eman ta zabal zazu
Universidad del País Vasco
Euskal Herriko Unibertsitatea
ZIENTZIA ETA TEKNOLOGIA FAKULTATEA
FACULTAD DE CIENCIA Y TECNOLOGÍA

Gradu Amaierako Lana
Biologia Gradua

Sex identification in zebrafish, *Danio rerio*, and thicklip grey mullet, *Chelon labrosus*, through transfer RNA expression analysis in gonads

Egilea:
Galder Garcia Gabiola
Zuzendaria:
Ibon Cancio Uriarte
Zuzendarikidea:
Oihane Diaz de Cerio Arruabarrena

Leioa, the 19th of July, 2021

INDEX OF THE CONTENTS

ABSTRACT

In the last decades, molecular methods are becoming increasingly relevant in the assessment of sex, sex differentiation and gametogenesis staging in teleost fish as they can provide more accurate estimates than histological or visual analysis of gonads. This is a requirement of the EU Data Collection Framework as part of the policy to improve fisheries management, where fecundity estimates of commercial fish stocks are of prime interest. In this study, we wanted to analyse the differential transcription distinguishing ovaries and testes in fish by studying the levels of transcription of amino acid-specific transfer RNAs (tRNA). Expression of tRNAs was hypothesised to be a lot higher in ovaries than in testes, as it has already been proved with the other major product of RNA polymerase III activity, 5S rRNA. The teleosts species selected for this study were the zebrafish, *Danio rerio,* as laboratory model species whose genome has been fully sequenced, and the thicklip grey mullet, *Chelon labrosus*, as pollution sentinel species that in the Basque coast has displayed cases of intersex with oocytes produced in testis due to exposure to xenoestrogens. Primers for PCR analysis were designed against the multiple genes displayed in the genome of zebrafish targeting specific amino acid tRNAs. Such primers allowed quantification of transcription levels of selenocysteine, suppressor, phenylalanine, tryptophan and alanine tRNAs showing higher transcription levels in testes than in ovaries. These results against expectations could be due to the different cross-amplification of primers across the more than 50 genes per analysed specific tRNA or due to tRNA structural properties that did not allow adequate PCR analysis. No amplification was obtained in the case of *C. labrosus* with the exception of suppressor tRNA. Small RNA capillary electrophoresis of total RNA, which allows identification of very small RNA fragments up to 200 bp in length, showed higher production of tRNAs, as well as of 5S rRNA, in the ovaries than in testes in both species. The calculated tRNA/5S rRNA index value showed no differences between gonads of both sexes but the tRNA/5.8S rRNA index clearly showed the highest levels in ovaries than in testes. These differences were only significant in the case of *C. labrosus* (analysed *C. labrosus* ovaries were all previtellogenic). This proves a high RNA polymerase III activity during the early phases of oogenesis in *C. labrosus* that results in high levels of tRNA and 5S rRNA. Conversely, RNA polymerase I activity and production of 5,8S, 18S and 28S rRNA is initiated with vitellogenesis which explains low levels in the studied mullet samples. In the meantime, zebrafish ovaries were showing oocytes in all stages of oogenesis and this did not allow significant differences in tRNA/5.8S rRNA index values between gonads. Bulk tRNA production has been proven to be a good marker to identify sex in teleost fish when comparing gonads in the early stages of gametogenesis. Similarly, the tRNA/5.8S rRNA index has shown to be a useful proxy to identify the oogenesis stage in fish ovaries. Further research is necessary to elucidate the usefulness of each specific tRNA in the monitoring and staging of ovarian gametogenesis in teleost fish.

LABURPENA

Azken hamarkadetan, metodo molekularrak gero eta garrantzi handiagoa dute sexu desberdintzapen eta gametogenesiaren faseen identifikazioan arrain teleosteoetan, aurreikuspen zehatzagoak eskaini ahal baitituzte gonaden analisi histologiko edo bisualak baino. Hori EBko "Data Collection Framework" betebeharra da, arrantzaren kudeaketa hobetzeko politika aurrera eramateko, non arrain komertsialen emankortasunaren estimazioa interes handia duen. Ikerketa honetan arrainetan obarioak eta testikuluak bereizten dituen transkripzio diferentziala analizatu nahi genuen amino azido espezifiko transferentziako RNA (tRNA) transkripzio maila aztertuz. tRNAren adierazpena obarioetan testikuluetan baino askoz altuagoa izateko hipotesia zegoen, polimerasa III aktibitatearen beste produktu nagusiarekin, 5S rRNA, frogatu den bezala. Analisi honetarako aukeratutako teleosteo espezieak, hauek izan ziren: zebra arraina, *Danio rerio*, genoma guztiz sekuentziatuta duen laborategiko eredu espezie gisa, eta hondoetako korrokoia, *Chelon labrosus*, poluzioaren monitorizatzaile gisa, Euskal kostaldean intersex egoera aurkeztu baitu xenoestrogenoen ondorioz obozitoak ekoiztuz testikuluetan. PCR analisirako primerrak diseinatu ziren *D. rerio* genomako amino azido espezifiko tRNA geneak kontuan izanda. Primer horiek selenozisteina, supresorea, fenilalanina, triptofanoa eta alanina tRNA transkripzio mailak kuantifikatzea ahalbidetu zuten testikuloetan obarioetan baino transkripzio maila handiagoak erakutsiz. Hondoetako korrokoiaren kasuan ez zen anplifikaziorik lortu supresore tRNAan izan ezik. RNA totalaren RNA txikiko kapilare elektroforesia, 200bp-ko RNA kate txikien identifikazioa ahalbidetzen duena, 5S rRNA eta tRNA produkzio handiagoa erakutsi zuen obarioetan testikuloetan baino bi espezieetan. Kalkulatutako tRNA/5S rRNA indizearen balioak ez zuen ezberdintasunik erakutsi gonaden artean, baina, tRNA/5.8S rRNA indizeak maila altuagoa argi erakutsi zuen obarioetan testikuloetan baino. Ezberdintasun horiek soilik izan ziren esangarriak *C. labrosus* kasuan (aztertutako *C. labrosus* obario guztiak prebitelogenikoak ziren). Horrek egiaztatzen du RNA polymerasa IIIren aktibitate handia *C. labrosus* oogenesiaren fase goiztiarretan tRNA eta 5S rRNA sortzen duena. Alderantziz, RNA polimerasa I-ko aktibitatea eta 5,8S, 18S eta 28S RNAren ekoizpena vitellogenesiarekin batera hasten da, korrokoietan lortutako balio baxuak azaltzen duena. Bitartean, *D. rerio* obuluak fase oogenesi guztietako obozitoak erakutsi dituzte eta horrek ez du ahalbidetu ezberdintasun esangarririk gonaden arteko tRNA/5.8S rRNA indizean. tRNA produkzioa sexua identifikatzeko markatzaile ona dela frogatu da arrain teleosteoetan gonadak alderatzean gametogenesiaren fase goiztiarretan. Era berean, tRNA/5.8S rRNA indizea arrain obulutegietako oogenesi fasea identifikatzeko baliogarria dela erakutsi da. Ikerketa gehiago egin behar dira zehazteko tRNA espezifiko bakoitzaren erabilgarritasuna monitorizazioan eta arrain teleosteoen obulutegi gametogenesiaren faseen adierazpenean.

1. INTRODUCTION

The study of the reproduction biology of fish from natural populations is a tedious and laborious task that, since the beginning steps of fisheries, has been receiving increasing attention. The study of fish reproduction and fecundity contributes to better management of commercial fish stock providing other benefits such as better monitoring of the environmental condition. The determination of teleostean fish sex ratios and the estimation of their maturity under different environmental conditions are also relevant for fisheries management, and they are a requirement of the European Data Collection Framework (https://datacollection.jrc.ec.europa.eu). In the scope of the global sustainability goals of UNESCO and in particular, in relation to the sustainability goal 14, life below water, such research is relevant as it contributes better biological resources, food, and health allowing for socio-economic growth.

Lately, molecular analysis of reproduction control mechanisms has acquired special relevance and teleost fish have become relevant laboratory study organisms. Not only they are studied as vertebrate models for the understanding of developmental processes but to study new, efficient, and more precise methods to better harvest this important resource. The analysis of molecular and cellular mechanisms regulating sexual differentiation can provide more accurate and unbiased information on fish development and reproduction than a simple visual or histological analysis of the fish gonads. It also allows the early monitoring of the possible effects of various external factors such as the effects of pollutants and reduced food availability before they are manifested at the histological level (Diaz de Cerio & Cancio, 2012).

Regarding the molecular mechanisms of sexual differentiation, the gender-related differences in gene transcription arising during gonad differentiation processes and, upon differentiation, in gametogenesis has allowed to identify different marker genes with diagnostic capability (Rojo-Bartolomé *et al.*, 2012; 2016). The typical marker gene of sex differentiation in oviparous vertebrates, and very especially in teleost fish is vitellogenin (Reading & Sullivan, 2011). Vitellogenin is produced in the liver of mature females, and then it is transported to the ovary forming the main storage protein during oocyte secondary growth and allow early embryo development (Rosanova *et al.* 2002). Thus, transcription of vitellogenin is the most used molecular marker of female differentiation and advanced gametogenesis in females. This can be analysed through analysing the levels of transcription of vitellogenin in the liver, or the level of circulating protein in the blood (Chu-Koo et al., 2008).

