Method for the molecular and quantitative identification of oocytes and their developmental stage in teleost fish gonads ☆

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ABSTRACT

Fish display diverse reproductive strategies and their gametogenesis is influenced by numerous genetic, physiological and environmental factors. The analysis of 5S rRNA expression levels in gonads has been proposed as useful method for the molecular identification of the presence of oocytes in fish tissues. The present method provides an easy and unbiased approach to analyse the expression of tRNAs and 5S rRNA in teleost gonads and stablish the presence and developmental stage of oocytes. Total RNA extracted from gonads is analysed through capillary electrophoresis in a Bioanalyzer 2100 (Agilent Technologies) using Small RNA Assays. Electropherograms allow quantifying the concentrations of tRNAs, 5S rRNA and 5.8S rRNA per sample and calculate their tRNA/5.8S rRNA and 5S/5.8S rRNA indices. Both indices clearly differentiate ovaries from testes and can be used to identify testes that present oocytes due to exposure to environmental xenoestrogens. The tRNA/5.8S and 5S/5.8S indices show the highest values in ovaries in previtellogenic stage, values decreasing as they advance towards maturity.

- Detailed molecular method to sex fish and quantitatively identify the maturity stage of females.
- tRNA levels in gonads can help in the study of teleost reproduction (female fecundity assessment, molecular gonad sexing) and environmental health assessment.

Specifications table

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Fisheries, aquaculture, environmental monitoring
Name of your method:	Molecular detection of oocytes at different developmental stages in teleost gonads
Name and reference of original method:	Masotti, A., Preckel, T. Analysis of small RNAs with the Agilent 2100 Bioanalyzer. Nat. Methods 3, 658 (2006). https://doi.org/10.1038/nmeth908
Resource availability:	**Reagents:**
	1. RNAlater® (Sigma-Aldrich)
	2. Liquid nitrogen
	3. TRIzol® Reagent Solution (Ambion; Life Technologies, Cat no: 15596018)
	4. Zirconia/silize beds 1.0 mm (Thermo Fisher Scientific, Cat no: 11079110z)
	5. Chloroform (Scharlab)
	6. Isopropyl alcohol (Scharlab)
	7. Ethanol (75%)
	8. RNAse/DNAse free Water (Invitrogen, Cat no: 10977)
	9. Small-RNA kit chips (Agilent Technologies, Cat no: 5067-1548)
	10. Agilent small RNA reagent part I (Agilent Technologies, Cat no: 5067-1548)
	11. RNase free microfuge tubes 1.5 mL (Invitrogen, Cat no: AM12400)
	12. RNase Zap (Invitrogen, Cat no: AM9780, AM9782)
	Equipment:
	1. Tissue Homogenizer (Precellys 24, Bertin Technologies)
	2. Microcentrifuges (Fresco™ 21, Thermo Fisher Scientific and Beckman Coulter MF16, DAI Scientific)
	3. Biophotometer (Eppendorf 6131) or a NanoDrop (Thermo Fisher)
	4. Agilent 2100 Bioanalyzer (Agilent Technologies).
	5. Heating block (AccuBlock™ Digital Dry baths, Labnet International)
	6. Chip priming station (Agilent Technologies).
	7. IKA vortex mixer (Agilent Technologies)
	8. Biomedical −80 °C Freezer (Haier Biomedical)

Background and value of the method

A developing oocyte needs to accumulate molecules that will be used for early embryo development, especially in externally fertilizing species. That is the whole logic behind the process of vitellogenesis during fish oogenesis and for maternally inherited RNAs [1,2]. Without doubt, a growing fish oocyte will need to sustain protein synthesis in the prospective embryo until its genome is activated. Ribogenesis has been estimated to be the most energetically-demanding process in the cell, so the regulation of the complex array of rRNAs and proteins necessary to produce new ribosomes is a major factor determining the potential of a cell to grow and divide [3,4] even more for a growing oocyte. RNA polymerase III (Pol-III) is in charge of synthesizing the 5S ribosomal RNAs and the tRNAs [1,5,6]. Fish accumulate high concentrations of 5S rRNA and tRNAs in previtellogenic oocytes; up to 90% of the total RNA in the ovary of some fish [5,6]. Later, during secondary growth and vitellogenesis, oocytes begin to accumulate the longest 5.8S, 18S and 28S rRNAs [1,5,6] under the regulation of RNA polymerase I (Pol-I). In previtellogenic oocytes 5S rRNAs are stored in cytosolic ribonucleoproteic particles. Then, after fertilization and together with the other rRNAs it is used in the assembly of new ribosomes that together with maternally inherited tRNAs will participate in embryo protein synthesis [5].

