# 5S rRNA and Accompanying Proteins in Gonads: Powerful Markers to Identify Sex and Reproductive Endocrine Disruption in Fish

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## ABSTRACT

In anuran ovaries, 5S rDNA is regulated transcriptionally by transcription factor IIIA (TFIIIA), which upon transcription, binds 5S rRNA, forming 7S RNP. 5S rRNA can be stockpiled also in the form of 42S RNP bound to 42sp43. The aim of the present study was to assess the differential transcriptional regulation of 5S rRNA and associated proteins in thicklip gray mullet (Chelon labrosus) gonads. Up to 75% of the total RNA from mullet ovaries was 5S rRNA. qPCR quantification of 5S rRNA expression, in gonads of histologically sexed individuals from different geographical areas, successfully sexed animals. All males had expression levels that were orders of magnitude below expression levels in females, throughout an annual reproductive cycle, with the exception of two individuals: one in November and one in December. Moreover, intersex mullets from a polluted harbor had expression levels between both sexes. TFIIIA and 42sp43 were also very active transcriptionally in gonads of female and intersex mullets, in comparison to males. Nucleocytoplasmatic transport is important in this context and we also analyzed transcriptional levels of importins-α1, -α2, and -β2 and different exportins. Importin-αs behaved similarly to 5S rRNA. Thus, 5S rRNA and associated proteins constitute very powerful molecular markers of sex and effects of xenosterogens in fish gonads, with potential technological applications in the analysis of fish stock dynamics and reproduction as well as in environmental health assessment.

## INTRODUCTION

The estimation of maturity stage and sex ratio of fish stocks in European waters is a requirement of the EU Data Collection Framework, as part of the policy to improve fisheries management (https://datacollection.jrc.ec.europa.eu). In the understanding of fish stock dynamics, it appears relevant to study possible sex-related differences in mortality, growth, reproduction, and migration patterns. Presently, the determination of maturity and the identification of sex in fish from natural populations is laborious, requiring a large number of individuals for visual/histological analysis of gonads, which can be challenging when oceanographic campaigns are not coincident with the spawning season.

Similarly, pollution monitoring and environmental health assessment are progressively increasing the focus on deciphering the mechanism of action of chemical compounds. (1) Molecular pathways relevant under chemical exposure, as studied using “omic” technologies, will commonly vary substantially between female and male individuals. (2) Therefore, there is a need to identify the individual sex of the studied pollution sentinel organisms; thus, reduce the noise introduced by sex, in biomarker response profiles. On the other hand, many xenobiotics and fluctuating environmental factors (i.e., temperature, $O_2$ availability, or pH) have important effects in sex determination/differentiation in many fish

species. (3) Some chemical compounds, known as endocrine disruptors, alter reproduction and development by changing hormonal homeostasis; in turn, altering gene expression related to reproduction, or interacting with hormonal signals. (4-6) Thus, changes caused by xenobiotics can be harmful not only for the health of the individual concerned, but also for its reproductive capacity significantly reducing the viability of affected populations, and potentially resulting in their disappearance. (6, 7)

Several cases of reproductive endocrine disruption have been described in aquatic vertebrates. The most comprehensive effect relates to the process of feminization of fish populations exposed to xenoestrogens. (3, 4, 6, 8) Recent studies undertaken in the Biosphere Reserve of Urdaibai have described the presence of intersex thicklip gray mullets, *Chelon labrosus*, with the presence of oocytes at different maturation stages in between sperm follicles (unpublished results). Similar xenoestrogenic effects have been reported in other mullet populations in contaminated estuaries. (8) Thus, the mullet *C. labrosus*, a fish with a wide distribution in European littoral/estuarine waters, constitutes an important sentinel for the study of pollution effects (9) and, more specifically, for biomonitoring endocrine disruption.

In teleosts, sex-determining genetic systems are diverse and involve either a polygenic control, dominant sex-determining factors combined with autosomal controls, or a sex chromosome control (XX/XY or ZZ/ZW) depending upon the species. (3) The process of sex determination is mainly about triggering a cell-differentiation process so as to create oocytes or sperm cells from undifferentiated primordial germ cells. Since spermatogenesis and oogenesis differ in their developmental gene transcription pattern, molecular biological techniques offer opportunities to molecularly identify sex in fish gonads. In fish, gene expression profiling during the first morphological changes of the differentiating gonad, has revealed many genes with sexually dimorphic transcription patterns. (5, 10-12) Estrogen treatments applied to male embryos during critical periods of development induce a phenotypic gonadal sex change from testis to ovary. (10, 12) Gonad *cyp19a1*, coding for the aromatase responsible for estrogen production, is the first unambiguous marker of ovarian differentiation. (12) Instead, during testicular development *cyp19a1* is downregulated.

In the process of oocyte differentiation/maturation, several molecules participate in the control of oocyte growth. These molecules will be incorporated from surrounding ovarian follicular cells, or from peripheral organs, such as the liver or muscle. This is the case of vitellogenin, synthesized specifically in female liver. Its expression in male liver has been implemented by organizations such as ICES or OECD as a biomarker of fish stock exposure to xenoestrogenic compounds. (7, 13, 14) Other molecules are produced by the oocyte itself such that, at the end of the process, the oocyte becomes competent to undergo fertilization; contains maternal mRNAs, RNAs, proteins, lipids, carbohydrates, vitamins, and hormones, which are important for the proper development of the embryo. (15, 16) One molecule involved considerably in this process is 5S rRNA. In the case of amphibian oocytes, rDNA is amplified ≈1000-fold early in oogenesis. (17) 5S rRNA expression changes during oogenesis, with the activity increasing dramatically during early vitellogenesis whereas transcription is slowed down in later stages.

