Marine Pollution Bulletin xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Transcription of ribogenesis genes in fish gonads: Applications in the identification of stages of oogenesis and in environmental monitoring of intersex condition

Iratxe Rojo-Bartolomé, Ainara Valencia, Ibon Cancio*

CBET Research Group, Dept. of Zoology and Animal Cell Biology, Research Centre for Experimental Marine Biology and Biotechnology of Plentzia (PiE-UPV/EHU), University of the Basque Country (UPV/EHU), Areatza, z/g, E-48620 Plentzia, Basque Country, Spain

ARTICLE INFO

Keywords:
5S rRNA
5S/18S rRNA index
Intersex severity
Oocytes
Oogenesis
Xenoestrogenicity

ABSTRACT

One of the best described effects of environmental xenoestrogens in fish is the generation of intersex gonads in males. Considering 5S rRNA a marker of the presence of oocytes, a 5S/18S rRNA index was calculated in 296 thicklip grey mullets (*Chelon labrosus*) from polluted environments. In addition, qPCR analysis of transcription factors *gtf3a* and *ubtf1*, related respectively to 5S and 18S rRNA synthesis, was conducted along female-oogenesis. 5S/18S rRNA index identified sex with a threshold value of 0.4521 separating males from females. Histological analysis identified 38 intersex individuals. Intersex severity and 5S/18S rRNA indexes were correlated. 5S/18S rRNA index identified ovarian developmental stage with high 5S rRNA levels during early oogenesis and 18S rRNA relative values increasing towards maturation. *gtf3a* and *ubtf1* transcription levels followed the pattern of 5S rRNA accumulation. Thus, ribogenesis genes provide easy/quantitative methods to molecularly identify the sex, female gametogenic stage and intersex severity in mullets.

1. Introduction

Organisms inhabiting estuarine waters are normally exposed to complex contaminant cocktails with anthropic origin (Boehm and Bischel, 2011). Among other contaminants detected in the aquatic environments, endocrine disrupting chemicals (EDCs) have received special attention after they were first highlighted by the European Environmental Agency in 1997 (EEA, 1997). EDCs were then defined as “chemical pollutants able to interfere with the normal functioning of hormones” (EEA, 2012) and they can cause alterations at different biological organisation levels, from the molecular one to the individual or the (sub)population one (Brander, 2013; WHO/UNEP, 2013). They include a complex array of substances, with different chemical structures and sources (Tyler et al., 1998; López de Alda and Barceló, 2001; Porte et al., 2006; Casals-Casas and Desvergne, 2011; Khetan, 2014) which in industrialized countries mainly arrive to the aquatic environment through the municipal, industrial and hospital wastewater treatment plants effluents (Campbell et al., 2006). Such compounds in complex mixtures, can interact enhancing their potency and biological activity, and in some circumstances, acting in an additive manner (Thorpe et al., 2003).

Xenoestrogens are considered EDCs with the ability to mimic estrogens or to cause estrogen-like responses in exposed organisms (Campbell et al., 2006). They alter hormonal homeostasis interfering with normal sexual differentiation and gametogenesis, which in consequence affects the development and reproduction of exposed individuals/populations (Tyler et al., 1998; Goksøyr et al., 2003; Mills and Chichester, 2005). The effects of xenoestrogenic compounds in some aquatic organisms are well known, one of the best described responses being the feminization of juvenile and male fish (WHO/UNEP, 2013; Tyler and Jobling, 2008; Goksøyr et al., 2003; Goksøyr, 2006; Bizarro et al., 2014). Sometimes this leads to the generation of intersex gonads, when oocytes differentiate within the normal testicular tissue in gonochoristic fish species (Matthiessen, 2003; Bahamonde et al., 2013; Bizarro et al., 2014; Ortiz-Zarragoitia et al., 2014). Intersex condition has been reported in both freshwater and marine fish related to chemical exposure in highly to moderately contaminated areas (Bahamonde et al., 2013; Ortiz-Zarragoitia et al., 2014). These intersex males display lower reproduction capacity than normal males, with logical consequences in population viability (Jobling et al., 2002b; Harris et al., 2011).

