion of androgens into estrogens. Since estrogens are essential for ovarian differentiation in all non-

mammalian vertebrates (Nagahama, 2006), aromatase function is then crucial for the establishment of the sex ratio.

Aromatase gene expression is susceptible of environmental temperature influences. Thus, and regardless of the sex ratio response pattern to temperature (Valenzuela and Lance, 2004; Ospina-Álvarez and Piferrer, 2008), in fish and reptiles, the two type of vertebrates with TSD, the effects of environmental temperature on sex ratios are mediated by changes in *cyp19a1* expression. More males are produced with increasing temperatures in all fish species analyzed so far (Ospina-Álvarez and Piferrer, 2008) and *cyp19a1* is always inhibited at male-producing temperatures and stimulated at female-producing temperatures (Guiguen et al., 2010). Nevertheless, information on gene expression shortly after temperature treatment is scarce in fish and almost limited to *cyp19a1* and a few other genes. In general elevated temperatures increase the expression of male-related genes such as *amh*, *dmrt1* or *arb* after the SD period, while it decreases the expression of female-related genes in addition to *cyp19a1a* such as *esr1*, *esr2*, *erb1, fshr* or *foxl2* (see Supplementary Table 1 for a summary on general thermal effects on gene expression).

Effects of temperature on fish sex ratios are more pronounced if animals that are exposed to elevated temperatures during early development and this is of crucial importance nowadays in a scenario of global change. However, the number of genes known to be affected is limited, as we have just seen above, and, further, the metabolic and signaling pathways affected are essentially unknown. Thus, a deeper knowledge of the effects of temperature is needed and this can be obtained not by analyzing a handful of cognate genes but with transcriptomic analysis. In this regard, several studies have explored the effects of temperature at the transcriptional level but these studies are usually concerned with tissues other than the gonads, such as liver, skeletal white muscle and gills (Gracey et al., 2004; Podrabsky and Somero, 2004; Cossins et al., 2006; Buckley et al., 2006; Kassahn et al., 2007a, 2007b; Chou et al., 2008; Vergauwen et al., 2010; Logan and Somero 2010, 2011; Magnanou et al., 2014); brain (Ju et al., 2002; Cossins et al., 2006; Magnanou et al., 2014) or heart (Vornanen et al., 2005; Gracey et al., 2004; Cossins et al., 2006). Furthermore, they are usually related to exposure to cold or to cold acclimation

rather than to heat (Somero, 1995, 2011). In general, a common response to cold, regardless of the considered tissue, involves the activation of cellular homeostasis, energy charge, ATP turnover and stress protein function. In addition, processes such as proteolysis and mitochondrial metabolism or membrane structure are also affected. Thermal acclimation studies, on the other hand, show an increase in expression of chaperons, of protein biosynthesis and of metabolism, with carbohydrate breakdown and lipid metabolism affected to cope with the membrane fluidity changes brought by low temperatures. Unfortunately, comparative transcriptomic studies on the effect of heat and on the gonads are essentially nonexistent (Supplementary Table 1). Furthermore, the studies on gene expression mentioned earlier and carried out on the effects on sex differentiation concern juvenile or adult gonads that have completed the process of sex differentiation. Thus, it is difficult to ascertain whether observed altered patterns of gene expression are the cause or the consequence of a given gonadal phenotype resulting from exposure to elevated temperature, a circumstance that complicates our understanding of, at least: 1) the transient vs. continued effects of temperature, 2) the molecular changes occurring not once the gonads have completed the process of sex differentiation but, importantly, at the moment when such process is taking place, and 3) the underlying possible epigenetic mechanisms that are responsible for the observed long-term changes in gene expression.

The European sea bass is a eurythermal marine teleost able to live between 8ºC to 27ºC and a gonochoristic species without sex chromosomes with a polygenic system of sex determination (Vandeputte et al., 2007), where genetics and temperature contribute essentially equally to sex ratios (Piferrer et al., 2005). Recent studies in the sea bass have shown that *cyp19a1a* and *cyp11b* are good markers of female and male sex differentiation, respectively (Blázquez et al., 2009). Also, elevated temperature during 0-120 days post hatch (dph) results in masculinization (Navarro-Martín et al., 2009b) although the expression of some steroidogenic enzymes and sex steroids receptors was not altered (Socorro et al., 2007; Blázquez et al., 2009). This is also the case with nuclear receptors such as *nrcb1* (Deloffre et al., 2009) and the sex hormone-binding globulin (Miguel-Queralt et al., 2007). Since the effect of elevated temperature on sex ratios is maximal during early development (Pavlidis et al., 2000; Koumoundouros et al.,

2002; Navarro-Martín et al., 2009b) when the sea bass gonads are still not formed even in the most rudimentary form, our lab studied the possibility of an epigenetic mechanism involved in linking environmental temperature and sex differentiation. Methylation levels of the *cyp19a1a* promoter of sea bass showed gender- and temperature-related differences at one year, with the concomitant hypermethylation of *cyp19a1a* gene expression suppression and increase in male percent (Navarro-Martín et al., 2011).

In the present study, we exposed European sea bass to either control (15ºC) or elevated temperature (21ºC) during the TSP and analyzed the thermal effects on the gonadal transcriptome 110 days after the treatment, i.e., when fish were going through sex differentiation, in an attempt to relate transcriptomal changes at this stage with the observed sex ratio at one year of age. Thus, the objective of this study was to assess the effects of temperature not on the juvenile gonads of exposed fish but at the time of sex differentiation, in order to gain a better understanding of the genes and the pathways involved in sex differentiation that are directly affected by temperature.

2. Materials and methods

2.1 Animals and rearing conditions

One-day-post hatch (dph) European sea bass larvae were obtained from a commercial hatchery (St. Pere Pescador, Girona, Spain) in May and were transported to the fish facilities at the Institute of Marine Sciences (ZAE) in PVC transport bags filled with oxygen and seawater. Rearing conditions and handling methods were as previously described (Díaz et al., 2013), except for the temperature treatment (see below). Larvae were fed three times a day with Artemia AF and then with amino acid enriched Artemia EG (INVE Aquaculture, Belgium). Juveniles and adults were fed *ad libitum* two times a day with dry food (ProAqua S.A., Spain) of increasing sizes according to the manufacturer's protocol as fish grew in size.

2.2 Experimental design

Larvae were divided in two 650-liter tanks and maintained at 17ºC, a temperature known to avoid temperature effects on sex ratio (Piferrer et al., 2005; Navarro-Martín et al., 2009b), for the first 20 dph. Then, in one tank the temperature was increased to 21ºC (high temperature, HT group), while in the other it was decreased to 15ºC (low temperature, LT group). In both cases, temperature was modified at a ratio of 0.5ºC/day. At 60 dph, temperature in the LT group was stepwise increased in order to match the temperature of the HT group. Then, at ~220 dph, temperature of both groups was left to follow the natural fluctuations. Thus, the only difference between the LT and the HT groups in terms of rearing conditions was in the temperature experienced during the 20–60 dph period (Supplementary Figure 1).

2.3 Samplings

Periodic samplings were carried out, where length (SL; precision 1 mm) and body weight (BW; precision 0.01 g) were assessed for all fish in each group by anesthetizing them with adjusted doses of 2-phenoxyethanol (2PE; 0.2ml·l^{-1}). After recovery, fish were returned to their tanks. At 170 dph, coinciding with the period of histological sex differentiation (Mylonas et al., 2005), and at 332 dph, when gonadal sex is firmly established, a sample of fish (n = 40 at 170 dph; n = 151 at 332 dph) were randomly taken from each group and sacrificed with an overdose of 2PE.

At 170 dph, sexually differentiating gonads were dissected out and snap-frozen in liquid Nitrogen for transcriptomic analysis of gene expression. At 332 dph, gonads were dissected out and weighed (precision 0.01 g) to calculate the gonadosomatic index (GSI) as previously described (Navarro-Martín et al., 2009b; Díaz et al., 2013). Gonads were fixed in 4% paraformaldehyde (PF) in PBS. Sex ratio of the population was visually assessed (n = 151 fish total; HT: n=85 and LT: n=66 fish) coinciding with the last sampling at 332 dph.

PF-fixed 332 dph gonads were used for sex assessment and to determine the stage of gonadal development. After dehydration, gonads were embedded in paraffin and 7 µm thick sections were obtained with the aid of a Reichert-Jung 2040 microtome. Sections were stained with hematoxylin-eosin (Supplementary Figure 2). Female and male developmental stages were assessed according to Brown-Peterson et al. (2011). Stages of oocyte maturation were classified as: cortical alveolar (CA), primary growth (PG) and primary vitellogenic stage (Vtg1). Male germ cells in different stages of spermatogenesis were classified as: primary spermatogonia (SpgA), primary spermatocytes (Scp1), secondary spermatocytes (Spc2), spermatids (Spd) and spermatozoa (Spz).

2.4 RNA extraction and cDNA synthesis

Total RNA was purified from 170 dph isolated juvenile sexually differentiating gonads by homogenizing the tissue with Trizol reagent (Invitrogen- Live Technologies, Scotland, UK). Briefly, homogenates were separated into phases by chloroform, precipitated with isopropanol and washed in 75% ethanol. The quality and concentration of the RNA were assessed with a ND-1000 spectrophotometer (NanoDrop Technologies) based on A_{260} absorbance and checked on a 1% agarose/formaldehyde gel.

Two hundred nanograms of total RNA were treated with *E.coli* RNAse H in order to remove complementary RNA. From this cleaned RNA, 100 ng were used for cDNA synthesis using SuperScript III RNase Transcriptase (Invitrogen, Spain) and Random hexamer (Invitrogen, Spain) following the manufacturer's instructions.

2.5 Quantitative real time PCR (qRT-PCR)

Real time PCRs were performed with two purposes: 1) to select 5 fish per treatment at 170 dph for further microarray analysis based on *cyp19a1a* expression levels. At this time, sex differentiation is taking place and differences in *cyp19a1a* expression are evident between presumptive future males and females since 120 dpf

(Blázquez et al., 2009). Thus, determination of *cyp19a1a* levels allowed to select fish with the highest *cyp19a1a* levels by increasing the chances to concentrate our efforts on the effects of temperature on presumptive future females, which are masculinized in response to temperature, and 2) to validate microarray results and analyze genes that are important either for sex differentiation or related to epigenetic regulatory mechanisms (see Supplementary Table 2 for a gene glossary). In both cases, cDNA was diluted 1:10 for the amplification of the target genes and 1:500 for the housekeeping, reference gene *r18S*. Primers were designed using Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Supplementary Table 3). A melting curve analysis (95ºC for 15 s, 60ºC for 15 s and 95ºC for 15 s) was performed after the amplification phase to analyze primer specificity. For every primer, the amplification efficiency (*E*) was calculated by analyzing the slope of a linear regression from a dilution series (mean threshold cycle Ct values plotted against log cDNA concentration) and calculated as $E=10^{(-1/\text{slope})}$, with *E* values between 1.99 and 2.38. Also, standard curves of target genes were calculated following the same formula, with slope values ranging from -2.7 to -3.3 and linear correlations (R^2) higher than 0.92 (Supplementary Table 3). Real-time PCR was performed on an ABI 7900HT (Applied Biosystems) with the following program: an initial UDG decontamination cycle at 50ºC for 2 min, followed by an activation step of 10 min at 95ºC and then 40 cycles of 15 s denaturation at 95ºC and a 1 min annealing/extension step at 60ºC. Finally, a dissociation step of 15 s at 95ºC followed by 15 s at 60ºC was added.

Samples were run in triplicate in 384-well plates in a final volume of 10 µl per well. Each well contained a mix of 5 µl of SYBRGreen Supermix (Applied Biosystems), 2 µl distilled water, 2 µl primer mix (forward and reverse primers at 10 µM concentration) and 1 µl of cDNA. Controls lacking either cDNA or primers were included per duplicate. Data was collected using SDS 2.3 software (Applied Biosystems) and gene expression levels were calculated using RQ Manager 1.2 (Applied Biosystems). Endogenous control gene *r18S* was used in all runs to calculate intra- and inter-assay variations. Ct values were adjusted for differences in *E* of each primer set when analyzing the results, and expression of target genes was normalized to the reference gene (*r18S*) based on the Schmittgen and Livak (2008) method.

2.6 Microarray analysis of gene expression

Microarray experiments consisted on the comparison of 5 individuals of each temperature group (LT and HT) sampled during the process of sex differentiation at 170 dph. Before microarray hybridizations, the integrity of the total RNA was verified in a 1 µl-sample with a Bioanalyzer 2100 fitted with the RNA 6000 Nano LabChip kit (Agilent, Spain) to assure consistency across samples. Only RNA samples of 100-200 ng/µl and RINs > 7 were used for microarray hybridizations. Hybridizations were carried out at the Institute of Biotechnology and Biomedicine (Autonomous University of Barcelona). RNA labelling, hybridizations, and scanning were performed according to the manufacturer's instructions. Briefly, total RNA (100 ng) was amplified and Cy3-labeled with Agilent's One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labelling kit), along with Agilent's One-Color RNA SpikeIn Kit. After labelling, cRNA was purified with RNeasy mini spin columns (Qiagen), quantified with the Nanodrop ND-1000 and verified using the Bioanalyzer 2100. Each sample (1.65 µg) was hybridized to a custom-made European sea bass microarray containing a total of 17,917 probes (Agilent ID 023790) at 65°C for 17h using Agilent's GE Hybridization Kit. Samples were evenly distributed among the slides to avoid batch effect. Washes were conducted as recommended by the manufacturer using Agilent's Gene Expression Wash Pack with stabilization and drying solution. Arrays were scanned with Agilent Technologies Scanner, model G2505B. Spot intensities and other quality control features were extracted with Agilent's Feature Extraction software version 10.4.0.0. The complete design has been submitted to Gene Expression Omnibus (GEO)-NCBI database (GSE52307) as well as the platform that validates the microarray (GPL13443).

2.7 Statistical analysis of data

2.7.1 General statistics

Prior to statistical analysis, the normality of data was checked with the Kolmogorov-Smirnov's test and the homoscedasticity of

variance with the Levene's test. Data of continuous variables was log-transformed when needed. Percentage data such as GSI were arcsine transformed. One-way analysis of variance (ANOVA) was performed to check statistical differences between temperature treatments for SL, BW and GSI data sets (including cyp19a1a expression levels; see section 2.7.2 below). If statistical differences were present, *post hoc* multiple comparisons were carried out using the Tukey's HSD test. The Student's *t*-test was used to pairwise compare high vs. low aromatase expressors between thermal treatments. The Chi-square test with Yates correction (Fowler et al., 2008) was used to analyze differences in sex ratios. Differences were accepted as significant when $P<0.05$. Unless otherwise stated, statistical analyses were performed using IBM SPSS Statistics v19.

2.7.2 qRT-PCR statistics

Quantitative RT-PCR statistical analysis was performed using 2DCt from the processed data (Schmittgen and Livak, 2008). 2DCt results were then checked for normality, homoscedasticity of variance and the Student's *t*- test was used to assess differences between treatments.

A two-step cluster analysis using 2DCt *cyp19a1a* qRT-PCR values was used to differentiate among high and low *cyp19a1a* expressors in both the LT and HT groups at 170 dph as previously described (Blázquez et al., 2009). These analyses were performed using PAST software (Hammer et al., 2001).

2.7.3 Microarray raw data normalization

Raw data was taken from the Feature Extraction output files and was corrected for background noise using the normexp (Ritchie et al., 2007) method. To assure comparability across samples, quantile normalization (Bolstad, 2001) was used. A probe or replicate was considered reliable if its raw foreground intensity was at least two times higher than the respective background intensity and if it was neither saturated nor flagged by the Feature Extraction software. On our custom array design, most probes (64.7%) were represented in two (or in some cases more) identical replicates. Median intensities

of probe replicates were taken in order to yield only one expression value per probe. A probe was considered reliable if at least half of its replicates were individually reliable, as defined above.

Differential expression analysis was carried out on all non-control probes with an empirical Bayes approach on linear models (limma) (Smyth, 2004). Results were corrected for multiple testing according to the False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995). Genes were selected as differentially expressed if they had an adjusted *P*-value <0.05, an absolute fold change (FC) >1.2 and were reliable, as defined above, in all samples.

All statistical analyses were performed with the Bioconductor project (http://www.bioconductor.org/) in the R statistical environment (http://cran.rproject.org/) (Gentleman et al., 2004).

2.8 Gene annotation enrichment analysis

Gene names, gene synonyms and gene functions were addressed using mostly Genecards (http://www.genecards.org/) and Uniprot (http://www.uniprot.org/). The web-based tool AMIGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) Gene Ontology (Carbon et al., 2009) was used to retrieve the differentially expressed (DE) gene sequences. After obtaining the sequences, Blast2GO software (Conesa et al., 2005) was used to enrich GO term annotation and to analyze the altered KEGG pathways (http://www.genome.jp/kegg/) that include those DE genes in order to extract a broader biological meaning. Using Blast2GO a reference set containing all the genes from the custom-made microarray was analyzed and used to check if the GO terms were enriched in a test group (DE genes set) when compared to it by a Fisher's Exact Test with Multiple Testing Correction of FDR (Benjamini and Hochberg, 1995). Also DAVID (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009a-b) was used to further analyze and verify the pathways to which the DE genes belong. Finally, DE genes official names were uploaded to the web-based tool STRING v9.1 (http://string-db.org/) (Franceschini et al., 2013) to analyze

physical and functional protein-protein interactions. Confidence associations were bibliographically confirmed.

3. Results

3.1 Growth and body indices

Because of the differences in rearing temperature between 20 and 60 dph, fish from the HT group were significantly (P<0.05) larger than those of the LT group until 170 dph in both SL and BW regardless of whether they were low or high *cyp19a1a* expressors (Table 1).

Table 1. Growth of European sea bass juveniles at 170 days post hatch, classified according to treatment and *cyp19a1a* expression levels, as shown in Figure 1A. Data as mean ± SEM.

		Low *cyp19a1a* expressors			High *cyp19a1a* expressors	
Treatment	N	Length (cm)	Weight (g)	N	Length (cm)	Weight (g)
LT	10	9.25 ± 0.196[a]	13.16 ± 0.967[a]	6	9.33 ± 0.061[a]	13.53 ± 0.581[a]
HT	9	9.86 ± 0.109[b]	17.41 ± 0.877[b]	7	10.28 ± 0.495[b]	19.35 ± 2.955[b]

Abbreviations: LT, low temperature; HT, high temperature; N, sample size. Different letters indicate significant (*P*<0.05) differences between treatments.

However, within any given treatment (LT or HT) there were no differences in SL or BW between low and high *cyp19a1a* expressors. Growth differences between groups had disappeared by 332 dph. Sexual growth dimorphism in favor of females (*P*<0.01) was present only in the LT group (Table 2).

Table 2. Growth of European sea bass juveniles at 332 days post hatch, classified according to treatment and sex. Data as mean ± SEM.

Treatment	N	Females		N	Males	
		Length (cm)	Weight (g)		Length (cm)	Weight (g)
LT	40	12.45 ± 0.180[a**]	33.16 ± 1.560[a**]	26	11.66 ± 0.223[a]	27.22 ± 1.867[a]
HT	16	12.22 ± 0.171[a]	33.38 ± 3.044[a]	60	12.36 ± 0.171[a]	33.79 ± 1.544[a]

Abbreviations: LT, low temperature; HT, high temperature; N, sample size. The same letter indicates lack of significant (P>0.05) differences between treatments. Asterisks indicate statistical differences (P<0.01) between females and males within the same treatment group, i.e., sexual growth dimorphism.

A two-step clustering based on *cyp19a1a* qRT-PCR expression levels at 170 dph was used to separate these fish in two groups: high (putative females) and low (putative males) expressors (Figure 1A). One-way ANOVA showed statistical differences due to expression levels, treatment and their interaction (P<0.001 in all three cases). Five 170 dph fish per group were selected among the high *cyp19a1a* expressors for the microarray hybridizations. Although HT fish were slightly larger and heavier (10.16±0.457 cm and 19.24±2.249 g) than LT fish (9.44±0.229 cm and 13.84±1.121 g), these differences were not statistically significant. Importantly, this meant that transcriptomic differences between HT and LT fish were due to temperature and not to size-related differences in gonadal development.

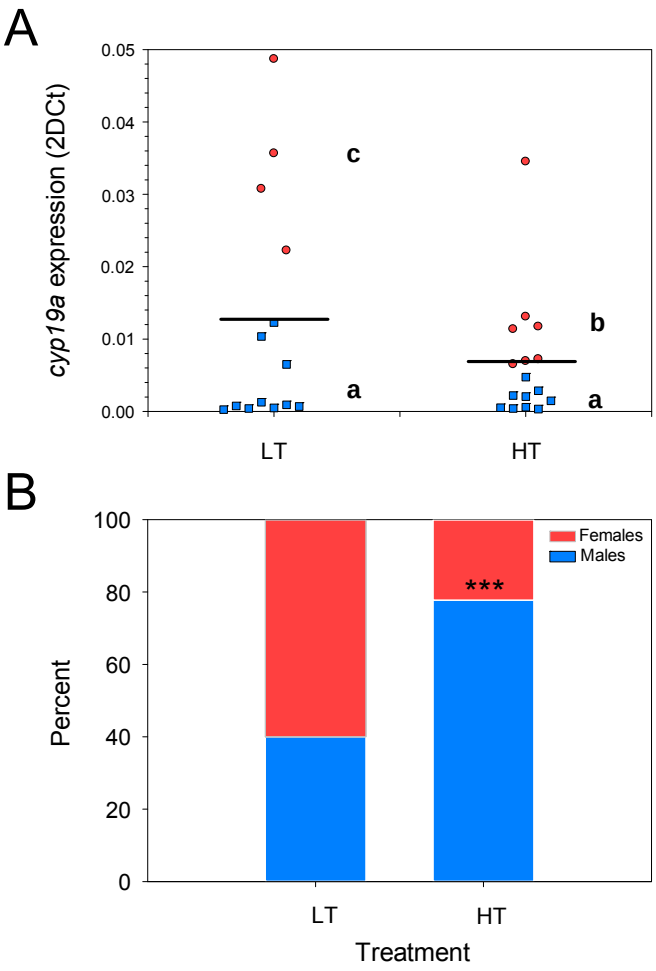


Figure 1. A) Individual gonadal aromatase (*cyp19a1a*) expression levels (2DCt) as assessed by qRT-PCR in the low (LT) and high (HT) temperature groups at 170 dph. Blue squares and red circles correspond to individual fish with low (putative future males) and high (putative future females) *cyp19a1a* levels, respectively. The horizontal line marks mean expression for each experimental group. Different letters indicate significant (ANOVA; $P<0.001$) differences between high and low *cyp19a1a* expressors of the LT and HT groups. B) Sex ratios of juvenile European sea bass sampled at 332 days post fertilization. Stacked bars showing male (blue) and female (red) percent in the low (LT) and high (HT) temperature groups. Statistical differences ($P<0.001$) between groups are marked with three asterisks. Sample size: LT: 66 fish; HT: 85 fish.

3.2 GSI and sex ratio

Visual assessment of the sex ratio of 332 dph juveniles combined with histological verification showed sex ratio differences since the LT group had 40.0% males while the HT group had 77.8% males (P<0.001) (Figure 1B). No differences were observed in the presence and abundance of the different cellular types between HT and LT fish. Females had immature ovaries containing oocytes at the cortical alveolar (CA) stage. On the other hand, males had testis containing all germ cell types, including spermatozoa although no running males were observed (Supplementary Figure 2).

The GSI values at 332 dph for LT males and females were 0.095±0.0004 and 0.111±0.0002, respectively, and for HT males and females were 0.090±0.0002 and 0.161±0.0003, respectively. A two-way ANOVA analysis showed statistical differences due to sex (P=0.032) but not for the thermal treatment or the interaction between sex and temperature.

3.3 Microarray analysis

Microarray analysis of sexually differentiating gonads at 170 dph obtained from fish with a high *cyp19a1a* expression, i.e., putative females, revealed the presence of 27 significantly and differentially expressed (DE) genes when comparing the HT *vs.* the LT group (Supplementary Table 4), of which 18 genes were upregulated (18/1,360 non repeated probes) and 9 were downregulated (9/4,789 non repeated probes). A heat map representation of the DE genes grouped fish according to their thermal history, with the exception of one LT fish, which had an intermediate position, and one HT fish, which was an outlier and was not further considered in the analysis (Figure 2).

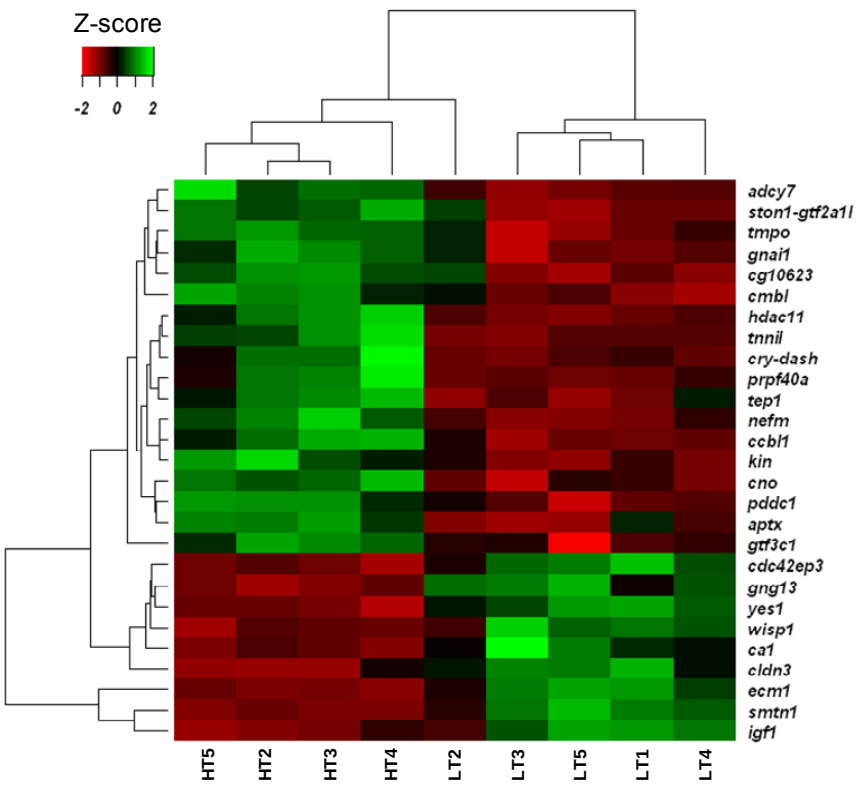


Figure 2. Heatmap of the microarray expression data for the 18 up- and 9 downregulated genes, where each row represents a gene and each column represents an individual fish. Key color representing the level of expression (green: high expression and red: low expression). The dendograms provide information of the similarity between genes and between the different samples. Notice that all HT samples and all but one LT samples cluster together. LT, low temperature group; HT, high temperature group. See supplementary table 2 for a complete list of gene names and abbreviations.

Some of the upregulated genes were related to reproduction, i.e., cryptochrome DASH (*cry-dash*) and troponin I (*tnnI*), or to epigenetic gene expression regulation, i.e., histone deacetylase 11 (*hdac11*). Some of the downregulated genes also showed reproduction-related functions such as cdc42 effector protein 3 (*cdc42ep3*), insulin-like growth factor (*igf1*) or smoothelin (*smtn1*) (see Table 3 for a complete list of DE genes and their functions).

Table 3. Differentially expressed genes summary results

Gene symbol	Gene name	Fold change (FC)	Adjusted *P*-value	Brief function description
adcy7	Adenylate cyclase 7	1.688	3.01e-02	Bound to the membrane and inhibitable by calcium.
aptx	Aprataxin	1.801	4.53e-02	DNA-binding protein involved in single- and soluble- strand DNA break repair and base excision repair.
ca1	Carbonic anhydrase	-3.173	4.53e-02	Interconverts carbon dioxide and bicarbonate to maintain acid-base balance in blood and other tissues, and to help transport carbon dioxide out of tissues.
ccbl1	Cysteine conjugate beta-lyase cytoplasmic, isoform CRA-a	1.324	4.53e-02	Metabolism of cysteine conjugates of certain halogenated alkenes and alkanes. Can form reactive metabolites leading to nephrotoxicity, neurotoxicity and reproduction problems.
cdc42ep3	Cdc42 effector protein 3	-1.986	2.41e-02	Development of germ lines in flies and worms. May be a target of PUMILIO2 in human male testis.
cg10623	DmeI-CG10623	1.980	4.53e-02	Homocysteine S-methyltransferase 1-like
cldn3	Claudin 3a	-4.265	2.08e-02	Integral membrane protein and a component of tight junction strands. Major role in tight junction-specific obliteration of the intercellular space, through calcium-independent cell-adhesion activity.
cmbl	Carboxymethylene-butenolidase homolog	2.377	4.53e-02	Cysteine hydrolase.
cno	Cappuccino homolog	1.369	4.53e-02	Maternal effect loci: anteroposterior and dorsoventral patterns. May play a role in organelle biogenesis (lysosomes, melanosomes).
cry-dash	Cryptochrome DASH	1.780	4.53e-02	May have a photoreceptor function. Circadian clocks input, UV-damage repair (single-stranded DNA). Responsible of massive spawning during full moon in corals.

ecm1	Extracellular matrix protein 1	-13.617	2.08e-02	Involved in endochondral bone formation as negative regulator of bone mineralization. Also, stimulates the proliferation of endothelial cells and promotes angiogenesis. Present in maturing trout ovaries and in a higher concentration when compared to testis or pre-spawning ovaries.
gnai1	Guanine nucleotide-binding protein G(i) subunit alpha-1	1.913	2.58e-02	Modulators or transducers in various transmembrane signaling systems. Involved in hormonal regulation of adenylate cyclase by inhibiting the cyclase in response to beta-adrenergic stimuli. May also play a role in cell division.
gng13	Guanine nucleotide binding protein G(I)/G(S)/G(O) subunit gamma-13	-1.795	2.08e-02	Modulators or transducers in various transmembrane signaling systems. Regulation of melatonin receptors in the brain.
gtf3c1	General transcription factor 3C polypeptide 1	2.697	2.08e-02	Required for RNA polymerase III-mediated transcription.
hdac11	Class 4 histone deacetylase 11 protein	1.647	2.08e-02	Deacetylates lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Giving a tag for epigenetic repression, thus playing an important role in transcriptional regulation, cell cycle progression and developmental events.
igf1	Insulin-like growth factor I	-7.119	4.53e-02	Related to insulin with a high growth-promoting activity. Sex steroids, growth hormone and igf1 play a role in neuroendocrine and metabolic regulation of puberty.
kin	DNA/RNA binding protein KIN17	1.422	2.86e-02	Involved in DNA replication and cellular response to DNA damage. May form a bridge between DNA replication and repair mediated by high molecular weight complexes. May also play a role in illegitimate recombination and regulation of gene expression.

nefm	Neurofilament medium polypeptide	1.472	2.08e-02	Involved in the maintenance of neuronal caliber. They may also play a role in intracellular transport to axons and dendrites.
pddc1	Parkinson disease 7 domain containing 1	1.666	2.08e-02	Regulation of cell cycle arrest and apoptosis following double strand DNA breaks.
prpf40a	Pre-mRNA processing factor 40 homolog A	2.00	2.08e-02	Plays a role in the regulation of cell morphology and cytoskeletal organization, in cell shape and migration control; and may be involved in pre-mRNA splicing.
smtn1	Smoothelin 1	-4.881	2.08e-02	A key factor governing sexual development and pregnancy. Induces adaptations in smooth and striated muscle. Produces infertility in KO mice phenotype.
ston1-gtf2a1l	Protein STON1-GTF2A	1.667	4.53e-02	Not determined yet.
tep1	Telomerase protein component 1	1.894	2.08e-02	Telomerase activity which catalyzes the addition of new telomeres on the chromosome ends. Its activity is related to a DNA-methylation increase and to a telomeric transcription reduction.
tmpo	Lamina-associated polypeptide 2, isoform alpha	2.182	2.58e-02	May maintain the structural organization of the nuclear envelope. May be involved in the structural organization of the nucleus and in the post-mitotic nuclear assembly. *tmpo1* and *tmpo5*may play a role in T-cell development and function.
tnnI	Troponin-I isoform 2	1.695	2.05e-02	Thin filament regulatory complex which confers calcium-sensitivity to striated muscle actomyosin ATPase activity. Controls ovulatory contraction of non-striated actomyosin network in gonads.
wisp1	WNT1 inducible signaling pathway protein 1	-1.684	3.44e-02	Associated with cell survival, p53-mediated apoptosis attenuation in response to DNA damage and with the up-

yes1	Tyrosine protein kinase Yes	-1.356	4.53e-02	regulation of the anti-apoptotic Bcl-X(L) protein. Sex hormone signaling in endometrial homeostasis. Involved in the regulation of cell growth and survival, apoptosis, cell-cell adhesion, cytoskeleton remodeling, differentiation and cell cycle progression (regulating the G1 phase by cdk4 phosphorylation and the G2/M progression and cytokinesis).

The AMIGO web-based tool was used to recover the sequence of these DE genes and were then uploaded to Blast2GO in order to enrich results with GO terms and extract more information from these DE genes. A Fisher's exact test with multiple testing corrections for False Discovery Rate (FDR) showed that five GO term categories were overrepresented when compared to a reference test containing all the annotated sequences from our custom-made array (Supplementary Table 5).

Further analysis of the GO terms provided their distribution among the three main categories: biological process, molecular function and cell component for the up- and downregulated genes separately (Supplementary Figures 3 and 4, respectively). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database provided more information on the functions of the DE genes and the interactions between them (Supplementary Table 6). Several pathways involved in protein synthesis as well as in immunological processes were found. Then, the STRING database (Franceschini et al., 2013) was used to predict protein-protein interactions. STRING integrates genomic, high-throughput and coexpression experiments with the existing literature, allowing filtering by organism. The STRING analysis using zebrafish as a background yielded too few protein-protein interactions due to the lack of studies in this fish. Then, the human dataset was used as a background and showed the predicted direct (physical) and indirect (functional) relationships between the proteins of some of our DE genes (Supplementary Figure 5). Despite the limitations of this approach, most of the possible interactions between these proteins have not been studied yet in fish.

3.4 Microarray validation

We selected eight DE genes either with a reproduction-related function in other species or with a relation to an epigenetic mechanism (4 up- and 4 downregulated genes) for validating the microarray. Importantly, the same cDNA used for microarray hybridizations (from 170 dph sea bass gonads) was used for qRT-PCR analyses. When analyzing HT versus LT individuals, all the genes showed the same tendency (more expressed at the HT group

for the upregulated genes and less expressed at the HT group for the downregulated genes) and significant differences (P<0.05) were observed for *cg10623 and hdac11* upregulated genes and for the *cdc42ep3* and s*mtn1* downregulated ones (Figure 3). Thus, qRT-PCR results validated microarray results.

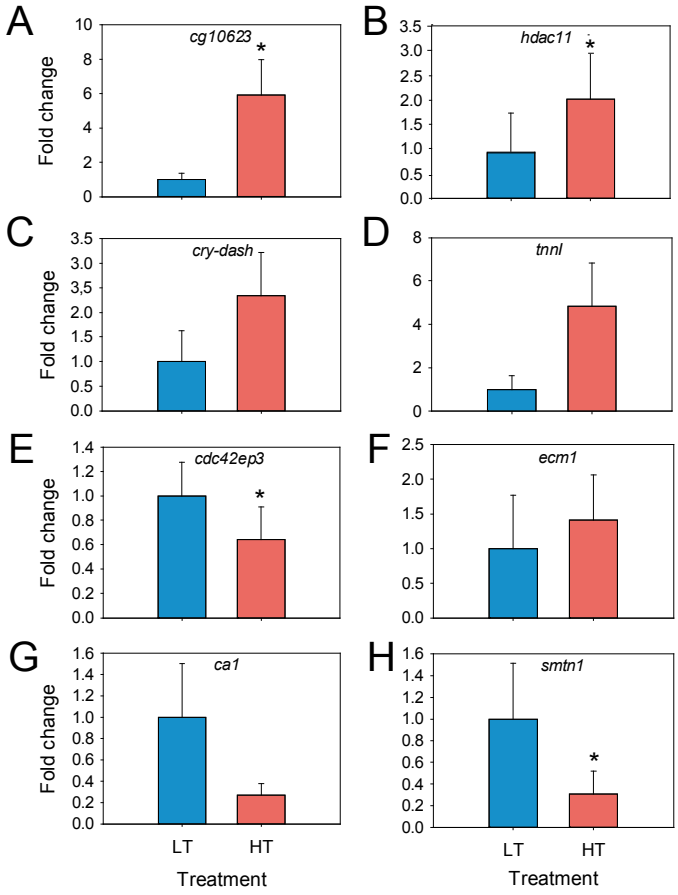


Figure 3. Validation of microarray results by qRT-PCR according to treatment (LT, low temperature group; HT, high temperature group). A-D, Four upregulated genes in the HT *vs*. the LT group comparison: DmeI_CG10623 (*cg10623*); histone deacetylase 11 (*hdac11*); cryptochrome DASH (*cry-dash*) and troponin I (*tnnI*). E-H, Four downregulated genes for the same comparison: cell division cycle 42 effector protein 3 (*cdc42eP3*); extracellular matrix protein 1 (*ecm1*); carbonic anhydrase 1 (*ca1*) and smoothelin (*smtn1*). Letters mark statistical significance (P<0.05) between groups.

