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SEX DIFFERENTIATION IN TELEOST FISH SPECIES: MOLECULAR IDENTIFICATION OF SEX AND OOCYTE DEVELOPMENTAL STAGE

International Ph.D Thesis submitted by
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“El tiempo no es importante.
Sólo la vida es importante.”

- Mondoshawan

Nire familiari, daudenei eta joan direnei,
urte hauetan nire alboan izan zaretenoi,
Eskerrik asko

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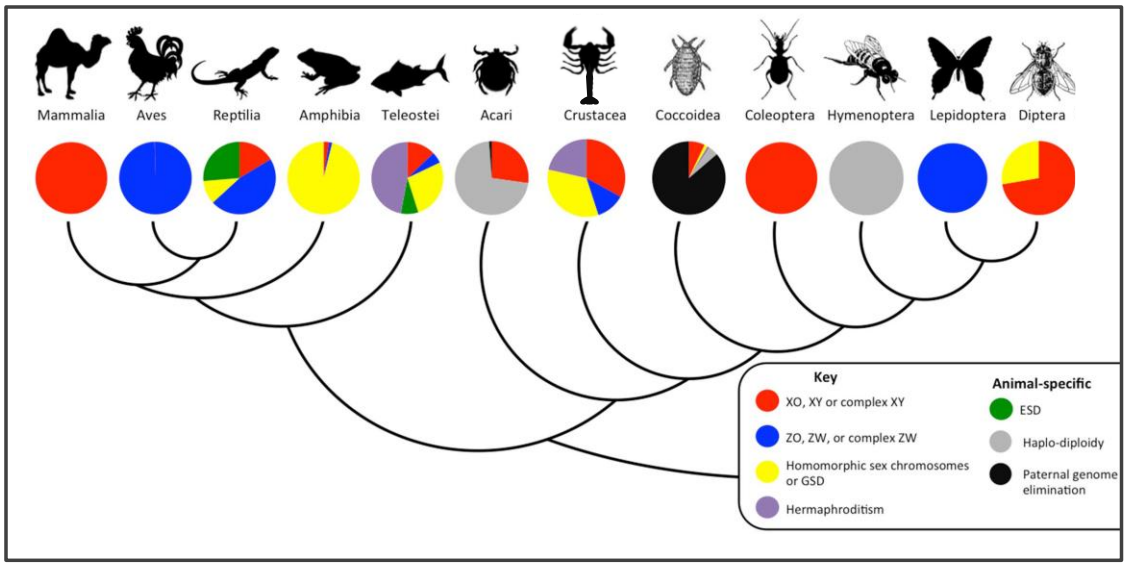
1. Sexu-determinazioa arrainetan

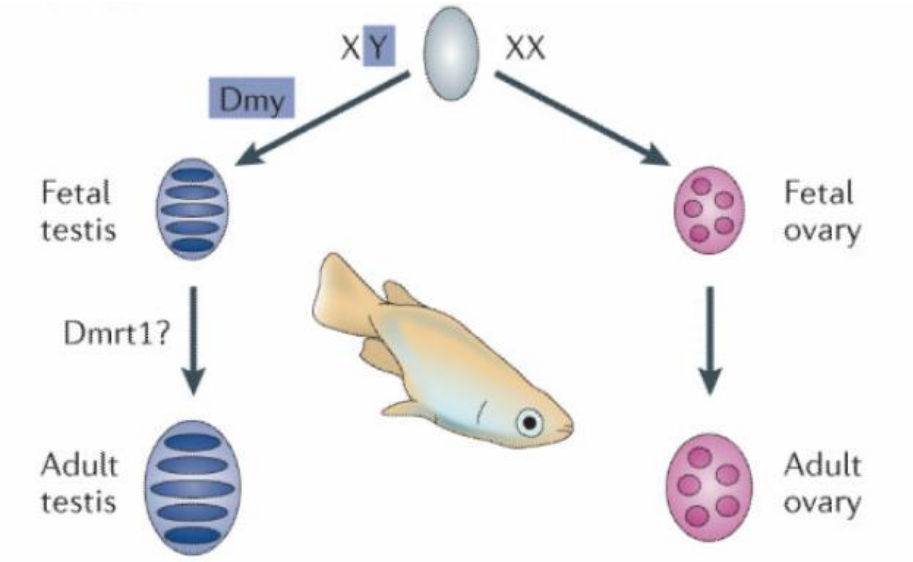
Ugalketa sexuala naturan existitzen denetik sexua determinatzen duten mekanismoak azkar eboluzionatu eta dibertsifikatu egin dira. Arrainetan sexu-determinazioa banako bat emea edo arra izango den erabakiko duten ingurune-faktore eta faktore genetiko eragileek eraentzen dituzten mekanismo molekular zein zelularren multzoari deritzo (Devlin & Nagahama, 2002). Katalogaturiko 33.400 arrain espezie baino gehiagoren artean (www.fishbase.org), %96-a teleosteoak dira, ornodunetan azaltzen den talderik handiena eta dibertsoarena. Teleosteoen arrain-taldean ornodunetan sexu-determinazio genetikorako deskribatu izan diren aldaera guztiak aurki ditzakegu (1. Irudia). Zenbait espeziek, sexu-kromosoma berezituak aurkezten dituzte, batzuk ar-heterogamia (XX/XY) erakutsiz, izokinak kasu, beste batzuk eme-heterogamia (ZZ/ZW) erakusten duten bitartean, *Gambusia* generoan esaterako. Ar-, eme-heterogamia gainera espezie bereko banakoen artean nahasturik ere ager daiteke, *Xinophorus maculatus* espeziean ikusi den bezala, ziurrenik berriki sortutako sexu-kromosomen dibergentzia prozesuaren ondorioz (Kallman, 1965; Baroiller et al., 1999). Sexu- kromosomak dituzten arrain-espezie askotan kromosomen arteko dibergentzia hain da berria, non ez den kromosomen arteko heterogamiarik bereizten. Nahiz eta zenbait espezietan sexu-kromosomen arteko morfologia ezberdintasunak topatu diren (aztertu direnen %10.4), gehienetan itxura oso antzekoa dute eta ezinezkoa da teknika zitogenetiko soilen bidez bereiztea (Devlin & Nahagama, 2002). Horrela sexu-kromosomak dituzten espezieen artean soilik bakan batzuetan aurkitu izan da gene bakarreko sexu-kontrola (kontrol monogeniko). Beste arrain batzuetan kontrol mekanismoak gene askok gidatzen dituzte(poligenikoa), esate baterako zebra arrainean (*Danio rerio*), zeinetan arra edo emea izatearen eragileak genoman hainbat kromosomatan sakabanatuta dauden guneak diren (Bachtrog et al., 2014).



1. Irudia: sexu-determinaziorako sistemen dibertsitatea animalia-klado adierazgarrietan. Ornodunak: Mammalia (plazentadunak, marsupialak eta monotrematuak), Aves (hegaztiak), Reptilia (dordokak, sugeak, krokodiloak, sugandilak), Amphibia (igel, apo, arrabioak) eta Teleostei (hezurdun arrainak). Ornogabeak: Acari (akaroak eta kaparrak), Crustacea (izkira, lanperna, karramarroak), Coccoidea (ezkata intsektuak), Coleoptera (kakalardoak), Hymenoptera (inurriak, erleak eta liztorrak), Lepidoptera (tximeletak) eta Diptera (euliak). GSD: sexu-determinazio genetikoa, ESD: ingurumenak baldintzatutako sexu-determinazioa (Batchrog et al., 2014-tik eraldatua).

Sexuaren kontrol monogenikoari dagokionez, ugaztunetan Y-kromosoman dagoen *sry* genea (ingelesezko sex-determining region Y) ar izaeraren edo determinazioaren eragilea kontsideratzen den bitartean (Sinclair et al., 1990), arrainetan espezie bakar batzuetan aurkitu dira sexu-kromosomei loturiko banakako gene determinatzaileak. Hain zuzen ere, 6 dira azken urteotan argitaratu diren adibideak; *Tetraodon rubripes*, *Cynoglossus semilaevis,* Patagoniako pejerrei (*Odontesthes hatcheri*), ostadar amuarraina (*Oncorhynchus mykiss*) eta *Oryzias luzonensis* eta *Oryzias* latipes medaka arrainenak hain zuzen ere (Batchrog et al., 2014). a*mhr2* geneak (ingelesezko anti-Müllerian hormone receptor 2) X-kromosomari loturiko dosiaren araberako sexu-determinaziorako mekanismoa baldintzatzen du tigre puxika-arrainean (*T. rubripes*). Aldiz, *C. semilaevis*-ean Z-kromosomari loturiko *dmrt1* (ingelesezko doublesex and mad 3 transcription factor-1) geneak du kontrola eta, Patagoniako pejerreian, ostadar amuarrainean zein *O. luzonensis*-en Y-kromosomari loturiko *amhy* (anti-Müllerian hormone genea), *sdY eta gsdf* (gonadal soma derived growth factor) geneek, hurrenez hurren, agintzen dute testikuluaren desberdintzapena (Kikuchi & Hamaguchi, 2013;

Batchrog et al., 2014). Azkenik, medakak Y-kromosomari loturiko DM-domeinu genea, edo *dmy* genea (ingelesezko Y-specific DM-domain gene) du, arrainetan urteetan zehar ezagutu den sexu-determinaziorako gene bakarra. Gene hau ere ar izaerari lotuta dagoen sexu-determinaziorako gene gidaria da (2. Irudia). *dmy* ere, *sry* bezala ugaztunetan, sexu-desberdintzapenaren hasieran gonadaren zelula somatikoetan, gerora Sertoli zelula desberdintzatuak izango direnak, adierazten da (Matsuda et al., 2002) eta bere adierazpena isiltzerakoan fenotipo emea aurkezten duten XY arrak sortzen dira (Devlin & Nagahama, 2002; Matson & Zarkower, 2013). Medakan ar izaerari loturiko *dmy* gene agintari honen jatorria *dmrt1a* gene autosomikoaren bikoizketan dago. Azterturiko arrain espezie guztietan *dmrt1* garatzen ari den gonada arran adierazten da, baita obario-testikulu trantsizioan dauden hermafrodito proteroginoetan ere. Medakan bikoizketaren ondorioz Y kromosoma berria sortu zen zeina ar garapenerako ezinbestekoa eta nahikoa den, berak agintzen baitu *dmy* bidez gerora ematen den *dmrt1* genearen adierazpena (2. Irudia). Hau da, genearen bikoizketaren ondorioz medakan sortutako gene berria desberdintzapen-turrustaren gidaria bilakatu zen. Antzeko zerbait gertatu da pejerreiren *amhY* genearekin (Kikuchi & Hamaguchi, 2013). *dmrt1* geneak bestelako arrain-espezieetan ere sexu-determinazioan eragin dezake, esate baterako, *dmrt1* genearen gain-adierazpenak XX Niloko tilapiatan (*Oreochromis niloticus*) emetik arrerako sexu-eraldaketa sortzen du. Zebra arrainetan gainera, nahiz eta sexu-determinaziorako sistema poligenikoa azaltzen duten, genoman egindako lotura analisiek *dmrt1* lokusak sexu-determinazioan paper garrantzitsua jokatzen duela ikusi da (Matson & Zarkower, 2013).



2. Irudia: Medakaren sexu-determinazio sistema monogenikoa. Y kromosomari loturiko *dmy* geneak, ugaztunetan *sry* geneak egiten duen moduan ar helduen testikuluaren desberdintzapena agintzen du (Matson & Zarkower, 2013).

Faktore genetikoez gain ingurune-faktoreek ere eragin handia dute arrainen sexu-determinazio eta -desberdintzapenean, Devlin eta Nagahamaren (2002) berrikustapenean laburtuta azaltzen den moduan. Besteak beste, tenperatura, oxigeno eskuragarritasuna, pH-a, inguruneko hormonek eta harreman sozialek arrainen sexu-fenotipoan eragin dezaketela ikusi da, askotan determinazio genetikoaren gainetik gailenduz. Orain dela 35 urte genotipoaz gain tenperaturak ere arrainen sexu-determinazioan eragin zezakeela ikusi zen lehenengoz *Menidia minidia* espeziearekin burututako ikerketan (Conover & Kynard, 1981). Harrezkeroztik, asko dira tenperaturaren menpeko sexu-determinazioa aztertzen jardun dutenak. Besteak beste, fletan japoniarrak (*Paralichthys olivaceus*) eta Niloko tilapiak (*Oreochromis niloticus*) tenperaturaren menpeko sexu-determinazioa aurkezten dute, non XX emeak tenperatura igoerarekin ar bilakatzen diren (D'Cotta et al., 2001; Kitano et al., 2000). Horrela, tenperaturaren bitarteko sexu-eraldaketa sexubakarreko populazioak lortzeko erabili izan da akuikulturan, hazkuntza-tasa altuena dueneko generoa faboratzeko asmoz (Devlin & Nagahama, 2002). Horrez gain, XX/XY eta ZZ/ZW sistema duteneko espezieetan hormonen bidezko tratamenduek ere sexu-eraldaketa eragin dezakete. Izan ere ikerketa arloan hormona-tratamenduen bidezko sexu eraldaketa sexubakarreko populazioak lortzeko oso hedatua dagoen hurbilketa da (Pandian & Sheela, 1995;

Vizziano et al., 2007). Harreman sozialek eragindako sexu-aldaketa inguruneak eragindako sexu-determinazio kontrolaren muturreko adibide moduan har daiteke, adibidez pailazo arrainean (*Amphiprioninae* subfamilia) (Casas et al., 2016).

2. Sexu-desberdintzapena eta ugalketarako sistema endokrinoa arrainetan

Arrainek ur-ekosistema osatzen duten nitxo ekologiko desberdinetan bizi ahal izateko asko dibertsifikatu deneko taldea osatzen dute. Horren adierazgarri, aurkezten dituzten sexu-desberdintzapen prozesuaren malgutasuna eta ugal estrategia ugariak nabarmentzen dira (Baroiller et al., 1999, Devlin & Nagahama, 2002). Sexu-desberdintzapena, sexu-determinazioaren ostean gertatzen deneko obario edo testikuluaren garapenari deritzo eta sexu-determinazioak bezala forma eta mekanismo ugari hartzen ditu arrain teleosteoetan. Arrain asko gonokoristikoak dira, hau da ar edo eme moduan garatzen dira eta bizi osoan zehar beraien sexu-fenotipoari eutsi egiten diote. Talde honen barnean dauden espezieak ere gonadaren garapen goiztiarrerako duten estrategiaren arabera multzoka daitezke: bereizitako arrain gonokoristikoak, gonada zuzenean obario edo testikulu moduan garatzen dute; bereizi gabekoak aldiz, zebra arraina kasu (*Danio rerio*), obarioa gazte bat garatzen dute testikulu edo obario helduen aurretik (Devlin & Nagahama, 2002). Azken talde honetan beraz, arrak izango direneko banakoek (gutxi-gora-behera populazioaren erdia) derrigorrez hasieran garatutako obario heldugabetik testikulurako aldaketan fase hermafrodita batetik igaro beharko dute. Gainera, bigarren mailako espezie gonokoristikoak ere topa daitezke, hala nola *Nemipterus bathybius*, zeintzuk heldu aurretik gonada hermafrodita garatzen duten (Devlin & Nagahama, 2002).

Espezie gonokoristikoekin alderatuta espezie hermafroditek euren bizitzan zehar oozitoak zein espermatozoideak gara ditzakeen gonada helduak aurkezten dituzte. Aldibereko hermafroditotan, aldi berean gameto ar eta emeak topatuko ditugu, normalean ehun konektiboz banaturiko gonada ar (testikulua) eta eme (obarioa) desberdindu banarekin, aldi berean esperma zein arrautzak askatzeko gaitasuna dutelarik. Hauen artean, gobioak (*Gobiidae* familia), meroak edota serranido asko (krabeak) aurkituko ditugu. Hala ere, auto-ernalketa soilik Amerikako mangladietan aurki

daitekeen *Rivulus marmoratus* arrain ziprinidoan deskribatu da (Devlin & Nagaham, 2002). Beste espezie asko hermafrodito sekuentzialak dira, kasu honetan espermatozoideak edo oozitoak txandaka ekoizteko ahalmena dute bizi zikloko momentu desberdinetan zehar. Hauen artean espezie proterandrikoak (muxarra, *Diplodus vulgaris* eta urraburua, *Sparus aurata* esaterako) eta proteroginoak (durdoia, *Labrus bergylta* edo dontzeila, *Coris julis* esaterako) bereiz ditzakegu, hau da, heldutasun sexualean lehenengo ar edo eme moduan garatzen direnekoak hurrenez hurren. Gerora, eta normalean tamaina jakin batetara heltzean, espezie proterandrikoetan (lehengo arra) gonada arraren endekapena gertatzen da eta obarioa garatzen dute. Arrain proteroginoen barnean bi joera nagusi bereiz daitezke, espezie monoandrikoak, non testikulua, aurretik obarioa izan den gonadatik garatzen den, eta diandrikoak, non testikulua, obariotik edo gonada hermafroditatik eratortzen den (Devlin & Nagahama, 2002). Esan bezala sexu-aldaketa horiek tamainaren emendioak bultzatuak izan daitezke edota harreman sozialek eragindakoak. Azken estrategia hau ohikoa da ar menperatzaile bakarreko taldeetan antolatutako arrain proteroginoetan, adibidez koral arrezifeetako zenbait arrain familiatan, edota mundu osoko labrido espezie askotan (meroak, dontzeila, txilibitua, durdoia), non ar nagusiaren desagerpenak eme handienaren sexu eraldaketa bultzatzen duen (Godwin, 2009). Populazio baten sexu-ratioaren oreka mantentzeak ere sexu eraldaketa bultza dezake zenbait arrainetan (Munday et al., 2006).

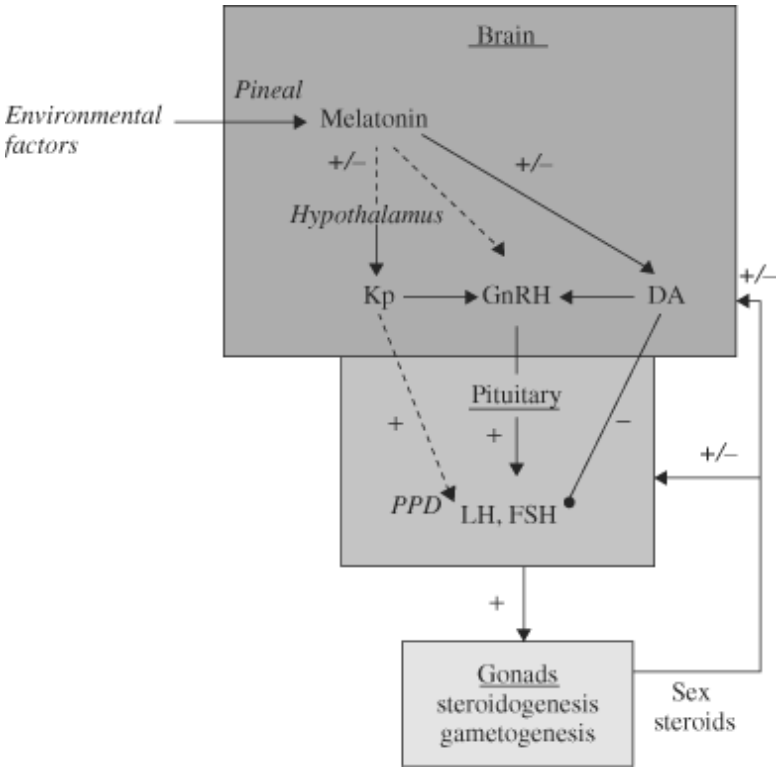
Arrainen sexu-desberdintzapena eta gametogenesia barne esteroideek eraentzen dituzte. Hainbat organo, guruin eta ehunek osatzen dute sistema endokrinoa. Oro har organismoaren funtzionamendua integratzeaz arduratzen den sistema barreiatua da; besteak beste, metabolismoa, garapena eta ugalketa kontrolatzen dituena. Horretarako, modu desberdinetan antolatutako zelula endokrinoek itu-zeluletara helduko diren hormonak odol-korrontera askatzen dituzte. Ornodunetan oso kontserbatua dagoen hipotalamo-guruin pituitario-gonada (HPG) ardatza hormona esteroideen metabolismoaz eta beraz ugalketaren kontrolaz arduratzen da (3. Irudia), banakoaren inguruneko kanpo informazioa (hala nola, informazio soziala, faktore klimatikoak, fotoperiodoa, elikagaien eta oxigenoaren eskuragarritasuna) barne fisiologiarekin

(homeostasia, metabolismoaren egoera eta banakoaren garapena) integratuz (Maruska & Fernald, 2011).

Arrainen hipotalamoa prosentzefaloan dago eta nerbio-sistema zentralaren erantzunaren ondorioz sorturiko hormona eta mezulari kimikoen sintesi eta askapenaz arduratzen da, nerbio-sistema zentrala nerbio-sistema autonomoarekin eta sistema endokrinoarekin konektatuz. Hipotalamoak kanpo estimulu eta faktore fisiologikoek sortutako neurotransmisoreen aurrean erantzuteko gai da, hartzaile espezifikoen bidez. Hauxe da kisspeptinen edo Kiss1 eta Kiss2 neuropeptidoen kasua, zeinak Gpcr (edo Kiss-R) hartzaileen bidez pubertaroa eraentzeaz arduratzen diren gonadotropinen hormona askatzaileak diren neuronak (GnRH neuronak) aktibatuz (Gopurappilly et al., 2013; Shahjahan et al., 2014). Kontrako eraginarekin, dopaminak (DA) pubertaroa eta arrain helduetan gametogenesi-ziklo bakoitzaren hasierako pausuen eta azkeneko oozitoaren hazkuntza eta obulazioaren inhibizioa eragiten du, zuzenean GnRH neuronetan dauden DA-D2 hartzaileen bidez. DA-ren askapena halaber melatoninak eraendua da. Melatoninak guruin pinealean ekoizten diren triptofano aminoazidotik eratorritako indoleaminak dira, zeinen ekoizpena fotoperiodoaren araberakoa den (Dufour et al., 2010). Melatoninak gauez ekoizten dira dopaminaren askapena inhibituz eta erritmo zirkadianoek sasoiek eta berari loturiko fotoperiodoak bere ekoizpen mailan eragiten dute. Bere eragina ere amets-zikloetara eta ugal zikloetara zabaltzen da.

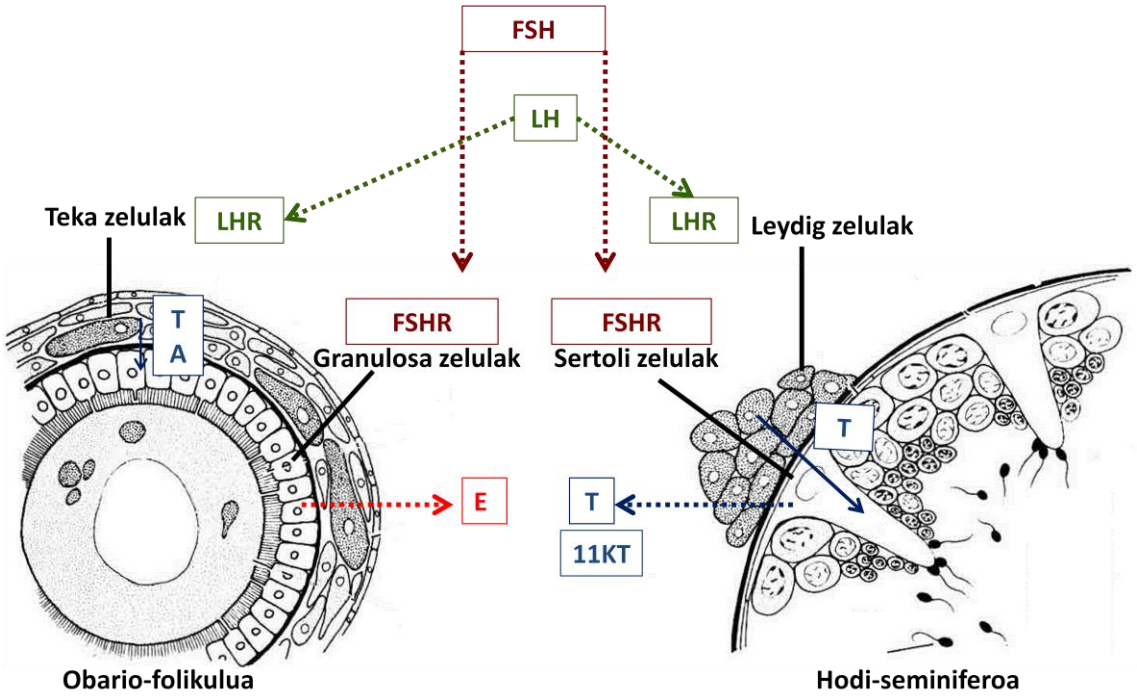
Kinada positiboen eraginpean (Kiss-Gpcr loturaren menpean) ugal neurohormona nagusia den GnRH (ingelesezko gonadotropin releasing hormone) ekoizten da hipotalamoan. GnRH-a dekapeptido bat da eta ornodunetan oso kontserbatua dago. Orain dela gutxi gainera ornogabeetan ere topatua izan da, bere garrantzi ebolutiboaren isla (Maruska & Fernald, 2011; Minakata, 2010; Tsai, 2006). Arrain teleosteoek bi edo hiru GnRH desberdinen ekoizpenerako gene paralogoak dituzte, GnRH3 arrainetan soilik topatu delarik. GnRH1-neuronen axoi-bukaerak zuzenean guruin pituitarioan dauden zelula gonadotropikoen ingurura jariatuko dute GnRH1 hormona. GnRH1-ek bi gonadotropinen, hormona luteinizatzailea (LH) eta hormona folikulu estimulatzailea (FSH), ekoizpena eta odol korronterako askapena bultzatuko dute pituitarioan (Levavi-Sivan et al., 2009). Odol korrontearen bidez gonadotropinek gonadetako zelula somatikoetaraino helduko dira, non sexu-hormona esteroideen sintesia eraenduko

duten (Kah & Dufour, 2011; Maruska & Fernald, 2011): emeetan 17β-estradiola (E) eta arretan testosterona (T) eta arrainetan bereziki 11-ketotestosterona (11-KT) ere (Le Menn et al., 2007; Hachfi et al., 2012). 11-KT arrain askoren testikuluetan frogatu denez, sexu-hormona nagusi gisa jokatzen duen T-ren aldaera oxidatua da (Nagahama et al., 1994). Horrez gain, gonadotropinek heltzearen eragile diren esteroideen sintesia kontrolatzen dute, batez ere, 17α,20β-dihydroxi-4-pregnen-3-ona bi sexuetan (Nagahama, 1997; Nagahama & Yamashita, 2008; Chen et al, 2013). FSH-k oogenesia eta espermatogenesia, hau da, lerro germinaleko zelulen proliferazioa, eragiten dituen bitartean, LH-k gametoen heltze prozesuan, obulazioaren indukzioan eta espermaren askapenean eragiten du beranduago (Ankley & Johnson, 2004; Rocha et al., 2009). Gonadotropinen askapena, gonadako esteroideek sorturiko "feedback" negatiboaren bidez eraen daiteke eta baita hipotalamoak askaturiko gonadotropinen hormona inhibitzailearen, GnIH-ren, bidez ere (Maruska & Fernald, 2011).



3. Irudia: Hipotalamo-pituitario-gonada (HPG) ardatzaren kontrol neuro-hormonala teleosteoetan. GnRH-k pituitarioaren bidezko gonadotropinen (LH eta FSH) askapena bultzatzen du, zeinek halaber, gonadetan gametogenesia eta esteroidogenesia aktibatzen dituzten. Dopaminak (DA) gonadotropinen sintesia eta askapena inhibitzen ditu eta kisspeptinak (Kp), GnRH eta gonadotropinen eraenketa positiboaren arduradunak dira. Melatoninak nerbio sistema zentralean jardun eta bereziki DA neuronen aktibitatea erregulatzen duten, ingurumen-faktoreen eraginaren bitartekariak dira (Dufour et al., 2010).

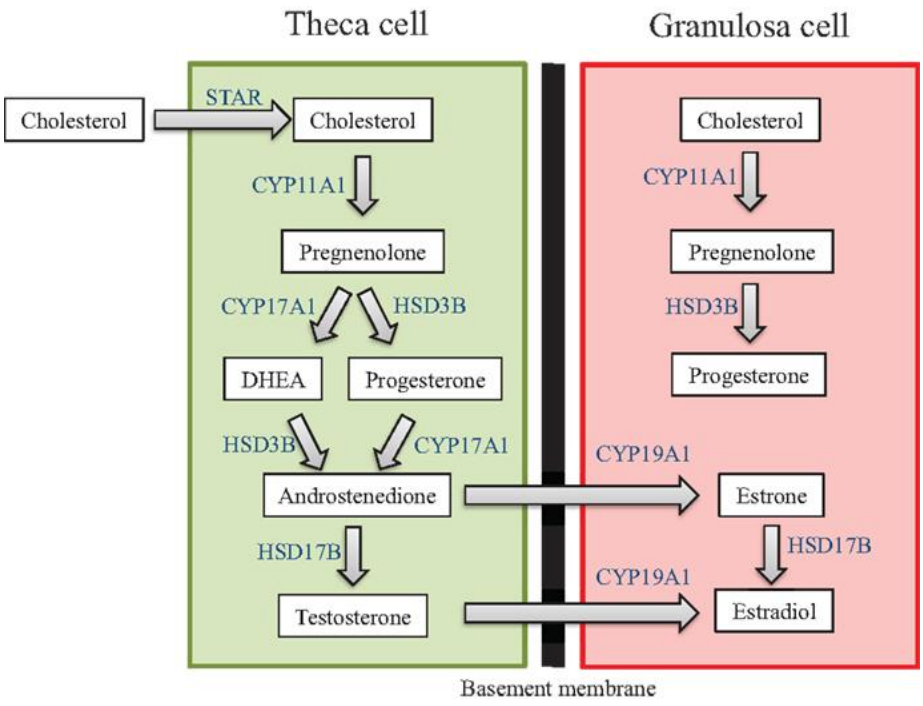
Sexu-hormona esteroideen sintesia gonaden zelula somatikoetan burutzen da. Horretarako, LH, teka zeluletan (obarioa) eta Leydig zeluletan (testikulua) dauden hartzaile espezifikoei (LHR) lotu behar zaie. Horrez gain FSH ere, granulosa (obarioa) eta Sertoli (testikulua) zeluletan adierazten diren eta mintz plasmatikoan txertatuak dauden FSHR hartzaile espezifikoei lotuko zaie (Hachfi et al., 2012) (4. Irudia).



4. Irudia: Hormona esteroideen sorrerarako gonadotropinen (hormona luteinizatzailea-LH eta folikulu estimulatzailea-FSH) bitarteko seinaleztapena arrain teleosteoen gonadetan. Laburdurak: T: testosterona, E: 17β-estradiola, 11-KT: 11-ketotestosterona, FSHR: hormona folikulu estimulatzailearen hartzaile espezifikoa, LHR: hormona luteinizatzailearen hartzaile espezifikoa (Hoar & Nagahama, 1978-tik eraldatuta).

Gonadaren zelula somatiko desberdindu horien mitokondrioetan kolesterolak eraldaketa sekuentzialak pairatuko ditu, testosterona, 11-ketotestosterona (androgenoak), zein 17β-estradiola (estrogenoa) ekoizteko (Devlin & Nagahama, 2002; Piferrer, 2011). Obarioan (5. Irudia) kolesterola teka zeluletan lipropoteina hartzaileen bidez sartu edota zuzenean zelula hauen zitoplasman *de novo* ekoitz daiteke. Orduan mitokondriatara garraiatua izango da StAR proteinaren (ingelesez steroidogenic acute

regulatory protein) laguntzaz. Bertan, Cyp11a1-ari (cytochrome-P450 cholesterol side-chain cleavage enzyme) esker pregnenolonan bilakatuko da. Pregnenolona mitokondriatik atera eta erretikulu endoplasmatiko leunera garraiatzen da progesterona edo dehidroepiandrosterona (DHEA) ekoizteko, Hsd3b (ingelesezko 3β-hydroxysteroid dehydrogenase) edo Cyp17a1-ri (esteroide 17-alfa-monooxigenasa) esker, hurrenez hurren. Progesterona eta DHEA androstenediona androgenoan bilakatuko dira, berriro ere Cyp17a1 edo Hsd3b-ri esker. Androstenediona orduan testosteronan eta bestelako androgenoetan bilakatuko da eta granulosa zeluletan estronan, HSd17b (17β-hydroxysteroid dehydrogenase) entzimari esker. Androstenedionatik eratorritako testosterona eta estrona estradiolean eralda daitezke aromatasa (*cyp19a1a* genearen produktua) edo Hsd17b-entzimei esker (Hannon & Flaws, 2015).



5. Irudia: estrogenoen ekoizpena obarioko folikuluen zelula somatikoetan. Esteroidogenesia obarioan folikuluaren teka zelulek eta granulosa zelulek bideratzen dute. Horrela, kolesterola estradiolean eta bestelako sexu-hormonetan bilakatuko da. Urdinez entzimak agertzen dira eta hormonak kutxa zurietan (Hannon & Flaws, 2015).

Hormona esteroideek arrainen sexu-desberdintzapenean jokatzen duten papera luze aztertu izan da (Pandian & Sheela, 1995). Hormona hauek zelulen hartzaile nuklearrekin, estrogeno- eta androgeno-hartzaileekin, elkarreragin dezakete (sexu-

desberdintzapenean eta gametogenesian parte hartzen duten geneen adierazpena aldatuz (Leet et al., 2011).

Jakina da estrogenoek arrainen obarioaren desberdintzapenean eta sorreran ezinbesteko funtzioa dutela. Are gehiago, oso posiblea da aromatasa entzima izatea sexu-determinazioan aritzen diren geneek eragiten duten etengailua, estrogenoen sintesia erregulatuz sexu-desberdintzapena eraentzeko (Chang et al., 2005). Aromatasa entzimak hormona esteroideen arteko oreka mantentzen du sexu-desberdintzapenean, garapenean eta ugalketan zehar (Devlin & Nagahama, 2002; Diotel et al., 2013). Arrainen genoman bi gene paralogo ezberdin daude aromatasaren sintesirako, *cyp19a1a* eta *cyp19a1b* (Chiang et al., 2001). Cyp19a1a-k, edo obarioteako aromatasak, oozitoa inguratzen duten teka eta granulosa zelulatan adierazpen altua duen bitartean, Cyp19a1b burmuinean adierazten da gehien bat, bai ar zein emeen kasuan (Chiang et al., 2001; Blázquez & Piferrer, 2004; Liu et al., 2007). Cyp19a1a estrogenoen sintesian duen eraginagatik obarioaren desberdintzapenean eta gibelean bitelogenesiaz arduratzen da. Cyp19a1b aldiz, neurogenesia eta ugal-garapenean jarduten du sexu-desberdintzapenean zein ugal-zikloan zehar (Blázquez & Piferrer, 2004; Diotel et al., 2013). Enbrioiaren garapenean *cyp19a1b* da lehenengo paralogoa adierazten baina *cyp19a1a*, nahiz eta beranduago adierazi, sexu-desberdintzapena ikusgarria izan aurretik adierazten hasten da; adibidez zebra arrainean, tilapian eta oztadar amuarrainean (Guiguen et al., 1999; Kwon et al., 2000; Trant et al., 2001; Kobayashi et al., 2003; Siegfried & Nüsslein-Volhard, 2008; Sawyer et al., 2006 ; Ijiri et al., 2008).

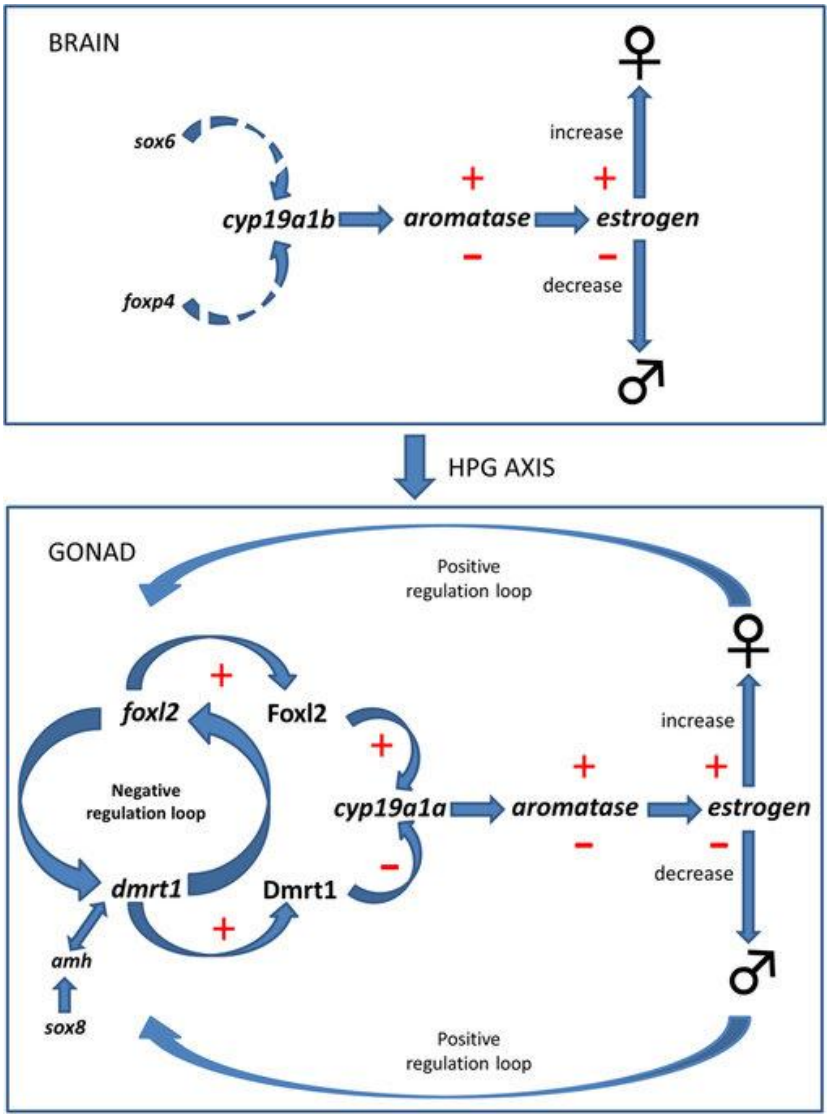
cyp19a1a-ren transkripzio-mailetan eta beraz estrogenoen sintesian eta sexu-desberdintzapenean eragiten duten hainbat gene deskribatu dira orain arte (6. Irudia). Horien artean, *sox9, amh, dmrt1 eta foxl2* aipa daitezke. Hauek azkenean *cyp19a1a*-ren aktibazioa edo desaktibazioa ondorioa duten bidezidorretan parte hartzen dute eta, adibidez zebra arrainetan, zeinek derrigorrezko obario garatugabea ekoizten duten *sox9*, *dmrt1* eta *amh*-ek paper garrantzitsua jokatzen dute obario garatugabe horretatik testikulua sortzeko. Gene hauek, ordurarte aktibo dagoen aromatasa jarduera ixilerazi behar dute, E-mailak jaitsi, T-mailak igo eta obarioaren ordez testikuluak garatu ahal izateko (Hofsten & Olsson, 2005). *sox* familia osatzen duten geneak DNA-loturarako HMG-kutxa (High Mobility Group) domeinua duten transkripzio-faktoreak kodetzen

dituzte (Leet et al., 2011). Familia honetako geneei funtzio ezberdinak esleitu zaizkie, sexu-determinazioan edo neurogenesian. Horien artean *sox9* genea, ugaztunetan pre-Sertoli zeluletan gain-adierazten dela ikusi eta *sf1* (steroid factor 1) eta *amh*-rekin (anti Müllerian hormone) elkarrekiten duela ikusi da. Oso antzera, *sox9* arrain teleosteoetan testikuluaren garapenarekin erlazionatuta dago (Rodríguez-Marí et al., 2005). Gonadak-desberdintzatu aurretik bi sexuetan adierazten da zelula germinalen inguruan dauden zelula somatikoetan (arretan gerora Sertoli zelulatan desberdinduko direnak) adierazten delarik. *Sox9*-aren gain-adierazpena gerora, testikuluaren garapenean azkeneko urratsetan ematen da, hodi seminiferoen azkeneko hazkuntzan parte hartuz (Nakamoto et al., 2005). Bestetik, pailaso arrainean (*Amohiprion bicinctus*) adibidez, sexu-desberdintzapenarekin erlazionatuta diruditen bestelako sox geneak deskribatu dira, horien artean, *sox6,* zeinak ornodunetan espermatogenesia erregulatzen duen eta *sox8,* testikuluen mantenimenduan diharduena (Casas et al., 2016). Horiez gain *foxp4*-ak (forkhrad box P4) ere, *sox6*-rekin batera burmuin mailan aromatasaren adierazpenaren erregulazioan jarduten dutela proposatu dute (Casas et al., 2016) (6. Irudia).

Ugaztunetan bezala, arrainetan ere Amh eta Dmrt1 proteina-mailak arren desberdintzapenarekin erlazionaturik daude (Kobayashi et al., 2004; Rodríguez-Marí et al., 2005). Arrerako sexu-desberdintzapenak ugaztunetan hormona anti-Mülleriarraren (Amh) adierazpenaren aktibazioa eskatzen du (Lasala et al., 2004). Amh da hain zuzen ere Sertoli zelulek testikulura askatzen duten lehenengo faktorea, duktu Mülleriarren atzeraka bultzatzeko ugaztunetan. Duktu Mülleriarrak arrain teleosteoetan egon ez arren, arrainek *amh* gene ortologoa aurkezten dute eta sexu-desberdintzapenaren garaian, *cyp19a1a*-ren erregulatzaile negatiboa da (Rodríguez-Marí et al., 2005). *amh*-k paper garrantzitsua jokatzen du espermatogonien proliferazioan ere (Yoshinaga et al., 2004; Vizziano et al., 2007; Ijiri et al., 2008). Ornodunetan *dmrt1* (ingelesez doublesex and mab-3 related transcription factor 1) ere kantitate altuetan adierazten da testikuluaren desberdintzapena gertatzean (Marchand et al., 2000). Dmrt1 Sertoli zelulatan ekoizten da desberdintzapenaren ostean eta espermatogonien proliferazioan jarduten du ere (Kobayashi et al., 2004). Medakan Y kromosomari loturiko *dmy* geneak, *dmrt1*-ren adierazpena bultzatzen du (Matson & Zarkower, 2013) eta zebra arrainean ere, zeinak sexu-determinazio poligenikoa duen, *dmrt1* adierazpena asko igotzen da

testikulu-desberdintzapenaren hasierako faseetan, baina obarioaren zelula germinaletan baita aurki daiteke. Arrainetan *dmrt1*-k fenotipo arra eragin dezake *adbp/sf1* (adrenal binding protein/steroidogenic factor 1) eta *foxl2* transkripzio faktorea (Fork-head transkription factor 2) inhibituz eta aromatasaren azpi-adierazpena bultzatuz. Horrela esteroideen bidezidorra androgenoen ekoizpenera bideratzen da, tilapian eta zebra espezieetan ikusi izan den bezala (Hofsten & Olsson, 2005; Wang et al., 2010). Horrez gain, *StAR, cyp17a1* eta *cyp11a1* aktiba ditzake, estrogenoen produkzioa isiltzeaz gain androgenoena bultzatuz (Wang et al., 2010).

foxl2 bestetik, feminizaziorako transkripzio faktorea kodetzen duen gene kontserbatua da. Granulosa zelulen desberdintzapenean, ugalketan eta obarioaren garapen eta mantentzean jarduten du, ornodunetan sexu-markatzaile goiztiarra kontsideratzen delarik. Tilapia eta medaka emeetan ikusi den bezala *foxl2*-ren adierazpena zelula germinalak inguratzen dituzten zelula somatikoetan hasten da obario-desberdintzapenarekin batera, eta oogenesi osoan zehar granulosa zeluletan adierazten da (Wang et al., 2004; Nakamoto et al., 2006). *foxl2*-k obarioaren sorreran c*yp19a1a*-ren transkripzioa eraentzen duela ikusi da (Yamaguchi et al., 2007). Izan ere, Foxl2 proteinak *cyp19a1*-aren promotorea lotzeko gaitasuna du bere adierazpena bultzatuz, adibidez japoniako platuxan eta tilapian ikusi den moduan (Yamaguchi et al., 2007; Wang et al., 2007; Ijiri et al., 2008).



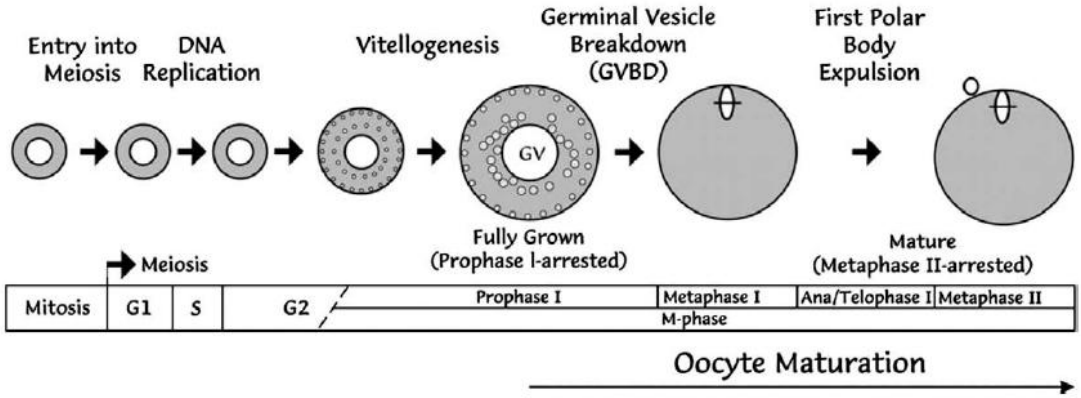
6. Irudia: Arrainen sexu-desberdintzapenaren eragile genetikoen bidezidorren proposamen eskematikoa. +/-: aktibazio/inhibizioa adierazten dute. Puntuztaturiko geziek bide hipotetikoak erakusten dituzte. Eskeman HPG-ean (hipotalamo-pituitario-gonada ardatza) estrogenoen ekoizpenaren orekan diharduten geneen aktibazio turrustek obario zein testikuluen desberdintzapena bultzatzen ikus daitezke. *cyp19a1b*, (burmuineko aromatasa), sox6 (SRY (sex determining region Y)-box 6), *foxp4* (forkhead box P4), *foxl2/*Foxl2 (forkhead box L2), *dmrt1* (doublesex and mab-3 related transcription factor 1),; *cyp19a1a* (obarioko aromatasa), *amh* (anti-Müllerian hormone); *sox8* (SRY (sex determining region Y-box 8) (Casas et al., 2016).

Inguruneko hormona exogenoek (ikus 5. atala), barne hormonek bezala, sexu-desberdintzapenean jarduten duten geneen adierazpen normala eralda dezakete. Androgenoekiko esposizioak adibidez obarioaren desberdintzapenean jarduten duten *foxl2*, *fst* (follistatina kodetzen duen genea) eta *cyp19a1a*- azpi-adierazten dituztela ikusi da, maskulinizazioa eragin dezaketelarik (Baron et al., 2008). 17β-estradiolak aldiz

arretan Dmrt1-mailak gutxitu ditzake, besteak beste, emeranzko eraldaketa bultzatuz (Marchand et al., 2000).

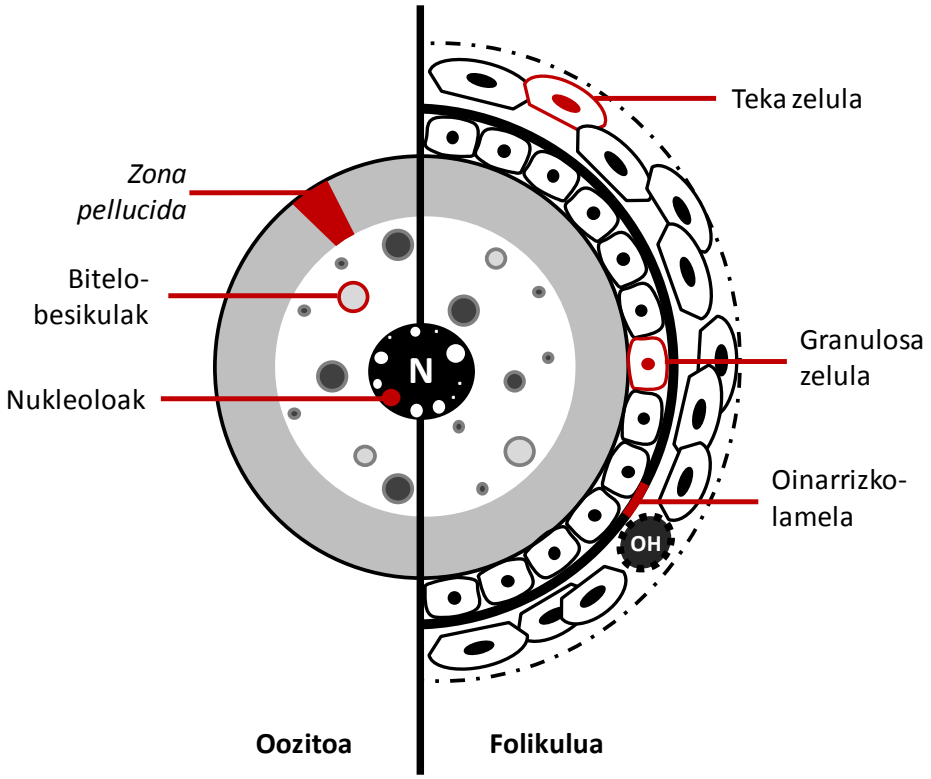
3. Oogenesia

Gametoak izaki zelulanitzetan meiosiz sortarazten diren zelula haploideak dira, ugalketa sexualaren bitartez izaki diploide berri bat sortzeko funtzioa dutenak. Gametoen garapenerako ematen den lehenengo urratsa zelula germinal primordialen edo PGC-en (ingelesezko primordial germ-cells) sorrera da. Arrainen PGC-ak enbriogenesian goiz, gastrulazioa gertatu eta gero, sortzen dira (Lubzens et al., 2010). PGC-ak arku genitaleraino migratu behar dute, non zelula somatikoekin elkartu eta gonada kolonizatzen duten. Arrain gonokoristikoen sexu-desberdintzapenean, PGC-ek euren izaera bipotentziala galdu eta meiosi hasierarekin batera gameto eme zein arretan desberdintzen hasten dira. PGC-a oogenesia edo espermatogenesira bideratzeko erabakia, kokatzen deneko mikroinguruneak edo ingurune zelularrak mugatzen du (Yoshizaki et al., 2010, 2012). Gonadaren zelula somatikoek emeen sexu-desberdintzapenean paper garrantzitsua jokatzen dute (Kurokawa et al., 2007; Siegrfried & Nüsslein-Volhard, 2008). Ugaztunetan ez bezala, eme teleosteoek lerro germinaleko zelula mitotiko desberdintsatugabeak (oogoniak) bizitza osoan zehar mantentzen dituzte, mitosirako eta arrautzen desberdintzerako (meiosirako) bidea aktibo mantenduz; ornodun ar guztietan espermatogonioekin gertatzen den bezala (Nakamura et al., 2010; Yoshizaki et al., 2010). Zebra arrainetan, obario heldugabea sortzen den momentuan, lerro germinaleko zelulek bidalitako seinaleak ezinbestekoak dira genetikoki emeak diren banakoetan obarioaren garapena eman dadin. Oozitoek inguruko zelula somatikoetan espezifikoak diren geneen (adib. *foxl2* eta *cyp19a1a*) transkripzio-mailak mantentzen laguntzen dute, testikuluekin erlazionatutako geneen azpi-erregulazioa bultzatuz. Zelula germinalen ekoizpena ekiditen denean, zebra arrainek testikulua eta arren kanpo ezaugarriak garatzen dituzte derrigorrez (Siegfried & Nüsslein-Volhard, 2008). Beraz, zebra arrainetan behintzat, testikuluak ekoiztutako esteroideak nahiko dira maskulinizazioa bultzatzeko (Siegfried & Nüsslein-Volhard, 2008). Bestetik, medakan, non gogoratu XY sexu-determinazio sistema dagoen, zelula germinalik gabeko XX emeek arrerako sexu itzulera pairatzen dute.