Xenoestrogenic effects have been reported also in mugilid fish populations from contaminated estuaries (Ferreira et al., 2004) and intersex gonads have been described in thicklip grey mullets (*Chelon*

* Corresponding author.
E-mail address: ibon.cancio@ehu.es (I. Cancio).

http://dx.doi.org/10.1016/j.marpolbul.2017.06.027
Received 20 March 2017; Received in revised form 23 May 2017; Accepted 7 June 2017

labrosus) used as pollution sentinel organisms in estuaries in the Southern Bay of Biscay (Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). Oocytes have been found in testes of mullets from Bilbao, Pasaia and Ondarroa harbours, and in estuaries in Deba and in the Biosphere Reserve of Urdaibai in Gernika (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). These sites have been associated to waste water treatment plant effluents and/or to industrial activities in harbour areas. Intersex mullets have been described all along the reproductive cycle, with percentages ranging from 3% to 60% of analyzed males depending on the site and month of capture (Ortiz-Zarragoitia et al., 2014). Most intersex mullets present low to moderate intersex severity indexes following the score developed by Jobling et al. (2006) that ranks the severity according to the number and distribution of oocytes presented in the testis. Furthermore, elevated vitellogenin transcript and protein levels have been measured in these males, accompanied by an up-regulation of the aromatase coding gene *cyp19a1b* in brain (Bizarro et al., 2014). Thus, thicklip grey mullet is considered an important sentinel species for the biomonitoring of exposure to reproduction EDCs in the Southern Bay of Biscay (Ortiz-Zarragoitia et al., 2014).

In spite of the increasing number of studies describing the intersex condition in fish in the last decades, the physiological and molecular mechanisms governing the process remain unknown (Abdel-Moneim et al., 2015). In addition, and although it is known that the transcription levels of several genes related to sex differentiation are altered after exposure to xenoestrogens (Bahamonde et al., 2015b), no direct relationship with the intersex condition has been established yet and more research is needed in order to find specific molecular markers of this condition (Bahamonde et al., 2013; Ortiz-Zarragoitia et al., 2014; Abdel-Moneim et al., 2015).

In this respect, recently, accumulation of 5S rRNA and of transcripts coding for 5S rRNA accompanying proteins have been studied in mullets from polluted areas (Diaz de Cerio et al., 2012; Valencia et al., 2017). The transcription levels of ribogenesis genes enabled the identification of sex of each individual in a comparative manner, when at least one individual of each sex is present in the study, irrespective of their site of collection and their stage within the reproductive cycle. In addition, such genes were up-regulated in intersex testis in comparison to normal testis. Strong 5S rRNA transcription, specific of ovaries, can be easily identified by a simple electrophoresis of total RNA extracted from the gonads (Diaz de Cerio et al., 2012). The relative amount of 5S to 18S rRNA calculated after electrophoretic analysis (5S/18S rRNA index) identifies the presence of oocytes in gonads and allows distinguishing not only the sex but also the oogenic stage in females, as demonstrated in different commercial fish species of the Bay of Biscay (Rojo-Bartolomé et al., 2016). This is so because 5S rRNA levels relative to 18S rRNA are higher in previtellogenic oocytes than in mature ones (Rojo-Bartolomé et al., 2016). In this context, the 5S/18S rRNA index was applied in the present study for the unambiguous molecular identification of sex, female reproductive stage and intersex severity in mullets collected from polluted sites of the Southern Bay of Biscay during a complete annual reproductive cycle. In addition, transcription levels of the general transcription factor IIIA (*gtf3a*) and upstream binding transcription factor 1 (*ubtf1*), genes related to ribogenesis through regulation of 5S and 18S rRNA synthesis, were studied in ovaries of females at different developmental stages. Such analysis could elucidate the possibility of using transcription levels of ribogenesis genes in an environmental monitoring context to assess the impact of xenoestrogenic compounds and the incidence of intersex condition in pollution sentinel fish species.