3.5 Reproduction and stress-related genes

Fifteen reproduction-related genes were selected to be analyzed by qRT-PCR (Supplementary Table 7). Results showed that some pro-male genes, i.e., known to be involved in testicular differentiation, such as doublesex-mab-3-related transcription factor 1 (*dmrt1*) were significantly ($P<0.05$) upregulated in HT fish, as also did the steroidogenic acute regulatory protein (*star*). In contrast, some pro-female, i.e., involved in ovarian differentiation, such as aromatase (*cyp19a1a*), were significantly ($P<0.05$) downregulated in HT fish, as also did aquaporin 1 (*aq1*). The expression of eight of these genes is shown in Figure 4.

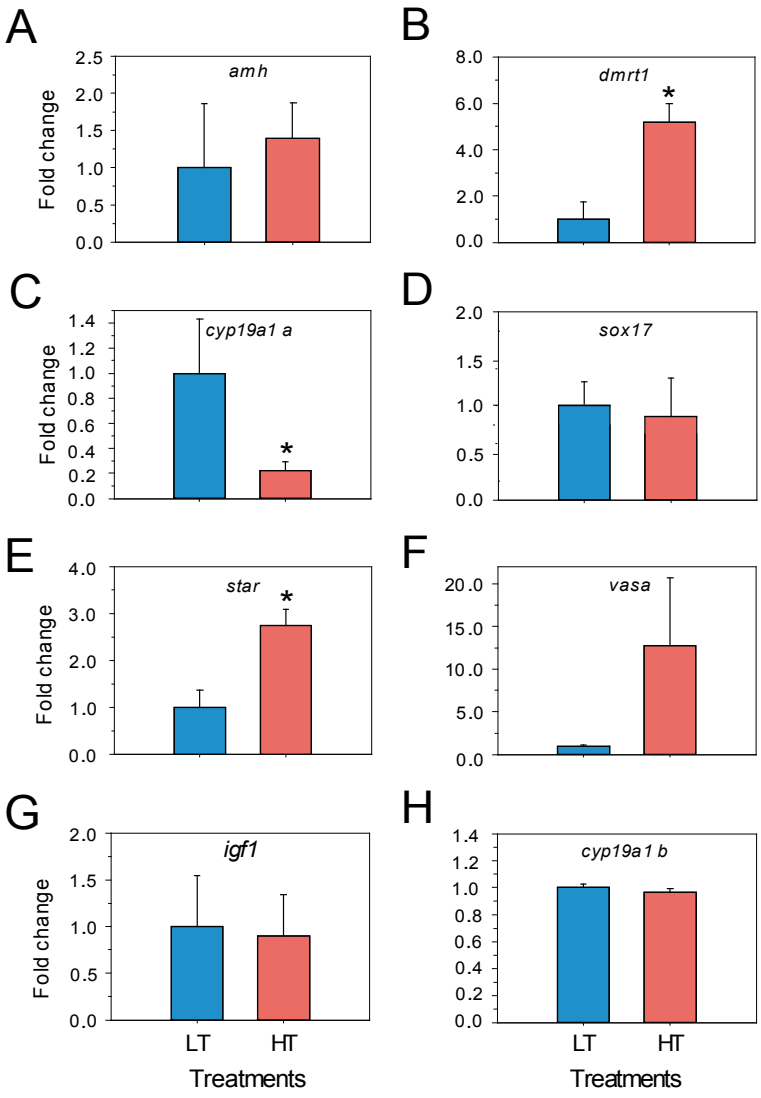


Figure 4. Quantitative RT-PCR results for eight known sex differentiation-related genes per temperature treatment groups. (A-D) Male pathway: anti-Müllerian hormone (*amh*), doublesex- and mab-3-related transcription factor 1 (*dmrt1*); female pathway: cytochrome P450, family19, subfamily A, polypeptide 1a (*cyp19a1a*) and SRY-related HMG-box transcription factor SOX17 (*sox17*), respectively. (E) steroidogenic acute regulatory protein (*star*), (F) vasa protein (*vasa*), (G) insulin-like growth factor 1 (*igf1*) and (H) cytochrome P450, family19, subfamily A, polypeptide 1b (*cyp19a1b*). Asterisk marks statistical differences between groups ($P<0.05$).

Since HT-induced masculinization is thought to also involve a stress response (Fernandino et al., 2012), the genes coding for 11ß-hydroxysteroid dehydrogenase (*hsd11b*), an enzyme that catalyzes the conversion of the stress hormone cortisol into the inactive cortisone, and the glucocorticoid receptor (*nr3c1*), the receptor to which cortisol among other glucocorticoids binds, were also analyzed by qRT-PCR. While *nr3c1* was significantly (P<0.05) upregulated in HT fish, *hsd11b* was downregulated, although in this case differences were not significant (Figure 5).

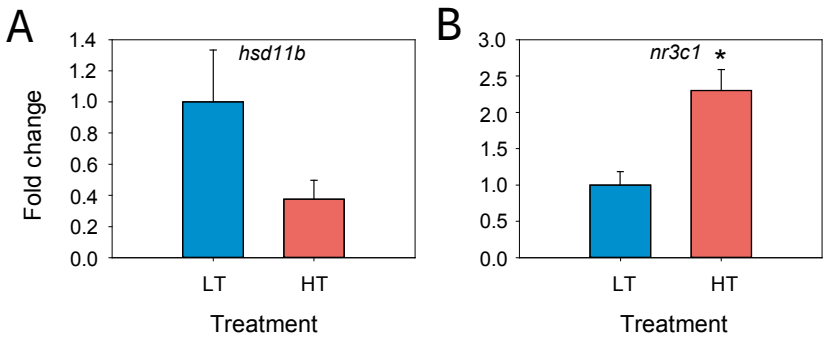


Figure 5. Quantitative RT-PCR results for (A) 11β-hydroxysteroid dehydrogenase (*hsd11b1*) and (B) glucocorticoid receptor (*nr3c1*). Asterisk marks statistical differences between groups (P<0.05).

3.6 Epigenetic regulatory mechanisms-related genes

According to a growing body of evidence, several genes involved in epigenetic regulatory mechanisms have been implicated in sex determination/differentiation (Piferrer, 2013, for a review). Based on the genes mentioned in this review, we analyzed the genes that were present in our microarray (Supplementary Table 8). Among them, seven genes were selected for qRT-PCR analysisin order to analyze a possible heat effect on their expression. These genes were representative of different categories and were: *dicer 1,* a helicase needed to produce an active small RNA component that represses gene expression; *ehmt2,* a histone methyltransferase; *jarid2a,* a DNA-binding protein that acts as a transcriptional repressor; *pcgf2,* contains a RING finger motif and forms protein-protein interactions to maintain transcriptional repression; *hdac11,* a histone

deacetylase); *mettl22,* a methyltransferase-like protein; and *suz12,* a suppressor of trithorax zeste 12 homolog gene. All of them were upregulated in the HT, with significant differences found (P<0.05) in four cases: *dicer1*, *jarid2a*, *pcgf2* and *hdac11* (Figure 6).

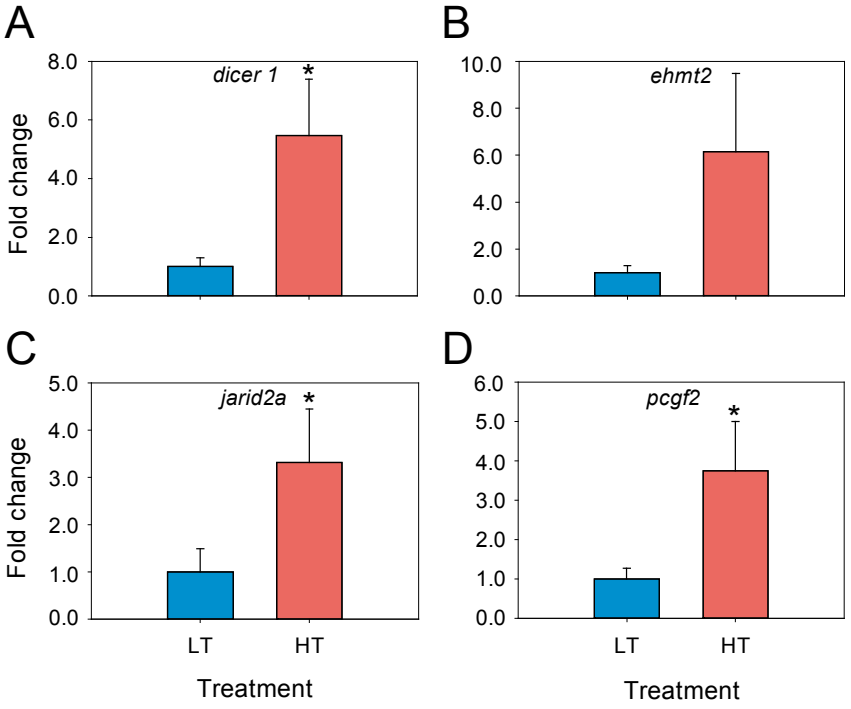


Figure 6. Quantitative RT-PCR results for the epigenetic regulatory mechanisms-related genes. (A-D) endoribonuclease Dicer (*dicer1*), euchromatic histone-lysine N-methyltransferase 2 (*ehmt2*), protein Jumonji (*jarid2a*) and polycomb group ring finger 2 (*pcgf2*). Asterisks mark statistical differences between groups (P<0.05).

3.7 Enrichment analysis

A GO enrichment analysis of the DE genes showed an upregulation and a downregulation of the same BP categories, albeit containing different DE genes (Supplementary Figure 3A and 4A). These altered categories contained more GOs that were upregulated and mainly related to metabolic and cellular processes (14.43% and

17.53%, respectively), while other processes such as reproduction (1.41%), growth (7.04%), immune processes (4.23%) or signaling (9.86%) were downregulated due to the elevated temperatures. Regarding MF GO categories (Supplementary Figure 3B and 4B), the catalytic and binding activities were the most represented subcategories for both up- and downregulated GO terms. Analysis of the CC categories showed that upregulated processes were taking place mainly into the organelle (14/57 GOs), macromolecular complex (9/57 GOs), membrane-enclosed lumen (8/57 GOs) or membrane (7/57 GOs) (Supplementary Figure 3C), while the downregulated processes were taking place in the membrane, organelle, macromolecular complex (6/36 GOs) or extracellular region (5/ 36 GOs) (Supplementary Figure 4C). Further analysis of the GO enriched terms of the DE genes in comparison to the microarray reference set (Supplementary Table 5), showed that terms related to the negative regulation of the nerve impulse and synaptic transmission were overrepresented as well as the adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway (implying a decrease in cAMP concentration).

Blast2GO analysis of the DE genes showed that eleven of the 13 differentially regulated pathways (Supplementary Table 6) had higher expression in HT fish with respect to LT fish and that these pathways were related to catabolism (amino acid metabolism), biosynthesis (tropane, piperidine and pyridine alkaloid) or signal transduction (phosphatidylinositol signaling system). However, the downregulated pathways were related, in agreement with the results of the GO term analysis, to immunology (T-cell receptor signaling pathway) and Nitrogen metabolism. DAVID analysis of the DE genes showed that there were 23 upregulated categories of which four were highly significant: 1) progesterone-mediated oocyte maturation (*gnai1*, *adcy7* and *igf1*), 2) tight junction (*gnai1*, *cldn3* and *yes1*), 3) chemokine signaling (*gnai1*, *adcy7* and *gng13*), and 4) hormone- mediated signaling (*adcy7* and *gng13*) pathways. In all of them, *adcy7* is involved as a signaling initiation factor. After running an annotation clustering with DAVID, seven clusters were downregulated and related to cell component, DNA binding, transcription and signaling processes.

STRING analysis (protein-protein interactions) of the data showed coincidences with Blast2GO and DAVID gene clustering into

pathways (Supplementary Figure 5). GnaiI, Gng13 and Adcy7 proteins exhibited direct interactions between them since they are in charge of receiving the external input and starting the different signaling processes, leading to, for instance, progesterone-mediated oocyte maturation (ending in meiosis), chemokine signaling pathway (cell growth and differentiation, among others), and the phosphatidylinositol signaling system present in tight junctions (proliferation). The aprataxin (Aptx) protein, related to single- and double-strand DNA repair, was directly interacting with Xrcc1 and Xrcc4 proteins, which have a DNA repair function. Capuccino protein (Cno) is in turn directly interacting with Bloc1s1 protein, and both of them are involved in organelle biogenesis (i.e., lysosomes). All protein-protein interactions had a combined association score higher than 0.8 (data not shown), except for the insulin receptor relationship with Yes1 protein (score = 0.63). These protein-protein interaction results come from the STRING integration of data from different sources, such as high throughput experiments, conserved protein coexpression, genomic context and previous knowledge using human as a background.

4. Discussion

Temperature is the main abiotic factor affecting biological functions at different levels including sex (Baroiller and D'Cotta, 2001; Schulte et al., 2011). As a poikilothermic ectotherm with a GSD sex determination mechanism influenced by environment, the European sea bass is masculinized by elevated temperature during early development (Blázquez et al., 1998a; Pavlidis et al., 2000; Koumoundouros et al., 2002; Saillant et al., 2002; Mylonas et al., 2005; Navarro-Martín et al., 2009b). In this study, we used a custom-made microarray to analyze the transcriptome of putative female gonads at 170 dph, i.e., during sex differentiation, 110 days after the end of the temperature treatment of two groups submitted either to low (15ºC) or to elevated temperature (21ºC) during the thermosensitive period. Thus, and to the best of our knowledge, this is the first study on the analysis of the gonadal transcriptome at the time of sex differentiation of fish previously exposed to elevated temperature during the thermosensitive period, since all previous studies involved the analysis of sexually differentiated gonads.

Temperature plays a main role controlling fish growth through its effects on behavior, feeding and metabolism (Person-Le Ruyet et al., 2004). Effects of temperature on sea bass growth had been previously described (Gardeur et al., 2001a; Person-Le Ruyet et al., 2004). In this study, due to differences in temperature between 0–60 dph, HT fish grew more than LT fish. However, once the LT group was switched to 21°C it started to converge in growth with the HT group. Although reduced, differences between the two groups were still statistically significant at 170 dph, during sex differentiation when samples were taken for microarray analysis. These differences disappeared at the time of the final sampling at 332 dph, when sex ratios were assessed. At this age, only sexual growth dimorphism in favor of females was present, as previously observed (Saillant et al., 2001a; Navarro-Martín et al., 2009b). Importantly, selected fish at 170 dph from both groups were of similar size. Thus, it can be safely concluded that observed differences in gene expression (see below) were due to the previous thermal history and not to differences in growth.

As expected, temperature induced male development in fish that otherwise would have developed as females (Navarro-Martín et al., 2009b; Navarro-Martín et al., 2011) resulting in a male-biased sex ratio (~80% males). Higher GSI values for HT-treated females may be due to the acceleration of gonadal growth relative to somatic growth. Histological analysis of sea bass gonads at one year showed no differences in maturation between fish of the two different groups. Sex-related differences were present since immature females were in a cortical alveolar stage while males had spermatozoa, although no running males were found. The lack of differences in the presence and abundance of the different cellular types between HT and LT fish further corroborates that observed transcriptomic differences arose as a consequence of the thermal treatment, and not because of a different cellular composition.

Among the genes analyzed by qRT-PCR, there were three patterns of response to heat (Figure 3, 4, 5, and Supplementary Table 7). Most of the genes showed increased levels of expression in the HT group regardless of whether these differences were significant or not. Among them, there were genes upregulated during normal testis differentiation such as *amh* or *dmrt1,* in accordance with the masculinizing effect of elevated temperatures (Navarro-Martín et

al., 2009b; Navarro-Martín et al., 2011). *Vasa* was also upregulated in the HT group. This helicase is a germ cell marker known to be more expressed in ovaries than in testis and previously found to be upregulated by elevated temperatures (Blázquez et al., 2011). There were also genes ultimately related to steroid hormone production that showed an increase in transcription, including gonadotropin-releasing hormone (*gnrh*) (Stocco et al., 2001; Kusakabe et al., 2002; Ings and Van Der Kraak, 2006; Nuñez and Evans, 2007) and *star,* which is involved in cholesterol import. Cholesterol is important in maintaining membrane integrity and sterol synthesis and its levels have previously been found to be also increased by temperature (Somero, 2011). In our study, genes such as *wisp1*, known to be involved in cellular growth were upregulated by heat, whereas genes such as *prl* and *nr3c1*, related to body growth regulation and immune system regulation responses, were downregulated, suggesting growth adjustment in the gonads. Nevertheless, these results may be more related to the immunological stress response rather than to reproduction since *prl* also controls immune cells growth and *nr3c1* plays a role in inflammatory responses.

There was a group of genes that showed a decrease in their expression due to heat. Among these there was *cyp19a1a,* confirming results of an independent experiment aimed to find molecular signature of male and female differentiation (Blázquez et al., 2009). Aquaporin 1 (*aqp1*), a water channel protein that plays a major role in oocyte hydration in fish (Fabra et al., 2005; Zapater, 2013) was also downregulated by heat. Moreover, the steroidogenic enzyme 11ß-hydroxysteroid-dehydrogenase 1 (*hsd11b*), which converts the stress hormone cortisol into the inactive metabolite cortisone, and also converts 11ß-hydroxy androgens such as 11ß-hydroxyadrostenedione into 11-ketotestosterone, a potent piscine androgen (Piferrer et al., 1993), was downregulated by heat, although differences in expression were not significant. In contrast, the glucocorticoid receptor (*nr3c1*) was upregulated in the HT group with significant differences with respect to the LT group. These results are interesting because in the pejerrey (*Odontesthes bonaeriensis*), a fish with TSD, Fernandino et al. (2013) observed an up-regulation of both *hsd11* and *nr3c1* in masculinized gonads by HT and attributed these observations to the fact that HT elicits both masculinizing and stress responses. In our study, the fact that

nr3c1 was upregulated 110 days after the end of the temperature treatment suggests a persistent stress response, probably maintained by an epigenetic regulatory mechanism. In fact, studies in rodents have shown that *nr3c1* is able to exhibit sustained expression, even a long time after the stimulus ended, through changes in methylation of its regulatory region (McGowan et al., 2009; Suderman et al., 2012). On the other hand, the fact that in our study *hsd11b* was downregulated in the HT group, hence contrasting with the observations of Fernandino et al. (2013) with the pejerrey, suggests that gender or developmental stage may be important in explaining these differences, since while Fernandino et al. (2013) sampled juvenile, sexually differentiated males, in our case we selected not only sexually differentiating fish but also those that exhibited *cyp9a1a* expression levels compatible with ovarian differentiation. In general, when comparing our results with those obtained in turtle microarray analysis of dimorphic gene expression (Chojnowski and Braun, 2012), genes that are normally highly expressed in the testis such as *dmrt1* and *amh* were also upregulated in our study, while genes such as *sf1* (steroidogenic factor 1), a transcription factor known to be an enhancer of *cyp19a1a* expression, or *cyp19a1a* itself, were downregulated due to temperature, highlighting the masculinizing effect of heat.

The third pattern of response was represented by genes whose expression was not affected by heat, including *sox17*, a gene that has been related with ovarian development in the European sea bass and other fish (Navarro-Martín et al., 2009c). Other genes belonging to this third group were the brain aromatase (*cyp19a1b*), thus corroborating earlier observations of our group (Navarro-Martín et al., 2011), and opposite to the increase observed in tilapia when applying HT during early development (Tsai et al., 2003); *tesc*, important during male gonadal development; *igf1*, involved in mediating growth and development; and *col18a1*, implicated in organ morphogenesis.

The masculinizing effect of elevated temperatures on European sea bass involved hypermethylation of the *cyp19a1a* promoter in both females and masculinized females at one year of age, accompanied with an increase of the male proportion. However, these changes were not so evident at 170 dph (Navarro-Martín et al., 2011). Thus, it may be that other epigenetic regulatory mechanisms were

responsible for the "memory" of early HT exposure and hence for the male-skewed ratio alteration towards a male bias found at one year. Increasing evidence shows that epigenetic regulatory mechanisms play crucial roles during sex differentiation (Piferrer, 2013). To further study the possible epigenetic changes due to heat, we examined the expression of seven epigenetic-related genes that have been directly or indirectly connected with sex determination and gonadogenesis (Piferrer, 2013) and were present in our microarray (Supplementary Table 8). The selected genes for qRT-PCR analysis were *hdac11*, *jarid2a*, *ehmt2*, *dicer1*, *suz12*, *pcgf2* and *mettl22*. All of them were upregulated in the HT group, with four of them displaying significant differences ($P<0.05$): *hdac11*, *jarid2a*, *dicer1* and*pcgf2*. Although further studies are clearly needed, it is interesting to note that, although in different ways, these genes have a transcriptional repression function, which here may be connected with the long-lasting effects of early heat exposure.

Whole gonad transcriptomic analysis showed 27 DE genes (18 up- and 9 downregulated; Figure 2 and Table 3). While some of these DE genes were related to metabolic processes, others were related to epigenetic regulatory mechanism including *cg10623* (methyltransferase), *hdac11* (histone deacetylase) or *tep1* (related to DNA methylation increase) or involved in reproductive processes in other species such as *cdc42ep3*, which its mRNA interacts with the human fertility protein PUMILIO2 in the testis (Spik et al., 2006); *cry-dash,* with an ancestral circadian role in light perception and related to massive spawning in corals during the Full Moon (Levy et al., 2007); *smtn1,* which is a regulator of the progesterone receptor during mice pregnancy (Bodoor et al., 2011); and *tnn1*, which controls ovulatory contraction of non-striated actomyosin networksin *C. elegans* (Obinata et al., 2010).

Enrichment analysis showed an upregulation of the overall catalytic activity, a process known to be affected by heat since elevated temperature produces changes on chemical reaction rates and increases protein denaturalization (Beitinger and Lutterschmidt, 2011). This corresponded to an overrepresentation of the catalytic pathways and of the signal transduction due to heat. The observation that most of the altered pathways were related to catabolism and signal reception-transmission corroborates the

hypothesis that the effects of heat were still persistent 110 days after the thermal treatment had finished.

In an attempt to further understand the biological meaning of our set of DE genes, DAVID clustering analysis showed that DNA binding and transcription were enriched, suggesting that despite protein and amino acid catabolism, protein synthesis and replacement was also occurring in HT gonads, most likely to compensate for the destabilizing temperature effects on protein structure. Likewise, downregulation of the immunology-related pathway may be due to the fact that our samples were obtained from differentiating females (progesterone-mediated oocyte maturation is affected as also it is the hormone-mediated signaling). A STRING analysis of protein-protein interactions corroborated the above mentioned relationships among some of these proteins. Main gene protein clusters were related to signal reception and transmission as well as to cellular and multicellular growth or to the oocyte maturation initiation pathway indeed. At first glance, the appearance of the oocyte maturation pathway may be surprising since our females were still immature. However, this pathway can also be activated by other ways such as by the insulin/IGF1 system (Andersen et al., 2003), where the IGF1 receptor (Igf1r) is in direct contact with the above mentioned proteins, or also by aldosterone/testosterone, as is the case in amphibian oocytes (Voronina and Wessel, 2003). This Igf1r is in turn related to IGF1, IGF-binding proteins (Igfbp1-Igfbp5) and Insulin receptor (Insr).

Cossins et al. (2006) investigated the transcriptomal response of seven carp tissues to cold. From that study, we selected the 15 genes that showed the greatest values in fold change increase in response to cold. Thirteen out of these 15 genes were present in our microarray. These genes are involved in protein turnover, unsaturated fatty acid synthesis, homeostasis or stress protein production. Interestingly, in general these genes exhibited a down-regulation tendency in the HT group, although without significant differences (Supplementary Table 9). Furthermore, the genes that Cossins et al., (2006) found upregulated by cold in the intestine with transport and regulatory functions were, when present, downregulated in the HT gonads. Therefore, these genes seem to behave in opposite ways depending on whether fish are exposed to heat or cold and regardless of species, which warrants their further

study in other species exposed to temperature changes and perhaps they could act as markers of a previous thermal history.

In contrast to what has been observed in killifish livers (Podrabsky and Somero, 2004), heat shock proteins were downregulated in our study, suggesting that the short heat exposure took place enough time ago to allow the return to their normal epxression levels. Also cholesterol and genes involved in the lipid metabolism were affected by heat. Thus, for example, cholesterol synthase and HMG-CoA reductase were downregulated as a result of chronic temperature elevation, as previously reported by Podrabsky and Somero (2004). The presence of translation elongation factors or proteasomes with a high level of expression due to heat is also in agreement with previous studies in other tissues (Gracey et al., 2004; Podrabsky and Somero, 2004).

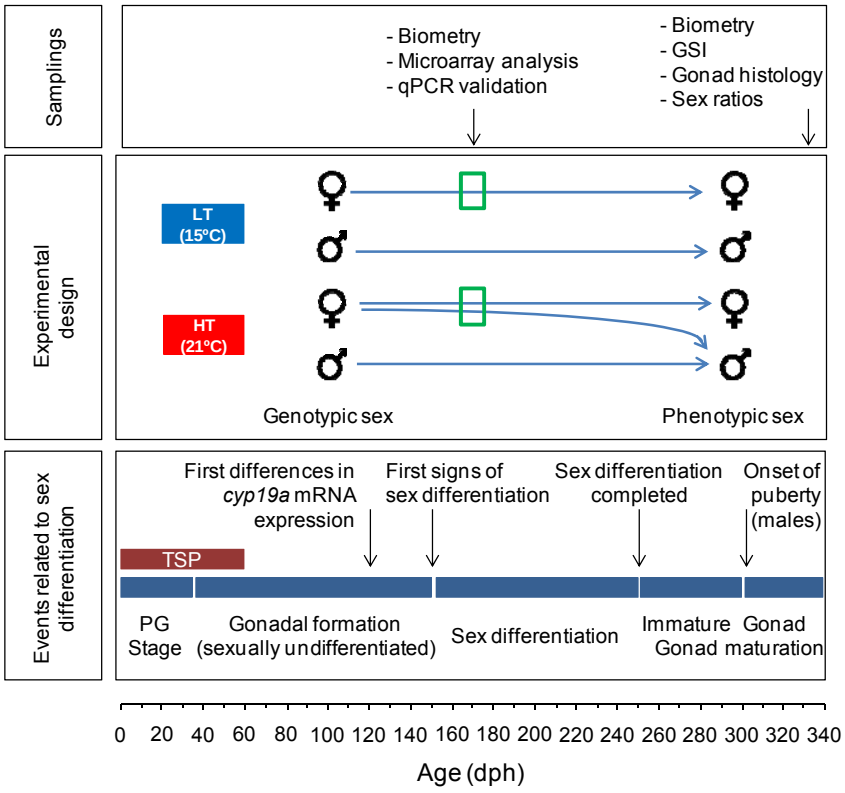
Apart from the common transcriptional response to heat, each tissue seems to have different strategies to cope with temperature changes: brain modulates glycolytic activity; liver turns on lipid metabolism; and muscle remodels its contractile apparatus (Gracey et al., 2004). From the present study, we can conclude that gonads increase catabolism and signal transduction, but reproductive and immune-related functions decrease. This is in agreement with the well documented deleterious effects of high temperature on gonadal function (Sloat and Reeves, 2014).

In conclusion, we have studied the effect of temperature at the whole gonadal transcriptomic level at the time of sex differentiation in a fish with mixed genetic and environmental sex determination. We show that about 3-4 months after the end of exposure to heat there is a downregulation of the expression of female-related genes such as *cyp19a1a* and an increase in male-related ones such as *amh* or *dmrt1*. Furthermore, some signaling, catabolic, biosynthetic, growth and reproduction pathways were still affected. Also, genes related to epigenetic regulatory mechanisms, involved in establishing and maintaining cell identity, were affected. Thus, the results of this study illustrate how the early environment sets permanent changes that result in long-lasting consequences in the sexual phenotype.

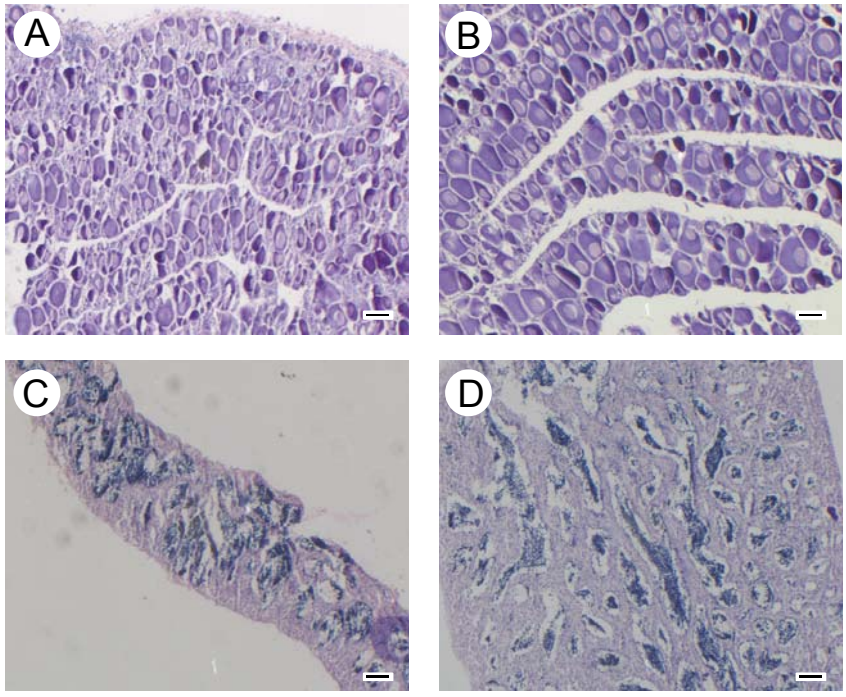
Acknowledgements

N.D. was supported first by a scholarship from the Government of Spain (BES-2007-14273) and then by a contract from the Epigen-Aqua Project (AGL2010-15939). Research was supported by Spanish Ministry Economy and Competitiveness grants SexGene (AGL2006-01359) and Consolider Aquagenomics (CDS2007-002) to F.P. Thanks are due to M. Blázquez, L. Navarro, L. Ribas and S. Joly for assistance with different aspects of this work. Thanks are also due to the fish maintenance staff at our Experimental Aquarium and Chamber facility (ZAE).

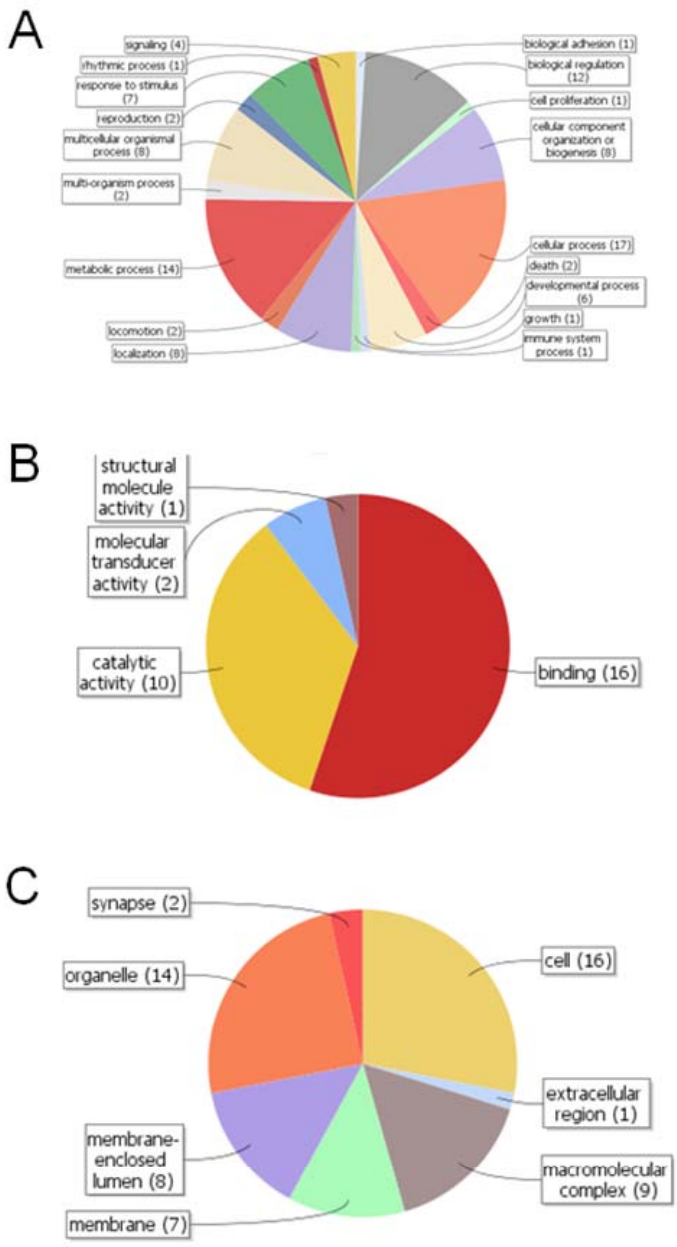
Supplementary figures



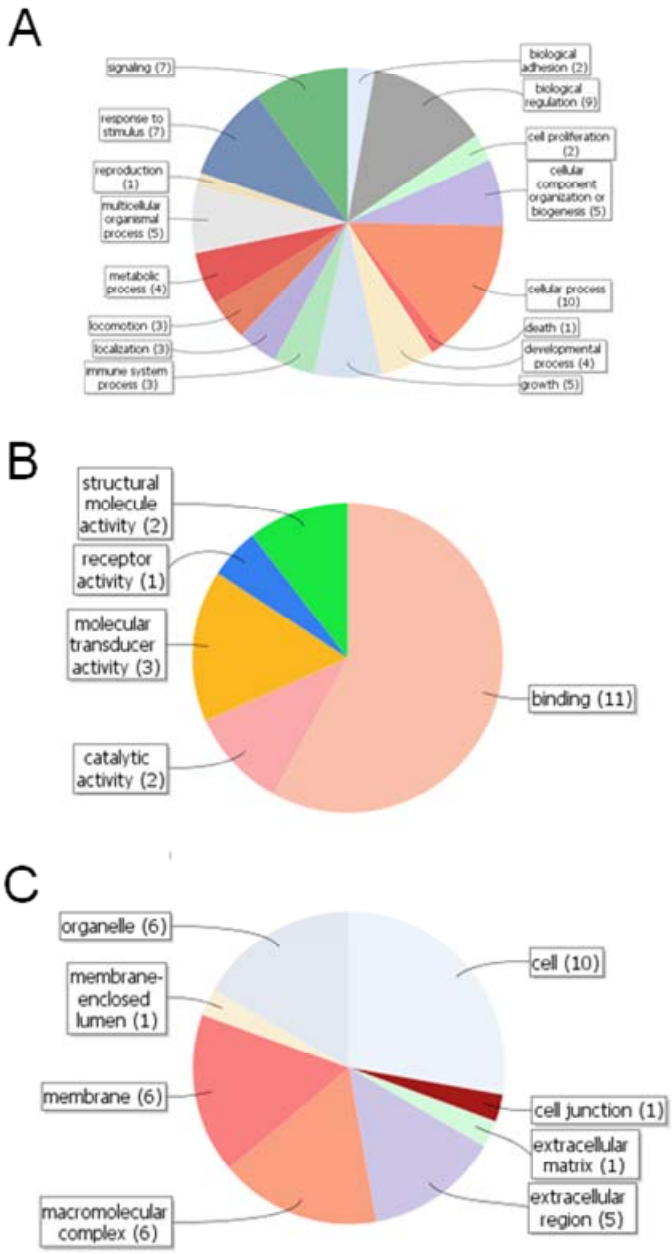
Supplementary Figure 1. Diagram on European sea bass sex differentiation events, experimental design and sampling strategy. On a calibrated age scale, the bottom panel illustrates the main events related to gonadal sex differentiation. The middle panel depicts the low (LT) and high (HT) temperature periods, matching the thermosensitive period (TSP). The boxes indicate the sampling for transcriptomic analysis in relation to age and events of sex differentiation. The top panel highlights the two main samplings of the experiment and the type of the performed analyses.



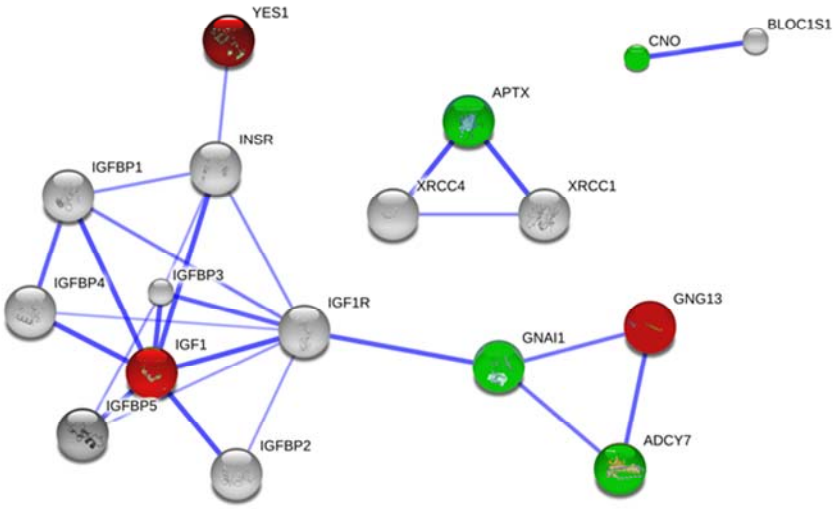
Supplementary Figure 2. Photomicrographs of one-year-old European sea bass gonads. (A) LT females, (B) HT females, (C) LT males and (D) HT males. Scale bar = 50 µm.