However vitellogenin is not the only marker of sex differentiation in fish, and to mention some we could call attention to the brain and gonadal aromatases (markers of female sex differentiation), dmrt1

and amh (markers of male sex) (Le Page *et al.*, 2010; Lin *et al.*, 2017). Several other genes can be seen in Figure 1. In the case of the Cell Biology in Environmental research Group of the University of the Basque Country, the gonadal transcription levels of some specific transcription factors associated with 5S rRNA production have been applied as powerful identifiers of sex on fish and of oogenesis stage in females (Diaz de Cerio *et al.*, 2012; Rojo-Bartolomé *et al.*, 2016). A clear difference in transcriptional profile has been proved between ovaries and testis in most teleost species, as that most represented transcript in the total RNA of ovaries is 5S rRNA while in testis, as in any other tissue it is 18S and 28S rRNA (Diaz de Cerio *et al.*, 2012; Rojo-Bartolomé *et al.*, 2016). In the same way, transcripts of genes associated with 5S rRNA transcription, transport and accumulation in the cytosol are more represented in ovaries than in testis (Diaz de Cerio *et al.*, 2012). However, there are some molecular markers on which the scientific community has not focused enough yet, one of those could be the transfer RNAs (tRNA). Take into account that while mRNAs are transcribed by RNA polymerase II and 18S and 28S rRNA are transcribed by RNA polymerase I, 5S rRNA but also tRNAs are transcribed by RNA polymerase III (Geiduschek & Tocchini-Valentini, 1988).

As highly stable cellular adaptors that efficiently translate genetic information into proteins on our biological systems, the importance of tRNA has been robustly proved. Not only that but their participation in other biochemical roles such as sensing the availability of certain amino acids, protein degradation, and biosynthesis of certain metabolites has proven to be of great interest. What makes all these functions possible is the network of interactions between tRNAs and enzymes (Phizicky and Hopper, 2010). tRNAs are also the most transcribed type of RNA by RNA polymerase III, as tRNA abundance allows higher translation efficiency and increases the most expensive metabolic process within a cell, protein production (Shah and Gilchrist, 2010).

tRNA sequence is usually composed of 73 to 90 nucleotides that make a cloverleaf secondary structure. The D-loop, T loop, variable loop, and anticodon loop form this structure, which further folds into an L-shaped tertiary structure. New functions are still emerging and, in recent years, the diverse roles of this kind of RNA have received a great deal of attention (Raina and Ibba, 2014).

tRNA, along with rRNA, has a special interest when we analyze the embryogenesis of some vertebrate species such as the amphibian *Xenopus laevis* or the teleost fish. tRNAs and 5S accumulate in the oocytes (Mazabraud, Wegnez & Denis, 1975; Diaz de Cerio *et al.,* 2012), most specifically in the cytosol, as a ribonucleoprotein complex termed the 42S RNP in the case of 5S rRNA.In the event of successful fertilisation these storage complexes fuel early embryo development allowing fast ribosome assembly and protein synthesis (Picard *et al.*, 1980).

One of the most studied teleosts is the laboratory model species zebrafish, *Danio rerio*. Its great similitude with the human genome, concise life cycle to reach maturity, great reproductive capability and transparency during the embryonic stage along with other advantages, has led *D. rerio* to be one of the most demanded animals in several disciplines worldwide, among them ecotoxicological research (Rougvie & O'Connor, n.d.).

For its characteristics, the zebrafish is a popular model organism in developmental biology. In typical laboratory conditions, this meaning a mean growing temperature of 28.5ºC and a light cycle of 14:10 light-dark hours, the embryogenesis takes 24 hours after the fertilisation, from a one-cell zygote above a yolk cell to a gastrula after 6 hours, and larvae hatch within 2-3 days (Ulloa, *et al.*, 2011). The transition from larva to juvenile is within 30 days postfertilization and they reach sexual maturity from 10 to 14 weeks and can live up to five years (D'Costa & Shepherd, 2009). The sex determination process in zebrafish starts at least ten days post fertilization (dpf) with all individuals developing first as immature females presenting previtellogenic early oocytes in their gonads. The mitotic activity starts at 15 dpf and sex differentiation starts with the appearance of perinuclear oocytes, around 17 dpf. At 21 dpf two patterns appear in the gonads and meiosis is going to initiate. By 25 dpf gonads are still bipotential, but, depending on the signalling network, some maintain the oogenic pathway and others start apoptosis of the ovarian tissue to initiate the process of differentiation from juvenile ovary to testis. At 40 dpf female gonads have various stages of the oogenesis, while degenerative oocytes are present, determining the testis (Fig. 1.).

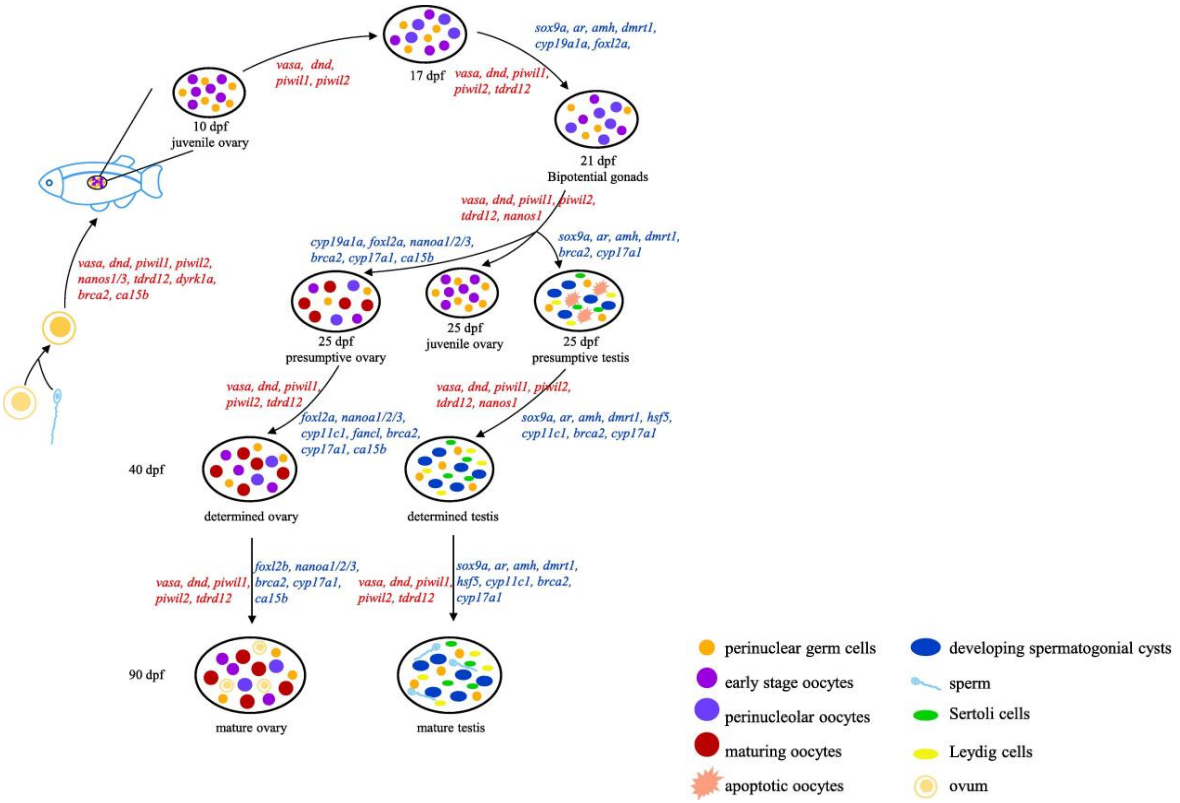


Figure 1: The process of sex determination and gonad development in teleost fish. Different cells are represented with different shapes and colours. The gene network associated with each pathway during gonad development is also indicated, depicting involved genes. Figure taken from Ye & Chen, 2020.

However, environmental health assessment can not rely on a laboratory model species, especially in relation to temperate estuarine and marine environments *Danio rerio* being a tropical freshwater species. In the Basque coast and its estuarine waters, the pollution sentinel species of interest is the thicklip grey mullet, *Chelon labrosus* (Diaz de Cerio *et al.*, 2012; Ortiz-Zarragoitia et al 2014). *C. labrosus* has a wide distribution in European waters and it is a gonochoristic fish species whose sex-determination system is not known yet, as it happens for most of the teleostean fish species. It has been extensively used as a sentinel of exposure to xenoestrogenic compounds in the Basque estuaries where high prevalences of intersex condition have been identified (Diaz de Cerio *et al.*, 2012; Ortiz-Zarragoitia et al 2014Bizarro et al., 2014), Intersex condition, is a pathological condition in which oocytes at varying degrees of development are produced in the testicular tissue of males due mainly to exposure to chemical compounds similar in structure to estradiol (Bizarro et al., 2014). In some places like in the estuary of Gernika or the harbour of Pasaia, prevalences as high as 80% have been described, this meaning that most of the males in those populations are suffering from severe symptoms of feminisation that could be affecting their fecundity and the viability of the populations.

