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High production of transfer RNAs identifies the presence of developing oocytes in ovaries and intersex testes of teleost fish

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ABSTRACT

5S rRNA is highly transcribed in fish oocytes and this transcription levels can be used to identify the presence of oocytes in the intersex testes of fish exposed to xenoestrogens. Similar to 5S rRNA, tRNAs are transcribed by RNA polymerase III (Pol-III) in eukaryotes, so this study focuses in the analysis of the levels of expression of tRNAs in the gonads (ovaries and testes) of eight teleost species as a possible new oocyte molecular marker. Total RNA extracted from gonads of six commercial teleost species in the Biscay Bay, from the pollution sentinel species thicklip grey mullet (*Chelon labrosus*) known present intersex testes in response to xenoestrogens in Gernika estuary and from the laboratory model species *Danio rerio* were analysed through capillary electrophoresis. Bioanalyzer electropherograms were used to quantify the concentrations of tRNAs, 5S and 5.8S rRNA. All studied ovaries expressed significantly higher levels of tRNAs and 5S rRNA than testes. A tRNA to 5.8S rRNA index was calculated which differentiates ovaries from testes, and identifies some intersex testes in between testes and ovaries in mullets. The tRNA/5.8S ratio was highest in ovaries in previtellogenic stage, decreasing towards maturity. Thus, strong oocyte expression of tRNAs is an additional proof of high activity levels of Pol-III during early stages of oocyte development in teleost ovaries. Incidentally, we observed that miRNA concentrations were always higher in testes than ovaries. The indexing approach developed in the present study could have multiple applications in teleost reproduction research and in the development of early molecular markers of intersex condition.

1. Introduction

Reproduction in teleost fish is highly diverse. We can find multiple sex determination and differentiation mechanisms, gametogenic control systems or spawning strategies (Devlin and Nagahama, 2002; Wootton and Smith, 2014; Jakobsen et al., 2016). However, little is known about the molecular determinants that define oocyte quality and reproductive output (Ma et al., 2019; Liu et al., 2022). In aquaculture significant achievements have been made in recent years to improve protocols for gonad quality assessment and to achieve high egg production efficiency, fertilization rates and increased progeny viability (Lubzens et al., 2010). Nevertheless, important gaps persist in understanding the dynamic processes associated with fish sex differentiation, gametogenesis and other reproductive traits (Murua et al., 2003; Lubzens et al., 2010; Ma et al., 2019).

Important commercial fish populations are declining because of deteriorating environmental circumstances and overexploitation, which reduce the number of viable offspring within spawning stocks significantly impacting the potential yield of many commercial fisheries (Wright and Trippel, 2009). It is thus essential to study the reproduction biology of commercial fish stocks in order to understand their population dynamics providing means for sound scientific advice to set up maximum catch limits for a sustainable management of commercial fish populations (Morgan, 2008; Jakobsen et al., 2016).

Sex determination and differentiation mechanisms in fish are very flexible and subjected to external environmental factors such as oxygen

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or food availability, changes in temperature, behavioural cues and exposure to chemicals including pollutants normally termed as endocrine disrupting compounds (EDCs) (Piferrer, 2001; Devlin and Nagahama, 2002; Kloas et al., 2009; Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2020). In many circumstances exposure to xenoestrogenic EDCs impairs gonad development in fish leading to the development of intersex gonads, that is, the development of oocytes in testes (Blazer et al., 2007; Ortiz-Zarragoitia et al., 2014).

Gametogenesis is a highly complex process in which germ line cells suffer strong differentiation steps, the most important being meiosis. Molecular changes during teleost oogenesis include growth through the incorporation of nutrients and molecules that will be necessary for embryonic development in case of successful fertilization (Lubzens et al., 2010; Diaz de Cerio et al., 2012). Differentiation pathways often require changes at the level of gene expression in the oocyte and its surrounding cells resulting in the accumulation of proteins, RNAs of different nature, hormones and polysaccharides in the oocytes (Jalabert, 2005; Lubzens et al., 2010).

Transfer RNAs (tRNAs) and 5S rRNA stored in nucleoprotein particles sedimenting at 42S can constitute up to 90% of the RNA content in the ovaries of teleost fish (Mazabraud et al., 1975) and by far 5S rRNA is the predominant RNA molecule in previtellogenic oocytes (Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2016). Therefore, it is important for the growing oocyte to accumulate high concentrations of 5S rRNA, possibly to aid rapid ribosome assembly in the case of successful fertilisation making protein synthesis possible during early embryo development (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014). 5S rRNA is the smallest ribosomal RNA molecule and forms part of the ribosome large subunit. Its gene structure and sequence has been often used in phylogenetic comparisons among fish species (Campo et al., 2009; Diaz de Cerio et al., 2012). In eukaryotes, 5S rRNA is coded by multiple copies of a single gene, which are transcribed by RNA polymerase III (Pol-III). Instead, large ribosomal RNA molecules which are producing the 28S, 18S and 5.8S rRNAs are transcribed by RNA polymerase I (Pol-I) while all mRNAs are transcribed by RNA polymerase II (Szymański et al., 2003).

tRNAs and some microRNAs (miRNAs) that play a decisive role in translation of mRNAs are also transcribed by Pol-III (Graczyk et al., 2018; Ramsay and Vannini, 2018). Amino acid specific tRNAs with a size of ~70–90 nucleotides are coded by multiple genes (isodecoders) and undergo extensive post-transcriptional maturation, mainly methylation of cytosines and other nucleotides (Pinkard et al., 2020; Behrens et al., 2021). Specific methylations are important for the stabilization of tRNA structure, reinforcement of the codon-anticodon interaction, prevention of frameshift errors and selection of the mRNA populations that are going to be translated (Pinkard et al., 2020; Behrens et al., 2021). In the context of a maternal transference of tRNA to newly formed embryos, the fluctuation of the functionally diverse tRNA transcriptome accumulated during oogenesis could heavily modulate mRNA translation and therefore protein production during early developmental stages. The understanding of this tRNA transcriptome during oocyte maturation could contribute to decipher the mechanisms that govern protein synthesis during early embryo development in a fluctuating environment. Little is known of tRNAs in fish genomes but there are indications that tRNAs are accumulated together with 5S rRNA in cytosolic ribonucleoproteins of oocytes during early oogenesis (Mazabraud et al., 1975).