7. Irudia. Arrain teleosteoetan ugalketa ziklo bakoitzean gertatzen diren oozitoen garapen faseak meiosi-faseekin erlazionatuta (Lubzens et al., 2010).

Oogenesia (7. Irudia, 1. Taula) gameto emeen garapenari deritzo, zeinaren azken produktua arrautza heldu bideragarria den (Lubzens et al., 2010). Obarioaren unitate-funtzionala folikulua da; oozitoa eta zelula somatikoz osatutako kanpo geruzak. Folikulua barrutik kanpora, oozitoak berak, granulosa zelulek, oinarrizko-lamelak eta teka zelulek osatzen dute (8. Irudia). Oogenesiaren hasieran desberdindu gabeko oogoniak mitosiz biderkatzen dira “oogonia-habia” sortuz eta granulosa zelulekin elkartuz. Era honetan, oogonia bakoitza granulosa zelulez osatutako geruzaz inguratuta geratzen da. Granulosa zelulek, oogoniak obarioaren estromatik bereizteko “oinarrizko lamela” jariatzen dute. Lamela geruzaren periferian teka zelulak lerrokatzen dira, zeinak odol hodiekin harremanetan jartzen dituzten oogoniak. Pubertaroa eta gero lehenengo zikloaren ostean, ingurune eta baldintza fisiologikoek eragindako seinale hormonalak jarraituz oogoniak lehenengo zatiketa meiotikoan sartuko dira, ugal-ziklo bakoitzaren hasieran lehenengo mailako oozitoak sortuz (Selman et al., 1993). Lehenengo mailako oozitoaren hazkundea nukleoan egitura perinukleolarren agerpenetik albeolo kortikalen sorrerara doa. Fase honetan zehar oozitoa lehenengo zatiketa meiotikoaren profasean (diplotenean hain zuzen) geldirik egongo da, eta beranduago beharrezkoak izango diren organulu eta molekulak ekoiztuko ditu. Egitura perinukleolarren agerpena, RNA erribosomikoen (rRNA) ekoizpenaren adierazpen morfologikoa da (Lubzens et al., 2010).



8. Irudia: Oozito eta obario-folikuluko zelula somatikoen arteko harreman espaziala azaltzeko irudi eskematikoa. N: nukleoa edo besikula germinala, OH: odol hodia (Hoar & Nagahama, 1978 eta Tyler & Sumpter, 1996-tik eraldatuta).

Oozitoa handituz doan heinean zitoplasman albeolo kortikalak deritzen proteinaz eta karbohidratoz beteriko besikulak biderkatu eta tamainan handitzen dira. Albeolo hauek oogenesiaren azkenengo faseetan eta zitoplasman gertatuko den bitelo-proteinen pilaketa dela eta oozitoaren periferiarantz mugituko dira. Ernalketaren ostean "erreakzio kortikala" izeneko prozesuan euren barruko osagaiak arrautzaren periferiara askatuko dira korion izeneko kanpo-geruza azelularra sortuz (Selman et al., 1993).

Bitelogenesia, bitelogeninak (VTG) oozitoan sartu eta bitelo proteinak ekoizteko jasan behar dituen aldaketa multzoari deritzo (Le Menn et al., 2007). VTG-ak edozein ornodun eme obiparoetan aurki daitezkeen oozitoen fosfolipoproteinak dira. VTG-ak arrainen gibelean ekoizten dira estradiolak erregulatutako prozesuaren bitartez (Badin et al., 2007). Arrain teleosteo espezie gehienetan VTG ekoizpenerako gutxienez hiru gene desberdin ezagutzen dira (*vtga*, *b* eta *c*). Gibelean sintetizatuak izan eta gero obariora garraiatzen dira odol hodietan zehar. Obarioan teka kapilarretatik igaro eta granulosa zelulen geruza era pasiboan zeharkatzen dute, azkenik hartzaile espezifikoen bidez

(bitelogeninaren hartzaileak) endozitoziz oozitoetan barneratzen dira. Sortutako besikulak lisosomekin bat egiten dute non txikiagoak diren bitelo-proteinatan zatituko diren (Wallace & Selman., 1990). Bitelogenesian zehar oozitoaren tamaina handitzen da eta prozesu honetara bideratuko den oozito-kopurua energia eskuragarritasunaren araberakoa izango da (Luckenbach et al., 2008a). Prozesu honetan VTG-z gain oozitoak ere bere matrize estrazelular berezia (*zona pellucida*) osatzen du, gibelean eta oozitoan bertan ekoizten diren koriogenina edo *zona pellucida* proteinen bitartez. Oozitoak baita bestelako erreserbak pilatuko ditu zitoplasman, hala nola, RNA-molekulak, proteinak, lipidoak, karbohidratoak, bitaminak eta hormonak, ernaldua izateko konpetentzia bereganatuz eta enbrioiaren garapenean zehar beharrezkoak diren osagaiak eskuratuz (Wallace & Selman, 1981, 1990; Brooks et al., 1997; Le Menn et al., 2007). Jatorriz amak ekoiztu dituen faktore hauek sustengatuko dute enbrioia garapen goiztiarrean zehar, zigotoa beraren genomaren transkripzioaren aktibazioa blastula garaian gertatu bitartean (Kane & Kimmel, 1993).

Bitelogenesiaren ostean heltze prozesua dator, non endozitosia eten, meiosia amaitu eta nukleoa edo besikula germinalaren apurketa ematen den. Albeolo kortikalak periferian kokatuta daudelarik, zitoplasmako bitelo egiturak disolbatuko dira orduan. Lehenengo meiosia amaitu ostean tamaina ezberdineko bi zelula lortzen dira, txikia den gorpuzkulu polarra eta handia den bigarren mailako oozitoa edo oozito heldua. Obulazioan II. metafasean dagoen oozitoa heldua folikulutik askatzen da eta bigarren meiosia amaituko du zelula haploidea bilakatuz. Orduan arrautza izena hartuko du. Arrautza pelagikoak ekoizten dituzten teleosteoetan gainera heltze-prozesuan zehar arrautzaren hidratazioa gertatzen da oozitoaren mintzeko ur-kanal edo akuaporinen bitartez (Cerdá et al., 2007). Azkenean arrautza pelagiko heldu baten pisuaren %90-95 eta bolumenaren %65-75 ura izango da (Cerdá et al., 2007). Hartutako urak flotagarritasuna emendatuko du eta enbrioia ingurune hiperosmotikotik babestuko du haren hedapenean eta barreiaduran lagunduz. Arrainetan ernalketa arrautzaren animalia-poloan dagoen mikropiloaren bidez gertatzen da. Espermatozoide bakarrak zeharka dezake mikropiloa. Orduan eta poliespermia ekiditeko, arrautza aktibatu eta mikropiloa ixten da. Ernalketa ostean korionak enbrioia ur ingurunetik babestuko du (Lubzens et al., 2010).

3.1. Oogenesiaren kontrol-hormonala

Oogenesiaren hasieran bi sexu esteroideek kontrolatzen dute oogonien proliferazioa eta meiosiaren hasiera; 17β-estradiolak (E) proliferazioan eragiten du eta 17,20β-dihidroxi 4-pregnen-3-ona-ek (17,20βP) proliferazioaz arduratzeaz gain meiosiaren lehenengo zatiketari hasiera ematen dio. Horrez gain, lehenengo zatiketa meiotikoan dauden oozitoen agerpenarekin LH eta FSH hormonen sintesia emendatzen da (Baron et al., 2005). Lehenengo mailako oozitotik bigarren mailako oozitorako trantsizioa pituitariotik heltzen diren hormonen menpekoa da, E-aren igoeraren aurrean erantzunda (Swanson et al., 1991; Swanson et al., 2003). Albeolo kortikalen sintesia FSH, E eta StAr pituitario eta plasma-mailen igoerarekin (Yamazaki, 1965; Tokarz, 1978; Khoo, 1979) eta aromatasa genearen adierazpenaren igoerarekin (Kwok et al., 2005) lotuta dagoela ikusi da. Gertatzen den lipido pilaketa ere FSHak sortutako erregulazioarekin erlazionaturik dago. Teka zelulek androgenoz hornitzen dituzte granulosa zelulak eta hauek halaber aromatasa adieraziko dute E ekoizteko. E-k gibeleko VTG sintesia eta FSH-k VTG-en folikulurako harrera sustatzen dute (Nagahama, 1994; Young et al., 2005; Tyler et al., 1991). Oozitoaren heltze prozesuan LH eta LH-hartzaileak plasman ugaritzen dira. LH-k E sintetizatzetik heltzearen esteroide eragileak sintetizatzera pasatuko da, 17,20βP adibidez (Nagahama & Yamashita, 2008; Lubzens et al., 2010), zeinek oozitoa obulaziorako prestatuko duten. Orduan ere estrogenoekin erlazionatutako geneen adierazpenak behera egingo du (Bobe et al., 2006). Oozitoaren obulazioa azido arakidonikoa eta bere metabolismoan sortzen diren subproduktuekin, hala nola protaglandinekin, erlazionatzen da eta beraz, ugaztunetan bezala, obulazioa hantura gertaerek bultzaturiko prozesua da, zeinetan hanturaren eragileak eta proteolisi, basodilatazio eta koagulazioarekin erlazionatutako geneak adierazten diren, besteak beste, *prss23* serina proteasa, *adam22* metaloproteasa-desintegrina, *cxxl14,f5* koagulazioa-faktorea, *fgl2* fibroblastoen hazkuntza-faktorea eta *ace2* angiotensina eraldatzailea den entzima (Bobe et al., 2006; Lubzens et al., 2010).

3.2. Oogenesian zehar obarioan ematen diren aldaketa molekularrak

Hainbat dira oogenesian zehar eta fase espezifikoei esleituta adierazten diren geneak eta metatzen diren molekulak (transkriptoak, proteinak, lipidoak, karbohidratoak,

bitaminak eta hormonak). *Pimephales promelas* arrain espezie asinkronikoan adierazpen diferentziala aurkezten duten 460 transkripto bereiztu dira, obarioan daudeneko oozito prebitelogeniko vs. bitelogenikoen proportzioaren arabera banatzen direnak (Villeneuve et al., 2010). Konposatu eta transkripto guzti hauek beraz, obarioan gertatzen diren prozesu ezberdinen eta noski, oozitoen presentziaren eta garapenaren adierazle gisa erabiltzeko kontuan hartu behar dira. Adibidez, zelula germinalekiko espezifikoak diren mRNA-en artean; euren garapen goiztiarreko fase mitotikoan ezinbestekoa diren *vasa* genea *nanos*, *tudor*, *dnd* eta *dazl* aurkitzen dira (Lubzens et al., 2016). Arrain helduen obarioetan oogenesian sartuko diren PGC-etan *Sox9* ere adierazten dela ikusi da (Nakamura et al., 2012). Horietaz gain, diplotene fasean dauden oozitoen inguruan dauden zelula folikularrek, euren markatzaile moduan erabil daitekeen *foxl2* adierazten dute.

Oncorhynchus kisutch izokinaren oozito prebitelogenikoetan *zona pellucida*-ren sorrera, bitelo harrera eta prozesamenduari lotutako proteinen sintesia gertatzen da, hala nola, *zona pellucida*-ren glikoproteinak (*zp*), Vldlr-ak, B-katepsinak, E-ziklinak, Dnaj eta ferritinaren 3 azpi-unitatearen sintesia; zeinen transkriptoen maila altuak agertzen diren oozito prebitelogenikoetan, albeolo kortikalen fasean dauden oozitoekin alderatuta (Luckenbach et al., 2008b). Badira ere meiosiarekin adierazten diren geneak eta beraz oozitoen lehen hazkuntzaren markatzaileak diren geneak, hala nola, *piwil* zeinak meiosi-prozesuan transposonen hedapena ekiditeko ezinbestekoa den (Diaz de Cerio et al., 2012). piRNA-k (Piwi-interaczio RNAk, RNA-bidezko geneen isiltzea burutzen duten zelula germinalekiko RNA espezifiko txikiak, Houwing et al., 2008), *mlh1* (mismatch repair gene MutL homolog1) eta *mps1* (monopolar spindle1, meiosian zehar kromosomen elkarketan eta segregazioaren arduradunak) ere fase honetan adierazten dira (Feitsma et al., 2007; Poss et al., 2004).

Albeolo kortikalen ekoizpenarekin eta bitelogenesiarekin batera, lipido desberdinen, arrautzaren izaeraren arabera, metaketa gertatzen da (Luckenbach et al., 2008b). Vtg-rekin batera barneratzen diren lipido polarrez gain (fosfoglizeridoak batez ere), *de novo* sintetizatuak diren lipido neutroak (batik bat triazilglizerolak) metatzen dira. Zenbait arrautzek, flotagarritasunean laguntzen duten ester esterilak diren lipido neutroak metatzen dituzte gehien bat (Yilmaz et al., 2016). Lipidoen sarrera eta prozesamenduari

loturiko markatzaileei dagokienez, *ldlr* geneak (low density lipoprotein recerptor) transkripzio-maila oso altuak aurkezten ditu prebitelogenesian, zeinak bitelogenesiaren hasierarekin jaitsi egiten den. Berdin gertatzen da gantz-azidoen garraioaz arduratzen diren proteinen transkriptoekin (Lubzens et al., 2016). *vtgr* (bitelogenina-hartzaileak) eta *vldlr* (low density lipoprotein receptor) transkripto mailak aldiz oogenesi hasierarekin igo eta bitelogenesian zehar mantentzen dira (Damsteegt et al., 2015). Bitelogenesian ere *lpl* (lipoprotein lipase) transkripto-mailak igo egiten dira zelula folikularretan (Ibañez et al., 2008).

Lipidoez gain, garatzen ari diren oozitoetan Vtg-rekin edo bestelako lipropoteinekin batera ere barneratzen diren, bitamina disolbagarriak (A, E bitaminak, bestelako retinoideak eta karotenoideak), mineralak (fosfato inorganikoa, kaltzioa, burdina, magnesioa eta bestelakoak), hormonak (hormona tiroideoa eta kortisola adibidez) eta bestelako erregulaziorako konposatuak metatzen dira (Lubzens et al., 2016). Aurretik esan bezala, Vtg-ak endozitosi bidez kantitate oso altuetan barneratzen dira oozitoan, non katepsina entzimak dituzteneko gorputz-multibesikularretan metatuko diren. Katepsinek Vtg-ak bitelo proteinetan prozesatuko dituzte. *lrp13* (Ldl hartzaileekin erlazionaturiko 13-proteina) transkripto-maila oogenesiaren hasieran aurkitzen diren bitartean, Lrp13 eta Vtgr proteinak bitelogenesian ekoiztu eta mintz zelularrera garraiatzen dira, oozitoaren heldutasuna bukatzeko (Lubzens et al., 2016). Hidratazioan, katepsinek bitelo proteinak amino-azido askeetan apurtzen dituzte, sortutako presio osmotikoarekin ura oozitoan sartzera behartuz. Gainera, ioi inorganikoen kantitatea eta amonio kantitateak ere igo egiten dira zitoplasman, presioan lagunduz. Uraren sarrera aurretik aipatutako akuaporinen bidez gauzatzen da; zehazki, Aqp1ab-ren (aquaporin 1ab) bidez. Akuaporina hau hazkuntza primarioan adierazten da eta bitelogenesia eta oozitoaren heltzearekin batera trankripzio-maila jaitsi egiten da; itzulpen osteko erregulazioa iradokiz (Zapater et al., 2011). LH-ren seinaleztapenarekin erlazionatutako transkriptoak, bitelogenesian gutxi adieraziak, obulazioan gain-adierazi egiten dira. Hauekin batera, aktibinak, inhibinak, *gdf9* (growth differentiation factor 9), *bmf7* (bone morphogenetic protein 7), neuregulin1, retinoikoaren X-hartzailea eta nerbio hazkuntza-faktoreen familia oozitoaren heltze prozesuan garrantzitsuak ere badirela frogatu izan da (Martyniuk et al., 2013).

3.3. Arrainen gonadaren garapen-bideak eta ugal-estrategiak

Ugal-ziklo bakoitzean obario eta oozitoen garapen-estrategia desberdinak aurki daitezke arrain espezie desberdinetan, folikuluen heltze-garaiaren edo oozitoen errekrutatze mekanismoen arabera sailkatzen direnak. Oozitoen errekrutatzea errute ostean eten egiten bada, orduan emankortasun zehaztua edo sinkronikoa duen espeziea kontsideratzen da; halakoak dira mugilidoak, klupeiformeak eta pleuronektidoak (Murua et al., 2003). Bestetik, oozitoen errekrutatzeak errun ostean jarraitzen badu, zehaztugabeko emankortasuna duten espeziez ari gara (asinkronikoak); hala nola, antxoak, legatzak eta txitxarroak. Lehenengo kasuan, oozito guztiak garapen fase berdinean edo gehienez bi fase desberdinetan aurkituko dira obario beraren baitan eta guztiz heltzean denak aldi berean askatuko dira. Kasu honetan, ugaltze ziklo bakoitzean errute eta ernaltze momentu zehatz mugatu eta bakarra dago. Bigarrengoan aldiz, garapen-fase ezberdinetan dauden oozitoak aurki daitezke obarioan zeinak ugal-ziklo bakoitzean zehar multzoka heldu eta errungo diren (Wallace & Selman, 1981; Murua et al., 2003). Espezie hauetan erruteak, eta beraz ernalketak, aste edota hilabetetan zehar luza daitezke.

Horrez gain, espezie batzuk burutzen dituzten migrazioak alde batera utzita, ugalketa-estrategiak anitzak dira arrainetan. Horrela, badaude bizitzan behin bakarrik ugaltzen diren espezieak, zeinei semelparo deritzen. Hauek normalean hil aurretik erruten dute eta ondorioz hil egiten dira. Gehienak aldiz iteroparoak dira, hau da, bizitzan zehar behin baino gehiagotan ugaltzen dira. Azkenengo hauek halaber, errute-maiztasunaren arabera sailkatzen dira eta isokronalak (urtean behin erruten dutenean), heterokronalak (ugalketa garaian maiztasun zehatzarekin behin baino gehiagotan erruten dutenean) eta zehaztugabeak (ugalketa garaian behin baino gehiagotan gameto taldeak askatu eta berriz ere berriak ekoitz ditzaketenean) izan daitezke (Miller & Nummela, 2009; Wootton & Smith, 2014).

1. Taula: Oogenesian zehar ematen diren gertaera nagusiak. n: kromosoma-kopurua, c: kromatida-kopurua.

Zelula germinalaren garapen fasea	Dotazio-kromosomikoa	Zatiketa-zelularra	Folikulua	Eraenketa
Oogonia	2n (2c)	Mitosia (oogonien proliferazioa)	1. folikulua	FSH, 17,20βP, E (folikuluen proliferazioa)
Perinukleolarra-Albeolo kortikala	2n (4c)	I. Meiosia (I. Profasea)		17,20βP (I. Meiosiaren hasiera)
Bitelogenikoa	n (2c)	I. Meiosia		FSH, E, StAr*, cyp19a1a* (Albeolo kortikalen agerpena)
Heldua		I. Meiosiaren amaiera, II. Meiosiaren hasiera (II. Metafasea)	2. folikulua	LH (*cyp19a1a*-ren azpi-adierazpena eta heltzea)
Arrautza (pelagikoak: hidratatua)	n (c)	II. Meiosiaren amaiera	Obulazioa	Akuaporinak* (hidratazioa), 17,20βP, azido arakidonikoa, prostaglandinak, proteasak* (heltzearen amaiera eta folikuluaren apurketa)

(*) Eraenketaren arduradun ez-hormonalak.

4. Erribogenesi-prozesua oozitoaren garapenean zehar

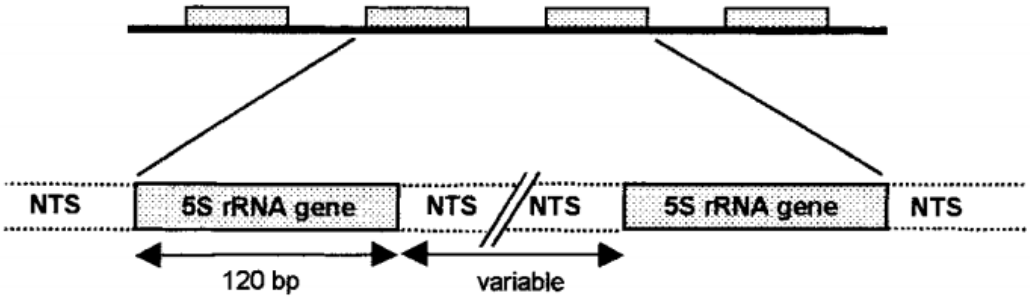
Zelula-hazkuntza eta ugaritzearen arteko uztarketa ezinbestekoa da organismo baten tamaina eta ehunen homeostasia mantentzeko. Zelula-hazkuntzaren ratioa proteinen sintesi-ratioaren eta beraz proteinak ekoizten dituzten erribosoma-kantitatearen araberakoa da (Donati et al., 2013). Erribosomen funtzioa proteinen ekoizpenean zehar lotura peptidikoen sorrerarako beharrezkoa den jarduera katalitikoa eskaintzeaz gain, mRNA-k (RNA mezulariak), tRNA-k (RNA garraiatzaileak) eta itzulpen-faktoreen uztarketarako ingurune fisiko egokia ziurtatzea ere bada (Szymański et al., 2003). Tamaina ezberdineko bi azpi-unitatez osatuta daude: azpi-unitate txikia edo eukariotoetan 40S jaulkipen koefizientea duen azpi-unitatea, 18S RNA molekula erribosomiko (rRNA) bakarraz eta 30 proteina erribosomiko (Rp) inguruz osatuta

dagoena. 60S azpi-unitatea handia aldiz, hiru rRNA-ek (28S, 5.8S eta 5S jaulkipen koefizientedunak) eta 49 Rp-ek osatzen dute (Lyman-Gingerich et al., 2007; Donati et al., 2013). Azpi-unitate txikia mRNA molekulen kodonak ezagutzeaz arduratzen den bitartean, azpi-unitate handiak lotura peptidikoa osatzearen funtzio katalitikoa betetzen du (Szymański et al., 2003). 5S rRNA izan ezik, bestelako rRNAk transkripto primario edo 45S pre-rRNA moduan sintetizatzen dira nukleoloan, rDNA gene bakar batetik abiatuta, zeinaren kopia ugari dauden animalien genomatan (Drygin et al., 2010). 45S pre-rRNA-ren sintesia I RNA Polimerasak (Pol I) bideratzen du Ubtf1 (Ingelesezko upstream binding transcription factor 1) deritzon transkripzio faktorearen kontrolpean. Ubtf1-ak rDNA promotore gunean tolestura bat sortzeko gaitasuna du, kromatinaren kondentsazioa ekidinez eta rDNA geneak aktibo mantenduz 45S pre-RNA-ren transkripzioa eman dadin (Bazzett-Jones et al., 1994; Reeder et al., 1995). Pre-rRNA honek prozesamendu eta moztitzazketa sekuentzialak pairatuko ditu nukleoloaren baitan rRNA helduak lortu ahal izateko. Heltze-prozesu honetan ere Rp-ak rRNA-ari batzen joango zaizkio 40S azpi-unitateko aitzindaria den 90S konplexu erribonukeoproteikoak (90S RNP) sortuz (Zhang et al., 2007; Henras et al., 2015).

Orokorrean goi-mailako eukariotoetan rRNA molekulen ekoizpenerako bi rDNA multigene-familia daude, 45S rDNA eta 5S rDNA. Gene hauek tandemetan banatzen dira eta bakoitzak ehundaka zein milaka kopia aurkezten dituzte genoman (Martins & Galetti, 2001). Anfibio eta teleosteoetan oogenesiaren hasieran 45S rDNA geneak anplifikatu egiten dira (Gall 1968; Brown & David, 1968; Vincent et al., 1969; Mazabraud et al., 1975), *Xenopus* oozitoetan 2*10^6 kopia izatera hel daitezkeelarik (Roger et al., 2002). Hala ere, anplifikatutako gene hauek inaktibo (Thomas 1970; Ford 1971; Mairy & Denis, 1971; Mazabraud et al., 1975) edo aktibitatea oso ahularekin mantentzen dira prebitelogenesian zehar, aktibitate maximoa bitelogenesian goiz aurkezten dutelarik (Roger et al., 2002).

5S rDNA-ren kasuan, sekuentzia kodetzailearen kopiak 120 bp-takoak dira eta 40 kDa-etako masa molekularra dute. Hauek transkribatzen ez direneko tarteko sekuentziez (NTS, ingelesezko non-trascribed sequence) banatzen dira (9. irudia). Eboluzioan gene hauek oso ondo kontserbatu diren bitartean NTS-ak azkar eboluzionatu dute, azterketa filogenetikoetan oso lagungarriak izanik espezie eta populazioen markatzaile gisa

(Gornung et al., 2007; Campo et al., 2009). 5S rRNA-k erribosomen gune funtzionalen arteko elkarrekintzen koordinazio eta eraentze prozesuetan paper garrantzitsua jokatzen duela ikusi den arren (Dokudovskaya et al., 1996; Bogdanov et al., 1995; Szymański et al., 2003), bere funtzio zehatzari dagokionez hutsune asko daude oraindik ere.



9. Irudia: Goi-mailako eukariotoen 5S rRNA geneen antolaketa. NTS (ingelesezko non-transcribed DNA segment) transkribatzen ez deneko DNA zatia (Martins & Galleti, 2001).

Anfibio eta arrainetan zelula somatikoetan eta oozitoetan era ezberdinean erregulatuta dauden bi 5S rDNA deskribatu dira (Komiya et al., 1986; Martins & Galleti, 2001). *Xenopus* igelaren genoman 5S rDNA somatikoak 400 kopia ditu genoma haploideko eta oozitoetako 5S rDNA-k 20.000 kopia (Peterson et al., 1980). Bi geneen arteko ezberdintasun nabariena barne-kontrol gunean (ICR, ingelesezko internal control region) kokatzen diren 5 bp-tan aurkitzen da, horien artean hiru transkripzioaren eragileen lotura gunean daudelarik (Song et al., 2005). 5S rDNA somatikoa zelula guztietan, oozitoak barne, eta garapen osoan zehar transkribatzen den bitartean (Wormington & Brown, 1983), oozitoetako 5S rDNA oozitoetan soilik da aktibo (Allison et al., 1995). Oozitoetan ekoizten den 5S rRNA somatikoa (oozitoetako 5S rRNA-rekin alderatuta oso maila baxuan agertzen dena) epe laburrean erabiliko den bitartean, oozitoetako 5S rRNA hilabeteetan metatu eta enbriogenesiaren lehenengo faseetan proteinen sintesian jardungo duten erribosomak ekoizteko erabiltzen dela proposatu da (Allison et al., 1995).

Bestelako rRNA-k ez bezala, 5S rRNA nukleolotik kanpo nukleoan transkribatzen da RNA Polimerasa III-ri (Pol III) esker (10. Irudia). Polimerasa honen jarduera eraentzeko IIIA transkripzio-faktore orokorra (Gtf3a) beharrezkoa da (Szymański et al., 2003). Gtf3a

bederatzi zink-besodun proteina bat da eta 5S rDNA-ren barne-kontrol guneari lotzen zaio. Horrez gain, 5S rRNA bera lotzeko gaitasuna ere badu, transkriptoaren garraio nukleo-zitoplasmatikoan eta geroko zitoplasmako metaketan lagunduz (Allison et al., 1995; Layat et al., 2013). Gtf3a-ren hatzamarrek taldeka jarduten dute, N-muturrean dauden hiru hatzamarrak DNA-rekin elkarrekiteko ezinbestekoak diren bitartean (Hanas et al., 1989; Liao et al., 1992), erdigunekoek 5S rDNA zein rRNA lotzeko gaitasuna aurkezten dute (Ogilvie & Hanas, 1997). *Xenopus* igelean *gtf3a* genearen promotorearen erabilera diferentzialaz bi transkripto ezberdin ekoizten dira zeinak halaber, proteinaren bi isoforma ezberdin ematen dituzten. 38 kDa-eko masa molekularrarekin proteinarik txikiena oozitoetan bakarrik azaltzen da eta Gtf3a oozitikoa deritzo. Bestea 40 kDa-eko pisu molekularrarekin, zelula somatiko guztietan eta baita zelula germinal mota bietan azaltzen da eta Gtf3a somatikoa deritzo (Layat et al., 2013). Oozito prebitelogenikoetan 38 kDa-ko Gtf3a kantitate altuan topatzen den bitartean, bitelogenikoetan bere kontzentrazioa jaisten da. 40 kDa-ko Gtf3a-ren kontzentrazioa bere aldetik, oogenesiak aurrera egin ahala gora egiten du. Proposatu izan da 40 kDa-etako Gtf3a-k 5S rRNA gene somatikoaren transkripzioa aktibatu eta 5S rRNA gene obarikoaren transkripzioa inhibi dezakeela enbriogenesian eta zelula somatikoetan (Blanco et al., 1989; Ghose et al., 2004; Malik et al., 2004). Arrainetan soilik *Ictalurus punctatus* siluriformean karakterizatu da Gtf3a, zeinetan 5S rRNA-rekin elkartuta agertzen den obarioetan (Ogilvie & Hanas, 1997). 38 kDa-eko Gtf3a proteinaren aminoazido-sekuentzia soilik %40-an *Xenopus*-aren oozitoko Gtf3a-ren berdina den arren, igelaren 5S rDNA lotzeko gai da. Gainera, siluroaren Gtf3a-n, anfibioetan oozitoetako Gtf3a proteinan soilik agertzen den sekuentzia kontserbatua aurki daiteke, hau da hasierako metioninarekin batera doan GER/K sekuentzia (Ogilvie & Hanas, 1997). Aldiz, 5S rDNA loturarako beharrezkoa den bederatzigarren zink besoaren aldameneko KRSLAS sekuentzia ez da siluroaren Gtf3a proteinan agertzen (Ogilvie & Hanas, 1997).

5S rRNA-ren transkripziorako hala ere, Gtf3a-z gain beste bi transkripzio faktore orokor, Gtf3b eta Gtf3c, behar dira (Wolffe, 1994). Prozesuan, Gtf3c-k Gtf3a-5S rDNA konplexua ezagutzen du. Orduan, Gtf3c-ek, Gtf3b errekrutatzen du eta azken faktore hau izango da hain zuzen ere Pol III-k ezagutuko duen proteina (Bieker et al., 1985; Setzer & Brown, 1985; Kassavetis et al., 1990). Eukariotoetan 5S rRNA-ren sintesia Gtf3a-k rDNA-ren

barne-kontrol gunearekin sortzen duen loturaren menpekoa da (Szymański et al., 2003) eta RNA tartekarien bitartez *gtf3a* isilaraztean 5S rRNA-ren sorrera eta erribosomen 60S azpi-unitatearen ekoizpena eten egiten dira (Donati et al., 2013).

Behin 5S rRNA sintetizatzen delarik, Gtf3a-k 5S rRNA lotu eta nukleotik zitosplasmara garraiatzen du. Bien elkarketak sortutako partikula 45 kDa-eko pisu molukarra gainditzen duenez, mintz-nuklearraren zeharkaketa garraio aktiboaren bidez burutzen da karioferinak deritzen proteinak erabiliz (Gen et al., 2008). Karioferinen taldean nukleotik molekula handien irteeran espezializatuak dauden proteinak edo exportinak, eta molekulen sarrerarako espezializatutakoak edo inportinak aurki ditzakegu (Gen et al., 2008). Behin zitoplasman, zelula somatikoetan 5S rRNA zuzenean L5 proteina erribosomikoari (RpL5) lotzen zaio, 5S RNP partikulak osatuz. 5S RNP partikulak orduan nukleora sartzen dira pre-erribosometan elkartzeko (10. Irudia, Zhang et al., 2007).

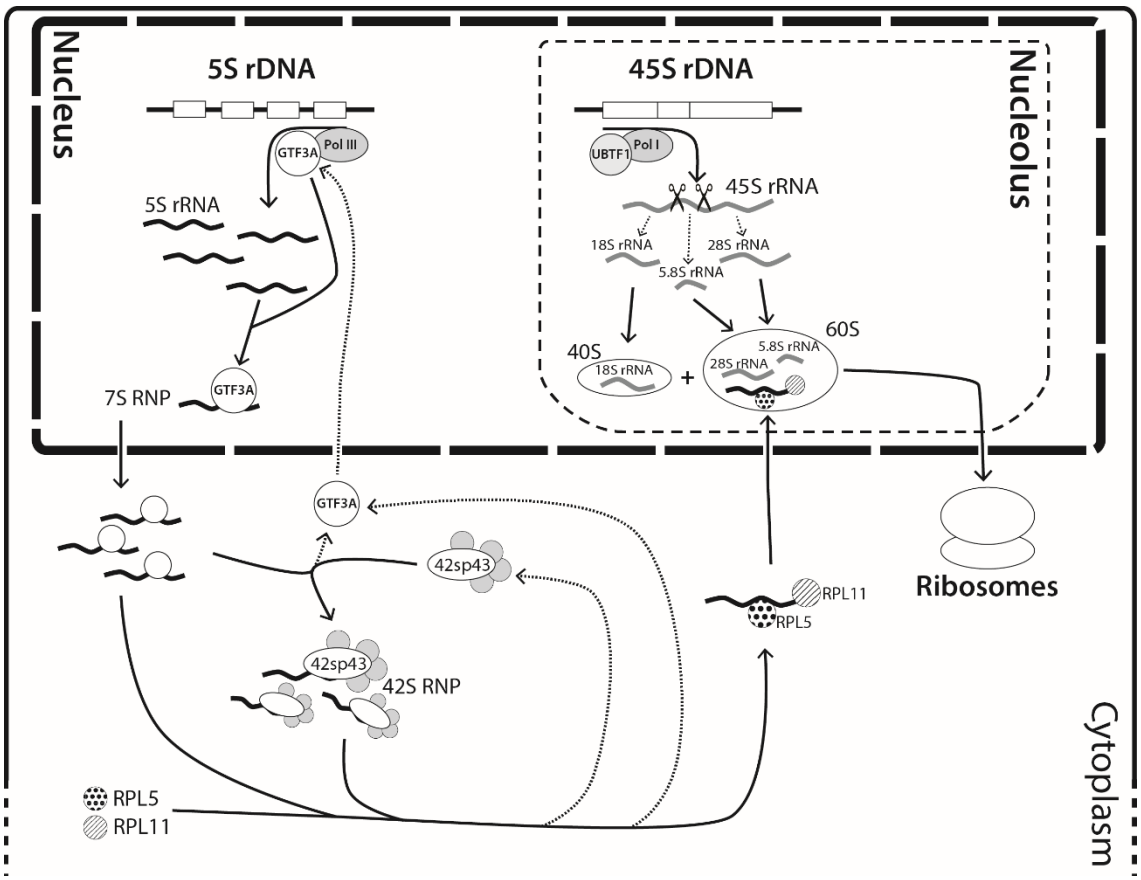
Xenopus igelaren oogenesiaren hasierako faseetan proteinen sintesia oso baxua dela ikusi da (Roger et al., 2002). Pol III-ren jarduera aldiz maximoa da oozito prebitelogenikoetan, 5S rRNA-ren sintesia bultzatuz. Igeletan bezala *Chelon labrosus* korrokoien obarioan 5S rRNA-ren mailak oso altuak direla frogatu da (Diaz de Cerio et al., 2012). Gauza bera ikusi da, tenka (*Tinca tinca*) arrain teleosteoaren eta katuarrain (*Scyliorhinus caniculus*) arrain kondriktioaren oozito prebitelogenikoetan (Denis & Wegnez, 1977; Wegnez et al., 1978). Baita errutilo kontinentalaren (*Rutilus rutilus*) obarioetan ere, non oozitoen hazkuntzaren hasieran eta albeolo kortikalen faserarte RNA molekula txikien kantitate oso altuak dauden. Oogenesiaren fase goiztiar hauetan oozitoen zitoplasman metatzen diren transkripto gehienek tRNA-k eta 5S rRNA dira. Ondoren, bitelogenesiaren hasierako faseetan kantitate baxuetan dauden 18S eta 28S rRNA-ren proportzioa igo egiten da (Kroupova et al., 2011).

Beraz, anuroen eta arrainen oogenesian 5S rRNA erribosomen bestelako konposatuak baino lehenago sintetizatzen da eta zitoplasman denbora luzez gorde egiten da. Horretarako, 5S rRNA, nukleotik irten eta gero, Gtf3a-ri loturik mantentzen da oozito prebitelogenikoen zitoplasman 7S partikula erribonukleoproteikoa (7S RNP) eran (10. Irudia). Zitoplasman, Gtf3a-k 5S rRNA hidrolisitik babestu egiten du (Szymański et al., 2003). 7S RNP-az gain anfibio zein arrainen oozitoetan 5S rRNA 42S RNP partikulak

eratuz ere metatzen da, tRNA, 42Sp50 eta 42Sp43 proteinekin batera. 42Sp43, Gtf3a-k bezala bederatzi beso ditu 5S rRNA lotzeko baina kasu honetan proteinak ez du rDNA lotzeko gaitasunik eta beraz ez du 5S rRNA-ren transkripzioan parte hartzen (Szymański et al., 2003). *Xenopus* igelaren oozitoetan dagoen 5S rRNA-ren erdia gutxi-gora-behera 42S RNP gisa topa daiteke, beste erdia 7S RNP gisa mantenduko delarik (Picard & Wegnez; 1979; Picard et al., 1980; Guddat et al., 1990; Viel et al., 1991; Szymański et al., 2003; Zhang et al., 2007). Gtf3a proteina eta mRNA-mailak anfibio zelula somatikoetan oso baxuak diren bitartean (Layat et al., 2013) oozitoetan *gtf3a* mRNA-mailak 1 milioi aldiz altuagoak dira (Penberthy et al., 2003; Ciganda & Williams, 2011), 5S rRNA-rekin mantendu behar duten erlazio estekiometrikoa dela eta. Modu berean, medakan bai *42sp50* zein *42sp43* geneen transkripzioak obario-espezifikoak direla ikusi da (Kanamori, 2000; Kinoshita et al., 2009). *42sp43*-ren transkripto-mailak altuak dira anfibio eta arrain teleosteoen oozitoetan, batez ere prebitelogenesian (Allison et al., 1995; Penberthy et al., 2003; Szymański et al., 2003; Layat et al., 2013) eta bai *gtf3a* zein *42sp43-maila*k obarioetan 5S rRNA-ren mailak islatzen dituzte (Penberthy et al., 2003; Diaz de Cerio et al., 2012).

Bitelogenesiaren hasierarekin eta bestelako rRNA-en ekoizpena maximoa denean, 5S rRNA zitoplasmako metaketa RNP partikula hauetatik askatzen da eta RpL5-ri lotuta 5S RNP gisa nukleora bideratuko da (10. Irudia), nukleoloan erribosomen azpi-unitate handiaren muntaian parte hartzeko (Zhang et al., 2007). 5S RNP-ren sorreraren ondorioz Gtf3a askatu eta nukleora bueltatuko da, inportinen bidez mintz-nuklearra zeharkatuz, 5S rRNA gehiagoren sintesia bultzatzeko (Szymański et al., 2003). Bitelogenesian zehar sintetizatzen den 5S rRNA berria zuzenean RpL5-ri lotu eta nukleora bideratzen dela ikusi da (Allison et al., 1995). RpL5-ak beraz Gtf3a-ren eskuragarritasunean ere dihardu (Layat et al., 2013). Ernalketa ostean enbrioi berriaren sorrerarekin batera oogenesian aktibo dagoen oozitoetako 5S rDNA isilarazten da. Orduan H1 histona transkribatzen hasten da (Wolffe, 1994; Panetta et al., 1998; Crane-Robinson, 1999). H1-ek oozitoko 5S rDNA-Gtf3a lotura ekiditen duen nukleosoma eratzen du, anuroetan behintzat. H1-ek ere 5S rDNA somatikoa lotzen duen arren, kasu honetan sortzen diren nukleosomak ez dituzte 5S rDNA-ren Gtf3a loturarako ezinbestekoak diren elementuak ukitzen eta promotorea

Gtf3a-rekin lotzeko eskuragarri mantentzen da (Bouvet et al., 1994; Panetta et al., 1999; Sera & Wolffe, 1998).



10. Irudia: Erribogenesi prosezua oozitoetan (Szymański et al., 2003-tik eraldatua).

5. Inguruneko hormona kutsatzaileak eta intersex egoera arrainetan

Ingurune urtarretan bizi diren organismoek gisa-jatorria duten hainbat konposatuen nahaste konplexuen eraginpean egoteko arriskua daukate (Boehm et al., 2011; DellaSala et al., 2013). Ingurune itsastarrean topa daitezkeen kutsatzaileen artean disruptore endokrinoak diren gai kimikoak edo EDC-ak (ingelesezko endrocrine disrupting chemicals) arreta handia bereganatu dute, Europako Ingurumen Agentziak lehenengoz aipatu eta ur-medioan duten ubikuotasuna iragarri zituenetik (EEA, 1997; Teijón et al., 2010; Murray et al., 2010; Cabeza et al., 2012; Gavrilescu et al., 2015).

Definizioz, EDC-ak organismo baten sistema hormonalaren funtzionamendu normala eraldatzeko gaitasuna duten kutsatzaile kimikoak dira (EEA, 2012). Konposatu hauek

antolaketa-maila biologiko ezberdinetan eragin dezakete, maila molekularretik hasita, banako mailatik jarraituz eta (azpi)populazio mailan ere eragin dezaketelarik (Brander, 2013; WHO/UNEP, 2013). EDC terminoak jatorri eta konposizio kimiko ezberdinak dituzten substantziak barneratzen ditu. Horien artean hormona naturalak eta sintetikoak, garbigarri industrialak, nekazaritzan erabiltzen diren pestizidak, fungizidak, alkilfenolak, substantzia poliklorinatuak, hidrokarburo aromatiko poliziklikoak, ftalato plastifikatzaileak, dioxinak, agente surfaktanteak, bromatutako gar atzekariak, perfluorokimikoak, musketa kimikoak, botikak, kosmetikoak, konposatu industrialak eta bestelako konposatuen apurketaz sortzen diren subproduktuak daude (Tyler et al., 1998; López de alda & Barceló, 2001; Porte et al., 2006; Casals-Casas & Devergne, 2011; Khetan, 2014).

EDC-ak ingurune urtarrera batez ere industriaren, nekazaritza eta abeltzaintza intentsiboaren isurketen ondorioz heltzen dira, askotan ospitale eta udal-hondakinen prozesamenduaz arduratzen diren ur-araztegien isurketetan agertzen direlarik ere (Campbell et al., 2006; Leet et al., 2011). Nahiz eta konposatu guzti hauek kontzentrazio baxuetan aurkitu ur-medioan, isurketa ezberdinetan mota askotako EDC-ak askatzen dira, isuritako substantziak nahaste kimiko konplexuetan agertzen direlarik. Honek eragin gehigarriak edo sinergikoak sortaraz ditzake biotarengan (Thorpe et al., 2003). Araztegietan EDC-ak ez dira guztiz ezabatzen beraien natura eta jatorri heterogenoa dela eta, eta askotan ur-mediora forma aktiboan askatzen dira. Modu honetan 90. hamarkadaz geroztik, araztegiek dituzten isurtze-puntu zehatzen inguruan dauden arrain-populazioengan eragin biologiko desberdinak ikusi izan dira, hauetatik eragin estrogeniko eta feminizatzaileak deigarrienak izanik (Sumpter & Jobling, 1995; Jobling et al., 1998; Puy-Azurmendi et al., 2013; Bahamonde et al., 2015; Valencia et al., 2016).

EDC-en artean konposatu estrogenikoak aurki daitezke. Estrogeno naturalen artean animalietatik datozen estrogenoak, onddoetatik datozen mikoestrogenoak eta landare jatorria duten fitoestrogenoak daude (WHO/UNEP 2012). Estrogeno sintetikoei aldiz xenoestrogeno deritzaie eta animalia ornodunen emeetan ugalketarako garrantzitsuena den hormona, estrogenoa, edo bere eragina, imitatzeko gai diren konposatuak dira. Batzuk, zuzenean estrogeno-hartzaileak lotzeko eta estrogenoen menpeko geneak aktibatzeko gai dira (WHO/UNEP, 2012). Beste batzuk, barne estrogenoen sintesia,

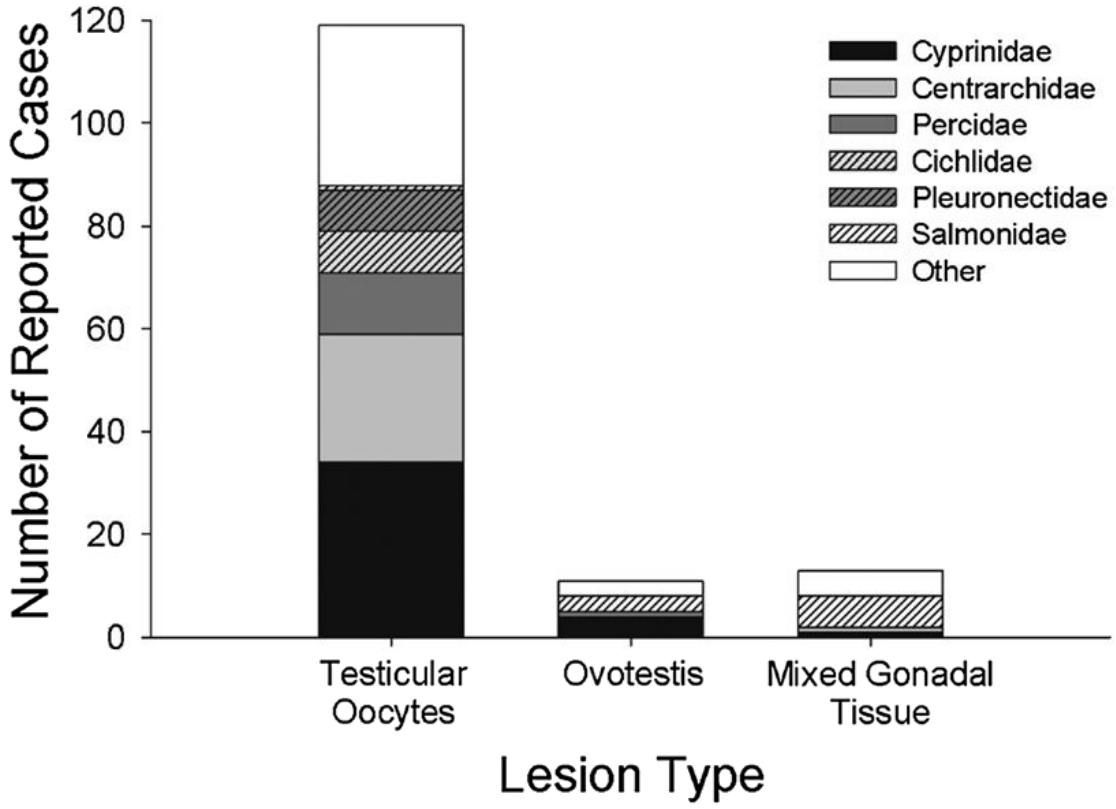
metaketa, askapena, garraioa, eragina edota metabolismoan eragin dezakete (Goksøyr et al., 2003; Mills & Chichester, 2005; WHO/UNEP, 2012).

Arrainetan, eta aurretik aipatu dugunez, sexu-determinazioa eta -desberdintzapena oso fenomeno malguak dira, faktore genetiko eta ingurune-faktoreen menpe daudenak (Devlin & Nagahama, 2002; Martínez et al., 2014). Janari-eskuragarritasuna, oxigeno-maila eta tenperaturaz aparte ur-medioan dauden kutsatzaileek ere, xenoestrogenoak kasu, arrainen sexu-determinazio eta -desberdintzapenean eragin dezakete esposizioa garapenaren garai kritikoetan ematen denean batez ere (Piferrer et al., 2001; Leet et al., 2011; Segner et al., 2013). Xenoestrogenoak, euren izaera lipofilikoari esker erraz biometatzen diren konposatu iraunkorrak dira (Langston et al., 2005; Porte et al., 2006) eta ugalketan eta banakoen/populazioen garapenean eragiten dute (Tyler et al., 1998; Goksøyr et al., 2003; Mills et al., 2005; Aris et al., 2014).