Supplementary Figure 3. GO terms results and classification in three main categories of upregulated genes in the HT group: A, biological process (BP); B, molecular function (MF); and C, cell component (CC).



Supplementary Figure 4. GO terms results and classification in three main categories of downregulated genes in the HT group: A, biological process (BP); B, molecular function (MF); and C, cell component (CC).



Supplementary Figure 5. Predicted protein-protein interactions based on the *Homo sapiens* database for the 18 up- and 9 downregulated genes in the HT vs. LT comparison. Seven genes (green = upregulated in the HT group; red = downregulated in the HT group) interact, three are directly (physically) related: guanine nucleotide-binding protein G(i) subunit alpha-1 (Gnai1), guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-13 (Gng13) and adenylate cyclase type 7 (Adcy7). Four of them are indirectly (functionally) related: insulin-like growth factor 1 (Igf1), tyrosine-protein kinase Yes 1 (Yes1), aprataxin (Aptx) and cappuccino homolog (Cno). Note: the zebrafish database, the only piscine database available in SPRING, was not used because preliminary tests showed that the number of meaningful interactions it returned was lower when compared to the human database.

Supplementary tables

Supplementary Table 1. Summary of the studies on the effects of temperature on gene expression in fish

Developmental stage	Methodology	Common name	Species	Treatment	Genes	Results	References	Temperature exposure	Sampling age
During sex differentiation	Candidate gene approach	African catfish	*Clarias gariepinus*	Heat	*cyp19a1a*	F>M	Valenzuela et al., 2013		
					cyp19a1a	HT<LT			
					sf1	F>M			
					sox9	F<M			
		Atlantic halibut	*Hippoglossus hippoglossus*	Heat	*wt1*	HT=LT	Valenzuela et al., 2013		
		European sea bass	*Dicentrarchus labrax*	Heat	*cyp19a1a*	HT<LT	Blázquez et al., 1998	57-137 dpf	21.4 months
				Heat	*arb*	HT=LT	Blázquez et al., 2009	0-120 dpf	120 dpf
					cyp11b	HT<LT		0-120 dpf	120 dpf
					cyp19a1a	HT<LT		0-120 dpf	195 dpf
					era	HT=LT		0-120 dpf	120 dpf
					erb1	HT=LT		0-120 dpf	120 dpf
					erb2	HT=LT		0-120 dpf	120 dpf
				Heat	*cyp19a1a*	HT<LT	Navarro-Martín et al.,	0-120 dph	170 dph

					2011		
			dax1	HT=LT			
Japanese flounder	*Paralichthys olivaceus*	Heat	*cyp19a1a*	HT<LT	Kitano et al., 1999	30-100 dph	10-100 dph (biweekly)
			cyp19a1a	HT<LT			
			cyp19a1a	HT<LT	Yamaguchi et al., 2007	30-100 dph	(biweekly)
			foxl2	suppressed by heat		30-100 dph	(biweekly)
			fshr	suppressed by heat		30-100 dph	(biweekly)
Nile tilapia	*Oreochromis niloticus*	Heat	*cyp19a1a*	HT<LT	D'Cotta et al., 2001	10-40 dpf	18-26 dpf
			cyp19a1b	HT<LT		10-40 dpf	18-26 dpf
		Heat	*amh*	HT>LT	Poonlaphdecha et al., 2013	10 dph and onwards	10 to 26 dpf
			dmrt1	HT>LT		10 dph and onwards	10 to 26 dpf
			foxl2	suppressed by heat		10 dph and onwards	10 to 26 dpf
		Heat	*cyp19a1a*	F>M	Valenzuela et al., 2013		
			dax1	F>M; after TSD F<M			
			sf1	F>M; after TSD F<M			
			sox9	F<M; mid-end TSD			
Pejerrey	*Odontesthes*	Heat	*amh*	HT>LT	Fernandino et	0-56 dph	0-42 dpf

			bonaeriensis				al., 2008a		(biweekly)
					cyp19a1a	HT<LT		0-56 dph	0-42 dpf (biweekly)
				Heat	*cyp19a1a*	HT<LT	Valenzuela et al., 2013	0-56 dph	0-42 dpf (biweekly)
					dmrt1	HT>LT	Fernandino et al., 2008b	0-56 dph	0-42 dpf (biweekly)
				Heat	*amh*	HT>LT	Hattori et al., 2009	0-126 dph	28-35-49 dph
					cyp19a1a	HT<LT		0-126 dph	28-35-49 dph
				Heat	*cyp19a1a*	HT<LT	Karube et al., 2007	0-70 dph	0-70 dph
					cyp19a1a	HT<LT	Valenzuela et al., 2013		
		Rainbow trout	*Oncorhynchus mykiss*	Heat	*wt1*	F<M; onset TSD	Valenzuela et al., 2013		
					cyp19a1a	F>M			
					dax1	F>M; onset TSD			
					sf1	F<M: onset TSD; F>M: mid TSD			
					sox9	F<M			
		Zebrafish	*Danio rerio*	Heat	*cyp19a1a*	suppressed by heat	Uchida et al., 2004	15-25 dph	40 dph
After sex differentiation is completed	Candidate gene approach	Atlantic halibut	*Hippoglossus hippoglossus*	Heat	*cyp19a1a*	HT<LT	Van Nes and Andersen., 2006	260 ddph	260, 600, 1100 ddph

		European sea bass	*Dicentrarchus labrax*	Heat	*cyp19a1a*	HT<LT	Navarro-Martín et al., 2011	0-120 dph	330 dph
					cyp19a1b	HT>LT		260 ddph	260, 600, 1100 ddph
					er1	HT>LT		260 ddph	260, 600, 1100 ddph
					er2	HT>LT		260 ddph	260, 600, 1100 ddph
Adult gonads	Candidate gene approach	Blue gourami	*Trichogaster trichopterus*	Heat	*gnrh3, igf1*	HT<LT; Females (male presence) affected under reproductive conditions	Levy et al., 2011		
					gnrh3, lh, gh	HT<LT; Females affected under normal conditions		not specified	adults
					pacap, prp-pacap, gh, gonadotropins	HT<LT; Females (no male presence) affected under reproductive conditions			
		Medaka	*Oryzias*	Heat	*cyp19a1a*	suppressed	Kitano et al.,	0-5 dph	adults

		latipes			by heat	2012		
				dmrt1	HT>LT	Hattori et al., 2007	embryo development	2-3 months
Transcriptomic analysis	Pejerrey	*Odontesthes bonaeriensis*	Heat	gsdf, *hsp90*, *cpa2*, *ctr*, *pretrypsinogen, etc…*	HT<LT	Fernandino et al., 2011	0-49 dph	49 dph
				ndrg3, pen2, gadph, p2xr1, kininogen, etc…	HT>LT		0-49 dph	49 dph
				wap56, *aldoa*	HT>LT; warm acclimation		0-49 dph	49 dph

Abbreviations: ddph, degree days post hatch; dpf, days post fertilization; dph, days post hatch; F, female; HT, high temperature; LT, low temperature; M, male

Supplementary Table 2. Gene abbreviation glossary

Gene abbreviations	Gene name
adcy7	Adenylate cyclase 7
Amh	Anti-Müllerian hormone
Aptx	Aprataxin
aqp1	Aquaporin 1
ca1	Carbonic anhydrase 1
ccbl1	Cysteine conjugate beta-lyase cytoplasmic, isoform CRA_a
cdc42ep3	Cdc42 efector protein 3
cg10623	DmeI-CG10623
cldn3	Claudin 3
Cmbl	Carboxymethylenebutenolidase homolog
Cno	Cappuccino homolog
col18a1	Collagen alpha-1 (XVIII) chain
cry-dash	Cryptochrome DASH
cyp19a1a	Cytochrome P450, family 19, subfamily A, polypeptide 1a
cyp19a1b	Cytochrome P450, family 19, subfamily A, polypeptide 1b
dicer1	Endoribonuclease Dicer
dmrt1	Doublesex- and mab-3- related transcription factor I
ecm1	Extracellular matrix protein 1
ehmt2	Euchromatic histone-lysine N-methyltransferase 2
gnai1	Guanine nucleotide-binding protein G(i) subunit alpha-1
gng13	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-13
Gnrh	Gonadotropin-releasing hormone
nr3c1	Glucocorticoid receptor
gtf3c1	General transcription factor 3C polypeptide 1
hdac11	Histone deacetylase 11
hsd11b1	11β-hydroxysteroid dehydrogenase
igf1	Insulin-like growth factor I
jarid2a	Protein Jumonji
Kin	DNA/RNA-binding protein KIN17
mettl22	Methyltransferase-like protein 22
Nefm	Neurofilament medium polypeptide
pcgf2	Polycomb group ring finger 2
pddc1	Parkinson disease 7 domain-containing protein 1
Prl	Prolactin

prpf40a	Pre-mRNA-processing factor 40 homolog A
r18S	r18S
smtn1	Smoothelin 1
sox17	HMG-box transcription factor SOX17
Star	Steroidogenic acute regulatory protein
ston1-gtf2a1l	Protein STON1-GTF2A
suz12	Suppressor of zeste 12 homolog
tep1	Telomerase protein component 1
Tesc	Tescalcin
Tmpo	Lamina-associated polypeptide 2, isoform alpha
tnnI	Troponin I
Vasa	Vasa protein
wisp1	WNT1 inducible signaling pathway protein 1
yes1	Tyrosine protein kinase Yes

Supplementary Table 3. Quantitative QRT-PCR primer characteristics

Gene	Gene abbreviation	Primer name	Primer sequence (5'→3')	Efficiency (*E*)	R^2
11β-hydroxysteroid dehydrogenase	*hsd11b1*	hsd11b1-Fw	CCTGGCAGCATATGGAGCAT	2.26	0.92
		hsd11b1-Rv	TACTGGTGCGACCTGTCCTA		
Anti-Müllerian hormone	*amh*	amh-Fw	TGCAGAGCAAAGCCTGAAAG	2.10	0.99
		amh-Rv	TCAACGGGGAACAAAGACAA		
Aquaporin 1	*aqp1*	aqua-Fw	GCCAGATCAGCGTGTTCAAG	2.27	0.98
		aqua-Rv	ACAGCACCAGCTGGAAGGTT		
Carbonic anhydrase 1	*ca1*	ca1-Fw	TGCCATAGTTGCTAACGCAC	2.22	0.97
		ca1-Rv	CTCATGGGACAGCCCTAACA		
Cdc42 efector protein 3	*cdc42ep3*	cdc42ep3-Fw	AGAGATCCTGCAGATGGACG	2.15	0.99
		cdc42ep3-Rv	TGTGTTGCTGTTCAGGCTTC		
Collagen alpha-1 (XVIII) chain	*col18a1*	col-Fw	AACTGCGACTCGGATCCTCA	2.01	0.98
		col-Rv	TATCCGGGTCTGCTCCACTG		
Cryptochrome DASH	*cry-dash*	cry-dash-Fw	GTTTGGGACAAAGCGTGCTA	2.29	0.96
		cry-dash-Rv	CTGTCTGTTGCAGGTCCTCT		
Cytochrome P450, family 19, subfamily A, polypeptide 1a (gonadal aromatase)	*cyp19a1a*	cyp19a1a-Fw	AGACAGCAGCCCAGGAGTTG	1.99	0.99
		cyp19a1a-Rv	TGCAGTGAAGTTGATGTCCAGTT		
Cytochrome P450, family 19, subfamily A, polypeptide 1b (brain aromatase)	*cyp19a1b*	cyp19a1b-Fw	CCCTTTTCAGCGCAGTGGTA	2.01	0.94
		cyp19a1b -Rv	CATTCGGCTTGTGGTGCTC		
DmeI-CG10623	*cg10623*	cg10623-Fw	AACTGGCCAAAGAGACGGTA	2.26	0.99
		cg10623-Rv	CAGAGCCGTTGAGCAGAAAG		

Doublesex- and mab-3- related transcription factor I	*dmrt1*	dmrt1-Fw	CCTTCACGCTACCCCACCTA	2.20	0.97
		dmrt1-Rv	GTTGTTGTCGTCCAGGCTGA		
Endoribonuclease Dicer	*dicer1*	dicer1-Fw	GCAGTACCGGAGCAGACTTA	2.04	0.98
		dicer1-Rv	AGAGGACGGTGCTCAACATT		
Euchromatic histone-lysine N-methyltransferase 2	*ehmt2*	ehmt2-Fw	TGTGTTTGATGCATGGTGCT	2.23	0.99
		ehmt2-Rv	AGACTTCATGTGTCAGGGCA		
Extracellular matrix protein 1	*ecm1*	ecm1-Fw	CAGAGCAGAGCACCCAGATA	2.57	0.96
		ecm1-Rv	TCAGTCTCACAGCATGAAGGA		
Glucocorticoid receptor	*nr3c1*	gr-Fw	CTTCCATCCAGCCCGTTGAT	2.07	0.98
		gr-Rv	GTAGTGGAGGTCTGCGTCTG		
Gonadotropin-releasing hormone	*gnrh*	gnRH-Fw	ACGCCCTGCAGAGTTTTAGG	2.04	0.99
		gnRH-Rv	AGAAGCACGAGGTCCTGACA		
Histone deacetylase 11	*hdac11*	hdac11-Fw	ACAGCACTACTGGAAGCACT	2.22	0.98
		hdac11-Rv	AGACGTTCTTCTCACCCGTT		
HMG-box transcription factor SOX17	*sox17*	sox17-Fw	CAAGAGACTGGCGCAGCAA	2.25	0.98
		sox17-Rv	TTTCCACGATTTCCCCAACAT		
Insulin-like growth factor I	*igf1*	igf1-Fw	TCCGTTTGTCACTTGTGTGAACT	2.16	0.95
		igf1-Rv	AGGCAATCAAGCACCATGAA		
Methyltransferase-like protein 22	*mettl22*	metll22-Fw	CCAGGAAGTGGCTGAAGCTC	2.18	0.97
		metll22-Rv	CTGCCTTGTGCTTCCTCTCC		
Polycomb group ring finger 2	*pcgf2*	pcgf2-Fw	CACTTCCACGGAATGAGACG	2.07	0.99
		pcgf2-Rv	GCTGCACTGAGAGACAAACC		
Prolactin	*prl*	prl-Fw	TATCCTGACCAGCGGATGTG	2.10	0.96
		prl-Rv	ACGCTGCCACCATGTACAAC		

Protein Jumonji	*jarid2a*	jarid2a-Fw	GGCTGAGCTCATGCATACAC	2.01	0.98
		jarid2a-Rv	ACCAGACGTTCTTCACACCA		
r18S	*r18S*	r18S-Fw	CCGCTTTGGTGACTCTAGATAACC	2.09	0.99
		r18S-Rv	CAGAAAGTACCATCGAAAGTTGA TAGG		
Smoothelin 1	*smtn1*	smtn1-Fw	CCTCAAGTTGGAAAGACGGC	2.18	0.98
		smtn1-Rv	TGTTCTTATCTGCAGCGCAC		
Steroidogenic acute regulatory protein	*star*	star-Fw	AGCAGAGGGGTGTTGTCAGA	2.10	0.99
		star-Rv	TGGTTGGCAAAGTCCACCTG		
Tescalcin	*tesc*	tesc-Fw	CAACATGGAGACCATCGCCC	2.10	0.96
		tesc-Rv	TGAACATCCGTCCTCGGTCA		
Troponin I	*tnnI*	tnnI-Fw	TGGTGTAGACAGCGGATACC	2.38	0.96
		tnnI-Rv	TGCCAGTGCTTCATATCGGA		
Vasa protein	*vasa*	vasa-Fw	CAGAAGCATGGCATTCCAATC	2,24	0.99
		vasa-Rv	TGCAGAATAGGGAGCAGGAAA		
WNT1 inducible signaling pathway protein 1	*wisp1*	wnt1-Fw	CATGCGAGTGTCCGAAGTCC	2.20	0.97
		wnt1-Rv	CGCACATCTTGCAGCAATCG		

Supplementary Table 4. Differentially expressed genes summary results

HT vs. LT	# downregulated genes	# upregulated genes	# Total
Total annotated genes	14,753	3,164	17,917
Without repetitions	4,789	1,360	6,149
Significant genes (without repetitions)	9	18	27

Supplementary Table 5. Fisher's exact test with multiple testing correction of FDR depicting the over-represented GO terms

GO Term	Name	Type	FDR	*P*-value	# in test group	# in reference group	# non annotated test group	# non annotated reference group	Over/Under
GO:0003008	System process	Biological process	1.8e-02	5.1e-06	9	342	19	6,672	Over
GO:0051970	Negative regulation of transmission of nerve impulse	Biological process	1.8e-02	6.6e-06	3	7	25	7,007	Over
GO:0050805	Negative regulation of synaptic transmission	Biological process	1.8e-02	6.6e-06	3	7	25	7,007	Over
GO:0031645	Negative regulation of neurological system process	Biological process	1.8e-02	6.6e-06	3	7	25	7,007	Over
GO:0007193	Adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway	Biological process	2.6e-02	1.2e-05	3	9	25	7,005	Over

Supplementary Table 6. Summary of the KEGG pathways from the up- and downregulated gene list

Pathway name	# sequences	# enzymes	Genes involved	Up/Down
Alanine, aspartate and glutamate metabolism	1	1	*ccbl1*	Up
Cysteine and methionine metabolism	2	2	*cg10623, ccbl1*	Up
Glyoxylate and dicarboxylate metabolism	1	1	*aptx*	Up
Inositol phosphate metabolism	1	2	*teP1*	Up
Nitrogen metabolism	1	1	*ca1*	Down
Phenylalanine metabolism	1	1	*ccbl1*	Up
Phenylalanine, tyrosine and tryptophan biosynthesis	1	1	*ccbl1*	Up
Phosphatidylinositol signaling system	1	2	*tep1*	Up
Purine metabolism	1	1	*adcy7*	Up
T cell receptor signaling pathway	1	1	*yes1*	Down
Tropane, piperidine and pyridine alkaloid biosynthesis	1	1	*ccbl1*	Up
Tryptophan metabolism	1	1	*ccbl1*	Up
Tyrosine metabolism	1	1	*ccbl1*	Up

Supplementary Table 7. Microarray versus qRT-PCR fold change results for 15 selected reproduction-related genes

	Microarray		qRT-PCR	
Genes	Fold change (FC)	Adjusted *P*-value	Fold change (FC)	*P*-value
Amh	6.36	0.288	1.40	0.236
aqp1	-5.47	0.169	0.11	0.023*
col18a1	2.56	0.259	0.59	0.566
cyp19a1a	-1.70	0.740	0.22	0.039*
cyp19a1b	1.06	0.756	0.97	0.388
dmrt1	1.73	0.339	5.19	0.017*
Gnrh	-1.08	0.965	13.61	0.083
igf1	-7.12	0.045*	0.90	0.901
mettl22	1.17	0.567	2.26	0.134
Prl	-1.02	0.974	3.24	0.039
sox17	-1.20	0.724	0.87	0.528
Star	1.56	0.401	2.75	0.036*
Tesc	1.09	0.900	1.06	0.219
Vasa	-	-	12.74	0.057
wisp1	-1.39	0,020*	-2.08	0.064

Supplementary Table 8. Epigenetic regulatory mechanisms- related genes implicated in sex differentiation as discussed in Piferrer (2013) that are present in the European sea bass custom-made microarray used in this study

			Upregulated		Downregulated	
Gene type	Gene name	Gene abbreviation	FC	Adjusted *P*-value	FC	Adjusted *P*-value
Heat shock proteins	heat responsive protein 12	*hrsP12*	1,50	0,536	-	-
	heat shock factor binding protein 1	*hsbP1*	-	-	-1,13	0,706
	heat shock protein 10	*hsPa10*	-	-	-1,15	0,869
	heat shock protein 14	*hsPa14*	1,20	0,694	-	-
	heat shock protein 4	*hsPa4*	-	-	-1,23	0,689
	heat shock protein 60	*hsPa60*	-	-	-1,15	0,824
	heat shock protein 70	*hsPa70*	1,29	0,513	-	-
	heat shock protein 70 binding protein	*hsPa70bP*	-	-	1,05	0,973
	heat shock protein 70 isoform 3	*hsPa70*	1,20	0,893	-	-
	heat shock protein 71	*hsPa71*	1,16	0,897	-	-
	heat shock protein 90B	*hsPa90b*	1,63	0,351	-	-
	heat shock protein transcription factor 2 binding protein	*hsf2bP*	-	-	1,0	0,906
Histone deacetylases	histone deacetylase	*hdac*	-	-	-1,1	0,949

	histone deacetylase 11	*hdac11*	1,64	0,424	-	-
	histone deacetylase 7 isoform D	*hdac7*	-	-	1,1	0,520
Dicer	dicer 1 (DCR-1 homolog)	*dicer1*	-	-	-1,1	0,384
Jarid (Jumonji)	Histone demethylase JARID1B	*kdm5b*	1,2	0,766	-	-
	Protein Jumonji	*jarid2a*	-	-	-1,1	0,762
JmjC-containing H3K9 demethylase	Lysine-specific demethylase 3a	*kdm3a*	1,2	0,647	-	-
	Lysine-specific demethylase 3b	*kdm3b*	1,3	0,259	-	-
	Lysine-specific demethylase 6b	*kdm6b*	-	-	1,1	0,973
Polycomb	Polycomb group RING finger protein 2	*pcgf2*	1,4	0,193	-	-
	Polycomb group RING finger protein 5	*pcgf5*	1,3	0,420	-	-
	Polycomb group RING finger protein 6	*pcgf6*	1,3	0,446	-	-
Thritorax suppressor	suppressor of zeste 12 homolog	*suz12*	-	-	1,0	0,934
Histone-lysine N-methyltransferase	Euchromatic histone-lysine N-methyltransferase 2	*ehmt2*	1,7	0,127	-	-

Supplementary Table 9. Comparison between the microarray results of this study and those reported in the literature on effects of temperature at the transcriptomic level (Gracey et al., 2004; Podrabsky and Somero, 2004; Cossins et al., 2006; Vergauwen et al., 2010; Chojnowski and Braun, 2012)

Probe ID	Description	Median	adjusted p-value
CUST_21190_PI416070213	40S ribosomal protein Sa-like protein	1,0638	9,62E-01
CUST_4351_PI416070213	acetyl-CoA acetyltransferase 2	1,0840	9,66E-01
CUST_12556_PI416070213	acidic leucine-rich nuclear phosphoprotein 32 family member A (PHAPI)	-1,0522	9,66E-01
CUST_3520_PI416070213	ADP-ribosylation factor-like 1	-1,1735	5,88E-01
CUST_1012_PI416070213	amh gene for anti-Müllerian hormone	4,8979	9,90E-01
CUST_20077_PI416070213	anti-Müllerian hormone	-1,0522	2,68E-01
CUST_5686_PI416070213	antizyme inhibitor 1 (AZI)	-1,2325	9,72E-01
CUST_11722_PI416070213	apolipoprotein A-I binding protein	1,0923	2,10E-01
CUST_4720_PI416070213	apolipoprotein A-I binding protein, isoform CRA_a	-3,8293	8,03E-01
CUST_8305_PI416070213	apolipoprotein AI precursor	-1,0262	4,67E-01
CUST_14611_PI416070213	apolipoprotein A-IV3	1,0615	9,87E-01
CUST_2026_PI416070213	apolipoprotein B	-1,6506	9,42E-01
CUST_2278_PI416070213	apolipoprotein E	-5,4703	7,69E-01
CUST_12361_PI416070213	aquaporin 1	1,3650	1,69E-01
CUST_14552_PI416070213	aquaporin 8	1,0026	7,86E-02
CUST_15718_PI416070213	arginase II	-1,0562	9,96E-01
CUST_13558_PI416070213	arginine-rich protein specific kinase 1	1,0688	9,44E-01
CUST_12544_PI416070213	ATP synthase	-1,0863	6,72E-01

CUST_3988_PI416070213	ATP synthase a chain	1,1256	9,07E-01
CUST_10513_PI416070213	ATP/GTP binding protein-like 4	1,7382	5,30E-01
CUST_2071_PI416070213	ATPase, Na+/K+ transporting, beta 1	-1,1077	7,12E-01
CUST_5095_PI416070213	basic transcription factor 3 isoform B	1,2555	8,58E-01
CUST_9550_PI416070213	basic transcription factor 3-like 4	-1,0223	6,38E-01
CUST_6508_PI416070213	beta-catenin	-1,9358	9,82E-01
CUST_17692_PI416070213	betainehomocysteine S-methyltansferase	-1,2578	2,45E-01
CUST_14122_PI416070213	btf3l4 protein	1,3894	8,05E-01
CUST_12253_PI416070213	C1q-like adipose specific protein	-1,1889	7,65E-01
CUST_18358_PI416070213	calmodulin (CaM)	1,0179	8,61E-01
CUST_1480_PI416070213	calreticulin	-1,1267	9,92E-01
CUST_110_PI416268254	carbonic anhydrase	-2,6397	7,93E-01
CUST_670_PI416070213	carboxypeptidase A1	-1,0225	8,97E-01
CUST_21325_PI416070213	cardiac myosin light chain-1	-1,1255	9,76E-01
CUST_5875_PI416070213	cathepsin H precursor	-1,1023	9,93E-01
CUST_16342_PI416070213	cds2 protein	-1,3620	7,54E-01
CUST_1666_PI416070213	chain A, Refined Solution Structure Of Human Profilin I	-1,3922	9,70E-01
CUST_7264_PI416070213	cofilin 2, muscle, isoform CRA_b	1,3753	5,99E-01
CUST_1303_PI416070213	cofilin protein	-1,1150	4,98E-01
CUST_139_PI416070213	cold-shock domain protein mRNA	1,0075	9,40E-01
CUST_14164_PI416070213	copper chaperone for superoxide dismutase	1,0576	6,00E-02
CUST_8065_PI416070213	copper/zinc superoxide dismutase	-1,0237	7,03E-01
CUST_2563_PI416070213	creatine kinase mitochondrial isoform	-1,1363	9,95E-01
CUST_4120_PI416070213	cyclin G1	-1,3054	9,85E-01

CUST_30_PI416268254	cytochrome b	-1,3147	4,25E-01
CUST_7084_PI416070213	cytochrome c oxidase subunitVb precursor	1,0107	8,53E-01
CUST_6961_PI416070213	cytochrome c oxidase subunit VIb isoform 1	-1,0921	8,53E-01
CUST_1_PI416268254	cytochrome P450	-1,2184	9,70E-01
CUST_535_PI416070213	cytochrome P450 aromatase B (cyp19b)	-1,1207	9,42E-01
CUST_16732_PI416070213	cytoplasmic polyadenylation element binding protein	-1,1492	7,70E-01
CUST_6385_PI416070213	cytosolic malate dehydrogenase thermolabile form	1,0476	3,05E-01
CUST_6439_PI416070213	dazap1 protein	-1,2408	4,49E-01
CUST_3067_PI416070213	dazap2-like protein	1,0065	9,47E-01
CUST_12025_PI416070213	death associated protein 1a	1,0243	7,02E-01
CUST_17401_PI416070213	delta-9-desaturase 1	-1,0487	3,08E-01
CUST_21223_PI416070213	dihydrolipoamide dehydrogenase	-1,1800	9,52E-01
CUST_17317_PI416070213	dihydropyrimidine dehydrogenase	1,0199	8,61E-01
CUST_12487_PI416070213	DM-related transcriptional factor Dmrt2b	1,2953	9,55E-01
CUST_10631_PI416070213	dmrt 1	1,2080	4,91E-01
CUST_3052_PI416070213	early growth response 1	-1,0577	9,40E-01
CUST_10501_PI416070213	eif1ad protein	-1,3994	9,67E-01
CUST_10678_PI416070213	elastase 1 precursor	-1,4463	9,22E-01
CUST_8971_PI416070213	elastase-like serine protease	-1,5505	8,89E-01
CUST_15238_PI416070213	elastin microfibrilinterfacer 2	-2,1761	2,66E-01
CUST_21118_PI416070213	elongation factor 1 alpha isoform 2	1,1757	2,53E-01
CUST_16189_PI416070213	ependymin	-1,0285	9,04E-01
CUST_12778_PI416070213	ependymin-2 precursor	-1,0623	9,27E-01
CUST_9475_PI416070213	eukaryotic translation initiation factor 2B	-1,0340	9,73E-01

CUST_18418_PI416070213	eukaryotic translation initiation factor 3	-1,0658	9,65E-01
CUST_13579_PI416070213	F-box only protein 2	-1,1159	9,77E-01
CUST_8251_PI416070213	ferritin-H subunit	-1,5972	8,49E-01
CUST_21229_PI416070213	filamin A interacting protein 1	-1,3786	5,06E-01
CUST_2752_PI416070213	filamin A, alpha	-3,4245	4,92E-01
CUST_11212_PI416070213	galectin	-1,8040	5,49E-01
CUST_16003_PI416070213	galectin 8	1,0283	9,18E-01
CUST_7216_PI416070213	galectin like protein	-1,1072	8,48E-01
CUST_6142_PI416070213	gelatinase	1,0543	9,73E-01
CUST_2614_PI416070213	glutathione S-transferase, theta 3	-2,3106	4,18E-01
CUST_16666_PI416070213	glycine dehydrogenase (decarboxylating)	1,4019	5,55E-01
CUST_13426_PI416070213	granulin-a	1,4162	6,66E-01
CUST_12714_PI416070213	granulin-like peptide	-1,0972	9,22E-01
CUST_3412_PI416070213	GTP-binding nuclear protein Ran (GTPase Ran)	1,0162	9,92E-01
CUST_2419_PI416070213	heat shock cognate 70	-1,0475	9,31E-01
CUST_17674_PI416070213	heat shock cognate 71	1,1065	9,37E-01
CUST_7477_PI416070213	heat shock factor binding protein 1	-1,0850	8,72E-01
CUST_6343_PI416070213	heat shock protein 10	-1,1418	8,95E-01
CUST_5653_PI416070213	heat shock protein 70 binding protein	1,0576	9,66E-01
CUST_18040_PI416070213	heat shock protein 90 beta	1,0685	9,54E-01
CUST_15709_PI416070213	heterogeneous nuclear ribonucleoprotein G	1,2192	6,96E-01
CUST_7765_PI416070213	high density lipoprotein (HDL) binding protein	-1,1989	9,08E-01
CUST_11980_PI416070213	high mobility group protein	-1,0976	8,69E-01
CUST_18037_PI416070213	High-mobility group box 1	-1,2261	4,73E-01

CUST_10831_PI416070213	high-mobility group box 2	-1,1422	8,93E-01
CUST_17146_PI416070213	high-mobility group protein 2-like 1	-1,0172	9,87E-01
CUST_13807_PI416070213	Histone H2B	-1,0702	9,85E-01
CUST_8773_PI416070213	histone H3	-1,0883	8,62E-01
CUST_283_PI416070213	HMG-CoA reductase mRNA	1,2728	9,83E-01
CUST_3016_PI416070213	hsp47	-1,1294	8,36E-01
CUST_10309_PI416070213	isocitrate dehydrogenase 1 (NADP+)	-1,0688	3,18E-01
CUST_17161_PI416070213	isocitrate dehydrogenase 3 (NAD+) alpha	-1,0518	9,41E-01
CUST_5272_PI416070213	isocitrate dehydrogenase 3 (NAD+) gamma isoform	1,0149	9,57E-01
CUST_13297_PI416070213	isocitratedehydrogenase 3, beta subunitisoform	1,3798	9,66E-01
CUST_8839_PI416070213	junB protein	-1,0054	9,64E-01
CUST_15787_PI416070213	kinesin-like protein KIF1B	-1,1594	9,91E-01
CUST_8515_PI416070213	lecithin cholesterol acyltransferase	1,0038	7,53E-01
CUST_10357_PI416070213	leucine rich repeat containing 42	-1,1614	9,95E-01
CUST_4639_PI416070213	malate dehydrogenase	-1,0179	6,37E-01
CUST_16465_PI416070213	malic enzyme 3, NADP(+)-dependent	1,0512	9,76E-01
CUST_9052_PI416070213	manganese superoxide dismutase	1,1171	9,74E-01
CUST_11593_PI416070213	mdh1b protein	1,1124	3,60E-01
CUST_19768_PI416070213	microsomal glutathione S-transferase	-1,0105	9,46E-01
CUST_11806_PI416070213	microsomal glutathione S-transferase 2	1,1775	9,86E-01
CUST_9199_PI416070213	mitochondrial ATP synthase gamma-subunit	-1,0344	8,91E-01
CUST_18255_PI416070213	mitochondrial creatine kinase	1,0804	9,04E-01
CUST_4891_PI416070213	mitochondrial ornithine aminotransferase	-1,0246	9,47E-01
CUST_3516_PI416070213	mitochondrial uncoupling protein 3	1,0542	9,76E-01

CUST_6304_PI416070213	mitosis-specific chromosome segregation protein SMC1 homolog	-1,2270	9,27E-01
CUST_18190_PI416070213	muc2 protein	-1,0744	7,77E-01
CUST_12637_PI416070213	mucin 2, oligomeric mucus/gel-forming, isoform CRA_a	-1,1538	7,88E-01
CUST_21289_PI416070213	myosin heavy chain	-1,3612	9,65E-01
CUST_1252_PI416070213	myosin light chain 1	-1,0918	9,86E-01
CUST_7768_PI416070213	NADH dehydrogenase subunit 4	-3,1728	7,13E-01
CUST_21013_PI416070213	obscurin	1,1429	7,65E-01
CUST_1846_PI416070213	ornithine aminotransferase	-1,0326	9,94E-01
CUST_11446_PI416070213	ornithine decarboxylase antizyme	1,0080	9,78E-01
CUST_1201_PI416070213	orphan nuclear receptor DAX2 (NR0B1b) gene	-1,0978	9,76E-01
CUST_17446_PI416070213	oxidoreductase NAD-binding domain containing 1	-1,0357	9,01E-01
CUST_1459_PI416070213	pc4 and sfrs1-interacting protein	-1,2041	8,50E-01
CUST_9442_PI416070213	pdia4 protein	1,1513	9,42E-01
CUST_2647_PI416070213	phosphoenolpyruvatecarboxykinase	1,1043	7,40E-01
CUST_18646_PI416070213	postmeiotic segregation increased 2 (pms2)	-2,7459	6,78E-01
CUST_1597_PI416070213	probable Bax inhibitor 1 (BI-1)	-1,1457	9,66E-01
CUST_10171_PI416070213	profilin 2 like	1,0323	9,42E-01
CUST_2938_PI416070213	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10	-1,0459	9,80E-01
CUST_7993_PI416070213	proteasome (prosome, macropain) subunit, alpha	1,0707	9,46E-01
CUST_13930_PI416070213	proteasome (prosome, macropain) subunit, beta type, 5	1,1885	9,33E-01
CUST_2773_PI416070213	ptp-IV1b, PTP-IV1 gene product	-1,0839	9,96E-01
CUST_17542_PI416070213	ptprd protein	-1,0032	6,66E-01
CUST_15433_PI416070213	ptprf interacting protein, binding protein 1	-1,1672	7,56E-01

CUST_14260_PI416070213	putative pyruvate dehydrogenase phosphatase isoenzyme 2	-1,0551	8,52E-01
CUST_16117_PI416070213	pyruvate dehydrogenase (lipoamide) beta	1,0572	6,31E-01
CUST_1510_PI416070213	pyruvate dehydrogenase E1 component subunit	-1,1418	8,81E-01
CUST_10711_PI416070213	pyruvate kinase	-1,0835	9,38E-01
CUST_19390_PI416070213	quiescin Q6 sulfhydryl oxidase 1 isoform a	1,0826	9,49E-01
CUST_18058_PI416070213	ran binding protein 10	1,0305	9,93E-01
CUST_7159_PI416070213	ran binding protein 3 isoform RANBP3-b	-1,0069	9,52E-01
CUST_2284_PI416070213	ran-binding protein 7	-1,0678	9,94E-01
CUST_7891_PI416070213	ranbp1 protein	1,0132	9,02E-01
CUST_4411_PI416070213	RNA-binding protein 1	-1,1543	6,03E-01
CUST_16006_PI416070213	rrm1 protein	-1,1065	8,24E-01
CUST_21007_PI416070213	sarcoglycan, gamma	1,2231	8,88E-01
CUST_19216_PI416070213	serine carboxypeptidase 1	-1,1060	9,78E-01
CUST_12704_PI416070213	skeletal muscle myosin heavy chain	-1,0691	1,09E-01
CUST_20632_PI416070213	slow myosin heavy chain 3	-1,0207	8,61E-02
CUST_14320_PI416070213	small nuclear ribonucleoprotein D2-like protein	1,2830	9,89E-01
CUST_1174_PI416070213	small nuclear ribonucleoprotein E-like mRNA	3,2432	4,22E-01
CUST_14152_PI416070213	sodium potassium ATPase alpha subunit	1,0153	5,77E-01
CUST_10027_PI416070213	solute carrier family	-1,6132	9,56E-01
CUST_16327_PI416070213	solute carrier family 3, member 2	-1,0341	4,23E-01
CUST_19864_PI416070213	splicing factor 3a, subunit 1, 120kDa isoform 1	1,3642	5,81E-01
CUST_15601_PI416070213	splicing factor 3b, subunit 1 isoform 1	1,2465	8,90E-01
CUST_3085_PI416070213	splicing factor arginine/serine-rich 3	-1,1149	9,27E-01
CUST_12128_PI416070213	T-complexprotein 1 subunit alpha	1,1564	4,64E-01

CUST_20779_PI416070213	tcp1 protein	-1,1055	9,35E-01
CUST_5194_PI416070213	tcp1-beta	-1,0641	9,33E-01
CUST_10183_PI416070213	tomm20	1,0960	8,60E-01
CUST_12044_PI416070213	transaldolase	1,0797	8,53E-01
CUST_17176_PI416070213	transcription factor SOX-8	1,3126	9,44E-01
CUST_12057_PI416070213	translation initiation factor eIF-2B precursor	-1,0425	9,63E-01
CUST_21553_PI416070213	tubulin alpha 6	1,0143	8,21E-01
CUST_7112_PI416070213	tubulin, alpha, ubiquitous	-1,1704	6,22E-01
CUST_17711_PI416070213	tubulin, delta 1, isoformCRA_c	1,2051	9,28E-01
CUST_13015_PI416070213	tubulin, gamma complex associated protein 2	1,1493	4,98E-01
CUST_88_PI416070213	U2AF1-RS2	1,2263	7,32E-01
CUST_6817_PI416070213	ubiquitin conjugating enzyme E2A	1,1512	7,07E-01
CUST_4522_PI416070213	UDP-N-acetylglucosaminetransferasesubunit	-1,0196	9,85E-01
CUST_3094_PI416070213	vacuolar ATP synthase 16 kDaproteolipid subunit-like protein	-1,0186	9,81E-01
CUST_13924_PI416070213	wnt1 inducible signaling pathway protein 1	-1,6458	6,48E-02

Chapter 4

PATTERNS OF GENE EXPRESSION IN EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*), A SPECIES WITH POLYGENIC SEX DETERMINATION, AT THE TIME WHEN GONADS WERE EXPERIENCING OPPOSITE PATHWAYS OF DIFFERENTIATION

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Abstract

The transcriptomic analysis of the process of sex differentiation has been carried out mostly in species with male or female heterogamety but such information is almost non-existent for species with other sex determination systems. The European sea bass is a gonochoristic teleost without sex chromosomes with a polygenic sex determination system influenced by temperature. Elevated temperatures masculinize genetic females, hence skewing sex ratios to ~3:1 in favor of males. In the present study, two groups of sea bass larvae were exposed to 21ºC from 20 to 220 days post-hatch (dph), comprising the period of early gonad formation. During the hormone sensitive period (90–154 dph), one group was fed with a Estradiol (E_2)-supplemented diet. A custom-made sea bass microarray (MA) was used to transcriptomically analyze the gonads of sexually differentiating putative females at 170 dph, identified by a cluster analysis based on *cyp19a1a* expression (a known marker of female differentiation in sea bass), comparing the E_2-treated fish against the untreated controls. MA results were validated by qRT-PCR. This strategy allowed us to compare patterns of gene expression in a species with polygenic sex determination at the time when gonads were experiencing opposite pathways of differentiation. As expected, elevated temperature masculinized genetic females (79% males in the control group) while E_2 treatment resulted in an all-female population, negating the

effects temperature. A total of 383 genes were differentially expressed (DE) due to E_2 treatment (92 up- and 291 downregulated) and both MA and qRT-PCR results showed a downregulation in the expression of genes involved not only in testicular but also in ovarian differentiation in the E_2-treated fish. This was mainly achieved by a shutdown of the first steps of steroidogenesis. GO enrichment analysis showed that E_2 administration affected pathways not only related to reproduction but also related to the immune response, xenobiotic metabolism, response to stimulus, signaling and growth. Together, these results show how E_2 had the power of negate the masculinizing effect of heat while decreasing at the same time the expression of the steroidogenic-related genes. Moreover, it also changed the expression of some genes related to epigenetic mechanisms, although these changes need further study.