A first approach to the tRNA study in fish gonads, taking into account the methodological constrains for their efficient sequencing due to their tertiary structure, extensive post-transcriptional modifications and difficulties for retrotranscription (Pinkard et al., 2020; Behrens et al., 2021), is the analysis of their bulk quantification through capillary electrophoresis of small RNA molecules. Within this context, the main objective of the present work was to study the levels of expression of tRNAs in teleost ovaries in comparison to testes to establish whether they could serve as markers of sex differentiation, oocyte growth and intersex condition in fish. In addition, this analysis could provide tools to explore the possible role of protein synthesis intermediates in defining oocyte quality in teleost fish.

2. Material and methods

2.1. Study area and fish samples

A total of eight teleost species were analysed in this study: Atlantic chub mackerel (*Scomber japonicus*); Atlantic mackerel (*Scomber scombrus*); horse mackerel (*Trachurus trachurus*); European hake (*Merluccius merluccius*); European anchovy (*Engraulis encrasicolus*); European pilchard (*Sardina pilchardus*); zebrafish (*Danio rerio*) and thicklip grey mullet (*Chelon labrosus*). The first six species are commercial fish species and were obtained from the local fish market between 2011 and 2014 (Table 1). Zebrafish (n = 13) instead were obtained from a local ornamental fish-shop. The thicklip grey mullet was selected due to its importance as sentinel species for the study of exposure to xenoestrogens in the Basque coast (Ortiz-Zarragoitia et al., 2014; Valencia et al., 2017). Mullet samples were obtained from the yearly monitoring studies (30 individuals per sampling) performed by the Biscay Bay Environmental Biospecimen Bank (BBEBB; PiE-UPV/EHU) in the estuary of Gernika (43°19′26″N, 2°40′26″W; SE Bay of Biscay). Animals were captured by fishing-rod in June 2021. In addition, intersex mullets from previous monitoring campaigns (2014–2021) in Gernika were explored for the incorporation of samples in the study. Immediately after being captured mullets were anaesthetized in a saturated ethyl-4-aminobenzoate water bath. All fish handling and procedures were approved by the UPV/EHU Ethics Committee on Animal Experimentation and by the regional authorities (Code: CEEA/337-2/2014/ORTIZ ZARRAGOITIA). Each gonad was dissected for molecular analysis and histological analysis. The portion for molecular studies was embedded in RNAlater® (Ambion, Life technologies), frozen in liquid nitrogen and stored at −80 °C until further used. The tissue portion for histological and sex identification was fixed in 4% neutral buffered formalin containing 1% glutaraldehyde. For all commercial species (individuals obtained in fish market were 10–30 per species) 15 individuals were histologically sexed (10 in the case of *T. trachurus*) to have enough testis and ovary samples for downstream analyses.

2.2. Histological analysis of fish gonads

Gonad samples were fixed for 24 h and embedded in paraffin wax. Tissue-blocks were sectioned to a thickness of 5 μm in a RM2125 RTS microtome (Leica, Germany). Sectioned tissue slides were stained with haematoxylin and eosin (H&E) in an automatic Leica autostainer (XL, CV 5030, Germany). Slide examination was done using an Olympus BX61 microscope (Tokyo, Japan) to sex and identify gametogenic stage of each individual. In the case of intersex individuals, intersex severity index for each fish was established according to Blazer et al. (2007) and depending on the average number of oocytes within each analysed testis section. For this study selected mullet intersex individuals were grouped into two study groups with lower (Intersex index 1 to 2) and higher (Index 3 to 4) intersex severity, eleven individuals all together.

2.3. RNA extraction and RNA quality check by electrophoresis

After thawing the gonad samples and carefully cleaning the RNAlater® solution from the tissue, 50–100 mg of sample was used for total RNA extraction using TRIzol® Reagent Solution (Ambion; Life Technologies, Carlsbard, California, USA) and following the manufacturer's instructions. After extractions, RNA pellets were dissolved in 40 μL RNase-free water and kept at −80 °C to preserve the RNA integrity. Concentration and quality were spectrophotometrically measured (good quality RNA established at 260/280 and 260/230 ratios around 2) in a biophotometer (Eppendorf, Hamburg, Germany). All samples displayed

Table 1
List of fish species studied in the present work, common and scientific names, capture locations and dates, number of individuals analysed (N) specifying how many males (M) and females (F) and the histological oogenic stage of the studied females (PV = previtellogenic, CA = cortical alveoli, AV = advanced vitellogenic). "*" indicates the number of intersex mullets analysed. Among 11 mullets analysed: six displayed an intersex severity index 1 to 2 and five an index 3 to 4.