Objectives

The main objective of the present research work is to develop new molecular markers of sex differentiation in the gonads of teleost fish, that could also be used to identify the presence of oocytes and also their maturation stage. The specific objective developed was to analyse the transcription pattern of transfer tRNAs and 5S rRNA in the gonads of zebrafish (*Danio rerio*) and thicklip grey mullet (*Chelon labrosus*) through PCR and total RNA quality analysis.

2. METHODS

2.1. Animals and RNA samples

Previously extracted and stored total RNA samples have been used in this experimental procedure. TRIZol extraction method was used to obtain the total RNA of both species. Ten RNA samples of zebrafish (*Danio rerio)* gonads were used during this experiment, of which five were females and the other half males. Total RNA samples of thicklip grey mullets (*Chelon labrosus)* gonads were also used. Five female, seven male, and two undetermined samples. All the samples were kept at -80ºC to preserve the RNA properly.

2.2. Small RNA quantification and index calculation

Total RNA was run in a capillary electrophoresis system using a Small-RNA kit along with the Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Alemania). Instructions provided by the kit were followed to run ten samples of *Danio rerio* on one chip and five male, four female, and two undetermined *Chelon labrosus* samples on a second chip with diluted samples at a concentration of 50ng RNA/µl H_2O.

Using the RNA profiling on the electropherograms the time corrected area of the tRNAs and the 5S and 5.8S RNA peaks were calculated. The areas were used to calculate the relative abundance of the tRNAs in two ways: A) tRNA/5S rRNA and B) tRNA/5.8S rRNA. Both indexes were calculated for each gender in each species. In the cases where the peak is unnoticeable a value of 0.1 was considered.

2.3.tRNA Primer design

The Genomic tRNA Database was used to get the tRNA scan-SE Analysis of *Danio rerio* (Genomic tRNA Database of zebrafish genome Zv9; (Chan, P.P. & Lowe, T.M., 2009). A multiple alignment process of the FASTA sequences of alanine, asparagine, phenylalanine, selenocysteine, tryptophan, tyrosine, and suppressor tRNA sequences was executed using the Multiple Sequence Alignment by CLUSTALW (Multiple Sequence Alignment by GenomeNet (Thompson et al., 1994)). Manually organization by similarity was made for each cluster. Having the division, a forward primer was sketched for each group. The primers were manually designed to theoretically attach to as many sequences as possible (Table 1).

To know how specific each primer pair was, and to calculate the probability of a primer attaching to a sequence, CLUSTALW was used again to see the similarity between the primers and each amino acid isotype. Finally, the coverage percentage of the forward and reverse primer couple on all the tRNA sequences of each isotype was calculated (Table 2).

2.4. Quantitative polymerase chain reaction (qPCR)

qPCR of *D. rerio* and *C. labrosus* were made for primer testing and sample analysis. For the first step, 2 µl from two female samples and 2 male samples on each species were used, at a concentration of 1 ng RNA/µl H_2O. For the amplicon confirmation, 2 µl of all samples were tested and each sample was amplified in triplicates.

For the amplification, a 7300 Real-Time PCR System (Applied Biosystems) was used. 10µl of FastStart Universal SYBR Green Master Mix (Rox) (Roche Diagnostics, Mannheim, Germany) was added to each sample. For the experiment, MicroAmp Optical 6-well reaction plates (Applied Biosystems) were used. The thermal cycler was adjusted in the following way: the activation stage at 50ºC 2 minutes and at 95ºC 10 minutes, 40 cycles at 95ºC 15 seconds and at the melting temperature (Tm) of the primers 1 minute (Table 1). The temperature was adjusted on the third phase of the qPCR in accordance with the melting temperature of each primer used. Finally, a dissociation stage was added at the end of the third phase of all qPCR which included 15 seconds at 95ºC, 1 minute at 60ºC, and 15 seconds at 95ºC.

Table 1: The identification, sequence and melting temperature of the primers used for this experiment. The identification is based on the amino acid coded by the tRNA: Tyr (Tyrosine), SeC (selenocysteine), Sup (Suppressor), Ala(Alanine), Trp (Tryptophan) and Asp (Aspartate). The melting temperature (TM) for each oligonucleotide is the temperature at which the RNA duplex will dissociate.

Forward primer identification	Sequence(5`→3`)	Tm (ºC)	Reverse primer identification	Sequence(5`→3`)	Tm (ºC)
Tyr Fw1	CCTTCGATAGCTCAGTTG	56.7	Tyr Rv1	TCCTTCGAGNCGGAGTCGN	59.4
SeC Fw1	TCAGGCCCAGTKGCCYAATGK	61.4	SeC Rv1	CAACCCAGRAACWGGGACTTGAACCCT	63.6
SeC Fw2	TCAAGCATRGTGGCCTAATGGA	59.3			
Sup Fw1	GCCTGGCTAGCTCAGTYGGTA	62.8	Sup Rv1	CGCCCAAMGTGGGGCTCRAA	63.5
Sup Fw2	GGTTCCATGGTGTAATGGTT	55.3	Sup Rv2	MNGTYCMACCAARATTTGARCT	53.2
Sup Fw3	GCCYGGATRGCTSAGTCGGTA	63.7	Sup Rv3	YGCCCRAACWGGGACTTGAACCCT	65.1
Ala Fw1	GTTTCYGTAGTGTAGTGGT	53.4	Ala Rv1	TGGAGATGCTGGGGATTGAACCCA	59.8
Ala Fw2	GGGGAATTAGCTCAARTGG	55.6	Ala Rv2	TGTTTCTGCTCRGTTTCRAA	53.2
Ala Fw3	GCATTGGTGGTTCAGTGG	56.0			
Trp Fw1	GACCTYSTGGYGCAACGGTA	61.4	Trp Rv1	TGACCCYGACGTGATTTGAA	54.2
Trp Fw2	GGACCTTTTGCGCAATGGTA	57.3			
Asp Fw1	TTCCTMGTTAGTATAGTGGAC	54.9	Asp Rv1	CCCCRTCRGGGWATCGAAC	58.2

The Ct values were also treated to obtain a relative quantification. This was carried out by comparing the Ct values of the female and male samples and normalizing the data ($2^{-\Delta\Delta Ct}$). For this method, the

mean of all testes and ovary Ct were used as calibrators and the mean of all testes Ct on each primer was used as a control.

Table 2: The coverage percentage (%) of the different forward and reverse primer combinations for each amino acid isotype. the combinations are indicated by the numbers of the first row: The first number indicates the Forward primers identification number and the second number indicates the reverse primers identification number. All the sequences coding each amino acid isotype on the Genomic tRNA Database were included in the comparison. Suppressor forward primers 1 and 3 (*) share multiple isotypes, thus, the resulting percentage is bigger than 100%. Alanine isotypes showed great variability, resulting in very specific primers that attach to few isotypes.

Primer combination (Forward.Reverse)	Asp(%)	Tyr(%)	SeC(%)	Sup(%)*	Ala(%)	Trp(%)	Phe(%)
1.1	98,73	86,96	52,94	31,25	1,59	78,95	82,74
1.2			14,29	0,00	0,00		
1.3				0,00			
2.1				0,00	0,88	13,16	
2.2				18,75	0,00		
2.3				0,00			
3.1				31,25	0,00		
3.2				0,00	0,71		
3.3				25,00			
Total aa isotype amplification prediction (%)	98,73	86,96	67,23	106,25*	3,18	92,11	82,74
Total different isotypes	59	230	14	17	572	38	227

2.5. Amplicon confirmation by agarose gel electrophoresis

The primer testing qPCR products were analyzed by running 2% agarose electrophoresis and Ethidium Bromide was used as a dye. 1µl of gel dye Bromophenol blue (MO BIO Laboratories Inc., Carlsbad, CA USA) was added to the qPCR products, from which 8µl of one of the three replicas of each sample was loaded on the gel. NZYDNA Ladder V (NZYTech, Lisboa, Portugal) was used as a reference. The gel was run for 20 minutes at 120V with Basic powerPack (BioRad, Spain). For the imaging, the system Vilber Lourmat EBOX VX5/20LM (fisher scientific, Canada) was used.

2.6. Statistical analysis

The statistical analyses were carried out with the SPSS software (IBM SPSS Statistics for Windows, Version 26). The two ratios obtained from the electropherogram and the $2^{-\Delta\Delta Ct}$ values of each selected primer couple were tested for normality and then a t-test, for normally distributed data, or a Mann-Whitney U test, for normally distributed data, was applied. The significance was established at a p-value of 0,05.