Intersex egoera, espezie gonokoristikoen testikuluetan oozitoak agertzeari deritzo eta nahiz eta hein txiki batean fenomeno naturala izan daitekeen, xenoestrogenoen eraginpean dauden organismoetan ezagutzen den alteraziorik deigarrienetarikoa da (Bahamonde et al., 2013; Bizarro et al., 2014). Intersex egoera larritasun-maila ezberdinetan gerta daiteke, kasu larrienetan sexu-eraldaketa osoa, feminizazio totala, gerta daitekeelarik (Devlin & Nagahama, 2002; Abdel-Moneim et al., 2015). Honekin batera, xenoestrogenoen eraginpean dauden arrainetan gibeleko *vtg* mRNA-maila eta plasmako VTG proteina-maila altuak topatu dira ar eta heldugabeetan, feminizazio molekular nabaria iradokiz (Bizarro et al., 2014). Intersex banakoek halaber, esperma-bolumena eta espermatozoide-kantitate baxuagoak ekoizten dituzte, euren ugaltzeko gaitasuna txikituz, honek, populazioen ugaltze-arrakasta eta bideragarritasuna arriskuan jar dezakeelarik (Jobling et al., 2002; Jobling & Tyler, 2006; Kidd et al., 2007; Harris et al., 2011).

Abdel-Moneim eta kideek (2015) 54 arrain espezieetan intersex egoera deskribatzen zuten 97 lan aurkeztu zituzten. Horietatik gehienak arrain-espezie teleosteoetan deskribatutakoak izan ziren. Aztertutako lan gehienak 1999-tik aurrera publikatuak ziren eta intersex egoeraren baitan beraiek, testikuluen barneko oozitoak (oozitoak dituzten testikuluak), obotestiak (testikulua ehuna duten obarioak, normalean androgeno edo

androgeno funtzioa duten substantzien eraginpean agertzen direnak) eta nahasturiko gonada (testikulua eta obario ehunak banatuta agertzen direnean) bereizten zituzten, lehenengoa egoera hedatuena izanik (11.irudia).

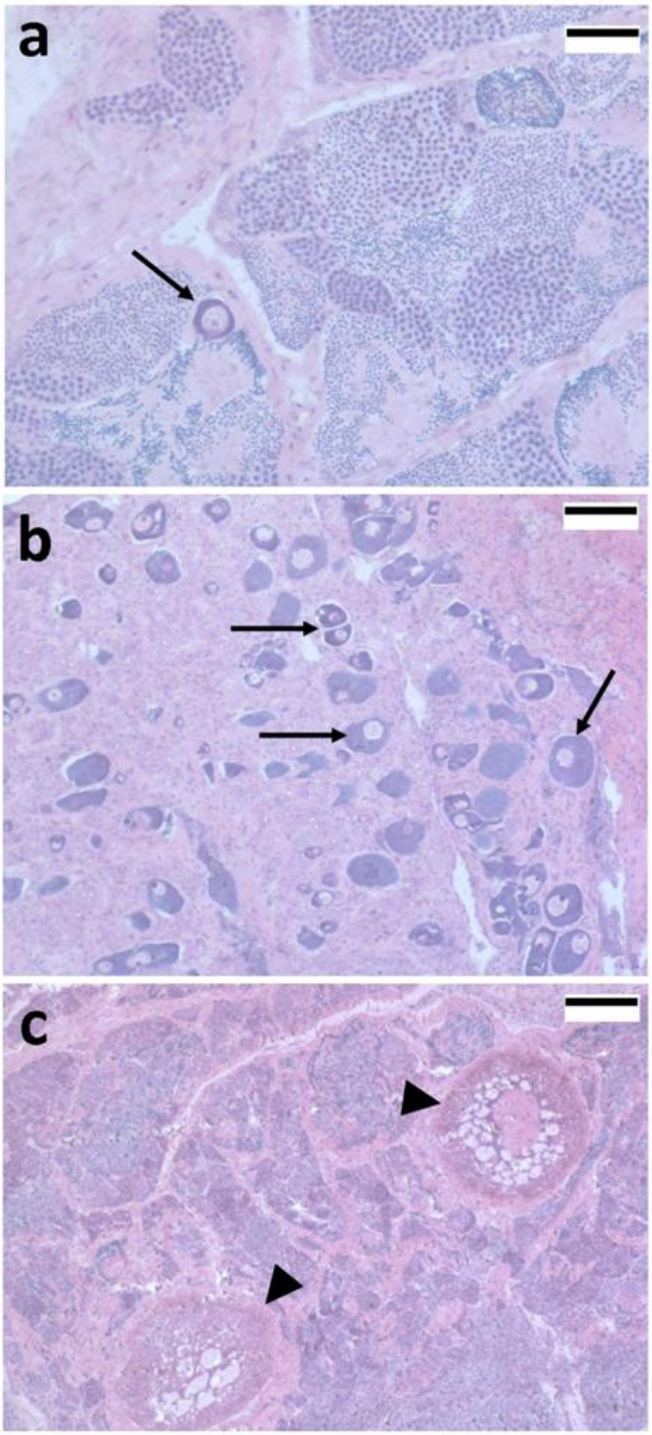


11. Irudia: Abdel-Moneim eta kideek (2015) arrainetan berrikusitako gonadaren garapenean gerta daitezkeen desorekak. Irudian intersex egoerarekin erlazionatutako "testicular oocytes" edo testikuluan oozitoen agerpena, ugariena da.

Intersex banakoak kutsatutako ur geza zein itsas ur guneetan bizi diren arrain espezietan aurkitu dira. Itsas arrainetan eragin xenoestrogenikoen presentzia aztertzen zuen lehenengo lanetariko bat 1990. urtean publikatu zen. Bertan, Erresuma Batuetako itsasadar industrializatuetan bizi zireneko platuxa latza (*Platichthys flesus*) arrek plasmako VTG-maila altuak eta intersex egoera aurkeztu zituzten (Matthiessen et al., 2002). Feminizatutako arrain hauek xenoestrogenoak isurtzen zituzten araztegien ondoko uretan bizi ziren. Geroztik, asko dira leku ezberdinetako itsasadar eta kostaldeko arrainetan xenoestrogenoen eraginak deskribatu dituzten lanak. Hala nola, Herbehereetan (Vethaak et al., 2002 eta 2005), Estatu Batuetan (Mills et al., 2003; Roy

et al., 2003) edota Japonian (Hashimoto et al., 2000; Ohkubo et al., 2003). Horietaz gain itsaso zabaleko arrainetan ere feminizazio efektuak ikusi dira, besteak beste, Mediterraneo Itsasoko eta Hegoafrikako ezpata-arrainean (*Xiphias gladius*) (De Metrio et al., 2003; Desantis et al., 2005), hegalaburrean (*Thunnus thynnus*), lanpo bularpikartan (*Euthynnus alletteratus*), Mediterraneo Itsasoko lohitako barbarinean (*Mullus barbatus*) (Fossi et al., 2002; Martin-Skilton et al., 2006; Macias et al., 2014) eta Ipar-ekialdeko Ozeano Atlantikoko bakailaoan (*Gadus morhua*) (Scott et al., 2006). Adibide guzti hauek xenoestrogenoen mundu mailako banaketa iradokitzen dute (intersex adibide gehiago ikusteko jo Bahamonde et al., 2013, 1.taulara eta Abdel-Monein et al., 2015, S1. taulara).

Euskal Herrian antzemandako lehenengo efektu xenoestrogenikoak Gernikako araztegiaren inguruneko *Chelon labrosus* korrokoi-populazioan deskribatu ziren. Bertan intersex arrak aurkitu dira 2007. urtez geroztik (Puy-Azurmendi et al., 2013; Valencia et al., 2016). Intersex egoeraz gain arrek *vtg*-maila altuak eta behazunean alkilfenol kontzentrazio altuak ere badituztela ikusi da (Puy-Azurmendi et al., 2013). Euskal kostaldean bestelako guneetan ere eragin xenoestrogenikoa duten kutsatzaileen presentzia aurkitu izan da ur-analisien bitartez (Bizarro et al., 2014), hala nola, Pasaian, Deban, Ondarroan eta Santurtzin. Kutsatzaile hauen eraginpean intersex egoera garatu duten korrokoi arrak aurkitu dira. Intersex prebalentziak, Euskal kostaldean aztertutako leku eta sasoi bakoitzean arrantzatutako ar guztien gainean kalkulatuak, %20-tik %50-ra bitartekoak izan dira eta intersex guztiek testikuluan barreiaturiko oozito prebitelogenikoak aurkeztu dituzte (12. Irudia). Oso kasu bakanetan ikusi izan dira oozito bitelogenikoak korrokoien testikuluetan. Kutsatutako gune horietan intersex egoera garatu ez duten arren artean ere *vtg* eta *cyp19a1b* geneen transkripzio-maila altuak, baina oso aldakorrak, ikusi dira (Bizarro et al., 2014).



12. Irudia. Euskal kostaldean harrapatutako korrokoi intersexak. Orokorrean folikulu espermatikoan barreiatuak agertzen diren oozitoak egoera prebitelogenikoan ageri dira (→) (a eta b). Oso arraroa da euskal kostaldeko korrokoi intersexetan c irudian agertzen den bezalako oozito bitelogenikoak (►) ikustea. Eskala-barrak dira (a) 50 µm, (b) 100 µm eta (c) 200 µm.

Intersex egoera beraz, konposatu xenoestrogenikoen eraginaren biomarkatzaile moduan erabiltzen da. Biomarkatzaileak epe luzera osasunean eman daitezkeen eraginen epe goiztiarreko adierazleak dira (Cajaraville et al., 2000). Tresna baliagarriak dira kutsatzaile

batek organismo batean izan ditzakeen eragin biologikoak ebaluatzeko, ingurumeneko kalitate-azterketak egitea posible bihurtzen delarik (Cajaraville et al., 200; Bizarro et al., 2014). Horrela, organismo bat kutsatzaile baten eraginpean egon ote den eta horren aurrean erantzuten ari denaren inguruko informazioa ematen dute. Biomarkatzaileekin aztertzen diren erantzun biologikoak molekula-, zelula- zein ehun-mailakoak izan daitezke, antolaketa-maila biologiko altuagoetan (fisiologia, organismo, populazio mailan) gerta daitezkeen eragin posibleei buruzko abisu goiztiarrak ematen dituztelarik.

Intersex egoeraren eta gonadaren morfologiaren azterketa histologikoarekin batera, hormonekiko esposizioaren ondorioen bestelako adierazleak ere erabil daitezke, hala nola, ezaugarri sexualen eraldaketa, indize gonadosomatikoa (GSI) eta plasma zein gibeleko bitelogenina mailen aldaketa (Seki et al., 2006). GSI arrainen ugalketaren osasun-egoeraren adierazle erabilienetariko bat da. GSI garapen sexuala, ugal-estrategia, ugalketa-fasea eta osasun orokorraren egoeraren isla da, gonadaren garapenaren eta mantentzearen egoera adierazten baititu (Barrett & Munkittrick, 2010). Orokorrean, ez da desberdintasunik ikusten GSI-mailari dagokionez, xenoestrogenoen eraginpean dauden arrain ar normal eta intersex banakoen artean. Biek testikuluaren tamaina maila berean murriztua dute esposizioaren ondorioz. Honek iradokitzen du gonadaren garapenean ematen diren aldaketak kutsatzailearen presentziari dagozkiola eta ez intersex egoera berari eta beraz, ezin da intersex egoera identifikatzeko biomarkatzaile moduan erabili (Bahamonde et al., 2013), bai ordea xenoestrogenoen presentziaren adierazle gisa.

Barne E mailen desorekak EDC esposizioaren isla izan daiteke (Bahamonde et al., 2013), E-mailak baitira, besteak beste bitelogenesi-prozesua kontrolatzen dutenak (Anderson et al., 1996). *vtg*-mailak arrain emeetan arretan baino askoz altuagoak dira, horrexegatik *vtg*-ren adierazpena arretan, EDC-ek eragin ditzaketen ugalketa mailako desorekak aztertzeko egokia da (Jobling et al., 1998; Routledge et al., 1998; Rotchell & Ostrander, 2003; Maltret-Geraudie et al., 2008), ingurumen-kalitatearen azterketarako programatan neurtu ohi delarik xenoestrogenoen eraginaren biomarkatzaile gisa (An et al., 2011; Puy-Azurmendi et al., 2013; Bizarro et al., 2014). Vtg eta *vtg*-maila altuak arretan eta intersexetan, kutsatzaile xenoestrogenikoen presentziarekin erlazionatuta dauden arren, orain arte ez da adierazpen-maila eta intersex egoeraren arteko

korrelaziorik ikusi (Hiramatsu et al., 2006; Bahamonde et al., 2013; Bizarro et al., 2014). Horiekin batera, koriogeninak, ere arrainen gibelean estrogenoei erantzunda sintetizatzen diren arrautza estalkiaren (korionaren) aitzindariak, ere EDC-en eraginaren ondorioz arren gibelean gain-adierazten direla ikusi da arrain ar eta intersexetan (Rhee et al., 2009; Yamaguchi et al., 2015) biomarkatzaile moduan plazaraziz. Bai Vtg-maila altuak zein koriogeninen adierazpena emeetan bitelogenesi fasean ematen dira eta ez prebitelogenesian, zeina arrain espezie gehienetan oogenesiaren faserik luzeena baita. Honek, markatzaile hauen neurketaren erabilgarritasuna mugatzen du, ugal-zikloan zehar momentu zehatzetan, hormonen barne dinamikari lotuta, adierazten baitira. Beraz, eta arrain-espezie desberdinetan intersex egoera aztertu dituzten hainbat lan berrikusi eta gero, argi dago gaur egun ez dagoela intersex egoeraren biomarkatzaile molekular egoki eta goiztiarrik biomarkatzaile hauek garatzea ezinbestekoa delarik (Bahamonde et al., 2013).

Hori dela eta, azken urteotan markatzaile tradizionaletatik haratago doazen geneak aztertu dira (2. Taula). Aztertutako gene hauek, sexu-determinazio, -desberdintzapen eta gametogenesian parte hartzen dute eta hori dela eta sexu-bereizketaren eta -eraldaketaren, naturala zein eragindakoa (hermafroditismoa/intersex), adierazle molekular gisa proposatu dira. Sekuentziazio paralelo masiboaren teknikek bestalde, transkripzio-mailetan ematen den dikotomia sexuala sakonki ikertu ahal izatea baimendu dute, gonadak edota gibela ez diren bestelako organoetan ere. Azterketa hauek burutzeko espezie hermafrodito proterandrikoak erabilgarriak izan daiteke, zeinak emerako sexu aldaketan intersex egoeran dagoen testikulua erakusten duten. Casas eta kideek (2016) pailazo arrain proterandrikoan egindako RNA-sekuentziazioan (RNA-seq) ar, eme eta sexu eraldaketa pairatzen ari diren banakoen burmuin zein gonadatan ezberdin adierazten diren geneak aztertu zituzten. Horien artean burmuinean adierazten diren sexuaren araberako hainbat gene berri identifikatu zituzten, hala nola, sexu eraldaketarekin batera isilarazten diren geneak; aurretik aipatutako *sox6* eta *foxp4*, *tenm2* (teneurin-2) eta *hdgfrp2* (hepatoma-derived growth-factor-related protein 2). Azken hau ere *Eretmodus cyanostictus* espeziean arren burmuinean gain-adierazita dago emeekin alderatuta (Bohne et al., 2014). Pailazo arrainean arretik emerako sexu-eraldaketarekin gain-adierazten diren geneei dagokienez, *adamts16* (metalloproteinase

with thrombospondin motifs 16), *phactr4a* (phosphatase and actin regulator 4A) eta *f13a* (coagulation factor XIII A chain-like) identifikatu zituzten. Azken hau ere guppy arrain emeen burmuinean gain-adierazten dela ikusi da (Gao et al., 2007). GnRH, Avt (arginine-vasotocin) eta Kiss *Amphiprion* espezieen hipotalamoan arretik emerako aldaketan gain-adierazi egiten direla ikusi da ere (Yaeger et al., 2014; Kim et al., 2014). Guzti hauek ere xenoestrogenizitatearen, eta agian intersex egoeraren, markatzaileak izan daitezke burmuinean.

Gonaden kasuan sexuen artean era desberdinean adierazten diren geneei dagokienez (2. taula), pailazo arrainetan esteroidogenesiarekin erlazionatutako geneak; *cyp19a1a*, *foxl2, star* eta *hsd17b* emeetan gain-adierazita agertzen diren bitartean, espermatogenesiarekin erlazionatutakoak (*sox8*, *dmrt1* eta *amh*) arretan gain-adierazita agertzen dira (Casas et al., 2016). Sexu-aldaketarekin transkripzio-patroia erabat aldatzen da. Modu honetan, asko dira gene hauen adierazpenaren aldaketa ikertu dituzten lanak. Izan ere, *sox9*, *amh*, *dmrt1 eta foxl2*-ren adierazpenean barne hormona-mailen menpe egoteaz gain, kanpo hormonen eraginpean ere aurkitzen direla ikusi da. Estrogenoetara esposatutako arrain arretan, obarioaren garapenarekin erlazionatutako geneen gain-adierazpena, eta hodi seminiferoen sorrera eta espermatogenesiarekin erlazionaturikoen azpi-adierazpena ikusi da. Horien artean, estrogenoen aurrean *foxl2* ostadar amuarrainean eta tilapian arretan gain-adierazten dela ikusi da (Baron et al., 2004; Guigen et al., 2010). Gainera, etinil-estradiolaren esposaketaren ondorioz *amh eta dmrt1* geneen transkripzio-mailak jaisten direla ikusi da zebra arrainetan (Schulz et al., 2007). Berdina ikusi da estrogenoekin trataturiko XY medaka arretan (Kobayashi et al., 2008), tilapian (Guiguen et al., 2010) eta ostadar amuarrainean (Marchanc et al., 2000). Aldiz, arrainak androgenoekin edo aromatasa inhibitzaileekin tratatzean, *foxl2, cyp19a1a* eta *fst* geneen azpi-adierazpena ikusi da ostadar amuarrainean (Vizziano et al., 2007). *Epirephelus mera*, tilapian eta medaka emeetan, androgeno eta aromatasa inhibitzaileen tratamenduek *dmrt1* adierazpena kitzikatu eta *foxl2* eta *cyp19a1a*-ena moteltzen dituzte (Alam et al., 2008; Kobayashi et al., 2008; Guigen et al., 2010). Baron eta kideek (2008) adibidez, ostadar amuarrainak androgenoetara esposatu ostean sexu-desberdintzapenarekin erlazionaturiko 103 geneen adierazpen aztertu zituzten. Horien artean, granulosa zeluletan adierazten diren geneak (*cyp19a1a, foxl2, fst*), I.-

meiosiarekin erlazionatutako geneak eta prebitelogenesiarekin erlazionatutakoak azpi-adierazita zeudela ikusi zuten, eta aldiz *dmrt1, sox9 eta dax1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) gain-adierazita (Baron et al., 2008). Tenperaturaren aldaketek ere, maskulinizazioarekin batik bat, baina baita feminizazioarekin erlazionaturiko geneen adierazpenean aldaketak eragiten dituela ikusi izan da (Shen & Wang, 2014).

Aipatutako guzti horietaz aparte, korrokoien gonadetan gainera oozitoen presentzia adieraz dezaketen zenbait markatzaile molekular berri aztertu dira ere, oozito-markatzaile posible gisa intersex egoeraren markatzaile espezifikoak izan daitezkeelakoan. Markatzaile hauek 5S rRNA eta bere proteina laguntzaileak kodetzen dituzten geneak dira, hala nola, *gtf3a*, *42sp43* eta *inportina α1* eta *α2* (Diaz de Cerio et al., 2012). Erribogenesiari loturiko gene guzti hauen transkripzio-mailek, beti ere neurketa berean arrak vs. emeak alderatuta, Pasaiako portuan (zeinak jatorri industriala duten kutsatzaileak aurkezten dituen) urte osoan zehar lagindutako korrokoi ar eta emeen artean bereiztea ahalbidetzeaz gain, intersex banakoen testikuluak bereiztea ere ahalbidetzen dute, beraietan ematen den adierazpen altua dela eta (Diaz de Cerio et al., 2012).

2.Taula: Arrain espezi ezberdinetan sexu-desberdintzapenaren markatzaileak izan daitezkeen geneen laburpen-taula.

PGC	**Sertoli zelulak**	**Leydig zelulak**	**Teka edota granulosa zelulak**	**Espermatogonio, esperma...**	**Oozitoak**
vasa, piwil1 (♂), piwil2, nanos1 (♀), nanos2, dnd, wt1a, dazl, buc (♀), cxcr4b, sdf-1α receptor, vg1-rbp(♀) , foxh1 (♀)	*amh, sox9a, dmrt1, ar, ff1d*	*cyp11b, hsd3b2, cyp17a, star, nr5a1*	*cyp19a1a, foxl2a, foxl2b, fst, fshb, bmp4, figα, lhr, fshr, star, P450scc, 3β-hsd, cyp17, 20β-hsd*	*gsdf, protamines, sycp3l*	*sox9b, zp1, zp2, zp3, vgr, 5S rRNA, tfIIIa, 42sp43, 42sp50, impα1, impα2, zar1, mos, gdf9, bmp15, btg3, lcal, lrham, alv, mPR*
Testikuluak			**Obarioa**		
ccng2, hsp70, sept4, tuba7, spermatogenesis associated 4, testis-specific A-kinase anchoring protein, tektin 1, igf1, nr0b1			*tpte, rbpms2, cx44.2, sox11b, ccnb2, activin β, tbp, sox3, fem1c*		

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GAIAREN EGOERA, HIPOTESIA ETA HELBURUAK

Ingurumen faktore desberdinek arrainen sexua zehazten parte har dezakete, askotan determinazio genetikoaren gainetik gailenduz. Honek, arrain populazioen sexu banaketan eragiteko aukera zabaltzen du, nahita, zenbait akuikultura jardueretarako onuragarria izan daitekeelako, zein nahi gabe, arrainak oharkabean baldintza arrotzetan hazten direnean. Arrain ar eta emeek esaterako, desberdintasun nabarmenak dituzte euren hazkuntza-tasatan eta akuikulturan larba-garapenaren fase zehatzetan hazkuntzarako baldintzak aldatu izan dira zenbait espezie komertzialetan sexubakarreko populazioak lortzeko eta hazkuntza azkarrena duen sexua faboratu ahal izateko (Devlin & Nagahama, 2002). Horrela amuarrainetan adibidez emeak faboratzen dira eta tilapien kasuan arrak (Devlin & Nagahama, 2012).

Enbrioi arren garapen-fase kritikoetan estrogenoen bitarteko tratamenduak aplikatuz erabateko eme fenotipoa eragin daiteke, testikuluen obariorako aldaketa bultzatuz (Loffler & Koopman, 2002; Vizziano et al., 2007). Gainera, enbrioi emeetan estrogenoen sintesiaren inhibizioak, adibidez *cyp19a1* genearekiko menpekoa den aromatasa entzimaren jarduera inhibituz, testikuluaren sorrera dakar (Fenske & Segner 2004, Siegfried & Nüsslein-Volhard, 2008; Vizziano et al., 2008). Hala ere, oraindik ezezagunak dira (xeno)estrogenoen esposizioarekin loturiko feminizazioaren ekintza mekanismo asko.

EDC-ek sistema endokrinoaren hormonen funtzioa betetzeko, zein sistema endokrino beraren funtzionamendu normala eraldatzeko gaitasuna duten substantzia exogenoak dira. Sortarazten dituzten erantzun molekularrek ondorio kaltegarriak ekar ditzake esposizioa pairatzen duten organismoen osasunarengan, zein beraien ondorengoengan (EEA 1997). EDC-en artean ugalketa eta gametoen garapenean eragin dezaketen konposatuak daude. Konposatu hauek hormona esteroideen orekan eragiten dute ugalketarekin erlazionaturiko geneen adierazpena aldatuz (Kime, 1995, Crews et al., 2000). EDC-ek eragindako aldaketek ez dute soilik alearen osasuna ezabatzen; gainera, bere ugalkortasunean eragin dezaketenez, populazio baten bideragarritasuna esanguratsuki murriz dezakete (WHO/IPCS, 2002, Bernanke & Köhler, 2009).

Ugalketa prozesua nahasten duten EDC horietako askok giza jatorria dute eta kaltetutako ekosistematan bizi diren organismoetan meta daitezke (WHO/IPCS, 2002).

Organismo urtarretan ugalketaren disrupzio endokrinoaren kasu asko deskribatu izan dira. Hobekien deskribatu den fenomenoa arrain populazioen feminizazioarena da, kasu batzuetan intersex egoeraren garapenean isla daitekeena. Egoera honetan genotipikoki arrak diren arrainei oozitoak hazten zaizkie testikuluen folikulu espermatikoetan barreiaturik. *Chelon labrosus* korrokoian ale intersexak topatu dira Urdaibaiko Biosfera Erreserban, Gernikan (Puy-Azurmendi et al., 2014; Valencia et al., 2016). Honez gain, intersex banakoak Bilbo, Ondarroa eta Pasaiako portuetan ere deskribatu izan dira, Euskal Herriko kostaldeko guneak soilik aipatzearren (Diaz de Cerio et al, 2012; Bizarro et al., 2014). Kasu hauetan, arrek eta arrain heldugabeek obarioko zelulen desberdintzapenaren markatzaileak diren geneak aktibatzen dituzte testikuluan (aromatasak, estrogeno hartzaileak, *foxl2, sox9*…) eta oozitoak ekoizteaz gain, emeen proteina espezifikoak ere metatzen dituzte. Testikulutatik kanpo ere ematen da emeen espezifikoak diren geneen transkripzioa eta proteinen ekoizpena, adibiderik argiena gibelean adierazten deneko oozitoetako biteloaren aitzindaria den bitelogenina da. Xenoestrogenoek arretan bitelogeninaren adierazpena eta sintesia bultzatzen dute (Arukwe & Goksøyr, 2003; Goksøyr et al., 2003) eta horregatik ingurumen biomonitorizazio programatan xenoestrogeno esposizioaren biomarkatzaile espezifiko moduan erabiltzen da askotan (OECD 2001; WHO / IPCS, 2002; Goksøyr et al., 2003). Hala ere bitelogenina emeetan, oozitoaren hazkuntza sekundarioarekin batera, bitelogenesi fasean zehar soilik detektagarria izateak zaildu egiten du biomarkatzaile honen erabilera ugal-ziklo osoan zehar. Derrigorrez, beharrezkoa da ooziotoen sorrera eta desberdintzapenaren markatzaile espezifikoak garatzea, intersex egoeraren azterketa zehatzagoa egin ahal izateko zelaiko baldintza arruntetan (Bahamonde et al., 2013).

5S rDNA, tandem eran errepikaturiko geneek osatzen dute eukariotoetan. *Xenopus* igelean bi mota 5S rRNA topatu dira; bat zelula somatikoetan adierazten dena eta bestea oozitoetan adierazten dena (Allison et al. 1995). Oso errepikatuak dauden gene paralogoen sistema hau hainbat arrain espezietan berretsi da ere (Denis & Wegnez, 1977; Campo et al., 2009). Hala ere, ez da frogatu bi geneen artean bat oozitoetan soilik transkribatzen denik. 5S rDNA bi mota hauek NTS-en luzeran desberdintzen dira, nahiz eta kasu batzuetan 120 basetako sekuentzia kodetzailean ere desberdintasunak

deskribatu izan diren (Denis & Wegnez, 1977; Campo et al., 2009). *Xenopus laevis* anuroan gertatzen den moduan, arrainen oozitoetan tRNA eta 5S rRNA metatu egiten dira (Mazabraud et al., 1975; Diaz de Cerio et al., 2012). Hauek garatuko den enbrioiaren proteinen sintesia mantentzeko erabiliko dira, ernalketaren ondorengo erribosomen muntaia azkarra ahalbidetuz.

Eukariotoetan, 5S rRNA-ren transkripzioa III RNA polimerasak baimentzen du, transkripzio orokorreko IIIA faktorearen (Gtf3a) eraenketaren menpean (Szymański et al., 2003). g*tf3a*-ren RNA-mailak, 5S rRNA-mailen adierazlea da *Xenopus*-en oozitoetan, zelula hauetan 1 milioi aldiz altuagoa izanik zelula somatiko eta espermatikoetan baino (Penberthy et al., 2003). Anuroetan, Gtf3a gain-adierazi egiten da oogenesi goiztiarrean eta gero bere adierazpena txikitzen da. Anfibioen oozitoetan 5S rRNA Gtf3arekin metatzeaz gain, 42Sp43-ari loturik ere metatzen da. Gtf3a eta 5S rRNA mintz nuklearra aktiboki zeharkatu behar dute horretarako kariofenina (inportinak eta exportinak) proteinak erabiliz. *Xenopus* igelaren oozitoetan eginiko inportina geneen transkripzio-mailen analisi sistematikoek Gtf3a eta 5S rRNA-ren antzeko adierazpen patroia dutela argitu dute (Wischnewski et al., 2004).

2012. urtean *Chelon labrosus* korrokoian oozitoetako 5S rRNA-ren metaketak arrain ar eta emeen artean desberdintzea ahalbide dezakeela ikusi zen. Honekin batera ar intersex-ak ere identifika zitezkeen, beraien adierazpen-maila bi sexuen artekoa zela ikusi zelarik (Diaz de Cerio et al., 2012). Korrokoian *42sp43* eta *gtf3a*-ren sekuentzia homologoak sekuentziatu ziren eta qPCR bidez gene hauen transkripzio-mailak aztertzean, 5S rRNA-ren antzekoak zirela ikusi zen (Diaz de Cerio et al., 2012). Berriro ere, 5S rRNA-rekin gertatzen zen bezala, intersex banakoek bi sexuen arteko transkripzio-mailak aurkeztu zituzten gene hauetarako. Horrez gain, garraio nukleo-zitoplasmatikoan jarduten duten *Impα1* eta *α2* gene biek *gtf3a*-ren transkripzio-patroia jarraitzen zuten. Honek guztiak 5S rRNA, *gtf3a, 42sp43* eta inportinak korrokoien sexua eta ar intersexak identifikatzeko biomarkatzaile moduan erabiltzeko aukera ematen du (Diaz de Cerio et al., 2012). Hala ere, galdera asko gelditzen dira zabalik. Zein da benetako 5S rRNA-ren funtzioa arrainen oozitoetan? 5S rRNA-ren transkripzio-patroia berdina da arrain espezie teleosteo desberdinetan? 5S rRNA-ren adierazpen diferentziala oozitoetan estrogenoen presentziaren/esposizioaren ondorioa da, edota

oozitoen desberdintzapen-prozesuaren ondorioa? Ba al du loturarik 5S rRNA metatze-mailak arrain teleosteoen oozitoen kalitatearen gainean? Zein da molekula erribosomiko desberdinen transkripzio- eta metatze-patroia oogenesian zehar?

Galdera hauek erantzuteko asmoarekin honako doktorego tesi proiektu hau garatu zen, hurrengo hipotesia gure garapen esperimentalerako iturburu gisa planteatuz.

HIPOTESIA

5S rRNA eta erribogenesirako berarekin erlazionaturiko proteinak arrain teleosteoen oozitoen desberdintzapenean zehar aktiboki eraentzen dira transkripzio-mailan. Eraenketa horrek arrainen sexua eta oozitoen heldutasun-maila aztertzeko, zein xenoestrogenoz kutsaturiko inguruneetan intersex arrainen identifikazio espezifikorako markatzaile molekular baliagarriak bilakatzen ditu.

HELBURUAK

Hipotesia honen ontasuna prozedura esperimentalen bitartez testatu ahal izateko honako ikerketa helburu nagusi hauek ezarri ziren:

1.- Gonadako 5S rRNA eta *gtf3a*-k Kantauriko kostaldean garrantzi komertziala duten arrain espezieen, beraien ugalketarako estrategia edozein dela ere, sexua eta emeen oogenesian zeharreko ugal heldutasuna kuantitatiboki identifikatzeko duten erabilgarritasuna baliozkotzea.

2.- Hormonen esposizioak 5S rRNA eta *gtf3a*-ren transkripzio-mailen eraentzean duten eragina aztertzea arrainen gonadogenesian eta gametogenesian zehar.

2.a.- Aingira europarrean (*Anguilla anguilla*) hormonalki (karparen pituitarioko estraktuaz) bultzatutako oogenesian zehar erribogenesiarekin loturiko markatzaile molekularrak oozitoen garapena aztertzeko duten baliagarritasuna ikertzea.

2.b.- Estradiol eta metiltestoreronaren tratamenduen bitartez feminizaturiko eta maskulinizaturiko zebra arrainetan (*Danio rerio*) *gtf3a* genearen, eta bestelako sexu-markatzaileen, transkripzio-mailak aztertzea.

3.- Arrain teleosteoen genoman *gtf3a* geneen edukia zein den aztertzea eta egon zitezkeen gene paralogoen ehunen araberako transkripzioa zebra arrainean (*Danio rerio*) aztertzea.

4.- Arrainen gametogenesian zehar qPCR bidezko itu-geneen transkripzio-mailen azterketarako erreferentzia-geneen bidezko zuzenketak vs. fluoreszentziaz kuantifikaturiko cDNA-maila zehatzen bidezko zuzenketak duten egokitasuna aztertzea.

5.- Korrokoietan (*Chelon labrosus*) gonadetako 5S rRNA-ren eta *gtf3a*-ren transkripzio-mailak xenoestrogenizitatearen ikerketan, eta espezifikoki intersex egoeraren azterketan, izan dezaketen balioa aztertzea.

6.- Arrainen akuikulturan ugalketa-protokoloen hobekuntzarako 5S rRNA eta *gtf3a*-ren transkripzio-mailen erabilpena aztertzea, erreboilo (*Scophthalmus maximus*) eta lupi (*Dicentrarchus labrax*) emeen oozitoen kalitatearen adierazle moduan izan dezaketen balioa baliozkotuz.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

5S rRNA and genes related to 5S rRNA production and ribogenesis are transcriptionally regulated during the process of oocyte differentiation and growth in teleost fish. This regulation can be applied in the identification of sex of fish individuals and to study the developmental stage of oocytes in females, these genes constituting also useful molecular markers in the specific identification of intersex condition in pollution sentinel fish under environmental exposure to xenoestrogens.

OBJECTIVES

In order to experimentally test this hypothesis, the next main research objectives were established:

1. To prove the usefulness of gonadal 5S rRNA and *gtf3a* for the quantitative identification of sex in commercially relevant teleost fish of the Cantabrian coast, and for ranking the ovarian developmental stage in females, independently of the reproductive strategy of the fish species under study.
2. To characterise the effect of hormone exposure in the transcription levels of 5S rRNA and *gtf3a* during fish gonadogenesis and gametogenesis:
    a. To study the usefulness of ribogenesis molecular markers to study oocyte development and growth during hormonally (carp pituitary extract) induced oogenesis in European eel (*Anguilla anguilla*).
    b. To investigate the transcription levels of *gtf3a* and other sex marker genes in zebrafish (*Danio rerio*) after feminisation and masculinisation induced through estradiol and methyltestosterone treatments.
3. To determine the context of *gtf3a* genes in the genome of different teleost fish, and study the developmental and tissue-dependant transcription levels of the possible *gtf3a* paralogues in zebrafish (*Danio rerio*).
4. To compare the suitability of normalizing fish gonadal target gene qPCR data along oogenesis, using either the transcription levels of reference genes or the exact levels of amplified cDNA as quantified fluorimetrically.

5. To investigate the usefulness of quantifying 5S rRNA and *gtf3a* transcription levels in gonads for the analysis of xenoestrogenicity in pollution sentinel thicklip grey mullets (*Chelon labrosus*), and more specifically for the study of the intersex condition.
6. To study the suitability of quantifying 5S rRNA and *gtf3a* transcript levels in spawned oocytes to improve the reproduction protocols applied in fish aquaculture, validating their usefulness as oocyte quality markers in turbot (*Scophthalmus maximus*) and european seabass (*Dicentrarchus labrax*) females.

RESULTS AND DISCUSSION

Chapter 1

Erreferentzia-geneen bidezko zuzenketa egokia al da arrainen obarioetan itu-geneen transkripzio-mailen azterketan aplikatzeko oogenesian zehar?

Parts of this chapter have been presented at:

30th ESCPB Congress-European Society for Comparative Physiology and Biochemistry. Barcelona, 2016. Changes on transcriptional levels of four common reference genes in fish ovary: are reference genes suitable for qPCR normalization during oogenesis? Rojo-Bartolomé, I, Ibañez, J, Bilbao, E, Ortiz-Zarragoitia,M. ICC, panel

LABURPENA

Denbora errealeko PCR kuantitatiboa (qPCR) oso tresna garrantzitsua bilakatu da zelulek estimulu ezberdinen aurrean nola erantzuten duten aztertzeko. Hala ere qPCR-ak sortutako itu-geneen inguruko datuak zuzenduak behar dute izan eta zuzenketa-metodo egokiaren aukeraketa oso eztabaidatua den gaia da. Orokorrean zuzenketarako metodoak itu-genearen transkripto-mailak hasierako RNA kantitatearekiko edo barne erreferentzia-gene baten edo hainbaten transkripto-mailekiko zuzentzean dautza. Hau guztia kontuan hartuta, lan honen helburua ugal-ziklo oso batean zehar korrokoien (*Chelon labrosus*) obarioetan geneen transkripzio-mailen zuzenketarako lau metodo frogatzea da: cDNA-kantitatearen, erreferentzia-gene bakarraren (18S rRNA, *actb*, *ef-1-α* eta *gapdh*), erreferentzia-gene guztien batezbestekoaren eta azterturiko gene guztien (erreferentzia-geneak + itu-geneak) batezbesteko geometrikoaren araberakoa. Erreferentzia-gene bakoitza eta euren batezbesteko geometrikoaren eta gene guztien batezbesteko geometrikoaren adierazpenen homogeneotasuna aztertu da. Itu-gene moduan gametogenesian eta esteroidegenesian parte hartzen duten hiru gene aukeratu dira: proteina erregulatzaile esteroidogeniko akutuaren genea (*star*), obarioaren aromatasa (*cyp19a1a*) eta 11β-hidroxilasa (*cyp11b1*). Helburuak betetzeko, urte oso batez arrantzaturiko 43 korrokoi eme histologikoki ezarritako gonadaren garapenaren arabera sailkatu ziren: erregresioa, prebitelogenesia, albeolo kortikalak eta bitelogenesia. Erauzitako RNA totaletik ekoiztutako cDNA-ren kantitatea fluoreszentzia bidez neurtu zen. Erreferentzia-geneen eta batezbestekoen egonkortasuna geNorm eta Normfinder tresna informatikoekin aztertu zen, eta itu-geneak aipatutako lau metodoen arabera zuzendu ziren. Gene batek ere ez zuen geNorm-en egonkortasun koefiziente minimo lortu eta soilik erreferentzia-geneen batezbestekoa baliogarri moduan sailkatu zuen. Normfinder-rek aldiz *actb* eta *ef-1-α* zuzentze-gene egokien moduan sailkatu zituen, 18S rRNA-ren erabilpena guztiz baztertuz bitelogenesian zehar dueneko gain-adierazpenagaitik. cDNA-kantitatea, *actb, ef-1-α*, erreferentzia-geneen eta gene guztien batezbestekoak datuen zuzenketarako erabilita *star*-en eta *cyp19a1a*-en transkripzio-mailek oogenesian zehar goranzko joera erakutsi zuten bitartean, *cyp11b1*-k oogenesian zehar transkripzio-maila konstanteak mantendu zituen. Gauzak horrela, lan honek korrokoien oogenesian zeharreko itu-geneen transkripzio-mailen zuzenketarako erabilgarriak izan daitezkeen hurbilketa desberdinak daudela frogatu duen arren, zuzenketa burutzeko cDNA-kantitatea erabiltzea gomendatzen du. Izan ere, erreferentzia-geneen erabilpenak qPCR gehigarriak egitera behartzeaz gain, geneak erabili aurretik horien aukeraketa eta baliozkotzea eskatzen du eta hau cDNA-ren kuantifikazioarekin ekidin daitekeen lana eta gastua da.

ABSTRACT

Real-time quantitative PCR (qPCR) has become a very important tool to study cell responses to different stimuli. Nevertheless, target gene qPCR data needs to be normalized, and the selection of a proper normalization method is an amply discussed topic. In general, these methods rely on the normalization in relation to the total amount of RNA in the sample, or to the transcription levels of a single or several reference-genes. Taking this into account the aim of this work was to test four different normalization methods applied to the quantification of transcription levels of genes in fish ovaries during a reproductive cycle in thicklip grey mullet (*Chelon labrosus*). Selected methods relied on the utilization of cDNA quantity, transcript levels of a unique reference gene (18S rRNA, *actb, ef-1- α* or *gadph*), the averaged level of all reference genes, and the averaged levels of all the studied genes (both reference and target genes). Three genes related to gametogenesis and steroidogenesis were selected as target genes: steroidogenic acute regulatory protein (*star*), ovary aromatase (*cyp19a1a*) and 11β- hydroxylase (*cyp11b1*). In order to achieve the objectives, 43 female thicklip grey mullets were fished and classified histologically according to their developmental stage: regression, previtellogenesis, cortical alveoli and vitellogenesis. The cDNA quantity, obtained from the extracted total RNA, was measured by fluorescence. The stability of the reference genes, individually, in couples or in conjunction, was analysed using the geNorm and Normfinder informatics tools. None of the genes obtained the minimum stability coefficient in geNorm and only the average of the reference genes was valid. On the contrary, when using Normfinder, *actb* and *ef-1- α* were classified as valid reference genes, while the use 18S rRNA was totally rejected due to its transcriptional up-regulation during vitellogenesis. When using the cDNA quantity, *actb*, *ef-1- α,* and the average of all reference genes and all of the studied genes for normalization, the transcription levels of *star* and *cyp19a1a* showed up-regulation during oogenesis progression, while *cyp11b1* transcription levels remained constant. Taking this into account, even if it has been demonstrated that different useful strategies can be used to correct the transcription levels of target genes during oogenesis in thicklip grey mullets, the obtained results recommend the use of cDNA quantity for qPCR data normalization. In fact, the use of reference genes does not only require additional qPCR experiments, but also the prior selection and validation of appropriate genes, with the additional work and expense that it demands, which can be avoided using cDNA quantity as normalization parameter.

SARRERA

Azken hamarkadetan denbora errealeko PCR kuantitatiboa (qPCR) oso tresna garrantzitsua bilakatu da zelulek estimulu ezberdinen aurrean nola erantzuten duten aztertzeko. qPCR gene zehatz baten RNA-mailak aztertzen dituen metodo azkar eta sentikorra da. Hala ere, teknika honek baditu bere zailtasunak. Izan ere, hain sentikorra da, ezinbestekoa dela hainbat baldintza betetzea lortutako emaitzak egokiak eta konparagarriak izan daitezen. Prozedura egokia jarrai dadin, qPCR-an oinarrituriko ikerketa lanak argitaratzeko ezinbesteko informazio minimoa zehazten duen MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) araudia egin da eskuragarri (Bustin et al., 2009). Bertan, besteak beste, emaitza biologikoki esanguratsuak eta konparagarriak lortzeko qPCR datuak zuzentzearen garrantzia azaltzen da. Zuzenketa-metodo egokiaren aukeraketa nahiko gai problematikoa da eta gaur egun ere eztabaida puri-purian darrai (Dheda et al., 2005, De Santis et al. 2011).

Nagusiki qPCR ostean lortutako CT balioen (CT, ingelesezko *cycle threshold*, anplifikazio prozesuan zehar neurtutako fluoreszentziak seinale basala gainditzen dueneko zikloari deritzo eta balioa itu-genearen kantitatearekiko alderantziz proportzionala da) zuzenketarako hiru metodo erabiltzen dira gaur egun. Lehenengoa, ΔCT metodoa (adibidez ikusi Kroupova et al., 2011), itu-genearen transkripto-mailak hasierako RNA kantitatearekiko zuzentzean datza. Bigarrengoa eta orain arte erabiliena, ΔΔCT metodoa da (Pffafl et al., 2001). Honek itu-genearen transkripto-mailak barne erreferentzia-gene baten transkripto-mailekin zuzentzen ditu. Hirugarrena ere ΔΔCT metodoan oinarritzen da, baina kasu honetan erreferentzia-gene bakarra erabili ordez, hainbat erreferentzia-geneen arteko batezbesteko geometrikoa erabiltzean oinarritzen da (Vandesompele et al., 2002).

Hiru metodo hauek badituzte euren abantailak eta desabantailak. RNA-mailan oinarrituriko ΔCT metodoa hiruretatik merkeena eta errazena da baina bi muga nagusi ditu. Alde batetik, RNA kantitatea neurtzeko normalean neurketa espektrofotometrikoak (A_{260} nm) erabiltzen dira. Irakurketa hau sentikortasun baxukoa da eta erauzketaren ondorioz laginean egon daitezkeen kutsatzaileak zein DNA genomikoa bereiztea

ezinezkoa da (Hashimoto et al., 2004; Lundby et al., 2005). Azken urteotan hau ekiditeko RNA kantitatea fluoreszentzia bidez irakurtzen hasi da, metodo askoz sentikorragoa delarik. Hala ere, RNA kantitatearen bidezko zuzenketa erabiltzean, ez dira kontuan hartzen ondoren prozesuan zehar egin daitezkeen akats esperimentalak. Besteak beste, cDNA-ren ekoizpenean (RT) sor daitezkeenak edo qPCR efizientzian gerta daitezkeenak ezin dira detektatu. Jakina da RT-an akats esperimental gehien egiten direneko urratsa dela (Stalhberg et al., 2004) eta RNA erauzketaren ostean doan prozesua izanik, qPCR emaitzak RNA kantitatearekiko zuzentzea ez da oso fidagarria (Lundby et al., 2005).

Barne erreferentzia-geneen bidezko zuzenketa-metodoak badu abantaila bat, prozedura esperimentalean zehar eginiko akatsak kontuan hartzen baititu. Urteetan zehar hauxe izan da zuzenketa-metodorik erabiliena eta MIQE araudiak gomendatzen duena. Hala ere, metodo honek ere baditu bere mugak. Alde batetik, erabilitako barne erreferentzia-geneak kontu handiarekin aukeratu behar dira. Edozein erreferentzia-genek hiru baldintza nagusi bete behar ditu: zelula ezberdinetan eta baldintza esperimental ezberdinetan modu konstantean transkribatu behar du eta transkripzio-mailek itu-genearenaren antzekoak izan behar dute (Zhu et al., 2008). Beraz, ez dago erreferentzia-gene unibertsalik eta esperimentu, espezie, zein organo/zelula berri bakoitzarekin balioztatze saioak egin behar dira (Gutierrez et al., 2008; Hugget et al., 2005). Izan ere, MIQE araudiak ez du onartzen balioztatu gabeko generik erabiltzea eta horrez gain, erreferentzia-gene bat baino gehiago erabiltzearen aldekoa da, gene bakarraren erabilpena zenbait kasu zehatzetan soilik onartuz (Bustin et al., 2009). Horrek guztiak, esperimentuaren kostua ikaragarri emendatzen du (Libus & Štorchová, 2006). Ondorioz, bibliografian erreferentzia-geneak erabiltzen dituzten ikerketa lan asko aurki daitezke (Rhinn et al., 2008).

Esate baterako, arrainetan buruturiko qPCR azterketen artean %50-ak zitoeskeletoko osagaia den β-aktina (*actb*) erabiltzen du erreferentzia-gene bakar moduan, %30-ak erribosomen parte den 18S RNA erribosomikoa (18S rRNA) erabiltzen du eta gainerako %10-ak proteinen biosintesian diharduen 1-α-elongazio faktorea (*ef-1-α*) (Jorgensen et al., 2006) eta glukosaren metabolismoan diharduen glizeraldehido-3-fosfato deshidrogenasa (*gapdh*). Hauetariko ikerketa gehienek ez dituzte erabilitako gene hauek balioztatu izanaren ebidentziarik ematen (Gutierrez et al., 2008) eta gaur egun jakina da

hauetariko gene askok baldintza ezberdinen aurrean transkripzio-maila aldakorrak dituztela. Arrainetan adibidez, gibel eta gonadetako *actb*-ren transkripzio-mailak estradiolera esposatzearen ondorioz aldatzen direla ikusi da (Filby & Tyler, 2007). Horrez gain, arrainen obarioa bezalako organo dinamikoa eta aldakorra aztertzeko 18S rRNA eta *ef-1-α* ez dirudite aukera egokiak (Mittelholzer et al., 2007; Deloffre et al., 2012; Diaz de Cerio et al., 2012).

Gauzak horrela, azken urteotan cDNA-kantitatearen bidezko qPCR datuen zuzenketa burutzen hasi da. Lan asko dira metodo honen erabileraren abantailak aipatzen dituztenak (Lundby et al., 2005; Filby & Tyler, 2007; Kroupova et al., 2011; De Santis et al., 2011; Rojo-Bartolomé et al., 2016). Izan ere, metodo hau erreferentzia-geneak erabiltzea baino merkeagoa da eta prozedura esperimentalean zehar egon daitezkeen akatsak kontuan hartzen ditu. qPCR-an lagin bakoitzarentzat erabilitiko cDNA kopurua zehatza kontuan hartzean, aurreko pausuetan sortu zitekeen aldakortasuna arbuiagarria da metodo honen bidez (Rhinn et al., 2008).

Hau guztia kontuan hartuta, lan honen helburua ugal ziklo oso batean zehar korrokoi (*Chelon labrosus*) emeen obarioetan geneen transkripzio-maila aztertzeko lau zuzenketa-metodo ezberdin frogatzea da. Zuzenketa-metodoak honako hauek izan dira: a) cDNA-kantitatearen araberakoa, b) banaka erabilitako erreferentzia-gene arrunten araberakoa (18S rRNA, *actb*, *ef-1-α* eta *gapdh*), c) erreferentzia-gene hauen batezbestekoaren araberakoa, eta azkenik d) azterturiko gene guztien batezbesteko geometrikoaren araberakoa (erreferentzia-geneak gehi itu-geneak) dira. Itu-gene moduan oogenesian parte hartzen duten hiru gene aukeratu dira (Sardi et al., 2015): proteina erregulatzaile esteroidogeniko akutuaren genea (*star*), mitokondriaren kanpo mintzetik barruko mintzera kolesterolaren translokazioa burutzeaz arduratzen den proteinaren gene kodetzailea; obarioaren aromatasa (*cyp19a1a*), obarioan estradiolaren ekoizpenarekin erlazionaturiko aromatasa kodetzearen gene arduraduna; eta 11β-hidroxilasa (*cyp11b1*), androgenoak ekoizteko behar deneko entzima mitokondriala kodetzearen arduraduna.