Common Name	Scientific Name	Capture location	Date	N (M/F)	Gametogenic Stage females
Thicklip grey mullet	*Chelon labrosus*	Urdaibai estuary, Gernika	June 2014–2021	12 (6/6) + 11*	PV
European hake	*Merluccius merluccius*	Bay of Biscay, ICES Fishing Subarea (VIIIb)	March 2011	10 (4/6)	PV
Atlantic mackerel	*Scomber scombrus*	Bay of Biscay, ICES Fishing Subarea (VIIIb)	Feb 2012	10 (4/6)	PV/CA
Atlantic chub mackerel	*Scomber joponicus*	Bay of Biscay, ICES Fishing Subarea (VIIIb)	May 2014	11 (5/6)	PV/CA
European anchovy	*Engraulis encrasicolus*	Bay of Biscay, ICES Fishing Subarea (VIIIc)	Feb 2012	15 (8/7)	AV
European pilchard	*Sardina pilchardus*	Bay of Biscay, ICES Fishing Subarea (VIIIb)	Nov 2011	11 (5/6)	AV
Horse mackerel	*Trachurus trachurus*	Bay of Biscay, ICES Fishing Subarea (VIIIb)	May 2014	7 (4/3)	AV
Zebrafish	*Danio rerio*	–	Aug 2021	13 (7/6)	AV

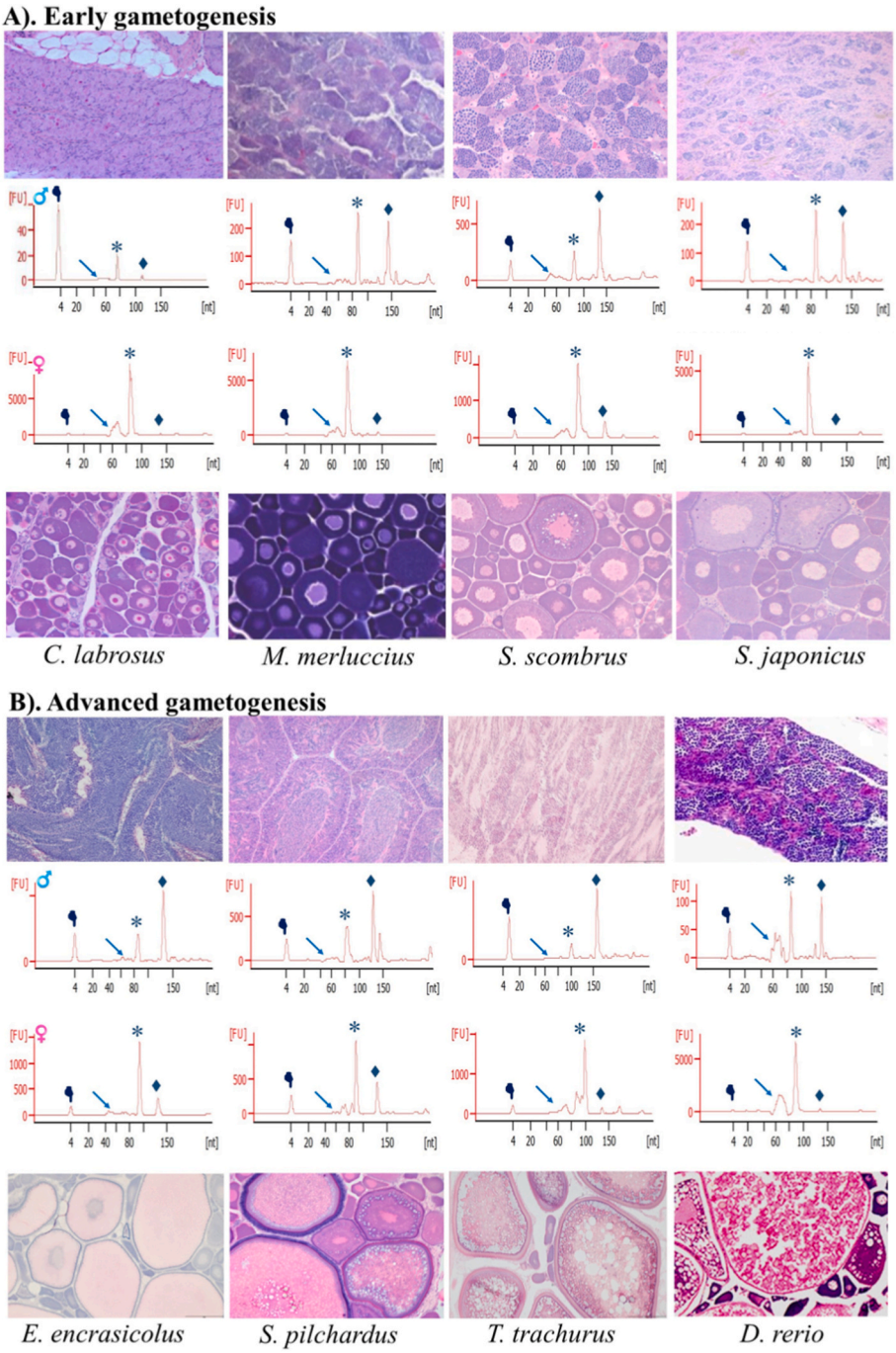

Fig. 1. Representative electropherograms showing the profile of different small RNAs in one representative testis (top) and one representative ovary (bottom) for each analysed fish species. Micrographs above (testes) or below (ovary) each electropherogram show the developmental stage of that particular fish from early (Fig. 1A) to late gametogenesis (Fig. 1B). Supplementary Fig. 1 better illustrates the developmental stage of the ovaries of the individuals studied for each species. Arrows in the electropherograms indicate the peak belonging to tRNAs; asterisks indicate the 5S rRNA peak; rhombs mark the 5.8S rRNA peak and fingers point to the Bioanalyzer chip internal marker.