3. RESULTS

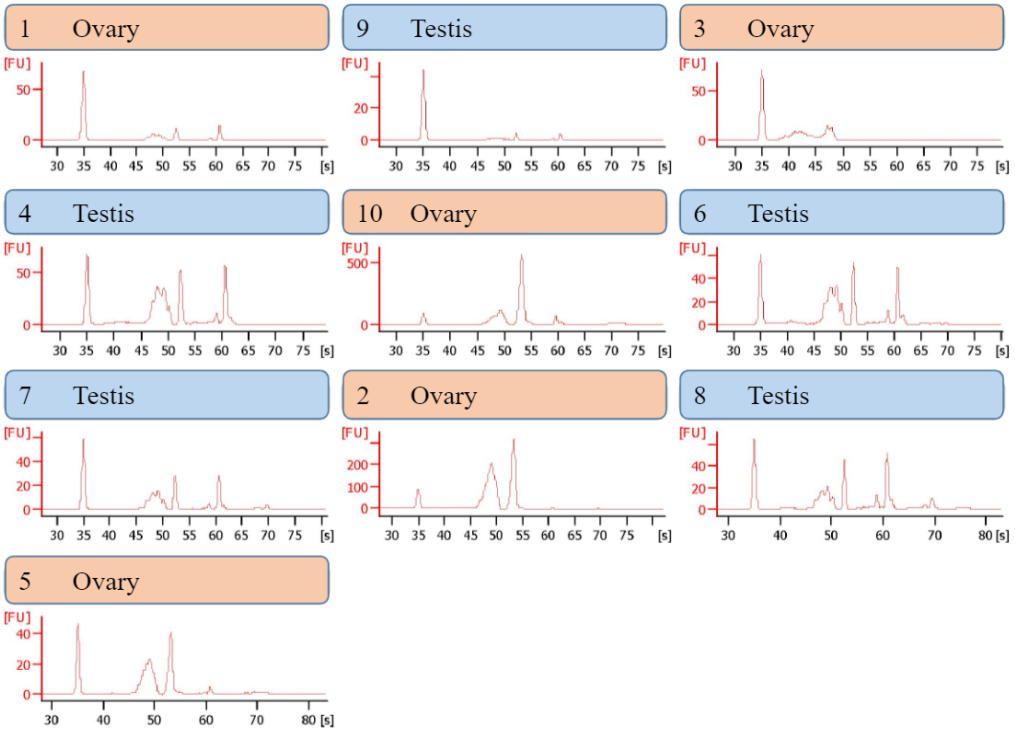
3.1.tRNA on bioanalyzer

The pattern of tRNA distribution is different in *Danio rerio* and *Chelon labrosus* gonads. In the samples of zebrafish, ovaries showed accumulation of tRNAs that appeared at 45-50 sec in a peak lower in height than that observed for the nano-chip markers, followed by a predominant peak for 5S rRNA (at 52 seconds). The peak for 5.8 rRNA was nearly unnoticeable (Figure 1a). The pattern differed in testes where the peak for 5S rRNA was similar to that for 5.8 rRNA. In testes, tRNAs accumulated to concentrations similar to those of 5S rRNA as seen by the height and width of the peak at 40-50 seconds. In the batch of samples shown in Fig. 1a, RNA in sample number 3 might be degraded.

In *C. labrosus* electropherograms, tRNAs were observed to accumulate mainly in the ovary samples (Fig.1b) the tRNA specific peak being followed by a very big peak of 5S rRNA, whereas the presence of 5.8S rRNA could not be noticed. The peak belonging to the tRNAs, and also the one for 5S rRNA, were significantly higher than the peak for the internal marker (around 35 seconds), unlike in zebrafish ovaries. In males, small rRNAs, 5S rRNA and 5.8 S rRNA were nearly unnoticeable.

When looking at the calculated concentration ratios (Fig. 2.), tRNA/5S rRNA ratio was higher in males than in females, but there were no significant differences between ovaries and testes in any of the two fish species studied. The tRNA/5.8S rRNA ratio instead showed clearly higher values in ovaries than in testes. In the case of *D. rerio*, there were no significant differences between means in both gonads but in the case of *C. labrosus*, significant differences became very evident indicative of an increased expression of tRNAs in the ovaries in comparison to the testes.

a) Danio rerio



b) Chelon labrosus

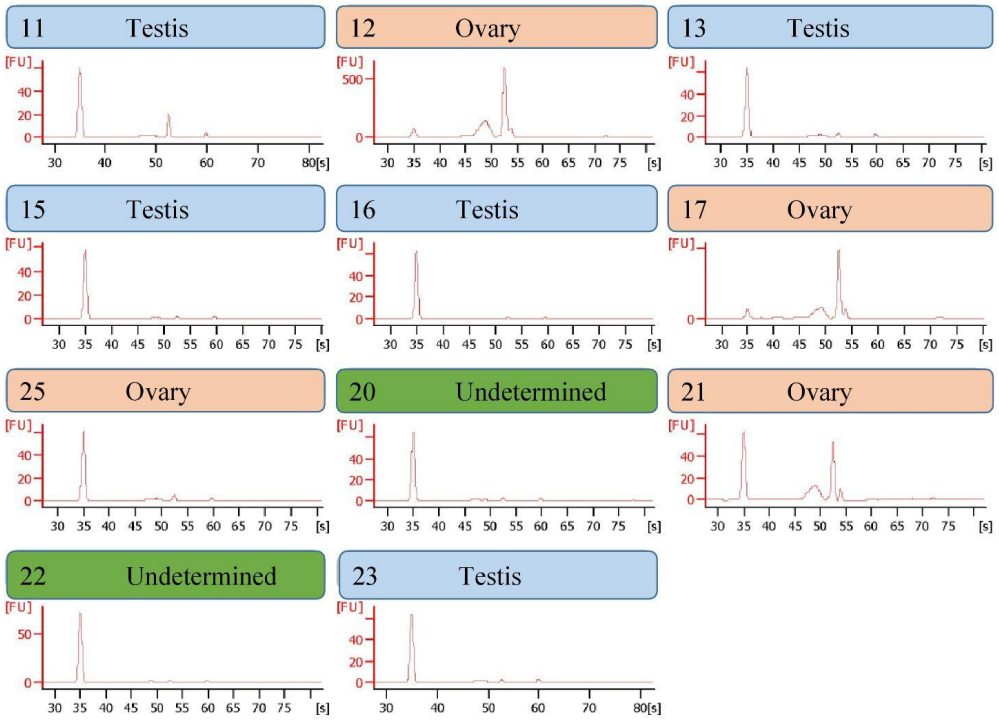


Figure 1: Electropherogram of Small RNA Agilent nano-chips for *Danio rerio* (sample 1 to 10) **(a)** and *Chelon labrosus* (sample11 to 25) **(b)**. The first peak in the electropherogram (35 seconds) belongs to the chip internal marker, the second peak (between 45 and 50 seconds) to tRNAs, the third (between 50 and 55 seconds) to 5S rRNA and the fourth one (60 seconds) belongs to 5.8S rRNA. Blue colour indicates testis, green gonad samples of undetermined sex and pink ovaries.

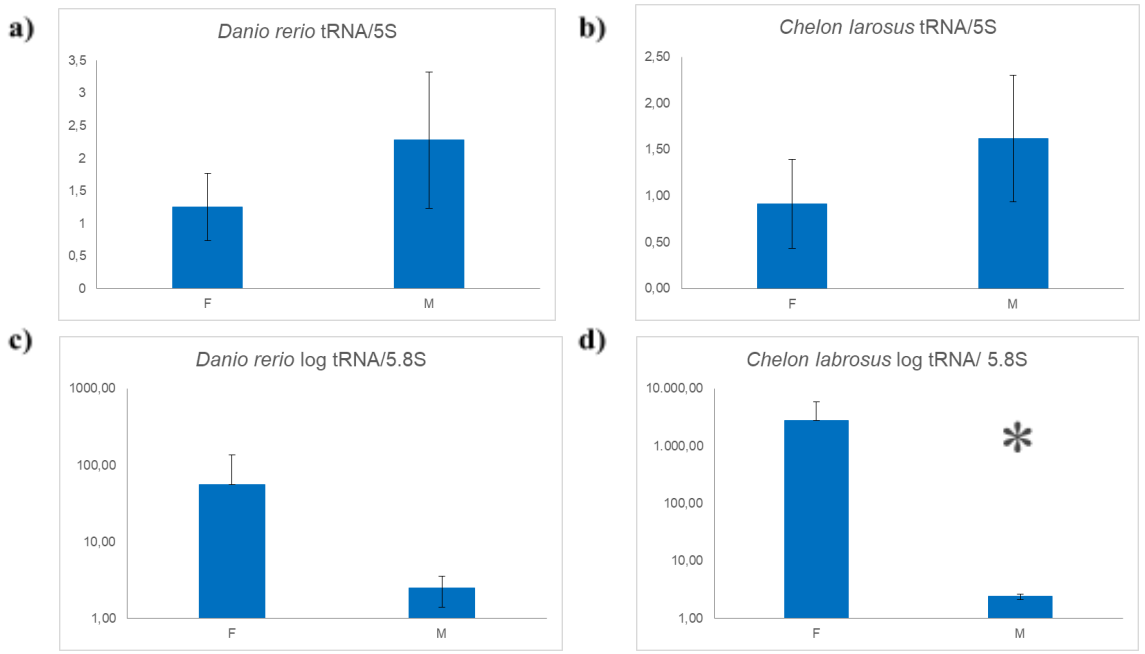


Figure 2: Histograms of the tRNA/rRNA ratios calculated in *Danio rerio* and *Chelon labrosus* ovaries and testes. **a)** and **b)** show the tRNA/5S ratio for testes (M) and ovaries(F) of *D. rerio* and *C. labrosus* respectively. **c)** and **d)** show log(tRNA/5.8S) ratio for testes (M) and ovaries(F) of *D. rerio* and *C. labrosus* respectively. Statistical differences between ovaries and testes are marked with an asterisk ($p<0,05$).