Horrez gain, MIQE araudiak gomendatu bezala, erreferentzia-gene bakoitzaren, euren batezbesteko geometrikoaren eta gene guztien batezbesteko geometrikoaren

adierazpenen homogeneitatea balioztatzeko geNorm eta Normfinder tresna informatikoak erabili dira.

MATERIALA ETA METODOAK

Lagin biologikoak

Lan honetan 2010ko irailetik 2011ko irailera bitartean arrantzatu ziren korrokoien (*Chelon labrosus*) obarioak aztertu dira. 43 korrokoi arrantzatu ziren Pasaia-Trintxerpeko amarra-lekuan (43°19'35''I,1°55'9''E). Ale guztiak *in situ* prozesatu ziren. Korrokoiak 4-etil-aminobenzoatoarekin (Fluka, Steinheim, Alemania) saturatuta zegoen ur-soluzio batean murgilduz anestesiatu eta burua moztuz sakrifikatu ziren. Gonadak osorik isolatu ondoren pisatu eta zatitu ziren. Gonadaren zati bat "RNA later"-ean (Life Technologies, Carslbard, AEB) sartu eta zuzenean nitrogeno likidotan izoztu eta gero -80ºC-tara gorde ziren laborategira eramandakoan. Beste gonadaren zati bat analisi histologikoak egiteko erabili zen. Horrela korrokoi emeak arrantzaturiko guztien artean identifikatu eta euren fase gametogenikoa zehaztu ahal izan zen.

Arrainen manipulazio lanak Espainian eta Europar Batasunean indarrean dauden animalia esperimentalen ongizaterako araudiak (Azaroaren 7ko legea 32/2007; Zuzendaritza 2010/63/UE) jarraituz egin ziren. Lan honetako prozedura osoak UPV/EHUko Animalien Ongizaterako Etika Batzordearen (AOEB) onarpena izan zuen (CEBA/152/2010) eta Bizkaiko Foru Aldundiaren baimena.

Obarioaren azterketa histologikoa

Korrokoi emeak fase gametogenikoaren arabera sailkatu eta taldekatu ziren. Horretarako, 24 orduz finkatzailean (%10-ko formalina neutroa) eduki ondoren histologiarako laginak etanol kontzentrazio ezberdinetan deshidratatu ziren (%70, %90 eta %96) eta metakrilatozko erretxinan (Technovit 7100; Heraeus Kulzer GmbH & Co., Werheim, Alemania) barneratu ziren. Erretxina gogortu ondoren, 5 µm-tako ebakiak egin ziren 2065 Supercut mikrotomoan (Leica Instruments GmbH, Wetzlar, Alemania). Azkenik, ebakiak hematoxilina/eosinaz koloreztatu eta argi mikroskopioaren bidezko (Olimpus BX61) behaketaz aleen sexua eta emeen fase obarikoa zehaztu ziren, Bizarrok

bere tesian (2005) eginiko sailkapenaren egokitzapena erabilita (1. Taula).

1. Taula: Emeen fase gametogeniko bakoitzaren ezaugarriak eta fase bakoitzean sailkaturiko lagin kopurua.

Faseak	Fasearen izena	Fasearen ezaugarriak	Eme kopurua
E	Erregresioa	Obarioa ia guztiz hustua. Oozito atretikoen agerpena. Muskulu eta ehun konektibo multzoen presentzia. Pareta obariko mehea.	6
Pb	Prebitelogenesia	Oozito perinukleolarren presentzia. Muskulurik ez da agertzen eta ehun konektiboa murriztua dago. Obarioren pareta mehea da.	19
Ak	Albeolo kortilalak	Oozito kortikalen presentzia. Zenbait oozito bitelogenikoen agerpena (%50 baino gutxiago).	9
B	Bitelogenesia	Erreserba tanten agerpena zitoplasman. Oozitoen mintz plasmatikoaren luzaketa nabaria da (oozitoen %50 baino gehiago bitelogenikoak dira).	9
H	Heldutasuna	Oozito hidratatuen agerpena. Lipido pikorren presentzia zitoplasman.	0
			Guztira 43

RNA erauzketa eta cDNAren sintesia

RNA totala TRIzol-aren (Life Technologies) bitarteko metodoa erabiliz eta hornitzailearen argibideak jarraituz erauzi zen. Erauzitako RNA 90 µL RNasa/DNasa-rik gabeko uretan disolbatu zen. Kalitate altuko RNA lortzeko asmoz, RNA totala DNasa-z tratatu (RNase-Free DNase Set, Quiagen, California, AEB) eta Quiagen RNeasy kit-komertziala (Quiagen) erabiliz garbitu zen. Azkenik, RNA kontzentrazioa eta kalitatea espektrofotometrikoki (Biophotometer, Eppendorf) zehaztu zen. Aurrera jarraitzeko 260/280 nm=1.80-2.0 eta 260 /230 nm=2.0-2.2 bitarteko absorbantzia ratioaren balioak zituzten laginak hautatu ziren.

cDNAren sintesia SuperScript Fist Strand Synthesis (Life Technologies) kit-komertzialerako ekoizleak zehaztutako argibideak jarraituz burutu zen, lagin bakoitzeko 1 µg RNA erabilita.

cDNA kontzentrazioaren neurketa

cDNA-kantitatea fluoreszentzia bidez neurtu zen. Quant-iT™ OliGreen® ssDNA Kit-aren (Invitrogen, Life Technologies) protokoloa jarraituz obario bakoitzetik ekoiztutako cDNA-ren kontzentrazioa kalkulatu zen. Neurketak, Synergy HT Multi-Made Microplate Reader (BioTek, Winoosky, AEB) irakurlean egin ziren 96 putzudun plakak erabiliz (Corning Incorporated; New York, AEB). 0.02-0.2 ng/µL tartean zegoeneko cDNA kontzentrazio teorikoa erabili zen neurketetarako 100 µL-ko erreakzio bolumenean. Uhin luzerak 485/2 nm kitzikadurara eta 528/20 nm igorpenera zehaztu ziren. Irakurketa "high-range standard curve" edo tarte altuko kurba estandarrarekin eta hornitzailearen argibideak jarraituz egin zen.

Interesezko sekuentzien lorpena

Gene bakoitzaren transkripzio-maila qPCR bidez kuantifikatu aurretik gene guztien sekuentziak eskuratu ziren. 18S rRNA (AY836368), *actb* (AY836369), *ef-1-α* (AY825252), *star* (JX294414), *cyp11b1* (JX294416) eta *cyp19a1a* (EF535845) sekuentziak NCBI-ko (National Center for Biotechnology Information) GenBank datu-basetik eskuratu ziren. *gapdh* sekuentzia ordea sekuentziatu behar izan zen. Horretarako, NCBI-n korrokoiarekin erlazio filogenetiko hurbila (Percomorpha) zuten arrainen sekuentziak lerrokatu ziren ClustalW2 programaren bidez (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Lortutako sekuentzien homologia handiko guneen gainean hasleak diseinatu ziren. Sekuentziaren anplifikazioa 35 zikloko PCR konbentzionalaz egin zen 2720 termozikladorean (Applied Biosystems). Horretarako Taq DNA Polymerase, recombinant Kit-a eta 100 mM dNTP Mix-a (Invitrogen) erabili ziren. Hasleak 0.8 mM-ko kontzentrazioan gehitu ziren 50 µL-ko erreakzio bolumenean. Termozikladorearen baldintzak honakoak izan ziren: 94°C-tako 2 minutuko hasiera urratsa; jarraian 35 ziklotan zehar, 30 segundo 94°C-tan harizpiak desnaturalizatzeko, beste 30 segundo hasleen hibridazio tenperaturan eta 8 minutuko elongazio urratsa 72°C-tan; azkenik 8 segundoko 72°C-ko azken pausu bat. PCR produktua etidio bromuroz tindaturiko %1.5 agarosa gel batean migratu eta UPV/EHU-ko Genomika Zerbitzuko Sekuentziazio eta Genotipo Azterketa Unitatera (SGIker) sekuentziatzera bidali zen.

Lortutako sekuentzia NCBI-ko BlastN nukleotido datu basearekin (http://blast.ncbi.nlm.nih.gov/Blast.cgi) alderatu zen beste arrainen *gapdh* geneekin homologia aztertzeko. Sekuentzia NCBI-ko Genbank (http://www.ncbi.nlm.nih.gov/genbank/) datu-basera igo zen (sarrera gakoa: KX132908). Azkenik, sekuentzia kontsentsuaren gainean qPCR-ak egiteko hasle espezifikoak diseinatu ziren (2. Taula).

qPCR bidezko geneen transkripzio-mailen neurketa

Geneen transkripzio-maila 7300 Real-Time PCR System (Applied Biosystems) termozikladorean 96 putzudun plakak (Applied Biosystems) erabiliz neurtu zen. cDNA lagin bakoitzeko 2 µL-ko 3 erreplika aztertu ziren 18 µL qPCR medioan. Medioaren osagaiak honako hauek izan ziren: 10 µL SYBR ®Green PCR Master Mix (Roche) eta hasleak, zegozkien kontzentrazioan, RNasa/DNasarik gabeko uretan diluituak (2. Taula).

2. Taula: Hasleen ezaugarriak eta qPCR baldintzak. Taulan geneei esleituriko kalifikazioa agertzen da; Eg: Erreferentzia-geneak adierazteko eta Ig: Itu-geneentzako. Hasleak, Fw: ingelesezko *forward*, hasle zuzenari deritzo eta Rv: ingelesezko *reverse*, alderantzizko hasleari. (°C): qPCR-an erabilitako hasleen hibridazio tenperaturari dagokio.

Genea	Kalifikazioa	Fw hasle-sekuentzia (5'-3')	Rv hasle-sekuentzia (5'-3')	Hasleen kontzentrarioa (nM)	Laginen diluzioa	(°C)
actb	Eg	AGCCAACAGGGAGAAGATGA	GAGCGTAGCCCTCGTAGATG	25	1/2000	56
ef-1-α	Eg	CAGGGATTTCATCAAGAACA	GTCCATCTTGTTGACACCA	25	1/200	53
18S rRNA	Eg	GAGGCCCTGTTAATTGGAATGAG	TAAGATACGCTATTGGAGCTGGAA	50	1/200	56
gapdh	Eg	ACCCCACCAACATCAAATGG	TGACAACCTTGAGGGAGTTG	12,5	1/200	55
star	Ig	GGCAGGTCCAGGCCCCAGTAA	TCCATCCGTCCTGCTCGCTGA	25	1/200	63.5
cyp11b1	Ig	GGAGGGGTCGACACGACAGC	CCGCCAGCCTTTGCCCATGAT	25	1/200	62.9
cyp19a1a	Ig	ACGCACCTGGACGACTTG	TGCAGCGCAGCAAACG	25	1/25	57

qPCR-ak 40 zikloz luzatu ziren eta termozikladorean ezarritako tenperatura eta denbora baldintzak honakoak izan ziren: 2 minutu 50°C-tan, 10 minutu 95°C-tan, eta gero 40 ziklotan zehar 15 segundoko desnaturalizazio pausu bat 95°C-tan eta minutu batez, hibridazioa baimenduz, hasle bakoitzari zegokion tenperaturan. Disoziazio pausu bat gehitu zen amaieran; 95°C-tan 15 segundoz, eta gene bakoitzari zegokion hibridazio tenperaturan minutu batez mantenduz. Azkenik, berriz ere laginak 95°C-tan 15 segundoz inkubatu ziren.

Aztertutako gene guztien qPCR emaitzak hainbat eratara zuzendu ziren, bai ΔΔCT metodoan (Pffafl et al., 2001) zein moldaturiko ΔCT metodoan oinarrituz (Rojo-Bartolomé et al., 2016): erreferentzia-geneen bidezko zuzenketak cDNA-kantitatearekiko eta gainontzeko erreferentzia-geneekiko egin ziren. Itu-geneen kasuan, cDNA-kantitatearekiko eta erreferentzia-gene bakoitzarekiko zuzentzeaz gain, erreferentzia-gene guztien batezbesteko geometrikoarekiko (Eg-k) eta aztertutako gene guztien batezbestekoarekiko (Elg-k) ere zuzendu ziren. Emaitzak fase gametogeniko bakoitzaren arabera sailkatu ziren (ikusi 1. Taula), H fasean zegoen emerik lortzea ezinezkoa izan zelarik.

cDNA bidezko qPCR emaitzen zuzenketa

Geneen cDNA-kantitatearen bidezko zuzenketa Rojo-Bartolomé eta kideek (2016) proposatutako ΔCT metodoaren adaptazioa jarraituz burutu zen. Horretarako lagin bakoitzeko qPCR-an kargaturiko cDNA-kantitate totala kontuan hartuta hurrengo formula erabili zen:

$$E = \left[10^{-1/m}\right] - 1$$

Non *E* efizientziaren zuzenketa eta *RQ* (ingelesezko *relative quantification*) edo kuantifikazio erlatiboa eta *m* kurba estandarraren malda diren.

$$RQ = Log_2\left[\frac{(1+E)^{-\Delta CT}}{ng\ cDNA}\right]$$

Non $\Delta CT = CT\ lagina - CT\ plakaren\ barne\ kontrola$

cDNA-kantitatea erreferentzia-gene hautagaien egonkortasuna aztertzeko eta gene guztien zuzenketarako erabili zen.

Erabilitako tresna informatikoak

Erreferentzia-gene hautagaien egonkortasuna aztertzeko genNorm (Vandesompele et al., 2002; https://genorm.cmgg.be/) eta Normfinder (Andersen et al., 2004; http://moma.dk/normfinder-software) tresna informatikoak erabili ziren. Erreminta bakoitzak erreferentzia-generik edo geneen arteko konbinaketarik egokiena zein den era ezberdinean kalkulatzen du. geNorm-ek geneak euren transkripzio egonkortasunaren arabera sailkatzen ditu, horretarako bikoteka konparatzen ditu geneak eta egonkortasun koefiziente bat (M) esleitzen die. Erreferentzia-gene bat onartzeko M<1.5 izatea ezinbesteko baldintza da. Zenbat eta M txikiagoa izan, orduan eta erreferentzia-gene egonkorragoa. Normfinder-ek aztertutako gene guztiak euren egonkortasunaren arabera ordenan jartzen ditu. Horrez gain, gene-bikote konbinaziorik egonkorrena zein den esaten du. Bi erremintekin eginiko datuen analisiak egileen argibideak jarraituz burutu ziren.

Estatistika

Lorturiko datuak SPSS.22 (SPSS Inc., Microsoft Co.) erabiliz aztertu ziren. Taldeen arteko ezberdintasun estatistikoak ezartzeko Kruskal Wallis testa erabili zen, Dunn-en post-hoc testaz jarraituta. Desberdintasun esangarriak p<0.05-an ezarri ziren. Emaitzak aurkezteko kutxa diagramak aukeratu ziren, non kutxa bakoitzak 25 eta 75 pertzentilen arteko datuak, erdiko marrak mediana eta goi eta beheko marrek maximo eta minimoak adierazten dituzten.

EMAITZAK

Hautatutako erreferentzia-geneen transkripzio-mailen egonkortasuna geNorm eta Normfinder tresna informatikoen arabera

Orokorrean geNorm-en, hautatutako erreferentzia-gene batek ere ez zuen M<1.5 ezinbesteko mugaren baldintza bete. Hala ere, hautagai gehienak M=1.51-1.581 tartean

zeuden, orden hau jarraituz: Eg-k < *actb* < *gapdh* < *ef-1-α* < EIg-k < 18S rRNA (A atala, 3.taula). 18S rRNA, M=1.814 balioarekin, generik ezegonkorrena izan zen. Erreferentzia-gene guztien batezbesteko geometrikoa egiterakoan (Eg-k) M=1.495-koa izan zen. Aldiz, itu-geneak analisian barneratzean (EIg-k) M=1.755-koa zen, onargarria den balioa gainditua (3. Taula). Gauzak horrela, erreferentzia-geneen egonkortasuna Normfinder-ekin aztertu zen, geNorm erabilita ezingo baikenuen datuen zuzenketarako hautatutako gene bat bera ere ez bakarka erabili. Normfinder tresnak emandako geneen adierazpen-egonkortasunaren araberako ordena honako hau izan zen: *ef-1-α* < *actb* < Eg-k < *gapdh* < Elg-k < 18S rRNA (B atala, 3.taula). Gene bikoterik egonkorrena 4.793-ko egonkortasun balioarekin, *actb* eta *ef-1-α* izan ziren. Generik ezegonkorrena ordea, berriz ere 18S rRNA izan zen.

3. Taula: geNorm (A) eta Normfinder (B) erreminten egonkortasun emaitzak: M: egonkortasun koefizientea. Stability: egonkortasun balioa. Eg-k: erreferentzia-geneen batezbesteko geometrikoa, EIg-k: gene guztien batezbesteko geometrikoa. Beltzez markaturiko balioa adierazpen egonkorreneko geneari dagokio.

A)

geNorm	**18S rRNA**	***actb***	***ef-1-α***	***gapdh***	**Eg-k**	**EIg-k**
M<1.5	1.814	1.510	1.581	1.552	**1.495**	1.755

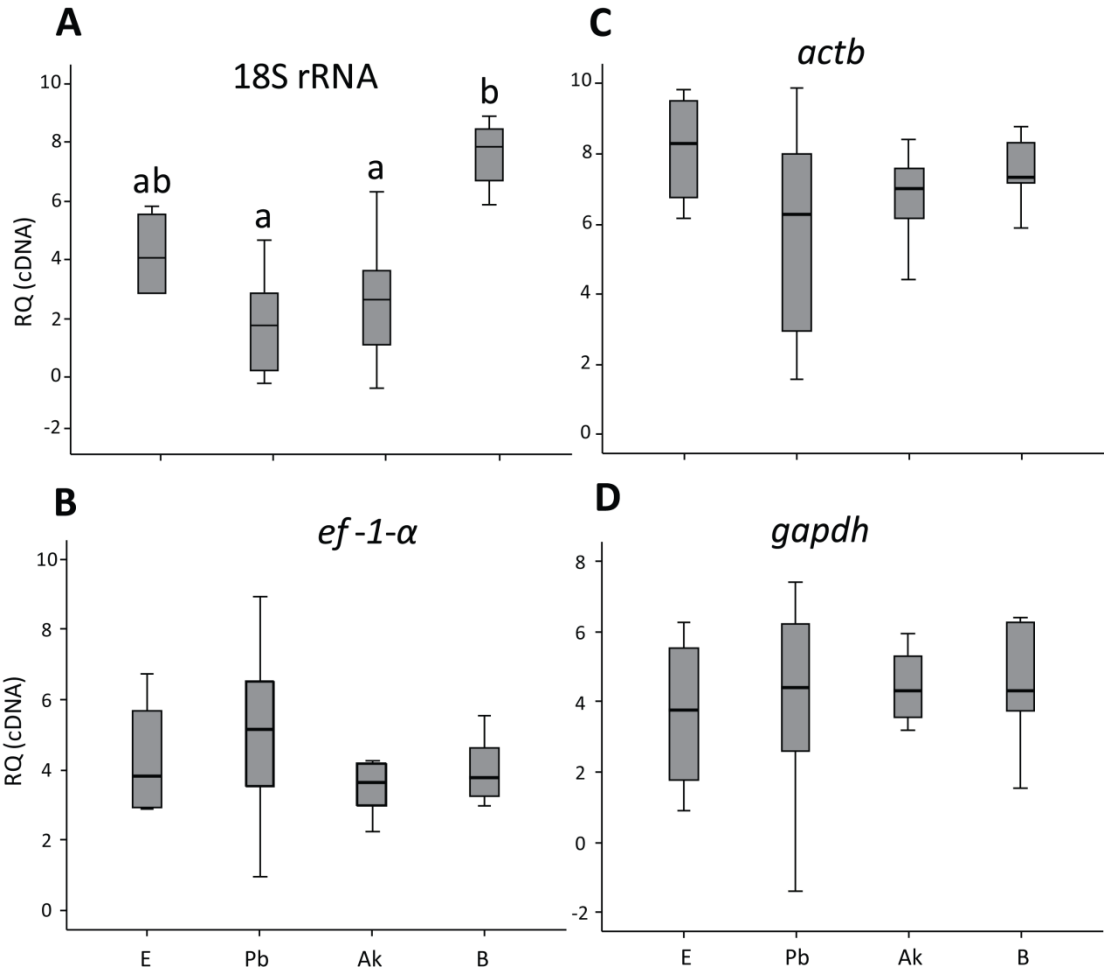
B)

Normfinder	**18S rRNA**	***actb***	***ef-1-α***	***gapdh***	**Eg-k**	**EIg-k**
Stability	7.168	6.798	**6.755**	7.013	6.867	7.136

Hautatutako erreferentzia-geneen transkripzio-mailen egonkortasuna cDNA-kantitatearen bitartez aztertua

cDNA erreferentzia-geneen egonkortasuna aztertzeko erabiltzean, *actb*, *ef-1-α* eta *gapdh* oogenesian zehar transkripzio-maila konstantea zutela ikusi zen (B, C eta D atalak, 1. Irudia). 18S rRNA-ren transkripzio-mailek ordea gora egiten zuten oogenesiak aurrera egin ahala, transkripzio-maila minimoak prebitelogenesi fasean eta maximoak

bitelogenesiarekin erakusten zituelarik (A atala, 1. Irudia). Gene egonkorrenetik desegonkorrenera lortzen deneko sailkapena honako hau da: *actb* < *ef-1-α* < *gapdh* < 18S rRNA.

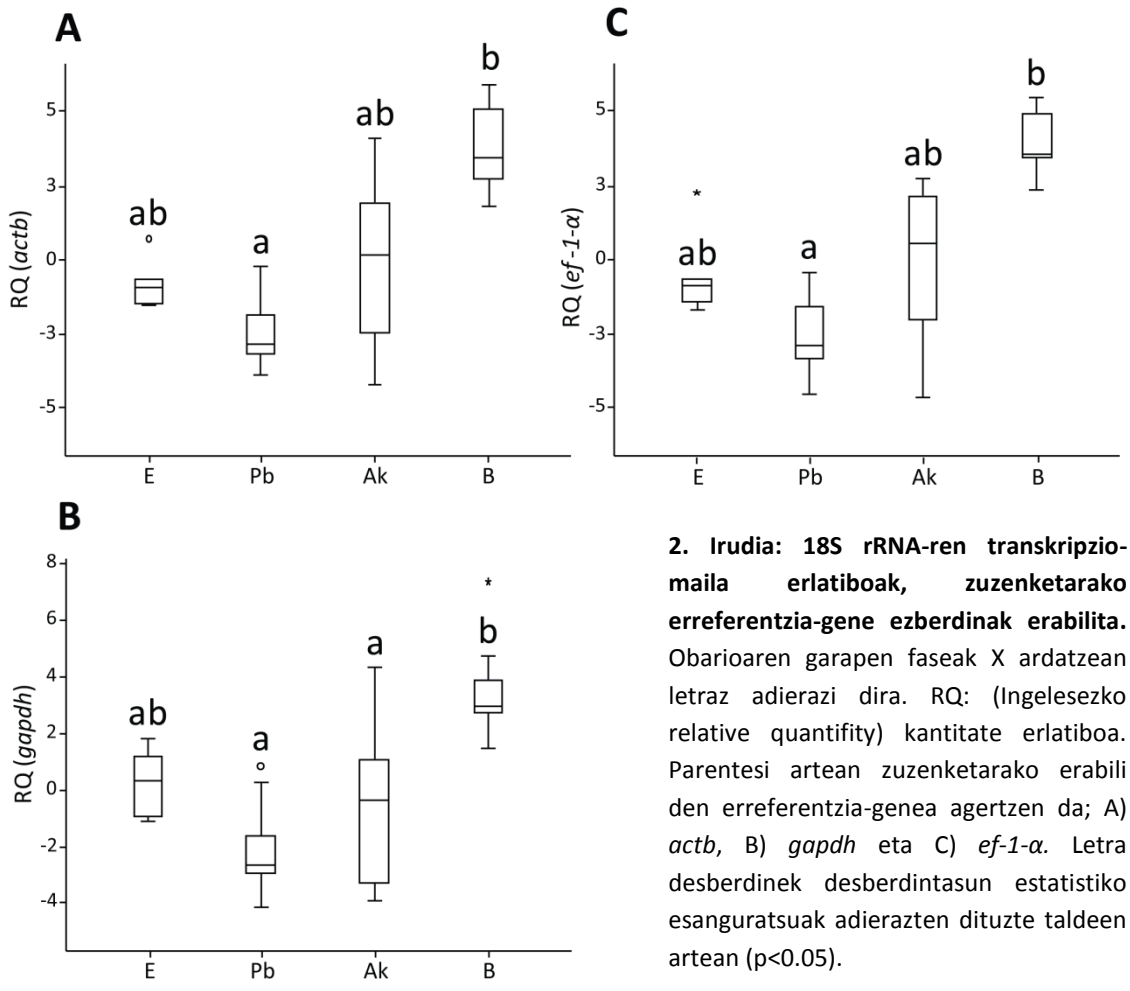


1. Irudia: Hautatutako erreferentzia-geneen transkripzio-mailen egonkortasuna cDNA-kantitatearen bitartez aztertua. Obarioaren garapen faseak X ardatzean letraz adierazi dira: E = erregresioa, Pb=prebitelogenesia; Ak=albeolo kortikalak eta B=bitelogenesia. RQ: transkripto bakoitzaren kantitate erlatiboa (ingelesezko relative quantity). A) *18S rRNA* B) *ef-1-α*, C) *actb* eta D) *gapdh*. Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte taldeen artean ($p<0.05$).

Korrokoiaren oogenesian zehar 18S rRNA-ren aldakortasun altua frogatuta ere, hurrengo ataletan erreferentzia-gene moduan erabili zen, berau erabiltzekotan geneen transkripzio-mailarekin gertatuko litzatekeena ikusi ahal izateko.

Oogenesian zeharreko erreferentzia-geneen transkripzio-mailak beste erreferentzia-gene hautagaien zuzenketaren bitartez aztertuak

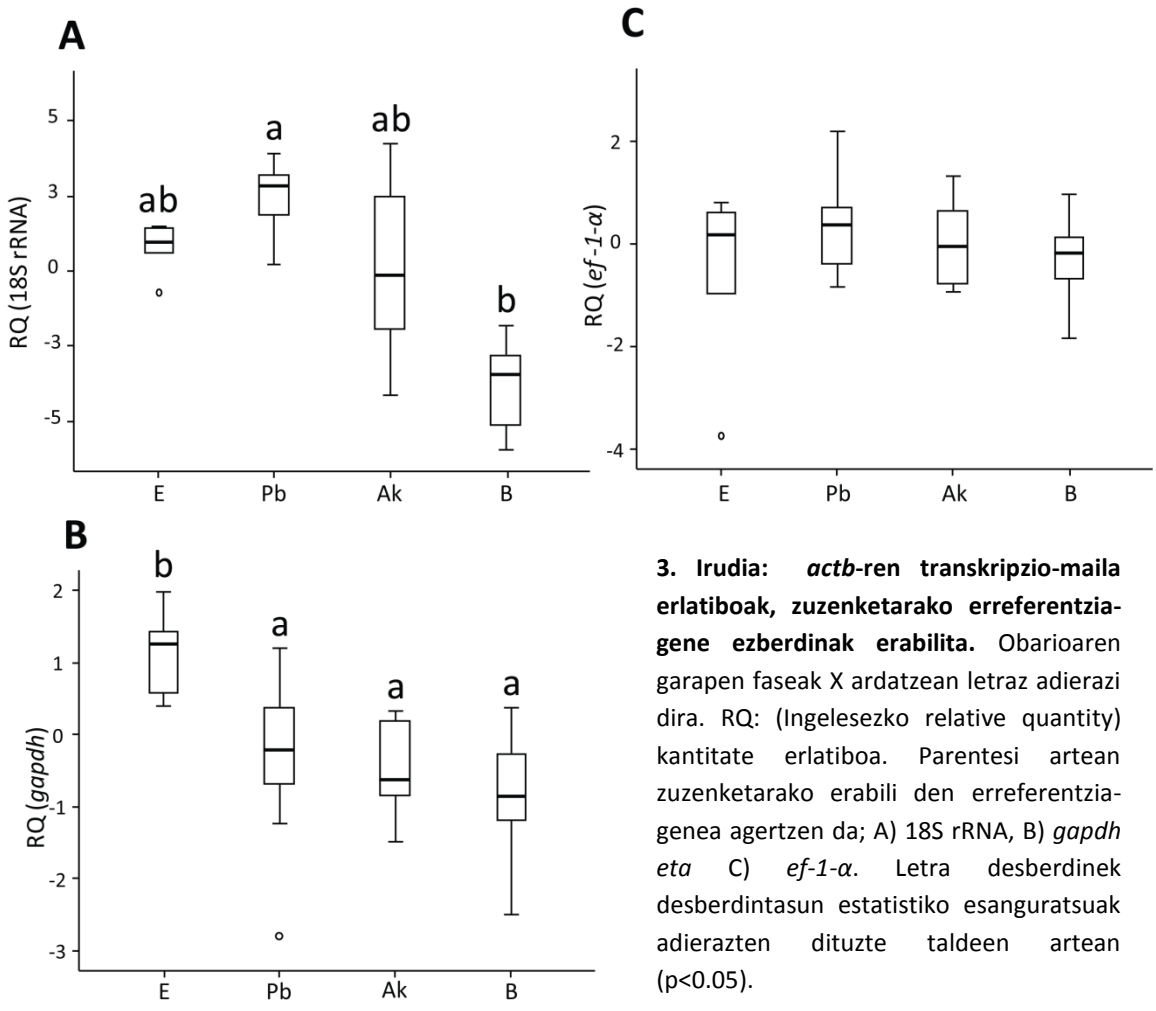
Orokorrean, erreferentzia-geneekin zuzentzerakoan 18S rRNA-ren transkripzio-mailak oogenesian zehar goranzko joera erakutsi zuen, zuzenketarako erabilitako genearekiko era independentean (2. Irudia). Transkripzio-mailarik baxuenak prebitelogenesian (Pb) eta altuenak bitelogenesi fasean behatu ziren (B).



2. Irudia: 18S rRNA-ren transkripzio-maila erlatiboak, zuzenketarako erreferentzia-gene ezberdinak erabilita. Obarioaren garapen faseak X ardatzean letraz adierazi dira. RQ: (Ingelesezko relative quantifity) kantitate erlatiboa. Parentesi artean zuzenketarako erabili den erreferentzia-genea agertzen da; A) *actb*, B) *gapdh* eta C) *ef-1-α.* Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte taldeen artean ($p<0.05$).

Bestelako geneei zegokienez (3., 4. eta 5. Irudiak), qPCR datuen zuzenketarako erabilitako genearen arabera emaitza ezberdinak lortu ziren. Horrela, *actb*-ren kasuan (3. Irudia), *ef-1-α* -rekiko (C atala, 3. Irudia) zuzentzerakoan oogenesian zeharreko transkripzio-maila konstanteak ikusi ziren. *gapdh*-rekiko zuzentzerakoan ordea, erregresio faseak (E) besteak baino transkripzio-maila altuagoa azaldu zuen (B atala, 3.

Irudia). 18S rRNA-rekiko zuzentzerakoan aldiz, *actb*-k beheranzko joera azaldu zuen (A atala, 3. Irudia), non baliorik altuenak prebitelogenesian eta baxuenak bitelogenesi fasean izan ziren, hurrenez hurren, 18S rRNAren transkripzio-maila baxu eta altuenekin bat zetozelarik (2. Irudia).

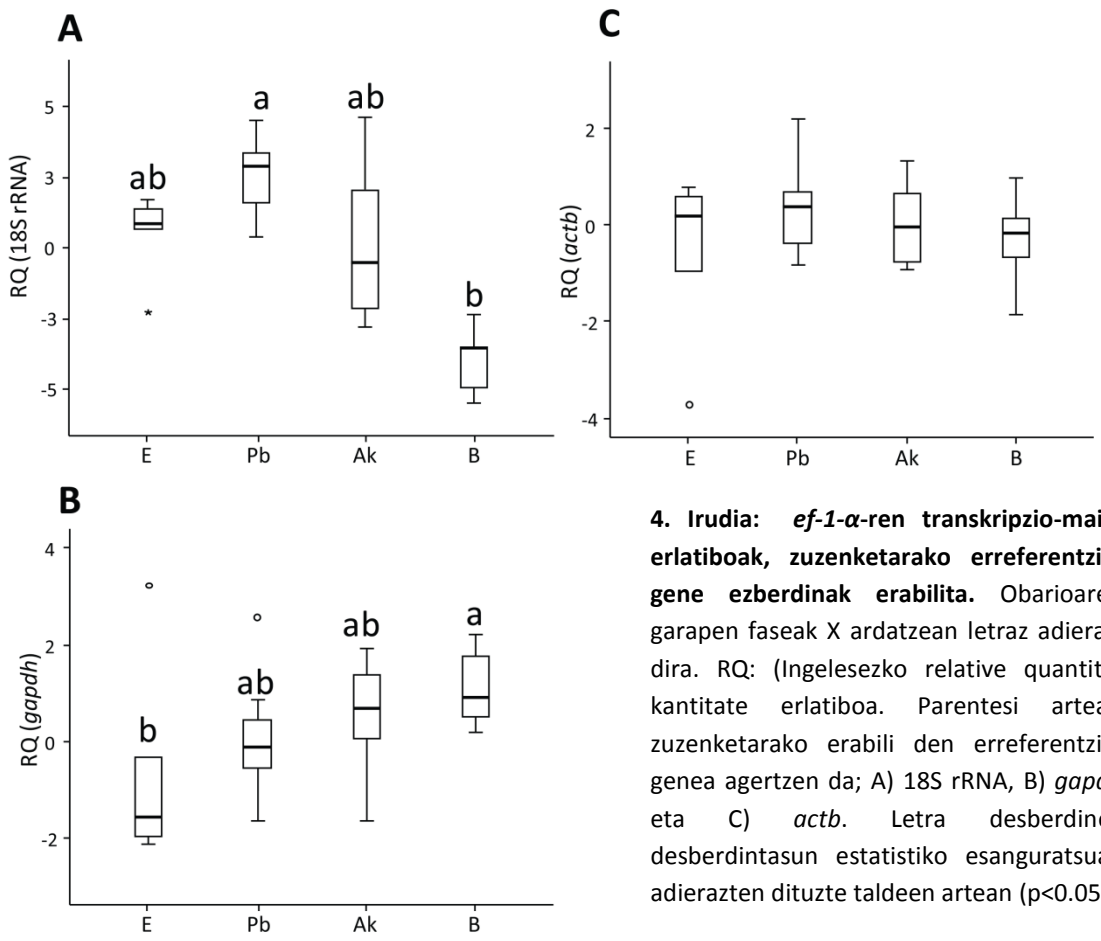


3. Irudia: *actb*-ren transkripzio-maila erlatiboak, zuzenketarako erreferentzia-gene ezberdinak erabilita. Obarioaren garapen faseak X ardatzean letraz adierazi dira. RQ: (Ingelesezko relative quantity) kantitate erlatiboa. Parentesi artean zuzenketarako erabili den erreferentzia-genea agertzen da; A) 18S rRNA, B) *gapdh eta* C) *ef-1-α*. Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte taldeen artean ($p<0.05$).

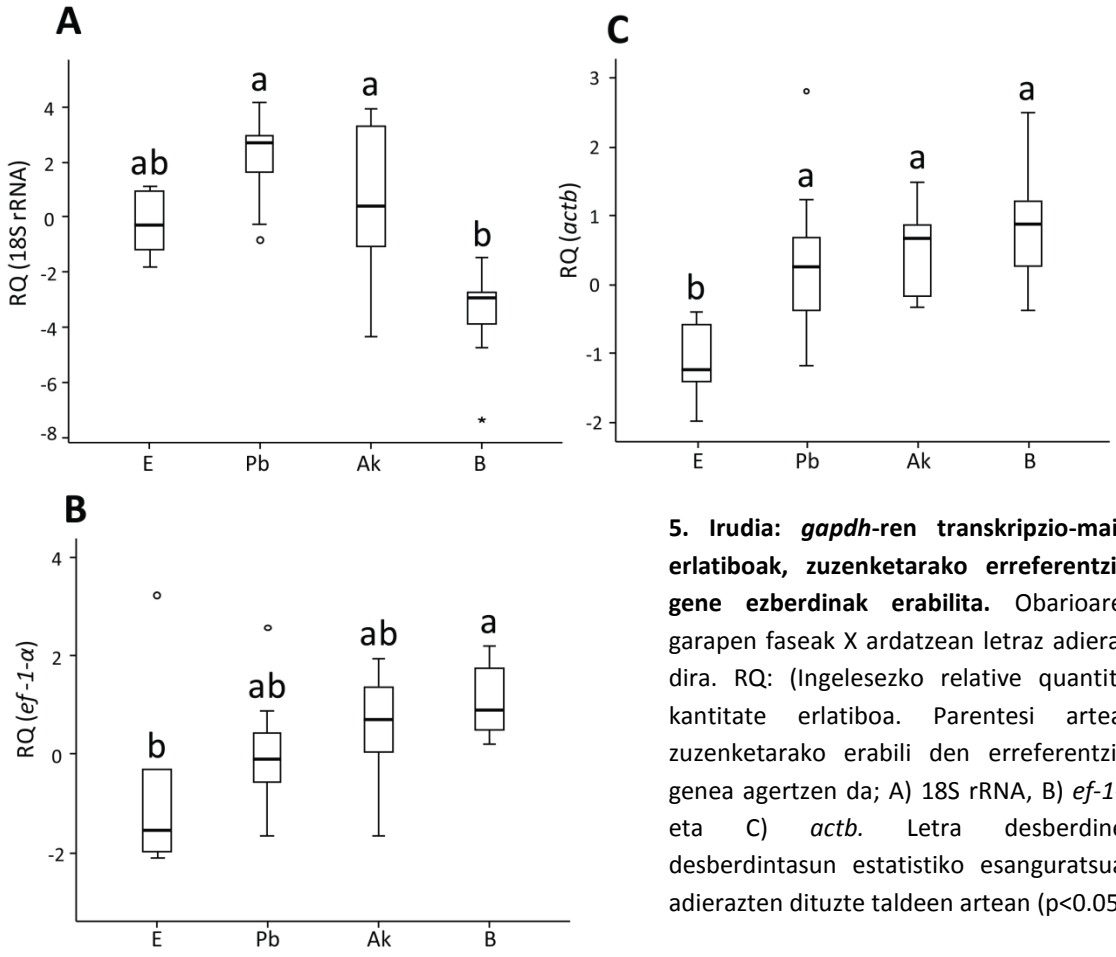
ef-1-α-ren kasuan (4. Irudia) emaitzak *actb*-k lortutakoen antzekoak izan ziren (C atala,4. Irudia). Baina *gapdh*-rekiko zuzentzerakoan, oogenesian zeharreko goranzko joera azaldu zuen, baliorik altuenak bitelogenesi fasean gertatu zirelarik (B atala, 4. irudia). 18S rRNA-rekiko zuzentzerakoan ere, *actb*-arekin gertatzen zen bezala, beheranzko joera azaldu zuen oogenesiak aurrera egin ahala (A atala, 4. Irudia).

gapdh-ren transkripzio-mailak *ef-1-α* eta *actb*-rekiko zuzentzerakoan (B eta C atalak, 5. Irudia), nahiz eta oogenesian zehar transkripzio-maila konstanteak mantendu, erregresio fasean (E) transkripzioaren beherakada ikusi zen. 18S rRNA-rekin zuzentzerakoan (A atala,

5. Irudia), transkripzio-mailak beherantz egin zuen. Baliorik baxuenak bitelogenesi fasean 18S rRNA-ren transkripzio-maila altuenekin bat zetozen (2. Irudia).



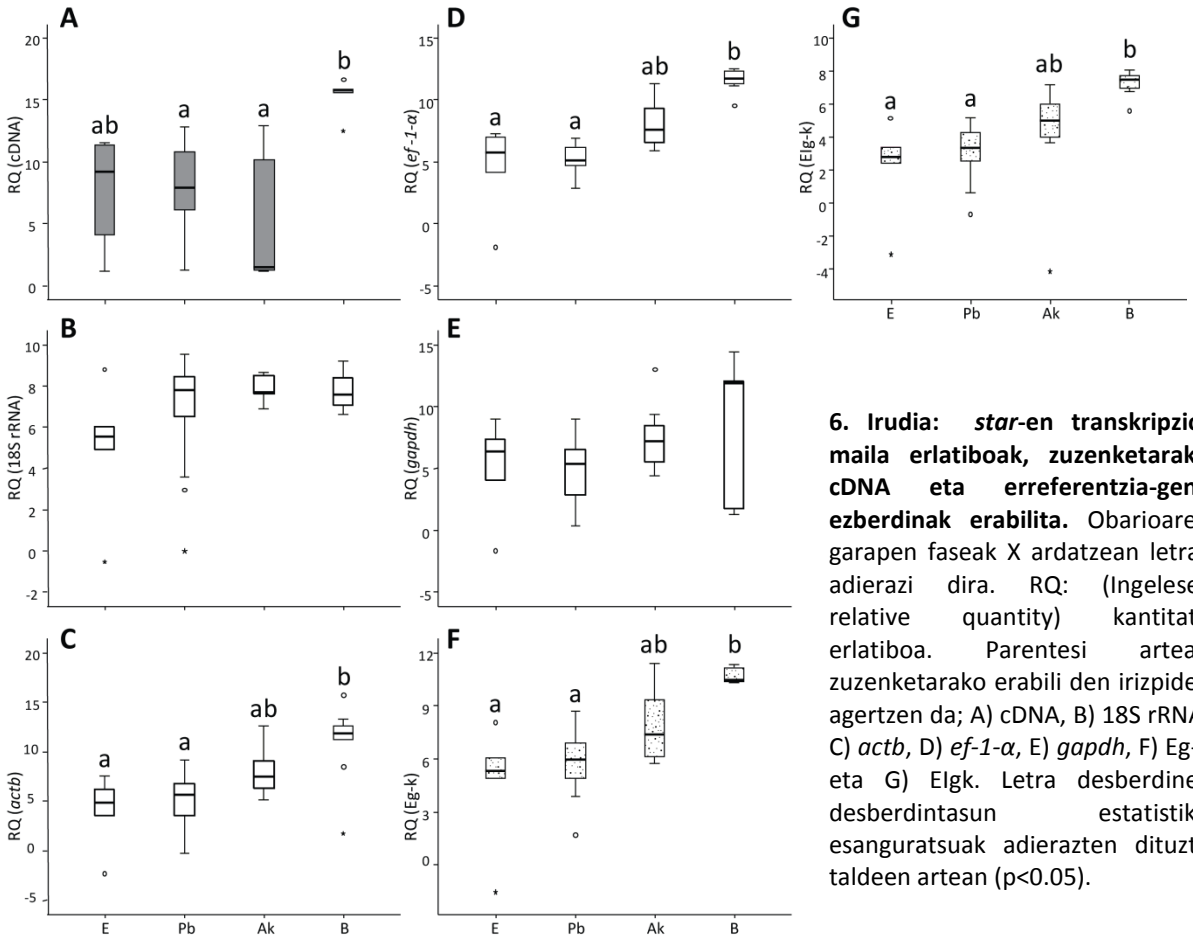
4. Irudia: *ef-1-α*-ren transkripzio-maila erlatiboak, zuzenketarako erreferentzia-gene ezberdinak erabilita. Obarioaren garapen faseak X ardatzean letraz adierazi dira. RQ: (Ingelesezko relative quantity) kantitate erlatiboa. Parentesi artean zuzenketarako erabili den erreferentzia-genea agertzen da; A) 18S rRNA, B) *gapdh* eta C) *actb*. Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte taldeen artean ($p<0.05$).



5. Irudia: *gapdh*-ren transkripzio-maila erlatiboak, zuzenketarako erreferentzia-gene ezberdinak erabilita. Obarioaren garapen faseak X ardatzean letraz adierazi dira. RQ: (Ingelesezko relative quantity) kantitate erlatiboa. Parentesi artean zuzenketarako erabili den erreferentzia-genea agertzen da; A) 18S rRNA, B) *ef-1-α* eta C) *actb.* Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte taldeen artean ($p<0.05$).

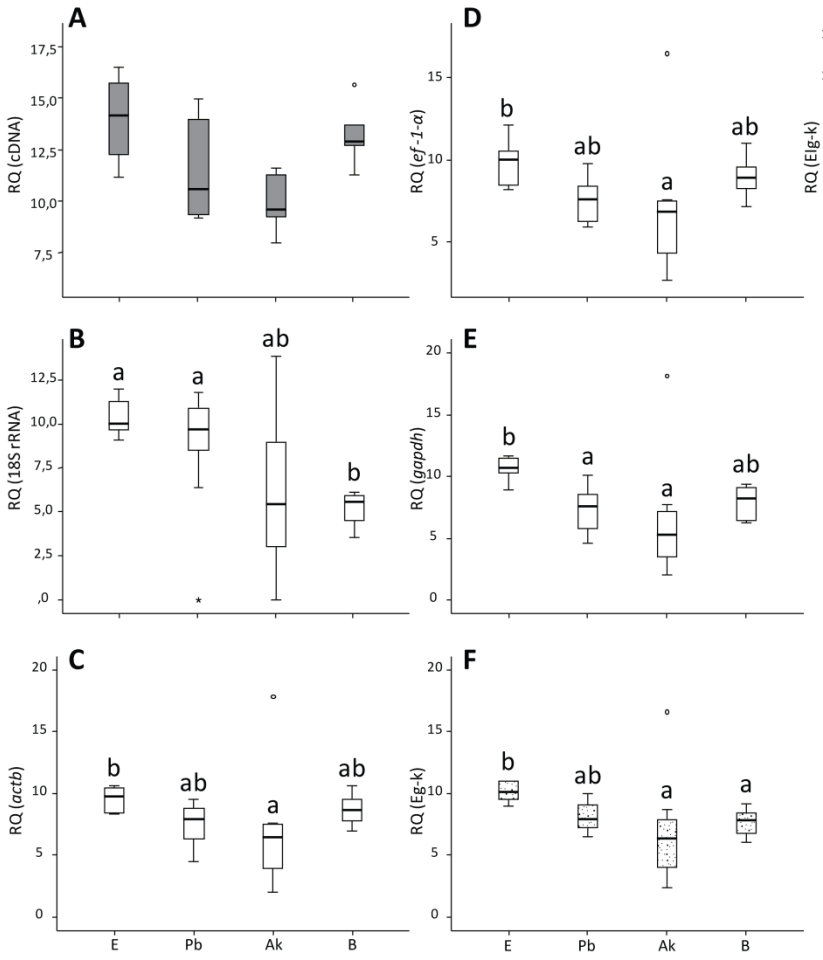
Oogenesian zeharreko itu-geneen transkripzio-mailak, cDNA-kantitatea eta erreferentzia-geneen bidezko zuzenketak erabilita

star geneak oogenesian zeharreko transkripzio-maila gero eta altuagoak azaldu zituen, B fasean maximoa zuelarik, bai cDNA-kantitatearekiko, baita *actb* eta *ef-1-α* erreferentzia-geneekiko zuzenduz (A, C eta D atalak, 6. Irudia), zein erreferentzia eta itu-gene guztien batezbestekoekiko zuzenduta ere (F eta G atalak, 6. Irudia). Aldiz, *gapdh* eta 18S rRNA-rekiko zuzentzerakoan oogenesian zehar ez zen *star* genearen transkripzio-mailen inongo aldaketarik ikusi (B eta E atalak, 6. Irudia).



6. Irudia: *star*-en transkripzio-maila erlatiboak, zuzenketarako cDNA eta erreferentzia-gene ezberdinak erabilita. Obarioaren garapen faseak X ardatzean letraz adierazi dira. RQ: (Ingelesez relative quantity) kantitate erlatiboa. Parentesi artean zuzenketarako erabili den irizpidea agertzen da; A) cDNA, B) 18S rRNA, C) *actb*, D) *ef-1-α*, E) *gapdh*, F) Eg-k eta G) Elgk. Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte taldeen artean ($p<0.05$).