A260/A280 absorbance ratios between 1.8 and 2.2 and thus were considered suitable for analysis.

2.4. Quantification of tRNA/5.8S rRNA, tRNA/5S rRNA, 5S rRNA/5.8S rRNA indices and the percentage of miRNAs

Total RNA was loaded in Small-RNA kit chips (Agilent Technologies, Santa Clara, California, USA) and analysed in the Agilent 2100 Bioanalyzer system following the instructions provided by manufacturers. All the samples were diluted at a final concentration of 100 ng RNA/μL RNAase/DNAse free water. The time corrected area of the peaks corresponding to tRNAs, 5S rRNA and 5.8S rRNA was measured and used to calculate the following ratios: tRNA/5.8S rRNA, tRNA/5S rRNA and 5S/5.8S rRNA. Finally, the ratios were Log10 transformed to plot the differences between testes and ovaries across all the species studied. Percentages (%) of miRNAs with a length of 20–40 nucleotides were automatically obtained from the analysis of the electropherograms as a default parameter provided by the Agilent 2100 Bioanalyzer.

2.5. Statistical analysis

All statistical analyses were performed with the SPSS statistical package (Version 28, SPSS Inc., Microsoft Co., Redmond, USA). Normal distribution was assessed with Shapiro-Wilk test. Significant differences in RNA levels between both sexes were evaluated by Student's T-test. In all cases, significant differences were established at $p < 0.05$.

3. Results

3.1. tRNAs in fish gonads

The electropherograms showed that the profile of RNA molecules in the total RNA differed dramatically when comparing testes and ovaries in different fish species. The ovaries displayed two main peaks; one between 60 and 80 nucleotides in length and belonging to tRNAs, and another one close to 100 nucleotides in length belonging to 5S rRNA (Fig. 1). Testes instead were characterised by one main peak around 150 nucleotides in length that belongs to 5.8S rRNA (Fig. 1).

Ovaries presenting oocytes mainly in previtellogenic stage (thicklip

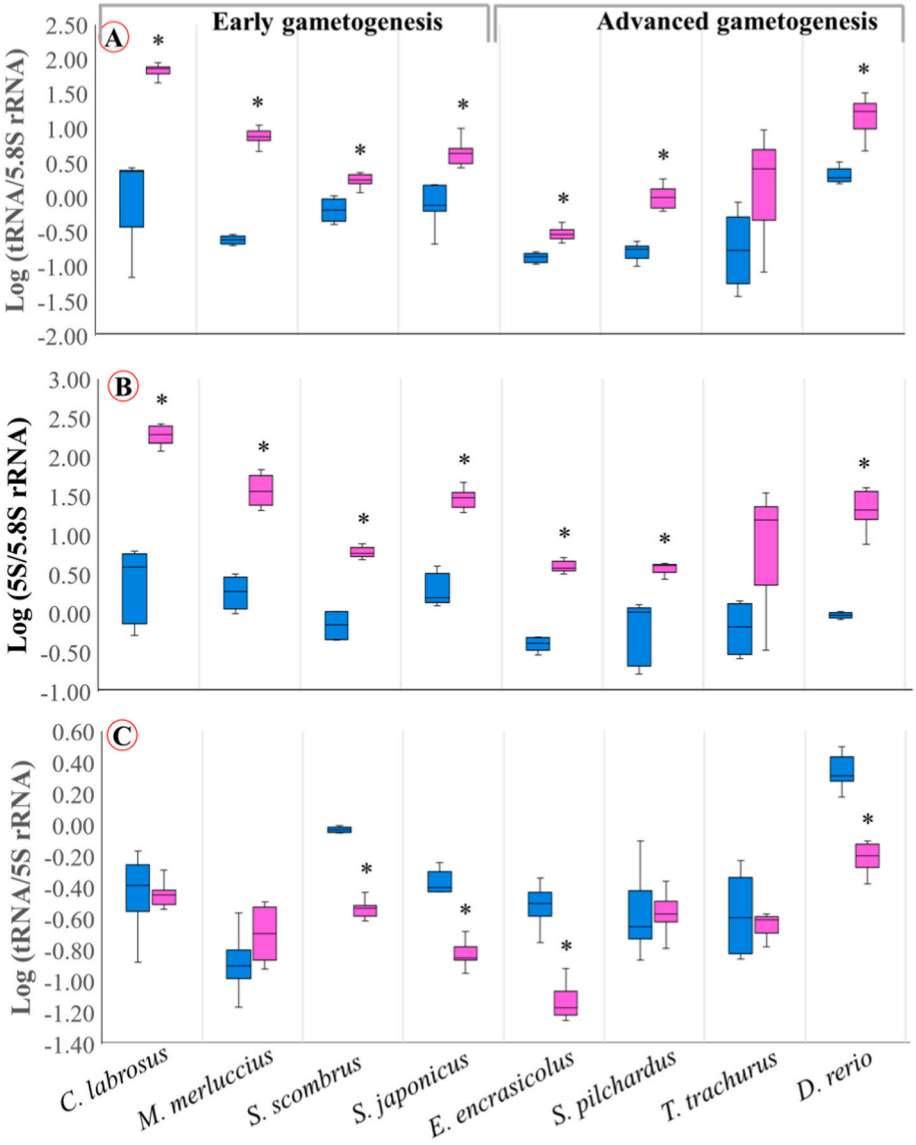

Fig. 2. Box plots of tRNA/5.8S rRNA (A), 5S/5.8S rRNA (B) and tRNA/5S rRNA indices (C) in the gonads of different teleost fish species. Box plots in pink identify ovaries with testes in blue. Boxes represent the data within the 25th and 75th percentiles; the line indicates the median value; and top and bottom whiskers indicate the minimum and maximum values. Asterisk within each species denote significant differences between pairs on means (Student's T-test, p < 0,05).

grey mullet, European hake, chub mackerel and Atlantic mackerel, Fig. 1A and Supplementary Fig. 1) showed levels of tRNAs and 5S rRNA higher than those in ovaries with oocytes in more advanced stage of oogenesis (anchovy, sardine, horse mackerel and zebrafish, Fig. 1B and Supplementary Fig. 1).