3.2. Levels of transcription of specific tRNAs: PCR and qPCR

Gel analysis of amplicons obtained after using all primer couples designed against specific amino acid tRNAs provided different results. In *D. rerio* selenocysteine, suppressor 3.1., phenylalanine, tryptophan, and alanine 2.1. specific tRNAs were successfully amplified in ovaries and testes showing higher expression levels in testes. However, the remaining primers designed for alanine, tyrosine, asparagine, and suppressor specific tRNAs did not produce any amplicons (Fig.3). The functioning primers were selected for qPCR analysis and to compare tRNA expression levels between testes and ovaries in *D. rerio*. In the case of thicklip grey mullets, successful amplification was only obtained with *D. rerio* primers for suppressor tRNA (Fig.4), both in testes and in ovaries.

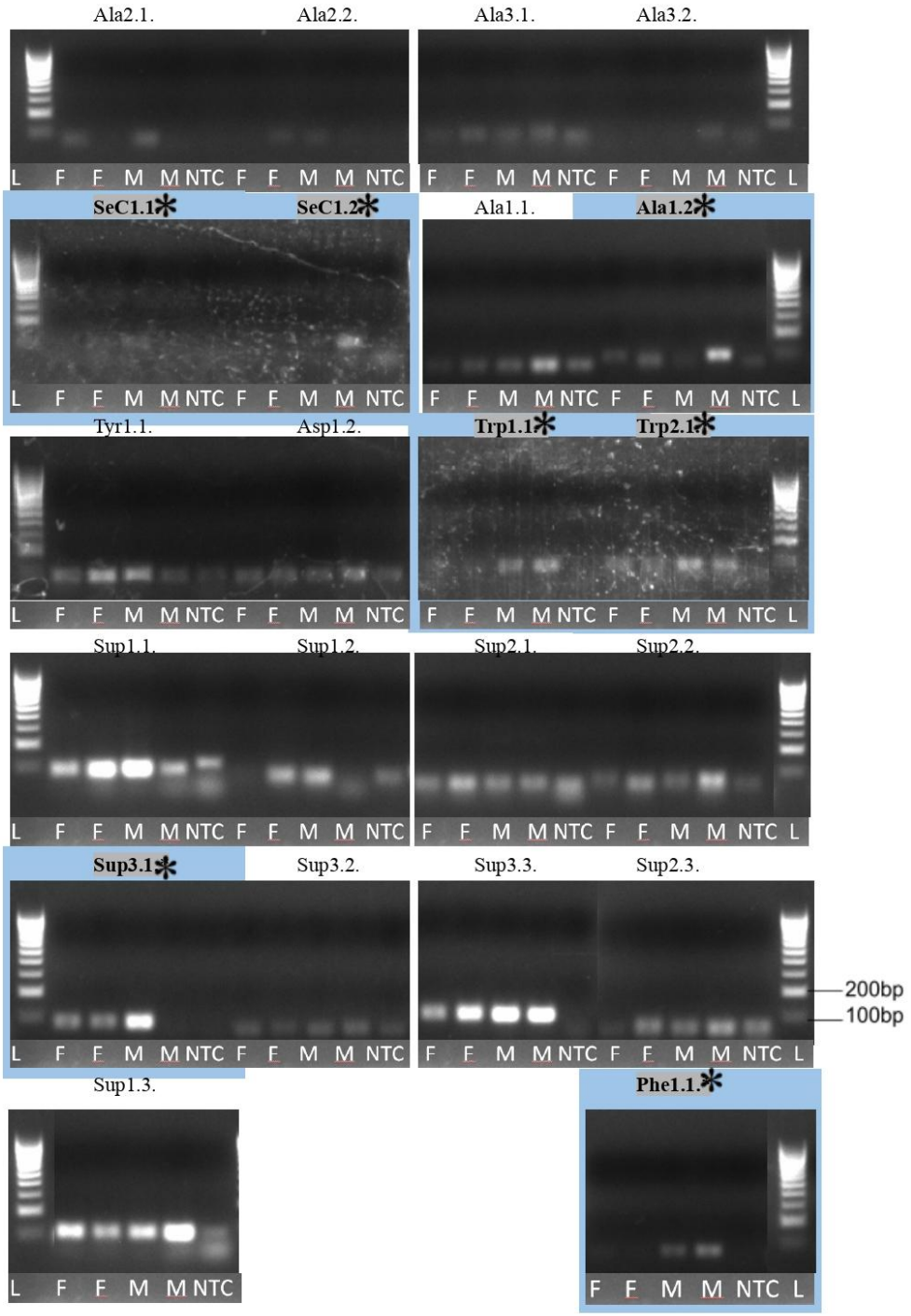


Figure 3: Agarose gels of tRNA amplicons obtained from two ovaries and two testes of *Danio rerio* obtained after utilizing all primer couples tested. F: ovary, M: testis, NTC: Non-template control, L: ladder. Asterisks and a blue highlight are used to mark the primer couples used on the tRNA to compare tRNA levels in ovaries vs testes.

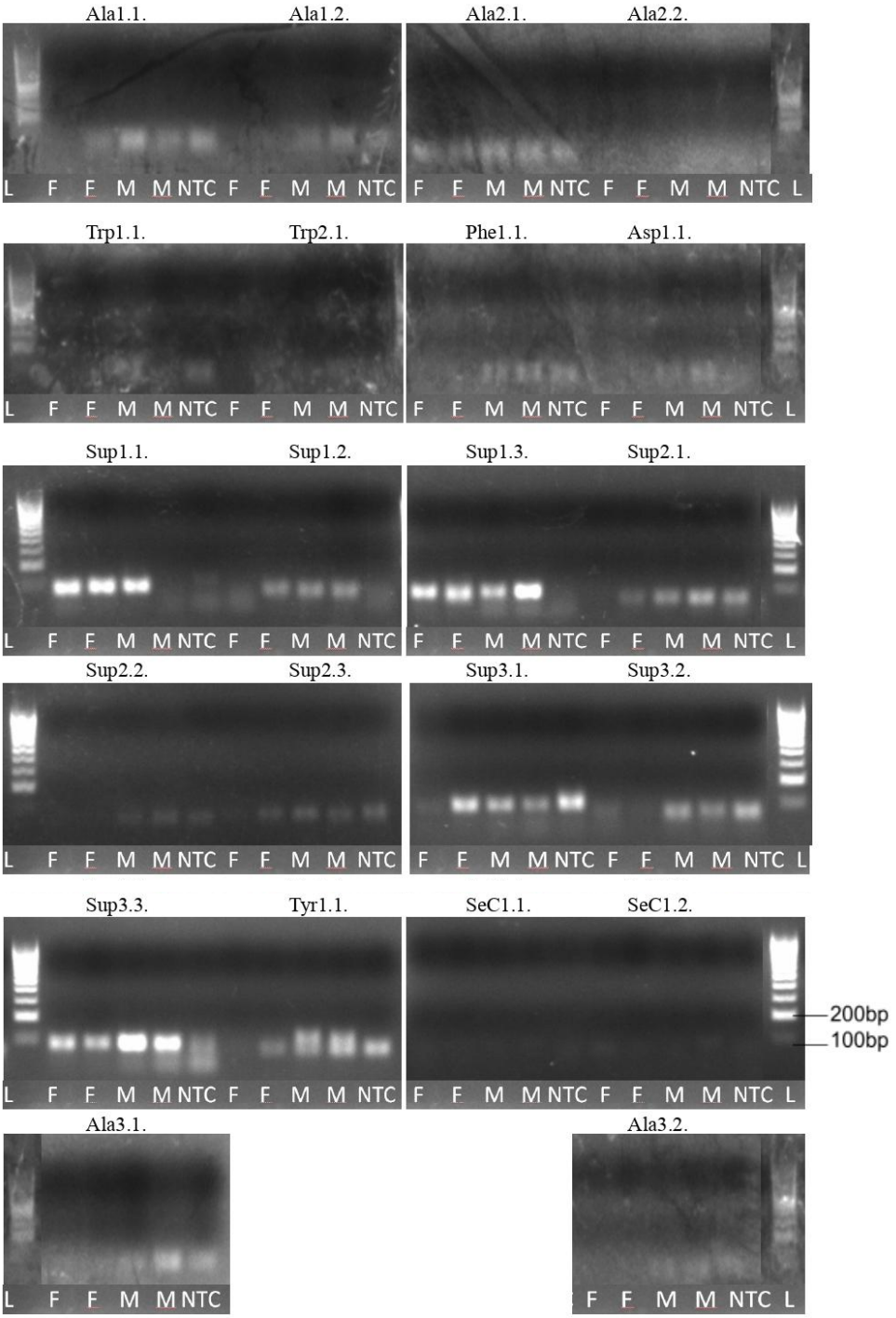


Figure 4: Agarose gels of the amplicons obtained from two ovaries and two testes of *Chelon labrosus* after utilizing all primer couples tested. F: ovary, M: testis, NTC: Non-template control, L: ladder

3.3. tRNA in ovaries vs testes (*Danio rerio)*

qPCR analyses revealed slightly higher transcription levels in *Danio rerio* testes than in ovaries when paying attention to the Ct values (Fig.5a) and, thus transcription of those particular tRNA genes would be higher in testes than in ovaries. The relative quantification showed a greater $2^{-\Delta\Delta Ct}$ mean value for testes than for ovaries for all tRNAs (Fig. 5b). These differences were only statistically significant for suppressor, phenylalanine and alanine tRNAs.