Two different types of 5S rDNA have been found in *Xenopus*; one expressed in somatic cells and the other one only in oocytes. (17, 18)*Xenopus* oocytes transcribe massive amounts of the gonad specific gene during oocyte maturation; this is something that does not occur in male gonads. (17, 18) This system of paralogous 5S rDNA genes has been confirmed also for the teleost fish *Tinca tinca* (19) and has been assumed to occur in other fish species. Mazabraud et al. reported in 1975 (20) high amounts of tRNA and 5S rRNA in ovaries of 9 teleost fish species. In 4 of these species, tRNA and 5S rRNA constituted more than 90% of the RNA content of the ovaries; as in *X. laevis* oocytes, it was stored in ribonucleoprotein particles (RNPs) of different size. (20) Although these observations have been overlooked since 1975, there are indications

that 5S rRNA could constitute a sensitive and universal marker of oogenesis and oocyte differentiation in fish. As an oocyte marker, 5S rRNA could have also important implications in sexing teleost species and in identifying reproductive endocrine disruption, leading to the appearance of intersex males. Here, our aim was to clarify whether 5S rRNA and the proteins that participate in its transcriptional control, stockpiling, and nucleo-cytoplasmic transport are regulated transcriptionally in a sex-specific way in ovary and testis of *C. labrosus*, throughout a complete annual reproductive cycle.

## MATERIALS AND METHODS

*Reagents*

All chemicals were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless specified otherwise.

*Animals and Experimental Procedure*

Mature thicklip gray mullets *Chelon labrosus* (34.22 ± 5.4 cm long and 485.28 ± 203.56 g weight) were collected from 5 different estuarine/harbor locations in the Bay of Biscay: Bilbao (43°20′24″ N; 3°0′45″ W); Plentzia (43°24′21″ N; 2°5647″ W); Urdaibai (43°23′42″ N; 2°40′48″ W); Ondarroa (43°19′19″ N; 2°25′10″ W); and Pasaia (43°19′18″ N; 1°55′53″ W). Mullets were collected in Ondarroa in May 2009, in Bilbao, Plentzia, Urdaibai, and Pasaia in July 2010, and then they were collected in Pasaia every month until May 2011. Pasaia was selected due to the wide availability of mullets and the high-polluting chemical burdens, including endocrine disruptor compounds. (21)

Nine-months-old adult zebrafish from our own stock were sampled also in April 2011. Similarly, adult European hakes (*Merluccius merluccius*), purchased from local fishermen on the same day they were angle-fished, were sampled in April 2011.

Mullets and zebrafish were anesthetized by immersion in a saturated solution of 3-aminobenzoic acid ethyl ester before being sacrificed for gonad dissection. Gonads were dissected as soon as possible after capture and weighed. A portion of each gonad was submerged in RNAlater (Sigma-Aldrich), frozen in liquid nitrogen, and then stored at −80 °C. Portions of each dissected gonad were also fixed in 10% neutrally buffered formalin containing 1% glutaraldehyde.

*Extraction of Total RNA and Capilary Electrophoresis*

Aliquots of 50–100 mg of gonad, from 5 females and 5 males per sampling point and month (from the monthly samplings in Pasaia only 7 months were analyzed) were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), using a Hybaid Ryboliser for 20 s at 4 m/s. Total RNA was also extracted from all intersex males identified for the harbor of Pasaia throughout the studied annual cycle (see Supporting Information (SI) for additional information). The same amount of RNA (250–500 ng, depending upon the species and month), as estimated through absorbance at 260 nm, was loaded in an Agilent 2100 Bionalyzer using RNA Nano LabChips (Agilent Technologies, Santa Clara, CA, USA) and used to estimate RNA quality and size distribution.

*Obtention of Sequences of Interest*

Sequences of genes of interest were obtained from a previous 454 sequencing of a multitissue normalized cDNA of *C. labrosus* (unpublished data). The obtained sequence information was searched though Blast analysis to annotate genes of interest in relation to 5S rRNA transcription, stockpiling, and nucleo-cytoplasmic transport. Gene sequences homologous to known vertebrate transcripts were found, in some circumstances belonging to different regions of a same gene. In such cases, specific primers were generated to lengthen the amount of sequence

information for those genes (see SI). Primer sequences and PCR conditions are depicted in SI Table S1.

*Real-Time RT-PCR*

Two µg of total RNA was used for cDNA synthesis by RT-PCR with SuperScript II (Invitrogen) using random hexamers. For each sample, SYBR Green real-time qPCR was undertaken in triplicate, using a 7300 Applied Biosystems Thermocycler (Applied Biosystems, Carlsbad, CA, USA). The 20-µL PCR reaction consisted of 10 µL of 2× SYBR Green PCR master mix (Roche, Basel, Switzerland), 10 µL of appropriate forward and reverse primers diluted in RNase-free water, and 2 µL of cDNA template. No-template well controls were performed also for each master mix prepared. Primer and template concentrations were optimized previously (Table S2). Specificity of amplification was checked by agarose electrophoresis after 40 cycles of amplification and through sequencing. In the case of *tfIIIa*, quantification was achieved using a custom Taqman assay (Applied Biosystems). Full description of the methodology is presented in SI.