3.2. tRNA/5.8S and 5S/5.8S rRNA indices in fish gonads

tRNA, 5S and 5.8S rRNA concentrations quantified from the electropherograms and the different indices calculated showed unequivocal differences between ovaries and testes in all species studied. Both tRNA/5.8S rRNA and 5S/5.8S rRNA indices displayed the highest values in ovaries in comparison to testes (Fig. 2A and 2B). These differences between sexes tended to be higher when the ovaries were in previtellogenesis (Fig. 2A and 2B). A tRNA/5S rRNA index was also calculated and four of the species studied (Atlantic and chub mackerel, European anchovy and zebrafish) displayed higher values in testes than in ovaries (Fig. 2C).

3.3. tRNA/5.8S rRNA values in intersex mullets

A total of 11 intersex testes were analysed in mullet samples separated in two discrete groups according to their intersex severity index. Six individuals showed low index values from 1 to 2 and five displayed more severe condition, from 3 to 4 (Supplementary Fig. 2). Comparatively, mullet ovaries displayed significantly higher tRNA and 5S rRNA levels than testes with intersex testes showing tRNA/5.8S rRNA index values in between (Fig. 3). The index values were higher the more severe the intersex condition. Very clearly six (three from each intersex severity index group) of the eleven intersex individuals showed higher values than normal testes.

3.4. Percentage of miRNAs in fish gonads

In all cases with the exception of pilchard the percentage of miRNA in comparison to the total amount of small RNAs resolved in the Bioanalyzer microchips was significantly higher in testes than in ovaries (Fig. 4A). In the particular case of the intersex testes of mullets the percentage of miRNA decreased with the severity index although differences with normal testes were not statistically significant (Fig. 4B).

4. Discussion

In the present study, the gonad specific production of small RNAs was analysed in eight teleost species that at the moment of sampling were each at different gametogenic stages within their specific reproductive cycle (Supplementary Fig. 1). A tRNA/5.8S rRNA index was developed that allows the molecular identification of sex in teleost fish. The electropherograms obtained analysing the total small RNA extracted from gonads identified sex due to the high levels of tRNA (and 5S rRNA) accumulated in the ovaries. In the same way, the developed index is useful to molecularly and numerically stablish the developmental stage of ovaries in fish and provides evidences of the presence of oocytes in the intersex testes of thicklip grey mullets exposed to environmental xenoestrogens.

Our previous studies have revealed that 5S rRNA is highly expressed in teleost ovaries (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2017). 5S rRNAs are transcribed by RNA polymerase III (Pol-III) so we hypothesized that ovaries could be also producing extremely high concentrations of tRNAs during early oocyte development because tRNA coding genes are also transcribed by Pol-III. This resulted to be the case in all the species analysed (Fig. 1). Meanwhile, 5.8S rRNA levels were higher in testes than in ovaries, indicating high Pol-I activity in testes. In fact, Pol-I transcribes the 45S rRNA polycistronic genes from which 5.8S, 18S and 28S rRNA molecules are derived (Szymański et al., 2003). These observations are in line with

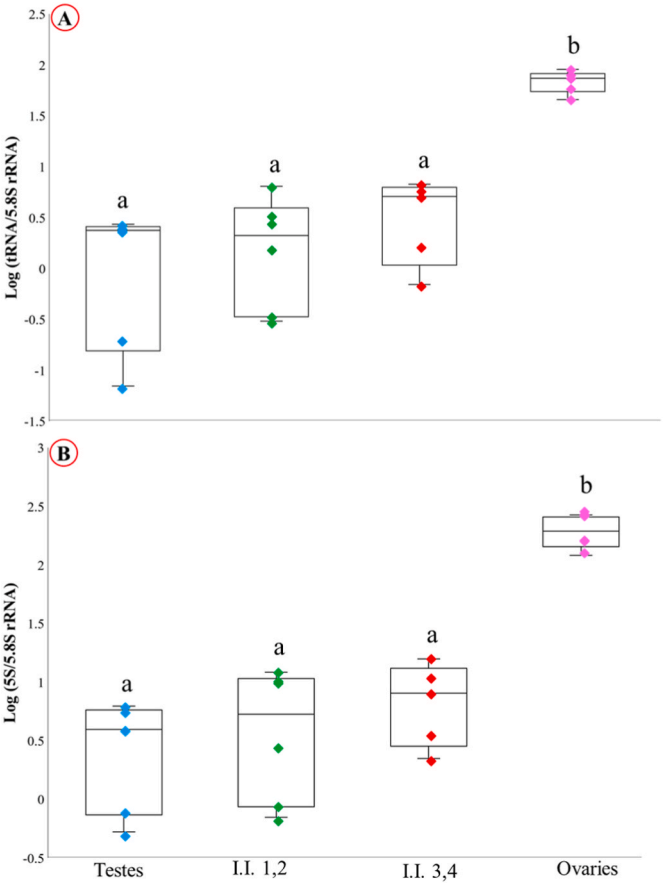

Fig. 3. tRNA/5.8S rRNA (A) and 5S/5.8S rRNA (B) index in mullet ovaries, testes and intersex testes with different intersex severity index values. Each dot represents the value of one individual. Boxes represent the data within the 25th and 75th percentiles; the line indicates the median value, and top and bottom whiskers indicate the minimum and maximum values. Different letters indicate significant differences among pairs of means (one-way ANOVA Tukey, $p < 0,05$).