These high levels of 5S rRNA and tRNAs transcription can be used to distinguish the presence of oocytes in teleost gonads, as demonstrated in multiple fish species [1,6,7]. In addition, this can be also used for the identification of intersex individuals, because transcription levels are intermediate between testis and ovary [6,7]. Some contaminants present in the marine environment behave as endocrine disrupting compounds (EDCs). These exogenous substances (plasticisers, pesticides, fungicides, surfactants, polychlorinated biphenyls, synthetic oestrogens) can alter the function(s) of the endocrine system and consequently cause adverse health effects in organisms or their progenies/(sub)populations" [8]. Among the effects of EDCs identified in aquatic organisms, the feminization of male and juvenile fish is one of the best described [8,9]. Male fish showing testis with individual or clustered oocytes (intersex condition), have been described in aquatic environments receiving feminizing EDCs [5,7–10]. For instance, up to 80% of the male mullets (*Chelon labrosus*) have been found to develop oocytes in their testes in some polluted estuaries in the South Bay of Biscay [10]. In some species, intersex fish have been shown to display lower reproduction capacity than non-impacted fish [11,12]. Oocytes in intersex individuals are mostly in the previtellogenic stage. At this stage oocytes accumulate 5S rRNA and tRNAs to their highest, this meaning that a simple electrophoresis of total RNA extracted from testis is enough to detect the presence of oocytes and the intersex condition [7].

Initiation of secondary growth in ovaries of female teleosts (accumulation of vitellogenin, glycogen and free glucose, mobilization of amino acids and resumption of meiosis) results in a sudden accumulation of 5.8S, 18S and 28S rRNAs synthesized by Pol-I [1,7]. Therefore, the dynamics of production of Pol-III and Pol-I products can constitute a powerful, quantitative and unbiased method to identify the gametogenic stage of female fish with application in commercial fish stock fecundity assessment [1]. We have generated a method to easily quantify the levels of rRNAs and tRNAs for the development of indices that could be employed to quantitatively identify the ovarian maturation-stage in any fish species and to identify the presence of intersex testes in pollution monitoring campaigns [6].

Method details

RNA extraction from fish gonads

- Dissect the gonads of the fish of interest and immediately immerse them into RNAlater® to freeze them in liquid nitrogen.
- Samples can be stored at −80 °C until further applying the method.
- For total RNA extraction homogenise the tissue in 1 mL of TRIzol® (per 50–100 mg tissue) using zirconia/silica beads in a tissue homogeniser (at 6500 rpm; 2 × 45 s) and following manufacturer´s instructions.
- Incubate the RNA tubes at room temperature for 5 min for complete dissociation of nucleoprotein complexes.
- Add 0.2 mL of chloroform and shake the tubes vigorously for 15 s, then incubate the tubes at room temperature for 2–3 min.
- Centrifuge the tubes at 12,000x g for 15 min at 4 °C for complete phase separation.
- Transfer the aqueous phase carefully to a new RNAse free tube.
- Add 0.5 mL of isopropanol (per 1 mL of TRIzol® reagent) to the aqueous phase and incubate the tubes for 10 min at room temperature.
- Centrifuge the tubes at 12,000 x g at 4 °C for 10 min to obtain a RNA pellet at the bottom of the tubes.
- Discard the supernatant from the top of the pellet and wash the RNA pellet with 1 mL of 75% ethanol per 1 mL of TRIzol® reagent.
- Mix the sample by vortexing and centrifuge at 7500 x g for 5 min at 4 °C.
- Remove the supernatant from the tube and dry the RNA pellet (air or vacuum dry) for 10–15 min at room temperature.
- Finally, elute the RNA pellet in 30–50 µl of RNAse/DNAse free water and store at −80 °C to preserve RNA integrity.