*Gonad Histology*

Gonads of 30 animals per sampling were fixed as described above. Samples were stored for 24 h in fixative, then dehydrated in a graded series of ethanol, and finally embedded in methacrylate resin (Technovit 7100; Heraeus Kulzer GmbH & Co. KG, Wehrheim, Germany). Resin sections (3 µm) were cut in a 2065 Supercut microtome (Leica Instruments GmbH, Nussloch, Germany). Subsequently, sections were stained with hematoxylin/eosin. Sex and gamete developmental stage were determined following the gametogenic stage grading for mugilids, as described by McDonough et al. (22) Five gonad reproductive stages were distinguished: 1 = immature; 2 = developing; 3 = running, ripe; 4 = atretic or spent; and 5 = inactive or resting. In addition, gonadosomatic index was calculated for each fish.

*Statistical Analysis*

The statistical analyses were undertaken using SPSS v. Thirteen (SPSS Inc., Chicago, Illinois). Significant differences in gene transcription levels between exposure groups were evaluated using the nonparametric Mann–Whitney's U-test and significant differences were established at $p < 0.05$.

## RESULTS

*Sequencing of Chelon labrosus 5S rRNA Related Sequences*

Sequencing of the multitissue normalized transcriptome of *C. labrosus*, through the 454 pyrosequencing approach and posterior data assembly, resulted in the annotation of more than 15 000 gene fragments (unpublished data). Among the annotated cDNA sequences we assembled fragments, or complete-coding domain sequences (cds), belonging to mullet transcription factor III A (*tfIIIa*), *42sp43*, importins *impα1*, *impα2*, *impβ2*, exportins *exp1*, *5*, *6*, and *7,* and piwi like protein coding genes *piwil1* and *piwil2*. They all show high homology with the limited number of available fish sequences and with homologue vertebrate (anuran, mammalian) sequences (Table 1).

**Table 1. Sequenced Gene Fragments, with the GenBank Accession Number Corresponding to Each Fragment, and e-Values Obtained with the Most Similar Available Homolog Protein Sequence, through BlastP Analysis**

| BlastP | | | | |
|---|---|---|---|---|
| **gene** | **ac number** | **species** | **e-value** | **length (bp)** |
| *Transcription factor III A* | JN257141 | *Ictalurus punctatus* (NP_001157205) | 2e-52 | 586 |
| *RNA binding protein 42sp43* | JN257142 | *Oryzias latipes* (NP_001098333) | 4e-45 | 366 |
| | — | | 2e-14 | 209 |
| *Importin α1* | JN257138 | *Pagrus major (*BAF36663) | 0.0 | 1672 (whole cds) |
| *Importin α2* | JN257139 | *Oreochromis niloticus (*AAD51751) | 0.0 | 1587 (whole cds) |
| *Importin β2* | JN257140 | *Homo sapiens* (NP_002261) | 0.0 | 1955 |
| *Exportin 1* | — | *Salmo salar* (ACN58686) | 2e-32 | 181 |
| *Exportin 5* | JN257146 | *Danio rerio* (XM_001921422) | 0.0 | 2365 |
| *Exportin 6* | JN257147 | *Equus caballus* (XP_001915933) | 0.0 | 1670 |
| *Exportin 7* | JQ684437 | *Danio rerio* (NP_001121702) | 9e-37 | 241 |
| | JQ684438 | *Homo sapiens* (BAA34465) | 2e-30 | 200 |
| *Piwi like protein 1* | JN257143 | *Oryzias latipes* (NP_001153908) | 3e-73 | 494 |
| | JN257144 | | 2e-24 | 219 |
| *Piwi like protein 2* | JN257145 | *Oncorhynchus mykiss* (NP_001117714) | 0.0 | 1262 |

[a]Length of each sequenced fragment is provided in base pairs. – means that exportin 1 sequence, being shorter than 200 nucleotides in length, has not been published in GenBank.

*5S rRNA as Sex Marker in Fish Gonads*

Total RNA extracted from female mullets, captured in May 2009 in Ondarroa and analyzed through electrophoresis, showed a distinctive single band around 120 bp (Figure 1). It constituted 75% of the total RNA content in the transcriptome of ovaries, as measured in the electropherogams provided by the Bioanalyzer (Agilent). This band, identified as belonging to 5S rRNA, was not conspicuous in somatic tissues, where a normal pattern of predominating 18S and 28S rRNA bands was observed.