1. Introduction

The sex ratio is an essential demographic parameter in population ecology (Bull, 1983) and its proper establishment is crucial for the perpetuation of all sexually reproducing species (Werren and Beukeboom, 1998). The establishment of the sex ratio depends on sex determination, i.e., the genetic or environmental process by which the gender of an individual is established (Penman and Piferrer, 2008), and sex differentiation, i.e., the transformation of an undifferentiated gonad into an ovary or testis (Piferrer and Guiguen, 2008). In fish, abiotic factors such as temperature can influence sex ratio, thus contributing to the observed high sexual phenotypic plasticity present when compared to other vertebrates (Devlin and Nagahama, 2002).

In contrast to the situation with master sex-determining genes, which are not conserved in vertebrates (Kikuchi and Hamaguchi, 2013), genes implicated in gonadal sex differentiation are fairly conserved (Cutting et al., 2013). Thus, gene expression analyses have shown that certain genes are typically associated with testis differentiation, e.g., *dmrt1, amh, sox9, wt1, nr5a2, nr0b1* (Ravi et al., 2014), whereas other genes are typically associated with ovarian differentiation, e.g., *cyp19a1a, foxl2, vasa, wnt4* (Ravi et al., 2014). However, interspecific differences in the temporal or spatial

expression of some of these genes are observed. Thus, for instance, while expression of *amh* precedes that of *sox9* in differentiating mammalian (De Santa Barbara et al., 1998) and zebrafish (*Danio reriosox9a;* Rodríguez-Marí et al., 2005) gonads, the opposite is observed in birds and crocodiles (Cutting et al., 2014; Western et al., 1999).

Furthermore, in contrast to mammals, in non-mammalian vertebrates estrogens are essential for proper ovarian differentiation (Nagahama, 2006; Guiguen et al., 2010). In fish, this comes from several lines of experimental evidence, including: 1) the study of the effects of administration of exogenous steroids, where estrogens such as estradiol-17ß (E_2) can feminize phenotypic males in many species (Piferrer, 2001; Saillant et al., 2001a; Devlin and Nagahama, 2002; Pandian, 2003; Navarro-Martín et al., 2009a), 2) blockade of aromatase, the steroidogenic enzyme that irreversibly converts androgens into estrogens such as E_2, by the use of aromatase inhibitors (AI) such as Fadrozole, which results in masculinization of genetic females (Piferrer et al., 1994, Navarro-Martín et al., 2009a), 3) blockade of the estrogen receptors by an antagonist (e.g., Tamoxifen) also results in masculinization of genetic females (Nagahama, 2006). Recent evidence shows that estrogens are important not only to initiate ovarian differentiation but also to maintain the gonadal female phenotype later on, since AI treatments on adult fish can sex-reverse some gonochoristic fish species (Bhandari et al., 2006; Ogawa et al., 2008). In this sense, many endocrine disrupting chemicals are known to act by mimicking the effects of estrogens. Thus, from a point of view of environmental toxicology the effects of exposure to estrogenic compounds are important for their consequences in development, growth and reproduction (Schiller et al., 2013a-b; Mills et al., 2014).

With the advent of high-throughput techniques, gene expression during normal or estrogen-induced gonadal differentiation has been studied at the whole transcriptome level. The transcriptomic analysis of the normal process of sex differentiation in fish has been carried out in established models such as the zebrafish (Zheng et al., 2013), or in medaka (*Oryzias latipes*) treated with E_2 (Kishi et al., 2006). With a similar approach, the study of rainbow trout (*Oncorhynchus mykiss*) oocyte maturation and ovulation showed

how E_2 synthesis was downregulated at the preovulatory period while pathways related to inflammation, vasodilation and coagulation were upregulated (Bobe et al., 2006). Recently, with the development of custom-made microarrays, similar studies on the identification of female- and male-enhanced genes have been extended to other species including the Nile tilapia (*Oreochromis niloticus*; Tao et al., 2013) or the half-smooth tongue sole (*Cynoglossus semilaevis*; Shao et al., 2014). Likewise, several transcriptomal studies have been conducted in fish to analyze the effects of E_2 administration, either in adults or embryos (Schiller et al., 2013a-b; Gunnarsson et al., 2007; Geoghegan et al., 2008); (Hao et al., 2013), demonstrating tissue- and gender-specific responses, as in Japanese medaka (Sun et al., 2011b) or biogeographically differences like in the Hornyhead turbot (*Pleuronicthys verticalis*; Baker et al., 2013). Nevertheless, few transcriptional studies have addressed the direct effects of exogenous estrogens on developing gonads. In this regard, is it still not clear if exogenous steroids such as E_2 or its derivate ethinylestradiol (EE_2) mimic physiological processes (Baron et al., 2007; Vizziano et al., 2008; Vizziano-Cantonnet et al., 2008) even though they inhibit the expression of several steroidogenic enzymes in some fish species (Govoroun et al., 2001, Vizziano-Cantonnet et al., 2008) and alter normal hormonal functions (Filby et al., 2007; Doyle et al., 2013). Santos and collaborators (2007a,2007b) analyzed the molecular mechanisms associated to reproductive disruption in breeding zebrafish, finding that fertility, fecundity and molecular pathways closely related to gametogenesis were compromised by the E_2 administration.

All but one of the species mentioned above have a well-established chromosomal system of sex determination. The exception is the zebrafish, in which sex determination is polygenic (Liew et al., 2012). In these systems, rather than a single master sex determining gene, there are several genes that promote either male or female differentiation. By virtue of this, environmental influences also are common in influencing the final sex ratio in species with a polygenic system of sex determination (Bull, 1983). To the best of our knowledge, with the exception of zebrafish (Santos et al., 2007a,2007b; Zheng et al., 2013; Ravi et al., 2014) no transcriptomic studies have been performed during normal or estrogen-induced gonadal sex differentiation in species with a

polygenic sex determination system. The question to be addressed is whether the same patterns of gene expression as previously seen in species with chromosomal systems will operate in species with a polygenic system.

The European sea bass is a gonochoristic species that lacks sex chromosomes and for which a polygenic system of sex determination involving a four-factor system has been proposed (Vandeputte et al., 2007). Furthermore, sex determination and differentiation are influenced by environment during early development (Piferrer et al., 2005). We took advantage of the fact that in the European sea bass elevated temperature applied during the thermosensitive period (0–60 dpf; Navarro-Martín et al., 2009b) masculinizes about half of the fish that under more natural temperatures (<17°C) would develop as females, thus increasing the proportion of males form ~50% to ~75% at one year of age. Elevated temperatures induce an epigenetic mechanism that links temperature and final sex ratio by differentially methylating the gonadal aromatase promoter. To this thermal masculinizing background, we superimposed feminization, achieved by E_2 treatment during the previously identified hormone-sensitive period (90–160 dph; Blázquez et al., 2001). The role of estrogens on ovarian differentiation is conserved among vertebrates (Devlin and Nagahama, 2002; Pieau and Dorizzi, 2004; Smith and Sinclair, 2004) and the exogenous estrogen administration is able to feminize sexually undifferentiated fish (Piferrer, 2001). In this way, we created a male-biased (78% males) and a female-biased (100% females) population through a thermal or hormonal treatment, respectively. However, rather than examining gene expression in juveniles, when gonads are already fully sexually differentiated, we sampled gonads at 170 dph, i.e., during the sex differentiation period, and analyzed their transcriptome by a custom-made oligomicroarray. This strategy allowed us to compare patterns of gene expression in a species with polygenic sex determination at the time when gonads were experiencing opposite pathways of differentiation.

2. Materials and methods

2.1. Animals and rearing conditions

One day post hatch (dph) larvae of European sea bass were obtained in May 2009 from a commercial hatchery (St. Pere Pescador, Girona, Spain) and transported to the Institute of Marine Sciences experimental aquarium facility (ZAE), stocked and reared following (Díaz et al., 2013). Fish were treated in agreement with the European Convention for the Protection of Animals used for Experimental and Scientific Purposes (EST Nu 123, 01/01/91).

2.2. Experimental design

Fish were divided in two tanks and maintained at 17ºC during the first 20 dph. Water temperature was then increased at a rate of 0.5ºC/day to 21ºC. Temperature was maintained at 21ºC until ~220 dph (fall) when water was left to follow the natural fluctuations in temperature. From 90 to 154 dph, the control group (Ctrl group) was fed *ad libitum* two times a day with a 96% ethanol-sprayed dry food, while the other group (E_2-group) was fed with Estradiol-sprayed dry food (10 mg/Kg E_2) (Supplementary Figure 1).

2.3. Samplings

Biometric data (standard length: SL with a 1 mm precision; body weight: BW with a 0.01 g precision) was collected periodically by anesthetizing fish with adjusted doses of 2-phenoxyethanol (2PE; $0.2ml \cdot l^{-1}$) and returning them afterwards to the tanks. For tissue biopsies, fish were anesthetized with an overdose of 2PE and tissues were either immediately frozen with liquid Nitrogen for subsequent molecular analysis of gene expression or fixed in 4% paraformaldehyde for histological analysis. Briefly, gonads were histologically analyzed as previously described (Díaz et al., 2013) and female stages of oocyte maturation and male progression of spermatogenesis were assessed (Brown-Peterson et al., 2011).

Coinciding with the end of the experiment (332 dph), eight fish were randomly taken from each group and gonadosomatic (GSI), hepatosomatic (HSI) and carcass indexes (CI) were determined to analyze the possible effects of temperature and hormonal treatment on fish maturation (for details on procedures see Díaz et al., 2013).

2.4. RNA extraction and cDNA synthesis

Total RNA was obtained from 170 dph sea bass gonads. Briefly, isolated gonads were homogenized with Trizol (Invitrogen, UK) and purified using a chloroform-isopropanol-ethanol protocol. RNA concentration and quality was measured with a ND-100 spectrophotometer (NanoDrop Technologies) and in a 1% agarose/formaldehyde gel, respectively. Two hundred nanograms of total RNA were RNAse-treated (*E. coli* RNAse H) and half of the volume was retrotranscribed to cDNA (SuperScript III RNase Transcriptase and Random hexamer both from Invitrogen, Spain).

A Bioanalyzer 2100, with the RNA 6000 Nano LabChip kit (Agilent, Spain), was used to further analyze total RNA integrity (1 µl/sample) before hybridizations. Samples with a 100-200 ng/µl concentration and RIN values>7 were selected for the hybridizations.

2.5. Quantitative real time PCR (qRT-PCR)

qRT-PCR was used for: 1) sample selection based on the *cyp19a1a* expression levels at 170 dph for microarray (MA) analysis and, 2) to validate MA results and check several genes related to sex differentiation (Supplementary Table 1 for a gene glossary). cDNA was always diluted 1:10 when analyzing target genes and 1:500 when measuring the housekeeping gene, *r18S*. Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used for primer design and quality checking. Primer specificity and performance was checked with a melting curve analysis (95ºC for 15 s, 60ºC for 15 s and 95ºC for 15 s) after the amplification (Supplementary Table 2: E: efficiency between 1.99 and 2.27; slope ranging from –2.6 to –3.3 and R^2: linear correlations higher than

0.94). Briefly, the qRT-PCR program consisted on an initial UDG decontamination cycle at 50ºC for 2 min, followed by an initial activation step of 10 min at 95ºC; 40 cycles of 15 s denaturation at 95ºC and a 1 min annealing/extension step at the 60ºC. Finally a dissociation step of 15 sec at 95ºC followed by 15 sec at 60ºC was added.

Samples were run in triplicate on an ABI 7900HT (Applied Biosystems) 384-well plates in a final volume of 10 µl per well (5 µl of SYBRGreen Supermix, 2 µl distilled water, 2 µl primer mix and 1 µl of cDNA). Negative controls lacking cDNA or primers were always included in duplicate. Data was collected using SDS 2.3 software and gene expression levels were calculated using RQ Manager 1.2 (both from Applied Biosystems). Ct values of the different genes were adjusted by primer *E* and the expression of target genes and the intra- and inter-assay variations were normalized to the endogenous reference gene, *r18S* (Schmittgen and Livak, 2008).

2.6. Microarray

2.6.1. Experimental design

Ten individuals, five from the Ctrl-group and five from the E_2-group were individually hybridized at the Institute of Biotechnology and Biomedicine (UAB, Barcelona). Samples were randomly distributed on the different slides to avoid batch effects.

2.6.2. RNA sample preparation and MA hybridization

Quality-checked RNA was Cy3-labeled with Agilent's One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labelling kit) along with Agilent's One-Color RNA SpikeIn Kit), cRNA was purified (RNeasy mini spin columns from Qiagen), quantified (Nanodrop ND- 1000), verified (Bionalyazer 2100), hybridized to sea bass arrays (Agilent ID 023790) at 65ºC for 17 h (Agilent's GE Hybridization Kit), washed (Agilent's Gene Expression Wash Pack with stabilization and drying solution) and

scanned (Agilent Technologies Scanner, model G2505B). Agilent's Feature Extraction software version 10.4.0.0 was used to check spot intensities and other control features. The complete design has been submitted to the Gene Expression Omnibus (GEO)-NCBI database (GSE52938) as it also has been submitted the platform that validates the MA (GPL13443).

2.7. Statistical analysis of data

2.7.1. Statistics of biometric data

Briefly, data normality was checked by the Kolmogorov-Smirnov's test, homoscedasticity of variance by the Levene's test and data was log-transformed when needed. GSI, HSI and CI data were arcsine transformed before any statistical analysis. One-way analysis of variance (ANOVA) was used to determine differences between *cyp19a1a* high and low expressors in Ctrl-and E_2-treated group for length, weight, GSI, HSI and CI data sets. *post hoc* multiple comparisons (Tukey's HSD test) were done when statistical differences were present. Data is expressed as mean ± SEM (standard error of the mean) and differences were accepted as significant when $P<0.05$. Sex ratio analysis was done using a Yates corrected Chi-square test (Fowler et al., 2008). Unless otherwise stated, statistical analysis were performed using IBM SPSS Statistics 19.

2.7.2. qRT-PCR statistics

A Student's *t*-test was used to analyze qRT-PCR results by taking 2DCt values (Schmittgen and Livak, 2008). These 2DCt values were previously checked for normality and homoscedasticity of variance. Also a two-step cluster analysis of 2DCt *cyp19a1a* values at 170 dph was used to select ten samples among the high *cyp19a1a* expressors for the hybridizations, as previously described (Blázquez et al., 2009).

2.7.3. Microarray normalization

Briefly, raw data from the Feature Extraction output files was corrected for background noise (normexp method: Ritchie et al., 2007) and quantile normalized (Bolstad, 2001). A probe was considered reliable if its raw foreground intensity was at least two times higher than the respective background intensity and if it was neither saturated nor flagged by the Feature Extraction software. In our custom-made array most probes (64.7%) were represented in two (or in some cases more) identical copies. Then, median intensities of replicated probes were used to obtain just one expression value per probe and were considered reliable if at least half of its replicates were reliable, as defined above.

An empirical Bayes approach on linear models (limma: Smyth, 2004) was used to analyze differential expression, and then corrected for multiple testing (False Discovery Rate method, FDR). Genes were considered differentially expressed when absolute fold change between the compared groups was higher than 1.5, the adjusted *P*-value was lower than 0.05 and were reliable in all samples.

Microarray statistical analysis was performed with the Bioconductor project (http://www.bioconductor.org/) in the R statistical environment (http://cran.rproject.org/) (Gentleman et al., 2004).

2.8. Gene annotation enrichment analysis

Genecards (http://www.genecards.org/) and Uniprot (http://www.uniprot.org/) were used to assess gene names, gene symbols, synonyms and functions. The web based tool AMIGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) (Carbon et al., 2009) was used to obtain sequences of the differential expressed (DE) genes. Then, Blast2GO software (Conesa et al., 2005) was used to enrich DE sequences with GO term annotation as well to provide information on the altered KEGG pathways (http://www.genome.jp/kegg/) associated to these DE genes. Also, using Blast2GO, a reference set containing all the genes from the

custom-made MA was analyzed and used as a background to checkDE genes GO term results by a two-tailed Fisher's Exact Test with Multiple Testing Correction of FDR (Benjamini and Hochkberg, 1995). Moreover, DAVID (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009a-b) was used to further analyze the pathways to which these DE genes belonged; specifically to assess the associated pathways of the nine different clusters generated by the heatmap analysis of the DE genes from the array.

Physical and functional protein interactions of the DE genes were analyzed with a web based tool STRING v9.1 (http://string-db.org/) (Franceschini et al., 2013). Confidence associations were bibliographically confirmed.

3. Results

3.1. Growth, including sexual growth dimorphism

Here we focus on the two main biometric samplings carried out in this study, at 170 dph, where SL and BW was determined and samples for MA hybridizations and qRT-PCR were taken, and at 332 dph, when the final sex ratio of the population was determined and SL and BW were analyzed again. At 170 dph, fish from both groups were separated into two subgroups based on *cyp19a1a* expression levels by qRT-PCR to select the samples to be used for MA analysis. The E_2-group *cyp19a1* levels were always lower than in the Ctrl group. Statistical analysis of these subgroups showed that the E_2-group had significantly lower length ($P<0.01$) and weight ($P<0.001$) when compared to the Ctrl-group, indicating a negative effect of E_2 on growth (Table 1).

Table 1. Growth of European sea bass juveniles at 170 days post hatch according to treatment and *cyp19a1a* expression levels by qRT-PCR

Treatment	Low *cyp19a1a* expressors			High *cyp19a1a* expressors		
	N	Length (cm) ± SEM	Weight (g) ± SEM	N	Length (cm) ± SEM	Weight (g) ± SEM
Ctrl	9	9.86 ± 0.109[a]	17.41 ± 0.877[a]	7	10.28 ± 0.495[a]	19.35 ± 2.955[a]
E_2	12	9.40 ± 0.176[b]	13.75 ± 0.875[b]	8	9.49 ± 0.193[a]	14.65 ± 0.862[b]

Results are shown as mean ± SEM. Different letters indicate statistical differences (P<0.05) between groups.

At 332 dph, sexual growth dimorphism (SGD) was not present in the Ctrl-group since there were no differences in BW between sexes. SGD data for the E_2-group is not available since this group had no males (Table 2). In contrast to the situation observed at 170 dph, at 332 dph Ctrl females despite being slightly bigger and heavier than E_2 ones, showed no differences for neither SL nor BW. Likewise, there were no differences of HSI values between females of the two groups although the E_2 group showed higher values (data not shown). Similarly, no differences in the CI were found between E_2 and Ctrl females, although values were higher in the latter. Male comparisons were ignored since no males were present in the E_2-group.

Table 2. Growth of European sea bass juveniles at 332 days post hatch according to treatment and gender

Treatment	Females			Males		
	N	Length (cm) ± SEM	Weight (g) ± SEM	N	Length (cm) ± SEM	Weight (g) ± SEM
Ctrl	16	12.22 ± 0.171[a]	33.88 ± 3.044[a]	60	12.36 ± 0.171	33.79 ± 1.544
E_2	41	11.82 ± 0.236[a]	29.45 ± 2.160[a]	0	-	-

Results are shown as mean ± SEM. Different letters mark statistical differences (P<0.05) between groups.

3.2. Sex ratio and the gonadosomatic Index

The Ctrl-group had 21% females while the E_2-group had 100% females (Figure 1A). Thus, the sex ratio between groups had significant differences (P<0.001). Regarding the GSI at 332 dph values were significantly higher in Ctrl females (P<0.05) (Figure 1B). Both Ctrl and E_2 females were still immature, with ovaries replete with oocytes at the cortical alveolar stage. Ctrl-group males were fully mature and presented seminiferous tubules filled with sperm (Supplementary figure 2 A-C, respectively).

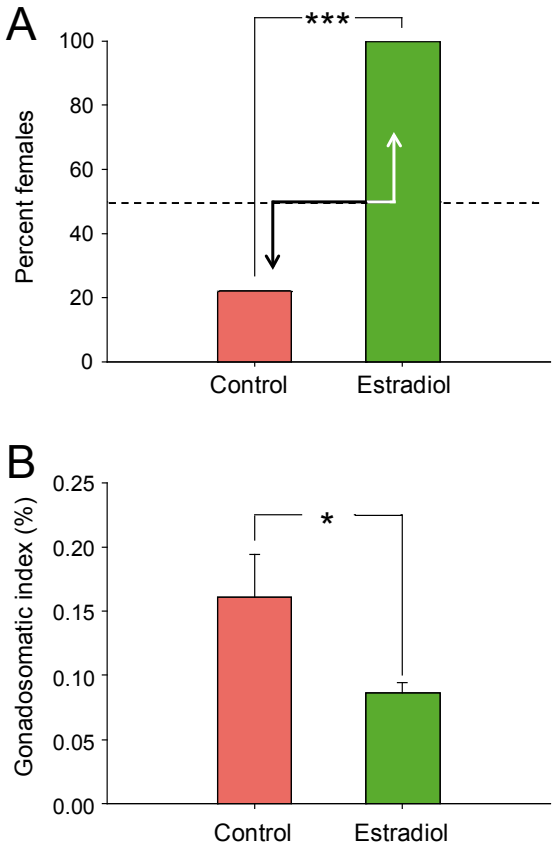


Figure 1. Sex ratio and gonadosomatic index (GSI) of juvenile, one-year-old European sea bass. (A) Female percent per group.Arrows indicate a decrease (black) or an increase (white) with respect to the Fisherian 1:1 balanced sex ratio.(B)Female GSI. Data as mean + SEM. Males were not included since no males were present in the E_2-treated group. * = P<0.05; *** = P<0.001.

3.3. Transcriptomic analysis of gene expression in sexually differentiating gonads

MA results are summarized in Table 3. A total of 383 genes were DE (92 up- and 291 downregulated) when comparing the E_2-group against the Ctrl.

Table 3. Number of differentially expressed genes in 170 dph European sea bass gonads when comparing fishes from the E_2- vs. Ctrl-group during sex differentiation

	# downregulated genes	# upregulated genes	# Total
Total annotated genes	12,947	4,312	17,259
Without repetitions	4,393	1,677	6,070
Significant genes (without repetitions)	291	92	383

A detailed list with all the DE expressed genes is shown in Supplementary Table 3**.** A heatmap representation of the DE genes where each column represents one individual, and where fish clustered in a treatment-related manner is shown in Figure 2. Ctrl1 fish was determined to be and outlier and eliminated of further statistical analysis. DE genes clustered forming nine gene groups and further analysis using DAVID yielded their associated functions (Supplementary Table 4).E_2 administration induced an upregulation of pathways related to DNA replication and repair, hormone-signaling (GnRH, erbB or Hedgehog), reproduction-related (progesterone-mediated oocyte maturation), lipid metabolism or immunological processes. In contrast, pathways related to oocyte meiosis, steroid biosynthesis, sugar metabolism or cytokine receptor interactions were downregulated (Figure 2).

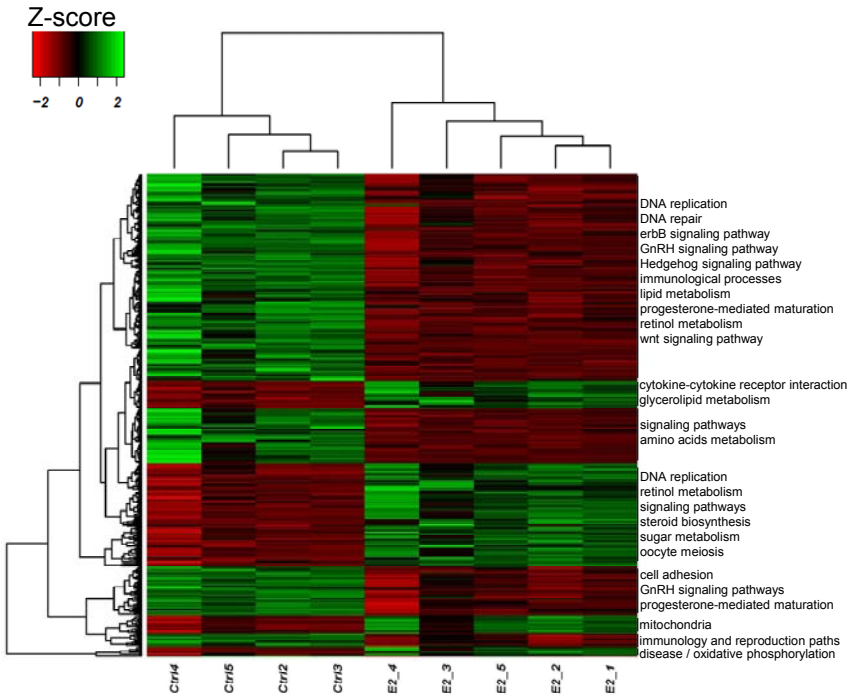


Figure 2. Individual heatmap representation of the DE genes for E_2 vs. Ctrl. High to low expression is shown by a degradation color from green to red, respectively. On the right, the DE genes clustering in pathways by DAVID is shown, although for space reasons only some of the altered pathways are highlighted (for a more detailed list of the altered pathways see Supplementary Table 4).

3.4. Validation of custom-made oligonucleotide microarray by quantitative real time PCR

qRT-PCR was used to validate MA results and to further explore the behavior of genes related to reproduction and growth. qRT-PCR validations results vs. MA results can be seen in Table 4, showing how E_2 treatment caused the downregulation of many of the analyzed genes. qRT-PCR analysis showed how among these selected genes E_2 treatment significantly ($P<0.05$) affected genes related to testis differentiation such as the anti-Müllerian hormone (*amh;* $P<0.05$), doublesex- and mab-3-related transcription factor 1 (*dmrt1;* $P<0.01$) and tescalcin (*tesc;*$P<0.05$) (Figure 3A-C,

respectively); to cholesterol import like the steroidogenic acute regulatory protein (*star; P*<0.01; Figure 3D); two female-related gene like the gonadal aromatase (*cyp19a1a*; Figure 3E) and the Wnt1-inducibe-signaling pathway protein1 (*wisp1*; Figure 3G). Nevertheless, two female-related genes, the transcription factor SOX-17 (*sox17*; Figure 3F) and the vasa protein (*vasa*; Figure 3H) showed no differences in expression among treatments. The neural isoform of aromatase (*cyp19a1b*) was significantly upregulated (*P*<0.05) by E_2 treatment (Figure 3I) while insulin-like growth factor-1 (*igf1*) was not significantly altered (Figure 3J**)**, in this case opposite to what has been found with the MA.

Table 4. Microarray validations by qRT-PCR

	Microarray		qRT-PCR	
Gene symbol	Fold change	Adjusted *P*-value	Fold change	Adjusted *P*-value
amh	-2.48	0.037*	0.11	0.011*
aqp1	2.16	0.349	1.63	0.094
col18a	-4.28	0.035*	0.58	0.174
cyp19a1a	-1.03	0.781	0.09	0.000***
cyp19a1b	-1.37	0.089	1.09	0.028*
dmrt1	-1.54	0.007**	0.19	0.004**
gnrh	1.11	0.129	0.28	0.051
igf1	5.95	0.035*	0.30	0.149
mettl22	-1.31	0.121	0.43	0.113
prl	-1.03	0.806	0.26	0.018*
sox17	1.08	0.779	1.28	0.570
star	-1.87	0.051	0.25	0.006**
tesc	-1.17	0.324	0.27	0.017*
vasa	-	-	0.64	0.721
wisp1	1.02	0.816	0.35	0.045*

Note: Asterisks denote statistical significant differences: * = *P*<0.05; ** = *P*<0.01; ***= *P*<0.001.

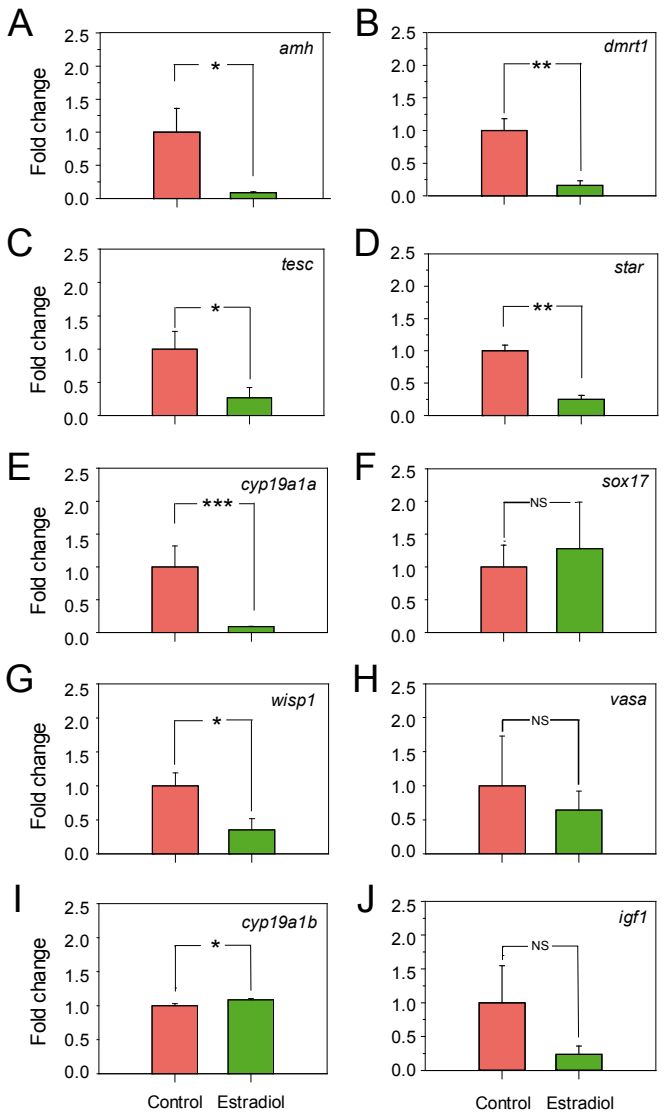


Figure 3. Quantitative RT-PCR validation results for ten genes related to male pathway (A-C): anti-*Müllerian* hormone (*amh*), doublesex- and mab-3-related transcription factor 1 (*dmrt1*) and tescalcin (*tesc*). (D) A gene in charge of cholesterol import at the beginning of the steroidogenic synthesis pathway: steroidogenic acute regulatory protein (*star*). (E-H) genes related to the female pathway: gonadal isoform of aromatase (*cyp19a1a*), SRY-related HMG-box transcription factor SOX17 (*sox17*), Wnt inducible signaling pathway protein 1 (*wisp1*), and vasa protein (*vasa*). (I) the neural isoform of aromatase (*cyp19a1b*), and (J) insulin-like growth factor 1 (*igf1*). Asterisks indicate significant statistical differences between groups (*P<0.05, ** P<0.01, *** P<0.001).

3.5. Gene ontology enrichment analysis of genes regulated by the Estradiol treatment

Blast2GO analysis enabled the identification of the associated GO terms for the up- and downregulated DE genes found at the MA, which are shown in Supplementary Figures 3 and 4, respectively. GO results are in turn subdivided into three categories: biological process (BP), molecular function (MF) and cell component (CC) with always more downregulated GO terms. The main subcategories were related to reproduction ([up]regulated: 13 different GO terms; representing 3.01% of total GO terms;[down]regulated: 31; 1.97%), signaling (up: 23; 5.32%; down: 118; 7.50%), responses to stimulus (up: 41; 9.49%; down: 150; 9.53%), growth (up: 9; 2.08%; down: 15; 0.95%), immune system (up: 10; 2.31%; down: 34; 2.16%) or developmental processes (up: 24; 5.55%; down: 99; 6.29%). While binding (up: 62; 48.82%; down: 234; 47.37%) and catalytic activities (up: 32; 25.20%; down: 122; 24.70%) were the most abundant among the MF subcategories.

In order to analyze the biological processes that were over- or under-represented due to the E_2treatment, a two-tails Fisher's exact test with multiple testing corrections of FDR (*P*-value filter of 0.05) was performed. There was an over-representation of the functions related to the genes that were downregulated at the MA analysis. There were 148 over-represented functions taking our MA as background (Supplementary Table 5). The 23CC GO-term categories were mainly related to cell location of the processes, male germ cell nucleus, transcription factor function, immunoglobulin complexes and high-density lipoprotein particles. The most interesting enriched MF GO-terms (26) were related to nuclear hormone receptor binding, growth factor and NADP-retinol dehydrogenase activity. Enriched GO-terms (97) were clearly related to: reproduction (ovulation cycle, response to E_2 stimulus, retinol metabolic process, spermatid development, sterol metabolic process or regulation of stem cell proliferation and steroid biosynthetic process), immunology (regulation of response to stress or positive regulation of T-cell activation) and growth (positive regulation of transcription, cellular response to epidermal growth or regulation of organ growth).

3.6. KEGG pathway enrichment analysis of genes regulated by the Estradiol treatment

DE genes analyzed with Blast2GO yielded a total of 46 affected pathways (10 up- and 36 downregulated). These pathways (Supplementary Table 6) were mainly related to amino acid biosynthesis (e.g., arginine and praline metabolism), sugar metabolism (e.g., galactose and sucrose metabolism), drug metabolism (e.g., cytochrome P450), immunological signaling pathways (e.g., motor and T-cell receptor), retinol metabolism and steroid hormone biosynthesis. Furthermore, a DAVID analysis on the GO-terms with the highest stringency showed that meiosis (two clusters with 1.47 and 1.06 enriched scores), reproduction (three clusters with 1.17, 0.81 and 0.4 enriched scores) and hormone regulation (one cluster with 0.33 enriched scores) were among the most enriched ones. Since genes involved with reproduction-related pathways were downregulated as a result of E_2 treatment, we further analyzed the MA expressions of the genes related to the ovarian steroidogenic pathway. Almost all the genes of the pathway showed the same tendency towards downregulation, with *star* (a gene in charge of cholesterol import into the mitochondria) and *gnrh* genes actually exhibiting a significant downregulation ($P<0.05$) or a significant increase in the expression ($P<0.05$) like *igf1* due to the E_2treatment (Figure 4).