what is known for the oocytes of *Xenopus* and it was published for the previtellogenic oocytes of nine teleost species in 1975. In fact, Mazabraud et al. (1975) reported a common accumulation of tRNAs and 5S rRNA in 42S ribonucleoprotein particles (RNPs) in oocytes making a total of 90% of the RNA content in the ovaries of *Tinca vulgaris, Labrus bergylta, Conger conger, Onos mustelus* and *Gobius paganellus* during early oorgenesis. Such analyses were done through polyacrylamide electrophoresis after isolation of the ovarian RNPs separated either by sucrose gradient centrifugation or by sephadex filtration (Mazabraud et al., 1975). With our results we assume that these observations can be generalized to other teleosts and their gonads.

tRNAs act as molecular adapters necessary for translation of the genetic code from mRNA to proteins (Arimbasseri et al., 2015) with more than one tRNA gene present for each specific amino acid. The existing codons are recognized by a combination of distinct tRNAs coded by a set of redundant tRNA genes or isodecoders (Schimmel, 2018). Even when only 61 codons exist, animals possess a redundant repertoire of tRNA genes ranging from 299 tRNA genes in *D. melanogaster* and 588 genes in humans to 15208 genes in *Danio rerio* and 35271 in *Bos taurus* (Bermudez-Santana et al., 2010). The Genomic tRNA Database of zebrafish, genome Zv9 (http://gtrnadb2009.ucsc.edu/Dreri/), reveals for instance 59 genes (isodecoders) coding for asparagine-tRNA but 573 for alanine-tRNA. The specific functions of the different tRNA isodecoders, during transcription is not understood yet. The normal approach to assess tRNA populations in a tissue would be high

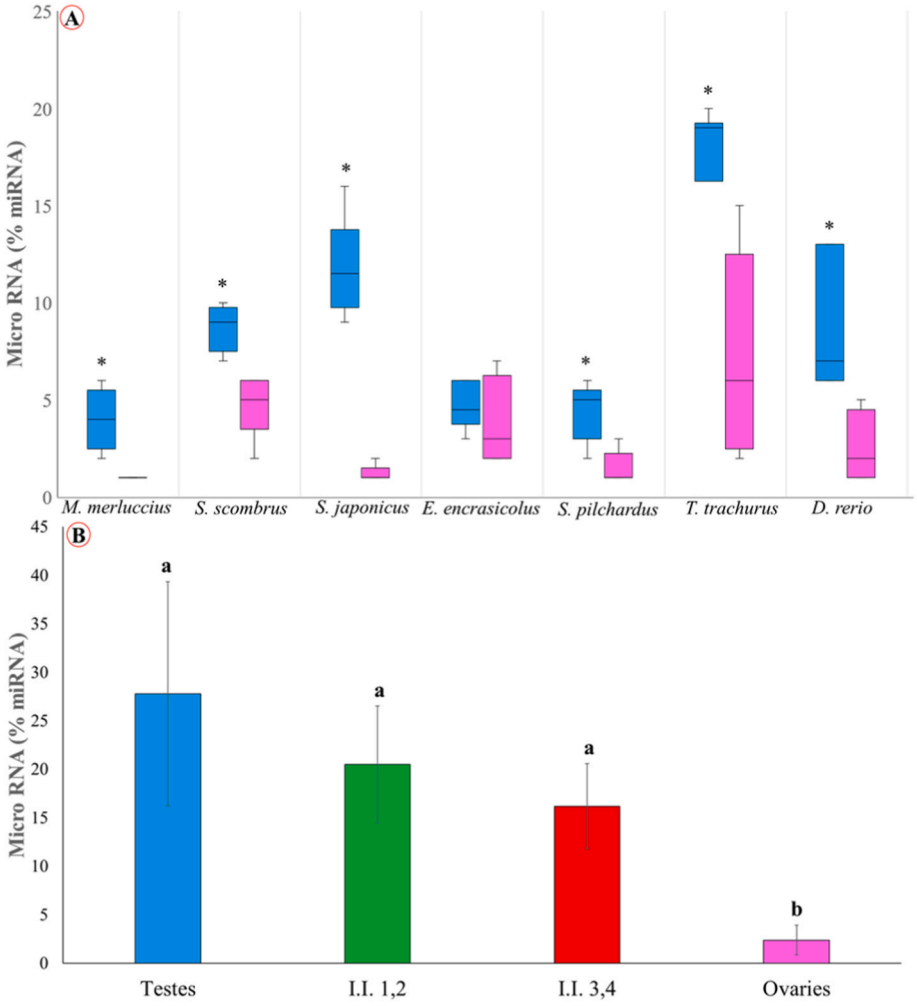

Fig. 4. Percentage of miRNA to total small RNA in ovaries and testes of different fish species (A) and in ovaries, testes and intersex testes of mullets (B). Boxes represent the data within the 25th and 75th percentiles, with the median indicated by a line, with top and bottom whiskers indicating the minimum and maximum values. Asterisks in A indicate significant differences among gonads in each species (Student's T-test $p < 0,05$). Different letters in B indicate significant differences among pairs of means in mullets (one-way ANOVA, Tukey $p < 0.05$). I.I. = Intersex severity index.