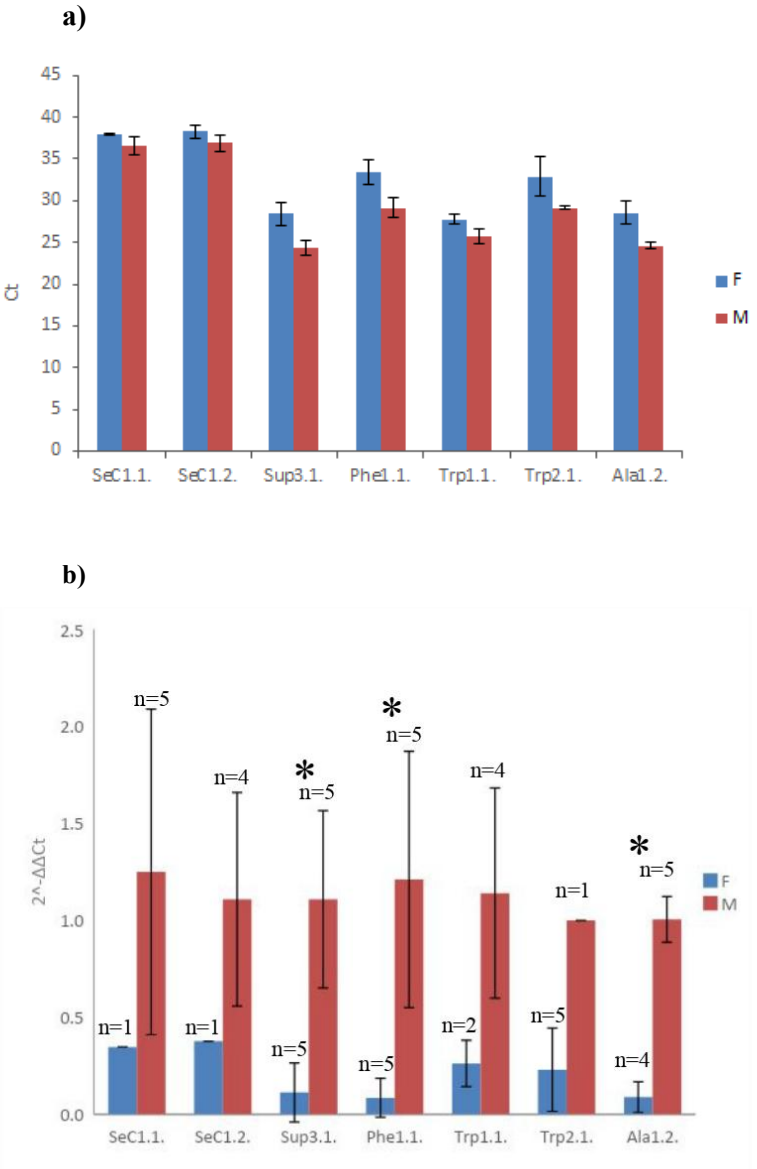


Figure 6: Two histograms showing qPCR results **a)** Mean and the standard deviation of the Ct values obtained in testes and ovaries of *Danio rerio* samples. On the longitudinal axis, the different combinations of primers used for the amino acid specific tRNAs: selenocysteine (SeC), suppressor (Sup), phenylalanine (Phe), tryptophan (Trp) and alanine (Ala) are displayed. **b)** Normalised transcription levels of the above mentioned tRNAs in ovaries (F) and testes (M) of *Danio rerio*. Sample size (n) is indicated on each bar representing mean transcription levels. Individual samples were excluded from the analysis if there was no transcription identified. Asterisks indicate significant differences between testes and ovaries for each specific tRNA ($p<0,05$).

4. DISCUSSION

In the present study, we have focused our attention on the analysis of the levels of transcription of tRNAs in the gonads of zebrafish *Danio rerio* and thicklip grey mullet *Chelon labrosus*. Primers generated for the amplification of specific amino acid tRNAs but coded by multiple genes per amino acid have not provided concluding results in any of both species. On the other hand, total RNA extracted from ovaries and testes and analysed using small Agilent Bioanalyzer RNA nano-chips has revealed strong transcription of tRNAs in ovaries, similar to observations for 5S rRNA, well above levels identified in testes.

There is a growing need for effective fish stock monitoring methods, some of them implying the analysis of molecular and cellular mechanisms for a precise and efficient identification of sex and gonad maturation (gametogenic) stage in teleosts (Ortiz-Zarragoitia et al., 2014; Devlin &Nagahama, 2002). Fecundity studies are employed to extrapolate population reproduction capacity and resilience under different environmental pressures, also overfishing. The above mentioned molecular approaches could be easily implemented to conduct such fecundity studies. On the other hand, sex differentiation can be altered in teleosts under exposure to xenobiotics, notably under exposure to xenoestrogens, and the above methods can be employed for early warning pollution biomonitoring studies and for the analysis of the effects of such exposures on fish populations.

Our initial hypothesis was that as it occurs with 5S rRNA (Diaz e Cerio et al., 2012, Ortiz Zarragoitia et al., 2014, Rojo-Bartolomé et al., 2017; Rojo-Bartolomé, 2017) ovaries produce extremely high concentrations of tRNAs in their early developing oocytes in order to fuel protein production during early embryo development. With the aim to quantify the levels of transcription of specific tRNAs in zebrafish the tRNA transcriptome sequence of zebrafish was explored and primers were designed maximising the possibilities to amplify the highest amount of different genes for each candidate amino acid tRNA. The same primers were used for *C. labrosus* whose genome has not been sequenced and for which no tRNA sequence is at hand. The Genomic tRNA Database was used to get the tRNA scan-SE Analysis of *Danio rerio*, Genomic tRNA Database of zebrafish genome Zv9 (Chan, & Lowe, 2009). A total of 1157 tRNA gene sequences are present in the zebrafish genome. In regard to specific amino acid specific tRNAs, there are for instance 59 genes coding for asparagine tRNA (73 nt in length), 230 for tyrosine tRNA (from 72 to 86 nt), 14 for selenocysteine (from 86 to 88 nt), 17 for suppressor tRNAs (from 72 to 82 nt), 572 for alanine (from 72 to 74 nt), 38 for tryptophan (72nt) and 227 for phenylalanine (from 70 to 74 nt), just to mention some.

The specific tRNAs to be analysed were selected for no specific reason and taking into account the different roles they could play. Some of the tRNAs selected, coded for non-essential amino acids such

as asparagine or alanine, an amino acid widely used for contention of energy as well as for protein production (PubChem, 2021) and tyrosine, that also serves as a precursor of catecholamines, thyroxine, and melanin (Fernstrom et al., 2007). Among the tRNAs coding for essential amino acids, we selected Selenocysteine-tRNA, selenocysteine being involved in the catalytic mechanism of selenoproteins and selenoenzymes (Steinbrenner et al., 2016) and tryptophan-tRNA precursor of serotonin (Höglund et al., 2019). Finally, suppressor tRNA isotypes were also selected. Suppressor tRNAs can arise by mutations in a gene encoding a tRNA. For example, if the wild-type gene encoding a tRNA recognizing a UAC codon and inserting tyrosine into the growing polypeptide chain when suffering mutation the anticodon could be changed so as to recognize the stop codon UAG in the mRNA and, instead of terminating the protein synthesis would insert the corresponding tyrosine. The mutant form of the tyrT gene is called supF (Leach, 1993; Herring & Blattner, 2004).

A slightly higher expression was observed in testes than in ovaries of *D. rerio* for selenocysteine, suppressor 3.1., phenylalanine, tryptophan, and alanine 2.1 specific tRNAs by traditional PCR. These results were further explored by qPCR with the comparison of Ct values and suppressor, phenylalanine and alanine specific tRNAs showed slightly higher expression in testes than in ovaries. In the case of *C. labrosus,* no expression was observed in most cases and in those in which amplification was achieved no differences were observed between gonads as the primers had been designed for *D. rerio* genes. The lack of concluding results for all the amino acid specific tRNAs in *D. rerio* and the contrast with our initial hypothesis could be due to different reasons. As said above, there are many different genes coding for each of the amino acid tRNAs analysed and the designed primers may have proved unable to amplify a significant amount of them. On the other hand, tRNAs have a peculiar two-dimensional structure (cloverleaf structure) that further takes a L-shaped three-dimensional structure to allow insertion into the ribosome (Holley et al., 1965). This tertiary structure, together with the short length of the sequence, typically between 70 and 90 nucleotides (Cramer et al., 1969), may complicate the PCR analysis using traditional ~20 nucleotide long primers. Moreover, the majority of tRNAs at least in mammalian cells are methylated by tRNA methylases, post-transcriptional modification that is important for tRNA activity in protein synthesis. Specific methylations are important for the stabilization of tRNA structure, reinforcement of the codon-anticodon interaction, regulation of wobble base pairing, and prevention of frameshift errors (Hori, 2014). This methylation state could hinder proper PCR based analysis of the target sequences. In the light of all these problems, further studies should focus on the use of more specific primers on properly linearised small RNAs extracted from tissues, followed by a demethylation step. In order to understand the complexity of tRNA genes in teleostean genomes, a different technical approach should be selected all together. A method that could offer a wide range of opportunities is RNA sequencing (RNA-Seq) modified for the analysis of small RNAs and more specifically for tRNAs

(tRNA-Seq) that would enable quantitative and qualitative examination of all the tRNA transcriptome of the tissue studied (Hrdlickova et al., 2016; Pinkard et al., 2020).