Estimation of RNA quality and concentration

- Defrost stored RNA samples.
- Estimate the RNA concentration by measuring the absorbance at A260 nm and A280 nm in a biophotometer after diluting in RNase-free water or measure directly in a NanoDrop.
- Samples with A260/A280 absorbance ratios between 1.8 and 2.2 are considered as good quality RNA and can be used for further analysis.

Preparation of RNA sample and reagents and sample loading Bioanalyzer RNA small chips

- Follow all the steps described in the Bioanalyzer protocol for gel generation in the Small RNA chips. Add 1 µL small RNA ladder in ad hoc well and RNA samples (1 µl per sample at a RNA concentration of 100 ng/µl) into each of the 11 sample wells. Vortex the chips at 2400 rpm for 60 s in the adapter of the IKA vortex mixer. Clean the electrodes with RNase Zap and then with 350 µl of fresh RNase-free water. Run the chip with the Agilent 2100 expert software by selecting small RNA series II assay.

Quantification of tRNAs, 5S and 5.8S rRNAs and calculations of indices

On finalisation of each run the Bionalyzer generates a chip data file (.xad) where capillary electrophoresis (Fig. 1) and electropherograms (Fig. 2) are provided. Data can be analysed in the computer modifying the settings as required. Concentrations of tRNAs, 5S and 5.8S rRNAs can be directly obtained from the electropherogram peaks specific to each RNA sample. The calculations need customisation for each fish species depending on the characteristics of their nucleic acids. Take into account that the peak of tRNAs comes from different amino acid specific tRNA isoacceptors, some of them unprocessed some of them mature of different sizes. Proceed as follows:

- In the electropherograms establish a baseline value for small RNAs and measure the representative time corrected areas of the peaks corresponding to tRNAs, 5S rRNA and 5.8S rRNA (Fig. 2).
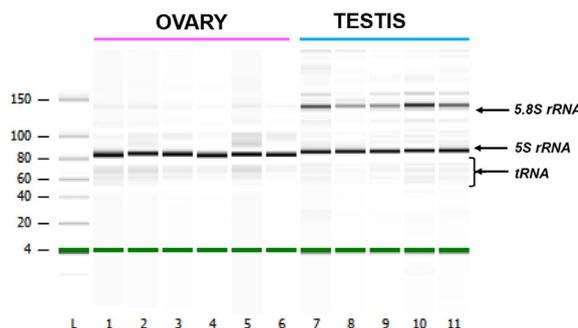
Fig. 1. Capillary electrophoresis of 11 total RNA samples (European hake *Merluccius merluccius*), six extracted from ovaries and five from testes. The position of tRNAs, 5S rRNA and 5,8S rRNAs is indicated. (L = ladder).
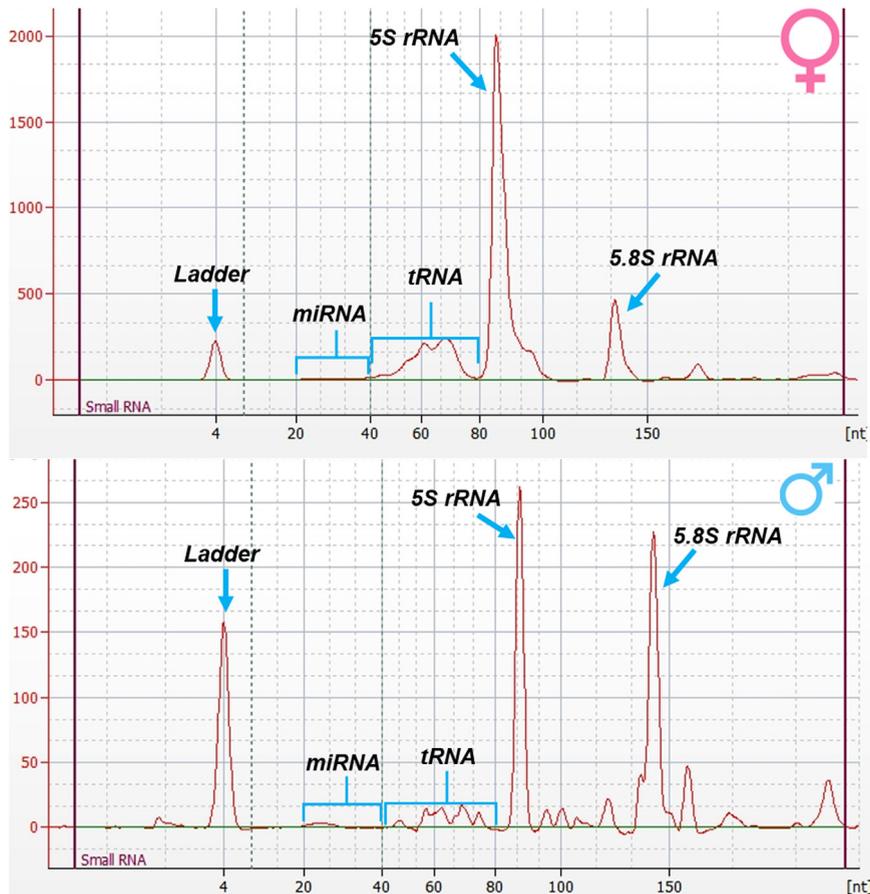
Fig. 2. Two representative electropherograms, one belonging to the RNA extracted from an ovary and the other one from a testis. Both belong to European hake *Merluccius merluccius* Samples. The peaks of the different RNA molecules studied are labelled.