throughput sequencing, but additionally to all the isodecoder variability mentioned above they present difficulties for specific sequencing. tRNAs possess a cloverleaf secondary structure with four loops which further fold into a L-shaped tertiary structure for insertion into the ribosome (Pinkard et al., 2020; Behrens et al., 2021). This structure together with the extensive post-transcriptional trimming and epitranscriptomic modifications (mainly methylations) make tRNAs not amenable to typical retrotranscription protocols and hinders effective sequencing of the tRNA transcriptome (Pinkard et al., 2020; Behrens et al., 2021).

Isodecoder genes present a big portion of their sequence differences in regions that are important for Pol-III transcription, and so they could be differentially transcribed during oogenesis. On the other hand, different isodecoders may alter mRNA translation and decay rates (Pinkard et al., 2020; Behrens et al., 2021). In this respect, the nature of the tRNAs pooled in the oocyte cytoplasm could greatly condition the pattern of mRNA translation and the protein yield in the early growing embryo, with large implications in survival rates, growth ratios etc. All these traits are of ecological interest but are also very important for fisheries and aquaculture to understand oocyte quality.

During oogenesis, oocytes in primary growth stages undergo the most significant changes in terms of volume, associated with the massive increase of Pol-III products within the ovary (Mazabraud et al., 1975; Kroupova et al., 2011; Diaz de Cerio et al., 2012; Shen et al., 2017). The accumulation of these intermediaries in protein synthesis, 5S rRNA and tRNAs, in early growing oocytes would contribute to rapid ribosome production and protein synthesis after fertilisation (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2016, 2017; Shen et al., 2017). Interestingly, RNA-Seq analyses comparing the transcriptome of good vs bad quality rainbow trout (*Oncorhynchus mykiss*) unfertilised oocytes identified that the largest cluster of differentially expressed genes was involved in ribosome biogenesis. 157 transcripts participating in ribogenesis were shown to be increased in high-quality eggs and only 7 in low-quality eggs (Ma et al., 2019). Additionally, other relevant gene clusters differentially expressed between oocytes of contrasting quality were associated with translation and protein synthesis (Ma et al., 2019). Recent papers applying RNA-Seq approaches to compare transcripts in ovaries vs testes in different fish species have highlighted ribosome biogenesis and assembly, ribonucleoprotein complex biogenesis, peptide biosynthesis process, translation and amino acid synthesis among the pathways enriched in ovaries in preparation for fertilisation (Boonanuntanasarn et al., 2020; Zhong et al., 2021). Similarly, the same pathways have been associated to growing oocytes in comparison to surrounding somatic cells in single cell RNA sequencing studies in ovarian cells of Chinese tongue sole *Cynoglossus semilaevis* and Asian seabass *Lates calcarifer* (Liu et al., 2021, 2022).

Our electropherograms showed a higher content of 5.8S rRNA in testes than ovaries of all species studied consistent with observations for 18S and 28S rRNA in previous studies (Rojo-Bartolomé et al., 2016; Shen et al., 2017). The tRNA/5.8S rRNA and 5S/5.8S rRNA index calculated showed that Pol-III products overwhelmingly exceed Pol-I products (5.8S or 18S rRNA as in Rojo-Bartolomé et al., 2016) in ovaries. This indexing approach also proved to be reliable to identify the ovarian developmental stage due to the high tRNA values displayed in ovaries at

previtellogenesis (*C. labrosus* and *M. merluccius*) that decrease as ovaries advance into cortical alveoli stage and vitellogenesis. This was already observed with the 5S rRNA/18S rRNA index in many different teleost fish species proving to be a useful method to quantitatively identify oogenic stage in fish (Rojo-Bartolomé et al., 2016; Shen et al., 2017). As oocytes begin to accumulate Pol-I products towards full maturation they begin to resemble the profile in testes. In asynchronous developing ovaries during advanced oogenesis, the case of zebrafish, horse mackerel, pilchard and anchovy in the present study, mature oocytes coexist with previtellogenic oocytes so the contribution of the different types of oocytes is represented in the index (Rojo-Bartolomé et al., 2016). In any case, the index presented hereby could be of considerable assistance in the characterisation of the reproductive stage of female fish.

When the two Pol-III products were quantified and used for the generation of the tRNA/5S rRNA index contradictory results were obtained. It was expected that no differences would be observed in ovaries and testes, but in the two mackerels, pilchard and zebrafish the levels of tRNAs respective to 5S rRNA were higher in testes than in ovaries. Additionally, high differences in index values were recorded across species. Mazabraud et al. (1975) who quantified the levels of tRNAs and 5S rRNA in nine different fish species reported that the molar ratio of tRNA to 5S rRNA was always high, tRNA always exceeding 5S rRNA levels in the cytoplasm of oocytes. This molar ratio was also very different across species, all of them being at previtellogensis at the moment of the study (Mazabraud et al., 1975), ranging from 4 in *Tinca tinca*, *Conger conger* and *Cottus bubalis* to 10 in *Spinachia spinachia*. This would imply that Pol-III transcribes tRNA and 5S rRNA genes at different speed or with different efficiency, this varying with developmental stage. In fact, Pol-III needs different associated transcription factors for its activity, and while tRNA genes need presence of general transcription factors III B and C (GTF3B and GTF3C), 5S rRNA additionally needs the participation of GTF3A (Szymański et al., 2003). Interestingly, fish genomes present a duplication of the *gtf3a* gene with an ovary specific paralog, *gtf3ab*, that is very strongly expressed during previtellogeneis (Rojo-Bartolomé et al., 2016, 2020). Recently, Liu et al. (2022) performing single cell transcriptomic analyses of the Chinese tongue sole ovaries showed a significant upregulation of *gtf3ab* in the transition from oogonia to oocytes confirming our previous suggestion of *gtf3ab* as a marker of oocyte differentiation (Rojo-Bartolomé et al., 2020). The analysis of GTF3B and GTF3C in fish deserves further investigations in the future, to better understand the different kinetics of 5S rRNA and tRNAs production.