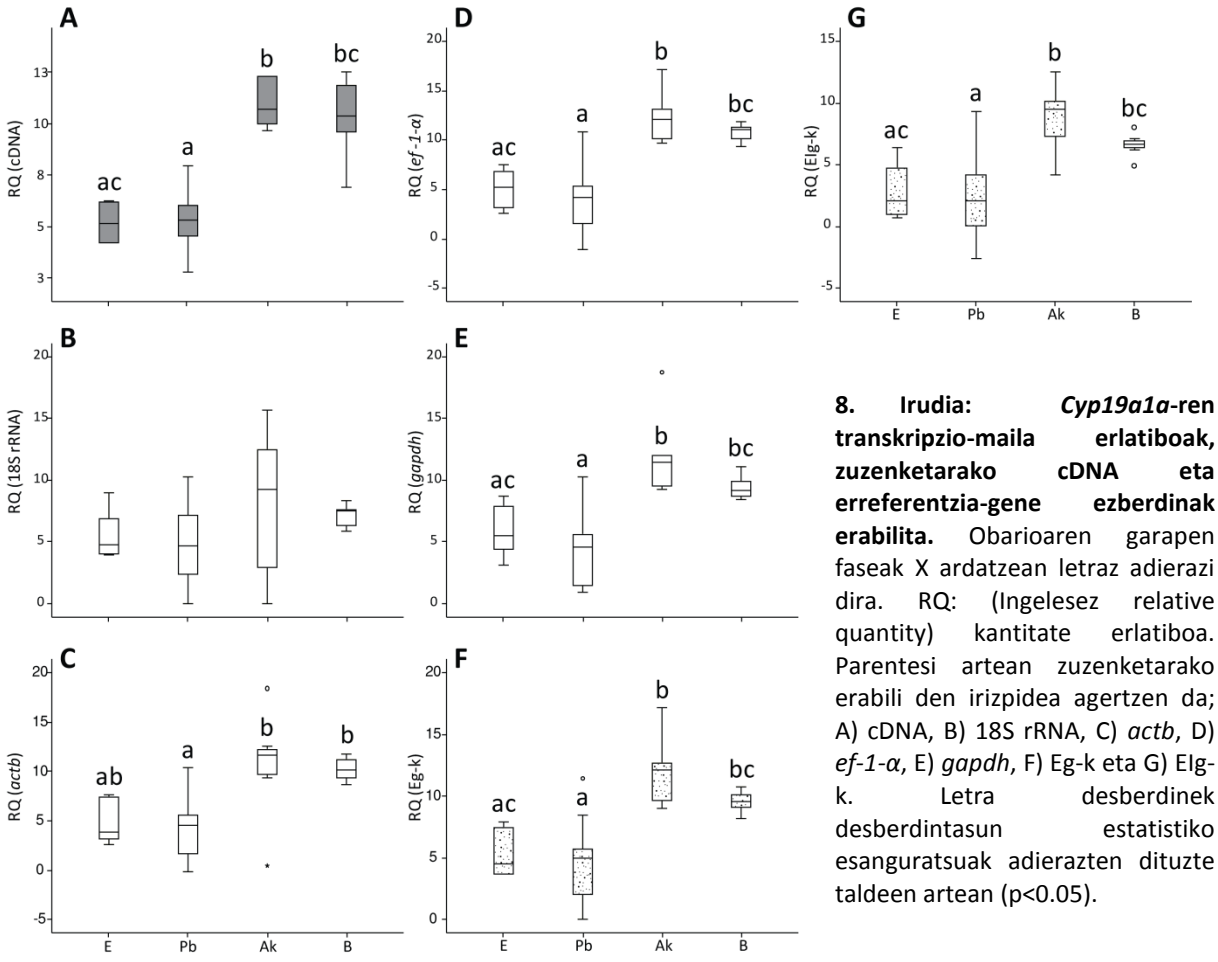
cyp11b1 genearen transkripzio-mailek ez zuten inongo aldaketa estatistikorik pairatu oogenesian zehar cDNA-kantitatearekiko zuzenketa metodoa aplikatzerakoan (A atala, 7. Irudia). Banakako erreferentzia-geneen adierazpenarekiko, zein Eg-k eta Elg-k-kiko zuzentzerakoan ere, nahiko konstante mantendu ziren genearen transkripzio-mailak ziklo osoan zehar, nahiz eta desberdintasun esanguratsuak ikusi ziren. Orokorrean harturik erreferentzia geneen bidezko zuzenketa-metodo guztiekin, 18S rRNA salbuespen (B atala, 7. Irudia), oogenesi hasieran eta fase aurreratuagoetan (Pb eta B) transkripzio-maila berdinak zeuden. Orohar, E fase inaktiboak erakutsi zituen transkripzio-maila esanguratsuki altuagoak, Ak faseak mailarik baxuenak erakutsiz.



7. Irudia: *cyp11b1*-ren transkripzio-maila erlatiboak, zuzenketarako cDNA eta erreferentzia-gene ezberdinak erabilita. Obarioaren garapen faseak X ardatzean letraz adierazi dira. RQ: (Ingelesez relative quantity) kantitate erlatiboa. Parentesi artean zuzenketarako erabili den irizpidea agertzen da; A) cDNA, B) 18S rRNA, C) *actb*, D) *ef-1-α*, E) *gapdh*, F) Eg-k eta G) Elg-k. Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte taldeen artean ($p<0.05$).

cyp19a1a-ren transkripzio-mailei zegokionez, bai cDNA kontzentrazioarekiko zein beste erreferentzia-geneen transkripzio-mailarekiko zuzentzerakoan oogenesian zeharreko transkripzio-mailaren igoera orokorra behatu zen, maximoa albeolo kortikal fasean izanik (A, C, D, E, F, G atalak, 8. Irudiak). Kasu honetan ere, 18S rRNA erreferentzia-genearekiko zuzenketak transkripzio-mailen aldaketak estali zituen, *cyp19a1a* eta 18S rRNA geneen kontrako transkripzio-mailen joeren isla (B atala, 8.irudia).

Azterturiko hiru itu-geneen transkripzio-mailak erreferentzia-geneen batezbestekoarekiko (Eg-k) eta gene guztien batezbestekoarekin (Elg-k) zuzentzerakoan, cDNA-kantitatearekiko zuzentzerakoan lortutako emaitzen parekoak izan ziren. Gainera, itu-gene bakoitzerako transkripzio-profila berdina izan zen batezbesteko metodo biekin zuzentzerakoan (F eta G atalak,6., 7. eta 8. Irudiak).



8. Irudia: *Cyp19a1a*-ren transkripzio-maila erlatiboak, zuzenketarako cDNA eta erreferentzia-gene ezberdinak erabilita. Obarioaren garapen faseak X ardatzean letraz adierazi dira. RQ: (Ingelesez relative quantity) kantitate erlatiboa. Parentesi artean zuzenketarako erabili den irizpidea agertzen da; A) cDNA, B) 18S rRNA, C) *actb*, D) *ef-1-α*, E) *gapdh*, F) Eg-k eta G) Elg-k. Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte taldeen artean ($p<0.05$).

EZTABAIDA

Zelula bakar edo zelula multzo batean ematen diren geneen qPCR bitartezko transkripzio-aldaketak aztertu nahi direnean ezinbestean datuen zuzenketa burutu behar da (Bustin et al., 2009). Zuzenketa hau erreferentzia-geneen bidez egiteko ohitura da hedatuena, non itu-genearen transkripzio-mailak erreferentzia-gene baten/batzuen transkripzio-mailekiko erlatibizatzen diren. Oro har, erreferentzia-geneek konstitutiboki eta modu egonkorrean transkribatu behar dute aztertutako lagin biologikoan eta normalean zelulen ohiko metabolismo- eta mantentze-lanetan parte hartzen dute (Zhu et al., 2008). Gene hauek kanpo zein barne faktore aldakorrekiko sentikortasun txikikoak izan behar dute. Azken baieztapen honek hala ere eztabaida handia sortu du azken urteotan; erreferentzia-geneen transkripzio-profila prozesu biologiko ezberdinetan zehar (Sellars et al., 2007; García-López et al., 2011), zein inguruneko kutsatzaileekiko esposizio baldintzen arabera, aldatzen dela frogatu delarik (Dheda et al., 2005; Arukwe, 2006;

Filby & Tyler, 2007).

Lan honetan korrokoien oogenesia izan da aztergai. Oogenesia oso prozesu aldakorra da, zeinetan obarioa sasoi bakoitzean berriztatu, hazi eta aldaketa estruktural, metaboliko, fisiologiko, zelular, zein molekular ugari jasaten dituen (Lubzens et al., 2010; Rojo-Bartolomé et al., 2016). Aldaketa prozesu guzti hauek direla eta, ulertzekoa da zaila izatea konstante mantentzen den erreferentzia-generen bat topatzea (Mauriz et al., 2012; Deloffre et al., 2012; Diaz de Cerio et al., 2012).

Erreferentzia-geneen egonkortasunaren azterketa

Lan honetan erreferentzia-geneen transkripzio egonkortasuna aztertzerakoan erabilitako metodoek 18S rRNA erreferentzia-genea aldakorrena dela ondorioztatu dute eta beraz, arrainen obarioetan oogenesian zehar geneen transkripzio-mailak aztertzerakoan erreferentzia-gene gisa erabiltzeko baztertu dute. Izan ere, nahiz eta 18S rRNA arrainetan erreferentzia-gene moduan oso erabilia izan (Jorgensen et al., 2006), oogenesiak aurrera egin ahala 18S rRNA transkripzio-mailek gora egiten dute (Kroupova et al., 2011; Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2016). Hain da aldakorra ezen oozitoen garapen fasea determinatzeko markatzaile molekular gisa ere erabili daitekeen 5S rRNA-rekin batera (Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2016).

Erreferentzia-generik egonkorrena hautatzerakoan, erabilitako tresna informatikoen arteko desadostasuna ikusi da. geNorm-ek aztertutako lau erreferentzia-geneen batezbestekoa aukeratu duen bitartean, Normfinder-rek *ef-1-α* aukeratu du zuzentze-gene egokiena moduan. Gene batek ere ez du geNorm-en egonkortasun koefiziente minimo lortu eta beraz emaitza honen arabera lan egin izan bagenu ezin izan genuen erreferentzia-generik erabili. Honen arabera, soilik hautagai guztien batezbestekoa erabilgarria izango litzateke zuzenketarako. Makailo (*Gadus morhua*) emeen garapenean 20β-hidroxiesteroide deshidrogenasa (*20ϐ-hsd*) eta *cyp19a1a* geneen transkripzioa zuzentzeko *ef-1-α* eta 18S rRNA erreferentzia-geneak aztertzerakoan ere batek ere ez zen behar bezain egonkorra (Mittelholzer et al., 2007). Are gehiago, Deloffre eta lagunek ere, geNorm erabilita, ez zuten erreferentzia-gene egokirik lortu Mozanbikeko tilapiaren

(*Oreochromis mossambicus*) oogenesia aztertzerako orduan eta soilik *actb, ef-1-α* eta D eta Z katepsinak (*ctsd, ctsz*) erreferentzia-geneen konbinaketarekin lortu zuten egonkortasun-maila onargarrietara jaistea (Deloffre et al., 2012).

Tresna informatikoen arteko desadostasun honen arrazoia, bakoitzak kalkuluak egiteko erabiltzen duen metodologian datza. geNorm-ek geneen transkripzio-profilaren antzekotasunean oinarritzen den bitartean (Vandesompele et al., 2002), Normfinder-rek taldeen arteko eta talde bakoitzaren barne aldakortasunak erabiltzen ditu (Andersen et al., 2004). Aukera bat edo bestea erabiltzeak askotan emaitza okerretara bidera gaitzake; adibidez,De Santis eta kideek (2011) barramundiaren (*Lates calcarifer*) muskulu galerarekin erlazionatutako *mstn-1* (miostatin-1) itu-genea aztertu zuten animaliak lau astez baraualdian edukita. *mstn-1* transkripzio-mailek denboran zehar igo egiten zirela ikusi zuten, cDNA eta erreferentzia-gene egonkor bakarrarekin zuzentzerakoan. Aldiz, *mstn-1* transkripzio-mailak denboran zehar egonkor mantendu ziren geNorm-ek hautatutako erreferentzia-geneekin, *ef-1-α, rpl8* (L8 proteina erribosomikoa) eta *ubq* (ubikitina), zuzenketa egiterakoan. geNorm-en aukera okerra zela ondorioztatu zuten, egonkor moduan sailkatutako geneek euren artean antzeko erregulazio patroia zutelako aukeratu baitzituen (De Santis et al., 2011). *Paralichthys olivaceus* arrainaren enbriogenesian zehar ere zortzi erreferentzia-gene aztertu ziren; *actb*, *gadph*, *ef-1- α*, 18S rRNA, *rpl7* (L7 proteina erribosomikoa), *ubce* (ubikitina-konjugazio entzima), *α-tub* (α-tubulina) eta *b2m* (β-2-mikrogobulina). Horietatik geNorm-ek *α-tub* eta *b2m* egonkor moduan sailkatu zituen ez baitzuen enbriogenesiaren garapen-fase ezberdinetan gene bakoitzak duen aldakortasuna kontsideratu. Aldiz, autoreek 18S rRNA enbriogenesian zehar erreferentzia-gene egonkorrena gisa hautatu zuten ΔΔCT metodo eraldatu bat erabiliz (Zhong et al., 2008).

Gero eta ikerketa-lan gehiagok egoera esperimental ezberdinetan erreferentzia-gene bakarraren ordez, aldi berean hainbat erreferentzia-gene erabiltzen hasi dira, MIQE araudiek gomendatzen dutena jarraituz (Vandesompele et al., 2002; Bustin et al., 2009).

cDNA-ren bidezko zuzenketa-metodoa eneen transkripzioa qPCR-an erabilitako cDNA-kantitatearekiko zatitzean datza (Rojo-Bartolomé et al., 2016). Horrela zuzendutako emaitzek generik egonkorrena *actb* dela esaten dute, *ef-1-α*-k jarraitua.. Nahiz eta

erreferentzia-geneen egonkortasuna aztertzeko metodo ez oso hedatua izan, badira lanak non cDNA-kantitatearen erabilera zeregin honetarako proposatu den. Esate baterako, Filby & Tyler-ek (2007) inguruneko hormona estrogenoek *Pimephales promelas* arrainaren gibelean eta gonadan azterketa transkripzionalak egin ahal izateko erreferentzia-gene ezberdinen erabilgarritasuna eta cDNA frogatu zituzten. Ikerketa honetan itu-geneak cDNA-kantitatearekiko eta erreferentzia-gene egonkorrekiko zuzentzerakoan transkripzio-profil berdinak eta biologikoki baliogarriak zituztela ohartu ziren.

Oogenesian zehar erreferentzia-geneen transkripzio-mailak cDNA-kantitatea eta erreferentzia-gene bakarraren bidezko zuzenketa-metodoak erabilita

Erreferentzia-gene bakoitza itu-gene moduan aztertuz lehenego ondorio nabari bat ateratzea dago; 18S rRNA erreferentzia-gene moduan erabiltzekotan, aztertu diren gene guztiak oogenesiak aurrera egin ahala, negatiboki erregulatuta daudela ondoriozta zitekeen, obarioan 18S rRNA-ren transkripzioa benetan erregulatuta dagoelako. 18S rRNA-rekin lorturiko emaitzek, egokia ez den erreferentzia-gene bat aukeratzeak emaitzen interpretazioan izan dezakeen eragina erakusten du (De Santis et al., 2011).

ef-1-α eta *actb*, ikerketa honetan generik egonkorrenak izanik, batak-bestearekiko zuzentzerakoan oogenesian zeharreko transkripzio-maila konstanteak ikusi dira, cDNA-rekin ere ikusi izan den moduan. Beraz, bi gene hauek erreferentzia-gene egokiak dira korrokoiaren oogenesian zehar geneen transkripzio-mailen azterketan aplikatzeko. *gapdh* itu-gene gisa aztertzerakoan ere, oogenesian zehar joera nahiko konstantea mantentzen duela ikusi da. Hala ere, *actb* eta *ef-1-α* erreferentzia-geneekiko zuzentzerakoan, erregresio fasean *gapdh* generaren azpi-adierazpen bat dagoela aditzera ematen dute. Transkripzio-mailaren aldaketa hau nabarmena da *actb* eta *ef-1-α* itu-gene moduan aztertzerakoan eta *gapdh* transkripzio-mailarekiko zuzentzerakoan ere. Beraz, nahiz eta *gapdh* transkripzio-mailak cDNA kantitatearekiko zuzentzerakoan konstante mantendu eta *gapdh*-k geNorm-en aldakortasun koefiziente txikienetarikoa eduki, gene honek kasu honetan erregresio fasean dueneko aldakortasun altua dela eta,

erreferentzia-gene ez egoki modura sailkatzera darama. *gapdh* beraz oogenesian aldakorra da bestelako ikerketa lanetan ere ondorioztatu den bezala (Filby & Tyler, 2007; Deloffre et al., 2012).

Oogenesian zeharreko itu-geneen (*star*, *cyp11b1*, *cyp19a1a*) transkripzio-mailak : zuzenketa-metodo ezberdinen arteko konparaketa

Ikerketa lan honetan frogatua geratu da erreferentzia-gene bakarra erabiltzeak ekar dezakeen arriskua. Pentsatzekoa da erreferentzia-geneen batezbestekoa egiterakoan egonkortasuna emendatzea (Bustin et al., 2009) eta aldiz, balioztatu ez diren itu-geneak zuzenketara gehitzerakoan egonkortasun hori galtzea. Hala ere, eta geNorm eta Normfinder-en emaitzak kontrajarriz, gene guztien batezbestekoa zuzenketara gehitzean ez da egonkortasunik galdu eta lortutako emaitzak cDNA-kantitatea eta erreferentzia-gene egonkorrekin lortutakoen berdinak dira.

18S rRNA-ren bidezko zuzenketak ematen dituen emaitza okerrak eta *gapdh*-rekiko zuzentzetak eragiten duen desberdintasun eza alde batera utzita, *star*-genearene transkripzio-mailek oogenesian zehar goranzko joera azaltzen dute zuzenketa-metodo guztiekin. Aurretik ikusi da *star*geneak korrokoiaren zein ortzadar-amuarrainaren (*Oncorhynchus mykiss*) obarioaren heltze-prozesuan zehar gain-adierazten dela (Nakamura et al., 2005; Sardi et al., 2015). *star* genearen mRNA-mailak bitelogenesian zehar igo egiten dira obulazioan mailarik altuenak azalduz. Igoera hauek plasma mailan sexu-hormona esteroideen igoerarekin bat dator (Sardi et al., 2015). *cyp11b1*-k transkripzio-mailak erregresio fasean dira altuen eta ez dira oogenesian zehar aldatzen edozein zuzenketa-gene erabiltzen delarik ere. cDNA-kantitatearekiko zuzentzerakoan aldiz, aztertutako fase guztietan transkripzio-maila konstanteak erakusten ditu. c*yp11b1* sexuaren araberako adierazpena duen genea da. Orokorrean, *cyp11b1* geneak kodeturiko entzimak androgenoen sorreran jarduten du eta arrainen testikuluan adierazten da, obarioetan adierazpen baxua edo adierazpenik erakusten ez duelarik oogenesi osoan zehar (Blázquez et al., 2009; Zhang et al., 2010; Sardi et al., 2015). *cyp19a1a* geneak ere, *star* geneak bezala gain-adierazpenerako joera erakusten du oogenesian zehar, hau zuzenketa-metodo guztiekin ikusten delarik. Gene honen transkripzio-maila maximoak bitelogenesiarekin bat datoz (Sardi et al., 2015). Errutearen

ostean transkripzio-mailak behera egiten dute. Aromatasa entzima kodetzen du gene honek, zeinak C19 androgenoak (testosterona eta androstenediona) C18 estrogeno (estradiol eta estrona) bilakatzeaz arduratzen den (Villeneuve et al., 2006). *cyp19a1a* obarioaren desberdintzapenerako ezinbestekoa da (Guigen et al., 2010) eta estrogenoen plasma mailak erregulatzeaz arduratzen da (Villeneuve et al., 2010).

Gauzak horrela, oso garrantzitsua da erreferentzia-geneak erabili aurretik balioztatzea eta ahal den heinean erreferentzia-gene bakarra edo soilik bibliografian oinarrituriko generik ez aukeratzea (Filby & Tyler, 2007; Rhinn et al., 2008; Deloffre et al., 2012). Izan ere, normalean erabiltzen diren erreferentzia-geneak unibertsalak ez direla argi geratu da (Deloffre et al., 2012). Adibidez, Filby & Tylerrek (2007) *Pimephales promelas* espezieko aleak estrogenoetara esposatu zituzteneko esperimentuan 18S rRNA erreferentzia-gene egokiena zela ondorioztatu zuten, aldiz *gapdh*-ren transkripzioa estrogenoen aurrean aldakorra zen, oraingo lan honetan gertatu ez den moduan. Mittelholzer eta kideek (2007) arrainen obarioen garapena aztertzeko 18S rRNA, *ef-1-α* eta *actb* testatu zituztenean, 18S rRNA eta *ef-1-α*-geneen transkripzio-mailen aldakortasuna iragarri zuten. Deloffre eta kideek (2012) arrainen oogenesian zehar erreferentzia-gene moduan *ef-1-α* eta *actb* testatu zituzten ere, aurretik zuzenketarako 18S rRNA eta *gapdh* geneen erabilpena baztertu ostean. Kasu honetan, geNorm eta Normfinderrek *ef-1-α* eta *actb* geneak adierazpen egonkorrekoak sailkatu zituzten arren, euren transkripzio-mailak oogenesian zehar aztertzerakoan, bitelogenesi fasean aldatu egiten zirela ikusi zuten. Fase horretan ematen den estradiol-maila altuari egotzi zioten aldakortasun hori (Deloffre et al., 2012).

Lan honek *actb, ef-1-α*, cDNA kantitatea, lau erreferentzia-gene eta gene guztien batezbestekoak (lau erreferentzia-gene eta hiru itu-gene), kasu praktiko honetan itu-geneen transkripzio-mailak zuzentzeko erabilgarriak direla frogatu du. Hala ere, itu-geneen transkripzioa erreferentzia-geneekiko zuzentzea aukera garestia eta astuna da (Libus & Štorchová, 2006). Horrez gain, ez da erreza arrain espezie ezberdinetan, kutsatzaileekiko esposizio-esperimentuetan (adibidez (xeno)estrogenoekin) eta batez ere garapenarekin edo oogenesiarekin erlazionaturiko geneen transkripzio-mailak zuzentzerakoan erreferentzia-gene egokiak lortzea (Von Schalburd et al., 2005; Filby & Tyler, 2007; Luckenbach et al., 2008a; Luckenbach et al., 2008b; Tingaud-Sequeira et al.,

2009), askotan sekuentzia informazio hori ez baitago eskuragarri interesezko espezierako (Mittelholzer et al., 2007). Horregatik, zuzenean aztergai diren itu-geneen transkripzio-mailen batezbestekoa zuzenketarako erabiltzea aukera berri moduan plazaratu da (Heckmann et al., 2011). Hala ere, metodo honek ere geneen konbinaketa posibleak testatzeko analisiak egitea eskatzen du kasu praktiko bakoitzean, eta horrek denbora kostua dakar.

Azkenik, cDNA-kantitatearen bidezko geneen transkripzioaren zuzenketa-metodoaren erabilirarekin, esperimentazioan zehar sor zitezkeen prozedura-akatsak saihesten dira, biologikoki koherenteak diren emaitzak lortuz baliabide gutxi erabiliz, modu errazean eta geneen transkripzio aldakortasunarekiko era independentean (Libus & Štorchová, 2006; Filby & Tyler,2007; Rhin et al., 2008; De Santis et al., 2012; Rojo-Bartolomé et al., 2016). Hau guztia dela eta, lan honek geneen transkripzioaren zuzenketa burutzeko cDNA-kantitatea erabiltzea gomendatzen du, bai arrainen oogenesian zeharreko azterketetarako (Rojo-Bartolomé et al., 2016), zein bestelako ikerketetan ere.

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Chapter 2

Identification of sex and female's reproductive stage in commercial fish species through the quantification of ribosomal transcripts in gonads

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Combined EAS (Aquaculture Europe Meeting) and WAS (World Aquaculture Meeting), Prague, (Check Republic), 2012. Identification of biomarkers for sex and fecundity and skeletal muscle texture in teleost fish. Cancio, I., Diaz de Cerio, O, Rojo-Bartolomé, I., Bizarro, C., Ortiz-Zarragoitia, M., Wang, P.A., Olsen, R.L., Erikson, U., Martinez, I. ICC, oral

17th INTERNATIONAL SYMPOSIUM ON POLLUTANT RESPONSES IN MARINE ORGANISMS (PRIMO17), Faro (Portugal). 2013. 5S/18S rRNA index in gonads; a powerful method to identify sex, female reproductive stage and xenoestrogenicity in fish. Rojo-Bartolomé I; Díaz de Cerio, O; Bizarro, B; Ortiz-Zarragoitia, M; Cancio I. ICC, panel

4th INTERNATIONAL WORKSHOP ON THE BIOLOGY OF FISH GAMETES. Albufeira, (Portugal). 2013. 5S/18S rRNA index in gonads as a universal method for the determination of oocyte maturity and female reproductive stage in fish. Rojo-Bartolomé I; Díaz de Cerio, O; Ortiz Zarragoitia, M; Cancio I. ICC, panel

10th INTERNATIONAL SYMPOSIUM ON REPRODUCTIVE PHYSIOLOGY OF FISH, Olhao, (Portugal). 2014. 5S rRNA in gonads and the molecular identification of sex and female reproductive stage in commercial fish species. Rojo-Bartolomé, I; Diaz de Cerio, O; Cancio, I. ICC, panel

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LABURPENA

Itsasoko baliabideen ustiaketa optimizatzeko politikaren bidean, gure arrain-erreserbetako aleen heldutasun-maila eta sexua estimatzea ezinbesteko baldintzak dira Europako Datu Bilketarako Programarako. Bestalde, gero eta gehiago arrainen biologiarekin erlazionatutako ikerketek biologia molekularrean oinarritzen hasi dira arrainen sexua eta ugalketa fasea identifikatzeko garaian. Arrainen sexu-desberdintzapenean eta gametogenesian zehar ematen diren geneen transkripzio-diferentzialak aprobetxatuz, 5S RNA erribosomikoa eta *gtf3a* transkripzio-faktore orokorrak Bizkaiko Golkoan arrantzatutako garrantzi ekonomikoko arrain espezieen sexua eta emeen gametoen garapena molekularki sailkatzeko zuten balioa ikertu zen. 9 arrain-espezieen (berdela, estornino, txitxarroa, lirioa, boga, antxoa, legatza, sardina eta gailoa) gonadak histologikoki sexatu ziren eta euren 5S rRNA eta 18S rRNA-ren RNA kontzentrazioak elektroforesi kapilarraren bidez neurtu ziren. Neurtutako rRNA-mailekin 5S/18S rRNA indizea kalkulatu zen. Hasle degeneratuek *gtf3a* genearen transkriptozatiak klonatzea eta sekuentziatzea ahalbidetu zuten ikertutako 7 espezieetan. 5S rRNA eta *gtf3a* transkriptoek, 5S/18S rRNA indizearekin batera, obarioak testikuluetatik bereizi zituzten ikertutako espezie guztietan, beti ere emeek arrek baino balio altuagoak aurkezten zituztelarik. Horrez gain, 5S/18S rRNA-ren balioak emeetan altuagoak zirela ikusi zen, arrainak obarioaren garapen-fase goiztiarretan harrapatuak izan zireneko kasuetan. Aldiz, arrainak bitelogenesian zeudenean, balio hauek baxuagoak izan ziren. Gailo eta antxoetan, non oogenesiaren fase ezberdinetako oozitodun obarioak lortu ziren, 5S/18S rRNA indizeak gametogenesi-fasea identifikatzeko balio duela erakutsi zuen. Hurbilketa honek, zeinak arrainak sexatzeko eta emeen gametogenesi-fasea identifikatzeko balio duen, arrain-stockaren kudeaketaren, arrainen ugalketaren eta emankortasunaren azterketan eta arrainen biologiaren ikerketan orokorrean lagungarria izan daiteke.

ABSTRACT

The estimation of maturity and sex of fish stocks in European waters is a requirement of the EU Data Collection Framework as part of the policy to improve fisheries management. On the other hand, research on fish biology is increasingly focused in molecular approaches, researchers needing correct identification of fish sex and reproductive stage without necessarily having in house the histological know-how necessary for the task. Taking advantage of the differential gene transcription occurring during fish sex differentiation and gametogenesis, the utility of 5S ribosomal RNA (5S rRNA) and General transcription factor IIIA (*gtf3a*) in the molecular identification of sex and gametogenic stage was tested in different economically-relevant fish species from the Bay of Biscay. Gonads of 9 fish species (Atlantic, Atlantic-chub and horse mackerel, blue whiting, bogue, European anchovy, hake and pilchard and megrim), collected from local commercial fishing vessels were histologically sexed and 5S and 18S rRNA concentrations were quantified by capillary electrophoresis to calculate a 5S/18S rRNA index. Degenerate primers permitted cloning and sequencing of *gtf3a* fragments in 7 of the studied species. 5S rRNA and *gtf3a* transcript levels, together with 5S/18S rRNA index, distinguished clearly ovaries from testis in all of the studied species. The values were always higher in females than in males. 5S/18S rRNA index values in females were always highest when fish were captured in early phases of ovary development whilst, in later vitellogenic stages, the values decreased significantly. In megrim and European anchovy, where gonads in different oogenesis stages were obtained, the 5S/18S rRNA index identified clearly gametogenic stage. This approach, to the sexing and the quantitative non-subjective identification of the maturity stage of female fish, could have multiple applications in the study of fish stock dynamics, fish reproduction and fecundity and fish biology in general.

INTRODUCTION

There are more than 30000 fish species inhabiting a wide range of aquatic habitats worldwide (Devlin & Nagahama, 2002), some of them being very important in the global economy and human diet. Moreover, as key integral members of ecosystems, fish are also becoming increasingly important sentinels of environmental health (Ortiz-Zarragoitia et al., 2014). Some fish are also relevant laboratory models for the analysis of different developmental processes in vertebrates.

In recent years, as part of the policy to improve fisheries management, the estimation of maturity and sex ratio of fish stocks in European waters has become a fundamental requirement of the European Data Collection Framework (http://ec.europa.eu/fisheries/cfp/fishing_rules/data_collection /index_en.htm). Due to the overexploitation of fish from ocean sources some extractive fisheries have reached a plateau or are depleted. The impossibility of fish populations to breed on time is resulting in a significant loss of potential yield. In this scenario, it is imperative to study the reproduction biology of fish stocks, understand the population dynamics and the different changes that can occur in their life history (Morgan, 2008). Moreover, as reproduction largely determines productivity and the resilience of populations, it is crucial to estimate the quality and quantity of gametes. These are indicators of the reproductive capacity of commercial fish populations towards scientifically-based fisheries management (Morgan, 2008; Murua et al., 2003).

Presently, the allocation of maturity and sex in some fish species is laborious and requires a large number of sampled target organisms for visual and/or histological analysis of their gonads (Morgan, 2008; Murua et al., 2003). This task is difficult when oceanographic campaigns do not coincide with the spawning season of the species under investigation and their gonads are poorly developed. In addition, many reproductive traits are highly variable and life history parameters (such as maturity at size or age, sex ratio, fecundity and spawning time/fraction) vary between species and populations or temporally within a single population (Morgan, 2008; Murua et al., 2003). It should not be overlooked either that sex-determining genetic systems are highly diversified in teleosts: environmental factors play a major role in sex differentiation

(Devlin & Nagahama, 2002). All of these factors together highlight the importance of studying the molecular and cellular mechanisms that drive sexual differentiation in fish. Most particularly, the growth and maturation of oocytes in females (Murua & Saborido-Rey, 2003; Prager et al., 2003) to determine the most efficient way to exploit and conserve different fish stocks.

On the other hand, fish biologists are increasingly applying molecular approaches in basic research studies to answer questions related to metabolism, reproduction, vertebrate development or to environmental stress and disease responses (Devlin & Nagahama, 2002; Ortiz-Zarragoitia et al., 2014; (WHO/IPCS, 2002; Lubzens et al., 2010). Many molecular approaches need that the sex and the developmental stage of the particular individual under study are known, sometimes in the absence of the histological material necessary to obtain this knowledge. In these circumstances a molecular method towards fish sexing, carried out in the same biological material prepared for the specific methodological approaches of interest, would be advantageous (Espigares et al., 2015).

Sex differentiation starts when primordial germ cells initiate their differentiation into either female or male germ line stem cells. During gametogenesis, oocytes and spermatozoids undergo diverse molecular and structural changes. Molecular changes during teleost oogenesis, for example, include variations in the level and nature of gene expression and in the accumulation of reserve substances (RNA, proteins, lipids, carbohydrates and hormones) necessary for early embryo development (Lubzens et al., 2010). These molecules will be incorporated into the oocytes from surrounding ovarian follicular cells and from other organs such as the liver, which mobilises vitellogenin, a phospholipoprotein that is used in pollution monitoring as a biomarker of exposure to xenoestrogens in males and juveniles (WHO/IPCS, 2002; Puy- Azurmendi et al., 2013).

5S ribosomal RNA (5S rRNA) is the smallest rRNA of the ribosome large subunit in eukaryotic ribosomes. Two types of tandemly-arranged 5S rRNA genes have been described for the anuran *Xenopus laevis*: one is expressed in somatic cells and testes; the other only in oocytes (Allison et al., 1995; Szymański et al., 2003). Similar organization with two paralogue gene classes has been described in fish, such as the

tench (*Tinca tinca*), hakes or mullets, amongst others (Denis & Wegnez, 1977; Campos et al., 2009). Intench, 5S rRNA has been described as constituting, together with tRNAs, 90% of the RNA content of the ovaries (Denis & Wegnez, 1977; Mazabraud et al., 1975). Whilst the precursor of the other ribosomal RNAs, 45S pre-ribosomal RNA (45S pre-rRNA) is transcribed by the RNA polymerase I (Pol I) in the nucleolus, 5S rRNA gene is transcribed by RNA polymerase III (Pol III) (Szymański et al., 2003). The activity of Pol III is regulated positively by the General transcription factor IIIA protein (Gtf3a), which also binds 5S rRNA transcript to assist in its stockpiling in the form of small 7S ribonucleoprotein particles in the cytoplasm (Szymański et al., 2003; Penberthy et al. 2003). In the case of being fertilized, the accumulated 5S rRNA molecules are incorporated into the nucleolus to initiate ribosome assembly. Thus, interactions of 5S rRNA with Gtf3a and ribosomal proteins are crucial for the regulation of 5S rRNA biosynthesis (Szymański et al., 2003); this implies that, during expression of 5S rRNA genes in oocytes, a massive accumulation of *gtf3a* transcript occurs (Ortiz-Zarragoitia et al., 2014; Diaz de Cerio et al., 2012). It has been shown that, in early oogenesis, *gtf3a* constitutes more than 10% of the total cytoplasmic protein. This decreases 5-10 folds in later stages (Szymański et al., 2003).

Based upon the different gene expression patterns in testis and ovary, these molecules can be diagnostic of the sex of the studied fish (Ortiz-Zarragoitia et al., 2014; Diaz de Cerio et al., 2012). We have shown previously that the accumulation of 5S rRNA and the high transcription levels of accompanying proteins in ovaries permit the identification of sex in thicklip grey mullets during their whole annual reproductive cycle (Diaz de Cerio et al., 2012). Moreover, as 5S rRNA and *gtf3a* transcript levels in oocytes constitute also strong markers of xenoestrogenicity, identifying intersex testis in mullets inhabiting polluted estuaries (Diaz de Cerio et al., 2012). It might be suggested that 5S rRNA and *gtf3a* constitute useful sex and oocyte maturity markers in teleost fish (Ortiz-Zarragoitia et al., 2014). Very recently, Espigares et al. (2015) used the accumulation of 5S rRNA to sex their experimental prepubertal European sea bass in the absence of biological material to carry out any histological analysis.

Within this context, the objectives of the present research are centered upon the study of 5S rRNA and *gtf3a* transcript levels as oocyte markers in several commercial teleost

fish species captured in the Bay of Biscay (ICES Subareas VIII a, b, c and d; http://www.fao.org/fishery/area/Area27/en), then landed in the Basque Country fishing ports. Amongst the most important species in 2013 anchovy and pilchard constituted for the artisanal fleet 57% of the landings and 69% of the income or first sale value. European hake and blue whiting represented, for the bottom trawler fleet, 64% and 70% of the landings and income, respectively. Therefore, these species were chosen for their additional interest as economically relevant species in the Bay of Biscay, and due to the possible implications that studies on their reproduction could have for fisheries stock management.

MATERIALS AND METHODS

Biological samples

All of the fish samples were obtained in the fish market of Ondarroa immediately after the fish were brought from the sea by commercial vessels that operate on working shifts of one day at sea. Samplings for each species depended on the season in which the different fisheries were open to the commercial fleet (Table 1). The species collected were: Atlantic mackerel (*Scomber scombrus*); Atlantic chub mackerel (*Scomber colias)*; blue whiting (*Micromesistius poutassou*); bogue (*Boops boops*); European anchovy (*Engraulis encrasicolus*); European hake (*Merluccius merluccius*); European pilchard (*Sardina pilchardus*); horse mackerel (*Trachurus trachurus*); and megrim (*Lepidorhombus whiffiagonis*).All individuals were dead on arrival at the harbour; they were kept on ice until dissection within 24 hours following their capture. On each sampling occasion around 20 individuals were measured and weighted, with the gonads being extracted (Table 1). Gonads were weighed and divided into two parts. One part was embedded in RNAlater® (Ambion, Life technologies), frozen in liquid nitrogen and then stored at -80°C until used further. The other part was fixed in 10% neutral buffered formalin containing 1% glutaraldehyde. All chemicals were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) unless specified otherwise.

Table 1. Fish species studied in the present work with common and scientific names, capture locations and dates within the Bay of Biscay, number (n) of sampled individuals, sex ratio and histologically identified gametogenic stage according to the developmental stage of the oocytes in females (PV= previtellogenic, CA= cortical alveoli, AV= advanced vitellogenesis and Hy: hydrated).

Common name	Scientific name	Origin (ICES Subarea)	Capture month	(n)	Sex ratio (F/M)	Gametogenic stage
Atlantic chub mackerel	*Scomber colias*	VIII b	November 2011 & May 2014	14	8/6	PV
Atlantic mackerel	*Scomber scombrus*	VIII b	February 2012	13	7/6	AV
Blue whiting	*Micromesistius poutassou*	VIII c	May 2012	15	13/2	PV
Bogue	*Boops boops*	VIII b	May 2014	10	8/2	PV
European anchovy	*Engraulis encrasicolus*	VIII c	April & May 2012	31	15/16	AV & Hy
European hake	*Merluccius merluccius*	VIII b	March 2011	11	7/4	PV
European pilchard	*Sardina pilchardus*	VIII b	November 2011	12	7/5	Hy
Horse mackerel	*Trachurus trachurus*	VIII b	February 2012 & May 2014	24	7/17	PV
Megrim	*Lepidorhombus whiffiagonis*	VIII b	February, May & November 2012	31	26/6	PV, CA & AV

Histological analysis

After 24 hours 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, Wehrheim, Germany). Resin sections (5 µm) were cut in a 2065 Supercut microtome (Leica Instruments GmbH, Nussloch, Germany) andstained with hematoxylin/eosin. Sex and gamete developmental stage were determined microscopically, following the gametogenic stage grading described by McDonough et al. (2005), and adapted for fish species with asynchronous developing gonads (Table 1).

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

Total RNA was extracted from 50-100 mg of tissue using TRIzol® (Invitrogen, Carlsbad, California, USA) 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 quality RNA established at 260/280 and 260/230 ratios around 2) was loaded in an Agilent RNA 6000 Nano Kit Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Electropherograms provided by the Bioanalyzer were used to quantify the concentration of the bands corresponding to 5S rRNA and 18S rRNA in each sample. The Time Corrected Area of each peak was used to calculate the 5S/18S rRNA ratio. When the presence of one of the rRNAs was below the levels of detection of the machine, 5S rRNA in the case of a few males and 18S rRNA in the case of a few females, a 0.1 value was given to each sample instead of 0 (the lowest recordable value was 0,2). The logarithm of the ratio was calculated in order to develop an index that allowed clear visualization of the differences between testes and ovaries.

This study was also extended to the gonads of 5 female and 5 male adult zebrafish (*Danio rerio*; UB Tubingen) from our own stock. Zebrafish individuals were euthanized by an overdose of MS-222 (tricaine methane-sulfonate) following the protocol authorised by the ethics commission of the University of the Basque Country CEEA/337-2/2014/ORTIZ ZARRAGOITIA. Gonads were dissected, immersed in RNA Later® (Ambion) and immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction. These zebrafish samples served as controls, whose RNA profiles could be compared with those extracted from the commercial fish species that were not dissected directly upon capture.

gtf3a cloning and sequencing

2 µg of total RNA from each individual gonad were used for cDNA synthesis with AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) using random primers.

gtf3a mRNA fragments were amplified using conventional PCR and employing 0.8 mM degenerate primers: forward 5´-TGGARGCTCATCTKTGCAAACACAC-3´ and reverse 5´-GTCYCCDCAGGCTYTCCTTCATG-3´. Primers were designed through alignment (Clustalw2, http://www.ebi.ac.uk/Tools/msa/clustalw2/) of piscine *gtf3a* sequences available in Ensembl and GenBank and searching for highly conserved nucleotide regions. Properties of designed primers were checked employing the IDT OligoAnalyzer Tool (https://eu.idtdna.com/calc/analyzer).

The amplification was run with commercial Taq DNA Polymerase, recombinant Kit and 100 mM dNTP Mix (Invitrogen) for 35 cycles in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad, California, USA). PCR procedure was as follows: 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 58°C (Tm) for 30 seconds, elongation at 72°C for 8 minutes and finally 72°C for 8 seconds. PCR products were stored at 4°C until they were analysed by electrophoresis in ethidium bromide stained agarose gels (1.5%) and sent to the SGIker Sequencing Service of the University of Basque Country for sequencing. Once sequenced, fragments were aligned to obtain a consensus sequence and analyzed using CAP3 (http://pbil.univ-lyon1.fr/cap3.php) and ClustalW2 tools.

All sequences obtained have been published in GenBank for Atlantic chub mackerel (JQ928632); Atlantic mackerel (JQ928631), blue whiting (KC191719), European hake (JQ928630), European pilchard (JQ928634), horse mackerel (KC191721) and megrim (JQ928633).

Real time PCR

cDNA obtained from megrim was quantified in the Synergy HT Multi-Made Microplate Reader (BioTek, Winoosky, USA) by Quant-iT™ OliGreen® ssDNA Assay Kit (Invitrogen, Life Technologies). The quantification was performed in a reaction volume of 100 µl with a theoretical cDNA concentration range of 0.02-0.2 ng/µL, at 485/20 nm excitation and 528/20 nm emission wavelengths. Real PCR input cDNA concentration was calculated using the high-range standard curve according to the manufacturer's instructions.

5S and 18S rRNA transcript levels were quantified in megrim females by SYBR® Green qPCR (Roche, Basel, Switzerland). qPCR was conducted in triplicates using a 7300 Applied Biosystems Thermocycler (Applied Biosystems). The 20 µl PCR reaction mixture consisted of 10 µl of 2× SYBR® Green PCR master mix, appropriate concentration of 5S and 18S rRNA primers diluted in RNase-free water (final primer concentration: 12.5 nM) and a 2 µl cDNA template. 5S rRNA primers are protected under Spanish patent P201130778 and international patent PCT/ES2012/070343. 18S rRNA primers were: forward 5'-CCTTTTAACGAGGATCCA-3'; and reverse 5'-ACGGCTACCACATCCAA-3' (Tm: 55°C). qPCR procedure was as follows: 50°C for 2 minutes, 95°C 10 minutes, then 40 cycles of denaturation at 95°C for 15 seconds and annealing temperature for one minute. Dissociation stage was added at the end with 95°C for 15 seconds, one minute in annealing temperature, 95°C for 15 seconds and a final step of 60°C for 15 seconds.

gtf3a transcript levels were quantified also in megrims using Probe #76 from the Universal Probe Library (Roche) according to the manufacturer's instructions; for 40 cycles and with 500 nM of the specific primers designed in Universal ProbeLibrary Assay Design Center (Roche): forward 5'-CAGCACCAAGAGAAGCGATA-3' and reverse 5'-TGGTTCCTCTTGTTGAAATCC-3', Tm: 60°C. All gene transcription results were normalized with the amount of cDNA charged in the qPCR (input cDNA, (Filby & Tyler, 2007)) using for that a ΔCT formula adapted from the ΔΔCT normalization method:

$$RQ = Log_2\left[\frac{(1+Efficiency)^{-\Delta CT}}{ng\ cDNA}\right]$$

Where $\Delta CT = CT\ sample - CT\ plate\ internal\ control$

Statistical Analysis

The statistical analyses were undertaken using SPSS (SPSS Inc., Chicago, Illinois). Significant differences in RNA levels between both sexes were evaluated by the non-parametric Mann-Whitney U-test. Significant differences in RNA levels, when ovarian developmental stages (and testis) in megrim were compared, were analysed applying one-way ANOVA. Significant differences between groups, in terms of qPCR gene transcription levels, were evaluated using the non-parametric Jonckhere-Terpstra test when *a priori* ordering was assumed for more than two independent samples (PV>CA>AV). In all cases, significant differences were established at $p< 0.05$.

RESULTS

5S rRNA in gonads: identification of sex in teleost fish

The electropherograms obtained from gonads of commercially-relevant fish species showed different RNA patterns when comparing total RNA extracted from adult testes and ovaries. No signs of RNA degradation, clearly observable in Agilent RNA chips run in the 2100 Bioanalyzer, were observed in any of the samples. In the case of ovaries, the relative 5S rRNA signal was always higher than in the testes. When ovaries presented oocytes in previtellogenic stages the 5S rRNA peak was higher than those of 18S and 28S rRNAs, which are prevailing in all eukaryotic cells (Figs 1, S1 and S2 Figs). The relative amount of 18S and 28S rRNA in ovaries becomes more similar to that of the testis the more advanced they are in the oogenesis process (Fig 1).

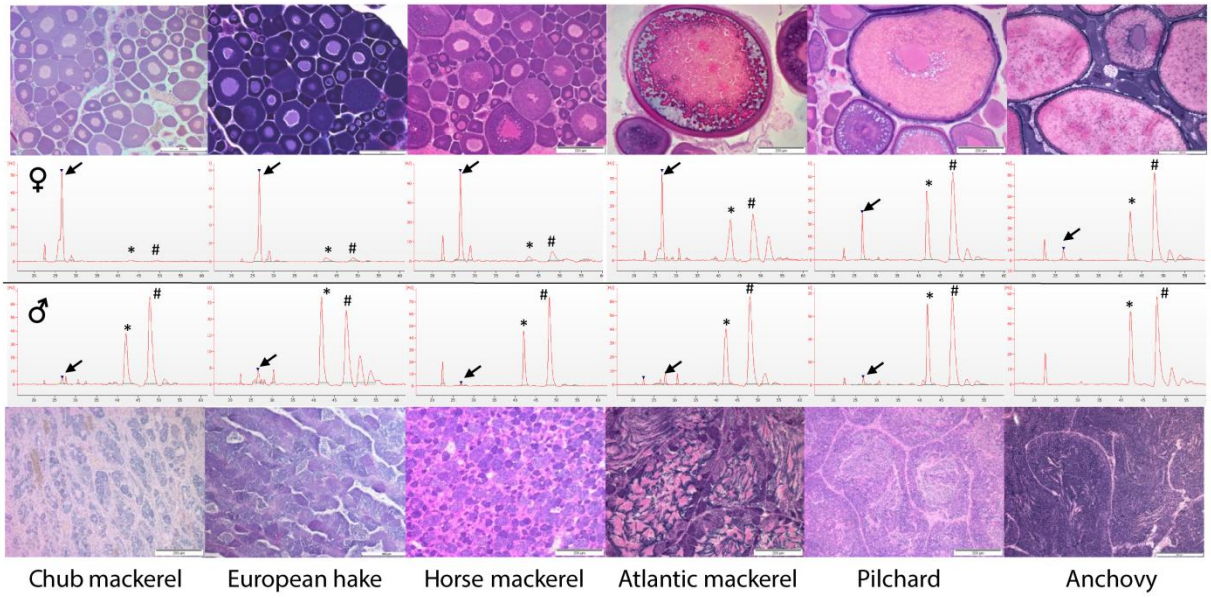

Fig 1. Representative electropherograms of gonad total RNA of different fish species. Electropherograms of gonad total RNA from previously histologically sexed male (bottom) and female (top) Atlantic chub mackerel, European hake, horse mackerel, Atlantic mackerel, European pilchard and European anchovy. Electropherograms and histological micrographs (in all cases scale bars = 200 μm) are representative of all the individuals sampled and analysed per species. Oocytes in ovaries of chub mackerel, hake, and horse mackerels were at previtellogenic stage, in advanced vitellogenesis in Atlantic mackerel and hydrated in the case of pilchard and anchovy. Notice that 5S rRNA in ovaries was relatively the most prominent in the species where ovary only displayed previtellogenic oocytes. Arrows indicate the 5S rRNA peak. * = 18S rRNA peak, # = 28S rRNA peak.

5S/18S rRNA index: identification of oogenesis stage

The quantification of the concentrations of 5S and 18S rRNA in the electropherograms permitted the calculation of a 5S/18S rRNA index. This index separated males from females in all of the species being studied (Fig 2), including the model species zebrafish. Zebrafish from our own stock allowed obtaining freshly prepared gonad samples. Electrophoretic profiles corroborated that profiles observed with RNA extracted from organs harvested from commercial fish species upon collection at the harbour were not the result of RNA degradation. When females were captured during advanced oocyte developmental stages (Atlantic mackerel, European anchovy and pilchard and zebrafish) the ratio was significantly lower than in those captured during resting/previtellogenic stages (blue whiting, bogue, chub and horse mackerel, European hake). The index

threshold value, which permitted sex discrimination, was species and gametogenic stage dependent (Fig 2). This observation was confirmed in megrim where, as a result of three different samplings, females with oocytes in previtellogenic, in cortical alveoli and in advanced vitellogenic stages were available (Fig 3). The 5S/18S rRNA index grouped vitellogenic ovaries together and separated them from previtellogenic ones (Figs 2 and 3B). Megrims in advanced vitellogenesis showed index levels closest but different to males (Fig 3B), as a consequence of the appearance of 18S rRNA. Similarly, two different samplings were carried out with anchovies. In both cases, females were captured during the spawning period but, in one case, oocytes were completely mature and index values were closest to those of males. In the other case, oocytes had not yet begun final maturation, with a higher 5S/18S rRNA index (S3 Fig).