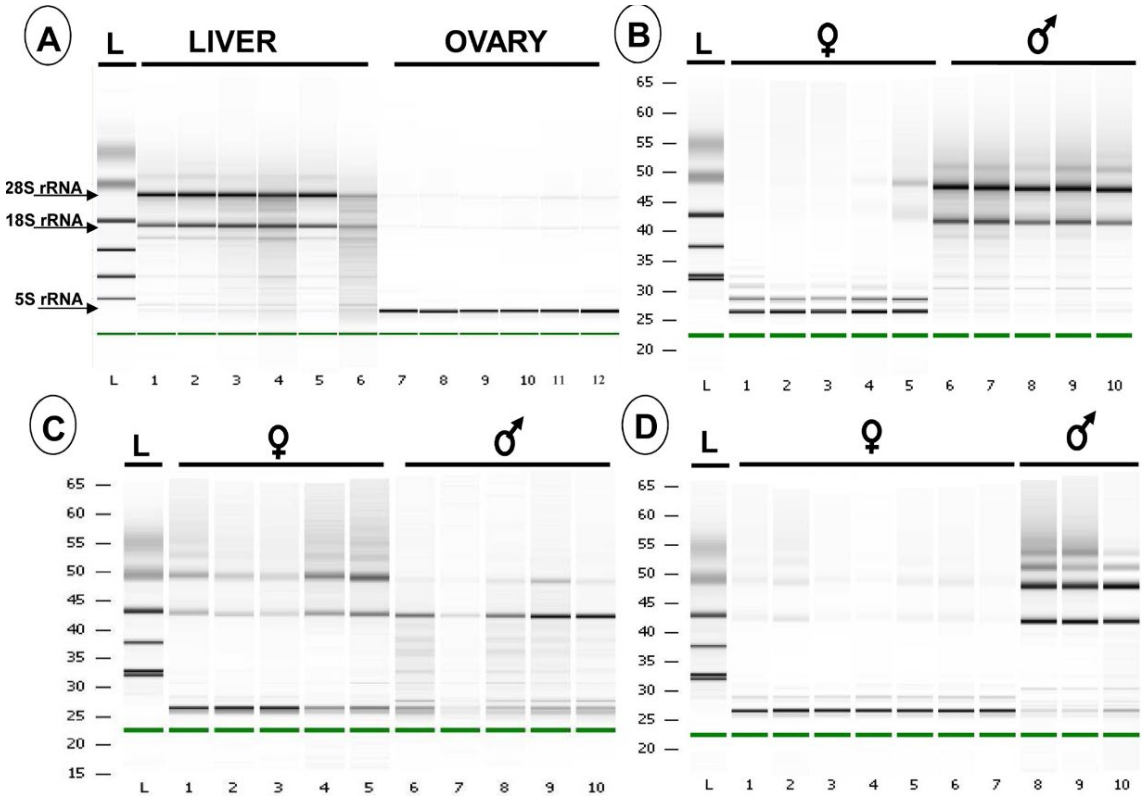


**Figure 1.** Total RNA analyzed through polyacrylamide electrophoresis, using the Agilent Bionalyzer 2100. (A) Band distribution of the total RNA extracted from 6 thicklip gray mullets *Chelon labrosus* (livers and ovaries), collected in the harbor of Ondarroa in 2009. Strongest bands in liver correspond to the 28 and 18S rRNA while strongest band in ovary belongs to 5S rRNA. (B–D). Different RNA band distribution in histologically sexed male and female gonads of mullets captured in July 2010 (B), zebrafish *Danio rerio* (C), and European hake *Merluccius merluccius* (D). L = molecular weight markers.

The sex-specific expression of 5S rRNA was studied through electrophoresis and qPCR analysis in the gonads of mullets captured in July 2010 in four estuaries with different pollution burdens and with different feeding and tidal regimes. 5S rRNA was expressed strongly (Figure 2) in ovaries of all animals tested, but not in testes (6–10 cycles of difference). Mullets from the polluted harbor of Pasaia were selected for analysis of 5S rRNA expression levels through an annual reproductive cycle. 5S rRNA was expressed strongly in females, but not in males, througout the reproductive cycle (Figures 1 and 2). The developmental stage of the gonads and the gonadosomatic index (Figure S1) demonstrated that spawning occurred in winter, while immature stages predominated in spring and summer. 5S rRNA could be used to discriminate between females and males in all cases, except one female individual in November and a second outlier in December. Results are shown in Figure 2, as CT counts for clear visualization of differences between sexes. Normalization to *ef-1α* as housekeeping gene did not introduce any significant alteration (Table S3) but allowed corroboration of robustness of the data and their interpretation, while keeping to terms with common practice in qPCR studies.

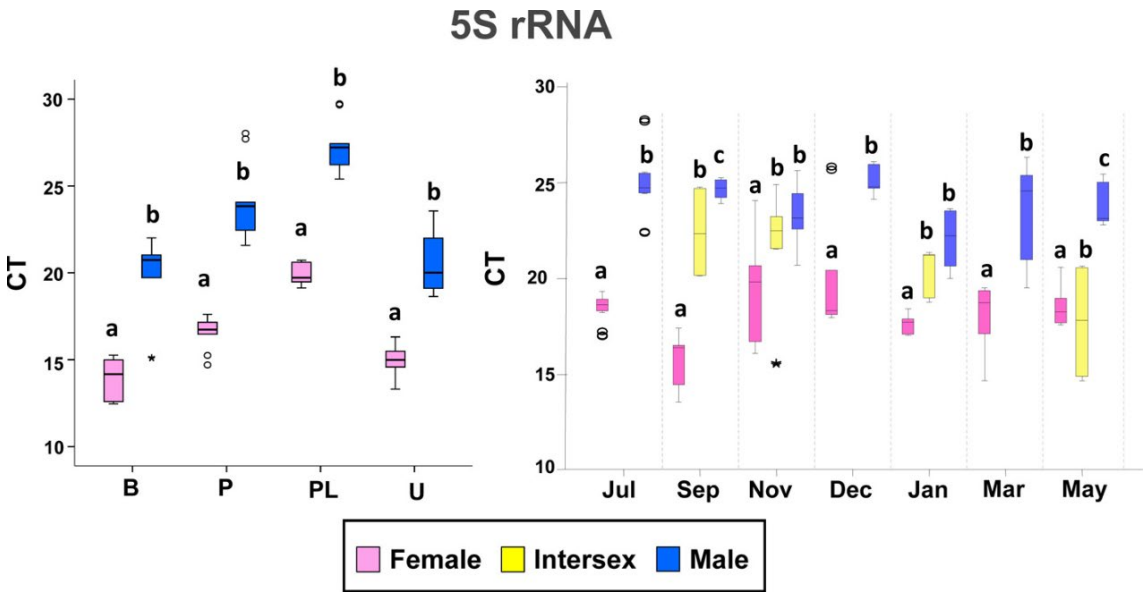


**Figure 2**. Box plots representing *5S rRNA* gene transcription levels in *C. labrosus* collected in 4 Basque estuaries in July 2010 (B = Bilbao, P = Pasaia, PL = Plentzia, U = Urdaibai) and in Pasaia throughout a complete annual reproductive cycle. Different letters indicate significant differences (Mann–Whitney U-test, $p < 0.05$) between sexes (histologically sexed) at each site and during each month ($n = 5$, each individual in triplicate in the annual study and in duplicate in the site study). Box-plots represent the data within the 25th and 75th percentiles, with the median indicated by a line. Whiskers include all data except outliers, which are identified by dots (defined as any data between 1.5 and 3 box lengths) and asterisks (extreme outliers). Data are represented as cycle thresholds for a clear visualization of differences between females and males. Data normalized to housekeeping genes as required for an adequate qPCR study are provided in Table S3 and confirm results shown here.