2. Materials and methods

2.1. Study area and biological samples

From September 2010 to September 2011 twelve to thirty adult (> 20 cm length) thicklip grey mullets (*Chelon labrosus*) were monthly collected by fishing-rod in the harbour of Pasaia (43°19′35″N, 1°55′9″W), located on the Basque coast (SE Bay of Biscay). Mullets were also sampled in June 2013 and in February 2014 in the estuaries of Gernika (43°19′26″N, 2°40′26″W) and Galindo (43°18′11″N, 2°59′55″W) close the points of discharge of the waste water treatment plants of Gernika and of the Bilbao metropolitan area. The total amount of individuals collected for this study was of 296.

All animal manipulations conducted were authorised by competent regional authorities after the evaluation and approval of all protocols by the Ethics and Animal Welfare Commission of the University of the Basque Country (CEBA/152/2010). After capture, individuals were immediately anaesthetized in a saturated ethyl 4-aminobenzoate, sacrificed by decapitation and gonads were removed. A portion of each gonad was embedded in RNA later (Ambion, Life Technologies, Carlsbard, USA), frozen in liquid nitrogen and then stored in the laboratory at − 80 °C until further used.

For histological analysis, a portion was taken from the middle part of the gonad from each fish (around 1 cm in length across the whole gonad). This was then fixed in 4% neutral buffered formalin and transported at 4 °C to the laboratory for further processing.

2.2. Histological analysis of the gonads

After 24 h in the fixative, gonads were dehydrated in a graded series of ethanol (70%, 90% and 96%) and embedded in methacrylate resin according to the manufacturer's instructions (Technovit 7100; Heraeus Kulzer GmbH & Co. KG, Werheim, Germany). 6 to 9 resin sections (5 µm) were cut in a 2065 Supercut microtome (Leica Instruments GmbH, Wetzlar, Germany) and stained with hematoxylin/eosin. Sex and the developmental stage of the oocytes in ovaries were determined microscopically, following the gametogenic stage grading described by McDonough et al. (2005) for *Mugil cephalus*. The stages were as follows. Resting (R), with the presence of atretic oocytes (> 20%) in an otherwise empty ovary with scarce oogonia and lamellae presenting some muscle and connective tissue bundles while ovarian wall look thickened. Perinucleolar (Pn), with inactive ovary containing perinucleolar oocytes and a very thin ovarian wall. Cortical alveoli (Ca), with oocytes at cortical alveoli stage some of them starting vitellogenesis (< 50%). Vitellogenesis (V), with oocytes full of yolk globules and enlarged plasma membrane. Mature/spawning (M), with hydrated oocytes showing coalescence of lipid droplets and very thick oocyte envelope, and presence of some atretic oocytes (< 20%). No full mature or spawning individuals could be identified as spawning occurs in the open sea (Ortiz-Zarragoitia et al., 2014) and samplings were always carried out in estuarine areas.

In the case of identified intersex individuals, the intersex severity was established microscopically. For that, up to 9 non-consecutive 5 µm histological sections were completely examined with a 20X objective, dividing each section in several fields of view. To determine the number of oocytes in each histological section, the field of view with the maximum oocyte amount recorded was considered, following the methodology described by Blazer et al. (2007). Then, the intersex severity for each fish was established depending on the mean number of oocytes within all histological sections analyzed per individual and following the index developed by Jobling et al. (2006).

2.3. Extraction of total RNA, capillary electrophoresis and 5S/18S rRNA index

Total RNA was extracted from 50 to 100 mg of tissue using TRIzol® (Invitrogen, Life Technologies) and following the manufacturer's instructions. Obtained RNA was purified using Qiagen RNeasy kit (Qiagen, California, USA) after a DNase digestion step (RNase-Free DNase Set, Qiagen). After purification, the same amount of RNA (250–500 ng), as estimated through absorbance at 260 nm (good