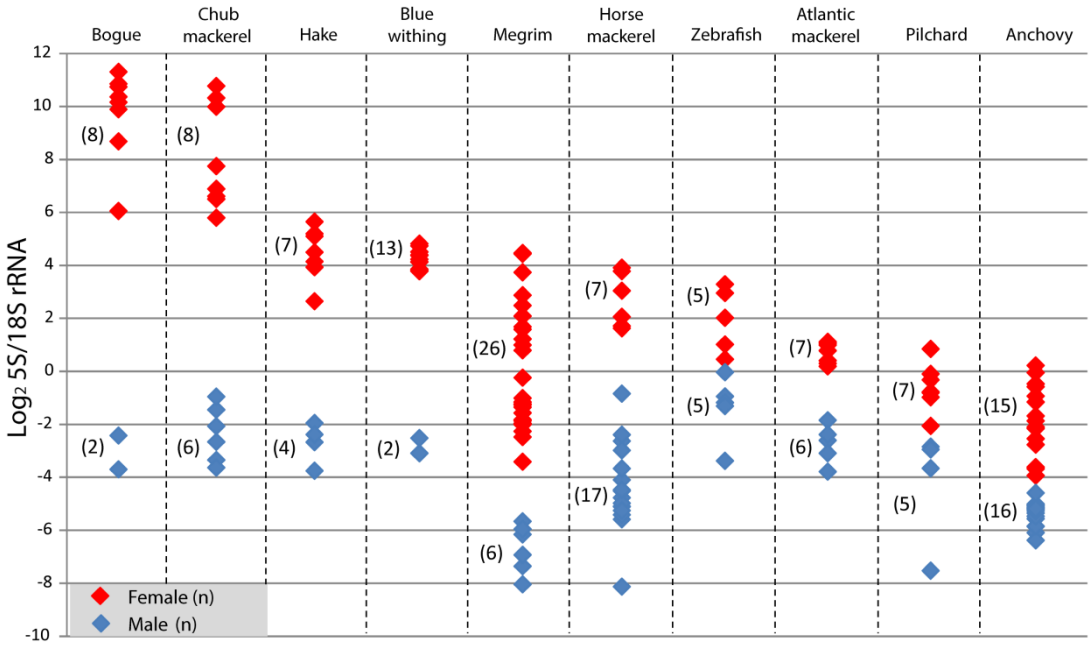

Fig 2. 5S/18S rRNA index in the gonads of different commercial fish species. Red dots identify ovaries and blue dots identify testes. Each dot corresponds to an individual and numbers between parentheses identify the number of individuals analysed per sex. Species are ordered from left (previtellogenic oocytes) to right (hydrated oocytes) as follows: bogue, Atlantic chub mackerel, hake, blue withing, megrim, horse mackerel , zebrafish, Atlantic mackerel, pilchard and anchovy . Female samples for megrim and anchovy displayed ovaries in different developmental stages (see Fig 3 and S3 Fig). Index values between both sexes were statistically different in all the 10 species studied (Mann-Whitney, $p< 0.05$).

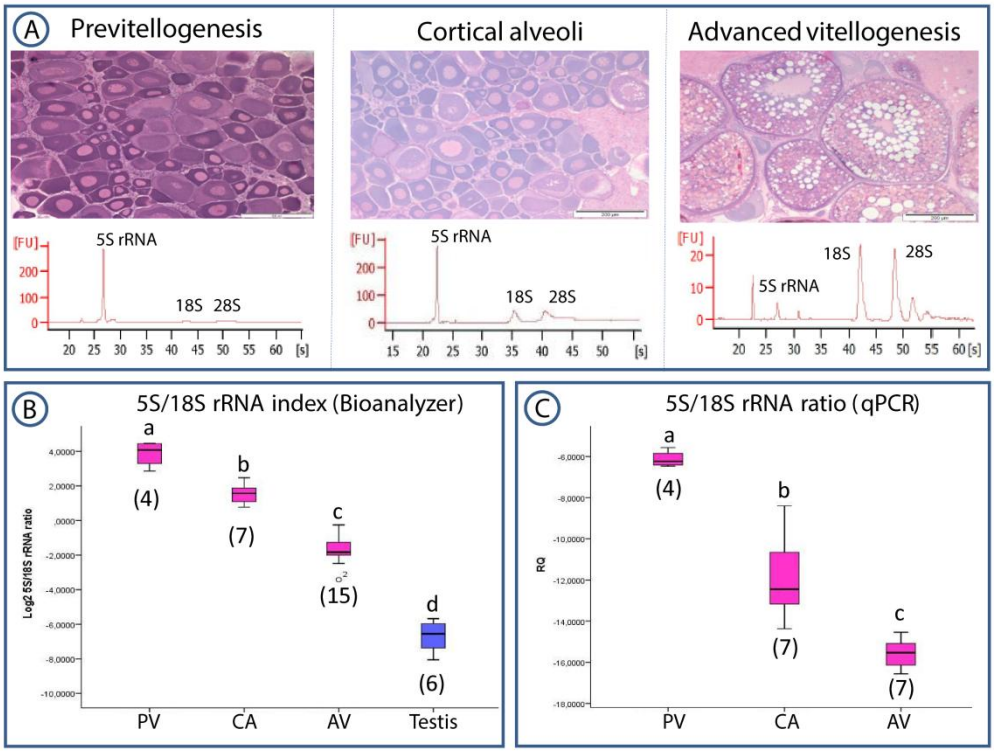

Fig 3. Total RNA electropherograms and 5S/18S rRNA index in the gonads of megrims. (A) Gonad histology (scale bars=200 µm) and electropherograms representative of total RNA distribution in megrim ovaries (captured in February, May and November) in different stages of oogenesis (previtellogenesis -PV-, cortical alveoli -CA- and advanced vitellogenesis -AV-). [FU] = Fluorescence, [s] = Time in seconds. The peak at around 20 seconds corresponds with the Agilent marker. 5S rRNA band appears between 25 and 30 seconds, 18S rRNA band around 40 seconds and 28S rRNA in between 45 and 50 seconds. (B) 5S/18S rRNA index in ovaries in different developmental stages and in testis as quantified from electropherograms. (C) 5S and 18S rRNA index as obtained after qPCR analysis of the levels of transcription of both genes in ovaries in different developmental stages. Numbers between parentheses indicate the number individuals analysed per group. Different letters indicate significant differences among pairs of means (one-way ANOVA, $p<0.05$ in B, Jonckheere-Terpstra, $p<0.05$ in C).

To corroborate that we were really measuring 5S and 18S rRNA levels in the electropherograms and no anything else, specific transcription was analyzed through qPCR in megrim. The 5S/18S rRNA index generated matched perfectly the results obtained with measurements on electropherograms, distinguishing ovaries on the basis of their gametogenic stage (Fig 3C).

gtf3a in commercial fish species; cloning and sex-specific transcript levels

Use of degenerate primers permitted the amplification and sequencing of *gtf3a* in ovaries of 7 of the studied fish species using conventional PCR. cDNAs used to obtain

these sequences were produced with RNA extracted from ovaries with previtellogenic (Atlantic chub and horse mackerel, blue whiting and hake,), advance vitellogenic (Atlantic mackerel and megrim) and hydrated (pilchard) oocytes. All of the fragments published are 523-707 nucleotides in length (S1 Table). *gtf3a* was transcribed strongly in the ovaries in all the species (developmental stages for each species as before and in Table 1), whilst nearly no transcription was detected in testes (Fig 4A). It was not possible to clone a *gtf3a* orthologue in anchovy and bogue.

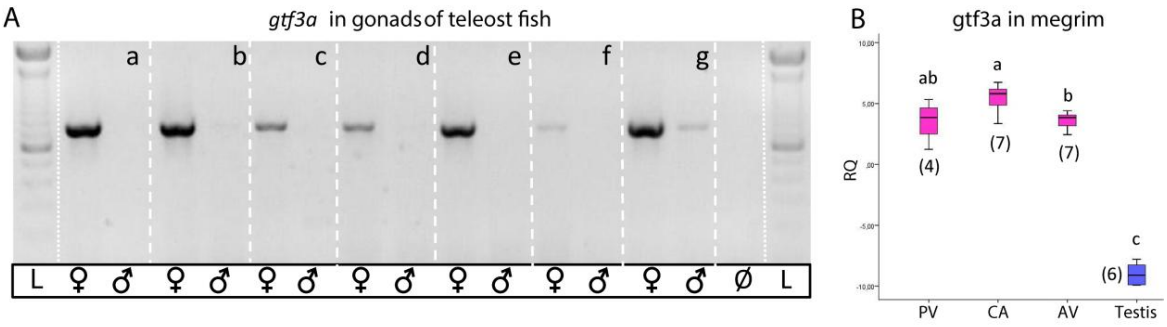

Fig 4. *gtf3a* transcription levels in ovaries and testis of fish. (A) Agarose gel electrophoresis of *gtf3a* fragments (around 700 nucleotides in length) amplified in one ovary and one testis through conventional PCR for 35 cycles. a: Atlantic mackerel; b*: chub mackerel*; c: megrim; d: blue whiting; e: hake; f: pilchard; g: horse mackerel. Developmental stages of ovaries as in Table 1, megrim in advanced vitellogenesis. Ø = no template control, L = Standard 100 bp (Invitrogen). (B) Box plots representing *gtf3a* transcript levels in megrim ovaries in different developmental stages (PV = previtellogenic, CA = cortical alveoli, AV = advanced vitellogenesis) and in testis. Numbers between parentheses indicate the number of individuals analysed per group. Different letters indicate significant differences (Jonckheere-Terpstra, $p<0.05$) between groups. Data normalized to cDNA ng per sample.

qPCR analysis of *gtf3a* was performed on megrim gonads. The results obtained showed significant differences between females and males (Fig 4B). Besides, ovaries in previtellogenic and cortical stages presented the highest *gtf3a* transcript levels, coincident with the oogenesis stages in which 5S rRNA production was at its highest and 18S rRNA at its lowest (Fig 4B). A significant down regulation of g*tf*3a was recorded when ovaries reached advanced vitellogenesis.

DISCUSSION

The results of the present study revealed marked sex-related differences in 5S rRNA and *gtf3a* transcript levels in gonads of fish species commercially-relevant in the Bay of

Biscay, irrespective of their reproductive stage. It has been shown that the electrophoretic analysis of total RNA extracted from gonads is sufficient to identify sex due to the high production and accumulation of 5S rRNA in the oocytes. This approach to sex fish only requires extraction of total RNA and running an electrophoresis so it is cheap, not as skill demanding as expert histological analysis and it requires a minimum amount of tissue. In 7 of these species *gtf3a* was sequenced and its quantification through PCR also permitted sex identification in all of the species analysed. Using this knowledge an index based on the quantification of 5S rRNA and 18S rRNA levels in electropherograms was developed; this allowing non-subjective female gametogenic stage identification in all the studied fish species.

5S rRNA: molecule useful in sex identification

Evidence that 5S rRNA gene is expressed always in higher amounts in ovaries than in testes in fish, independent of gametogenic stage during the seasonal reproductive cycle, was presented firstly in thicklip grey mullets (Diaz de Cerio et al., 2012). This observation was hypothesised as corresponding to a mechanism to accumulate rRNA intermediates that should be available in the oocyte to, in the event of being fertilised, allow rapid assembly of ribosomes and sustain protein production during early embryo development (Ortiz-Zarragoitia et al., 2014; Diaz de Cerio et al., 2012). It was confirmed here that this observation can be generalized to other teleosts, at least temperate marine fish species plus zebrafish, as 5S rRNA gene was also highly expressed in the ovaries of all of the commercially-relevant fish species that were studied. Therefore, it was confirmed that high levels of 5S rRNA in gonads could be diagnostic of teleost fish sex, identifying females independent of the period within their reproductive cycle and of the species studied. Such a molecular approach could provide an easy tool for the allocation of sex in other fish species, which is otherwise somewhat laborious and not easy when samplings do not coincide with the spawning season (Diaz de Cerio et al., 2012), when enough gonad tissue is not available or when tissue has not been processed for histological analysis (Espigares et al., 2015). For instance, in molecular biology laboratories where transcriptomic studies are carried out, electrophoretic analysis of RNA quality should be standard procedure, giving this additional information on the sex

of the fish (Espigares et al., 2015). It should be taken into account though, that this is a post-mortem approach and that requires gonad dissection. Moreover this sexing approach is only effective when sex differentiation has already occurred, as high 5S rRNA transcription is the consequence of having oocytes.

5S rRNA accumulation in fish ovaries has been shown already elsewhere by other authors in different teleost fish (Denis & Wegnez, 1977; Mazabraud et al., 1975). Very recently it was also proved in prepubertal European sea bass (Espigares et al., 2015). This observation might have important implications in transcriptomic studies using fish ovaries. The RNA Integrity Number (RIN), which has become the gold standard method to evaluate total RNA quality (Schroeder et al., 2006), proves to be not useful to analyze RNA extracted from ovary due to the prevailing presence of 5S rRNA and other small-sized RNA molecules (Mittelholzer et al., 2007). For example, in sea bream (*Diplodus puntazzo*), and due to the presence of prevailing RNA peaks around 100 nucleotides, Manousaki et al. (2014) concluded that it is not possible to use total RNA electrophoresis and the RIN number to estimate the RNA quality in ovaries that are in early developmental stages. Similar conclusion was reached by Kroupova et al. (2011).

The fish species studied in this work presented different reproductive strategies, which have strong implications in the analysis of their fecundity. Following the classification proposed by Murua et al. (2003) females can be classified as asynchronous indeterminate batch spawners (Atlantic chub and horse mackerels, blue whiting and European anchovy, hake and pilchard), asynchronous determinate batch spawners (Atlantic mackerel) and group-synchronous batch spawners (megrim). No data are presented here on any synchronous-determined total spawner, but the previous study by Diaz de Cerio et al. (2012) focused on thicklip grey mullet, which presents this reproductive strategy. In all cases, 5S rRNA distinguished males independent of the reproductive strategy.

gtf3a: sex specific transcription levels

gtf3a was cloned and sequenced in 7 species of the 9 studied; and its high transcription levels in ovaries in comparison to testes also allowed easy identification of sex. In this way, *gtf3a* could be considered also a potent molecular sex marker for teleost fish.

In all of the cells, ribosome biosynthesis monopolizes up to 80% of the cellular transcription activity and requires the synthesis of RNAs by three nuclear RNA polymerases: Pol I, which produces the precursor of 5.8S, 18S and 28S RNAs; RNA polymerase II (Pol II), which produces all mRNAs including those that encode ribosomal proteins; and Pol III, responsible for 5S rRNA gene transcription (Layat et al., 2013). Activity of Pol III requires Gtf3a, which has the ability to bind both 5S rRNA gene and 5S rRNA itself. Firstly, it recognizes and binds the promoter sequence 5S rRNA gene helping in the assembly of the transcription machinery of Pol III (Penberthy et al. 2003). Subsequently, Gtf3a binds 5S rRNA for stockpiling in the cytoplasm in the form of 7S ribonucleoprotein particles, formed by one molecule of 5S rRNA and one Gtf3a protein molecule (Penberthy et al. 2003).

While low *gtf3a* levels appear to be a common feature of somatic cells, extremely abundant *gtf3a* transcript levels have been long known in amphibian oocytes (Pfaff et al., 1991; Allison et al., 1995). Levels of *gtf3a* mRNA mirror those of 5S rRNA; in *Xenopus* these are about 1 million times higher in oocytes than in somatic cells (Penberthy et al. 2003). Consistent with this observation, it has been reported that Gtf3a predominantly binds the oocyte-type and not the somatic-type 5S rRNA in *Xenopus*, even when both sequences only diverge in three nucleotides (Allison et al., 1995). Moreover, *gtf3a* is overexpressed early in oogenesis, constituting even 10% of the total cytoplasmic protein in anurans, then decreasing 5-10-fold by later stages (Penberthy et al. 2003; Wischnewski et al., 2004).

Little is known about *gtf3a* transcription dynamics in fish (Ortiz-Zarragoitia et al., 2014). Functional aspects of Gtf3a were studied only in catfish (*Ictalurus punctatus*) oocytes, showing a similar capacity to bind oocytic 5S rRNA (Ogilvie & Hanas, 1997). Diaz de Cerio et al. (2012) demonstrated, through qPCR analysis for the first time in fish, that *gtf3a*

transcriptional regulation resembles that of 5S rRNA in ovaries. Interestingly, *gtf3a* appeared also in lists of zebrafish and flounder (*Paralichthys olivaceus*) ovary enriched transcripts (Sreenivasan et al., 2008; Fan et al., 2014). Our qPCR results clearly identified *gtf3a* early expression in megrim oocytes. *gtf3a* transcript levels were at their highest in the early stages of ovary maturation at cortical alveoli stage, decreasing during vitellogenesis, as in *Xenopus* (Pfaff et al., 1991). No reference genes were used to normalize the target gene transcription data; instead, the amount of input cDNA per sample was used (Espigares et al., 2015). The traditional qPCR normalization method includes the use of reference or housekeeping genes with presumably invariant levels of transcription in each particular experimental system (Dheda et al., 2005). Ovaries suffer profound changes in terms of cell composition, physiological status, hydration level, or meiotic stage; as such their RNA content differs in amount and composition throughout growth and maturation. No valid reference gene exists in these circumstances. Libus and Štorchová (2006) and Mittelholzer et al. (2007) proposed the use of total amount of cDNA to normalize qPCR data in fish gonads. In addition, Filby and Tyler (2007) used also ΔCT method with input cDNA amount for normalization purposes when studying the usefulness of reference genes in fish ovaries. Also, De Santis and co authors (2011) used cDNA for the normalization of muscle data in barramundi (*Lates calcarifer*). These investigations concluded that this new normalization approach may produce the most biologically-valid results when studying very dynamic tissues where reference genes are not stably expressed. Similar conclusions were reached by other authors when studying different tissues and organs (Lundby et al., 2005; Rhinn et al., 2008).

5S/18S rRNA index: female developmental stage identification

5S rRNA quantification provides an easy way to sex teleost fish species in a comparative basis (comparing a female and a male individual on the same electrophoresis) (Ortiz-Zarragoitia et al., 2014; Diaz de Cerio et al., 2012). However, it would be useful to set threshold expression levels to unequivocally identify sex. For this purpose, a 5S/18S rRNA index was developed after quantifying the concentration of 5S and 18S rRNAs in the electropherograms provided by the Bionalyzer RNA nanochips. 18S rRNA was selected for the quantification of the index since 28S rRNA could be more prone to

cleavage because of its bigger size. The logarithmic transformation of this ratio allowed improved visualization of the differences between both sexes.

The index did not only allow absolute identification of sex without the need of comparing two individuals but also ranking the development stage of the oocytes present in the ovary due to changes in the relative amount of RNA species throughout oogenesis. Kroupova et al. (2011) analyzed in detail the stage-dependent RNA composition in roach (*Rutilus rutilus*) ovaries, concluding also that during primary growth and early cortical alveoli stages smaller-size RNAs were accumulated. In megrim females the index was highest in previtellogenic females and lowest in advanced vitellogenic ones. Thus, a developed 5S/18S rRNA index could constitute a new approach to study the reproductive stage in females, independent of their reproductive strategy. It is true that as 5S rRNA predominated in previtellogenic oocytes this index should work better in identifying the reproductive stage in ovaries with synchronous development. In any case, in the present investigation, where most species studied displayed an asynchronous development with different type of oocytes mixed within the ovary, the index proved to be useful in grading the maturity of females. Thus, the developed index could be of considerable assistance in the characterization of reproductive potential; this is extremely important in the study of fish stocks dynamics (Murua & Saborido-Rey, 2003) and fish biology research in general.

The data presented here could also contribute to studies of fish fecundity, essential to develop an understanding of the stock and recruitment relationship in fish, or in the assessment of spawning stock biomass. Nowadays, methodological approximations to fish population fecundity studies depend on the spawning strategy of females; likewise, then are very laborious and required skilled histological capabilities (Murua & Saborido-Rey, 2003; Witthames et al., 2009; Armstrong & Witthames, 2012). In any case, the methodology presented here could be a very useful approach for laboratories that use molecular approaches in their research, and that could need of proper identification of sex and reproductive stage of their samples as supporting parameter (Espigares et al., 2015).

Why did 5S rRNA and 18S rRNA relative amounts change during the development of the oocytes?

Previous studies on *Xenopus* have shown that during early previtellogenic stages rRNA genes are amplified to a final content of ~$2x10^6$ per oocyte; this provides sufficient rRNA gene templates to produce $1x10^{12}$ ribosome particles per mature oocyte (Roger et al., 2002). Despite this amount of rRNA genes, the transcription of 45S pre-rRNA (precursor of 5.8S, 18S and 28S rRNA) takes place at very low levels in previtellogenic stages but increases dramatically in vitellogenic stages. 18S and 28S rRNA transcription occurs in response to the activation of Pol I during vitellogenesis (Szymański et al., 2003). On the contrary, Pol III activity is maximal in the previtellogenic stages in *Xenopus* (Szymański et al., 2003). The results described here, together with those presented for roach ovaries (Kroupova et al., 2011), would indicate the same trend as in the case of anurans. Ovaries at previtellogenic and cortical alveoli stages would accumulate small-sized RNAs. It could be hypothesised that, during early oogenesis, energetic investment in reproduction takes a decision towards the least energetically demanding production of 5S rRNA. When reproduction is envisaged to occur under favourable conditions, oocyte secondary growth is initiated. The real energetic investment in reproduction commences with the production of the large ribosomal RNA molecules and vitellogenesis. Thus, the 5S/18S rRNA index changed during differentiation, with the inactivation of Pol III activity (observed in megrim as decreased *gtf3a* transcript levels in advanced vitellogenic ovaries) and activation of Pol I at vitellogenesis.

Overall, the results presented here confirmed that 5S rRNA and *gtf3a* are useful molecular sex markers in teleost fish; at least in the ones studied to date. Moreover, these molecular markers could be used easily to establish the gonad developmental stage in females. Presently attempts are being made to develop a fish sexing molecular kit, with technological transfer potential, based upon the qPCR assessment of the transcript levels of 5S rRNA. This molecular approach could be relevant for the study of basic fish biology (Espigares et al., 2015) and for the analysis of fish populations and reproduction dynamics setting management policies to maximize exploitation whilst protecting the spawning potential of the stocks (Prager et al., 2003). At the same time, it

must be considered that different methods to quantify egg production are used to estimate spawning stock biomass, towards the implementation of scientifically-based fisheries management (Morgan, 2008; Armstrong & Witthames, 2012).

Further investigations are needed to understand the molecular mechanisms that govern transcription of 5S rRNA and accompanying genes during gametogenesis and sex differentiation in fish. In addition, according to the development stage of ovaries, it will be crucial to understand the relationship between the activation of Pol I and III and the levels of 5S and 18S rRNA. It is also necessary to explore the possibilities to enlarge the scope of teleost fish species (with different reproductive strategies, geographical and habitat distribution, developmental history...) where the 5S/18S rRNA index could be successfully used as in the present work. The present authors consider that the pattern of accumulation of these ribosomal molecules could define the quality of the spawned oocytes, as levels of 5S rRNA can also be measured in in them. In this way, 5S rRNA quantitative analysis could be useful also in determining the quality of reproductive females in aquaculture hatcheries. Finally, 5S/18S rRNA index could be also used to study the environmental xenoestrogenicity and identify intersex individuals, as 5S rRNA levels can identify the presence of oocytes (mainly previtellogenic) in gonads.

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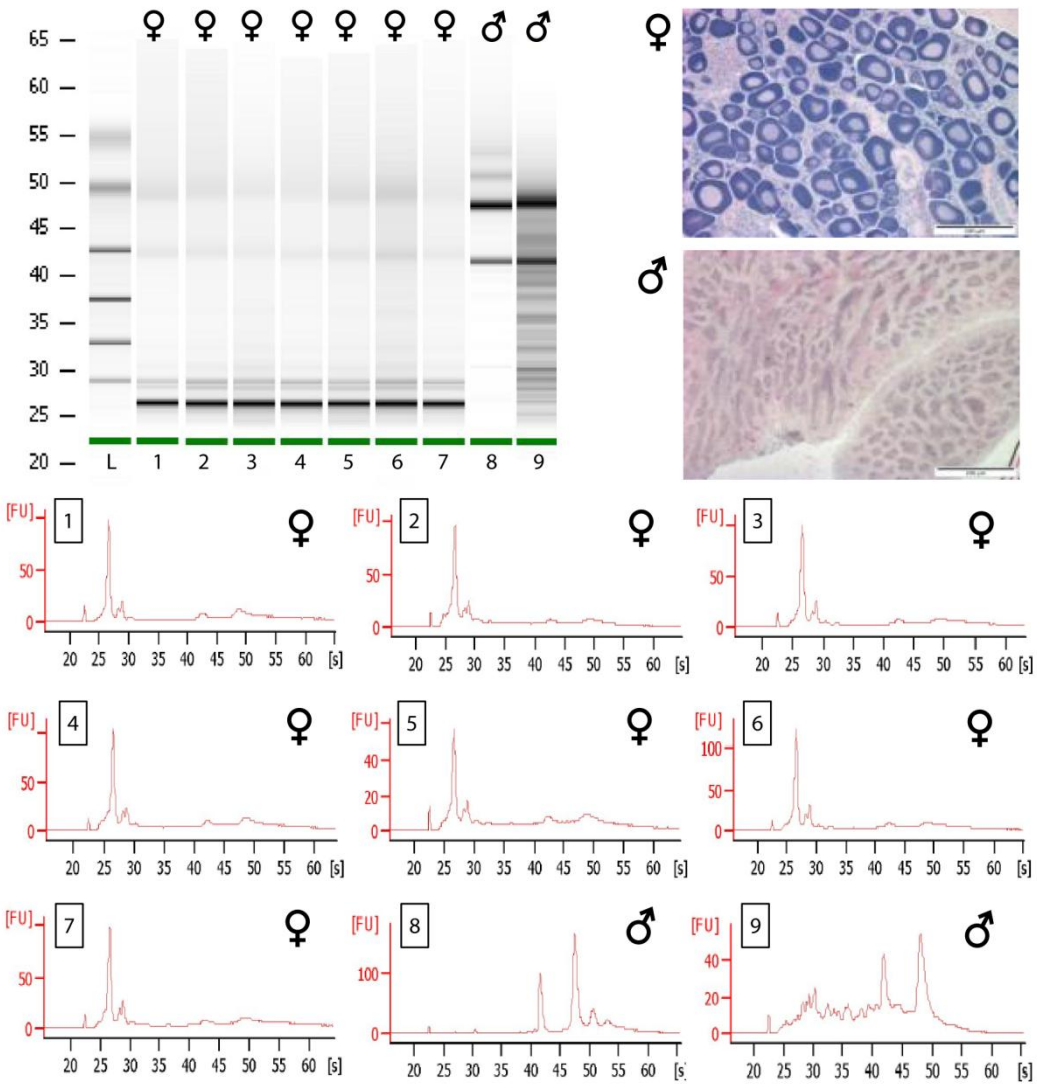
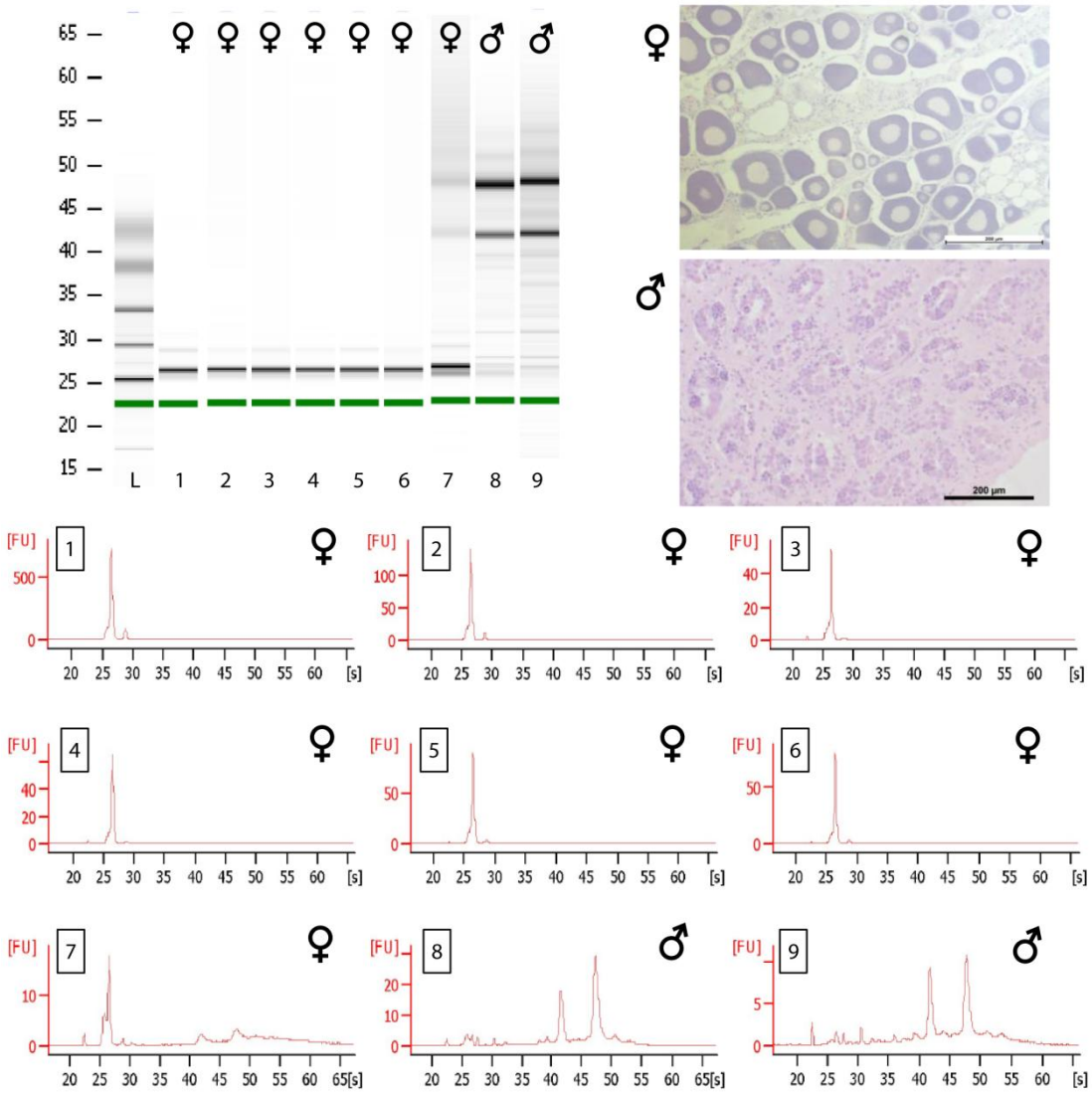
SUPPORTING INFORMATION

S1 Table. *gtf3a* sequences obtained from the different fish species studied. Table depicts size of amplified and sequenced fragments and e-value through BlastX analysis with the most similar sequences in Genbank. For comparative purposes the *Danio rerio gtf3ab* sequence (NP_001083013) appears in all the cases.

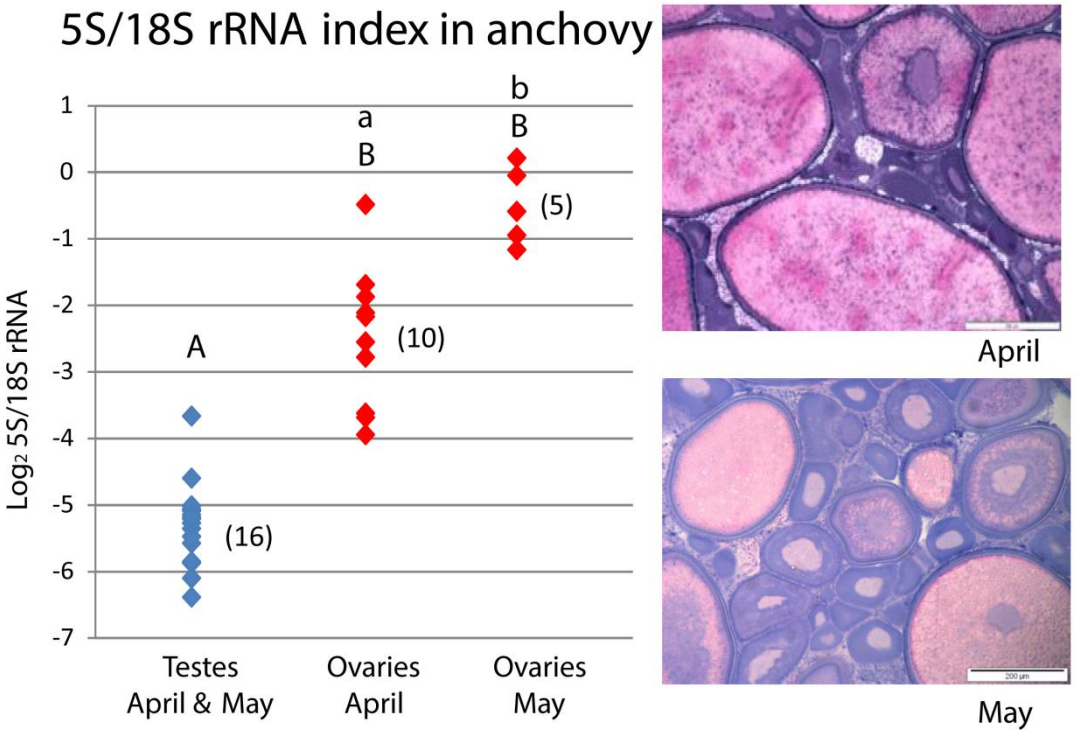
Species	Genbank accession number	Size (bp)	E-value	BlastX Most similar sequence and Genbank accession number
M. merluccius	JQ928630	523	3e-76 7e-54	*Oreochromis niloticus* PREDICTED TFIIIA,XP_003443591 *Danio rerio,* TFIIIA, b NP_001083013
S. scombrus	JQ928631	699	3e-133 5e-93	*Oreochromis niloticus* PREDICTED TFIIIA,XP_003443591 *Danio rerio,* TFIIIA, b NP_001083013
S. colias	JQ928632	707	2e-124 3e-96	*Oreochromis niloticus* PREDICTED TFIIIA,XP_003443591 *Danio rerio,* TFIIIA, b NP_001083013
L. whiffiagonis	JQ928633	691	2e-134 1e-93	*Oreochromis niloticus* PREDICTED TFIIIA,XP_003443591 *Danio rerio,* TFIIIA, b NP_001083013
S. pilchardus	JQ928634	636	6e-99 2e-91	*Danio rerio,* TFIIIA, b NP_001083013 *Oreochromis niloticus* PREDICTED TFIIIA,XP_003443591
M. poutassou	KC191719	594	2e-70 3e-46	*Takifugu rubripes*, PREDICTED TFIIIA like, XP 003968009 *Danio rerio,* TFIIIA, b NP_001083013
T. trachurus	KC191721	594	1e-112 2e-68	*Maylandia zebra,* PREDICTED TFIIIA like, XP 004546742 *Danio rerio,* TFIIIA, b NP_001083013



S1 Fig. **Total RNA electrophoresis and electropherograms of 9 blue whitings captured in May 2014.** Samples 1 to 7 belonged to females during early oogenesis with previtellogenic oocytes. Only 2 male individuals were available (samples 8 and 9). 5S rRNA predominated in females, while peaks belonging to 18S and 28S rRNA were very small. L: RNA 6000 Nano Kit Ladder. Two micrographs representative of the ovaries and the testes (early gametogenic stages) in the fish studied are shown. Scale bars = 200 µm.



S2 Fig. **Total RNA electrophoresis and electropherograms of 9 bogues captured in May 2014.** The band belonging to 5S rRNA was clearly observable in females (individuals 1 to 7), where nearly no 18S or 28S rRNA was observed. Only two males were available for the study (individual 8 and 9). All individuals were in an early gametogenic stage, as it could be observed in micrographs of a representative ovary and a testis. L: RNA 6000 Nano Kit Ladder. Scale bars: 200 µm.



S3 Fig. **5S/18S rRNA index in testes and ovaries of anchovies from two samplings.** 15 female and 16 male anchovies sampled during the spawning season in April and May 2012. Anchovies normally spawn on the weekly basis for a period of a few months. So upon capture females could present many mature close to hydration oocytes, as it was the case in the sampling carried out in April, or could be initiating a new maturation cycle, as it was the case of the ovaries in May. In this last case less mature oocytes were observed. The 5S/18S rRNA index distinguished ovaries according to the maturation stage; ovaries with less mature oocytes showing highest index values. Males of both samplings are combined to simplify the graph. Different capital letters indicate significant differences between sexes and different lower case letters indicate differences between the two analysed ovary stages (Mann-Whitney, $p<0,005$). Scale bars in both micrographs = 200 µm.

Chapter 3

Molecular markers of oocyte differentiation and maturation in European eel (*Anguilla anguilla*) during artificially induced oogenesis

Parts of this chapter have been presented at:

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5th INTERNATIONAL WORKSHOP ON THE BIOLOGY OF FISH GAMETES. Ancona, (Italy). 2015. Ribogenesis molecular markers of oocyte differentiation in european eel *Anguilla anguilla*: transcriptional regulation during artificially induced oogenesis. Rojo-Bartolomé, I; Martínez-Miguel, L; Lafont, A.G; Peñaranda, D.S; Vílchez, M.C.; Asturiano, J.F.; Pérez, L; Cancio, I**.** ICC, oral

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LABURPENA

Ugalketa aingira europarrean (*Anguilla anguilla*) ikertu behar den gai gakoa da, gatibutasunean ugaltzeak aingira populazioak jasaten ari den murrizketari aurre egiteko irtenbide bakarra izan daitekeela jakinik. Oozitoen heltze artifizialean zehar erribosomen azpi-unitateen ekoizpena kontrolatzen duten mekanismoak ulertzea interes berezikoa izan daiteke. Horrela, erribosomen sorrerarekin erlazionaturiko geneen transkripzio-mailen aldaketak hormonen bidez bultzatutako oogenesian zehar oozitoen garapen eta heltzearen jarraipenerako markatzaileak izan daitezke. Horretarako, Valentziako Albuferatik aingirak arrantzatu eta karparen pituitario estraktuarekin tratatu ziren 15 astez. Obarioak histologikoki prozesatu ziren euren garapen-maila aztertzeko eta RNA erauzteko. Prebitelogenesian (PV, tratatu gabeko emeak), bitelogenesi goiztiar, tarteko bitelogenesian eta bitelogenesi berantiarrean (EV, MV, LV) eta migrazio nuklearrean (MN) zeuden obarioak aztertu ziren. 5S rRNA eta 18S rRNA mailak RNA totalaren elektroforesi bidez kalkulatu ziren eta 5S/18S rRNA indizea kalkulatu zen lagin bakoitzeko. Gero *gtf3a*, 18S rRNA, *ubtf1*, *42sp43*, *rpL5* eta *rpL11*-ren transkripto-mailak kuantifikatu ziren qPCR bidez. 5S rRNA eta erribogenesiarekin erlazionatutako geneak oso transkribatuak ziren PV oozitoak zituzten obarioetan, baina oozitoak garatu ahala, 5S rRNA-ren sorrera (*gtf3a*), pilaketa (*gtf3a, 42sp43*) eta garraio nukleo-zitoplasmatikoarekin (*rpl5, rpl11*) erlazionatutako geneen transkripzio-mailek eta 5S/18S rRNA indizeak behera egin zuten (PV>EV>MV>LV>MN). Aldiz, 18S rRNA MN fasean aurkeztu zuen transkripzio-mailarik altuena. Bitelegonesiaren hasiera 18S rRNA eta 28S rRNA-ren ekoizpenaren igoerarekin batera ematen da beraz, nahiz eta *ubtf1*, rRNA hauen sintesirako ezinbestekoa dena, 5S rRNA eraentzen duten geneen transkripzio-profil berdina aurkeztu zuen. Hormona tratamendura erantzun ez zuten aingirei begiratuta (NR), PV emeen antzeko 5S/18S rRNA indizea eta EV/LV banakoen antzeko erribogenesiko geneen transkripzio-mailak aurkeztu zituzten. Beraz, NR emeek huts egiten dute rRNA molekula handiak adierazteko garaian, eta beraz bitelogenesi-arrakastaren markatzaileak izan daitezke. Ondorioz, erribogenesiarekin erlazionatutako geneen transkripzio-dinamikak tresna molekular erabilgarria direla frogatu dugu aingira europarrean artifizialki induzituriko obarioaren garapena aztertzeko. Etorkizunean, markatzaile hauek hormona-tratamenduaren aurrean aingirek duten erantzuna eta artifizialki lortutako oozitoen kalitatean azterketarako kontuan har daitezke.

ABSTRACT

Reproduction is a key issue to be studied in European eel (*Anguilla anguilla*) as reproduction in captivity could be a solution for the dwindling population of this species. Understanding the mechanisms controlling the production of ribosomal building blocks during artificially induced oocyte maturation in eels could be of particular interest. Changes in the transcription levels of genes associated with ribosomal biogenesis could be used as marker to monitor oocyte growth and maturation during hormonally induced oogenesis. Eels from the Valencia Albufera Lagoon were treated with injections of carp pituitary extract for 15 weeks. Ovaries were processed for histological staging of gonad maturation and for RNA extraction. Ovarian samples in previtellogenic stage (non-injected), and in early, mid or late vitellogenesis (EV, MV, LV), as well as in nuclear migration (MN) were analysed. Levels of 5S and 18S rRNA were quantified after total RNA electrophoretic analysis and the 5S/18S rRNA index calculated for each sample. Then, transcription levels of *gtf3a*, 18S rRNA, *ubtf1*, *42sp43*, *rpL5* and *rpL11* were quantified by qPCR. 5S rRNA and ribogenesis related genes were highly transcribed in ovaries with PV oocytes. As oocytes developed, transcriptional levels of genes related to 5S rRNA production (*gtf3a*), accumulation (*gtf3a*, *42sp43*) and nucleocytoplasmic transport (*rpl5, rpl11*) and the 5S/18S rRNA index decreased (PV>EV>MV>LV>MN). On the contrary, 18S rRNA was at its highest at MN stage. Entry into vitellogenesis is thus accompanied by an up-regulation of 18S and 28S rRNA in oocytes although *ubtf1*, necessary to this rRNA synthesis, showed the same transcription profile as 5S rRNA regulating genes. Regarding individuals that did not respond (NR) to the treatment, they showed 5S/18S rRNA index values similar to PV females while studied genes showed EV/LV-like transcription levels. Therefore, NR females fail to express the largest rRNA molecules, which could thus be taken as markers of successful vitellogenesis progression. In conclusion, we have been able to prove that the transcriptional dynamics of ribosomal genes provide useful molecular tools to characterize the process of artificially induced ovarian development in European eels. In the future, such markers should be studied as putative indicators of the level of response to hormonal treatments and of the quality of artificially obtained eel oocytes.

INTRODUCTION

The European eel stock has been in gradual decline for at least half a century (van Ginneken et al., 2005; Pujolar et al., 2012; ICES 2013) with numbers dropping as much as 99% since the 1980s (ICES 2013). Understanding the mechanisms triggering sexual maturation in European eels has become a focus of economic and scientific interest (van Ginneken et al., 2005). Advances in methodologies to control maturation could allow establishing a self-sustained aquaculture rather than the nowadays applied culture system dependent on fishing and growing of wild glass eels (Dirks et al., 2014).

In order to establish an efficient aquaculture activity, high-quality eggs and sperm are needed to produce viable juveniles (Babin et al., 2007). Attempts to reproduce eels in captivity have largely been unsuccessful (Boëtius and Boëtius, 1980; Pedersen 2004, Palstra et al. 2005 Palstra and van den Thillart 2010, Pérez et al. 2011). In this respect, Tanaka and co-workers were able to obtain leptocephali larvae of *Anguilla japonica* in captivity in 2003, taking them through metamorphosis to obtain glass eels (Ijiri et al., 2011; Okamura et al., 2014). In the case of the European eel successful fertilization and hatching, taking the larvae through the yolk-sac stage was recently reported (Butts et al., 2014; Sørensen et al. 2014). However, with the existing breeding protocols, most fertilized eggs do not develop and all larvae die prematurely. Considering this, it is imperative to enhance the knowledge base on eel reproduction and develop the technology needed to produce good quality gametes and viable offspring that would allow rear larvae beyond the first feeding stage.

Gonadotropins (luteinising hormone, LH; and follicle-stimulating hormone, FSH) positively control the development and activity of gonads in all vertebrates (Vidal et al., 2004; Rocha et al., 2009). In some teleosts, gonadotropin secretion at the pituitary is under the control of gonadotropin-releasing hormone (GnRH) that exerts a stimulatory control, and dopamine, kisspeptins and gonadotropin inhibitory hormone, with an inhibitory effect (Dufour et al. 1991; Vidal et al., 2004; Pasquier et al., 2011). In eels, this gonadotropic production and/or release is blocked during the yellow and silver stages becoming active during their oceanic reproductive migration. Eels do not mature in their continental water habitats due to a strong dopaminergic inhibition and a deficient

stimulation of gonadotropin-releasing hormone (GnRH) release (Dufour et al., 1991: Vidal et al., 2004). Therefore, eels will not become sexually mature until they are in the open ocean, under the influence of still unknown environmental factors (Bruijs et al., 2009; Mazzeo et al., 2014). Research on the control of this blockade of sexual maturation has allowed to artificially inducing maturation in captivity, applying hormonal treatments mainly consisting of injections of fish pituitary extracts to farmed or silver eels captured in transition from freshwaters to the ocean (reviewed in Okamura et al., 2014). In this way, the maturation in female European eels is based on weekly injections of such extracts administered for periods of 10-20 weeks (Butts et al. 2014). The same is applied to males injecting human chorionic gonadotropins (Asturiano et al., 2005; Gallego et al. 2012; Okamura et al., 2014). There are obvious disadvantages to this method such as individuals need to be manipulated weekly, cost being high and gamete quality unpredictable (Okamura et al. 2014). Besides, in many circumstances some individuals do not respond even after 6 months of injections (Dirks et al., 2014).

Oocyte differentiation and maturation in fish relies on an intense incorporation of a large quantity of molecules into the cell. This partially involves a specific expression regulation of the oocyte genome, but many of the molecules are incorporated from surrounding ovarian cells or from other organs. Our previous studies have revealed that 5S ribosomal RNA (5S rRNA) and accompanying proteins are good markers of female fish oogenesis and oocyte maturation progress due to their high transcription levels in oocytes (Diaz de Cerio et al. 2012; Rojo-Bartolomé et al., 2016). 5S rRNA levels are also high in the testis of intersex individuals that due to exposure to xenoestrogenic chemicals develop oocytes in their spermatic cysts (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014).

All the molecular machinery necessary for 5S rRNA transcription and accumulation is also required in great quantities in the oocyte (Song et al., 2005; Lyman-Gingerich et al., 2007). Ribosomes are formed by the assembly of ribosomal rRNAs (28S, 18S, 5.8S and 5S rRNA) and ribosomal proteins (RpL) (Lyman-Gingerich et al., 2007). With the exception of the 5S rRNA, all other rRNAs are produced as a single 45S pre-rRNA precursor transcript in the nucleolus by RNA polymerase I (Pol I) which is controlled by the upstream binding transcription factor 1 (Ubtf1). In contrast, 5S rRNA is transcribed in the

nucleus by RNA polymerase III regulated by the general transcription factor IIIA (Gtf3a) (Szymanski et al., 2003; Ortiz-Zarragoitia et al., 2014). Gtf3a binds 5S rRNA within the nucleus and the complex is transported to the cytoplasm where it is accumulated in the form of small 7S ribonucleoprotein particles (RNP) (Szymanski et al., 2003). Although a big proportion of the cytoplasmic 5S rRNA appears as 7S RNP, it can also be accumulated as 42S RNP associated to p43 (or 42sp43) (Picard et al., 1980; Zhang et al., 1995; Ortiz-Zarragoitia et al., 2014). Ribosomal protein 5 (RpL5) can then bind 5S rRNA accumulated as 7S or 42S RNPs to stabilize the 5S rRNA and forming a pre-ribosomal RNP that will migrate to the nucleus for ribosome assembly when bound to yet another ribosomal protein, RpL11 (Ciganda and Williams, 2011).

In *Xenopus*, *gtf3a* mRNA levels are approximately 1 million times higher in oocytes than in somatic cells, *42sp43* transcript levels being also very high in oocytes (Allison et al., 1995; Penberthy et al., 2003; Szymanski et al., 2003). The levels of *gtf3a* and *42sp43* mRNA mirror those of total 5S rRNA also in fish ovaries (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014). *gtf3a* is overexpressed early in oogenesis, constituting a high proportion of total cytoplasmic mRNA and protein in oocytes of anurans and fish, and then decreases manifold during vitellogenesis (Penberthy et al., 2003; Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2016). In this way, *gtf3a* is also a potent molecular marker of oocytes in many teleost fish species (Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2016). Additionally, it has been observed that the expression of 5S rRNA predominates in ovaries with oocytes in previtellogenic stages, while vitellogenesis marks the onset of the transcription and accumulation of 18S rRNA in fish. Therefore, a simple calculation of the ratio 5S to 18S rRNA allows ranking fish ovaries according to their developmental stage, at least in *Engraulis encrasicolus* and *Lepidorhombus whiffiagonis* (Rojo-Bartolomé et al., 2016).

In this molecular context, it could be hypothesised that oocytes in fish need to accumulate ribosomal intermediates in order to quickly assemble ribosomes in case of being fertilized (Diaz de Cerio et al., 2012). This would allow sustaining protein synthesis during embryogenesis, and, 5S rRNA being a small molecule, the cost of its synthesis would be low enough to be expandable in case the oocyte would need to interrupt maturation and suffer atresia.

Our aim in the present study was to characterize the profile of ribosomal RNA incorporation into experimentally matured European eel *Anguilla anguilla* oocytes. For that purpose, we calculated the ovarian 5S/18S index using total RNA as a molecular biomarker of oocyte development and differentiation, and quantified the transcription levels of different genes related with ribosomal biogenesis, chosen according to their association with the activity of RNA polymerases I and III (*18S rRNA, ubtf1* and *gtf3a*) and 5S rRNA accumulation in the cell (*gtf3a*, *42sp43*, *rpL5* and *rplL11*). Such transcripts could give quantitative information of the effect of hormonal treatments on the quality of the artificially matured oocytes in European eels.

MATERIALS AND METHODS

Samples and hormonal treatment

Wild female European eels were captured in the Valencia Albufera lagoon during their migrating phase as silver eels and transferred to the Aquaculture Laboratory from the Universitat Politècnica de València, where they were maintained in 500 l tanks equipped with recirculation system, heating/cooling systems, and black covers to reduce light intensity. After acclimation from freshwater to seawater conditions at 15-20 °C, weekly hormonal treatments started. Female eels were treated with weekly intraperitoneal injections of carp pituitary extract (CPE, Catvis, Ltd.) at a dose of 20 mg/kg body weight (Pérez et al. 2011; Mazzeo et al. 2014), until the end of the experiment, after 15 weeks. During the experiment the fish were starved, and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

Histological analysis and staging

Animals (n=33) were dissected, as described in Mazzeo et al et al (2014), previous to the first injection (0) and after 4, 8 and 12 weeks of hormonal treatment with CPE. For histological analysis the ovaries were fixed with 10% buffered formalin and embedded in paraffin wax. Then, sections of 5 to 10 μm in thickness were produced. Sections were stained with haematoxylin and eosin using standard procedures. Slides were observed

using a Nikon Eclipse E-400 microscope and the evaluation of the maturation stages was performed according to Pérez et al. (2011). After histological observation the samples were classified into five stages of ovarian development (Fig 1).