Intersex gonads have been described in *Chelon labrosus* from different estuaries in the SE Bay of Biscay. In most of the estuaries mullets showed intersex gonads during different stages of the reproductive annual cycle, with intersex prevalence up to 90% in the Biosphere Reserve of the Urdaibai estuary in Gernika (Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2017; Valencia et al., 2017). Intersex condition in mullets has been associated to xenoestrogenic EDCs, detected in the bile of analysed fish, suggesting exposure to chemicals discharged from the Wastewater Treatment Plants (WWTPs) (Puy-Azurmendi et al., 2013; Ortiz-Zarragoitia et al., 2014; Valencia et al., 2017).

In this study we showed that the tRNA/5.8S rRNA and the 5S rRNA/5.8S rRNA indexes identified the presence of oocytes in some intersex mullets, not in all. As the severity of the condition increases (more oocytes present in the testis) the indexes also increased, as previously described for the 5S rRNA/18S rRNA index (Rojo-Bartolomé et al., 2017). The interindividual variability detected in the tRNA/5.8S rRNA and 5S rRNA/5.8S rRNA index values within each intersex group probably reflects the sensitivity of this analysis, reflecting the amount and developmental stage of oocytes in each intersex testis sample. Thus, the tRNA/5.8S rRNA index could provide additional information to identify intersex condition in fish, in combination with the 5S rRNA/18S rRNA index (Rojo-Bartolomé et al., 2017) or the 5S rRNA/5.8S rRNA index.

In the last few years, microRNAs (miRNAs) are receiving special attention due to their role in post-transcriptional control of gene expression (Tao et al., 2016; Presslauer et al., 2017; Best et al., 2018). miRNAs are highly conserved small non-protein-coding RNA molecules (20–24 nucleotides) that target specific mRNAs and control their translation (Neilson et al., 2007; Presslauer et al., 2017; Best et al., 2018; Bhat et al., 2021). Mature miRNAs derive from longer primary transcripts (pri-miRNAs) containing hairpins and are transcribed by Pol-I and also by Pol-III (Tao et al., 2016; Gao et al., 2019). The possible function of miRNAs during the early stages of fish sex differentiation is currently a subject of debate but their physiological roles in fish are mostly unknown and concentrated in zebrafish (Presslauer et al., 2017; Bhat et al., 2021). Mishima et al. (2008) and Torley et al. (2011) described expression of miRNAs in testes and ovaries in fish defending that they play a vital part in sex differentiation and growth.

Dynamics of miRNA production during development in gonads are also very poorly understood in fish. In recent years, few studies have reported the different expression during sex differentiation and sexual maturation in teleost gonads (Bizuayehu et al., 2012; Jing et al., 2014; Presslauer et al., 2017; Bhat et al., 2021). In the current study we observed that the percentage of miRNA produced in relation to the concentration of small RNAs was consistently higher in testes than ovaries. Similar high miRNA levels were observed in the testes of zebrafish (Presslauer et al., 2017; Bhat et al., 2021), common carp, *Cyprinus carpio* (Xu et al., 2014; Wang et al., 2017), and medaka, *Oryzias latipes*, (Lau et al., 2014). Bizuayehu et al. (2012) studied the Atlantic halibut finding that the expression of some important miRNAs was higher in adult testes as compared with ovaries or testes of immature males. We are not able to envisage the possible functional implications of the differences in miRNA production that we have observed here. It could be also that the highest percentage identified in testes than ovaries is due to a lower overall absolute production of small RNAs in testes in comparison to ovaries and not to a higher production of miRNAs in testes.

5. Conclusions

Overall, the findings of the present work establish that tRNA, 5S and 5.8S rRNA and proposed indexes are useful molecular markers to identify sex in teleost fish and these markers could easily serve as a simple and robust approach to rank the gonad developmental stage during the assessment of fish reproduction dynamics carried out towards sustainable fisheries management. Our results suggests that the activity of Pol-III is essential during fish early oogenesis as ribogenesis is vital for protein synthesis during embryo development. Accumulation of Pol-III products inevitably has implications in the generation of good quality oocytes that can be successfully spawned, fertilised and give rise to viable offspring. Further analysis of the nature of the tRNA pool, its differential transcription, characteristics and post-transcriptional modifications could provide means to assess the quality of teleost ovaries with applications in fisheries research and in broodstock management in aquaculture facilities.

Finally, and in a context of xenoestrogenic pollution monitoring, the highest levels of tRNA/5.8S rRNA and 5S/5.8S rRNA index values reported in intersex mullets in comparison to non-intersex males, suggest the utility of these ratios to molecularly identify intersex condition in fish testes.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ibon Cancio reports financial support was provided by Spain Ministry of Science and Innovation. Ibon Cancio reports financial support was provided by Basque Government.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2023.105907.

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