- Set the time corrected area corresponding to tRNAs, that could be in the range in between 40 and 80 nucleotides in length (Fig. 2).
- Set the time corrected area corresponding to 5S rRNAs that will be in the range in between 84 and 96 nucleotides in length (Fig. 2).
- Set the time corrected area corresponding to 5.8S rRNAs that could be in the range in between 135 and 150 nucleotides (Fig. 2).
- Calculate the tRNA/5.8S rRNA ratio by dividing the putative concentration obtained for tRNA with that obtained for 5.8S rRNA within each sample.
- Do the same to calculate 5S/5.8S rRNA ratios.
- Log transform the values obtained for both ratios to obtain the corresponding tRNA/5.8S rRNA and 5S/5.8S rRNA indices allowing a better graphical visualisation of differences between sexes and ovarian developmental stages.

Validation of the method

The consistency of the proposed method was confirmed by Bir et al. [6] quantifying the concentrations of total small RNAs (tRNAs, 5S rRNA and 5.8S rRNA) in gonads of eight different marine and freshwater fish species, including intersex testes of thicklip grey mullet *Chelon labrosus*. The gonads were previously histologically analysed for sex identification and gametogenic staging. The capillary electrophoreses and electropherograms (Fig. 2) showed that tRNAs and 5S rRNA levels are significantly higher in teleost ovaries than in testes [6]. Also, the tRNA/5.8S and the 5S/5.8S rRNA indices clearly differentiated ovaries from testes in teleosts [6] (Fig. 2) and can be also used to identify *C. labrosus* testes that due to environmental exposure to xenoestrogens present oocytes [6].

Ovaries at different developmental stages showed different levels of tRNAs and 5S rRNA and the calculated indices significantly varied with the oogenic stage in females. Ovaries presenting mainly oocytes in previtellogenic stage showed index values higher than those in ovaries with oocytes in more advanced stages of oogenesis [6].

In conclusion, the proposed method proved to be reliable to molecularly identify sex in mature teleosts, intersex testes in fish exposed to xenoestrogens and to rank the ovarian developmental stage of studied fish species.

Ethics statements

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Joyanta Bir: Methodology, Data curation, Writing – original draft. **Iratxe Rojo-Bartolomé:** Methodology, Writing – review & editing. **Oihane Diaz de Cerio:** Methodology, Writing – review & editing. **Maren Ortiz-Zarragoitia:** Supervision, Writing – review & editing. **Ibon Cancio:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Data availability

Data will be made available on request.

Acknowledgments

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