To obtain a response to our initial hypothesis we decided to take an inspecific alternative approach to try to calculate the total amount of tRNA produced in ovaries and testes. Strongly expressed small RNAs can be quantified in total RNA analysed through small RNA nano-chips. These Agilent Bioanalyzer chips allow separation, detection and quantification by electrophoresis of RNAs of sizes below 200 nt, 5S rRNA being 120 nt and tRNAs 70 to 90 nt long. The results for each gonad were visualized through electropherograms, in which the fluorescently labelled RNA fragments separate according to size. The electropherograms showed a very strong production of tRNAs in *C. labrosus* together with a strong expression of 5S rRNA, which in all cases was stronger than that observed in testes. This is easily quantifiable through calculating the area of the peak for all tRNAs (coded by pol III) and for 5,8S rRNA (coded by polI as part of the 45S rRNA precursor that is cleaved into the 5,8S, 18S and 28S rRNAs), a proxy of the concentration of each of them. The tRNA/5,8S rRNA index calculated in that way shows a strong presence of tRNA in ovaries and a significant difference with testes in thicklip grey mullets. The results on *D. rerio* were not so marked and although the production of tRNA was high in ovaries the differences between testes and ovaries in tRNA/5,8S rRNA index were not statistically significant. In this context, the different gamete development mechanisms in the ovaries of both studied species need to be taken into account.

D. rerio follows an asynchronous mechanism of development during oogenesis, oocytes at different stages of development co-occurring in the same ovary at any given moment and spawning occurring regularly (Devlin & Nagahama, 2002; Rojo-Bartolomé et al., 2020). Meanwhile, oogenesis in *C. labrosus* is synchronous, meaning that all oocytes develop simultaneously and are at the same developmental stage, with one single spawning episode in each reproductive cycle (Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2017). In the study of Rojo-Bartolomé in 2017, *C. labrosus* was shown to have different ovarian transcriptome profiles in Agilent Bioanalyzer microchips depending on the oogenesis stage. In this sense, 5S rRNA was predominant in the total RNA of ovaries in previtellogenesis, 18S and 28S rRNA (products of pol I as 5,8 S rRNA) beginning to accumulate after oocytes entered vitellogenesis. In 2016 Rojo-Bartolomé *et al.* demonstrated that testis and ovaries accumulated different levels of 5S rRNA in nine teleost species (Atlantic, Atlantic-chub and horse mackerel, blue whiting, bogue, European anchovy, European hake, pilchard and megrim), with ovaries always showing higher values than testes, but 5S rRNA expression levels being at their highest when ovaries were at the earliest phases of development. The mullet ovaries studied in this work were all belonging to individuals in previtellogeneis with only perinucleolar oocytes present, so at the moment of peak 5S rRNA production, while in zebrafish the level of 5S rRNA (and tRNA)

produced in previtellogenic oocytes was compensated by the high levels of 5,8S rRNA in more developed oocytes.

All these results point in the same direction suggesting that during oogenesis from previtellogenic oocytes to the cortical alveolar stage, polymerase III activity is maximal at previtellogenesis (Denis and Wegnez, 1977; Rojo-Bartolomé, et al., 2016, 2017; Rojo-Bartolomé, 2017). This causes short RNA molecules 5S rRNA but, as proved hereby, also tRNAs to accumulate in immature oocytes. showed. According to calculations dating back to the 1970s in some teleosts tRNAs and 5S RNA can make up to more than 90% of the RNA content of the ovaries (Mazabraud et al. 1975). It is evident then, that the production of 5S rRNA and tRNAs is co-regulated and that, as observed hereby, the tRNA/5S rRNA index does not show any differences between ovaries and testes.

Overall, this proves that the activity of polymerase III is important during oogenesis as the process of ribogenesis and protein synthesis will be important during embryo development in case of successful fertilization. As previously mentioned, its key role stands on the previtellogenic stage, when its activity is maximal. The produced transcripts are stockpiled in the cytoplasm in the form of 7S and 42S ribonucleoprotein particles (42S RNP). These 42S RNPs are formed by 5S rRNA associated to mainly two proteins 42Sp50 and 42Sp43 contributing importantly in the ribogenesis and protein synthesis of the early stages of embryogenesis (Allison et al., 1995). Meanwhile, Gtf3a is the protein that forms the smaller 7S RNPs (Szymansky et al., 2003, Diaz de Cerio et al., 2012). Gtf3a is a protein with a dual function, first as a transcriptional activator of polIII for the specific transcription of 5S rRNA and then as 5S rRNA binding protein (Szymansky et al., 2003, Diaz de Cerio et al., 2012). It has been demonstrated that teleost genomes possess two paralog gtf3a genes, *gtf3aa* and *gtf3ab*. *gtf3ab* having been subfunctionalised to become specifically transcribed in ovaries (Rojo-Bartolomé et al. 2020). In that respect, and showing the same pattern of transcription of 5S rRNA, *gtf3ab* has become a useful molecular marker of female sex and of the gametogenic stage in ovaries of teleost fish (Ortiz-Zarragoitia et al., 2014, Rojo-Bartolomé et al. 2020). They are also good molecular markers of the presence of oocytes in testes of fish feminised due to the exposure to xenoestrogenic chemicals, something quite common for instance in Gernika or in Pasaia on the Basque coast (Ortiz-Zarragoitia et al., 2014, Rojo-Bartolomé et al. 2020).

The transcription of tRNAs does not require the participation of Gtf3ab, but it does require the participation of other general transcription factors, GTF3b and GTF3c. These transcription factors are polypeptidic so they are coded by different genes and in the ground of Cell Biology and Environmental Toxicology of the University of the Basque Country it has been recently demonstrated that many of these genes are also duplicated in the genomes of many teleosts, preliminary results pointing towards specific transcription of one of the paralogues in ovaries (Bir et al., 2021).

Further studies are necessary in order to comprehend the dynamics of specific tRNA transcription in teleost gonads and somatic tissues. It will be crucial to understand the relation with other polymerase I and III related molecular markers, as well as to enlarge the scope of teleost fishes, including species with different habitat distribution and reproductive strategies where tRNA accumulation can be proved. In any case, a simple electropherogram of total RNA allows obtaining sufficient information to infer the sex of a teleost fish, provided that total RNA from its gonads can be obtained and to calculate the stage of development in the case of females.

5. CONCLUSIONS

We can conclude that fish ovaries show higher bulk tRNA expression levels than testes and thus, that the relative quantification of tRNA levels can be used as a method to identify sexing in teleosts species. The introduced tRNA/5,8 S rRNA index holds promise as a useful mechanism to quantitatively monitor the level of maturation in teleost gonads, with high index values reflecting immature previtellogenic stage ovaries and low levels reflecting close to spawning ovaries, especially in synchronously developing species.

Part of the work will be presented in the Iberian Association for Comparative Endocrinology (AIEC) XIII congress by Bir, Joyanta, in the work named “5S rRNA and tRNA in fish ovaries: a history of duplications”.

6. BIBLIOGRAPHY

Allison, L., North, M., & Neville, L. (1995). Differential Binding of Oocyte-Type and Somatic-Type 5S rRNA to TFIIIA and Ribosomal Protein L5 in Xenopus Oocytes: Specialization for Storage versus Mobilization. *Developmental Biology*, *168*(2), 284-295. doi: 10.1006/dbio.1995.1080

Bizarro, C., Ros, O., Vallejo, A., Prieto, A., Etxebarria, N., Cajaraville, M., & Ortiz-Zarragoitia, M. (2014). Intersex condition and molecular markers of endocrine disruption in relation with burdens of emerging pollutants in thicklip grey mullets (*Chelon labrosus*) from Basque estuaries (South-East Bay of Biscay). *Marine Environmental Research*, *96*, 19-28. doi: 10.1016/j.marenvres.2013.10.009

Chan, P.P. & Lowe, T.M. (2009) GtRNAdb: A database of transfer RNA genes detected in genomic sequence. *Nucl. Acids Res.* 37(Database issue):D93-D97.

Chu-Koo, F., Dugué, R., Alván Aguilar, M., Casanova Daza, A., Alcántara Bocanegra, F., & Chávez Veintemilla, C. et al. (2008). Gender determination in the Paiche or Pirarucu (*Arapaima gigas*) using plasma vitellogenin, 17β-estradiol, and 11-ketotestosterone levels. *Fish Physiology And Biochemistry*, *35*(1), 125-136. doi: 10.1007/s10695-008-9211-8

Cramer, F., Erdmann, V., Von Der Haar, F., & Schlimme, E. (1969). Structure and reactivity of tRNA. *Journal Of Cellular Physiology*, *74*(S1), 163-178. doi: 10.1002/jcp.1040740416

D'Costa, A., & Shepherd, I. (2009). Zebrafish Development and Genetics: Introducing Undergraduates to Developmental Biology and Genetics in a Large Introductory Laboratory Class. *Zebrafish*, *6*(2), 169-177. doi: 10.1089/zeb.2008.0562

Denis, H., & Wegnez, M. (1977). Biochemical research on oogenesis. *Developmental Biology*, *59*(2), 228-236. doi: 10.1016/0012-1606(77)90256-1

Devlin, R., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, *208*(3-4), 191-364. doi: 10.1016/s0044-8486(02)00057-1

Diaz de Cerio Arruabarrena, O., & Cancio Uriarte, I. (2012). *Transcriptomic tools and gene expression profiles of toxic chemical exposure in aquatic pollution sentinel organisms*. Leioa.