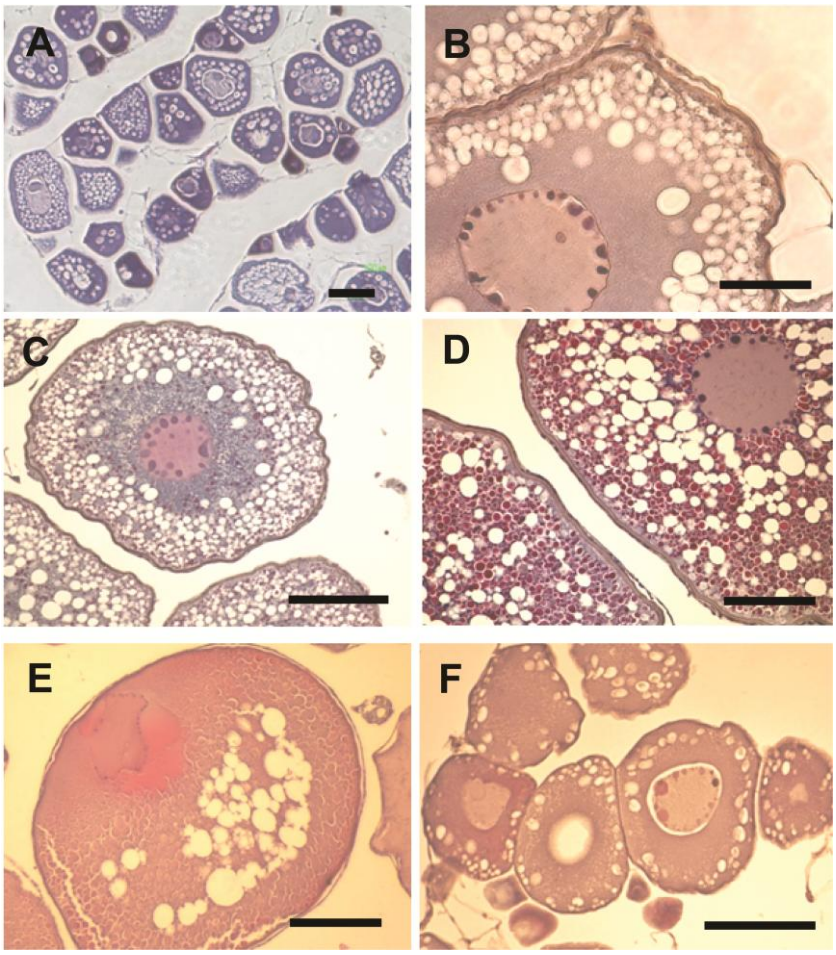

Fig 1. European eel ovarian developmental stages as analysed through ovary histology. Micrographs illustrate the developmental stage of oocytes in the eel ovaries along the hormonal treatment. (A) Ovary with PV oocytes in perinucleolar stage in a control female that received no CPE injections. (B) Ovary with oocytes in EV stage after 4 CPE injections. (C) Oocytes in MV stage after 8 CPE injections. (D) LV stage oocytes after 12 CPE injections. (E) Ovary with oocytes in MN stage (12 CPE injections). (F) Ovary after 8 CPE injections in a NR female showing previtellogenic stage oocytes. Scale bars: A, C, D, E and F=100 µm; B=50 µm.

The samples of non injected eels showed previtellogenic oocytes (PV), displaying lipid droplets but without observable yolk vesicles (Fig 1A). Females in EV stage were obtained after 4 weeks of injections, and their gonads showed EV oocytes with small yolk vesicles restricted to the cell cortex (Fig 1B). Ovaries with MV oocytes were obtained after 8 CPE injections (Fig 1C), and showed abundant yolk vesicles. Characteristic and abundant yolk vesicles were distributed throughout the cytoplasm

advancing inwards towards the nucleus. LV ovaries were obtained after 12 CPE injections, showing oocytes with enlarged and more abundant yolk vesicles (Fig 1D). Post-vitellogenic ovarian samples were obtained after 12-15 weeks; they showed the nucleus migrating from the centre of the oocyte to the periphery, during the process leading to final oocyte maturation (Fig 1E). Some ovaries showed no evidence of having initiated oocyte differentiation after 8 and 12 weeks of injection (NR), with the oocytes resting in early oogenesis stages, most of them at PV stage (Fig 1F).

RNA extraction, quantification and quality assessment: 5S/18S rRNA index

A portion (less than 40 µg) of each of the ovaries analysed histologically was embedded in RNA Later (Sigma-Aldrich) and frozen in liquid nitrogen until needed for RNA extraction. Total RNA was then isolated using TRI® reagent (Invitrogen) according to manufacturer's instructions. Dry RNA pellet was resuspended in 80 µL RNase-free water and stored at –80°C.

RNA amount and quality were assessed with a cuvette photometer (Biophotometer plus, Eppendorf AG) and also using 2100 Bioanalyzer Agilent RNA 6000 Nano Kit (Agilent Technologies). A_{260}/A_{280} ratio values around 2 and A_{260}/A_{230} ratio values around 1.8 were considered acceptable. The RIN value of all samples was calculated when possible with the Bioanalyzer (RIN values in the range of 7.2-9.4 are considered acceptable). This ratio was impossible to measure in PV, EV and NR ovaries due to the high signal detected in the 5S rRNA region. In these cases the 28S/18S ratio was used as quality indicator, with values around 2 indicating good quality.

Electropherograms provided by the Bioanalyzer were also used to quantify the relative concentration of the bands corresponding to 5S rRNA and 18S rRNA in each sample. The Time Corrected Area of each rRNA peak was used to calculate the 5S/18S rRNA ratio and Log_2 of this value provided the 5S/18S rRNA index (Rojo-Bartolomé et al., 2016).

cDNA synthesis and quantitative PCR (qPCR) analysis

First-strand cDNA was synthesized using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen) in the 2720 Applied Biosystems Thermal Cycler (Life

Technologies). Retrotranscription was performed according to manufacturer's instructions using a maximum of 2 µg total RNA in a reaction volume of 20 µl (100 ng/µL final theoretical cDNA concentration).

The concentration of single stranded cDNA (ssDNA) was quantified by fluorescence in the Synergy HT Multi-Made Microplate Reader (BioTek) using Quant-iT™ OliGreen® ssDNA Assay Kit (Invitrogen, Life Technologies). The quantification was run in triplicates, in a reaction volume of 100 µl, with a theoretical cDNA concentration of 0.2 ng/µL. The fluorescence was measured at 485/20 nm excitation and 528/20 nm emission wavelengths. Real cDNA concentration was calculated using the high-range standard curve according to the manufacturer's instructions. Once cDNA concentration was calculated, the exact amount of cDNA loaded in the qPCR reactions was calculated adjusting the dilution used for each gene.

Gene transcription analyses by qPCR

Sequences for *A. anguilla utbf1* (GeneBank Accession number: KX132907), *rpL5* (KU140416) and *rpL11* (KU140415) were obtained from the European eel transcriptome database available in our laboratory after pyrosequencing the multi-tissue eel transcriptome (data not published). 18S rRNA sequence was obtained from NCBI (FM946070). Information allowing to clone and sequence partial fragments of *A. anguilla gtf3a* (KX132905) and *42sp43* (KX132906) sequences were obtained from the European eel genome sequence repository (http://www.zfgenomics.com/sub/eel). Annotation of sequences was performed through homology search using the BlastN and BlastX analysis (Table 1).

Table 1. European eel sequences cloned and sequenced for this study and identity values (through Balstx) with the most similar ortholog sequences available in GenBank. The percentage of the sequenced total gene cds is provided for each gene.

Gene name & NCBI accession number	Aminoacid identity (%)	E value (Blastx)	Most similar ortholog in GenBank	CDS%
A. anguilla upstream binding factor 1 (*ubtf1*) KX132907	97	2E-145	Upstream binding transcription factor, RNA polymerase I [*Danio rerio*] NP_001005395	73
A. anguilla transcription factor III A (*gt3a*) KX132905	62	E-116	General transcription factor IIIAa [*Danio rerio*] AAH95553	97
A. anguilla 42sp43 KX132906	64	3E-143	PREDICTED: P43 5S RNA-binding protein-like [*Esox lucius*] XP_012991469	96
A. anguilla ribosomal protein L5 (*rpL5)* KU140416	95	0	60S ribosomal protein L5 [*Salmo salar*] ACI66198	96
A. anguilla ribosomal protein L11 (*rpL11*) KU140415	99	2E-54	60S ribosomal protein L11 [*Platichthys plesus*] CAH57695	92

Primers 15-25 bp long were designed to obtain amplicons of around 200 bp. They were also designed to span exon-exon boundaries to avoid amplification of genomic DNA. The obtained primer sequences (Table 2) were evaluated for homo/hetero-dimmers and hairpin formations using the IDT online primer design tool. Primers were purchased from Eurofins MWG. To verify specificity of the primers, conventional PCRs were performed as follows: 94°C for 2 min, denaturation at 94°C for 30 s, annealing step (temperature for each primer set in table 2) for 30 s, elongation at 72°C for 8 min and finally 72°C for 8 s. PCR products were visualized in 1.5% agarose gels stained with ethidium bromide.

Table 2. Primer sequences used for the qPCR analysis of the transcription levels of 18S rRNA, *42sp43*, *gtf3a*, *rpL5*, *rpL11* and *ubtf1*in the ovaries of European eel.

Gene	Forward sequence (5'-3')	Reverse sequence(5'-3')	Amplicon size (bp)	Annealing temp (°C)
18SrRNA	GAGGCCCTGTAATTGGAATGAG	TAATATACGCTATTGGAGCTGGAATT	110	60
42sp43	CCTGCTTCTCCACCACCTT	CAGACTCCCTTGCATGGCA	124	58
gtf3a	AGGGTTGCGACAAGAGTTTCTGC	GAACACTTTTCCACAGCCCTCATA	214	61
rpL5	AGCAGTTCTCCCGCTTCAT	GACTGGGTTCTCACGGATA	96	56
rpL11	ATCGGCATCTACGGCTTGGA	GCCTCCTCCTTGCGGATG	119	59
ubtf1	ACCACTGCTAAAGATCAAGCCTG	CTGCAGAGTAGTGATTGAATGCCT	154	61

Transcription levels of *gtf3a*, *ubtf1*, *42sp43*, *rpL5*, *rpL11* and 18S rRNA were determined using SYBR® Green PCR Master Mix (Roche). Optimal concentrations of primers (25 mM for *ubtf1* and 12.5 mM for other genes) and samples (20 ng/µL) were used for each gene. Samples were run in triplicates in a 7300 PCR thermal cycler (Applied Biosystems)

using a final reaction volume of 20 µL, containing 2 µL of appropriately diluted sample. Reaction conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and annealing step of 60 s at appropriate temperature (Table 2). Amplification reaction was followed by a dissociation stage to obtain a dissociation curve, which would allow checking the specificity of each primer set and ensuring that only the specific transcript was amplified.

Transcription levels were normalized taking into account the amount of cDNA loaded for each sample as measured by fluorescence. All gene transcription results were normalized with the amount of cDNA charged in the qPCR according to Rojo-Bartolomé et al. (2016) using as adapted ΔCT formula (*RQ*) with efficiency correction (*E*):

$$E = \left[10^{-1/m}\right] - 1$$

m being the slope of the standard curve of the qPCR reaction.

$$RQ = Log_2\left[\frac{(1 + Efficiency)^{-\Delta CT}}{ng\ cDNA}\right]$$

Where $\Delta CT = CT\ sample - CT\ plate\ internal\ control$

Statistical analysis

The statistical analyses were undertaken using SPSS (SPSS Inc.). For the statistical analysis data were tested for normality by the Shapiro-Wilk (n<30) test. Equality of variance was also tested applying the Levene's test, both at a 0.05 significance level ($p<0.05$). Then, data were subjected to analysis of variance by One-way ANOVA to identify significant differences between the ovarian developmental stages. The one-way ANOVA analyses were followed by a Tukey post-hoc test to compare all of the groups in pairs.

RESULTS

RNA quality and 5S rRNA

RNA quality was checked by spectrophotometry and capillary electrophoresis. The mean value of A_{260}/A_{280} ratio was 1.71, with quite similar values for all the samples (Table 3). The total RNA quality assessment provided by the RIN algorithm could not be calculated for any of the PV and NR samples, and for some of the EV, MV and LR ones. In the cases when the RIN was measurable, only ovaries with LV and MN oocytes displayed values acceptable under the commonly used standards (Table 3).

Table 3. RNA quality parameters as they were estimated spectrophotometrically measuring the absorbance at A_{260}/A_{280} and at A_{260}/A_{230} and as measured through the calculation of the RNA Integrity Number (RIN) after capillary electrophoresis in the 2100 Bioanalyzer.

Sample	A_{260}/A_{280}	A_{260}/A_{230}	RIN
PV1	1.85	2.22	-
PV2	1.89	2.03	-
PV3	1.89	1.97	-
PV4	1.76	2.35	-
PV5	1.83	1.89	-
EV1	1.80	2.14	2.5
EV2	1.61	0.56	4.9
EV3	1.80	0.97	5.5
EV4	1.89	0.37	2.8
EV5	1.73	1.56	-
EV6	1.65	1.98	-
EV7	1.79	2.08	-
EV8	1.63	1.36	5.5
MV1	1.71	2.06	-
MV2	1.65	2.03	7.4
MV3	1.68	1.89	-
MV4	1.63	2.23	-
MV5	1.74	1.54	8.7
MV6	1.75	1.84	8.2

Sample	A_{260}/A_{280}	A_{260}/A_{230}	RIN
LV1	1.70	1.04	2.4
LV2	1.63	1.31	8.7
LV3	1.63	2.05	8.5
LV4	1.55	2.05	8.8
LV5	1.61	1.19	9.1
LV6	1.63	1.42	8.9
LV7	1.67	1.65	-
MN1	1.60	1.18	9.4
MN2	1.89	1.98	7.2
MN3	1.75	1.87	9.0
MN4	1.69	1.67	9.3
MN5	1.70	1.65	9.0
NR1	1.63	1.78	-
NR2	1.59	2.30	-
NR3	1.59	2.30	-
NR4	1.68	1.20	-

In the less developed ovaries total RNA electrophoresis always showed a prominent band in the region around 120 nucleotides that corresponds to 5S rRNA. Meanwhile the typical bands for 18S and 28S rRNA were very faint. Electropherograms provided by the Agilent 2100 Bioanalyzer allowed to distinguish different transcript profiles for ovaries depending on the developmental stage of the oocytes they contained (Fig 2). 5S rRNA peak was very high in ovaries with previtellogenic oocytes (PV and NR). Subsequently, 18S and 28S rRNA peaks progressively gained importance with respect to the 5S rRNA peak as vitellogenesis (EV<MV<LV) advanced towards oocyte maturation (MN) (Fig 2).

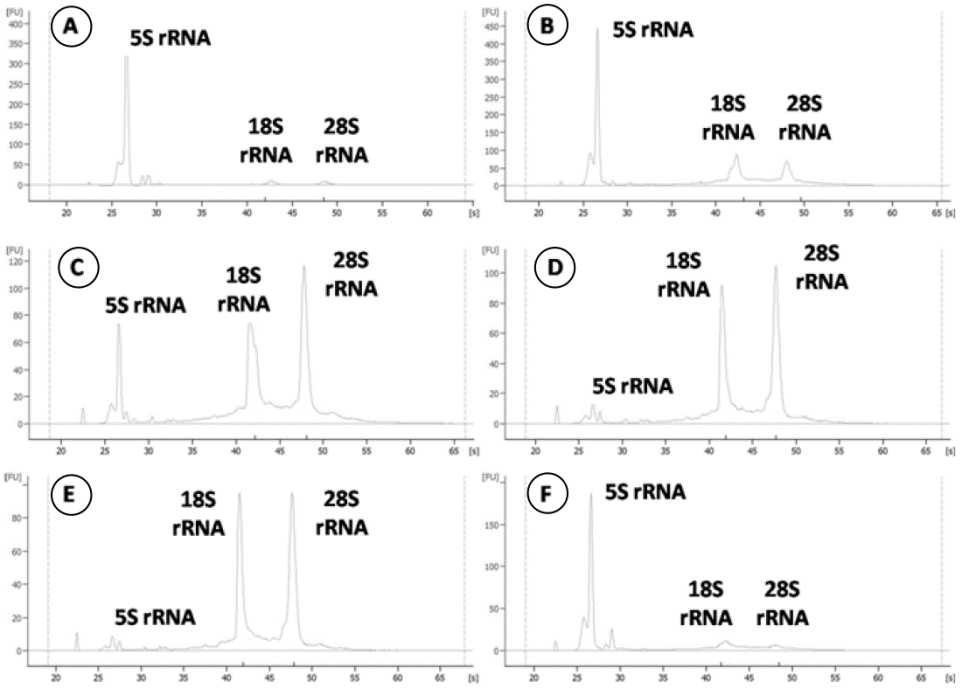

Fig 2. Total RNA extracted from eel ovaries at different developmental stages after hormonal treatment and analyzed through capillary electrophoresis using the 2100 Agilent Bionalyzer. (A) Electropherogram corresponding to an ovary with oocytes in PV stage with a prominent peak (high RNA concentration) belonging to 5S rRNA. 18S and 28S rRNA levels are so low that they can be hardly recognised. (B, C and D) Electropherograms of ovaries with oocytes at EV (B), MV (C) and LV (D) stages. (E) Electropherogram of an ovary with oocytes in MN stage. (F) Ovary from a NR female with previtellogenic oocytes.

5S/18S rRNA index during oocyte development

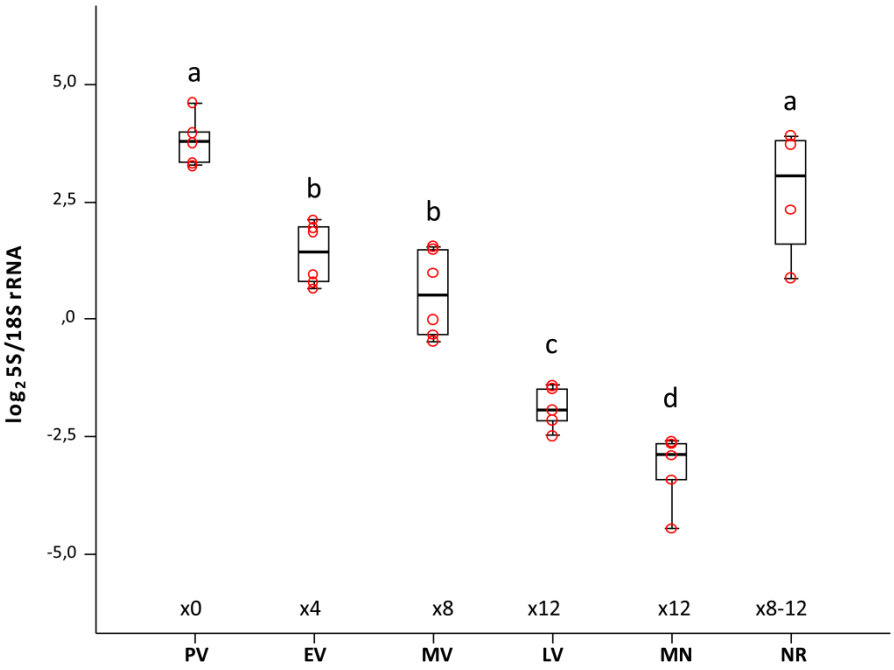

Fig 3. **5S/18S rRNA index during the ovarian development as calculated from the total RNA electropherograms**. Each dot identifies the index value of one individual. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between groups (Kruskal-Wallis, $p<0.05$). The number of CPE injections received by each individual is indicated "x number". In the NR group, 3 of the eels received 8 injections while the forth one received 12 injections.

The 5S/18S rRNA index allowed distinguishing ovaries according to their developmental stage (Fig 3). The highest index values were shown by the ovaries with oocytes in PV stage, differing significantly from ovaries with oocytes at other developmental stages as a result of the high prevalence of 5S rRNA. The index value decreased as the oocytes entered vitellogenesis and started to produce 18S and 28S rRNA, till reaching final maturation. Some of the animals did not respond to the hormonal stimulus and did not progress from early oogenesis, this being reflected in a lack of activation of 18S rRNA production. In these NR samples, the 5S/18S rRNA index was high, resembling the index values of non-treated ovaries (Figs 1, 2 and 3).

Transcription levels of ribosome biogenesis related genes through ovary development

The transcription levels of genes related with the 5S rRNA production by RNA Polymerase III (*gtf3a*), cytoplasm stockpiling (*gtf3a* itself and *43sp42*) and nuclear transport for ribosome assembly (*rpL5* and *rpL11*) were measured (Fig 4A). All genes showed identical transcription pattern. The highest transcription levels in all the cases were recorded during early oocyte developmental stages (PV and EV). On the contrary, when the oocytes were mature, at MN stage, the lowest transcription levels were measured. NR individuals showed transcription levels among EV, MV or LV groups (Fig 4). Also, genes related with RNA Polymerase I activity and 18S rRNA production were studied transcriptionally (*ubtf1* and 18S rRNA) (Fig 4B). In the case of *ubtf1*, the transcription levels followed the same trend observed for the genes related to 5S rRNA. The NR group showed the same transcription levels of LV and MN groups. On the contrary, 18S rRNA transcription levels were at their lowest at PV stage and increased while oogenesis progressed to maturity. Transcription levels in the NR individuals resembled those recorded in PV individuals (Fig 4B).

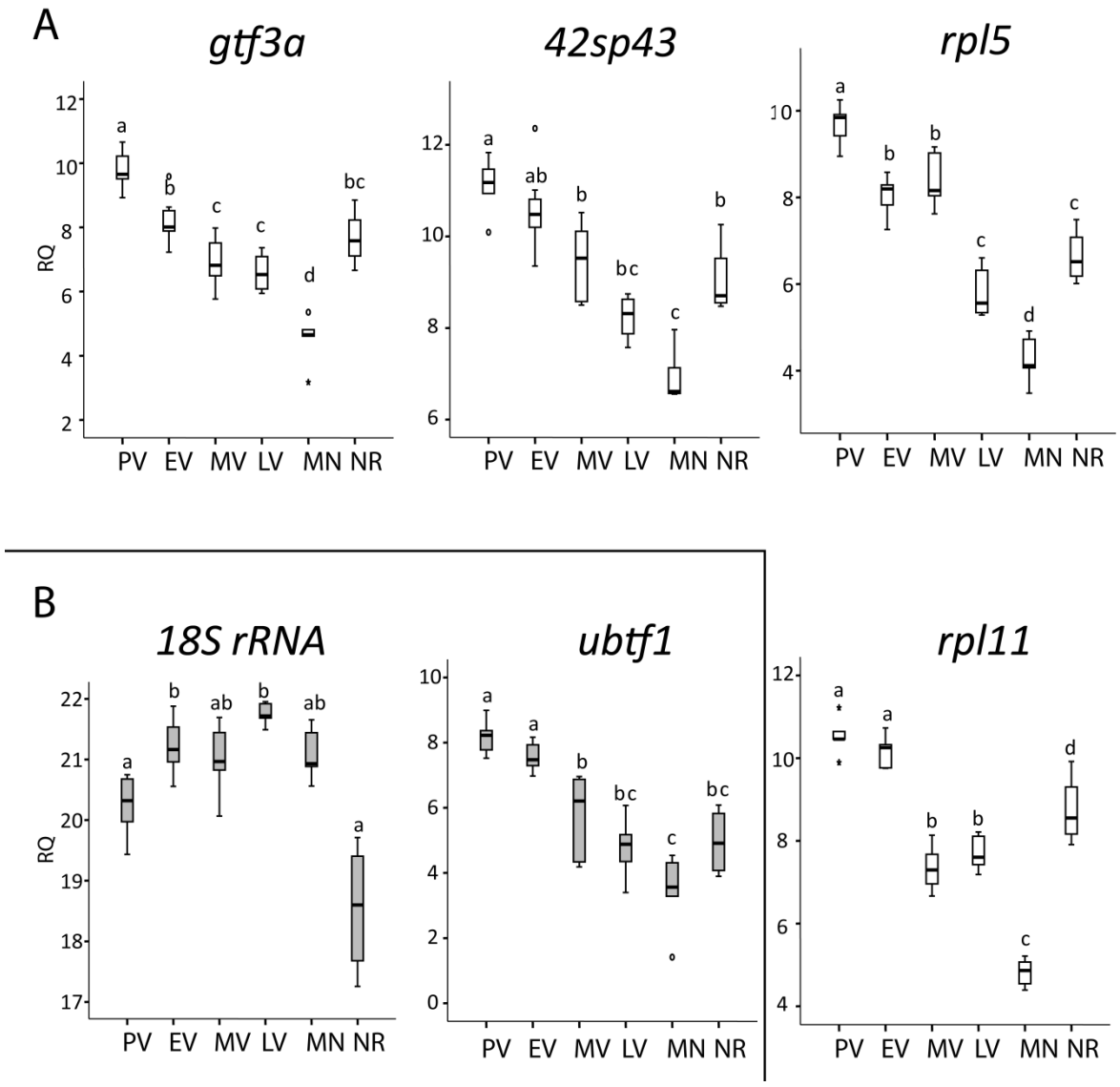

Fig 4. **Box plots representing relative quantification of the transcription levels of different genes related to the biogenesis of ribosomes in ovaries of European eels injected with CPE**. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. (A) Transcript levels of 5S rRNA related genes (*gtf3a, 42sp43, rpL5 and rpL11*) (B) Transcript levels of RNA Polymerase I related genes (ubtf1 and 18S rRNA). Different letters indicate significant differences between means (ANOVA, $p<0.05$).

DISCUSSION

In the present study we have quantified the changes in the transcription levels of ribogenesis related genes in ovaries of European eel, *Anguilla anguilla*, artificially matured through weekly intraperitoneal injections of CPE. 5S rRNA predominates in the

total rRNA of ovaries containing PV stage oocytes. Then, 18S rRNA begins accumulating as vitellogenesis advances until reaching a maximum with the presence of migratory nucleus stage oocytes after 12 weeks of treatment. NR ovaries, still with PV oocytes after 8-12 weeks of injections, show a rRNA profile similar to that of the non-hormonally treated ones. In association with these changes we have observed a transcriptional regulation of g*tf3a*, *42sp43*, *rpl5* and *rpl11* which follows the same pattern as that of the 5S/18S rRNA index along oocyte development and through hormonal treatment. In contrast, the transcription profile of *ubtf1* does not follow the pattern shown by 18S rRNA and it also resembles that of the index.

Ovarian development and 5S rRNA accumulation in European eel

Sexual maturation in freshwater eels has been studied trying to decipher the cell signals and molecular mechanisms that trigger gametogenesis (Durif et al., 2009; Palstra & van den Thillart 2010). When female European eels initiate reproductive migration, and leave the upper continental streams and lakes to enter transitional waters, oocytes in the ovaries are generally in PV stage (perinucleolar and/or cortical alveolar oocytes). During this late phase of prepuberty, eels begin the silvering process as a consequence of many hormonal, developmental, morphological and behavioural changes (Aroua et al., 2005; Pasquier et al., 2011). In any case, vitellogenesis continues to be blocked and it is oceanic migration, in a process that it is believed to be controlled by light, temperature and swimming exercise, that removes the blockade (Palstra & van den Thillart, 2010; Pérez et al., 2011; Mazzeo et al., 2014). This blockade is mainly due to dopaminergic inhibition (Dufour et al., 2003; Aroua et al., 2005). Thus, hormonal treatments with fish pituitary extract can artificially initiate oogenesis in eels (Pasquier et al., 2011; Pérez et al., 2011), obtaining gonads with gametes at different development stages (Pérez et al., 2011). Hormones within pituitary extracts allow ovarian steroid synthesis, stimulating oocyte growth and activating the reproductive endocrine axis.

The electrophoretic analysis of total RNA extracted from ovaries showed that previous to initiation of vitellogenesis, oocytes accumulated high relative amounts of 5S rRNA in perinucleolar PV oocytes; whereas 18S and 28S rRNA progressively gained in importance in comparison to 5S rRNA as vitellogenesis advanced towards final oocyte maturation.

This oocyte specific accumulation of 5S rRNA was first described in anuran frogs, where 5S rRNA can constitute 75% of the ovarian total RNA content (van den Eynde et al., 1989), and it has been also demonstrated in many teleost fish species with different ovarian maturation mechanisms; asynchronous vs. synchronous (Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2016).

These results have important methodological implications when applied to the molecular analysis and gene transcription profiles in fish ovaries. The gold standard for the analysis of the quality of the total RNA extracted from a tissue is given by the RNA Integrity Number (RIN) obtained using the 2100 Bioanalyzer of Agilent Technologies. This value is obtained using the 28S to 18S rRNA ratio. Acceptable values for downstream gene transcription analysis are above 7-7.8. In our eel samples, with oocytes in stages MV, LV and MN, calculated RIN values were normally above 7. In contrast, in many samples with oocytes in PV, EV and NR, RIN values could not be calculated or they were bellow 7, due to the lack of prominent 18S and 28S rRNA bands. We have previously seen in ovaries of different fish species that RIN values cannot be calculated when ovaries are in early oogenesis stages (Rojo-Bartolomé et al., 2016). Kroupova et al. (2011) and Manousaki et al. (2014) have also described this problem. Manousaki et al. (2014) concluded that it was not possible to calculate the RIN value in the ovaries of the proterandric hermaphrodite fish *Diplodus puntazzo*. They even mentioned that specific efforts were done to improve the RIN number of the ovaries, whose values were always sub-optimal due to the presence of a prevalent peak of around 100 nucleotides (5S rRNA). In the future, fish reproductive physiologists and endocrinologists should consider that RIN calculation is not representative of RNA quality in fish ovaries, especially in immature individuals.

5S/18S rRNA index during oogenesis in European eels

5S/18S rRNA index, calculated on electropherograms obtained after total RNA electrophoresis, showed that each stage of ovarian development can be quantitatively differentiated in eels. As described in other teleost species; *Engraulis encrasicolus* and *Lepidorhombus whiffiagonis* (Rojo-Bartolomé et al., 2016), PV stages in eels displayed the highest index values, while ovaries containing mature oocytes ranked the lowest,

due to the increased transcription of 18S rRNA during vitellogenesis. Therefore, 5S/18S rRNA index values are diagnostic to identify quantitatively the developmental stage of the ovaries.

Oogenesis involves oocyte growth, meiosis, and synthesis and storage of organelles and new molecules (Song et al., 2006; Kleppe et al., 2014; Ortiz-Zarragoitia et al., 2014). This is evident in the case of 5S rRNA in PV oocytes, RNA showing an electrophoretic profile different from any other eukaryotic cell. Ribosome biogenesis is dependent of rRNA synthesis, which is itself subjected to growth and nutrients availability (Murray et al., 2003). It seems that oocytes would need to accumulate rRNAs in order to quickly assemble ribosomes in case of being fertilized and, in this way, allow protein synthesis during early embryogenesis (Ortiz-Zarragoitia et al., 2014). Early production and stockpiling of 5S rRNA could be explained attending to the reduced energetic demand in comparison to the energy that needs to be invested in the production of the biggest rRNA molecules. In this way, the oocytes would save energy during previtellogenesis until reproduction is envisaged to occur under favourable conditions (Rojo-Bartolomé et al., 2016). At this point, with the onset of vitellogenesis, the production of more energetically demanding molecules, such as 18S and 28S rRNA, would be put in place. This is more evident in yellow eels that live in continental waters at prepubertal stage for years. Mechanistically, the present study suggests that blockade of vitellogenesis in European eels, exerted at the level of dopamine and gonadotropin production, inhibits transcription via RNA polymerase I (transcription of 45S rRNA genes and synthesis of 18S and 28S rRNA). Thus, gonadotropins (or the sex steroids induced by them) would release this blockade during gonadal recrudescence.

Transcription levels of genes controlling rRNA synthesis in European eel ovaries

5S rRNA is produced by RNA Polymerase III (Pol III) in eukaryotic cells. In turn, Pol III activation is controlled by Gtf3a (Szymanski et al., 2003). Additionally, Gtf3a also binds 5S rRNA product for its stockpiling in the cytoplasm as 7S RNP and it has been shown to be strongly transcribed in ovaries in contrast to testis in fish (Rojo-Bartolomé et al., 2016). Our results hereby demonstrate the accumulation of *gtf3a* transcripts in ovaries

of eels. Moreover, as it occurs in ovaries from megrim (Rojo-Bartolomé et al., 2016), *gtf3a* was downregulated at vitellogenesis in European eel allowing also to identify the ovarian developmental stage. PV individuals displayed the highest transcription levels of *gtf3a*, and MN ones the lowest. NR females, despite suffering hormonal treatment for 8 to 12 weeks, showed *gtf3a* transcription levels similar to those observed in EV and LV, and displayed oocytes at PV-EV stages. Thus, transcription of *gtf3a* seems somehow regulated by the hormonal treatment itself, although full regulation is consequence of oocyte differentiation occurring after hormonal treatment.

18S rRNA and other ribosomal RNAs, in contrast to 5S rRNA, are synthesised by RNA polymerase I (Pol I) which produces the 45S pre-rRNA transcript in the nucleolus (Drygin et al., 2010). Pol I is regulated by Ubtf1 that has the ability to form an 'enhancesome' in rDNA and enable the access of the Pol I machinery (Bazett-Jones et al., 1994; Reeder et al., 1995). In cancer cells, where a high demand of ribosomes for protein production occurs, transcriptional upregulation of *ubtf1* has been observed (Drygin et al., 2010). On the other hand, a possible postranslational regulation of Ubtf1 activity by de/phosphorilation and de/acetilation processes has been defended to predominate (Russell & Zomerdijk, 2005). For instance, Ubtf1 phosphorilation at serine 358 is required for the activation of rDNA transcription in mammalian fibroblasts (Voit & Grummt, 2001). Ubtf1 phosphorilation directly regulates rRNA elongation by inducing the remodelling of rDNA chromatin in the nucleolus (Stefanovsky & Moss, 2008). In the present study, qPCR analysis showed no match between the profiles of *ubtf1* and 18S rRNA transcription. In European eel ovaries *ubtf1* transcript levels were at their highest during PV stage, when, as reported in *Xenopus,* Pol I activity would be very low (Roger et al., 2002). On the contrary, it was significantly downregulated during MN phase, when Pol I activity should be maximal, according to 18S rRNA levels in the present study and as reported for *Xenopus* oocytes (Ginsberg et al., 1984). Thus, these results reinforce the idea that postranlational events, such as phosphorilation of Ubtf1, are responsible for activation of the Pol I machinery during vitellogenesis. The production of 45S pre-rRNA precursor must be fast before meiosis resumption, so Pol I would be controlled mainly by posttranslational modifications of Ubtf1 as proposed by Voit & Grummt (2001) or Stefanovsky & Moss (2008). Thus, we propose that CPE, and possibly gonadotropins

therein or the ovarian estradiol production triggered by them, would regulate Pol I activation during secondary oocyte growth. In NR females the impossibility to respond to the gonadotropin challenge would be reflected in an impossibility to activate Pol I and to produce 18S rRNA.

Transcription levels of other genes involved in 5S rRNA subcellular localization

It could be expected that genes related to ribosome biogenesis and 5S rRNA handling would be also transcribed very early during oocyte development (Diaz de Cerio et al., 2012). For instance, 42Sp43 forms 42S RNP storage particles with 5S rRNA in the cytoplasm (Ciganda & Williams, 2011; Ortiz-Zarragoitia et al., 2014). As expected, and as previously observed in thicklip grey mullet ovaries (Diaz de Cerio et al., 2012), *42sp43* showed the same transcription pattern during artificially induced eel maturation as that of 5S rRNA and *gtf3a*.

Ribosomal proteins RpL5 that binds 5S rRNA in the cytoplasm to allow its re-entry into the nucleus to begin ribosome formation; and RpL11, that also interacts with 5S RNA in the process of transportation towards the nucleus (Szymański et al., 2003; Donati et al, 2013; Tang et al., 2015) were analysed as well. *rpL5* and *rpL11* showed the same transcription pattern as *gt3a* and *42sp43* during maturation. The lowest levels were recorded in the most mature ovaries, and the highest levels in ovaries with PV oocytes. NR females always showed higher transcription levels than responding ones. It must be remembered that RpL5 and 11 are the main responsible for the incorporation of 5S rRNA into the nucleolus (Szymanski et al., 2003), which is the main morphological and functional feature of fish PV (perinucleolar) oocytes.

From looking at the overall gene transcription profile hereby, it could be postulated that all genes, with the exception of 18S rRNA, were downregulated during oocyte development as a consequence of a dilution effect caused by oocyte growth. Moreover, it could also be deduced that this downregulation would be consequence of a general turning off of the transcription machinery, occurring with whole chromatin assembly prior to final meiotic division in oocytes. However, fish oocytes are known to be

transcriptionally active until the MN stage, and many genes are actively up-regulated during vitellogenesis (Kleppe et al., 2014). In a microarray based transcriptome comparison of Atlantic cod (*Gadus morhua*) follicles in different oogenesis stages, including ovulated eggs, Kleppe et al., (2014) demonstrated a continuous pattern of mRNA accumulation and degradation along oogenesis. In general, more transcripts were downregulated than upregulated from PV to EV stage follicles (555 transcripts down *vs* 349 up), or from EV to LV stages (532 *vs* 376), but transcription was still active at LV. The general shut down of transcription occurred at ovulation (MN) but with still 149 upregulated genes (647 downregulated). Transcriptional studies, performed with the same eel batch studied here, have demonstrated constant transcription of housekeeping or zona pellucida genes until the MN stage (Mazzeo et al., 2012; 2014).

Thus, we can conclude that 5S rRNA is highly expressed in European eel ovaries with the highest relative expression levels in ovaries with PV oocytes and the lowest in ovaries with MN oocytes. Carp pituitary extract, probably gonadotropins therein or the estrogen they could induce to produce in the ovary, positively regulates vitellogenesis in European eel, controlling also the inactivation of RNA polymerases III and the activation of the RNA polymerase I pathway. In female eels therefore, identification of a decrease in 5S/18S rRNA index and in *gtf3a* transcription levels could be taken as an indication of ovarian recrudescence and initiation of vitellogenesis. This could be a very useful tool in the research of eel reproduction, endocrinology and physiology.

However, the observed regulation of ribosomal genes studied herein cannot be considered a direct consequence of CPE treatment, but a consequence of the oocyte differentiation/maturation triggered by the hormonal treatment. NR females, treated during 8-12 weeks, never reach the gene transcription levels measures in the MN group. In any case, non-differentiated NR females show transcription levels more similar to MV suggesting that hormonal treatment in itself, or the estrogen synthesis that it triggers in the ovaries, has some transcription regulation role in such genes. The 5S/18S rRNA index identifies NR females after 4 weeks of injection. Long-term hormonal treatments currently necessary to mature eels in captivity (Okamura et al., 2014) are expensive, and maturing one single female can cost between 50 and 100 € (taking into account only the CPE used in this experiment, Mazzeo et al., 2014). Being able to predict in a batch of

female eels, that they will not be able to mature properly after just 5 injections, could save a considerable amount of money.

This study, has provided important basic knowledge on the transcriptional regulation of ribosomal genes during oocyte development in fish and could have important implications for the study of European eel endocrinology and future aquaculture production.

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Chapter 4

Duplication of the transcription factor IIIA (*gtf3a*) gene in teleost genomes, and oocyte-specific transcription of *gtf3ab*

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5th INTERNATIONAL WORKSHOP ON THE BIOLOGY OF FISH GAMETES. Ancona, (Italy). 2015. Duplication of the transcription factor IIIa (TFIIIA) gene in teleost genomes, and oocyte-specific transcription of TFIIIab: aplications in the environmental monitoring of xenoestrogenicity. Rojo-Bartolomé, I; Cancio, I. ICC, oral

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LABURPENA

Oogenesia arrainetan ugalketa sasoi bakoitzean gertatzen den oozitoaren hazkuntza masiboa du bereizgarri. Zenbait molekulen pilaketak, RNA erribosomikoak adibidez, etorkizuneko enbrioiaren garapen egokia ziurtatzen du. Pilatutako rRNA hauek esaterako, sortu berria den enbrioian erribosomen eta beraz proteinen sintesian laguntzen dute. 5S rRNA-ren gain-adierazpena oozitoetan, zeina Gtf3a transkripzio-faktoreak bideratzen duen, xenoestrogenoen eraginpean agertutako intersex banakoak identifikatzeko markatzaile gisa erabil daiteke ingurumenaren osasun-jarraipen programatan. Arrain-genomen analisiak (Ensembl) arrain teleosteoekiko espezifikoa izan zen genomaren bikoizketaren ondorioz (sintenia analisia) sortutako bi *gtf3a* gene paralogo identifikatzea ahalbidetu du. *gtf3ab*, zebra arrainean (*Danio rerio*) eta tilapia (*Oreochromis niloticus*) espezieetan frogatu den bezala, arrainen oozitoetan adierazten da soilik, *gtf3aa* ehun guztietan adierazten den bitartean. Zebra arrainak 100 ng/L 17β-estradiola-rekin 61 egunez (ernalketa egunetik hasita) tratatu ostean eme bilakatzea lortu zen. Eme hauek obarioko *gtf3ab* adierazi zuten bitartean, ar bilakatutako zebra arrainek (100 ng/L 17α-metiltestosteronarekin tratatuak) soilik *gtf3aa* adierazi zuten. *gtf3ab*-ren transkripzioa emeetan *cyp19a1a*-ren transkripzioarekin bat zetorren eta *amh* eta *dmrt1*-ren kontrara. Zebra arrainen enbrioietan, *gtf3ab* transkripzioa oogenesiarekin batera hasi zen, oozitoen ekoizpenaren markatzaile goiztiar gisa jardunez. Aldiz, *gtf3aa* transkripzioa enbrioietan zigotoaren genoma piztearekin batera hasten dela ikusi zen. Beraz, *gtf3ab*-ren transkripzioa oozitoen desberdintzapenaren ondorioa da eta ez estrogenoen esposizioaren eragin zuzena, eta oozitoen adierazlea izanik, intersex banakoen markatzaile baliogarria izan daiteke.

ABSTRACT

Oogenesis in fish is characterized by a massive growth of the oocytes for spawning each reproductive season. Stockpiling of certain molecules, such as ribosomal RNAs ensures proper development of the future embryo and in this way, accumulated rRNAs assist ribosomal assembly and protein synthesis in the newly formed embryo. The massive 5S rRNA expression in oocytes, which is allowed by transcription factor IIIA (Gtf3a), serves as marker of intersex condition in fish exposed to xenoestrogens in environmental monitoring programmes.

Analysis of fish genomes (Ensembl) has allowed the identification of two *gtf3a* paralog genes. *gtf3ab*, aroused from the teleost specific genome duplication event (synteny analysis) and it has been maintained as a gene with specific transcription in oocytes as demonstrated here for zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*). Instead *gtf3aa* is ubiquitously expressed in zebrafish and tilapia organs. Under laboratory exposure to 100 ng/L 17β-estradiol, 61 dpf zebrafish were fully feminised showing transcription of ovarian *gtf3ab*, while masculinised zebrafish (exposed to 100 ng/L 17α-methyltestosterone) only showed transcription of *gtf3aa*. The transcription of g*tf3ab* in females coincided with the transcription of ovarian *cyp19a1a* and opposite to *amh* and *dmrt1*. In normal developing zebrafish embryos, *gtf3ab* transcription begins only at the onset of oogenesis, functioning as an effective early marker of oocyte production. On the other hand, the transcription of *gtf3aa* in normal developing zebrafish begins with the activation of the zygotic genome (~8 hpf). Thus, *gtf3ab* transcription is a consequence of oocyte differentiation and not a result of estrogen exposure and could constitute a very potent marker of intersex condition and gonad feminisation.

INTRODUCTION

A great variety of anthropogenic chemicals present estrogen- or androgen-like properties displaying biological activities similar to those of endogenous hormones. Upon bioaccumulation such chemicals interfere with the normal hormonal function altering, for example, the normal control of sexual differentiation, gametogenesis and reproduction. They are known as reproductive endocrine disrupting compounds (reproductive EDCs) (Leet et al., 2011¸ Segner et al., 2013; Ortiz-Zarragoitia et al., 2014) and they have become a global concern due to their ubiquity in aquatic environments (Örn et al., 2006). EDCs reach the aquatic environment from wastewater treatment plants, and strongly influence fish development, especially in sensitive early life stages even at concentrations as low as parts per trillion (Godwin et al., 2003; Guerrero-Estévez & Moreno-Mendoza, 2010; Leet et al., 2011; Valencia et al., 2016).

Sex determination systems in teleost fish are extremely plastic but poorly understood with most of the karyotyped species not showing differentiated sex chromosomes (Devlin & Nagahama, 2002). In addition, teleost genomes show a partially duplicated genome after the fish specific genome duplication event and this further complicates the identification of potential sex linked genes (von Hofsten & Olsson, 2005). Both sex determination and differentiation are also environmentally driven in fish. Among others, environmental factors, such as hypoxia (Shanget al., 2006), food availability (Lawrence et al., 2008), and temperature (Uchida et al., 2004; Ospina-Álvarez & Piferrer, 2008) have been shown to strongly influence sex determination and differentiation. Sex and reproduction in fish can also be influenced by EDCs (Segner et al., 2013). Exposure to EDCs during critical periods of development in zebrafish (*Danio rerio*) has been reported to impair among others gonadal development (Xu et al., 2008; Baumann et al., 2014a; Luzio et al., 2015a), alter sex phenotypes (Uchida et al., 2004; Fenske et al., 2005; Luzio et al., 2015a) and to feminise and/or masculinise individuals (Fenske et al., 2005; Larsen et al., 2008; Soares et al., 2009, Leet et. al., 2011).

The zebrafish (*Danio rerio*) is recommended as a test species in many existing standard ecotoxicological guidelines and it is probably the most studied fish in developmental biology (von Hofsten & Olsson, 2005). Zebrafish is considered to be an undifferentiated

gonochoristic species (Yamamoto, 1969), with both sexes passing through an ovary-like or juvenile-ovary stage, before differentiation into both phenotypic mature sexes. In males, this includes a type of juvenile hemaphroditism at around 25 days post hatching (Takahashi, 1977; Chan & Yenung, 1983; Maack & Segner, 2003). Sexual determination in zebrafish is polygenic (Bradley et al., 2011; Anderson et al., 2012; Liew et al., 2012; Liew & Orban, 2014) and is secondarily influenced by environment (Devlin & Nagahama, 2002; Ospina-Álvarez & Piferrer, 2008). Exposure of different chemical compounds considerEDCs at relatively low concentrations) can cause sex reversal and disturbances in gonad development and in reproduction capacity (Andersen et al., 2000; Hill & Janz, 2003; Soares et al., 2009; Luzio et al., 2015).

Traditional biomarkers of fish exposure to (xeno)hormones include alterations in gross morphology and sex characteristics, changes in gonadosomatic index, in plasma or liver vitellogenin levels, and apparition of gonad histopathological alterations (Seki et al., 2006). One of the most notable effects identified in fish inhabiting polluted sites is the onset of intersex condition, where male testis develops oocytes within the spermatic follicles (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014). Exposure to reproductive EDCs also alters the normal expression pattern of genes involved in sex differentiation. Some of the genes whose transcription levels are dimorphic in relation to sex phenotype and whose transcription is altered after exposure to EDCSs are the anti-Müllerian hormone (*amh*), doublesex and mab-3 related transcription factor 1 (*drmt1*) and gonadal aromatase (*cyp19a1a)*. It is well known that exposure to androgens down-regulates *cyp19a1a* expression (Baron et al., 2008), a well recognized player in ovarian development, that exhibits sexually dimorphic expression (Leet al., 2011). In contrast, male marker genes, such as *amh* and *dmrt1,* can be down-regulated after estrogen exposure leading to feminising effects (Marchand et al., 2000; Schulz et al., 2007). In an environmental context of xenoestrogen exposure in which intersex males appear it remains to be elucidated if transcription alteration of such genes reveals estrogen exposure or oocyte formation in testis. It follows, that generating biomarkers of oocyte differentiation could be important for the early identification of intersex condition in environmental monitoring programs.

In addition to the mentioned genes, the general transcription factor IIIA (*gtf3a*), controlling the transcription of 5S rRNA in eukaryotes (Szymański et al., 2003), has been shown to be up-regulated in testis of intersex male thicklip grey mullets (*Chelon labrosus*) from a polluted harbour in the Bay of Biscay (Diaz de Cerio et al., 2012). These mullets also showed elevated vitellogenin transcript and protein levels in liver and plasma and up-regulation of *cyp19a1b* in brain (Bizarro et al., 2014). 5S rRNA is strongly expressed and accumulated in teleost oocytes working as a very efficient molecular marker of sex in teleost fish (Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2016). *gtf3a* transcription levels consequently are higher in ovaries than in testes in all teleost fishes tested to date, as Gtf3a besides acting as a transcription factor that activates RNA polymerase III it also acts as a 5S rRNA binding protein for its stockpiling in the cytoplasm (Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2016). In *Xenopus*, a single *gtf3a* gene codes for two different transcripts corresponding to an oocyte and a somatic form of the protein, synthesized through differential promoter usage (Penberthy et al., 2003; Layat et al., 2013). In fish, a single *gtf3* gene was first characterized in the catfish, where it was shown to code for a protein associated to 5S rRNA in the ovary (Ogilvie & Hanas, 1997).

Thus, the objectives of the present study were to elucidate the nature of *gtf3a* sexually dimorphic transcription in fish gonads. It remains to be elucidated whether transcription in ovaries (and in intersex testis) is a consequence of estrogen exposure or of oocyte differentiation. In the same way, it remains to be known whether fish present two differentially expressed *gtf3a* transcripts, one specifically expressed in ovaries and another one in somatic tissues, as it is the case in anuran frogs. In order to pursue such objectives we selected the laboratory model species *Danio rerio*, whose genome is fully sequenced, in order to study the pattern of *gtf3a* transcription under exposure to 17β-estradiol and 17α-methyltestosterone from fertilization to 60 days post-fertilization (dpf).