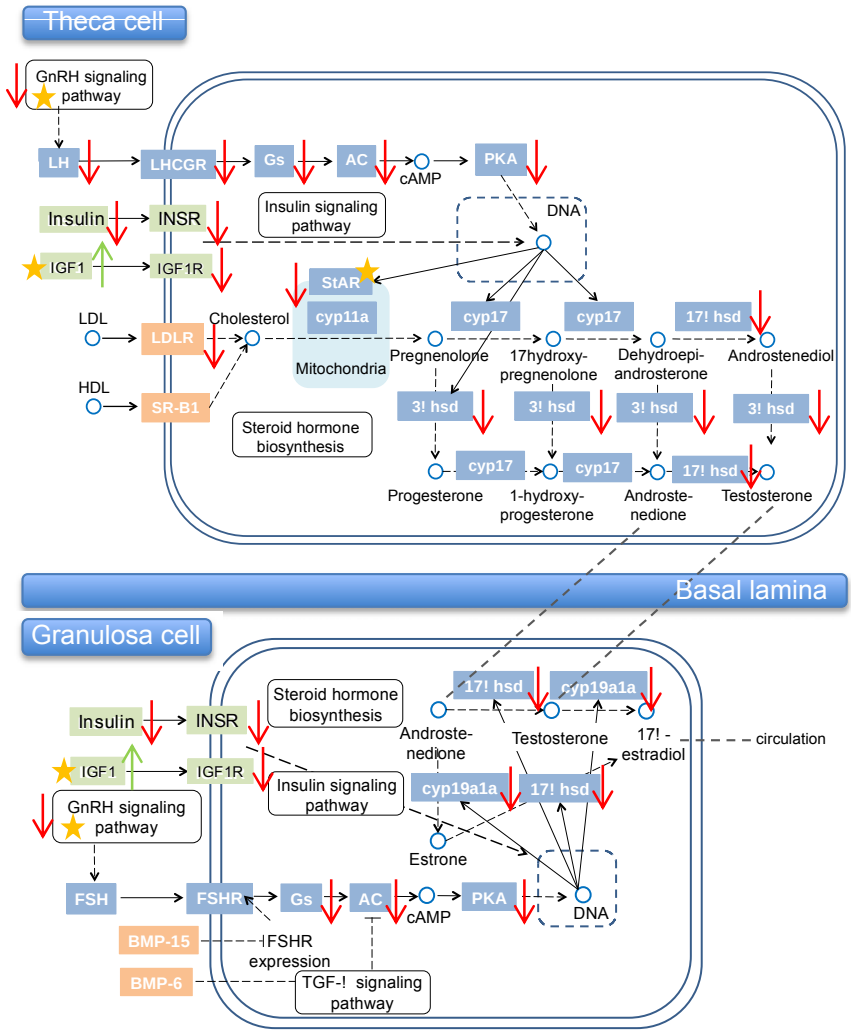


Figure 4. A KEGG pathway-based figure depicting the ovarian steroidogenesis pathway. Microarray results are marked with arrows. Green arrows pointing up indicate a fold change (FC) higher than 1.5, while red arrows pointing down indicate a FC lower than 1.5. Yellow stars denote DE genes in the microarray E_2 vs. Ctrl comparison.

3.7. Protein-protein interaction analysis

Using the STRING web tool to further analyze protein association networks, we found that the proteins coded by the genes DE present in the MA were highly enriched in interactions ($P< 0.001$).

From the list of upregulated genes due to the E_2 treatment six clusters of protein relationships including transcriptional activation (range of combined scores of interactions 0.579–0.935), DNA repair (0.634–0.999), immunity (0.567–0.999), catabolism (0.494–0.601), oxidative phosphorylation (0.546–0.999) and muscle contraction (0.400–0.475) were identified (Supplementary Figure 5). On the other hand, the proteins coded by the downregulated genes yielded seven clusters of protein relationships, including: 1) apoptosis, immunity and inflammation, histone demethylases and inhibition of histone deacetylase 1 (0.456-0.970); 2) cytoskeleton adhesion properties (0.457-0.927); 3) cell adhesion, morphology and motility (0.557-0.960); 4) protein complex assembly and intracellular trafficking and secretion (0.700-0.800); 5) Rho and Rac GTPases activators producing intracellular signaling cascade and signal transduction (0.688-0.968); 6) pathogen recognition, inflammatory process and signal transduction activator of transcription (0.402-0.999); and 7) response to steroid or peptide hormone stimulus and reproductive structure development (0.505-0.971) (Supplementary Figure 6).

3.8. Genes related to epigenetic regulatory mechanisms

In a recent study from our lab (Díaz and Piferrer, submitted), we found that some genes related to the epigenetic regulatory mechanisms were upregulated by heat. Here, we set out to analyze their patterns of expression after treating one group with E_2. If the same criteria applied in our previous study (FC of 1.2 and *P*-value=0.05) is applied here in order to compare the expression patterns of both studies, we observed that all the demethylases, dicer1, helicases, most histone deacetylases, polycomb complex members, as well as DNA-methyltransferases 1 and 3 were downregulated in the E_2-group. In contrast, most histone acetyltransferases and methyltransferases were upregulated. Finally,

histone deacetylase 11 (*hdac11*) and euchromatic histone lysine N-methyltransferase (*ehmt2*), two genes previously analyzed by qRT-PCR and that showed a heat-relatedupregulation were still upregulated even when applying E_2 (Supplementary Table 7).

4. Discussion

In this study we carried out an analysis of the European sea bass gonadal transcriptome by using a custom-made MA applied to two groups of European sea bass submitted both to the masculinizing effects of elevated temperatures and one of them also submitted to a treatment with the natural E_2, a compound known to have feminizing effects on undifferentiated fish (Piferrer, 2001).

The Ctrl-group experienced the expected masculinization due to high temperatures since it had 79% males, a value previously found in farmed sea bass (Koumoundouros et al., 2002; Piferrer et al., 2005; Navarro-Martín et al., 2009b). On the other hand, as previously reported (Saillant et al., 2001a; Gorshkov et al., 2004; Navarro-Martín et al., 2009a), E_2 treatment completely feminized the E_2-group without affecting the histological structure of the immature ovary (cortical alveolar oocytes in both groups) (Sea bass: Navarro-Martín et el., 2009a. Zebrafish: Santos et al., 2007a-b). GSI values showed that E_2 treatment reduced ovarian growth, as previously found in rainbow trout after EE_2 treatment (Filby et al., 2007), and in contrast with previous studies in the sea bass (Navarro-Martín et al., 2009a). Nevertheless, the Ctrl-group GSI values are in agreement with what has been previously described for natural sea bass females, where most of the females of one year of age have higher energy investment in gametogenesis (Saillant et al., 2001a).

E_2 treatment also affected body growth since Ctrl fish right after the hormonal treatment were bigger and heavier that E_2 treated fish. But at one year these differences were no longer present, in agreement with what has been previously described for feminized sea bass (Saillant et al., 2001a; Navarro-Martín et al., 2009a; Leal et al., 2013). Inhibitory effects on ovarian growth have been observed after estrogen treatments (Brion et al., 2004; Pawlowski et al., 2004;

Filby et al., 2007) Also, higher GSI values in sea bass are found in animals with a lower fat content and a higher HSI (Saillant et al., 2001a). In our experiment, in contrast, GSI levels for E_2-group females were higher than in the Ctrl group without any effect on fat content. Despite not being significantly different, HSI levels were slightly higher in E_2-group females, in agreement with some studies on fish exposure to xenoestrogens (Schultz et al., 2003; Parrott and Blunt, 2005; Osachoff et al., 2013).

MA analysis showed that E_2 treatment caused an alteration in the expression of 383 genes (92 up- and 291 downregulated) mainly affecting reproduction, immunity, signaling, responses to stimulus and growth. Some of those effects are discussed below.

Reproduction

cyp19a1a expression and its aromatase enzyme activity are necessary for ovarian differentiation and maintenance in fishes (Piferrer and Guiguen, 2008; Guiguen et al., 2010). In agreement with our findings, *cyp19a1a* decreased after E_2 administration in rainbow trout and Nile tilapia (Guiguen et al., 2010). A qRT-PCR analysis of *cyp19a1a* expression at 170 dph, right after the hormonal treatment finished, showed that levels in the E_2-group were ten times lower than those of controls, also in agreement with MA results. That would mean that exogenous estradiol signal is enough to induce female pathway in genetic males and able to feminize a population that suffered from heat-induced masculinization.

Exogenous E_2 downregulated *cyp19a1a* expression, explaining the low levels of aromatase found in feminized fishes, as also has been found in rainbow trout (Vizziano-Cantonnet et al., 2008; Urbatzka et al., 2012). Since *cyp19a1a* expression was not affected at the end of the E_2 treatment period in previous sea bass studies (Navarro-Martín et al., 2009a), this would suggest that E_2-induced feminization may not directly involve *cyp19a1a*. Furthermore, in many fishes including the sea bass the *cyp19a1a* promoter does not contain estrogen response elements (EREs), so E_2 may exert its effects by regulating other genes involved in ovarian differentiation

(Piferrer and Blázquez, 2005). This fact is reinforced at a transcriptomic level by the downregulation of the whole ovarian steroidogenesis pathway. Moreover, *star* was significantly downregulated by E_2, in agreement with previous results in zebrafish (Urbatzka et al., 2012), suggesting that E_2 shutdowns the first steps of steroidogenesis by blocking *star* expression. This blockage is believed to be dose- and species-dependent, since the E_2 treatment decreases its expression in zebrafish (Urbatzka et al., 2012), European sea bass (present study) and fathead minnow (Filby et al., 2006 and 2007). In contrast, increases its expression in rainbow trout (Nakamura et al., 2009). This steroidogenesis shutdown by *star* blocking is also present in zebrafish males treated with E_2 (Urbatzka et al., 2012), but it must also exist another mechanisms of E_2 action since further downstream genes such as *cyp19a1a* and *17β-hsd* are also affected in females but not in males (Zebrafish: Sawyer et al., 2006; Urbatzka et al., 2012. Rainbow trout: Nakamura et al., 2009. Sea bass: females from the present study). Downstream shutdown of *cyp19a1a* expression does not involve changes in its promoter DNA methylation since the comparison between natural females and feminized females by E_2 showed no differences in the gonadal aromatase promoter methylation levels (Navarro-Martín et al., 2011a). Other genes related to the female pathway such as *wisp1* or *vasa;* and *amh*, *dmrt1* and *tesc,* male-related genes, were downregulated after E_2 treatment, in contrast to what has been found in fathead minnow for these genes and for the above mentioned *cyp19a1a*, *17β-hsd* and *star* (Filby et al., 2007). Moreover, genes like *cyp19a1b* and *sox17* showed an upregulation due to E_2 in accordance to what happens in fathead minnow.

Immunity

The innate immune system in fish is the first defense against pathogens and exogenous materials and is thought to be of main importance in primary defense (Sun et al., 2011a). Also, recent studies have pointed out the possibility that sex steroids that modulate the reproductive tissues may affect the immune system (Cuesta et al., 2007). Our MA was enriched with immunity-related terms such as defense response to Gram-negative bacterium,

leukocyte cell-cell adhesion, positive regulation of cytokine biosynthetic process, immunoglobulin complex, and also in a group of signaling pathways responsible of generating the immune response: Toll-like, NOD-like, RIG-I-like or T-cell receptor signaling pathway. The latter being significantly downregulated in our array. Also, the corresponding DE proteins were enriched inprotein-protein interactions (immunity, inflammation and pathogen recognition). Our results showed a downregulation of most of the genes involved in immunity, just like previous studies on Japanese medaka (*Oryzias latipes*), where genes of the complement component, some cytokines and lysozymes were downregulated after E_2 treatment (Sun et al., 2011a-2011b). Also, several terms referring to response to stimulus were enriched incuding: response to estradiol, to mechanical stimulus, to lipopolysaccharide or to regulation of response to stress.

Xenobiotic metabolism

From the DE genes, three pathways related to drug metabolism and drug metabolism or xenobiotic metabolism by cytochrome P450 were downregulated. Furthermore, analyzing MA results for Glutathione S-transferases (GSTs), a family of proteins able to metabolize xenobiotics (Blanchette et al., 2007), two GSTs out of five were upregulated (*gst* and *gstθ*). On the contrary, and against expected, *gstα*, *gstk*, *gstm* were downregulated as also happened for *gstα* in goldfish exposed to a hepatotoxin (Li et al., 2008) or to *gstπ* in Atlantic salmon exposed to tributyltin (Mortensen and Arukwe, 2007).

Growth

Studies in the sea bream (*Sparus aurata*) suggest that sex steroids may influence fish growth by altering the GH-IGF system (Carnevali et al., 2005). Also an increase in the steroid plasma levels during sexual maturation correlate to GH plasma levels, demonstrating a crosstalk between reproduction and growth-related pathways (Rainbow trout: Norbeck and Sheridan, 2011. Salmon: Bjornsson et al., 1994. Goldfish: Trudeau et al., 1992). In our array,

and in contrast to what has been described for the fathead minnow (Filby et al., 2007), all the genes related to growth hormone and its receptors were downregulated. The same ocurred with the insulin-like growth factor II gene, its receptors, *igf1* receptor and its associated binding proteins. Our MA was enriched in growth related terms such as positive regulation of transcription, cellular response to epidermal growth and regulation of organ growth.

Lipids

Similar to what has been observed for the mummichog (Doyle et al., 2013), terms related to lipid metabolism were over represented. Some of the terms referred to white fat cell differentiation, regulation of fat cell differentiation, apolipoprotein binding, plasma lipoprotein particle clearance or very low density and high density lipoprotein particle remodeling. Moreover, DE genes were associated with pathways with an enhanced expression due to E_2 treatment such as either lipid metabolism or fatty acid biosynthesis. In this regard, downregulation was observed for apolipoproteins, the proteins responsible of the lipid transport, including *apoh*, *apom*, (these genes in the mummichog showed no significant changes on expression due to E_2 treatment), *apoa1* (also had a decreased expression in other species; Zebrafish: Hoffman et al., 2008. Carp: Moens et al., 2006. Rainbow trout: Skillman, 2006), and *apoe,* a protein related with lipid uptake by oocytes (Zebrafish: De Witt et al., 2010). In contrast, the apolipoproteins *apoa4*, *apob*, *apoc2*, *apoh* and *apoa1* precursor were upregulated.

Epigenetic regulatory mechanisms-related genes

We analyzed the MA results for the genes related to epigenetic mechanisms that has been previously checked in sea bass gonads (Díaz and Piferrer, submitted) and that showed an upregulation due to high temperatures. Five of the genes previously tested by qRT-PCR (*dicer1*, *jarid2a*, *pcgf2*, *suz12*, *mettl22*) were affected by E_2 since they showed a downregulation of its expression, overriding temperature effects. In return, *ehmt2* and *hdac11* were E_2-insensitive. Also, applying the same filtering criteria (FC of 1.2 and

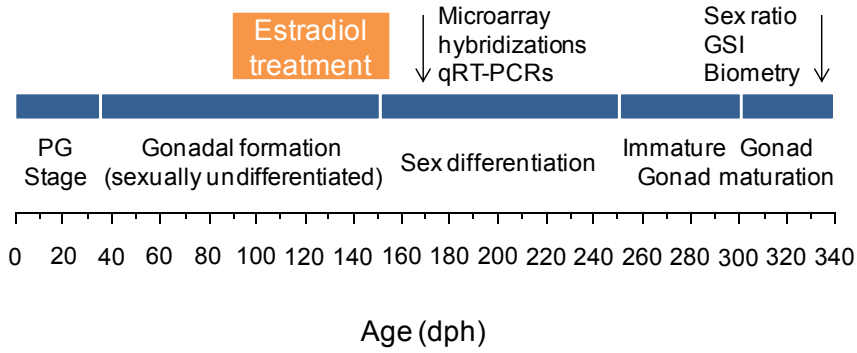
P-value=0.05) as in aprevious MA thermal study on sea bass gonads, we observed that six heat shock proteins (*hrsp12*, *hsbp1*, *hsp10*, *hsp60*, *hspa14* and *hsp70*) were upregulated due to the constant heat treatment, implying that early exposure to elevated temperatures had persistent effects on the gonadal transcriptome, effects that were not override by subsequent E_2 treatment.

Taken together, the results of this study show that, at the population level, exposure to estrogen during early juvenile development is able to completely negate the masculinizing effect of exposure to elevated temperature during larval development, since eventually all fish developed as females. However, these fish did so despite the observation that at the time of sex differentiation some genes involved in steroidogenesis were downregulated, including not only those at the early stages, such as *star*, but also downstream genes such as *cyp19a1a* and *17β-hsd*. Furthermore, pathways related to reproduction, immunity, xenobiotic and lipid metabolism, signaling, responses to stimulus and growth were also affected. Thus, exposure to exogenous estrogens can have profound reprograming effects on the gonadal transcriptome of sexually differentiating fish, resulting in complete feminization of the population and disturbing normal steroidogenesis in the developing females. However, at one year of age feminized fish exhibited a normal gonadal histology, suggesting that once the female phenotype is imposed gonads can apparently continue their normal development. The role of E_2 in maintaining this situation needs further study.

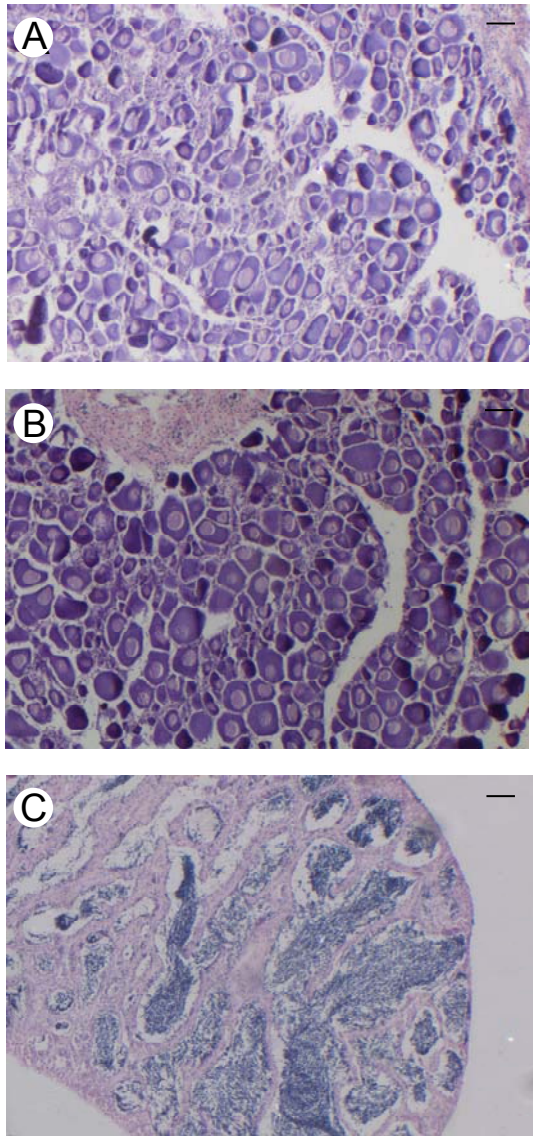
Acknowledgements

N.D. was supported first by a scholarship from the Government of Spain (BES-2007-14273) and then by a contract from the Epigen-Aqua Project (AGL2010-15939). Research was supported by Spanish Ministry Economy and Competitiveness grants SexGene (AGL2006-01359) and Consolider Aquagenomics (CDS2007-002) to F.P. Thanks are due to M. Blázquez, L. Navarro, L. Ribas and S. Joly for assistance with different aspects of this work. Thanks are also due to the fish maintenance staff at our Experimental Aquarium and Chamber facility (ZAE).

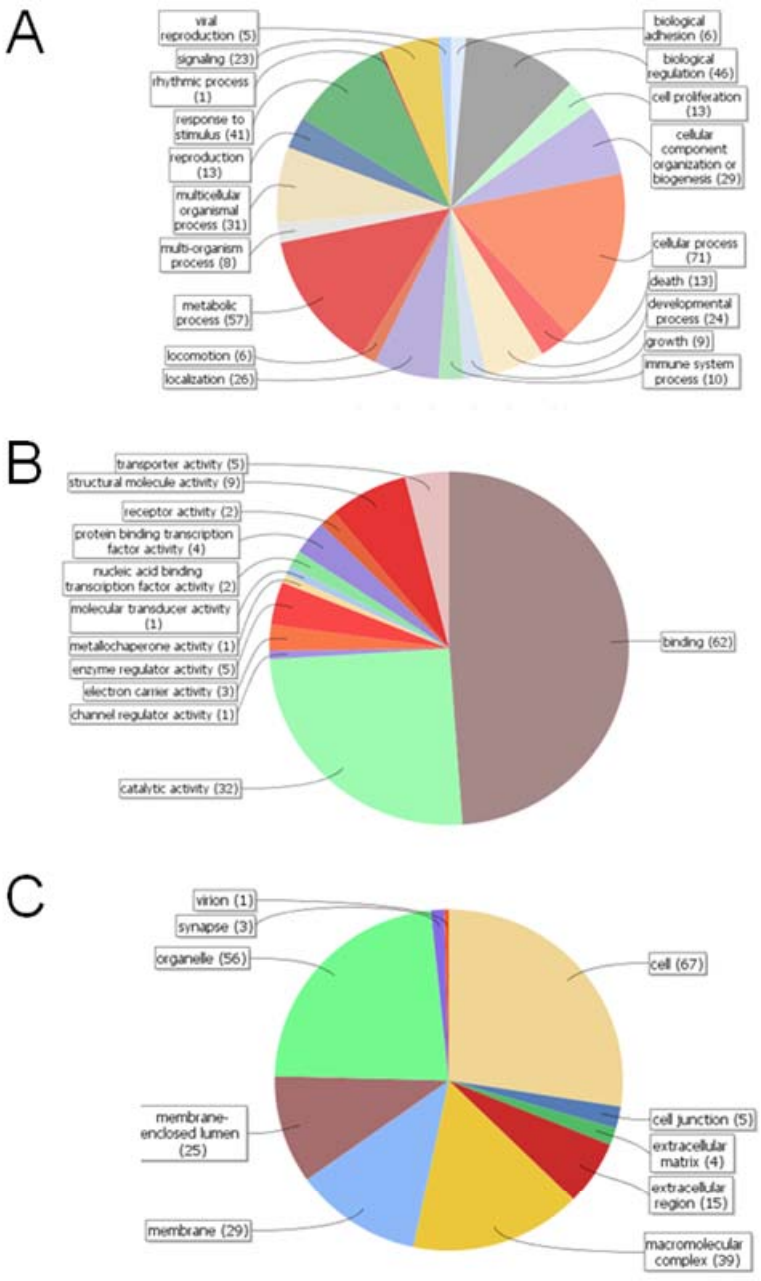
Supplementary figures



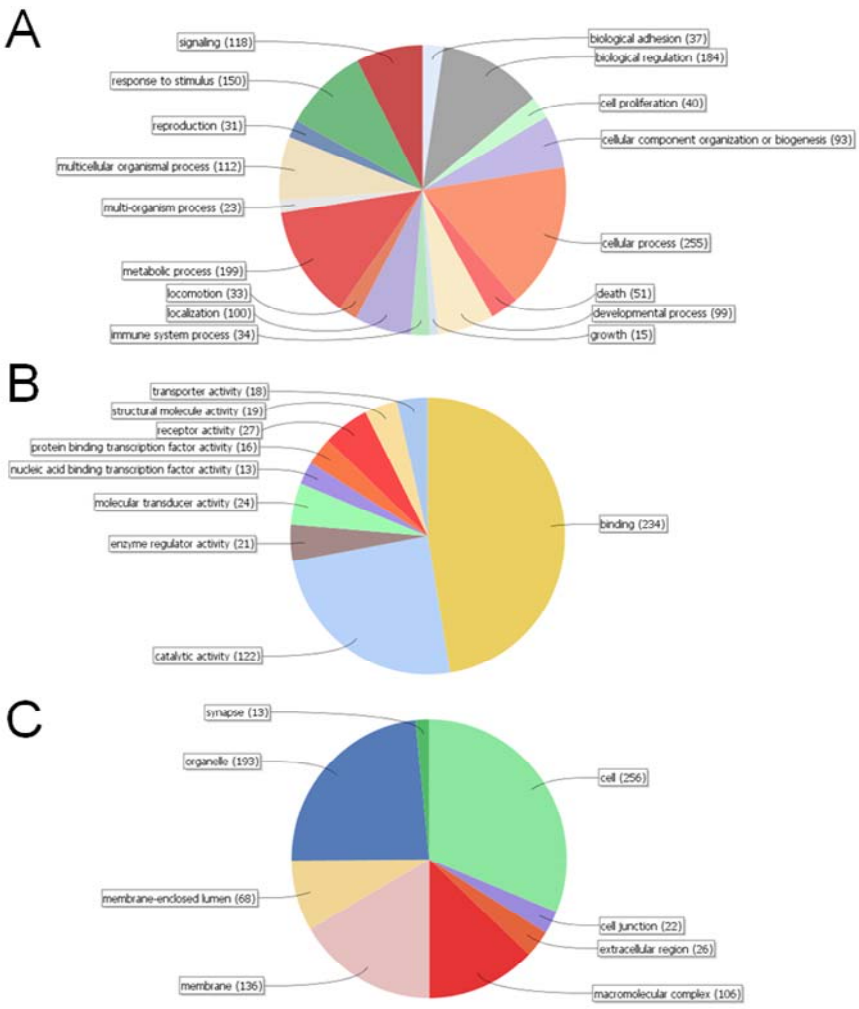
Supplementary Figure 1. Experimental design. All fish were reared at 21ºC from 20 to 220 dph, covering the gonadal formation and part of the sex differentiation process. Then, fishes were switched to natural temperature to allow gonadal maturation. Control fish (Ctrl) were fed with an Ethanol-sprayed dry food and treated fish with an Estradiol-sprayed (E_2) dry food. Treatment took place from 90 to 154 dph (marked with a light orange box). Vertical arrows indicate sampling times, either for molecular biology analyses at 170 dph or biometries, GSI and sex ratios at 332 dph.



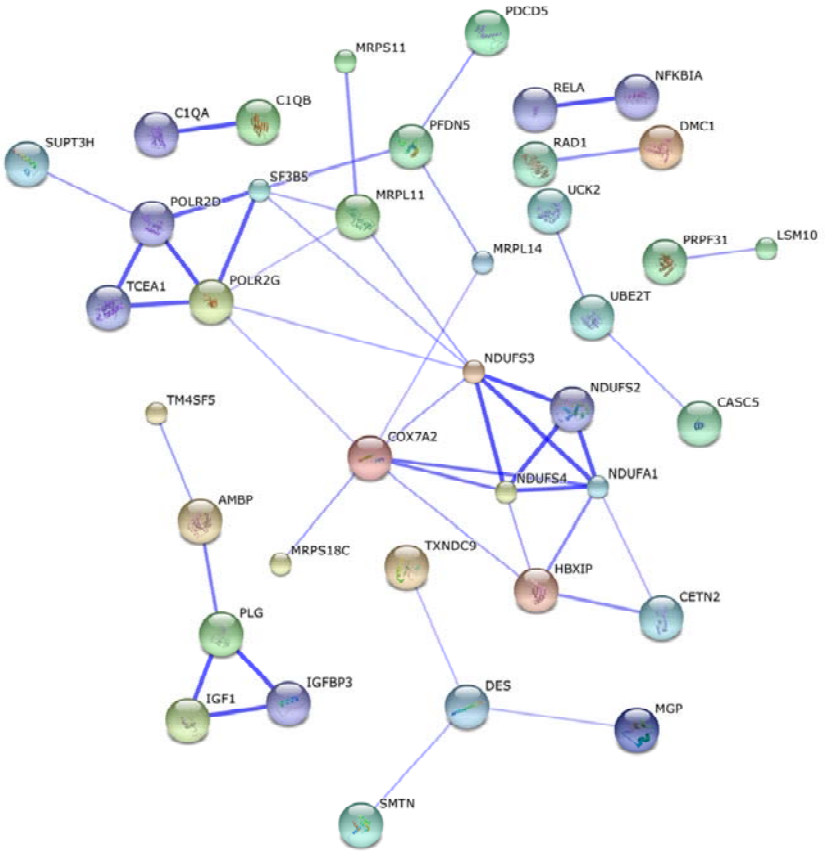
Supplementary Figure 2. Histological images of one-year-old European sea bass. (A) Control females, (B) E_2-treated females and (C) Control males. Scale bar = 50 μm.



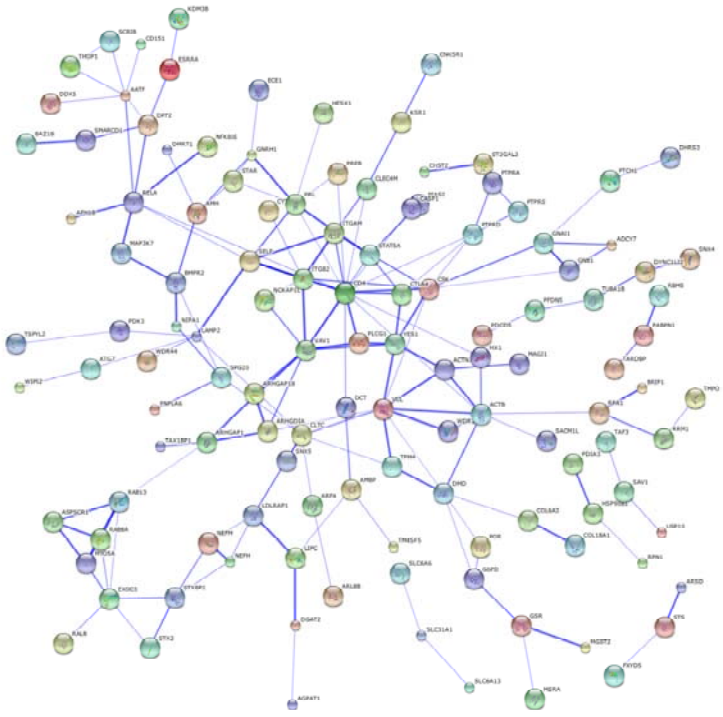
Supplementary Figure 3. GO terms results from the upregulated DE genes classified in the three main categories: A, biological process (BP); B,molecular function (MF); and C, cell component (CC).



Supplementary Figure 4. GO terms results from the downregulated DE genes classified in the three main categories: A, biological process (BP); B, molecular function (MF); and C, cell component (CC).



Supplementary Figure 5. Predicted protein-protein interactions by STRING for the 92 upregulated genes in the E_2 vs. Ctrl comparison.



Supplementary Figure 6. Predicted protein-protein interactions by STRING for the 291 downregulated genes in the E_2 vs. Ctrl comparison.

Supplementary tables

Supplementary Table 1. Gene abbreviation glossary of the genes analyzed either by qRT-PCR or selected from the microarray DE gene list

Gene abbreviation	Gene name
Amh	Anti-Müllerian hormone
aqp1	Aquaporin 1
col18a1	Collagen alpha-1 (XVIII) chain
cyp19a1a	Cytochrome P450, family 19, subfamily A, polypeptide 1a
cyp19a1b	Cytochrome P450, family 19, subfamily A, polypeptide 1b
dicer1	Endoribonuclease Dicer
dmrt1	Doublesex- and mab-3- related transcription factor I
ehmt2	Euchromatic histone-lysine N-methyltransferase 2
Gnrh	Gonadotropin-releasing hormone
hdac11	Histone deacetylase 11
igf1	Insulin-like growth factor I
jarid2a	Protein Jumonji
mettl22	Methyltransferase-like protein 22
pcgf2	Polycomb group ring finger 2
Prl	Prolactin
r18S	r18S
sox17	HMG-box transcription factor SOX17
Star	Steroidogenic acute regulatory protein
suz12	Suppressor of zeste 12 homolog
Tesc	Tescalcin
Vasa	Vasa protein
wisp1	WNT1 inducible signalingpathway protein 1

Supplementary Table 2. Quantitative RT-PCR primer characteristics

Genes	Gene abbreviation	Primer name	Primer sequence (5'→3')	Efficiency (*E*)	R^2
Anti-*Müllerian* hormone	*amh*	amh-Fw	TGCAGAGCAAAGCCTGAAAG	2.10	0.99
		amh-Rv	TCAACGGGGAACAAAGACAA		
Aquaporin 1	*aqp1*	aqua-Fw	GCCAGATCAGCGTGTTCAAG	2.27	0.98
		aqua-Rv	ACAGCACCAGCTGGAAGGTT		
Collagen alpha-1 (XVIII) chain	*col18a1*	col-Fw	AACTGCGACTCGGATCCTCA	2.01	0.98
		col-Rv	TATCCGGGTCTGCTCCACTG		
Cytochrome P450, family 19, subfamily A, polypeptide 1a	*cyp19a1a*	cyp19a1a-Fw	AGACAGCAGCCCAGGAGTTG	1.99	0.99
		cyp19a1a-Rv	TGCAGTGAAGTTGATGTCCAGTT		
Cytochrome P450, family 19, subfamily A, polypeptide 1b	*cyp19a1b*	cyp19a1b-Fw	CCCTTTTCAGCGCAGTGGTA	2.01	0.94
		cyp19a1b -Rv	CATTCGGCTTGTGGTGCTC		
Doublesex- and mab-3- related transcription factor I	*dmrt1*	dmrt1-Fw	CCTTCACGCTACCCCACCTA	2.20	0.97
		dmrt1-Rv	GTTGTTGTCGTCCAGGCTGA		
Gonadotropin-releasing hormone	*gnrh*	gnRH-Fw	ACGCCCTGCAGAGTTTTAGG	2.04	0.99

		gnRH-Rv	AGAAGCACGAGGTCCTGACA		
HMG-box transcription factor SOX17	*sox17*	sox17-Fw	CAAGAGACTGGCGCAGCAA	2.25	0.98
		sox17-Rv	TTTCCACGATTTCCCCAACAT		
Insulin-like growth factor I	*igf1*	igf1-Fw	TCCGTTTGTCACTTGTGTGAACT	2.16	0.95
		igf1-Rv	AGGCAATCAAGCACCATGAA		
Methyltransferase-like protein 22	*mettl22*	metll22-Fw	CCAGGAAGTGGCTGAAGCTC	2.18	0.97
		metll22-Rv	CTGCCTTGTGCTTCCTCTCC		
Prolactin	*prl*	prl-Fw	TATCCTGACCAGCGGATGTG	2.10	0.96
		prl-Rv	ACGCTGCCACCATGTACAAC		
r18S	*r18S*	r18S-Fw	CCGCTTTGGTGACTCTAGATAACC	2.09	0.99
		r18S-Rv	CAGAAAGTACCATCGAAAGTTGA TAGG		
Steroidogenic acute regulatory protein	*star*	star-Fw	AGCAGAGGGGTGTTGTCAGA	2.10	0.99
		star-Rv	TGGTTGGCAAAGTCCACCTG		
Tescalcin	*tesc*	tesc-Fw	CAACATGGAGACCATCGCCC	2.10	0.96
		tesc-Rv	TGAACATCCGTCCTCGGTCA		
Vasa protein	*vasa*	vasa-Fw	CAGAAGCATGGCATTCCAATC	2.24	0.99
		vasa-Rv	TGCAGAATAGGGAGCAGGAAA		
WNT1 inducible signaling pathway protein 1	*wisp1*	wnt1-Fw	CATGCGAGTGTCCGAAGTCC	2.20	0.97
		wnt1-Rv	CGCACATCTTGCAGCAATCG		

Supplementary Table 3. Differentially expressed gene list

Description	Gene symbol	Fold change	adjusted *P*-value
Extracellular matrix protein 1	*ecm1*	20.964	1.14E-02
Desm protein	*desma*	11.015	3.46E-02
Tetraspanin 13	*tspan13*	7.804	4.70E-02
Insulin-like growth factor I	*igf1*	5.476	3.62E-02
Desmin	*des*	5.373	3.55E-02
chemokine (C-C motif) ligand 13	*ccl13*	4.809	3.64E-02
RecA homolog Dmc1	*dmc1*	4.351	4.13E-02
C-myc binding protein	*mycbp*	3.996	4.00E-02
Hypoxanthine phosphoribosyltransferase like	*hprt1l*	3.966	3.85E-02
Male-specific protein	*msp*	3.873	3.46E-02
Adrenomedullin-1	*adm1*	3.859	3.47E-02
Matrix Gla protein	*mgp*	3.751	3.47E-02
DNA-damage-inducible transcript 4	*ddit4*	3.668	4.83E-02
CDC28 protein kinase regulatory subunit 2	*cks2-cdc28*	3.638	4.27E-02
Virus-induced protein 5	*ebi5*	3.510	4.31E-02
Signal recognition particle 9kDa	*srp9*	3.341	4.67E-02
AGAP011243-PA	*agap_agap011243*	3.202	4.44E-02
Chain A, Structure Of A Human Prp31-15.5k-U4 Snrna	*prp31*	3.110	4.67E-02
Pleiotrophin	*ptn*	3.037	3.53E-02
Smoothelin	*smtn*	2.968	3.95E-02
SMT3 suppressor of mif two 3 homolog 2	*sumo2-smt3*	2.959	4.55E-02
Frataxin	*fxn*	2.915	4.62E-02
Biotinidase precursor	*btd*	2.845	4.76E-02
p65 transcription factor	*rela*	2.828	4.83E-02
Claudin 3a	*cldn3*	2.762	3.62E-02
Phosphatidylethanolamine-binding protein 4	*pebp4*	2.759	3.64E-02
Si:busm1-211o13.10	*si:busm1-211o13.10*	2.745	4.63E-02
Zinc finger, MYND domain containing 17	*zmynd17*	2.667	3.55E-02
Suppressor of Ty 3 homolog	*supt3h*	2.581	4.31E-02
Cenpk protein	*casc5*	2.507	4.78E-02
Plasminogen	*plg*	2.491	4.31E-02
Chain A, Ubiquitin-Conjugating Enzyme Hspc150	*ube2t*	2.342	4.83E-02
Mediator complex subunit 27	*med27*	2.196	4.31E-02