To examine the usefulness of 5S rRNA to determine sex in fish, the 120 bp band in the gonads of histologically sexed adult zebrafish (*Danio rerio*) and European hake (*Merluccius merluccius*) was analyzed. As in mullets, female individuals had a distinctive and strong sex-specific expression of 5S rRNA in all cases (Figure 1). Electropherograms are provided for zebrafish in Figure S2, for an improved discrimination of females and males. 5S rRNA transcription levels were analyzed also, through real-time PCR in zebrafish, and significant differences were observed between females and males (CT values 21.8 ± 0.37 in females and 25.07 ± 1.2 in males; $p < 0.05$)

*5S rRNA Genes and Intersex Gonads*

Males with intersex gonads were sampled in Pasaia in September, November, January, March, and May. Through qPCR analysis these individuals were found to have 5S rRNA expression levels between females and males, although the difference to males was only significant in September (Figure 2 and Table S3). A detailed analysis revealed that the melting curves of the resulting final amplicons were different in somatic tissues and testes in comparison to ovaries. The melting curve in the ovary was 80.5 °C, while in somatic tissues and testes it was 81.5 °C (Figure S3). The melting curve in intersex testes, even in the cases in which expression levels were identical to those of normal testes (November), showed a main curve at 81.5 °C, with a shoulder at 80.5 °C. Sequencing of ovarian and hepatic amplicons revealed the existence of a moderately polymorphic 5S rRNA cds, but it was not possible to sequence any ovary- or somatic-specific

sequence (Figure S3). The appearance in intersex *C. labrosus* of a melting peak of 80.5 °C, with the primers employed in this study, is a molecular indication of the presence of oocytes in testes.

*Gene Transcription Levels in Gonads: tfIIIa, 42sp43, Importins, and Exportins*

*tfIIIa* and *42sp43* had exactly the same profile and levels of transcription as found for 5S rRNA, with strong transcriptional activity in ovary and very low activity in males (Figure 3). The differences in threshold cycles were large and in the same range as described above for 5S rRNA. Intersex individuals again had intermediate transcriptional profiles. Differences with respect to females and males were significant in November and January for *tfIIIa* and in September and November for *42sp43* (Figure 3). It was noted that intersex individuals in January had *42sp43* transcription patterns identical to those of females. Normalization to *ef-1α* as housekeeping provided the same results (Table S3).