Diaz de Cerio, O., Rojo-Bartolomé, I., Bizarro, C., Ortiz-Zarragoitia, M., & Cancio, I. (2012). 5S rRNA and Accompanying Proteins in Gonads: Powerful Markers to Identify Sex and Reproductive Endocrine Disruption in Fish. *Environmental Science & Technology*, *46*(14), 7763-7771. doi: 10.1021/es301132b

Drygin, D., Rice, W., & Grummt, I. (2010). The RNA Polymerase I Transcription Machinery: An Emerging Target for the Treatment of Cancer. *Annual Review Of Pharmacology And Toxicology*, *50*(1), 131-156. doi: 10.1146/annurev.pharmtox.010909.105844

Espigares, F., Rocha, A., Molés, G., Gómez, A., Carrillo, M., & Zanuy, S. (2015). New insights into the factors mediating the onset of puberty in sea bass. *General And Comparative Endocrinology*, *224*, 176-185. doi: 10.1016/j.ygcen.2015.08.013

Fernstrom, J. D., & Fernstrom, M. H. (2007). Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. *The Journal of nutrition*, *137*(6 Suppl 1), 1539S–1548S. doi.org/10.1093/jn/137.6.1539S

Geiduschek, E., & Tocchini-Valentini, G. (1988). Transcription by RNA Polymerase III. *Annual Review Of Biochemistry*, *57*(1), 873-914. doi: 10.1146/annurev.bi.57.070188.004301

Herring, C., & Blattner, F. (2004). Global Transcriptional Effects of a Suppressor tRNA and the Inactivation of the Regulator frmR. *Journal Of Bacteriology*, *186*(20), 6714-6720. doi: 10.1128/jb.186.20.6714-6720.2004

Höglund, E., Øverli, Ø., & Winberg, S. (2019). Tryptophan Metabolic Pathways and Brain Serotonergic Activity: A Comparative Review. *Frontiers In Endocrinology*, *10*. doi: 10.3389/fendo.2019.00158

Holley, R., Apgar, J., Everett, G., Madison, J., Marquisee, M., & Merrill, S. et al. (1965). Structure of a Ribonucleic Acid. *Science*, *147*(3664), 1462-1465. doi: 10.1126/science.147.3664.1462

Hori, H. (2014). Methylated nucleosides in tRNA and tRNA methyltransferases. *Frontiers In Genetics*, vol. *5*. doi: 10.3389/fgene.2014.00144

Hrdlickova, R., Toloue, M., & Tian, B. (2016). RNA-Seq methods for transcriptome analysis. *Wiley Interdisciplinary Reviews: RNA*, *8*(1), e1364. doi: 10.1002/wrna.1364

Le Page, Y., Diotel, N., Vaillant, C., Pellegrini, E., Anglade, I., Mérot, Y., & Kah, O. (2010). Aromatase, brain sexualization and plasticity: the fish paradigm. *European Journal Of Neuroscience*, *32*(12), 2105-2115. doi: 10.1111/j.1460-9568.2010.07519.x

Leach, D. (1993). A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria. *Genetical Research*, *62*(2), 159-159. doi: 10.1017/s001667230003175x

Lin, Q., Mei, J., Li, Z., Zhang, X., Zhou, L., & Gui, J. (2017). Distinct and Cooperative Roles of amh and dmrt1 in Self-Renewal and Differentiation of Male Germ Cells in Zebrafish. *Genetics*, *207*(3), 1007-1022. doi: 10.1534/genetics.117.300274

Mazabraud, A., Wegnez, M., & Denis, H. (1975). Biochemical research on oogenesis. RNA accumulation in the oocytes of teleosts. *Developmental Biology*, *44*(2), 326-332. doi: 10.1016/0012-1606(75)90403-0

National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 5950, Alanine. Retrieved July 9, 2021 from https://pubchem.ncbi.nlm.nih.gov/compound/Alanine.

National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 6267, Asparagine. Retrieved July 9, 2021 from https://pubchem.ncbi.nlm.nih.gov/compound/Asparagine.

Ortiz-Zarragoitia, M., Bizarro, C., Rojo-Bartolomé, I., de Cerio, O., Cajaraville, M., & Cancio, I. (2014). Mugilid Fish Are Sentinels of Exposure to Endocrine Disrupting Compounds in Coastal and Estuarine Environments. *Marine Drugs*, *12*(9), 4756-4782. doi: 10.3390/md12094756

Phizicky, E., & Hopper, A. (2010). tRNA biology charges to the front. *Genes & Development*, *24*(17), 1832-1860. doi: 10.1101/gad.1956510

Picard, B., Maire, M., Wegnez, M., & Denis, H. (1980). Biochemical Research on Oogenesis. Composition of the 42-S Storage Particles of Xenopus laevis Oocytes. *European Journal Of Biochemistry*, *109*(2), 359-368. doi: 10.1111/j.1432-1033.1980.tb04802.x

Pinkard, O., McFarland, S., Sweet, T., & Coller, J. (2020). Quantitative tRNA-sequencing uncovers metazoan tissue-specific tRNA regulation. *Nature Communications*, *11*(1). doi: 10.1038/s41467-020-17879-x

Raina, M., & Ibba, M. (2014). tRNAs as regulators of biological processes. *Frontiers In Genetics*, *5*. doi: 10.3389/fgene.2014.00171

Reading, B., & Sullivan, C. (2011). THE REPRODUCTIVE ORGANS AND PROCESSES | Vitellogenesis in Fishes. *Encyclopedia Of Fish Physiology*, 635-646. doi: 10.1016/b978-0-12-374553-8.00257-4

Rojo-Bartolomé, I. (2017). Sex differentiation in teleost fish species: molecular identification of sex and oocyte developmental stage. [Doctoral thesis, University of the Basque Country]. http://hdl.handle.net/10810/26173

Rojo-Bartolomé, I., Diaz de Cerio, O., Diez, G., & Cancio, I. (2016). Identification of Sex and Female's Reproductive Stage in Commercial Fish Species through the Quantification of Ribosomal Transcripts in Gonads. *PLOS ONE*, *11*(2), e0149711. doi: 10.1371/journal.pone.0149711

Rojo-Bartolomé, I., Santana de Souza, J., Diaz de Cerio, O., & Cancio, I. (2020). Duplication and subfunctionalisation of the general transcription factor IIIA (gtf3a) gene in teleost genomes, with ovarian specific transcription of gtf3ab. *PLOS ONE*, *15*(1), e0227690. doi: 10.1371/journal.pone.0227690

Rojo-Bartolomé, I., Valencia, A., & Cancio, I. (2017). Transcription of ribogenesis genes in fish gonads: Applications in the identification of stages of oogenesis and in environmental monitoring of intersex condition. *Marine Pollution Bulletin*, *121*(1-2), 292-301. doi: 10.1016/j.marpolbul.2017.06.027

Rosanova, P., Romano, M., Marciano, R., Anteo, C., & Limatola, E. (2002). Vitellogenin precursors in the liver of the oviparous lizard,Podarcis sicula. *Molecular Reproduction And Development*, *63*(3), 349-354. doi: 10.1002/mrd.90019

Rougvie, A., & O'Connor, M. (2013). Preface. *Current Topics In Developmental Biology*, xiii-xv. doi: 10.1016/b978-0-12-396968-2.09990-5

Shah, P., & Gilchrist, M. (2010). Effect of Correlated tRNA Abundances on Translation Errors and Evolution of Codon Usage Bias. *Plos Genetics*, *6*(9), e1001128. doi: 10.1371/journal.pgen.1001128

Steinbrenner, H., Speckmann, B., & Klotz, L. (2016). Selenoproteins: Antioxidant selenoenzymes and beyond. *Archives Of Biochemistry And Biophysics*, *595*, 113-119. doi: 10.1016/j.abb.2015.06.024

Szymański, M., Barciszewska, M., Erdmann, V., & Barciszewski, J. (2003). 5 S rRNA: structure and interactions. *Biochemical Journal*, *371*(3), 641-651. doi: 10.1042/bj20020872

Thompson, J., Higgins, D., & Gibson, T. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*(22), 4673-4680. doi: 10.1093/nar/22.22.4673

Ulloa, P., Iturra, P., Neira, R., & Araneda, C. (2011). Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes. *Reviews In Fish Biology And Fisheries*, *21*(4), 649-666. doi: 10.1007/s11160-011-9203-0

Ye, M., & Chen, Y. (2020). Zebrafish as an emerging model to study gonad development. *Computational And Structural Biotechnology Journal*, *18*, 2373-2380. doi: 10.1016/j.csbj.2020.08.025