Allantoicase	*allc*	2.163	4.60E-02
ATP binding protein associated with cell differentiation	*txndc9*	2.152	3.74E-02
Cysteine conjugate-beta lyase	*ccbl1*	2.112	4.37E-02
Cytochrome c oxidase subunit VIIa polypeptide 2	*cox7a2*	2.112	4.87E-02
PHD finger protein	*phf*	2.099	4.52E-02
Polymerase (RNA) II (DNA directed) polypeptide G	*polr2g*	2.075	4.84E-02
Mrps11 protein	*mrps11*	2.060	3.74E-02
Proteasome (prosome, macropain) 26S subunit, ATPase, 1a	*psmc1a*	1.985	3.86E-02
Hepatitis B virus x interacting protein	*hbxip*	1.975	3.64E-02
Mitochondrial ribosomal protein L11	*mrpl11*	1.970	4.00E-02
Mitochondrial ribosomal protein L14	*mrpl14*	1.950	4.76E-02
Programmed cell death 6	*pdcd6ip*	1.880	3.60E-02
Probetacellulin precursor	*btc*	1.872	4.40E-02
NADH dehydrogenase (ubiquinone) Fe-S protein 4	*ndufs4*	1.858	4.83E-02
NADH dehydrogenase ubiquinone Fe-S protein 3	*ndufs3*	1.853	4.55E-02
Centrin2	*cetn2*	1.853	4.29E-02
MORN repeat containing 3	*morn3*	1.848	4.63E-02
Nucleolin	*ncl*	1.830	4.01E-02
Small nuclear ribonucleoprotein D2-like protein	*ot02g07600*	1.806	3.86E-02
High-mobility group protein 2-like 1	*hmgxb4*	1.799	4.82E-02
C-14 sterol reductase	*erg3*	1.780	4.03E-02
Testis antigen 76	*syce1*	1.774	3.46E-02
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	*ndufa1*	1.768	3.46E-02
Glycine-gated ion channel alpha3 subunit	*glra3*	1.755	3.46E-02
Uridine-cytidine kinase 2	*uck2*	1.753	4.00E-02
Ubiquitin carboxyl-terminal esterase L3	*uchl3*	1.750	4.25E-02
Lecithin cholesterol acyltransferase	*lcat*	1.734	4.25E-02
BolA-like 1	*bola1*	1.730	4.09E-02
Prefoldin subunit 5	*pfdn5*	1.727	4.34E-02
Splicing factor subunit 5	*sf3b5*	1.723	4.89E-02
Fermitin family homolog 1	*fermt1*	1.706	4.32E-02
Alpha 1 microglobulin/bikunin	*ambp*	1.677	3.46E-02
DTW domain containing 1	*dtwd1*	1.676	4.93E-02
Glutathione synthetase	*gss*	1.671	4.37E-02
C-type lectin domain family 4, member E	*clec4e*	1.671	4.87E-02
RAB, member RAS oncogene family-like 5	*rabl5*	1.659	4.14E-02
Fumarylacetoacetate hydrolase	*igf 1*	1.658	4.34E-02

Interferon-inducible protein Gig1	*gig1*	1.655	4.07E-02
Squint	*sqt*	1.648	3.46E-02
C1q-like adipose specific protein	*c1q*	1.644	3.88E-02
Transcription elongation factor A (SII) 1	*tcea1*	1.636	4.00E-02
U7 small nuclear RNA associated	*lsm10*	1.601	4.00E-02
Adf-1 protein	*adf1*	1.596	3.73E-02
Ribokinase	*rbsk*	1.584	3.46E-02
dehydrogenase/reductase (SDR family) member 3	*dd83.1*	1.577	3.53E-02
Sorting nexin-3	*snx3*	1.574	4.35E-02
Rad1 protein	*rad1*	1.574	4.55E-02
Histidine triad nucleotide binding protein 3	*hint3*	1.564	3.64E-02
NIPA1 protein	*nipa1*	1.563	4.42E-02
Mitochondrial ribosomal protein S18C	*mrps18c*	1.551	3.62E-02
Nucleosome assembly protein 1-like 4	*nap1l4*	1.549	4.69E-02
Deoxyhypusine synthase	*dhps*	1.545	3.73E-02
Heat shock factor binding protein 1	*hsfbp1*	1.542	3.86E-02
Programmed cell death 5	*pdcd5*	1.540	3.46E-02
ATP synthase, H+ transporting	*atp5s*	1.538	4.81E-02
Ap1s1 protein	*ap1s1*	1.534	4.07E-02
ADP-ribosylation factor-like 1	*arl1*	1.522	4.31E-02
CDGSH iron sulfur domain 2	*cisd2*	1.509	3.46E-02
Transmembrane 4 L six family member 5	*tm4sf5*	1.506	3.64E-02
Nonstructural protein P125	*nlrc*	1.487	4.78E-02
Ribokinase	*rbsk*	1.486	4.52E-02
C-14 sterol reductase	*tm7sf2*	1.486	4.80E-02
Sperm associated antigen 8	*spag8*	1.485	3.46E-02
Ankyrin repeat and SOCS box-containing 12	*asb12*	1.473	4.55E-02
Dickkopf 1	*dkk1*	1.472	4.72E-02
Transmembrane 6 superfamily member 1	*tm6sf1*	1.468	4.09E-02
Microsomal glutathione S-transferase 2	*mgst2*	1.464	3.53E-02
Programmed cell death 5	*pdcd5*	1.463	3.64E-02
Mitochondrial ribosomal protein S18C	*mrps18c*	1.462	4.56E-02
Solute carrier family 31	*slc31a1*	1.459	4.11E-02
ADP-ribosylation factor-like 1	*arl1*	1.458	4.06E-02
Magnesium transporter NIPA1	*nipa1*	1.455	4.42E-02
Heat shock factor binding protein 1	*hsbp1*	1.455	4.03E-02
C21orf59-like	*alp56*	1.452	4.49E-02
Alpha 1 microglobulin/bikunin	*ambp*	1.449	3.64E-02
Calcium and integrin binding family member 2	*cib2*	1.444	3.46E-02
Radixin isoform 1	*slc93r1*	1.443	4.55E-02
FRG1 protein	*frg1*	1.442	4.51E-02
Nuclear factor, interleukin 3	*p125*	1.438	4.16E-02

Dehydrogenase/reductase (SDR family) member 3	*dhrs3*	1.431	4.32E-02
Prefoldin subunit 5	*pfdn5*	1.423	3.62E-02
Transmembrane 4 L six family member 5	*tm4sf5*	1.397	4.01E-02
D3Mm3e protein	*wdr54*	1.394	4.86E-02
SET and MYND domain containing 3	*smyd3*	1.392	3.64E-02
Signal peptide, CUB and EGF-like domain-containing protein 2	*scube2*	1.391	4.99E-02
A kinase (PRKA) anchor protein 14 isoform a	*akap14*	1.390	3.46E-02
Transmembrane protein 42	*tmem42*	1.373	4.51E-02
Cytochrome c-a	*cyc-a*	1.356	3.77E-02
PEST proteolytic signal containing nuclear protein	*pcnp*	1.354	4.34E-02
Trafficking protein particle complex 6b-like	*trappc6a*	1.338	3.46E-02
AGAP011847-PA	*agap011847*	1.335	4.34E-02
MYND-type containing 12	*zmynd12*	1.334	4.67E-02
MYO5A variant protein	*myo5a*	1.300	4.78E-02
Regulator of telomere elongation helicase 1	*rtel1*	1.294	3.46E-02
Spastic paraplegia 20, spartin	*spg20*	1.282	3.46E-02
Estrogen receptor-related receptor alpha	*err-alpha*	1.243	4.12E-02
Hepatic lipase	*lipc*	1.186	4.76E-02
Receptor-interacting factor 1 isoform 1	*loc539067*	-1.167	4.06E-02
Thimet oligopeptidase	*thop1*	-1.190	4.95E-02
Mitochondrial ribosomal protein L1	*mrpl1*	-1.201	3.85E-02
TGF-beta-activated kinase TAK1	*map3k7*	-1.204	3.95E-02
Kinase suppressor of ras 1	*ksr1*	-1.211	3.78E-02
Histidine acid phosphatase domain containing 2A	*ppip5k1*	-1.212	4.73E-02
Caspase-1	*casp1*	-1.227	4.09E-02
ATP-binding cassette, sub-family B	*abcb8*	-1.241	3.46E-02
Dopachrome tautomerase (Dct) gene	*dct*	-1.247	4.87E-02
Pyruvate dehydrogenase kinase, isoenzyme 3	*pdk3*	-1.250	4.83E-02
Ribosomal protein S8	*prs8-40s*	-1.275	4.55E-02
DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	*ddx41*	-1.293	4.08E-02
Neurofilament medium polypeptide	*nefm*	-1.300	4.01E-02
Autophagy related protein 7-like protein	*atg7*	-1.307	4.83E-02
1-acylglycerol-3-phosphate O-acyltransferase 5	*agpat1*	-1.308	3.77E-02
GTP-binding protein	*gtpbp*	-1.311	4.31E-02
Histone demethylase JARID1B (Jumonji/ARID)	*kdm5b*	-1.311	4.52E-02
Retinoblastoma protein-binding zinc finger protein	*prdm2*	-1.325	4.36E-02

Dihydrolipoamide S-succinyltransferase	*dlst*	-1.328	4.38E-02
Interferon-induced GTP-binding protein Mx	*mx1*	-1.330	4.37E-02
Brevican isoform 1	*bcan*	-1.332	4.68E-02
Ankyrin repeat-containing protein Asb-2	*asb2*	-1.333	3.66E-02
Alpha M integrin	*itgam*	-1.334	4.09E-02
Cytoplasmic dynein intermediate chain 2B	*dync1li2*	-1.336	3.46E-02
Diacylglycerol acyltransferase	*dgat2*	-1.338	3.46E-02
Carbohydrate sulfotransferase 10	*chst10*	-1.341	4.70E-02
Major facilitator superfamily domain	*mfsd3*	-1.341	4.09E-02
NOD-like receptor C	*narg2l*	-1.341	4.91E-02
MAP kinase kinase 4	*mapk4*	-1.343	4.42E-02
Nucleotide binding protein 1	*gnbp*	-1.343	4.13E-02
Partial prl gene for prolactin	*prl*	-1.344	4.42E-02
Ribonucleotide reductase M1 polypeptide	*rrm1*	-1.347	3.46E-02
Syntaxin binding protein 3	*stxbp1*	-1.348	3.76E-02
Carbonic anhydrase-like	*ca*	-1.348	4.74E-02
testis-specific kinase 1	*tssk1b*	-1.351	4.36E-02
Signal transducer and activator of transcription 5 gene	*stat5a*	-1.359	3.55E-02
Guanine nucleotide binding protein beta polypeptide 1-like	*gnb1*	-1.362	4.85E-02
NIMA (never in mitosis gene a)-related kinase 6	*nek6*	-1.365	3.46E-02
Hs-CUL-3	*cul3*	-1.368	3.47E-02
Glycogen storage disease type IV	*gsd 4*	-1.370	4.44E-02
ADP-ribosylation factor-like 8B	*arl8b*	-1.371	4.00E-02
Aquaporin 8	*aqp8*	-1.374	4.81E-02
Osteopetrosis associated transmembrane protein 1	*ostm1*	-1.376	3.31E-02
Glycerate kinase	*glyctk*	-1.393	4.31E-02
Ankyrin-3	*ank3*	-1.395	4.31E-02
CMP-sialic acid transporter	*slc35a1*	-1.395	4.89E-02
Fasciculation and elongation protein zeta 2	*fez2*	-1.397	3.64E-02
Exocyst complex component 3	*exoc3*	-1.398	4.93E-02
Actin binding LIM protein family, member 3, isoform	*ablimi3*	-1.399	3.46E-02
FYVE, RhoGEF And PH Domain Containing 6	*fgd6*	-1.400	4.87E-02
WD repeat domain 44	*wdr44*	-1.403	3.70E-02
Polypeptide N-acetylgalactosaminyltransferase 5	*galnac-t5*	-1.406	4.01E-02
WD repeat domain 1	*wdr1*	-1.410	3.46E-02
YTH domain family 3	*ythdf3*	-1.414	4.17E-02
Sirtuin	*sirt4*	-1.413	4.00E-02
JMJD1B protein	*kdm3b*	-1.416	4.87E-02

Mitochondrial peptide methionine sulfoxide reductase	*msra*	-1.416	3.99E-02
Low density lipoprotein receptor adaptor protein	*ldlrap1*	-1.419	4.54E-02
Hydroxysteroid (17-beta) dehydrogenase 4	*hsd17ba*	-1.430	4.15E-02
UPF0184 protein C9orf16 homolog	*c9orf16*	-1.430	3.64E-02
RNA binding motif protein 5	*rbm5*	-1.434	4.09E-02
Ajuba	*jub*	-1.436	4.55E-02
BRCA1 interacting protein C-terminal helicase 1	*brip1*	-1.436	3.65E-02
C10orf12 protein	*c10orf12*	-1.437	4.15E-02
KIAA0280	*fam168a*	-1.443	4.17E-02
Ssu72 RNA polymerase II CTD phosphatase homolog	*ssu72*	-1.443	3.91E-02
TAR DNA binding protein	*tardbp*	-1.446	4.00E-02
WW45 protein	*sav1*	-1.448	3.62E-02
TAF3 RNA polymerase II	*taf3*	-1.449	3.64E-02
STON1-GTF2A1L protein	*alf*	-1.451	4.38E-02
Glutathione reductase	*gsr*	-1.452	3.46E-02
Vav 1 oncogene	*vcl*	-1.452	4.29E-02
Prpf3 protein	*prpf3*	-1.453	3.46E-02
ASC	*asc*	-1.454	4.08E-02
RB-binding protein	*aatf*	-1.454	4.87E-02
E3 SUMO-protein ligase PIAS2	*pias2*	-1.455	4.00E-02
Mesoderm induction early response 1 isoform N1	*mier1*	-1.455	3.57E-02
Neuroligin 2	*nlgn2*	-1.457	4.22E-02
SH2 domain-containing protein 4A	*sh2d4a*	-1.461	4.29E-02
WW and PDZ domain containing 1	*magi1*	-1.464	4.87E-02
Neuralized-like	*neurl*	-1.467	3.86E-02
Src-family tyrosine kinase SCK	*csk*	-1.467	3.77E-02
Huntington's disease gene homologue	*hdh*	-1.471	3.73E-02
CD4 protein	*cd4*	-1.472	3.46E-02
Beta-actin	*actb*	-1.477	3.77E-02
Alpha inhibiting activity polypeptide 1	*gnai1*	-1.479	3.62E-02
RNA binding motif protein 7	*rbm7*	-1.488	4.70E-02
NADPH-P450 reductase	*por*	-1.490	4.67E-02
ATP-binding domain-containing protein 4	*atpbd4*	-1.491	3.85E-02
Serine/threonine protein kinase	*srsf8*	-1.503	4.17E-02
Poly(A) binding protein, nuclear 1	*pabpn1*	-1.506	4.74E-02
MAP7 domain containing 2	*map7d2*	-1.510	4.67E-02
Pyruvate dehydrogenase kinase, isoenzyme 2	*pdk2*	-1.511	3.46E-02
DEAD-box RNA-dependent helicase p68	*ddx5*	-1.514	3.82E-02
Odd-skipped related 1	*osr1*	-1.516	4.62E-02

Deleted in bladder cancer 1	*dbc1*	-1.517	4.16E-02
Hyperparathyroidism 2 homolog	*cdc73*	-1.518	3.64E-02
Snail homolog 2	*snai2*	-1.520	3.64E-02
SH3-domain binding protein 1	*sh3bp1*	-1.521	4.37E-02
Ribophorin I	*rpn1*	-1.522	4.76E-02
TPA: arylsulfatase D	*arsd*	-1.528	3.46E-02
SAP47 homolog	*syap1*	-1.534	3.46E-02
Qrich1 protein	*qrich1*	-1.534	3.84E-02
Syntaxin 3	*stx3*	-1.538	3.78E-02
Rho GTPase activating protein 18	*arhgap18*	-1.538	4.87E-02
Glycine-gated ion channel alpha3 subunit	*glra3*	-1.538	4.61E-02
NFKBIE protein	*nfkbie*	-1.539	4.02E-02
WDR40A protein	*wdr40a*	-1.542	3.65E-02
Adducin 3 (gamma)	*add3*	-1.543	3.64E-02
Aldo-keto reductase family 7 member A5	*akr7a5*	-1.544	4.82E-02
Nuclear receptor co-repressor/HDAC3 complex	*nfil3*	-1.545	4.87E-02
Glucoseregulated protein 94	*grp94*	-1.548	4.67E-02
Phosphoinositide interacting 2	*wipi2*	-1.558	4.51E-02
3-sialyltransferase 4	*st3gal3*	-1.558	3.58E-02
CTCL tumor antigen HD-CL-09	*ctcl*	-1.567	3.46E-02
V-ral simian leukemia viral oncogene homolog B	*vav 1*	-1.568	3.65E-02
Rho-GTPase-activating protein PS-GAP-a	*arhgap1*	-1.576	4.13E-02
130 kDa EDT-soluble extracellular protein	*dap5*	-1.576	3.81E-02
Actinin, alpha 1	*actn1*	-1.578	3.74E-02
Pleckstrin homology, Sec7 and coiled/coil domain	*cytip*	-1.579	4.51E-02
DNA-binding protein PREB	*preb*	-1.580	3.46E-02
Connector enhancer of kinase suppressor of Ras 1	*cnksr1*	-1.583	4.87E-02
Mel transforming oncogene	*rab8a*	-1.588	3.72E-02
Dystrophin (Dyst) gene	*dmd*	-1.584	3.62E-02
Gamma-butyrobetaine dioxygenase	*bbox1*	-1.587	3.87E-02
Tax1 binding protein 1	*tax1bp1*	-1.592	4.87E-02
Keratinocytes-associated transmembrane protein	*kct1*	-1.594	3.54E-02
Family with sequence similarity 40, member A	*fam40a*	-1.599	4.31E-02
Ubiquitin specific protease 10	*usp10*	-1.604	4.45E-02
Myosin phosphatase-Rho interacting protein	*mprip*	-1.610	3.77E-02
Neuralized-like protein 2	*neurl 2*	-1.611	3.64E-02
Carbohydrate sulfotransferase 2	*chst2*	-1.613	4.87E-02
Vinculin	*scn4ab*	-1.627	3.98E-02

Dmd gene for putative dystrophin	*dmd*	-1.629	3.46E-02
Msx2 interacting nuclear target protein	*spen*	-1.629	3.46E-02
KIAA0494 protein	*kiaa0491*	-1.644	4.01E-02
Patatin-like phospholipase domain containing 6	*pnpla6*	-1.646	4.87E-02
Alveolar soft part sarcoma chromosome region	*aspscr1*	-1.646	4.29E-02
Sorting nexin 4	*snx4*	-1.648	3.47E-02
Copine III	*cpne3*	-1.650	4.30E-02
Carboxymethylenebutenolidase-like	*cmbl*	-1.650	4.83E-02
Mgea5 protein	*mgea5*	-1.651	4.28E-02
Lysosomal membrane glycoprotein lamp-2 homolog	*lamp2*	-1.652	3.46E-02
Filamin A interacting protein 1	*filip*	-1.652	3.87E-02
Triple functional domain (PTPRF interacting) (TRIO)	*trio*	-1.653	3.64E-02
Anterior pharynx defective 1B	*aph1b*	-1.661	3.46E-02
Biliverdin reductase A	*blvra*	-1.668	4.75E-02
Sorting nexin 5	*snx5*	-1.673	4.88E-02
MDN1, midasin homolog	*mdn1*	-1.673	3.47E-02
Protein FAM114A2	*fam1141*	-1.678	3.46E-02
Immunoglobulin mu heavy chain	*ighm*	-1.681	3.64E-02
Calcium/calmodulin-dependent serine protein kinase	*dmi3*	-1.681	4.00E-02
PTPRD protein	*ptprd*	-1.687	3.77E-02
Type 7 adenylyl cyclase	*adcy7*	-1.691	3.17E-02
Proline rich 12	*prr12*	-1.703	3.64E-02
Replication protein A1	*rpa1*	-1.703	4.14E-02
Protocadherin 2A33	*pcdh 2a33*	-1.709	3.64E-02
Centaurin, alpha 1	*adap1*	-1.715	3.56E-02
NADPH-cytochrome P450 reductase	*cpr*	-1.725	3.46E-02
Protein phosphatase 1, regulatory (inhibitor) subunit 13B	*ppp1r13b*	-1.726	3.46E-02
Neurofilament, heavy polypeptide	*nefh*	-1.727	3.62E-02
NCK-associated protein 1-like	*nckap1l*	-1.7307	4.51E-02
Protein tyrosine phosphatase a	*ptpra*	-1.739	4.57E-02
Growth hormone receptor type II (GHR-II) gene	*ghr-ii*	-1.741	3.64E-02
Phospholipase C, gamma 1	*plcg1*	-1.741	3.64E-02
Subunit VIIa 2	*cox7a2l*	-1.741	4.31E-02
D4, zinc and double PHD fingers family 2	*dpf2*	-1.747	4.00E-02
C-type lectin domain family 4 member M	*clec4m*	-1.752	4.60E-02
Selectin P	*selp*	-1.757	3.98E-02
Purinergic receptor P2Y1	*p2r41*	-1.758	4.86E-02

RAB13, member RAS oncogene family	*rab13*	-1.765	4.37E-02
Tubulin, alpha	*tuba1b*	-1.779	3.98E-02
p65 transcription factor	*rela*	-1.784	4.19E-02
Transmembrane 9 superfamily member 2	*tm9sf2*	-1.797	3.64E-02
SWI/SNF-related matrix-associated actin-dependent	*smarcd1*	-1.802	4.87E-02
Ribosomal protein S6 kinase, polypeptide 4	*rps6ka4*	-1.808	4.37E-02
Thymopoietin	*tmpo*	-1.809	4.65E-02
ADP-ribosylation factor 4	*arf4/arl4*	-1.813	4.85E-02
ESCO1 protein	*esco1*	-1.825	3.46E-02
p53 binding protein	*tp53bp*	-1.826	4.21E-02
Crystal Structure Of Holo-CrbP	*rdh16*	-1.834	4.34E-02
Activating transcription factor 7 interacting protein	*atp7ip*	-1.844	4.31E-02
Splicing factor 3b, subunit 1 isoform 1	*sp3b1*	-1.844	4.00E-02
Secretion protein HlyD	*vc_a1080*	-1.861	4.34E-02
CTLA4-like protein	*ctla4*	-1.872	4.83E-02
Basic transcription factor 3-like 4	*btf3l4*	-1.874	4.36E-02
Protein phosphatase methylesterase 1	*ppme1*	-1.883	4.55E-02
Glucose-6-phosphate 1-dehydrogenase (G6PD)	*g6pd*	-1.889	4.37E-02
Coronin-7	*crn7*	-1.892	4.45E-02
SPEN homolog	*spen*	-1.895	4.89E-02
Rho GDP dissociation inhibitor (GDI) alpha	*arhgdia/rhogdi1*	-1.899	3.46E-02
DNA (cytosine-5-)-methyltransferase 6	*dnmt3ab*	-1.910	3.46E-02
Longevity assurance homolog 1	*cers1*	-1.917	3.59E-02
Protein phosphatase type 2C alpha 2	*ptc1*	-1.930	3.46E-02
Glutamate oxaloacetate transaminase 2	*cot2*	-1.931	4.17E-02
Fibronectin type III domain containing 3A	*fndc3a*	-1.937	4.86E-02
Nuclear receptor coactivator 5	*ncoa5*	-1.948	3.61E-02
Sacm1l protein	*sacm1l*	-1.957	3.46E-02
Gonadotropin-releasing hormone gene	*gnrh1*	-1.957	4.25E-02
CD18 protein	*itgb2*	-1.958	3.65E-02
Transposase	*tnp*	-1.964	3.55E-02
GTP-binding protein PTD004	*ola1*	-1.976	4.34E-02
Peptidylglycine alpha-amidating monooxygenase	*pam*	-1.992	4.76E-02
Disabled homolog 2 interacting protein isoform	*dab2ip*	-1.992	4.83E-02
Serine-rich 8	*stpk*	-1.993	3.73E-02
Plasma membrane calcium ATPase 1 isoform a	*atp2b1*	-1.994	3.21E-02
Meteorin	*metrnl*	-1.999	3.46E-02
Von Willebrand factor A domain-containing	*g7c*	-2.040	3.55E-02

protein 7			
Transmembrane BAX inhibitor motif containing 1	*tmbim1*	-2.060	4.67E-02
EF hand calcium binding protein 1	*efcab1*	-2.067	3.65E-02
Transcription factor Sox12	*sox12, sox-12*	-2.068	4.81E-02
4732435N03Rik protein	*rik*	-2.095	4.34E-02
Elongation of very long chain fatty acids-like	*elovl*	-2.113	3.64E-02
Voltage-gated sodium channel Nav1.4b	*ralb*	-2.131	4.27E-02
Heavy polypeptide (Hc)	*cltc*	-2.164	4.85E-02
Sb:cb825 protein	*pdia3*	-2.167	3.97E-02
Formin-like 1	*fmnl1*	-2.179	4.86E-02
Collagen, type VI, alpha 3	*col6a3*	-2.182	3.72E-02
RNA binding protein CD151	*cd151*	-2.195	4.62E-02
Bone morphogenetic protein receptor type II-like	*bmpr2*	-2.198	4.44E-02
Fibulin 1	*fbln1*	-2.208	3.46E-02
Scribble-related protein 1	*scrib*	-2.212	4.92E-02
Transmembrane protein 16E	*ano5*	-2.257	4.23E-02
Tropomyosin 4 isoform 2	*tpm4*	-2.262	4.45E-02
Tyrosine-protein kinase Yes	*yes1*	-2.276	3.46E-02
Neuralized homolog	*neurl*	-2.280	3.64E-02
Steroidogenic acute regulatory protein	*star*	-2.291	4.19E-02
Receptor protein-tyrosine phosphatase sigma	*ptprs*	-2.301	4.83E-02
GRAM domain containing 3	*gramd3*	-2.341	3.31E-02
Endothelin converting enzyme 1	*ece1*	-2.357	3.46E-02
Phospholipase A2, group IVF	*pla2g4f*	-2.366	4.52E-02
Anti-*Müllerian* hormone	*amh*	-2.401	3.85E-02
TPA: zinc finger protein	*anf*	-2.406	3.64E-02
Myocardin	*myocd*	-2.446	3.68E-02
Erythrocyte membrane protein band 4.1 like 5	*epb41l5*	-2.465	3.46E-02
Taurine transporter like	*slc6a6*	-2.475	3.46E-02
Ephrin A5a	*efna5a*	-2.508	3.76E-02
FBP32 gene	*fbp32*	-2.611	3.64E-02
Doublesex- and mab-3-related transcription factor 1A	*dmrt1*	-2.634	4.18E-02
Bromodomain adjacent to zinc finger domain, 2B	*baz1b*	-2.784	3.46E-02
Endothelin receptor A	*endra*	-2.868	4.07E-02
Cytochrome c oxidase subunit VIa precursor	*mt-coi*	-2.930	4.83E-02
General transcription factor polypeptide 4	*gtf4*	-2.933	3.92E-02
Slow myosin heavy chain 3	*smyhc3*	-2.300	3.84E-02
Heat shock protein 70 isoform 3	*hsp70-3*	-3.079	4.70E-02
N-acetyl lactosaminide beta-1,3-N-acetyl	*lytd*	-3.310	3.46E-02

glucosamide			
Taurine transporter	*taut*	-3.321	3.64E-02
Magi1 protein	*magi1*	-3.691	3.46E-02
Embigin homolog	*emb*	-3.946	4.67E-02
GMP reductase 2-like protein	*gmpr2*	-4.448	4.73E-02
Collagen type XVIII alpha 1	*col18a1*	-4.561	3.46E-02
Sodium- and chloride-dependent GABA transporter 2	*slc6a13*	-5.238	4.09E-02
CD11-1	*cd11-1*	-6.766	3.82E-02

Supplementary Table 4. Differentially expressed gene clusters from the heatmap analyzed by DAVID

DE genes clusters	Associated pathways
Cluster 1	ABC transporters
	Adherens junction
	Alzheimer's disease
	Amino sugar and nucleotide sugar metabolism
	Amyotrophic lateral sclerosis (ALS)
	Antigen processing and presentation
	Biosynthesis of alkaloids derived from histidine and purine
	Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid
	Biosynthesis of alkaloids derived from shikimate pathway
	Biosynthesis of alkaloids derived from terpenoid and polyketide
	Biosynthesis of phenylpropanoids
	Biosynthesis of plant hormones
	Biosynthesis of terpenoids and steroids
	Biosynthesis of unsaturated fatty acids
	Butanoate metabolism
	Calcium signaling
	Cardiac muscle contraction
	Cell adhesion molecules (CAMs)
	Citrate cycle (TCA cycle)
	Cysteine and methionine metabolism
	Cytokine-cytokine receptor interaction
	DNA replication
	Dorso-ventral axis formation
	ECM-receptor interaction
	Endocytosis
	ErbB signaling pathway
	Ether lipid metabolism
	Fatty acid metabolism
	Fc epsilon RI signaling pathway
	Fc gamma R-mediated phagocytosis
	Focal adhesion

Fructose and mannose metabolism
Gap junction
Glioma
Glutathione metabolism
Glycerolipid metabolism
Glycerophospholipid metabolism
Glycine, serine and threonine metabolism
Glycolysis / Gluconeogenesis
Glycosphingolipid biosynthesis
GnRH signaling pathway
Hedgehog signaling pathway
Hematopoietic cell lineage
Homologous recombination
Huntington's disease
Inositol phosphate metabolism
Insulin signaling pathway
Jak-STAT signaling pathway
Keratan sulfate biosynthesis
Leukocyte transendothelial migration
Lysine degradation
MAPK signaling pathway
Melanogenesis
Mismatch repair
Natural killer cell mediated cytotoxicity
Neuroactive ligand-receptor interaction
Neurotrophin signaling pathway
N-Glycan biosynthesis
NOD-like receptor signaling pathway
Non-small cell lung cancer
Notch signaling pathway
Nucleotide excision repair
Oxidative phosphorylation
Pancreatic cancer
Parkinson's disease
Pathways in cancer
Pentose phosphate pathway

	Phosphatidylinositol signaling system
	Porphyrin and chlorophyll metabolism
	Primary immunodeficiency
	Progesterone-mediated oocyte maturation
	Prostate cancer
	Proteasome
	Purine metabolism
	Pyrimidine metabolism
	Regulation of actin cytoskeleton
	Regulation of autophagy
	Renin-angiotensin system
	Retinol metabolism
	Ribosome
	RIG-I-like receptor signaling pathway
	Selenoamino acid metabolism
	SNARE interactions in vesicular transport
	Spliceosome
	Synthesis and degradation of ketone bodies
	T cell receptor signaling pathway
	Tight junction
	Toll-like receptor signaling pathway
	Tyrosine metabolism
	Valine, leucine and isoleucine degradation
	VEGF signaling pathwaypathway
	Wnt signaling pathway
Cluster 2	Cytokine-cytokine receptor interaction
	Glycerolipid metabolism
	Wnt signaling pathway
Cluster 3	Adherens junction
	Aminoacyl-tRNA biosynthesis
	Arrhythmogenic right ventricular cardiomyopathy (ARVC)
	Autoimmune thyroid disease
	Biosynthesis of alkaloids derived from shikimate pathway
	Biosynthesis of phenylpropanoids
	Biosynthesis of plant hormones
	Cell adhesion molecules (CAMs)

	Chemokine signaling pathway
	Cytosolic DNA-sensing pathway
	Focal adhesion
	Glycerolipid metabolism
	Glycine, serine and threonine metabolism
	Glyoxylate and dicarboxylate metabolism
	Jak-STAT signaling pathway
	Leukocyte transendothelial migration
	Neurotrophin signaling pathway
	NOD-like receptor signaling pathway
	Pathways in cancer
	Phenylalanine, tyrosine and tryptophan biosynthesis
	Porphyrin and chlorophyll metabolism
	Purine metabolism
	Regulation of actin cytoskeleton
	Small cell lung cancer
	Spliceosome
	Systemic lupus erythematosus
	T cell receptor signaling pathway
	Tight junction
	Ubiquitin mediated proteolysis
Cluster 4	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) degradation
	Adipocytokine signaling pathway
	Aldosteroneregulated sodium reabsorption
	Alzheimer's disease
	Amino sugar and nucleotide sugar metabolism
	Apoptosis
	Biosynthesis of terpenoids and steroids
	Biosynthesis of unsaturated fatty acids
	Biotin metabolism
	Cell adhesion molecules (CAMs)
	Cell cycle
	Chemokine signaling pathway
	Complement and coagulation cascades
	Cytokine-cytokine receptor interaction

Cytosolic DNA-sensing pathway
Dilated cardiomyopathy
DNA replication
Drug metabolism
Endocytosis
ErbB signaling pathway
Focal adhesion
Folate biosynthesis
Fructose and mannose metabolism
Gliomacardiomyopathy,
Glutathione metabolism
Glycerophospholipid metabolism
Glycosphingolipid biosynthesis
Huntington's disease
Hypertrophic cardiomyopathy (HCM)
Limonene and pinene degradation
Long-term depression
Lysosome
MAPK signaling pathway
Meiosis
Melanoma
mTOR signaling pathway
Neuroactive ligand-receptor interaction
NOD-like receptor ceptor signaling pathway
Oocyte meiosis
Oxidative phosphorylation
p53 signaling pathway
Parkinson's disease
Pathways in cancer
Pentose phosphate pathway
Progesterone-mediated oocyte maturation
Prostate cancer
Protein export
Purine metabolism
Pyrimidine metabolism
Retinol metabolism

	RIG-I-like receptor signaling pathway
	Spliceosome
	Starch and sucrose metabolism
	Steroid biosynthesis
	Tight junction
	Toll-like receptor signaling pathway
	Tryptophan metabolism
	Two-component system
	Adherens junction
Cluster 5	Adipocytokine signaling pathway
	Antigen processing and presentation
	Arrhythmogenic right ventricular cardiomyopathy
	B cell receptor signaling pathway
	Calcium signaling pathway
	Chemokine signaling pathway
	Cytokine-cytokine receptor interaction
	Dilated cardiomyopathy
	Focal adhesion
	Gap junction
	GnRH signaling pathway
	Hypertrophic cardiomyopathy
	Leukocyte transendothelial migration(HCM)
	Melanogenesis
	Neuroactive ligand-receptor interaction
	Neurotrophin signaling pathway
	Oocyte meiosis
	Other glycan degradation
	Progesterone-mediated oocyte maturation
	Purine metabolism
	Regulation of actin cytoskeleton
	t cell receptor signaling pathway
	Terpenoid backbone biosynthesis
	TGF-beta signaling pathway
	Tight junction
	Vascular smooth muscle contraction
	Viral myocarditis

Cluster 6	Mitochondria
	Acute myeloid leukemia
Cluster 7	
	Axon guidance
	Chemokine signaling pathway
	Chronic myeloid leukemia
	Cytokine-cytokine receptor interaction
	Endocytosis
	ErbB signaling pathway
	Gap junction
	Huntington's disease
	Jak-STAT signaling pathway
	Leukocyte transendothelial migration
	Long-term depression
	Lysosome
	Melanogenesis
	Pathways in cancer
	Progesterone-mediated oocyte maturation
	Taste transduction
	TGF-beta signaling pathway
	Tight junction
	Ubiquitin mediated proteolysis
Cluster 8	Alzheimer's disease
	Huntington's disease
	Oxidative phosphorylation
	Parkinson's disease
	Proteasome

Supplementary Table 5. Two-tail Fisher's exact test with multiple testing corrections of FDR results

GO Term	Name	Type	FDR	Single Test *P*-Value
GO:0005887	integral to plasma membrane	C	1.63E-08	5.28E-11
GO:0051240	positive regulation of multicellular organismal process	P	1.04E-05	9.18E-08
GO:0019904	protein domain specific binding	F	9.57E-05	1.20E-06
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	P	2.84E-04	4.10E-06
GO:0005792	microsome	C	5.11E-04	8.08E-06
GO:0043066	negative regulation of apoptotic process	P	5.18E-04	8.28E-06
GO:0097458	neuron part	C	8.12E-04	1.39E-05
GO:0050872	white fat cell differentiation	P	1.42E-03	2.65E-05
GO:0050829	defense response to Gram-negative bacterium	P	1.42E-03	2.65E-05
GO:0007568	aging	P	1.81E-03	3.47E-05
GO:0045667	regulation of osteoblast differentiation	P	1.97E-03	3.82E-05
GO:0005794	Golgi apparatus	C	2.08E-03	4.09E-05
GO:0005829	cytosol	C	2.73E-03	5.69E-05
GO:0030335	positive regulation of cell migration	P	3.04E-03	6.39E-05
GO:0050839	cell adhesion molecule binding	F	3.51E-03	7.56E-05