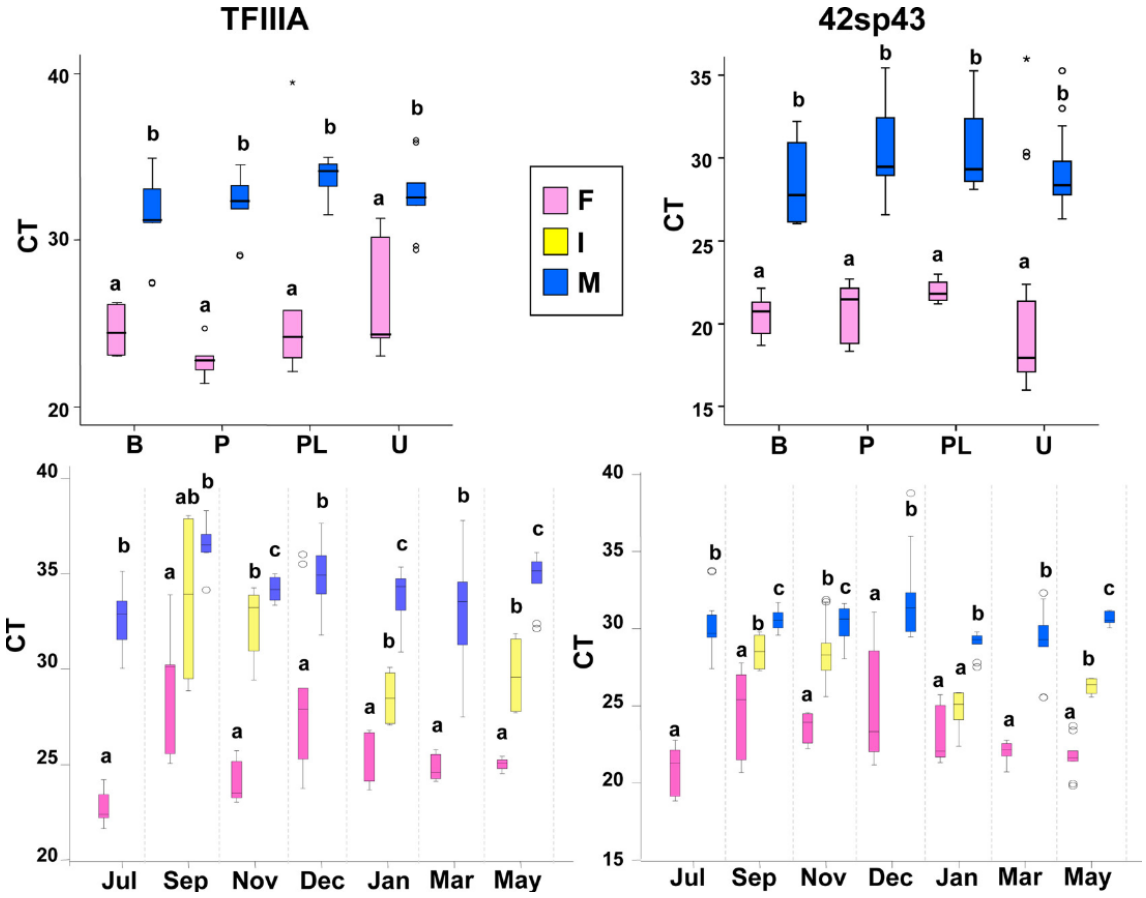


**Figure 3**. Box plots representing *tfIIIa* and *42sp43* gene transcription levels in *C. labrosus* collected in July 2010 in Bilbao, Pasaia, Plentzia, and Urdaibai, and in Pasaia throughout a complete annual reproductive cycle. Different letters indicate significant differences (Mann–Whitney U-test, $p < 0.05$) between sexes (histologically sexed) at each site and during each month ($n = 5$, each individual in triplicate in the annual study and in duplicate in the site study). Data are represented as cycle thresholds for a clear visualization of differences between females and males. Data normalized to housekeeping genes as required for an adequate qPCR study are provided in Table S3 and confirm results shown here.

Both *impα1* and *impα2* followed the same pattern of transcription of *5S rRNA, tfIIIa* and *42sp43* in *C. labrosus*, constituting also potent mullet sex markers. There were even more than 10 cycles of difference between male and female samples, in the 4 localities studied in July (Figure S4). On the other hand, *impβ2* and *exp5* were transcribed similarly in both sexes, while *exp6* was slightly, but significantly, more expressed in females in Bilbao, Pasaia, and Plentzia (Figure S5).

IMPα-s, especially *impα1*, identified sex in all the months studied throughout the reproductive cycle of the mullet (Figure S4 and Table S3). In addition, *impα1* was also a potent marker of intersex.

*Gene Transcription Levels in Gonads: piwi-Like Proteins*

In July, no differences in the transcription levels of *piwil1* were observed at any of the 4 locations studied (Figure S6). With *piwil2*, female gonads were again transcriptionally most active, but differences between sexes were more moderate than for the other genes. Considering the monthly differences at Pasaia, *piwil1* appeared to be transcribed mainly in males in the months of December and February, while *piwil2* was transcribed similarly in both sexes (Figure S6 and Table S3). A marked seasonality was detected in the case of *piwil1* transcription.

## DISCUSSION

A series of molecular markers linked to 5S rRNA transcriptional regulation, stockpiling, and nucleo-cytoplasmic trafficking, transcribed differently in the gonads of male, female, and intersex male thicklip gray mullets (*Chelon labrosus*), have been sequenced. All these molecular markers were specific in the identification of fish sex (only one female individual in the month of December, identified as an outlier, could not be discriminated with our markers) and they were intersexuality-linked to xenoestrogen exposure, throughout an annual reproductive cycle and in all of the sampling areas.

*Sex Differentiation in Fish and 5S rRNA as a Sex Marker in Fish Gonads*

The first stage of gamete development into either egg or sperm involves the formation of primordial germ-cells (PGCs); these then have to initiate a differentiation protocol, following sex determination. (3) In females, the transformation of PGCs into oogonia is followed by their transformation into primary oocytes, with the onset of meiosis. (15, 16) Subsequently, these immature/previtellogenic stage oocytes show proliferation of perinucleolar structures; structural manifestation of rRNA production. This is followed by the massive growth of the oocyte during vitellogenesis, whereby the oocyte accumulates nutritional reserves, completing the differentiation of its cellular and noncellular envelopes. Then, maturation processes begin, being characterized by the resumption of meiosis, germinal vesicle breakdown, formation of a monolayer of cortical alveoli, yolk platelet dissolution, and, in the case of pelagic oocytes, hydration. (15, 16) In the process of oocyte differentiation/maturation, different molecules (for instance rRNAs) have to be incorporated to the oocyte, as such, it becomes competent to undergo fertilization and sustain embryo development.

During 2010, it was observed that a simple electrophoresis of total RNA extracted from the gonads of thicklip gray mullets *Chelon labrosus* was sufficient to sex them according to the difference in a low molecular weight band of 120 bp. Later analysis has revealed that this band belonged to the smallest rRNA molecule, 5S rRNA. It has been confirmed that this observation can be generalized to other teleosts, as 5S rRNA is also expressed highly in the ovaries of the commercially relevant European hake (*Merluccius merluccius*) and of the laboratory model species *Danio rerio*. In particular, 5S rRNA can constitute 75% of the total RNA content in the transcriptome of a mullet ovary (Figure 1). Moreover, intersex *C. labrosus* individuals collected in a polluted estuary could be identified due to their intermediate level of 5S rRNA expression between males and females. Thus, 5S rRNA expression in mullet gonads is diagnostic of the presence of maturing or mature oocytes. This observation, that only needs to run an agarose

electrophoresis with total RNA from gonads, has only been reported once in the literature, by Mazabraud and co-workers in 1975. They studied RNA accumulation in the oocytes of immature ovaries in 9 teleost fish species belonging to different families, and reported that in 4 of these species tRNA and 5S rRNA made up more than 90% of the total RNA content in the ovaries. The present study confirms this observation and concludes that the differential expression of 5S rRNA in gonads is diagnostic of fish sex, irrespective of the site of collection and time in the reproductive cycle, at least in the case of mullets. Further, when exposure to xenoestrogens results in the appearance of oocytes within testes 5S rRNA can also be diagnostic.