GO:0071364	cellular response to epidermal growth factor stimulus	P	3.51E-03	7.64E-05
GO:0007595	lactation	P	4.22E-03	9.59E-05
GO:0009897	external side of plasma membrane	C	4.30E-03	9.84E-05
GO:0010740	positive regulation of intracellular protein kinase cascade	P	4.47E-03	1.05E-04
GO:0019901	protein kinase binding	F	4.82E-03	1.15E-04
GO:0004620	phospholipase activity	F	5.64E-03	1.38E-04
GO:0001726	ruffle	C	5.68E-03	1.40E-04
GO:0044463	cell projection part	C	6.04E-03	1.52E-04
GO:0042803	protein homodimerization activity	F	6.37E-03	1.61E-04
GO:0050798	activated T cell proliferation	P	6.70E-03	1.71E-04
GO:0007600	sensory perception	P	6.97E-03	1.82E-04
GO:0045121	membrane raft	C	7.37E-03	1.95E-04
GO:0008092	cytoskeletal protein binding	F	7.68E-03	2.06E-04
GO:0045202	synapse	C	9.03E-03	2.57E-04
GO:0042698	ovulation cycle	P	9.57E-03	2.75E-04
GO:0007159	leukocyte cell-cell adhesion	P	1.10E-02	3.30E-04
GO:0035066	positive regulation of histone acetylation	P	1.10E-02	3.30E-04
GO:0003682	chromatin binding	F	1.24E-02	3.90E-04
GO:0050870	positive regulation of T cell activation	P	1.24E-02	3.92E-04
GO:0009395	phospholipid catabolic process	P	1.24E-02	3.92E-04

GO:0019012	virion	C	1.35E-02	4.40E-04
GO:0001673	male germ cell nucleus	C	1.35E-02	4.40E-04
GO:0071305	cellular response to vitamin D	P	1.35E-02	4.40E-04
GO:0000255	allantoin metabolic process	P	1.35E-02	4.40E-04
GO:0055012	ventricular cardiac muscle cell differentiation	P	1.35E-02	4.40E-04
GO:0032403	protein complex binding	F	1.37E-02	4.46E-04
GO:0005912	adherens junction	C	1.49E-02	4.94E-04
GO:0003712	transcription cofactor activity	F	1.50E-02	4.97E-04
GO:0048646	anatomical structure formation involved in morphogenesis	P	1.51E-02	5.03E-04
GO:0043235	receptor complex	C	1.51E-02	5.04E-04
GO:0032330	regulation of chondrocyte differentiation	P	1.55E-02	5.30E-04
GO:0043565	sequence-specific DNA binding	F	1.58E-02	5.40E-04
GO:0023061	signal release	P	1.61E-02	5.58E-04
GO:0060090	binding, bridging	F	1.61E-02	5.66E-04
GO:0006929	substrate-dependent cell migration	P	1.61E-02	5.71E-04
GO:0005001	transmembrane receptor protein tyrosine phosphatase activity	F	1.61E-02	5.71E-04
GO:0043278	response to morphine	P	1.61E-02	5.71E-04
GO:0007416	synapse assembly	P	1.75E-02	6.33E-04
GO:0007409	axonogenesis	P	1.75E-02	6.38E-04

GO:0032355	response to estradiol stimulus	P	1.98E-02	7.41E-04
GO:0070252	actin-mediated cell contraction	P	1.98E-02	7.41E-04
GO:0043043	peptide biosynthetic process	P	1.98E-02	7.41E-04
GO:0042572	retinol metabolic process	P	1.98E-02	7.41E-04
GO:0022603	regulation of anatomical structure morphogenesis	P	2.01E-02	7.56E-04
GO:0006935	chemotaxis	P	2.11E-02	8.08E-04
GO:0009612	response to mechanical stimulus	P	2.11E-02	8.10E-04
GO:0030522	intracellular receptor signaling pathway	P	2.15E-02	8.30E-04
GO:0007286	spermatid development	P	2.22E-02	8.65E-04
GO:0005539	glycosaminoglycan binding	F	2.22E-02	8.65E-04
GO:0005856	cytoskeleton	C	2.30E-02	9.11E-04
GO:0007157	heterophilic cell-cell adhesion	P	2.30E-02	9.15E-04
GO:0046620	regulation of organ growth	P	2.30E-02	9.15E-04
GO:0043065	positive regulation of apoptotic process	P	2.30E-02	9.17E-04
GO:0070013	intracellular organelle lumen	C	2.35E-02	9.41E-04
GO:0045598	regulation of fat cell differentiation	P	2.47E-02	1.01E-03
GO:0090090	negative regulation of canonical Wnt receptor signaling pathway	P	2.47E-02	1.01E-03
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	P	2.52E-02	1.04E-03

GO:0034185	apolipoprotein binding	F	2.52E-02	1.06E-03
GO:0007185	transmembrane receptor protein tyrosine phosphatase signaling pathway	P	2.52E-02	1.06E-03
GO:0072132	mesenchyme morphogenesis	P	2.52E-02	1.06E-03
GO:0002162	dystroglycan binding	F	2.52E-02	1.06E-03
GO:0045213	neurotransmitter receptor metabolic process	P	2.52E-02	1.06E-03
GO:0055024	regulation of cardiac muscle tissue development	P	2.52E-02	1.06E-03
GO:0035414	negative regulation of catenin import into nucleus	P	2.52E-02	1.06E-03
GO:0005667	transcription factor complex	C	2.58E-02	1.10E-03
GO:0016125	sterol metabolic process	P	2.59E-02	1.10E-03
GO:0003012	muscle system process	P	2.69E-02	1.15E-03
GO:0005543	phospholipid binding	F	2.77E-02	1.19E-03
GO:0005911	cell-cell junction	C	2.77E-02	1.20E-03
GO:0010720	positive regulation of cell development	P	2.78E-02	1.20E-03
GO:0000982	RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity	F	3.04E-02	1.34E-03
GO:0042593	glucose homeostasis	P	3.04E-02	1.34E-03
GO:0072091	regulation of stem cell proliferation	P	3.08E-02	1.38E-03

GO:1901888	regulation of cell junction assembly	P	3.08E-02	1.38E-03
GO:0050810	regulation of steroid biosynthetic process	P	3.08E-02	1.38E-03
GO:0005100	Rho GTPase activator activity	F	3.08E-02	1.38E-03
GO:0042108	positive regulation of cytokine biosynthetic process	P	3.08E-02	1.38E-03
GO:0007628	adult walking behavior	P	3.08E-02	1.38E-03
GO:0034381	plasma lipoprotein particle clearance	P	3.08E-02	1.38E-03
GO:0007010	cytoskeleton organization	P	3.12E-02	1.40E-03
GO:0004713	protein tyrosine kinase activity	F	3.15E-02	1.42E-03
GO:0005625	soluble fraction	C	3.23E-02	1.46E-03
GO:0035257	nuclear hormone receptor binding	F	3.29E-02	1.49E-03
GO:0050731	positive regulation of peptidyl-tyrosine phosphorylation	P	3.44E-02	1.57E-03
GO:0008083	growth factor activity	F	3.44E-02	1.57E-03
GO:0080134	regulation of response to stress	P	3.44E-02	1.58E-03
GO:0035023	regulation of Rho protein signal transduction	P	3.72E-02	1.72E-03
GO:0007422	peripheral nervous system development	P	3.74E-02	1.75E-03
GO:0040014	regulation of multicellular organism growth	P	3.74E-02	1.75E-03
GO:0048771	tissue remodeling	P	3.74E-02	1.75E-03
GO:0030055	cell-substrate junction	C	3.77E-02	1.77E-03
GO:0006897	endocytosis	P	3.86E-02	1.81E-03

GO:0031329	regulation of cellular catabolic process	P	3.95E-02	1.86E-03
GO:0051090	regulation of sequence-specific DNA binding transcription factor activity	P	3.98E-02	1.89E-03
GO:0030500	regulation of bone mineralization	P	4.15E-02	1.99E-03
GO:0010657	muscle cell apoptotic process	P	4.15E-02	1.99E-03
GO:0019814	immunoglobulin complex	C	4.15E-02	1.99E-03
GO:0033629	negative regulation of cell adhesion mediated by integrin	P	4.16E-02	2.04E-03
GO:0045123	cellular extravasation	P	4.16E-02	2.04E-03
GO:0010839	negative regulation of keratinocyte proliferation	P	4.16E-02	2.04E-03
GO:0045885	positive regulation of survival gene product expression	P	4.16E-02	2.04E-03
GO:0005200	structural constituent of cytoskeleton	F	4.20E-02	2.07E-03
GO:0030168	platelet activation	P	4.31E-02	2.14E-03
GO:0050679	positive regulation of epithelial cell proliferation	P	4.42E-02	2.24E-03
GO:0051347	positive regulation of transferase activity	P	4.42E-02	2.30E-03
GO:0050852	T cell receptor signaling pathway	P	4.42E-02	2.33E-03
GO:0010518	positive regulation of phospholipase activity	P	4.42E-02	2.33E-03
GO:0034332	adherens junction organization	P	4.42E-02	2.33E-03
GO:0021629	olfactory nerve structural organization	P	4.42E-02	2.36E-03

GO:0072365	regulation of cellular ketone metabolic process by negative regulation of transcription from RNA polymerase II promoter	P	4.42E-02	2.36E-03
GO:0052650	NADP-retinol dehydrogenase activity	F	4.42E-02	2.36E-03
GO:0006700	C21-steroid hormone biosynthetic process	P	4.42E-02	2.36E-03
GO:0006583	melanin biosynthetic process from tyrosine	P	4.42E-02	2.36E-03
GO:0051684	maintenance of Golgi location	P	4.42E-02	2.36E-03
GO:0042985	negative regulation of amyloid precursor protein biosynthetic process	P	4.42E-02	2.36E-03
GO:0031088	platelet dense granule membrane	C	4.42E-02	2.36E-03
GO:0004167	dopachrome isomerase activity	F	4.42E-02	2.36E-03
GO:0030260	entry into host cell	P	4.42E-02	2.36E-03
GO:0046449	creatinine metabolic process	P	4.42E-02	2.36E-03
GO:0045906	negative regulation of vasoconstriction	P	4.42E-02	2.36E-03
GO:0034372	very-low-density lipoprotein particle remodeling	P	4.42E-02	2.36E-03
GO:0034364	high-density lipoprotein particle	C	4.42E-02	2.36E-03
GO:0021847	ventricular zone neuroblast division	P	4.42E-02	2.36E-03
GO:0007567	parturition	P	4.42E-02	2.36E-03
GO:0071375	cellular response to peptide hormone stimulus	P	4.42E-02	2.36E-03

GO:0032496	response to lipopolysaccharide	P	4.79E-02	2.59E-03
GO:0001701	in utero embryonic development	P	4.79E-02	2.59E-03
GO:0032101	regulation of response to external stimulus	P	4.79E-02	2.59E-03
GO:0043169	cation binding	F	4.91E-02	2.67E-03
GO:0051093	negative regulation of developmental process	P	4.91E-02	2.68E-03
GO:0032553	ribonucleotide binding	F	4.91E-02	2.68E-03

Abbreviations: C, cell component; M, molecular function; P, biological process.

Supplementary Table 6. KEGG pathways derived from the up- and downregulated DE genes

Pathway name	# sequences	# enzymes	Genes involved	Up/ Down
Alpha-linolenic acid metabolism	1	1	*lcat*	Up
Amino sugar and nucleotide sugar metabolism	1	1	*por*	Down
Arginine and proline metabolism	1	1	*got2*	Down
Biotin metabolism	1	1	*btd*	Up
Carbon fixation in photosynthetic organisms	1	1	*got2*	Down
Citrate cycle (TCA cycle)	1	1	*dlst*	Down
Cutin, suberine and wax biosynthesis	1	1	*dgat2*	Down
Drug metabolism	2	2	*nlgn2, ppme1*	Down
Drug metabolism - Cytochrome P450	1	1	*mgst2*	Down
Drug metabolism - Other enzymes	1	1	*uck2*	Up
Ether lipid metabolism	1	1	lcat	Up
Fatty acid biosynthesis	1	1	*acc1*	Up
Fructose and mannose metabolism	1	1	*akr1b7*	Down
Galactose metabolism	1	1	*akr1b7*	Down
Glutathione metabolism	4	4	*g6pd, mgst2, gsr, rrm1*	Down
Glycerolipid metabolism	5	4	*dgat2, agpat1, glyctk, akr1b7*	Down

Glycine, serine and threonine metabolism	1	1	*glyctk*	Down
Glycosaminoglycan degradation	1	1	*mgea5*	Down
Glyoxylate and dicarboxylate metabolism	1	1	*glyctk*	Down
Inositol phosphate metabolism	3	3	*sacm1l, ednra, plcg1*	Down
Isoquinoline alkaloid biosynthesis	1	1	*got2*	Down
Linolenic acid metabolism	1	1	*lcat*	Up
Lysine degradation	4	4	*prdm2, smyd3, box1, dlst*	Down
Melanogenesis	2	1	*dct*	Down
Metabolism of xenobiotics by Cytochrome P450	1	1	*mgst2*	Down
mTOR signaling pathway	1	1	*mapk4*	Down
Mucin type O-glycan biosynthesis	1	1	*galnt5*	Down
Nitrogen metabolism	2	1	*ca7*	Down
Novobiocin biosynthesis	2	2	*got2, ASP1*	Down
Other types of O-glycan biosynthesis	1	1	*st3gal3*	Down
Oxidative phosphorilation	4	2	*cox7a2, ndufa1*	Up
Pentose and glucuronate interconversions	1	1	*akr1b7*	Down
Phosphatidylinositol signaling system	3	3	*sacm1l, ednra, plcg1*	Down
Porphyrin and chlorophyll metabolism	1	2	*fxn, cp*	Up
Purine metabolism	6	6	*gmpr2, star, ATPase, adcy7, gask, rrm1*	Down
Pyruvate metabolism	1	1	*akr1b7*	Down

Retinol metabolism	2	2	*dgat2, rdh2*	Down
Sphingolipid metabolism	3	2	*cers1, arsd*	Down
Starch and sucrose metabolism	1	1	*gbe1*	Down
Steroid biosynthesis	1	1	*tm7sf2*	Down
Steroid hormone biosynthesis	1	1	*arsd*	Down
T cell receptor signaling pathway	4	1	*ssu72*	Down
Tropane, piperidine and pyridine alkaloid biosynthesis	1	1	*ccbl1*	Up
Tropane, piperidine and pyridine alkaloid biosynthesis	1	1	*got2*	Down
Tryptophan metabolism	1	1	*got2*	Up
Various types of N-glycan biosynthesis	1	1	*st3gal3*	Down

Supplementary Table 7. Epigenetic regulatory mechanisms-related gene list

	Gene	Gene name	Fold Change	adjusted *P*-value
	cxxc1	CpG-binding protein	-1.029	0.798
demethylases	*jarid2*	jumonji, AT rich interactive domain 2	-1.172	0.166
	jmjd1c	probable JmjC domain-containing histone demethylation protein 2C	-1.012	0.906
	kdm3b	lysine-specific demethylase 3B	-1.355	0.103
	kdm5b	lysine-specific demethylase 5B	-1.311	0.045
	kdm6b	lysine-specific demethylase 6B	-1.196	0.295
dicer1	*dicer1*	endoribonuclease Dicer	-1.110	0.267
	PCON_06426	similar to protein kinase/endoribonuclease (IRE1)	-1.145	0.098
helicases	*Brm*	ATP-dependent helicase BRM	-1.377	0.099
	ddx39b	spliceosome RNA helicase DDX39B	-1.01937	0.842
histone acetyltransferases	*hat1*	histone acetyltransferase type B catalytic subunit	1.354	0.273
	kat8	histone acetyltransferase KAT8	1.504	0.162
	myst2	MYST histone acetyltransferase 2	-1.312	0.064
histone deacetylases	*hdac3*	nuclear receptor co-repressor/HDAC3 complex subunit	1.170	0.270
	Hdac	class 4 HDAC protein	1.062	0.774
	hdac1	histone deacetylase 1	1.266	0.428
	hdac11	histone deacetylase 11	1.122	0.217
	hdac7	histone deacetylase 7	-1.271	0.101

methyltransferases	*dmap1*	DNA methyltransferase 1 associated protein 1	-1.283	0.130
	dnmt3b	DNA (cytosine-5)-methyltransferase 3B	-1.910	0.035
	ehmt2	euchromatic histone lysine N-methyltransferase 2	1.755	0.152
	mettl1	tRNA (guanine-N(7)-)-methyltransferase	1.312	0.324
	mettl15	probable methyltransferase-like protein 15	1.014	0.890
	mettl2b	methyltransferase-like protein 2B	1.138	0.700
	mettl4	methyltransferase-like protein 4	-1.067	0.816
	mettl7a	methyltransferase-like protein 7A	-1.178	0.516
	mettl8	methyltransferase-like protein 8	1.263	0.353
	n6amt1	hemK methyltransferase family member 2	1.049	0.567
	setd7	histone-lysine N-methyltransferase SETD7	1.037	0.876
	skmBOP	skmBOP	-1.032	0.779
	smyd3	histone-lysine N-methyltransferase SMYD3	1.442	0.182
	trdmt1	tRNA (cytosine(38)-C(5)-methyltransferase	-1.354	0.202
	prmt3	protein arginine N-methyltransferase 3	1.271	0.154
	prmt7	protein arginine N-methyltransferase 7	1.355	0.169
	setd8	N-lysine methyltransferase SETD8	-1.442	0.115
	set1	putative histone-lysine N-methyltransferase 1	-1.758	0.147
	smyd3	histone-lysine N-methyltransferase SMYD3	1.442	0.182
polycomb	*pcgf2*	polycomb group RING finger protein 2	-1.005	0.986
	pcgf5	polycomb group RING finger protein 5	-1.147	0.333
	pcgf6	polycomb group RING finger protein 6	1.343	0.134
	suz12	polycomb protein SUZ12	-1.090	0.369

Discussion

The overall objective of this thesis was to study the effects of several environmental factors on the European sea bass growth and gonadal transcriptome at the time of sex differentiation. The effects of food ration on the juvenile testis transcriptome were also studied. For that purpose, we carried out different experiments taking into account the effects of altered growth rates through food availability, the effect of elevated temperature during early development or of the exposure of exogenous estrogen.

The results of these experiments have been discussed in detail in the corresponding preceding chapters 1 through 4, each having its particular and specific goals. However, there might be more to be learnt when experiments are contemplated together rather than separately. Thus, here we attempt to extract further information by comparing the results of the different experiments on relevant aspects (growth, sex ratios and gonadal transcriptomics) together with the aim that the result is larger than the sum of its parts.

1. Growth

In the European sea bass, the onset of sex differentiation is more related to the attained length than to age (Blázquez et al., 1999). Gonads remain undifferentiated until about 8 cm SL, when females start to differentiate, earlier than males (~9 cm SL). However, females mature later than males (Blázquez et al., 1999; Navarro-Martín et al., 2009a; Díaz et al., 2013). Although the sex differentiation process proper does not finish until fish attain ~12 cm SL, at ~10 cm SL undifferentiated fish are no longer present (Figure 1A). Furthermore, in European sea bass sexual growth dimorphism is present in a population of mature males and immature females (Gardeur et al., 2001a; Blázquez et al., 2001; Saillant et al., 2001a; Díaz et al., 2013).

In fish of a given batch, individual variability in growth is high even if they are maintained under the same environmental conditions (Gardeur et al., 2001a, 2001b; Campeas et al., 2009). This is also the case of European sea bass, where differences in growth performance of the three families studied in this thesis varied due to different parental origins, since the environmental parameters were

under control. Aside from differences in growth performance between the three used families, their growth trajectories fit within the maximum and minimum growth values found in the literature (Figure 1B). However, these three families grew less than other families previously raised in our facilities. That may be in part due to differences in the genetic growth potential inherited from their parents (Carter et al., 1993; Gardeur et al., 2001a), but also to the fact that the female percent in our families was, in general, lower than in previously used families (Navarro-Martín et al., 2009b). It should also be remembered that in the European sea bass there is a size advantage in families with high female proportions, even when correcting for sex dimorphism (Vandeputte et al., 2007).

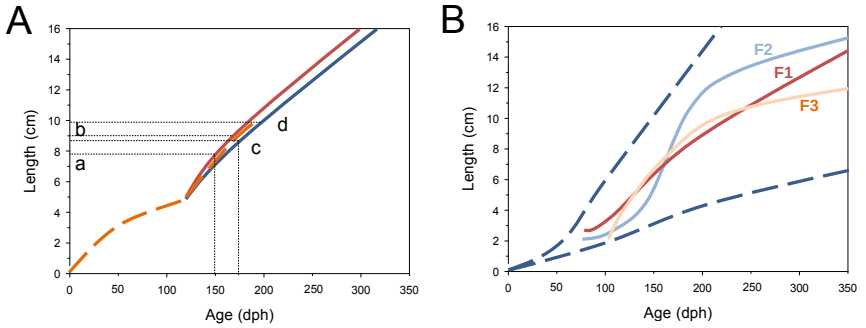


Figure 1. A) Diagram of the growth and sex differentiation relationship in the European sea bass based on data from the literature as well as our own observations. The dashed orange line indicates the undifferentiated fish, the red solid line females and blue solid line males. a-b mark the period when first females are distinguishable, while c-d mark the appearance of first males. Growth is represented by a two-cycle Gompertz curve (Olivar et al., 2000). B) Blue dashed lines indicate the European sea bass growth range under different natural or artificial environmental conditions. Colored solid lines represent the growth trajectories of the three families (F1, F2 and F3) used in this thesis.

In order to study differences among the fast- and the slow-growing fish we compared the differentially expressed (DE) genes observed in two of our microarray studies: undifferentiated fish from the Chapter 2 and Chapter 3. Unexpectedly, the analysis of the common DE genes in the fast- vs. slow-growing fish comparison, on one hand, and the HT vs. the LT comparison on the other hand, gave no coincidences (data not shown). This suggests that either the underlying mechanisms controlling growth rate differences are not

conserved or that temperature is influencing growth through a different mechanism (most probably more related to the future sex of the fast growing fish, since a high female percent is found among the fast growing European sea bass).

The growth of the three families at three different times: 134 dph (point 1: before sex differentiation), at 150 dph (point 2: first histological differences) and at 170 dph (point 3: in the middle of the sex differentiation period) was different. At point 1, different growth performances were already visible between families, with SL ranging from 3.5 cm (group S, family 2 or F2) to 6 cm (group HT, family 3 or F3). Later, at point 2, there were less differences between families 1 and 2 (Chapter 1), with SL ranging from 5 cm (Chapter 1, family 1 or F1) to 6.5 cm (Chapter 1, F2), while family 3 showed higher growth (7.0– 8.5 cm SL). Finally, at point 3, F3 still showed the greatest growth performance (F3>F2>F1). On the contrary, F1 (Chapter 1) had lower growth since its fast-growing group showed the same mean SL than the slow-growing fish from F2 (Chapter 1) and 2 cm less than the slow-growing group from F3. Furthermore, we have also compared the growth of the three families at the moment when we conducted microarray experiments: 134 dph (Chapter 1, F2), 170 dph (Chapter 3, F3) and at an intermediate point for Chapter 1-Experiment 1 (F1).As expected, differences in length and weight were present among the groups ($P<0.01$).

Table 1. Growth at the time of sex differentiation in the three different families used in this thesis

Experiment	Group	Age (dph)	Length (cm) ± SEM	Weight (g) ± SEM	Condition factor (*K*)
Chapter 1-Experiment 2	S	134	3.5 ± 0.003[a]	0.7 ± 0.002[**]	1.64
Chapter 1-Experiment 2	F	134	5.0 ± 0.005[a]	1.9 ± 0.005[**]	1.52
Chapter 1-Experiment 1	S1	150	5.3 ± 0.014[b]	2.7 ± 0.017[**]	1.81
Chapter 1-Experiment 1	F	150	5.5 ± 0.016[c]	3.8 ± 0.022[**]	2.28
Chapter 3	LT	170	9.2 ± 0.008[d]	13.3 ± 0.038[**]	1.71
Chapter 3	HT	170	9.8 ± 0.009[e]	17.4 ± 0.045[**]	1.85

Note: Different letters indicate statistical differences for length (P<0.01); asterisks indicate statistical differences for weight (P<0.01).

The specific effects of three different environmental factors have also been studied in this thesis:

1.1. Effect of amount of food on growth (Chapter 1-Experiments 1 and 2)

European sea bass growth can be modified by changing the amount of food provided or the composition of the diet (Paspatis et al., 2000; Lupatsch et al., 2001; Azzaydi et al., 2007). We carried out two growth-related experiments including both Chapter 1-Experiment 1, a non size-graded European sea bass population homogenously divided into four groups, and Chapter1-Experiment 2, a size-graded population divided into three groups.

In both experiments, growth rates were altered as desired. In the first experiment, growth of the four groups was homogeneous for about three months after the start of the experiment (225 dph), when statistical differences for both length and weight were evident. The highest differences among the four groups (F>S3>S2>S1; although no statistical differences between S2 and S1 groups) were more evident at the end of the food restriction period (368 dph). Similarly, in the second experiment, fish were size-graded and

hence resulting groups had marked differences throughout the course of the study until 337 dph.

Sexual growth dimorphism and compensatory growth after a period of food restriction were also analyzed. Sexual growth dimorphism with larger females was present only when growth rates were artificially altered by different feeding protocols during the period of sex differentiation (Chapter1-Experiment 2) since imposed fasting at different moments of this process eliminated sexual growth dimorphism (Chapter 1-Experiment 1). This may be due to the fact that, in juvenile European sea bass, sexual growth dimorphism is present in populations with mature males and immature females (Gardeur et al., 2001a) since at one year precocious males are bigger than immature females (Felip et al., 2006). In the second experiment there were no precocious males, although males had mature gonads whilst females were still immature.

In the past years, several studies have focused on European sea bass growth compensation after starvation, some of them showing full compensation after a single starvation phase (Pastoureaud, 1991). Conclusions that one can take from these studies include: 1) there is a trade-off between compensatory growth and the genetic ability of food deprivation tolerance (Dupont-Prinet et al., 2010), 2) European sea bass has a rapid response to cycling starvation/re-feeding and 3) that a 25% reduction of diet is insufficient to trigger a catch-up growth response (Turkmen et al., 2012). By contrast, in Chapter1-Experiment 1 while it is true that European sea bass quickly responded to re-feeding by increasing their SGR (in length and weight), their inherent genetic predisposition towards food deprivation tolerance/intolerance was the same. And it was the same since they came from the same parental origin but they still showed catch-up growth in different degrees after an *ad libitum* re-feeding period (Jobling, 2010). In Chapter 1-Experiment 1, catch-up growth was triggered by "only" a 20% of food reduction, which contrast with what was stated by Turkmen et al., (2012) where a 25% of food reduction was not enough to generate a compensatory growth response in European sea bass. This may be due to the fact that previous compensatory growth experiments on European sea bass were always conducted on the starvation/re-feeding basis and for short periods (from days to few weeks) while our experiment

consisted on food restriction not only during a longer period but during the critical period of sex differentiation. More studies are needed to further understand growth compensation mechanisms since European sea bass microsatellite selection based on feed efficiency after starvation (high versus low weight loss after starvation) have shown no differences in feeding efficiency and growth performance (Daulé et al., 2014).

1.2. Temperature effect on growth (Chapter 3)

Fish are poikilotherms and as such are vulnerable to environmental temperature changes that in turn regulate growth by changes in metabolic, physiological, ecological and behavioral patterns (Moyle and Cech, 2003; Person-Le Ruyet et al., 2004). Acute thermal changes affect fish physiology, with cold temperatures slowing physiological processes and warm temperatures accelerating them (Somero, 2011; Beitinger and Lutterschmidt, 2011). In European sea bass, high temperatures (25ºC) cause a decrease in swimming velocity (Koumoundouros et al., 2002, 2009; Almeida et al., 2014) hence affecting prey capture and predator avoidance (Moyle and Cech, 2003), a deterioration of fish health by increasing antioxidant enzyme function (Almeida et al., 2014), an acceleration of growth (Gardeur et al., 2001a; Koumoundouros et al., 2002; Person Le-Ruyet et al., 2004) and a switch in final sex ratios (Blázquez et al., 1998a; Pavlidis et al., 2000; Koumondouros et al., 2002; Saillant et al., 2003b; Navarro Martín et al., 2009b).

With our thermal treatments (Chapter 3) growth was strongly affected by rearing temperature with differences (P<0.05) already present when a common temperature was attained (60 dph) and with HT fish growing larger than LT (control) fish. This was in agreement with what has been previously described for European sea bass thermal treatment effects (Blázquez et al., 1998a; Pavlidis et al., 2000; Saillant et al., 2002; Koumoundouros et al., 2002; Person-Le Ruyet et al., 2004). Furthermore, when the thermal treatment is suppressed, growth rates for HT fish decreased and SL or BW differences with LT group were no longer present at one year (Saillant et al., 2002; Koumoundouros et al., 2002). Nevertheless, sustained low temperatures irreversibly affect length

and weight not only during the thermal period (Blázquez et al., 1998a; Saillant et al., 2002; Koumoundouros et al., 2002) but thereafter (Blázquez et al., 1998a) and highly masculinize the populations, since European sea bass males grow less than females (Blázquez et al., 1998a, 1999; Pavlidis et al., 2000; Piferrer et al., 2005; Navarro-Martín et al., 2009b; Navarro-Martín et al., 2011). Moreover, as previously reported for European sea bass (Person-Le Ruyet et al., 2004), cold temperatures cause a growth depression marked by a dramatic reduction of SGR values. However, these SGR differences among the thermal treatments are eliminated after raising both groups at high temperatures (at 21ºC, the SGR was about 79% lower in HT with respect to the LT group), probably due to an acclimation of LT fish to high temperatures and the subsequent increase in food intake.

1.3. Hormone effect on growth (Chapter 4)

It is known that some sex-reversal chemicals can affect fish growth rates by inducing different GnRH and somatostatin-relasing inhibitory factor patterns, hence affecting hypothalamic neurons and appetite control centers (Hassan et al., 2001). Normally, estradiol (E_2) addition affects fish growth negatively (for a list of reported cases see Król et al., 2014). Nevertheless, in other species such as the yellow perch (Malison et al., 1986; Mandiki et al., 2005) E_2 administration promoted growth by an increase in appetite, although high doses produced the opposite effect. On the contrary, E_2 produced no effect on growth when applied to European catfish (Król et al., 2014).

Previous studies in European sea bass showed that E_2 administration to control sex ratios did not affect growth (Saillant et al., 2001a; Navarro-Martín et al., 2009a; Leal et al., 2013). However, differences ($P<0.05$) between E_2-treated groups and controls were present at 170 dph between low and between high aromatase expressors in our experiment involving E_2 administration (Chapter 4), where controls were growing larger than E_2-treated fish. Afterwards, at one year, these differences had disappeared, in agreement with previous studies (Saillant et al., 2001a; Navarro-Martín et al., 2009a; Leal et al., 2013).

On the other hand, in Chapter 1-Experiment 2, E_2-treated fish grew less than controls right after the hormonal treatment (165 dph), but also at 232 dph, and these differences were maintained until the end of the experiment (337 dph). These size differences between our E_2-treated fish and previous studies on E_2 administration (Saillant et al., 2001a; Navarro-Martín et al., 2009a; Leal et al., 2013) may be due to a direct estradiol effect on diminishing growth (Piferrer, 2001; Viñas et al., 2013), or by the fact that our controls in this case had a male-biased sex ratio (Gardeur et al., 2001a).

We also treated one group of European sea bass (Chapter 1-Expermient 2) with Fadrozole (Fz), an aromatase inhibitor known to inhibit estrogen synthesis previously tested in the European sea bass (Navarro-Martín et al., 2009a). Fz has already been used to masculinize females into phenotypic males in several fish such as Pacific salmon (Piferrer et al., 1994), European sea bass (Navarro-Martín et al., 2009a), Zebrafish (Uchida et al., 2004), Nile tilapia (Kobayashi et al., 2003; Kwon et al., 2000), halibut (Babiak et al., 2012) or Japanese flounder (Kitano et al., 2000) with a variety of responses on growth, including no effects (Navarro-Martín et al., 2009a), transient early growth retardation (Babiak et al., 2012) or accelerated growth with Fz-treated fish growing larger than controls (Uchida et al., 2004; Mandiki et al., 2005 Experiment 2). These differences may be related to dosage differences (high doses of Fz administered to Eurasian perch had a negative effect on growth: Mandiki et al., 2005 Experiment 1), different periods of administration, or to differences in the sex determination and differentiation processes among these fish.

In our experiment (Chapter 1-Experiment 2) Fz-treated fish showed no differences in growth with controls neither at 165 (right after the hormone treatment) nor at 232 dph. This is in agreement with previous studies on Fz administration in European sea bass (Navarro-Martín et al., 2009a) and possibly related to the fact that Fz and control groups showed the same male-biased population. Surprisingly, at the end of the experiment (337 dph) there were no growth differences between the Fz and the E_2 treated group being both groups larger than control.

2. Sex ratios

Fish show high plasticity in response to the changing environment. This plasticity is also visible when analyzing sex ratios, the product of two processes: sex determination (Penman and Piferrer, 2008) and sex differentiation (Piferrer and Guiguen, 2008). Both processes have shown to be highly labile in fish not only in ESD species but also in GSD species with environmental influences.

In this thesis we have analyzed the effects that environmental factors such as food ration control, temperature and external treatments (E_2 and Fz) had on the final sex ratio. For that purpose, we used three families of European sea bass which showed, in their respective control groups different sex ratios (black bars in Figure 2).

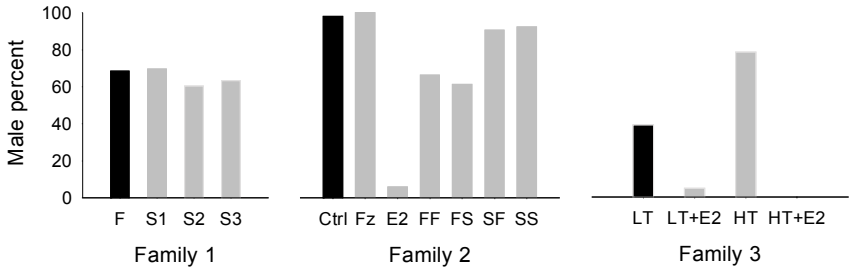


Figure 2. Sex ratio of the three families of European sea bass used in this thesis. Family 1 was used in Chapter 1, Family 2 in Chapters 1 and 2 and Family 3 in Chapters 3 and 4. Treatment abbreviations details in the corresponding chapter.

First, we analyzed the relationship between growth and sex differentiation in European sea bass during the sex differentiation period to determine whether the above mentioned relationship was established at that point or earlier. For that purpose, we altered growth rates by giving fish different amounts of food during the sex differentiation period in two different experiments: a non size-graded (F1, Chapter 1-Experiment 1) and on a size-graded population (F2, Chapters 1 and 2) under the same controlled environmental conditions. For F1, food reduction starting at three different moments during the sex differentiation period

(corresponding to fish of 8, 10 and 11 cm SL) did not alter the final sex ratio. Thus, gonadal sex differentiation does not depend on the growth rate or the attained length during the sex differentiation process.

Then a second family (F2) was used to analyze the final sex ratio after size-grading the fish. Again, altering growth rates during the sex differentiation process did not alter sex ratios, confirming the previous findings with a distinct family.

Thus, and in contrast to what was previously stated (Blázquez et al., 1999; Papadaki et al., 2005), the sex ratio of the European sea bass does not depend on the growth rates *during* sex differentiation. Instead, the sex ratio of a European sea bass population is established before the start of the sex differentiation process and we identified the 3-4 cm SL as the size range most likely established in relation to attained length, making this size an interesting point where to look for early sex-related differences. This affirmation is supported by the observation that at the moment of size-grading in Chapter 1-Experiment 2 fish from the control group had ~4 cm SL and yielded ~2% females, while F-derived groups (FF and FS) were ~6 cm SL and gave ~36% females, indicating that although no visual or histological differences could be observed at that time, molecular differences between sexes were already taking place.

Next, we analyzed the effects of thermal treatment on the sex ratio of F3 (Chapter 3). As previously observed (Blázquez et al., 1998; Pavlidis et al., 2000; Koumoundouros et al., 2002; Saillant et al., 2002; Mylonas et al., 2005; Papadaki et al., 2005; Navarro-Martín et al., 2009b), high temperature (21ºC) increased male percent by masculinizing fish that otherwise would have developed as females, while low temperature (17ºC) allowed the maximum female percent (Navarro-Martín et al., 2009b, 2011). Also part of the siblings from this family (Chapter 4) were treated with both, temperature and E_2, and E_2 managed to completely negate the masculinizing effect of high temperatures (HT-E_2 group) by completely feminizing HT fish (HT-E_2 group yielded 100% females).

A further analysis of the sex ratio of all experimental groups showed that many deviated from the Fisherian 1:1 sex ratio (Table 2).