The expression of rDNA genes changes during amphibian oogenesis. In early vitellogenesis, the activity increases dramatically whereas transcription slows down again in later stages. These variations in rDNA gene activity during oogenesis are accompanied also by well-characterized ultrastructural modifications of the amphibian nucleolus. (23) Similarly, a succession of nucleolar transformations have been observed to take place in fish, including morphological changes of the nucleolus from the oogonia up to the mature oocyte, very similar to those in anurans. (24) The previtellogenic oocytes in stages I to III (perinucleolar oocytes) are very rich in nucleoli. (16) The differences observed within each sex in the mullets of the annual and site-specific studies, e.g. lower transcription levels in female mullets from Plentzia, in comparison to other sites, would reflect the differences in 5S rRNA transcriptional activity throughout the gonad development; these would be linked to differences in food availability, physicochemical conditions, and the reproductive cycle.

In eukaryotes, the 5S rRNA gene family is arranged in several thousands of copies of tandemly repeated units. Each unit consists of a highly conserved coding sequence of 120 bp, together with a flanking nontranscribed spacer (NTS). (13, 25-27) The NTS is neutral but the 5S rRNA cds fits in a concerted evolution model, thus decreasing intraindividual and intrapopulation heterogeneity and permitting its utilization as a molecular marker for species identification. Many studies have been published dealing with the structure and chromosomal location of *5S rRNA* genes in animals, including fish species such as gray mullets or hakes of the genus *Merluccius*. (26, 27) Two different types of 5S rDNA have been found in anurans: one transcribed in somatic cells and the other one in oocytes. (18) In fish at least 2 types of *5S rRNA* genes exist, *5S rRNA* Class-I and Class-II, distinguishable mainly by differences in length and sequence in the NTS. (25, 26) These 2 types of 5S rDNA have been interpreted to belong to the somatic and the ovarian genes, always based on studies involving gene structure analysis in DNA extracted from somatic tissues. (25-29) In all these cases, small intraspecific differences in the 120 bp long cds have been identified; these could belong to polymorphisms within the tandemly repeated gene unit. The only study to focus on transcribed rRNA molecules stored in oocytes and in somatic tissues was carried out in the teleost fish *Tinca tinca*. (19) This study reported the existence of an oocyte- and a somatic-type of 5S rRNA, which differed in 3 nucleotides. Our qPCR analysis of ovarian total RNA resulted in amplification of a fragment with a melting temperature of 80.5 °C, while in nonovarian tissues the melting temperature was 81.5 °C, thus clearly identifying the presence of two different transcripts. Sequencing attempts have revealed a certain level of polymorphism, but none of the nucleotide substitutions detected was specific either for the ovary or for the nonovarian tissues. In any case and for *C. labrosus,* with the PCR primers employed in the present study, a melting curve peaking at 80.5 °C was indicative of the presence of oocytes (ovary or intersex testes).

*Transcriptional Regulation and Stockpiling of 5S rRNA in Fish Gonads*

In eukaryotes, the transcription of 5S rRNA by RNA polymerase III is controlled by general transcription factor IIIA (TFIIIA). (17, 18, 28) In *Xenopus* oocytes, TFIIIA is found in a complex with 5S rRNA (7S RNP), which serves as a storage particle for 5S rRNA. (17, 18) Thus, TFIIIA recognizes specifically and binds the 5S rDNA promoter sequence, and binds also the resulting transcription product. Levels of TFIIIA mRNA mirror those of 5S rRNA; in *Xenopus* they are about 1 million times higher in oocytes than in somatic cells. (30) TFIIIA is overexpressed early in *Xenopus* oogenesis (Stages I–III), the protein constituting even 10% of the total cytoplasmic protein in anurans, then decreasing 5–10-fold by Stage IV–V. (30-32) TFIIIA has been observed to bind predominantly the oocyte-type 5S rRNA and not the somatic-type in *Xenopus*, even when both sequences only diverge in 3 nucleotides. (18)

In our case, among the annotated sequences obtained through 454 pyrosequencing a mullet TFIIIA homologue was found. In fish, only channel catfish (*Ictalurus punctatus*) TFIIIA has been characterized previously. (33) TFIIIA protein was identified through its association with 5S rRNA in the form of 7S RNPs in catfish ovaries, and subsequent cloning revealed only 40% identity with *Xenopus* protein. Our mullet TFIIIA cDNA sequence, together with 109 nucleotides of the 3'-UTR, contained 477 nucleotides of the cds 3'end, that code for the first 159 amino acids. Total protein size is 322 amino acids in the channel catfish and 344 in *Xenopus laevis*, amino acid identity with mullet TFIIIA being 62% and 49% respectively. Although catfish TFIIIA was found to bind *Xenopus* 5S rDNA, it was not able to promote gene transcription in a rodent RNA polymerase III transcription system. (33) Mullet *tfIIIa* qPCR analysis demonstrated, for the first time in fish, with the exception of *tfIIIa* appearing in a list of ovary-expressed genes in zebrafish, (11) that its transcriptional regulation resembles that of 5S rRNA. Intersex individuals had transcription patterns between both sexes. In contrast, TFIIIA did not show any significant transcriptional differences between the testis and ovary of zebrafish (CT values 28.9 ± 1.14 in females and 31.8 ± 2.76 in males). This observation requires further study as maturation in mullet ovary is synchronous (all oocytes at the same developmental stage) and zebrafish is asynchronous (all oocyte developmental stages coexist in the ovary).