Table 2. Sex ratio of the experimental groups used in this thesis

Experiment	Group	N	Percent males	Sex ratio differences vs. Control	Differences from 1:1 sex ratio
Chapter 1- Experiment 1	F	76	68.5	-	$P>0.05$
	S1	53	69.7	$P>0.05$	$P>0.05$
	S2	56	60.4	$P>0.05$	$P>0.05$
	S3	51	63.3	$P>0.05$	$P<0.05$
Chapter 1- Experiment 2	Ctrl	46	97.8	-	$P<0.001$
	Fz	52	100.0	$P>0.05$	$P<0.001$
	E_2	67	6.0	$P<0.001$	$P<0.001$
	FF	65	66.1	$P<0.001$	$P>0.05$
	FS	67	61.2	$P<0.001$	$P>0.05$
	SF	53	90.6	$P>0.05$	P<0.001
	SS	64	92.2	$P>0.05$	P<0.001
Chapter 3	LT	66	39.4	-	P>0.05
	HT	76	78.9	$P<0.001$	P<0.001
Chapter 4	LT-E_2	83	4.8	$P<0.001$	P<0.001
	HT-E_2	41	0.0	$P<0.001$	P<0.001

Treatment abbreviations details in the corresponding chapter.

Moreover, if we do not include thermal or hormonal treated groups, there were statistical differences ($P<0.001$) between the sex ratio of the three families.

3. Thermal and hormonal (E_2) effects on reproduction

3.1. Microarray analysis of ovarian steroidogenesis

In non-mammalian vertebrates, steroidogenic enzymes and sex steroids play a crucial role during the sex differentiation process (Bogart, 1987) and specifically, aromatase is essential for ovarian differentiation in fish (Piferrer et al., 1994; Guiguen et al., 1999; Baroiller and D'Cotta, 2001) and reptiles (Lance, 2009). In contrast, 11-β hydroxylase (*cyp11b*), an enzyme involved in 11-ketotestosterone synthesis, is involved in fish testicular differentiation (Wang and Orban, 2007; Socorro et al., 2007; Ijiri et al., 2008).

In European sea bass, expression studies on genes related to sex differentiation submitted to different thermal conditions have been conducted (Blázquez et al., 2009; Navarro-Martín et al., 2009b and 2012). It is known that high water temperatures affect aromatase expression (Navarro-Martín et al., 2011) and its catalytic activity (González and Piferrer, 2002). In contrast, elevated temperature does not appear to affect the expression of other sex differentiation related genes (*cyp11b, arb, era, erb1, erb2*) although they are detected during early development (Blázquez et al., 2009).

The analysis of the combined thermal and hormonal (E_2) effects on ovarian steroidogenesis yields interesting results (Figure 3). First, high temperatures and E_2 administration caused the downregulation of most of the enzymes present in the ovarian steroidogenic pathway, even those upstream of *cyp19a1a*, meaning that elevated temperature and E_2 treatment may affect sex differentiation through different mechanisms, including others than the DNA methylation-mediated suppression of *cyp19a1a* transcription (Navarro-Martín et al., 2011). This is reinforced by the observation that the gonadal aromatase gene promoter does not contain estrogen response elements. Thus, exogenous E_2 may be regulating other genes involved in ovarian differentiation (Piferrer and Blázquez, 2005). Furthermore, there seems to be an additive effect of temperature and estradiol since the combination of both treatments (HT-E_2) decreased GnRH and StAR expression in a significant way. Since

StAR is in charge of cholesterol translocation to the inner mitochondria, its changes suggest that, as also found for zebrafish (Urbatzka et al., 2012), steroidogenesis is blocked at its first steps. This blockage is not only dose-dependent but also species-dependent (Filby et al., 2006-2007; Nakamura et al., 2009; Urbatzka et al., 2012). In any case, E_2 effects on steroidogenesis appear to be more complicated, since *star* is also blocked in E_2-treated males (Urbatzka et al., 2012) and other downstream genes (*17B-hsd* and *cyp19a1a*) of this pathway were also affected by both treatments (Chapters 3 and 4).

igf-1 expression was upregulated by E_2 treatment but downregulated by elevated temperatures. *Igf-1* plays a central role in growth, differentiation and reproduction (Shved et al., 2007). Moreover, prolonged exposure of developing fish to exogenous E_2 is known to negatively affect growth (Jobling et al., 2002; Fenske et al., 2005), suggesting an influence of estrogens on the IGF-1 system during development (Shved et al., 2007). Experiments in tilapia (Shved et al., 2007) have shown a long-lasting effect of estrogen administration by indirectly inhibiting pituitary GH and directly suppressing IGF-1. In this thesis, we found the opposite to what has been shown in tilapia (Shved et al., 2007), since exogenous E_2 increased *igf-1* expression in ovaries, but elevated temperature caused the opposite response. Differences in our results may be due to the different applied estrogen compounds (EE_2 in Shved´s study and E_2 in our experiments) or to the fact that thermal and E_2 combination could be affecting GnRH and Insulin signaling pathways through different mechanisms.

Cholesterol is transported in blood by low- (LDL) and high-density (HDL) lipoproteins and is delivered into the cells through different mechanisms. Our microarray results showed a downregulation of LDL and HDL receptor expression by high temperature, E_2 treatment or the combination of both. In addition, HDL was downregulated due to high temperatures or the combined treatment but upregulated when E_2 was applied without temperature. Studies on mammalian HDLs (Attie, 2006) have shown that steroidogenic cells take cholesterol esters from HDL particles in a selective uptake manner, mediated by the scavenger receptor B-1 (SR-BI). Disruption of this receptor causes infertility in female mice (Miettinen et al., 2001) due to a lack of cholesterol ester supply. In

this sense, E_2 by downregulating HDL expression may be another limiting step, since as *star* blockage does, also controls the amount of cholesterol needed for the ovarian steroidogenesis process.

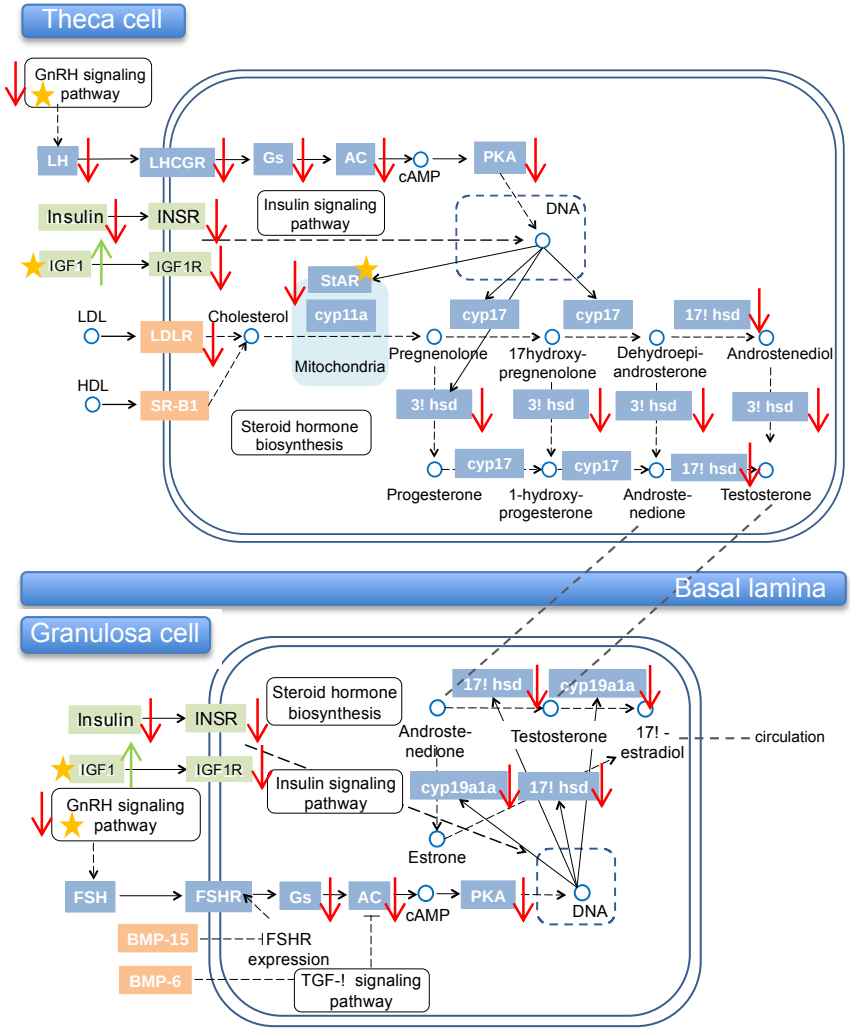


Figure 3. Environmental effects on the European sea bass ovarian steroidogenesis from a transcriptomic point of view. A KEGG pathway-based figure depicting the ovarian steroidogenesis pathway. Microarray results are marked with arrows. Green arrows pointing up indicate a fold change (FC) higher than 1.5, while red arrows pointing down indicate a FC lower than 1.5. Yellow stars denote DE genes in the microarray E_2 vs. Ctrl comparison.

3.2. qRT-PCR analysis of reproduction-related genes

qRT-PCR analysis was conducted for six reproduction-related genes: three male-related genes (*amh*: Anti-Müllerian hormone, *dmrt1*: doublesex- and mab-3-related transcription factor I, and *tesc*: tescalcin), and three female-related genes (*cyp19a1a*: gonadal aromatase, *sox17*: HMG-box transcription factor SOX17, *vasa*: vasa protein). In general, the expression of male-related genes was reduced after E_2 administration regardless of thermal treatment (Saillant et al., 2001a; Navarro-Martín et al., 2009a), while it was increased, as expected (Blázquez et al., 1999; Navarro-Martín et al., 2009b), as a consequence of elevated temperatures (Figure 4A-C). Thus, E_2 had a stronger and opposite effects with respect to elevated temperatures in changing the expression of male-related genes.

Gonadal aromatase expression (Figure 4D) was reduced after E_2 treatment and also decreased, as expected, when applying high masculinizing temperatures. With respect to the other two female-related genes (Figure 4E-F), their expression was more irregular and a pattern cannot be established.

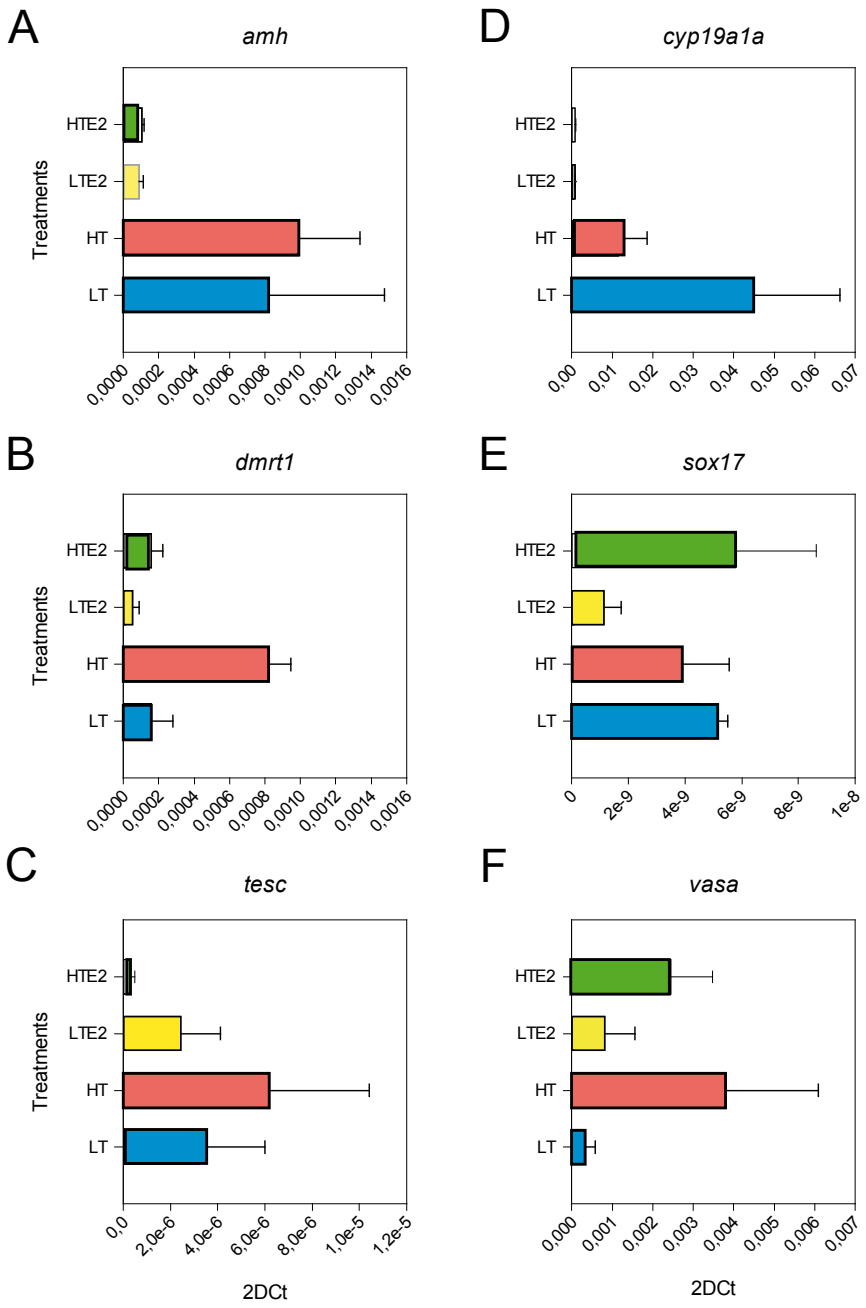


Figure 4. 2DCt values from the qRT-PCR analysis of the reproduction-related genes: A-C, male related genes (*amh, dmrt1* and *tesc*, respectively). D-F, female-related genes (*cyp19a1a, sox17* and *vasa*, respectively).

3.3. Effects of food ration on the sex steroid plasma levels

Groups F and S1 (Chapter 1-Experiment 1) had histological differences regarding the degree of gonadal maturation, and these differences were more pronounced in females. Consequently, plasma levels of testosterone (T) and 11-ketotestosterone (11-KT) were determined in males, while E_2 plasma levels were determined in European sea bass females using EIA kits (Cayman Chemical, USA). No differences in the levels of T and 11-KT were found between S1 and F males. However, E_2 plasma levels were significantly ($P<0.05$) higher in F than in S1 females (data not shown). These results suggest that differences in the degree of gonadal maturation are reflected in the corresponding plasma levels and that after a period of food restriction followed by a re-feeding period, energy was allocated to growth recovery.

T and 11-KT plasma levels were determined in the same manner in Ctrl, FF, FS, SF and SS males (Chapter 1-Experiment 2).While 11-KT levels were not affected by the growth rate manipulation during the sex differentiation process, T plasma levels were significantly higher ($P<0.05$) in SF than in SS males, thus being its levels more related to the final size of the fish since histological differences were not present.

4. Microarray pathway comparisons

Transcriptomic analysis of gene expression using high throughput technologies, such as microarrays, typically yield large lists of differentially expressed (DE) genes. However, extraction of meaningful information just from these lists of genes is not trivial. In order to understand high-level functions and get a better picture of what processes are affected by a given experimental condition, pathway analysis is required. In this thesis, we have used two programs to this end: a) Blast2GO, which is connected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), which provides a list of different types of pathways in which the DE genes are involved, and b) the DAVID Bioinformatic database, which connects with KEGG and also with the PANTHER and REACTOME databases in order to

generate more complete pathway lists (http://david.abcc.ncifcrf.gov/). The use of both databases might seem redundant but they are useful since together they cover a broad range of species and they allow for a Fisher's Exact Test with Multiple Correction of the false discovery rate (FDR). This has the objective of reducing the number of false positives and to increase the chances of identifying all the differentially expressed genes and pathways (Benjamini and Hochberg, 1995).

The analysis of the three microarray experiments conducted in this thesis has been discussed in detail in their corresponding chapters (Chapters 2, 3 and 4). In this section we attempted to compare the results from these three experiments at a glance to explore differences and similarities of the effects that the three studied environmental factors had on the European sea bass gonadal transcriptome (Figure 5).

From the different experiments presented in this thesis, four conditions were selected to be further compared by using Venn diagrams: a) the effects of high temperature on sexually differentiating gonads (HT vs. LT; Chapter 3), b) the combined effects of temperature and E_2 treatment on sexually differentiating gonads (HT-E_2 vs. HT; Chapter 4), c) sexually undifferentiated gonads from fish with different growth rates (F vs. S; Chapter 2), d) juvenile testis of males subjected to different growth trajectories (FF vs. SS; Chapter 2).

Results showed that very few pathways were common among the different experimental conditions tested. Among the 26 upregulated pathways (Figure 5A) nine were exclusively due to elevated temperature and mainly related to amino acid metabolism, eight were exclusively due to E_2 administration and mainly related to lipid metabolism, four were related to natural differences in growth rates, and one was exclusive of fish with forced opposite growth trajectories and related to drug metabolism. Moreover, there were two common pathways between high temperature comparisons, and two more between fast and slow growth rates of undifferentiated gonads and differentiated testis. (see further discussion below).

On the other hand, 43 pathways were downregulated among the four different comparisons (Figure 5B): 30 specific pathways were

altered due E_2 treatment, two were related to natural differences on growth rates and five were exclusive of fish with opposite growth rates. Among the common pathways one was due to high temperature effects, one was due to faster growth either through high temperatures or naturally fast growing fish and there were four pathways in common among the three environmental factors (see further discussion below).

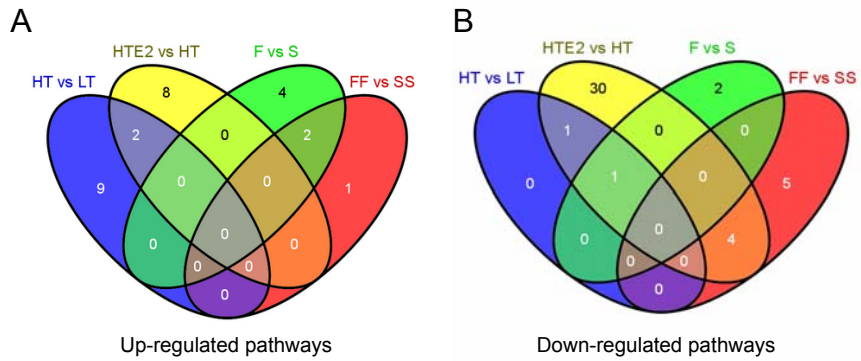


Figure 5. Venn diagrams showing the exclusive and common differentially expressed pathways among the three microarray experiments conducted in this thesis: the effects of high temperature on sexually differentiating gonads (HT vs. LT; Chapter 3), the combined effects of temperature and E_2 treatment on sexually differentiating gonads (HT-E_2 vs. HT; Chapter 4), sexually undifferentiated gonads from fish with different growth rates (F vs. S; Chapter 2), and juvenile testis of males subjected to different growth trajectories (FF vs. SS; Chapter 2).

The application of *high temperatures* (HT vs. LT) resulted in the upregulation of amino acid metabolism related to low energy reserves and to gluconeogenesis. The increase in the metabolism of some essential amino acids is also in agreement with the fact that high temperatures accelerate growth and therefore increase the energy demand. Furthermore, elevated temperatures activated the phosphatidylinositol signaling system, which is known to regulate a vast variety of signal transduction processes such as growth, apoptosis, cell migration, endocytosis, cytoskeletal reorganization, membrane transport or cell proliferation and differentiation (Nelson and Cox, 2013).

The *E_2 treatment* (HTE_2 vs. HT) on one hand resulted in the upregulation of the lipid and fatty acid metabolism related to the increase in the energy demand due to the high temperatures and, on the other hand, generated a downregulation of sugar metabolism, steroid and steroid hormone biosynthesis pathways as well as the mTOR signaling pathway that is known to favor catabolic processes during stress conditions (Nelson and Cox, 2013). Furthermore, they show a common thermal response (with HT treatment) by downregulating the T-cell receptor signaling pathway and then shutting down the immune system response what has been associated with reproduction processes.

Contrasting growth rate differences on undifferentiated gonads showed, opposite to what was expected, no sex-related differences but a high energy demand of the F group when compared to the S group. This energy demand is exemplified by an upregulation of the ketone body metabolism, a mechanism active after the glucose and fatty acids are no longer available as a source of energy (Nelson and Cox, 2013). This fact is reinforced by the downregulation of two pathways related to lipid metabolism, which were linoleic acid and alpha-linoleic acid metabolism. Also fast growing fish showed an increase of two pathways related to detoxification, the drug-metabolism through cytochrome P450 and the metabolism of xenobiotics.

Pathways exclusive of juvenile testis from males subjected to different growth trajectories (FF and SS groups). Growth rates differences on differentiated testis were also smaller than expected. While FF testis were characterized by a high transcription levels or DNA binding, SS testis were undergoing proteolysis, protein modifications and processes related to the fatty acid metabolism. Being these differences markedly related to their differences in food availability (opposite natural growth rates before the size-grading plus opposite feeding regimes during and after the sex differentiation period). Although despite their differences, they shared two pathways that were overexpressed in fast growing fish (no matter the age) when compare to slow growing fish: the glutathione and xenobiotic metabolisms. Glutathione plays an important role in antioxidant defense, nutrient metabolism and regulation of cellular events such as gene expression, immune response or signal transduction (Wishart et al., 2010). The above

mentioned functions of glutathione are in accordance with an accelerated growth scenario, since in a non-limiting environment, fish tend to show growth rates near to its maximum potential conditions. Furthermore, the xenobiotic metabolism is also connected to the glutathione one, since it is in charge of detoxification of external lipophilic compounds in three phases: modification by introducing reactive and polar groups (one of the most common modifications being a hydroxylation by the cytochrome P450 oxidase system), conjugation with an endogenous substrate to form a highly polar conjugate (such as glutathione or the methyltransferase S-adenosyl-L-methionine) and a third and final step of further modification and excretion.

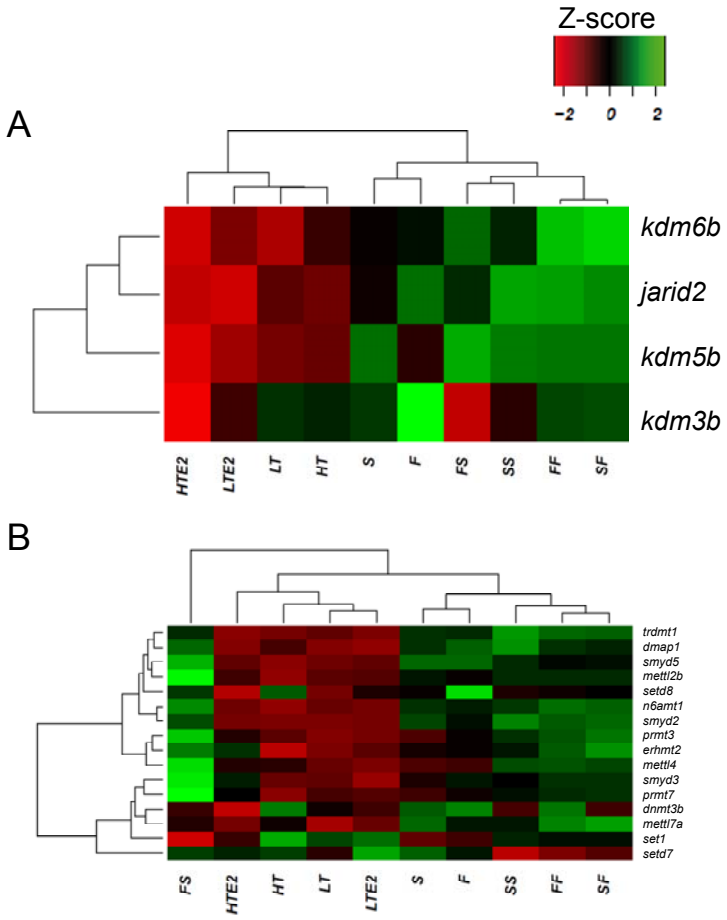
5. Epigenetic regulatory mechanisms-related genes

Epigenetic mechanisms allow organisms to integrate genomic and environmental information on a particular phenotype (Turner, 2009). Thereby and based on previous results from our laboratory, where the European sea bass gonadal aromatase promoter was found to be differentially methylated in a gender- and temperature-dependent manner at one year but not necessarily at 170 dph (see General Introduction), we conducted a screening of the genes related to epigenetic regulatory mechanisms for which there is a direct or indirect evidence of a possible role in sex determination/differentiation in other models (Piferrer, 2013) and that were present in our custom-made European sea bass microarray.

First, we compiled a list of such genes and examined their behavior in the different microarray comparisons carried out in this thesis regardless of whether they were DE or not. In Figure 6, median corrected values of genes subdivided according to their function and treatment group are shown on a heatmap shape.

Results of these selected genes are based on the corrected median values of the different probes present in the microarray for the different genes and taking into account a Fold Change (FC) of 1.2 and an adjusted *P*-value ≤ 0.05. In general, thermal or hormonal

(E_2) treatments produced a downregulation of the genes belonging to the category of demethylases, helicases, histone deacetylases or methyltransferases. In contrast, genes related to the polycomb group were, in general, upregulated by heat but downregulated by E_2. qRT-PCR analysis of six epigenetic regulatory mechanisms-related genes (*dicer1, ehmt2, jarid2a, pcgf2, hdac11* and *suz12*) showed this general tendency in upregulation in response to heat. This illustrates long-lasting consequences of early elevated temperatures on the epigenome, although per se they do not provide an answer on how the temperatures elicit a sustained response that is still maintained when animals are one year old. Thus, the meaning of these modifications need further study.



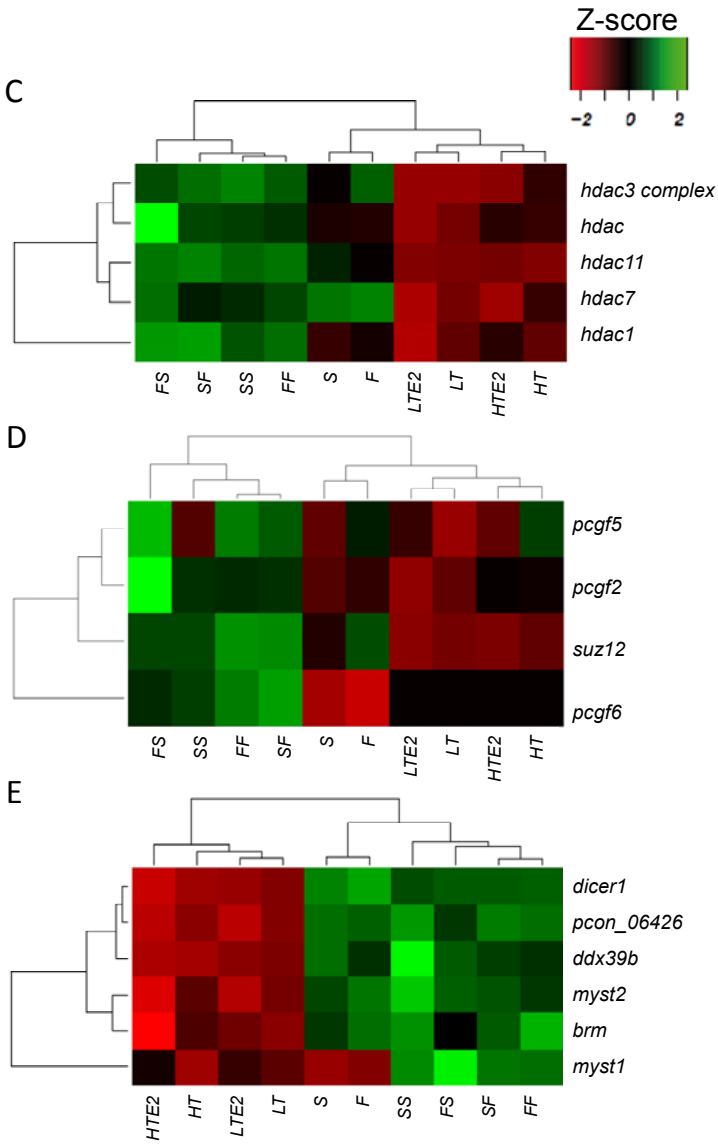


Figure 6. Heatmap analysis of the genes related to epigenetic regulatory mechanisms present in the European sea bass custom microarray used in this thesis, comparing the three experiments together and an extra experimental group (LTE_2) that consisted on fish grown at LT temperatures and fed with E_2-sprayed dry food. Genes were subdivided according to their functions: A) genes with a demethylase function, B) genes with a methyltransferase function, C) genes with a histone deacetylase function, D) polycomb complex genes and E) a mix of dicer 1 related genes (*dicer1* and *pcon-06426*), helicases (*brm* and *ddx39b*) and histone acetyltransferases (*myst1* and *myst2*) genes.

Table 3. Epigenetic regulatory mechanisms-related genes

Gene abbreviation	Description
A. demethylases	
jarid2	jumonji, AT rich interactive domain 2
kdm3b	lysine-specific demethylase 3B
kdm5b	lysine-specific demethylase 5B
kdm6b	lysine-specific demethylase 6B
B. methyltransferases	
dmap1	DNA methyltrasnferase 1 associated protein 1
dnmt3b	DNA methyltransferase 1 associated protein 1
ehmt2	euchromatic histone lysine N-methyltransferase 2
mettl2b	methyltransferase-like protein 2B
mettl4	methyltransferase-like protein 4
mettl7a	methyltransferase-like protein 7A
n6amt1	hemK methyltransferase family member 2
prmt3	protein arginine N-methyltransferase 3
prmt7	protein arginine N-methyltransferase 7
set1	putative histone-lysine N-methyltransferase
setd7	histone-lysine N-methyltransferase SETD7
setd8	N-lysine methyltransferase SETD8
smyd2	N-lysine methyltransferase SMYD2
smyd3	histone-lysine N-methyltransferase SMYD3
smyd5	*SET and MYND domain-containing protein 5*
trdmt1	tRNA (cytosine(38)-C(5)-methyltransferase
C. histone deacetylases	
hdac	class 4 HDAC protein
hdac1	histone deacetylase 1
hdac11	histone deacetylase 11
hdac3 complex	nuclear receptor co-repressor/HDAC3 complex subunit
hdac7	histone deacetylase 7
D. polycomb complex	
pcgf2	polycomb group RING finger protein 2
pcgf5	polycomb group RING finger protein 5
pcgf6	polycomb group RING finger protein 6
suz12	polycomb protein SUZ12

E. mix	
brm	ATP-dependent helicase BRM
ddx39b	spliceosome RNA helicase DDX39B
dicer1	endoribonuclease Dicer
myst1	MYST histone acetyltransferase 1
pcon_06426	similar to protein kinase/endoribonuclease (IRE1)

Demethylases (Figure 6A): the expression pattern depicted by the heatmap analysis clearly shows that their expression pattern is more related to family than to treatment (Family 3 groups on the left; Family 2 on the right) or age. Next, age at sampling rather than treatment subdivides groups within Family 2. Finally, it is interesting to note that groups of experiment 3 cluster according to growth rates at the time of sampling. It is also interesting to note the inhibitory effect of E_2 on DNA demethylase expression, which is in accordance with the general downregulation of gene expression observed in the E_2 treated group.

Methyltransferases (Figure 6B): in general, the expression pattern, although with some exceptions, seems to follow the one for DNA demethylases. However, the FS group, has a completely different pattern of expression, as previously described for the other transcriptomic comparisons (Chapter 2), suggesting that decreasing growth rates in general upregulate these genes. Since DNA methylation is believed to be related to transcriptional inhibition, these results may reflect the lack of proper energy supply to prepare the juvenile testis for reproduction, thus suppressing gene expression and conserving energy for non-reproductive functions.

Histone deacetylases (Figure 6C): histone deacetylase expression patterns appears to be more related to age or to the degree of differentiation (the two possibilities cannot be resolved apart) than to treatment, since undifferentiated gonads (groups F and S) sampled at 134 dph cluster with sexually differentiating gonads of fish treated with either temperature or E_2 (170 dph gonads), albeit with different polarity of gene expression, but distinct from juvenile testis (332 dph). Next, temperature has more clustering power than E_2 treatment. Finally, within juvenile testis the behavior of the FS group is again highly different from that of the rest of the groups.

Polycomb complex genes (Figure 6D): their expression pattern follows that of the histone deacetylases (discussed above). In this case, however, the F and S groups behave in the same direction as the groups with sexually differentiating gonads.

Mixed group (Figure 6E): this group is formed by genes of the following three functions: dicer 1 (*dicer 1* and *pcon-06426*), helicases (*brm* and *ddx39b*) and histone acetyltransferases (*myst1* and *myst2*). The expression pattern matches that of DNA methyltransferases and DNA demethylases, i.e., the clustering is dependent first on family, then on age and finally on treatment. The combination of E_2 and high temperature treatment is separating HT-E_2 group from the rest, as age is subdividing family 2 into undifferentiated and differentiated gonads. Moreover, SS testis showed more transcriptomic differences when compared to the other differentiated groups. Also there is an exception to this generalization, since the expression pattern of *myst1* is first dependent on family but also on the age of the individuals (F and S expression cluster with that of the undifferentiated gonads from Chapter 3 and 4 experiments).

In conclusion, here we have shown that the expression patterns of a suite of genes related to epigenetic regulatory mechanisms have different degrees of dependencies according to their functional category, which is interesting. Thus, while the expression patterns of genes related to DNA methylation and demethylation are more related, in that order, to genetic background, developmental time and finally to external influences, on the other hand, genes related to histone modifications and the polycomb complex are more related to development, genetic background and finally to treatment. To the best of our knowledge, such broad metaanalysis, combining two genetic backgrounds, three developmental times, four environmental conditions and four gene categories, has not been made before in any organism. It is clear that more research is needed to better understand the patterns identified here and, more importantly, the consequences for the actual epigenetic regulation of genes implicated in essential biological functions including those that have consequences for aquatic production. We believe that the information provided here represents a good foundation for future studies.

Conclusions

1. Parental and thermal influences aside, the sex ratio in the European sea bass does not depend on the growth during or after the sex differentiation period but it is established before. Sex-related differences at the molecular level are most probably established when fish attain a length of 3–4 cm.

2. European sea bass displays different catch-up growth and gonadal maturation responses if there is food restriction during the sex differentiation period. Food restriction at the onset allows a complete recovery in growth while causing gonadal development retardation. On the contrary, food restriction at the end of the sex differentiation period does not affect gonad development but prevents full growth recovery. Thus, growth recovery has priority over reproduction if growth is affected early, while the opposite is true if growth is affected later. This indicates the existence of a threshold in size ~10 cm, where only above it is worth investing in reproduction.

3. Differences in growth prior to the onset of sex differentiation do not translate into substantial gonadal transcriptomic differences. Regardless of the degree of gonadal development, slow-growing fish gonadal transcriptomes exhibit an altered protein turnover with a higher catabolism, represented by a reduction in transcription and translation, a decreased immunological response, and a metabolism based on lipids and gluconeogenesis. On the other hand, fast-growing fish show enhancement of anabolic processes such as transcription, translation, protein synthesis and elongation, and a metabolism based on glucose.

4. Transcriptomic analysis of 11-month-old juvenile testes, suggested that fish are able to recover from an initial slow growth if during the sex differentiation period the access to food is unrestricted. However, the opposite is not true since, in naturally fast-growing fish, a "good start" does not provide any advantage in terms of transcriptomal fitness in case of food restriction during the sex differentiation period. These results have implications for natural fish populations subjected to fluctuating food supply as well as for populations of farmed fish under suboptimal feeding regimes.

5. Exposure to elevated temperatures during early development sets permanent changes that result in long-lasting consequences that can reprogram the sexual phenotype. Transcriptomic analysis of sexually differentiating gonads shows that the masculinization by elevated temperatures involves not only the upregulation of male-related genes but also a general downregulation of the genes involved in the steroidogenic pathway related to both testicular and ovarian differentiation.

6. The examined genes related to epigenetic regulatory mechanisms were upregulated by elevated temperature, which has deleterious effects on gonadal development. Although the significance of this overall upregulation is at present unknown, the fact that these genes share gene transcription repression functions suggests that the permanent deleterious effects of elevated temperatures involve transcription repression through epigenetic processes.

7. Exposure to exogenous estrogen during early juvenile development has profound reprogramming effects on the

gonadal transcriptome of sexually undifferentiated fish, affecting both up- and downstream genes within the steroidogenic pathway of developing females, and resulting in the complete feminization of the population. However, at one year of age, the gonadal morphology is undistinguishable from that of untreated females, suggesting that once the female phenotype is imposed by estrogen, gonads can continue normal development.

8. The expression patterns of a suite of genes related to epigenetic regulatory mechanisms have different degrees of dependencies according to their functional category. Thus, while the expression patterns of genes related to methylation and demethylation are more related, in that order, first to genetic background, then to developmental time and finally to external influences, on the other hand, genes related to histone deacetylation and the polycomb complex are more related to, first, development, then to genetic background, and finally to external influences. To the best of our knowledge, such broad metaanalysis, combining genetic backgrounds, developmental times, environmental conditions and four types of functional gene categories, has not been made before in any organism. It is clear that more research is needed to understand better the patterns identified here and, more important, the consequences for the actual epigenetic regulation of genes implicated in essential biological functions, including those that are relevant for aquatic production. We believe that the information presented here provides a good foundation that warrants future studies.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This thesis was sent to print on July 17th 2014, Barcelona