In amphibian oocytes, 5S rRNA is stored also in larger 42S RNPs. (17) In *Xenopus*, approximately 50% of the oocyte 5S rRNA is associated with TFIIIA, with the other half being present in 42S RNPs. These 42S RNPs consist of two proteins: 42sp50 (also p48), 42sp43 (p43 5S rRNA binding protein) and 5S rRNA. (17, 34) This 42sp43 is a nine-zinc-finger protein responsible for 5S rRNA binding, similar to TFIIIA, however unlike TFIIIA, it is not implicated in its transcriptional regulation. (17) The only reference in fish relates to *O. latipes,* where both 42sp50 and 42sp43 transcripts were found to be ovary-specific. (35, 36) A transgenic medaka line with a reporter driven by the *42sp50* promoter permitted the fluorescent labeling of oocytes. Fluorescence began as early as 5 days posthatch, (36) consistent with *42sp50* transcription in Stage I oocytes. Testis-ova oocytes induced artificially in the testes were also labeled fluorescently. (36) In our study, *42sp43* behaved very similarly to *tfIIIa* and 5S rRNA; its massive transcriptional activity in the ovary permitted fish gonads to be sexed without exception throughout the entire reproductive cycle. Similarly, male gonads with oocytes showed 42sp43 transcript levels that allowed their identification through PCR.

*Nucleo-Cytoplasmatic Transport in the Ovary*

In eukaryotic cells, nucleo-cytoplasmic bidirectional transport of molecules relies upon access through nuclear pore complexes. Molecules larger than 45 kDa can not passively pass through

the nuclear envelope and must be transported via carrier proteins, karyopherins. (37) Karyopherins include members of the importin (IMP) and exportin (EXP) protein families. The best-characterized import mechanism is mediated by IMPα and IMPβ heterodimers. Nuclear proteins bind to an IMPα, and this complex is targeted by an IMPβ to the nuclear pore. (37) Upon translocation through the pore, IMPβ binds to the small GTPase Ran, releasing the cargo. (38) In this way, hundreds of different proteins can be targeted specifically into the nucleus. The IMPα gene family has expanded during evolution and 5–8 different IMPα genes have been identified in *Xenopus*, mice, and humans. (32, 38)

Up until now, the only reference to this transport system in fish relates to characterization of an IMPα in the red seabream (*Pagrus major*) ovary. (37) RT-PCR analysis showed transcription of this *P. major impα* in testis and, especially, in the ovary; not in other tissues. IMPα mRNA levels in males increased in association with testicular development, whereas those in females remained high throughout sexual maturation. (37) A systematic analysis of all five known IMPα variants known in *Xenopus laevis* revealed that only *impα1* and *impα2* are transcribed in a pattern similar to *tfIIIa* during its massive synthesis stages in early oogenesis. (32) Not only that, while *impα3*, *4*, and *5* are transcribed in all tissues, IMPα1 and 2 mRNAs are mainly detectable in the ovary. Thus, TFIIIA may be imported into the nucleus via interaction with these 2 proteins. (32) In our hands, both *impα1* and *impα2*, but not *impβ2* or *exp5* and *exp6*, followed the same pattern of transcription of *tfIIIa* in *C. labrosus*; these constitute also potent sex and intersex markers.

*Testicle Markers in Chelon labrosus*

As a control to our mainly female-specific transcription results we decided to study the transcription levels of the piwi-like protein coding genes, as *piwil1* (*ziwi* in zebrafish) has been reported in zebrafish to be expressed preferentially in testis. (39, 40) This argonaute family of proteins is responsible for the proliferation and maintenance of primordial germ cells during adulthood. In adult zebrafish they are expressed exclusively in gonads (39-41) and participate in RNA-mediated transposon-silencing pathways. (40)*Piwil1* was transcribed to a higher extent in mullet testis than in ovary, in all months except July; *piwil2* was transcribed similarly in both sexes except in July (females > males). Interestingly, *piwil1* shows 2 clearly distinctive seasonal transcription-profiles in mullets, with transcription being higher from July to November than from December to May. In zebrafish, ovary *piwil1* is only expressed in oogonia and in Stage I and II oocytes. (39) These are the developmental stages that predominate in mullets from July to November (Figure S1). In contrast, *piwil2* (*zili*, in zebrafish), which did not show such seasonality in mullets, has been reported in zebrafish to be expressed in oocytes in all stages. (40)

Thus, gonad-specific 5S rRNA, and its accompanying proteins (TFIIIA, 42sp43 and IMPαs), have demonstrated to be useful molecular markers to identify sex and oocyte maturity as well as xenoestrogenicity in male mullets displaying testis-ova. To our knowledge, these are the most powerful molecular markers of sex and xenostrogenicity described to date in fish gonads, and have been shown to be useful throughout the entire annual reproductive cycle of the thicklip gray mullet *C. labrosus*. There is a need to study whether these observations can be generalized to fish with different sexual determination/differentiation strategies and with different ovary maturation programs (synchronous/asynchronous). The variability of typical housekeeping genes thoughout the reproductive cycle means that there is a need to find ways to precisely quantify the transcript levels. Hence results can be used to compare individuals from different populations and establish threshold values discriminating males from females

and from intersex males. In this context, we believe that quantification of a battery of 5S rRNA and accompanying proteins can be used also to develop a female fish maturity index to better estimate commercial fish fecundity. These molecular markers and derived fecundity indexes could also be applied to spawned eggs in aquaculture fish species. All this would be very relevant to many research and commercial applications, such as the study of fish stock dynamics and reproduction, selection of reproductive females in aquaculture, and the assessment of environmental health and the management of xenoestrogenicity. Use of this methodology has been protected under Spanish patent (P201130778).

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