MATERIALS AND METHODS

Synteny analysis of the general transcription factor 3A gene (*gtf3a*)

The Emsembl genome repository was (http://www.ensembl.org/index.html) searched looking for vertebrate *gtf3a* sequences ortholog to the known *Chelon labrosus* (JN257141) and *Xenopus laevis* (BC129561) sequences. When such sequences were found the possible presence of paralog sequences in each of the genomes was checked. Then, a synteny analysis was carried out using human *gtf3a* (Ensembl ENST00000381140) as template and comparing all the flanking genes for each of the identified *gtf3a* genes in the Genomicus v84.01 browser (Louis et al., 2013). A phylogenetic tree was created with the protein sequences coded by the found fish (teleosts, non-teleost actinoptygian *Lepisosteus oculatus* and basal sarcopterygian fish *Latimeria chalumnae*), human, *Xenopus* (oocyte and somatic Gtf3a) and chicken *gtf3a* orthologs using the Phylogeny.fr platform (www.phylogeny.fr).

Biological samples and hormonal treatment

Chemicals

In order to obtain monosex female and male populations, zebrafish were exposed from fertilization to 61 dpf to 100 ng/L of 17β-estradiol (E) and 17α-methyltestosterone (MT) taking into account the procedures described by Andersen et al., (2000). Hormones (purity >98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). E and MT stock solutions were prepared fresh weekly dissolving them in absolute ethanol at 1 g/L (stock solution). Then, 2 mL of stock solutions were daily diluted in 20 L water to obtain a final E or MT nominal concentration of 100 ng/L and 0.01% ethanol in the total volume.

Danio rerio egg production and exposure to hormones

Adult zebrafish (*Danio rerio*; wild type AB Tübingen) were maintained at a water temperature of 24°C with a 14 h light/10 h dark cycle in 100 L tanks with mechanical and biological filters following standard protocols as described in Vicario-Parés et al., 2014). The fish were fed with Vipagran Baby (Sera, Heinsberg, Germany) and artemia nauplii (*Artemia salina*) twice per day. 23 breeding couples were selected and placed separated by sex through a barrier in a single tank. The day prior to the beginning of the exposure experiment, females and males were independently coupled in breeding traps separated by

a barrier (S1A Fig). Before turning on the light in the following morning, the barrier was removed. 18 couples reproduced and the newly fertilized eggs were collected selecting fertilized viable eggs under a stereoscopic microscope (Nikon smz800, Kanagawa, Japan).

Obtained embryos (n=830 initial amount) were separated in groups of 40 to 60 individuals and placed in glass Petri dishes, to obtain three Petri dishes per experimental group (S1B Fig). The Petri dishes were filled with 50 mL of corresponding experimental solution: water (water control), water with 0.01% ethanol (ethanol control, ET), and hormone solution (100 ng/L E or MT depending on the experimental group). Once a day half of the volume was replaced with fresh solution. Additionally, embryos were examined daily until 5 dpf to detect and discard any embryo with malformations or dead. Criteria for normal zebrafish embryo development morphology were based on Kimmel et al. (1995).

After 5 dpf 100 larvae were transferred to 10 L tanks (filled with 8 L control or spiked water) and exposed in constant drip flux until day 61. The experiment was carried out in duplicated tanks for each experimental condition. The exposure regime was 10 L/day replaced with new solution (Figs S1C and S1D), and pH, conductivity, ammonia, nitrates and nitrites were weekly measured using commercial Sera tests (Hersteller, Germany) for the chemical analysis. The juveniles were fed *ad libitum* twice a day with corresponding fish food and artemia nauplii (see annex 1). Waste produced by fish was carefully removed with suction and lost volume immediately replaced. After 26 and 61 dpf, 22 individuals from each experimental condition were euthanized by an overdose of MS-222 (tricaine methane-sulfonate, Sigma-Aldrich) following the protocol authorised by the ethics commission of the University of the Basque Country (CEEA/337-2/2014). 12 whole individuals (6 from each experimental group replicate) were independently embedded in RNA later (Ambion, Life Technologies, Carslbard, USA) and frozen at -80ºC for molecular analysis. 61 day juveniles were frozen after decapitation. The rest 10 individuals were fixed in 4% neutral buffered formalin (NBF) and stored at 4°C for 24h before paraffin embedding for histological analysis. All individuals were measured before processing. After 61 dpf remaining fish were kept in clean water for 1 year and then sexed. All along the experiment no appreciable differences in mortality were observed between groups.

Sampling of adult zebrafish and tilapia individuals

Brain, gonad and muscle were dissected from three female and three male adult zebrafish (UB Tubingen) from our own stock. Adult tilapias (*Oreochromis niloticus*), five females and four males, were purchased from BREEN, Ltd., (NER group, Hondarribia, Spain). Fish were anaesthetized in a saturated ethyl 4-aminobenzoate (Fluka, Steinheim, Germany) water bath following the protocol authorised by the ethics commission of the University of the Basque Country (CEBA/152/2010). Each fish was sacrificed by decapitation and gonad, liver, muscle, eye and brain were dissected. A portion of each tissue was embedded in RNA later (Ambion, Life Technologies), frozen in liquid nitrogen and then stored in the laboratory at -80°C until further used.

Procurement and sampling of non-exposed zebrafish embryos

Additionally, 6 zebrafish couples were paired to obtain embryos. A total of 390 embryos were obtained and pooled in glass Petri dishes (≈30 embyos per dish) with clean water. Embryos suffering malformations or with retarded development were removed. Three groups with around 100 embryos were collected after 2, 8 and 30 hours post fertilization (hpf), immersed in TRizol (Invitrogen, Carlsbad, California, USA) and maintained at 4°C to proceed immediately with RNA extraction.

Histological analysis and staging

After 24 hours in the fixative samples were dehydrated in a graded series of ethanol (70%, 96%, and 100% ethanol) in a Leica ASP 300 tissue processor (Leica Biosystems, Wetzlar, Germany) and embedded in paraffin (see annex 2). 5 µm thickness sections were cut in a 2065 Supercut microtome and stained with hematoxylin/eosin using the Leica Autostainer XL workstation and mounted with the aid of the Leica CV 5030 workstation. The slides were microscopically examined under an Olympus BX61 light microscope (Tokyo, Japan).

RNA extraction and cDNA synthesis

Total RNA was extracted from 50-100 mg of tissue, or whole body in the case of exposed zebrafish larvae, using TRIzol® (Invitrogen) and following the manufacturer´s instructions.

Obtained RNA quality was checked in an Agilent RNA 6000 Nano Kit Bioanalyzer (Agilent Technologies, Santa Clara, California, USA).

First-strand cDNA was synthesized using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen) in the 2720 Applied Biosystems Thermal Cycler (Life Technologies). Retrotranscription was performed according to manufacturer's instructions using a maximum of 2 µg total RNA in a reaction volume of 20 µl (100 ng/µL final theoretical cDNA concentration). The concentration of single stranded cDNA (ssDNA) was quantified by fluorescence in the Synergy HT Multi-Made Microplate Reader (BioTek) using Quant-iT™ OliGreen® ssDNA Assay Kit (Invitrogen, Life Technologies). The quantification was run in triplicates, in a reaction volume of 100 µl, with a theoretical cDNA concentration of 0.2 ng/µL. The fluorescence was measured at 485/20 nm excitation and 528/20 nm emission wavelengths. Real cDNA concentration was calculated using the high-range standard curve according to the manufacturer's instructions. Once cDNA concentration was calculated, the exact amount of cDNA loaded in the qPCR reactions was calculated adjusting the dilution used for each gene.

gtf3aa and *gtf3ab* transcription pattern in zebrafish and tilapia

gtf3aa and *gtf3ab* mRNA fragments were designed for tilapia an zebrafish using sequences obtained from Ensembl and NCBI (Table 1). They were designed in exon-exon boundaries to avoid amplification of genomic DNA and amplified using conventional PCR employing 0.8 mM primers (Table 1). Properties of designed primers were checked employing the IDT OligoAnalyzer Tool (https://eu.idtdna.com/calc/analyzer) and purchased from Eurofins MWG.

Amplifications were run with commercial Taq DNA Polymerase, recombinant Kit and 100 mM dNTP Mix (Invitrogen) for 35 cycles in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad, California, USA). PCR procedure was as follows: 94°C for 2 minutes, and 35 cycles of denaturation at 94°C for 30 seconds, annealing step (temperature for each primer set in table 1) for 30 seconds and elongation at 72°C for 30 seconds, and a final step at 72°C for 8 minutes. PCR products were visualized in 1.5% agarose gels stained with ethidium bromide.

Quantitative PCR (qPCR) analysis

Sequences for *Danio rerio actb, amh, cyp19a1a, dmrt1, gtf3aa* and *gtf3ab* were obtained from NCBI (Table1).

Table 1. Primer sequences used for the PCR and qPCR analysis of different target genes in zebrafish and tilapia. Amplified fragment size in bp and PCR annealing temperature (**°C**) are indicated.

	Species	Gene	NCBI accession number	Forward sequence (5'-3')	Reverse sequence (5'-3')	bp	°C
PCR	*O. niloticus*	*gtf3aa*	XM_003454117	ATCTGTTCGTTTAGCGGCTGCT	GTATTTCCTCGGCTCCAGGC	276	60
		gtf3ab	XM_00344354	CACCCGCTACCAACTCACCA	CTGATGGACTCGGGCAATGT	133	60
	D. rerio	*gtf3aa*	NM_001003866	CACACTCAGCTTCTACCTTTCT	GGTCTCACAAGAGTAGCCTTTAT	114	59
		gtf3ab	NM_001089544	TTGCATGTGGAGACTGTGAGAAGA	CTGACTGAACACAGGTAAGGCTT	100	60
qPCR	*D. rerio*	*amh*	NM_001007779	AGGCTCAGTACCGTTCAGTGTT	TCTTCATCAGCTCTCGCTGCT	100	59
		actb	NM_131031	CATCTATGAGGGTTACGCTCTT	TCTCTTTCGGCTGTGGTGG	129	58.8
		cyp19a1a	NM_131154	CTCAATGAGCACGATCTGCTT	CTCCTGAGCATCTCTTTTGTG	129	57.9
		dmrt1	NM_205628	CAGGTTCCTCGTGCCAACA	GGGACGGTTTCCTGATGGA	173	58.8
		gtf3aa	XM_003454117	CATCCCGTCTGAGTGGCTACA	CTACACTAAGAAGGGCTCTAATAGG	224	61
		gtf3ab	XM_00344354	TAGGAAGCTGCATGAAGGTTA	ACATGGAAGGTTTACTCTGTG	115	55

Transcription levels were determined using SYBR® Green PCR Master Mix (Roche). Optimal concentrations of primers (12.5 mM) and theoretical sample concentrations of 8 ng/µL were used for each gene. Samples were run in triplicates in a 7300 PCR thermal cycler (Applied Biosystems) using a final reaction volume of 20 µL, containing 2 µL of appropriately diluted sample. Reaction conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and annealing step of 60 s at appropriate temperature (Table 1). Amplification reaction was followed by a dissociation stage to obtain a dissociation curve, which would allow checking the specificity of each primer set and ensuring that only the specific transcript was amplified.

Transcription levels were normalized taking into account the amount of cDNA loaded for each sample as measured by fluorescence. All gene transcription results were normalized with the amount of cDNA charged in the qPCR according to Rojo-Bartolomé et al. (2016) using an adapted ΔCT formula (*RQ*) with efficiency correction (*E*):

$$E = \left[10^{-1/m}\right] - 1$$

m being the slope of the standard curve of the qPCR reaction.

$$RQ = Log_2\left[\frac{(1+Efficiency)^{-\Delta CT}}{ng\ cDNA}\right]$$

Where $\Delta CT = CT\ sample - CT\ plate\ internal\ control$

In the hormone exposure experiment final RQ values were obtained as follows:

$$RQ\ sample\ + \overline{RQ}\ corresponding\ reference\ group$$

Reference group in each case was chosen according to the nature of the studied gene since exposure control groups contained both female and male individuals. This was done assuming lowest transcription levels for each gene in the corresponding reference group. In this way; MT group was selected as reference group in the case of female marker genes (*gtf3ab* and *cyp19a1a*), ET group for *gtf3aa* and *actb*, and E group in the case of male marker genes (*amh* and *dmrt1*).

Statistical analysis

The statistical analyses were undertaken using SPSS (SPSS Inc., Chicago, Illinois). Data failed in normality and variance equality after applying the Shapiro-Wilk (n<30) test and Levene's test, both at a 0.05 significance level ($p<0.05$). Significant differences between replicates were thus established using the non-parametric Mann-Whitney test and differences between experimental groups were then evaluated using the non-parametric Kruskal-Wallis test. In all the cases, significant differences were established at $p< 0.05$.

RESULTS

Synteny analysis of the general transcription factor 3A gene (*gtf3a*)

To elucidate the *gtf3a* evolutionary history, we compared the adjacent genomic regions of *gtf3a* in all curated vertebrate genomes incorporated in Ensembl. In non-teleost genomes a single *gtf3a* was identified, localized in different chromosomes depending on the species (Fig 1; Table S1). *gtf3a* gene was observed to be duplicated (*gtf3aa* and *gff3ab*) in teleost genomes (zebrafish, tetraodon, fugu, stickleback, Amazon molly, tilapia and Atlantic cod),

only one gene, identified as *gtf3ab*, being present in cave fish, medaka and platyfish. The teleost paralog *gtf3ab* sequences clustered together, separated from the cluster formed by the protein sequences of teleost *gtf3aa* genes and the non-teleost *gtf3a*-s (S2 Fig). Synteny analysis, revealed that teleost *gtf3ab* neighbouring genes (for instance; *wasf3*, *rpl21*, *usp12*, *lnx2* and *pdx1*) coincided greatly with those surrounding the *gtf3a* genes in all vertebrate species studied (Fig 1). In turn, *gtf3aa* in teleost genomes did not conserve any of the non-teleostean *gtf3a* neighbouring genes, with the exception in zebrafish of *wasf3, mtif3, gsx1 and scl7a* present close to *gtf3aa* in chromosome 5. Curious enough *wasf3* is also duplicated in the zebrafish genome, where *wasf3a* and *wasf3b* are linked to *gtf3aa* and *gtf3ab,* respectively.

(Next Page: Fig 1. Conserved genomic synteny of vertebrate *gtf3a* genes.)


Tetrapoda
Human
Chr13
spata13 c1qtnf9 mtmr6 nup58 atp8a2 shisa2 rnf6 cdk8 wasf3 gpr12 usp12 rpl21 rasl11a gtf3a mtlf3 lnx2 gsx1 pdx1 cdx2 urad flt3 pan3 flt1 slc7a1
Frog
Chr: GL173431.1
cdk8 wasf3 gpr12 usp12 mtlf3 rpl21 rasl11a gtf3a lnx2
Sarcopterygi
Coelacanth
Chr: JH126705.1
wasf3 gpr12 usp12 rpl21 rasl11a gtf3a mtlf3 lnx2 gsx1 pdx1 cdx2 urad pan3 flt1
Actinopterygi
Spotted gar
Chr: LG3
mtmr6 nup58 shisa2 atp8a2 cdk8 wasf3 gpr12 usp12a rpl21 rasl11a mtlf3 gtf3aa lnx2a gsx1 pdx1 cdx2 urad flt3 pan3 flt1 spata13 c1qtnf9
Teleostei
Zebrafish
Chr 24
Chr 5
shisa2 rnf6 atp8a2 cdk8 wasf3b gpr12 usp12a rpl21 gtf3ab lnx2a pdx1 urad flt3 pan3 flt1 spata13 c1qtnf9
wasf3a gtf3aa mtlf3 gsx1 slc7a2
Tetraodon
Chr6
Chr18
atp8a2 rnf6 cdk8 wasf3b gpr12 usp12a rpl21 gtf3ab lnx2a pdx1 urad flt3 pan3 flt1 spata13 c1qtnf9
gtf3aa
Fugu
scaffold_95
scaffold_206
shisa2 atp8a2 rnf6 cdk8 wasf3b gpr12 usp12a rpl21 gtf3ab lnx2a pdx1 urad flt3 pan3 flt1 spata13 c1qtnf9
gtf3aa
Stickleback
Chr group XXI
Chr group IX
shisa2 atp8a2 rnf6 cdk8 wasf3b gpr12 usp12a rpl21 gtf3ab lnx2a pdx1 urad pan3 flt1 spata13 c1qtnf9
lpar2b
gtf3aa
Amazon molly
Chr:KI519674.1
Chr:KI519954.1
shisa2 rnf6 cdk8 wasf3b gpr12 usp12a rpl21 gtf3ab lnx2a pdx1 urad flt3 pan3 flt1 spata13 c1qtnf9
gtf3aa
lpar2b
Tilapia
Chr:GL831157.1
Chr:GL831280.1
shisa2 atp8a2 rnf6 cdk8 wasf3b gpr12 usp12a rpl21 lnx2a gtf3ab pdx1 urad pan3 flt1 spata13 c1qtnf9
gtf3aa
Atlantic cod
scaffold_733
scaffold_4115
shisa2 atp8a2 rnf6 cdk8 wasf3b usp12a rpl21 gtf3ab lnx2a pdx1 urad pan3 flt1 spata13 c1qtnf9
gtf3aa
Platyfish
Chr: JH556681.1
shisa2 atp8a2 rnf6 cdk8 wasf3b gpr12 usp12a rpl21 gtf3ab lnx2a pan3 flt1 spata13 c1qtnf9
Medaka
Chr: 20
shisa2 atp8a2 rnf6 cdk8 wasf3b gpr12 usp12a rpl21 gtf3ab lnx2a pdx1 urad pan3 flt1 spata13 c1qtnf9

Fig 1. Conserved genomic synteny of vertebrate *gtf3a* genes. Genomic synteny maps comparing localisation *gtf3a* and neighbouring genes in the genomes of tetrapods (human and frog), a basal sarcopterygian (coelacanth), a non-teleost actinopterygian (spotted gar) and different teleost species (zebrafish, tetraodon, fugu, stickleback, amazon molly, tilapia, Atlantic cod, cave fish, platyfish and medaka). In teleosts two *gtf3a* paralogs exits (*gtf3aa* and *gtf3ab*) and the neighbouring genes are shown in comparison to the genes surrounding human *gtf3a*. Orthologs for each gene are represented with the same colour and displayed in the order and direction in which they are placed in each chromosome or scaffold as indicated in the right side of the figure (detailed genomic locations for each gene are given in Table S1).

Organ specific transcription of *gtf3a* paralog genes in zebrafish and dynamics of *gtf3aa* and *gtf3ab* transcription levels during zebrafish early embryo development

Transcription levels of both *gtf3a* paralog genes were measured in brain, gonads and muscle of adult zebrafish (Fig 2A). While *gtf3aa* was strongly transcribed in all the tissues studied, *gtf3ab* showed an ovary specific transcription with a hint of transcription in the testis. The same transcription pattern was observed in gill, eye, ovary and testis (S3 Fig) of tilapia. Results were verified by *gtf3ab* qPCR analysis in muscle, testis and ovaries of zebrafish (Fig 2B). No brain was studied by qPCR due to the inexistent transcription signal in the electrophoresis. *gtf3ab* transcript levels were high in ovaries and nearly non-existent in muscle and testis, confirming the specific transcription of *gtf3ab* in ovaries. *gtf3aa* and *gtf3ab* transcription levels were analysed during early embryonic development, and transcript levels were detected at 2 hpf (64-cell blastula), 8 hpf (gastrula period) and 30 hpf (Prim-16 period) for both genes (Fig 2C). The transcription levels of *gtf3aa* suffered a reduction at 8 hpf increasing again at 30 hpf. In contrast, *gtf3ab* transcript level decreased after 2 hpf and were not observable after 8 hpf.

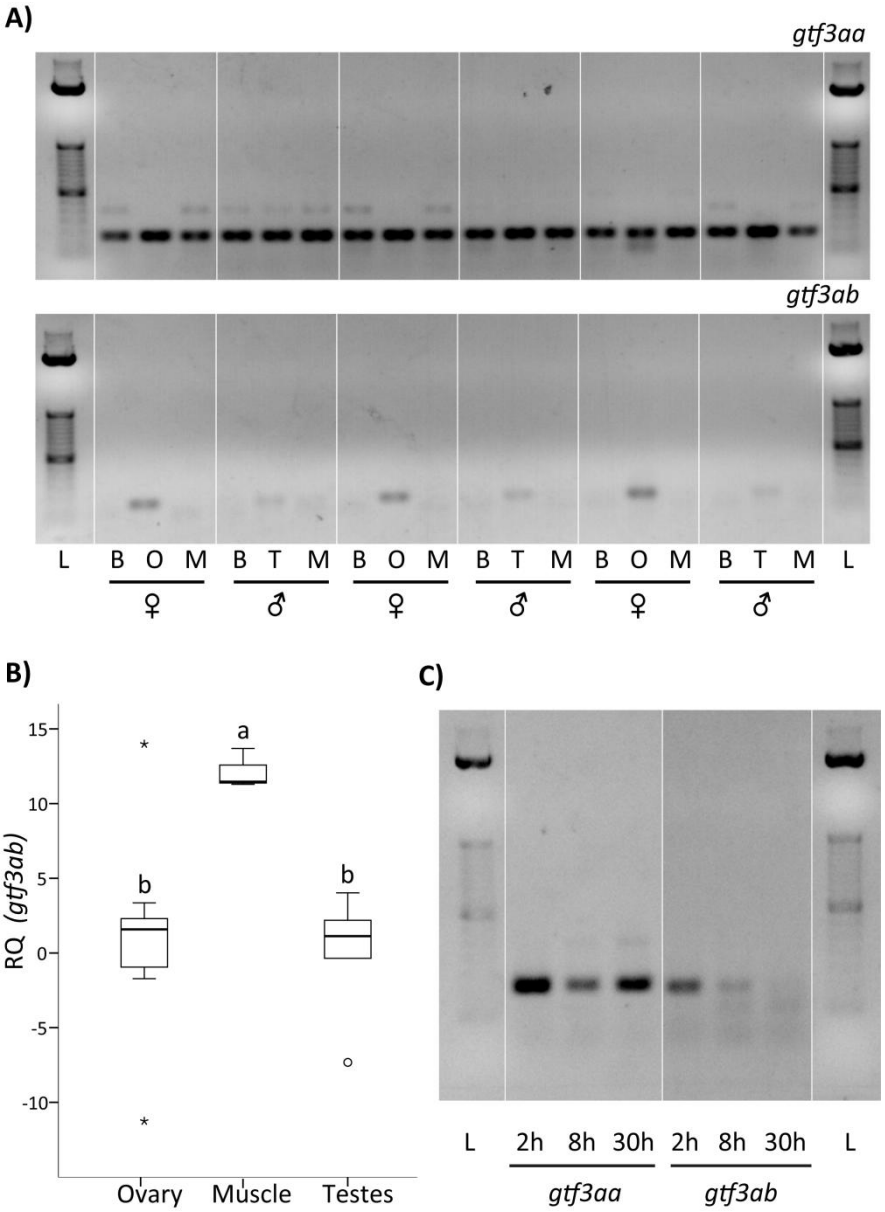

Fig 2: Transcription levels of *gtf3aa* and *gtf3ab* in different tissues and developmental stages of zebrafish. A) Agarose gel electrophoresis showing *gtf3aa* and *gtf3ab transcript levels* after conventional PCR in organs of three male and three female individuals. Brain (B), ovary (O), testis (T) and muscle (M). Amplified fragments were around 100 nucleotides in both cases. L = Standard 50 bp (Invitrogen). B) Transcription levels of *gtf3ab* in different tissues of adult zebrafish through qPCR. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between means (Mann Whitney, $p<0.05$). Number of samples, 6 for ovary, 3 for muscle and 4 for testes. C) Agarose gel electrophoresis after conventional PCR amplification showing transcription levels of *gtf3aa* and *gtf3ab* in zebrafish embryos 2, 8 and 30 hpf. Amplified fragments were around 100 nucleotides in size. L = Standard 50 bp (Invitrogen).

Gonad development in zebrafish exposed to esteroid hormones

Histological analysis was performed in 10 individuals from each experimental group after 26 and 61 dpf (Fig 3). No gonad was present in any of 26 dpf individuals with the exception of an ovary observed in one individual in the ET control group (data not shown). After 61 dpf differentiated gonads were observed in all individuals with 60% of individuals showing ovaries and 40% showing testes in the case of ethanol control group. 100% of the individuals were females displaying well differentiated ovaries in the 17β-estradiol (E) treatment group. 100% were males in 17α-methyltestosterone (MT) treatment group. There was no difference regarding gonad development between control and treated groups. Ovaries always showed previtellogenic oocytes and testis were always in early-mid spermatogenesis. After a year in clean water both E and MT group maintained monosex populations.

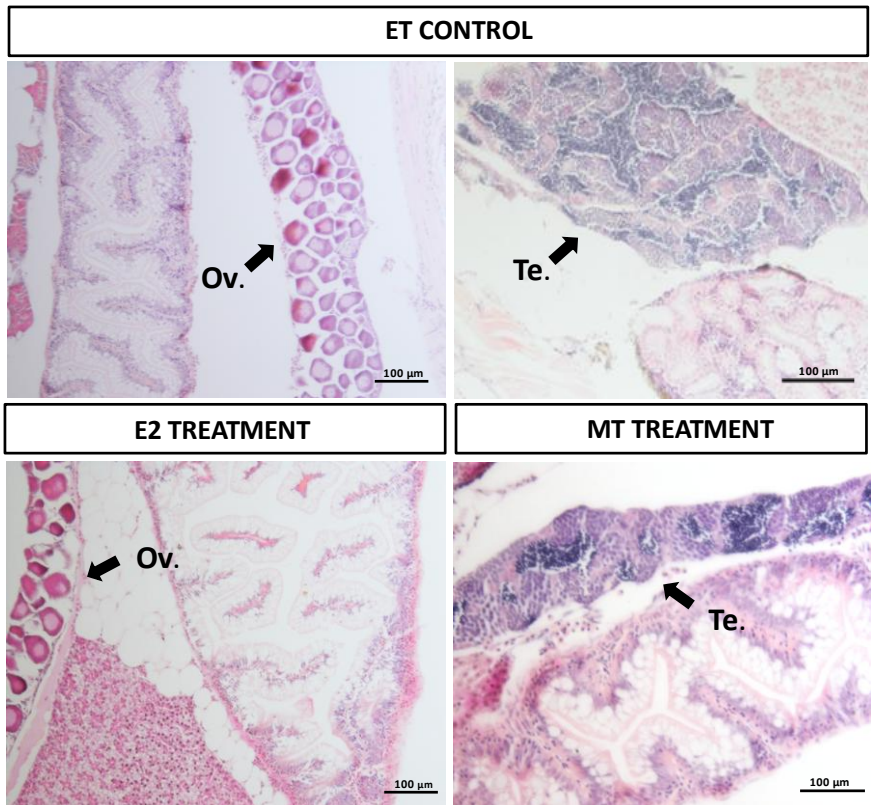

Fig 3: Histological analysis of hormone treated zebrafish at day 61. Micrographs (scale bars = 100 µm) are representative of 10 individuals analysed for each experimental group. ET: ethanol control group (6 females with well formed ovaries and 4 males with testis), E: 17β-estradiol treatment (100% of the individuals with ovary) and MT: 17α-methyltestosterone treatment (100% of the individuals with testis). Black arrows mark the presence of well differentiated gonad (ovaries in the two micrographs in the left and testes in the right).

Gene transcription profiles in feminised and masculinised zebrafish

The results of all the individuals from both replicate tanks per experimental group were used together (n=12) as no differences were observed between tanks in growth (Table S2) (Mann-Whitney, $p<0.05$) or in gene transcription levels. Target gene transcription levels in larvae at day 26 showed no differences between feminised and masculinised groups (Fig 4). Only *gtf3ab* showed higher transcription levels in the ET control group than in the E exposed group. This difference existed also with the MT group although it was not significant. Differences in transcription levels between hormonally feminised and masculinised fish were observed at day 61 with higher transcription levels of *gtf3ab* in the E exposure group. These analyses were performed on RNA extracted from whole organisms without dissection of organs. Transcription levels of *amh* and *dmrt1* were higher in the MT exposure group than in the E group. *cyp19a1a* was maintained constant in all groups, as it was the case also for *gtf3aa* and *actb*. The control group showed values in between the two treated groups at 61 dpf. *dmrt1* was up-regulated in the MT group after 61 dpf in comparison to 26 dpf. In contrast, *gtf3ab* was up-regulated in the E group at day 61 vs. day 26 (Fig 4).

The high variability observed for some of the genes within the ET61 control group should be due to the fact that some of the individuals were female and others male. Attending to the bimodal levels of transcription of *gtf3ab*, high levels indicating females (S4 Fig), we identified the female and male individuals within ET control group. ET females identified in this mode showed significantly higher *cyp19a1a* transcription levels than ET males, with a down-regulation associated to E and MT treatments (S4 Fig). ET group females and males showed no differences in *amh* and *dmrt1* transcription levels, but E treatment resulted in a down-regulation of *dmrt1* when compared to control males and females and a down-regulation of *amh* when compared to ET control males (S4 Fig).

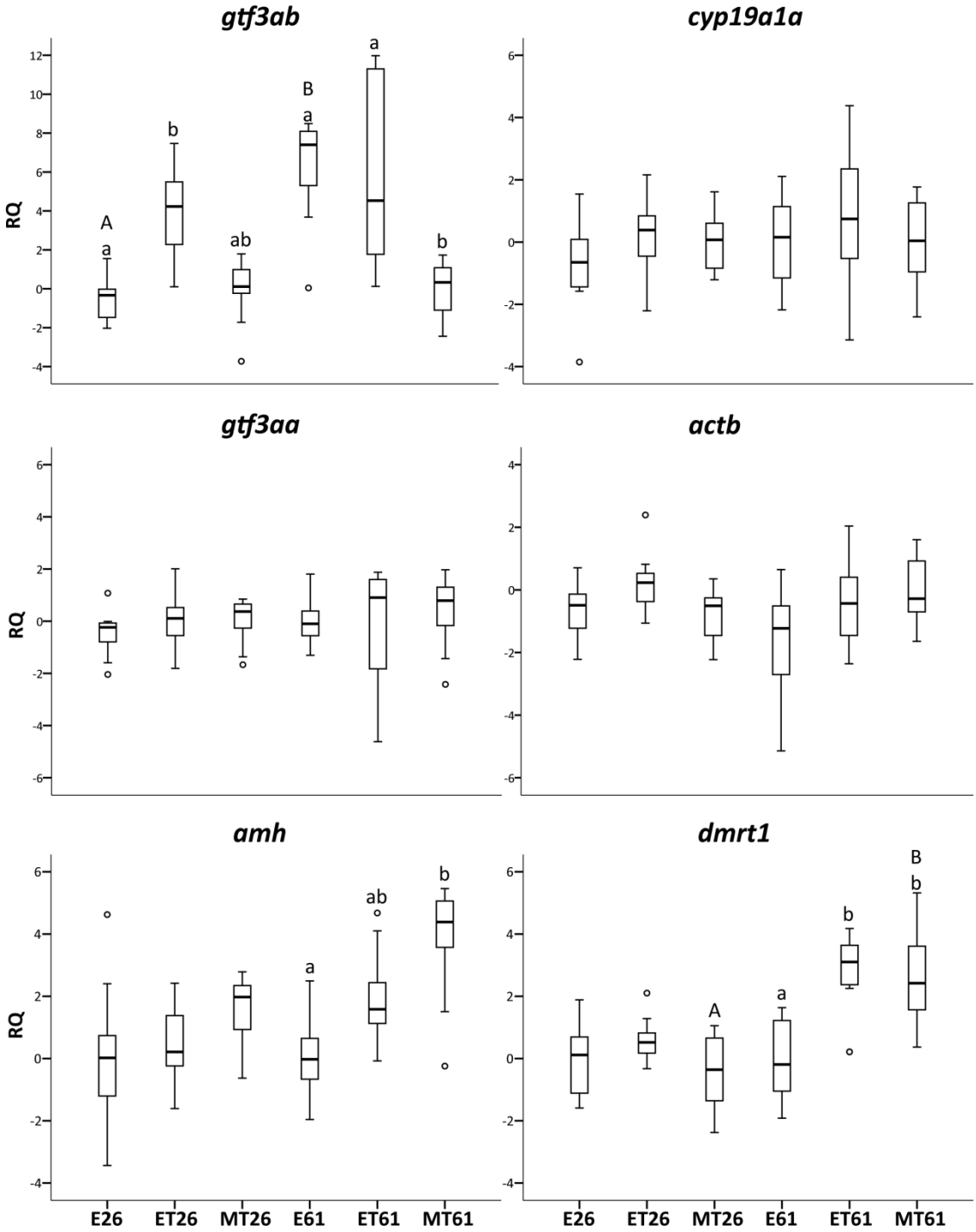

Fig 4: Transcription levels of sex related genes in zebrafish exposed to hormones for 26 and 61 days. Fish exposed to 17β-estradiol (E) and 17α-methyltestosterone (MT) for 26 and 61 days (E26, E61 and MT26, MT61). Ethanol control group at 26 and 61 days (ET26 and ET61). Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values (n=12 individuals per experimental group). Different lower case letters indicate significant differences between groups within each sampling day and different capital letters indicate significant differences within each exposure group either comparing days 26 and 61 (Kruskal-Wallis, $p<0.05$).

DISCUSSION

gtf3aa and *gtf3ab,* paralog genes in fish genomes: organ specific transcription

Phylogenetic and synteny analysis have demonstrated the presence of a single *gtf3a* in coelacanth, spotted gar and in all tetrapods. Actinopterygians, diverged before the teleost-specific third whole genome duplication (3R), present a single *gtf3a* gene while teleost fish genomes present two *gtf3a* genes (*gtf3aa* and *gtf3ab*). In the zebrafish genome these two paralogs, *gtf3aa* (ENSDARG00000030267) in chromosome 5 and *gtf3ab* (ENSDARG00000071583) in chromosome 24, code for two different proteins with a deduced protein size of 367 aa and estimated molecular weight of 42.6kDa in the first case , and 318 aa with 37.15 kDa for the case of *gf3ab* (Table S3). This is very close to the two protein sequences obtained through alternative promoter usage of the unique *gtf3a* gene in *X. laevis* genome (Ogilvie & Hanas, 1997; Layat et al., 2013). The smallest transcript giving rise to a 38 kDa protein is observed in all studied frog tissues while the biggest one, 40 kDa, is observed only in the ovary (Penberthy et al., 2003; Layat et al., 2013). The deduced aminoacid sequence of all teleostean Gtf3ab proteins allows to observe the conserved initiating sequence MGER(K) (Table S3), typical of the oocyte form of the protein in all frog species (Ogilvie & Hanas, 1997). On the other hand, the KRSLAS domain behind the protein las Zn finger (Table S3), required for Gtf3a-dependant 5S gene transcription (Ogilvie & Hanas, 1997) is only present in teleostean Gtf3aa-s but not in Gtf3ab-s. This would suggest a sub-functionalisation of *gtf3ab* in fish, with an oocyte specific function for Gtf3ab in 5S rRNA binding and stockpiling without a role as transcription factor. This function would be retained only by Gtf3aa in all cell types.

Synteny analysis reveals that the region neighbouring *gtf3ab* is most similar to that of other vertebrates, something that does not occur with *gtf3aa*, pointing to a specific gene duplication event. In any case, the *gtf3aa* neighbouring region in zebrafish conserves some neighbouring genes that clearly point to the 3R as the cause of the appearance of two paralog genes. Similar results have been obtained through synteny

analysis of other genes, as for instance the *hox* cluster in the European eel (*Anguilla anguilla*) (Henkel et al., 2012). The existence of duplicated genes in relation to reproductive endocrinology and sex differentiation control has been extensively documented in many fish species (Maugars & Dufour, 2015). The fact that no *gtf3aa* orthologs are found in cave fish, medaka and platyfish is due to sequencing gaps in the genome sequence released in Ensembl, as *gtf3ab* transcript sequences can be found for these species in NCBI (XP_007235992, XP_005800526.1 and XP_004084267).

The possibility of a gene specifically transcribed in ovaries, *gtf3ab,* and another gene, *gtf3aa*, transcribed in somatic tissues and testis has been confirmed. PCR results demonstrated that both paralogs are differentially transcribed in different tissues of zebrafish and tilapia. In both species *gtf3ab* was highly transcribed in ovaries but not in other tissues. In contrast, *gtf3aa* was transcribed in all the studied tissues, including ovaries. This is exactly what it has been described in frogs, but in this case with two proteins produced through alternative promoter usage of one single gene (Penberthy et al., 2003, Layat et al., 2013). Previous studies performed in our laboratory comparing gonads of thicklip grey mullets along a complete reproductive cycle demonstrated high transcription levels of *gtf3a* (identity analysis in the light of the present results reveals that this sequence,JN257141, in fact belongs to mullet *gtf3ab*) in ovaries and not in testis, all along the cycle (Diaz de Cerio et al., 2012). Moreover, studies performed in megrim (*Lepidorhombus whiffiagonis*) and European anchovy (*Engraulis encrasicolus*) have demonstrated that, *gtf3ab* is not only differentially transcribed in ovaries comparing with testis, but also in ovaries at different developmental stages. *gtf3ab* transcription is at its highest early in oogenesis to decrease during later stages, in association with a decrease in 5S rRNA transcription and an activation of 18S and 28S rRNA production (Rojo-Bartolomé et al., 2016).

Transcription levels of *gtf3aa* and *gtf3ab* during zebrafish early embryo development

Transcription levels during the first hours of zebrafish embryo development demonstrate the maternal oocyte origin of *gtf3ab*. At 64-cell stage (2 hpf), when zygotic genome is not transcribed in zebrafish yet, *gtf3ab* transcript levels were high, decreasing

as embryogenesis proceeded towards Prim-16 stage (30 hpf). After fertilization, maternal mRNA factors support early embryonic development until activation of zygotic transcription (Westerfield, 2000). The initiation of zygotic transcription occurs during the "maternal-embryo transition" (MET). In fish MET occurs at the mid-blastula stage and is also known as "mid-blastula transition" (MBT). In zebrafish MBT is well characterised and takes place at 512-cell stage after 2.75 hpf (Kane & Kimmel, 1993). At this time and until 50% epiboly stage (5.25 hpf) a major transition in gene regulation and transcriptional activity takes place (Vesterlund et al., 2011). The disappearance of *gtf3ab* after 2 hpf reveals the maternal (oocytic) origin of *gtf3ab*, which is linked to ovarian 5S rRNA production in oocytes and thus ribosome formation to ensure fast protein production during early embryonic developmental.

In contrast, *gtf3aa* transcript levels decreased slightly from the 2 hpf stage to the gastrula stage (8 hpf). Then, and as a consequence of MET at 30 hpf *gtf3aa* transcript levels increased to levels observed at 2 hpf. At this stage (18 hours before hatching) the 2.5 mm embryo is undergoing the last organogenesis processes (Westerfield, 2000). Obviously, no gonad has been formed yet and thus until sex differentiation leading to ovarian formation takes place, no zygotic *gtf3ab* transcription was observed.

E and MT exposure: gonad development in feminised and masculinised zebrafish

Gonad differentiation in zebrafish occurs between 25 and 45 dpf (Takahashi, 1977) and is completed after 60 dpf (Takashi, 1977; Andersen et al., 2003; Wang et al., 2007). In our case, no visible gonad was observed in treated and non-treated individuals after 26 dpf. This absence of gonad could have been caused by the suboptimal growth water temperature of 24°C during the experiment. Zebrafish optimal growth temperature ranges from 26 to 32°C (Engeszer et al., 2007) and it has been previously described that lower temperatures could cause a delay in general growth of zebrafish, as well as in gonad differentiation and maturation (Luzio et al., 2016). In addition, exposure to estrogens has been shown to reduce fish growth in a concentration-dependent way (Versonnen & Janssen, 2004; Silva et al., 2012; Segner et al., 2013). Androgens such as 11-ketotestosterone on the other hand, used for 96 hours to obtain a medaka male

monosex populations (Örn et al., 2003) increased growth rates. In the present study no significant differences in growth were observed among individuals in the control, the E or the MT groups.

No sex reversal was observed in both generated monosex zebrafish groups after 1 year in clean water. Baumann et al. (2014b) reported that the irreversibility of the androgenic effects on sexual development in zebrafish is a consequence of loss of primordial germ cells during early testis development, making the later development of ovaries impossible. In contrast, it has been described that feminised genetic males could develop testis after withdrawal of estrogenic compounds (Nash et al., 2004; Baumann et al., 2014b). This reversible effect could be dependent on exposure, timing concentration and on duration of the treatment (Maack & Segner, 2004). Exposure during gonad differentiation at early life stages, as in our experiment, would make the process irreversible.

E and MT exposure: gene transcription profiles in feminised and masculinised zebrafish

Many studies have demonstrated that fish exposure to hormones during sex differentiation disrupts the normal expression of genes involved in gonadogenesis (Leet et al., 2011, Liao et al., 2009; Maack & Segner, 2004). In the present study, where it is convenient to remember that whole body transcription levels were analysed without dissection of specific organs, no changes in transcription levels of studied genes were observed after 26 days of exposure, with the exception of *gtf3ab,* down-regulated in both hormone treatment groups. This lack of transcriptional responses seems linked to the fact that in the present experiment zebrafish did not present developed gonads at 26 dpf. In normal conditions, zebrafish gonad differentiation starts with a juvenile ovary phase from 2.5 weeks post-fertilization (wpf) to around 4 wpf (Takahashi, 1977; Maack & Segner, 2003). Possibly some individuals from the control ET group might have initiated gonad development as juvenile females after 26 dpf and this would be reflected in the higher transcription levels of ovary specific *gtf3ab* in comparison to hormone treatment groups.

After 61 days of exposure E feminised group showed up-regulated *gtf3ab* in comparison to E at day 26 and MT at day 61, MT group in turns, showed a down-regulation in respect to the control group. In the control group with individuals of both sexes being present a strong variability in *gtf3ab* transcription levels was observed resulting from a bimodal *gtf3ab* (male *vs* female) transcription pattern.

In contrast, E exposure showed a suppressive effect on *amh* and *dmrt1* transcription levels, which are associated with male sex differentiation (Kobayashi et al., 2004; Rodríguez-Marí et al., 2005). Schulz and colleagues (2007) observed that 5 ng/L ethinyl estradiol exposure during zebrafish early life stages suppressed both *amh* and *dmrt1* expression and caused an inhibition of male gonad development. The MT masculinised group on the other hand showed up-regulation of both genes. Transcription levels for these two male marker genes were recorded in the ET group, due to the presence of both female and male individuals in this group. Male-specific differentiation in mammals includes activation of *amh* expression as the first factor secreted by differentiated Sertoli cells in the testis which leads the regression of Müllerian ducts. Despite de absence of Müllerian ducts in teleosts, they present an *amh* ortholog (Rodríguez-Marí et al., 2005) that is mainly expressed in males during sex differentiation, suggesting that it has a function during testis differentiation and spermatogonial proliferation (Yoshinaga et al., 2004; Vizziano et al., 2007; Ijiri et al., 2008). *Dmrt1* is expressed in Sertoli cells after testicular differentiation for spermatogonial proliferation (Kobayashi et al., 2004). In zebrafish, *dmrt1* expression is up-regulated during the early testicular differentiation, but it is also observed in ovarian developing germ cells. *dmrt1* induces male phenotypic development via a down-regulation of aromatase, shifting the steroidogenic pathway towards androgen production (Wang et al., 2010).

In this sense*, cyp19a,* encodes the enzyme aromatase, which is responsible for catalyzing the aromatization of androgens to estrogens, being a key gene in ovarian differentiation in teleosts (Kitano et al., 1999). Fish genomes contain two aromatase genes (*cyp19a1a* and *cyp19a1b*), *cyp19a1a* being highly expressed in the steroidogenic Theca and granulosa cell layers surrounding the oocytes (Chiang et al., 2001; Blázquez & Piferrer, 2004; Liu et al., 2007). This role of *cyp19a1* genes in ovarian differentiation has been demonstrated in several studies that have treated fish with aromatase inhibitors,

resulting in the suppression of estrogen biosynthesis and induction of sex-reversal of genetic females to phenotypic males (Piferrer et al., 1994; Guiguen et al., 1999; Kitano et al., 2000; Kwon et al., 2000; Fenske & Segner 2004). In any case, *cyp19a1a* promoter region does not display an estrogen response element in teleosts and it is not transcriptionally activated by estrogens as observed in the present study, in contrast to what occurs with *cyp19a1b* is in the brain (Kitano, 2000, Bizarro et al., 2014; Valencia et al., 2016). Contrary, phenotypic masculinisation when fish in early life stages have been treated with androgens is similarly accompanied by a down-regulation of *cyp19a1a* (Kitano et al., 2000; Fenske & Segner 2004). However, since aromatase converts androgens to estrogens, exposure to aromatizable androgens (i.e. MT) may induce both masculinising and feminising effects (Koger et al., 2000; Seki et al., 2004) and this could explain the lack *cyp19a1a* down-regulation in the MT group in comparison to the E group seen hereby. It has to be recalled again that present studies are showing transcription levels using RNA extracted from whole individuals, pointing out also to the added value of *gtf3ab* as a marker of oocyte differentiation in fish.

No differences were observed in *gtf3aa* transcription levels, as it happened with *actb*, not being affected by sex or hormone exposure. Several studies use *actb* as a reference gene in PCR analyses due to its constant transcription throughout tissues and experimental conditions in fish (Jorgensen et al., 2006). In any case, *actb* transcription levels have been reported to change in gonads after E exposure in fish (Filby & Tyler, 2007). This is why, and in spite of the lack of variability of *actb* transcription in the present study involving whole body RNA, we suggest when working with fish gonads to refer qPCR results to the amount of cDNA amplified per sample (Rojo-Bartolomé et al., 2016, Valencia et al., 2016).

All together**,** two *gtf3a* paralog genes are present in teleost genomes as consequence of fish specific 3R event. As a consequence of sub-functionalisation of the new gene products, while *gtf3ab* displays ovarian specific transcription associated with ovarian 5S rRNA stockpiling, *gtf3aa* has been maintained as a gene for the transcriptional regulation of 5S rRNA in somatic tissues, testis and ovary. Exposure to hormones (E and MT) affecting sex differentiation in zebrafish demonstrate that transcription of *gtf3ab* is a consequence of oogenesis and a marker of oocyte differentiation, not a marker of

estrogen or androgen exposure *per se* (differences observed at day 61, not at day 26). This circumstance, that needs to be proved true in other fish species, has important consequences for pollution monitoring programmes. Oocyte specific transcription of *gtf3ab* has already been described in intersex testis of *C. labrosus* from polluted Basque estuaries (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014; Valencia et al., 2016), further defining *gtf3ab* transcripts as specific molecular markers of intersex condition, and not of mere xenoestrogen exposure.

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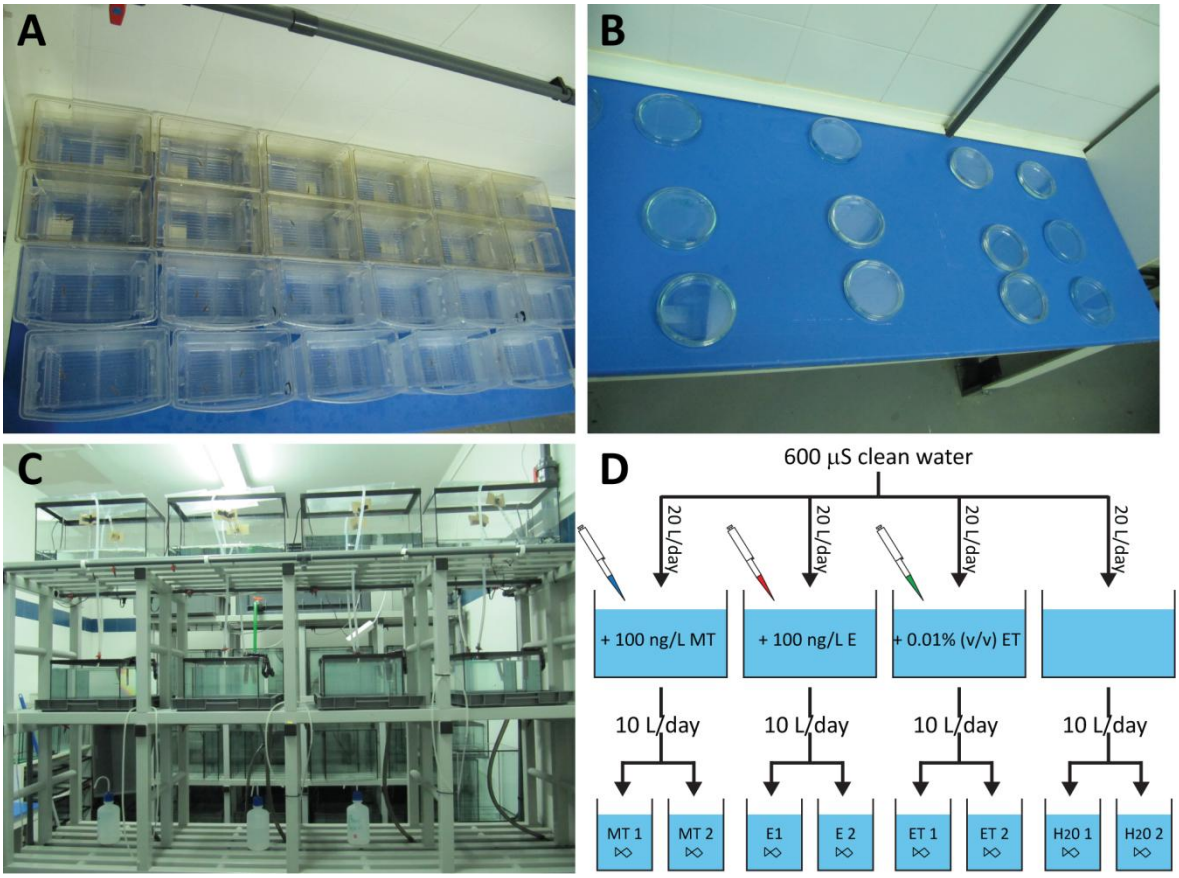
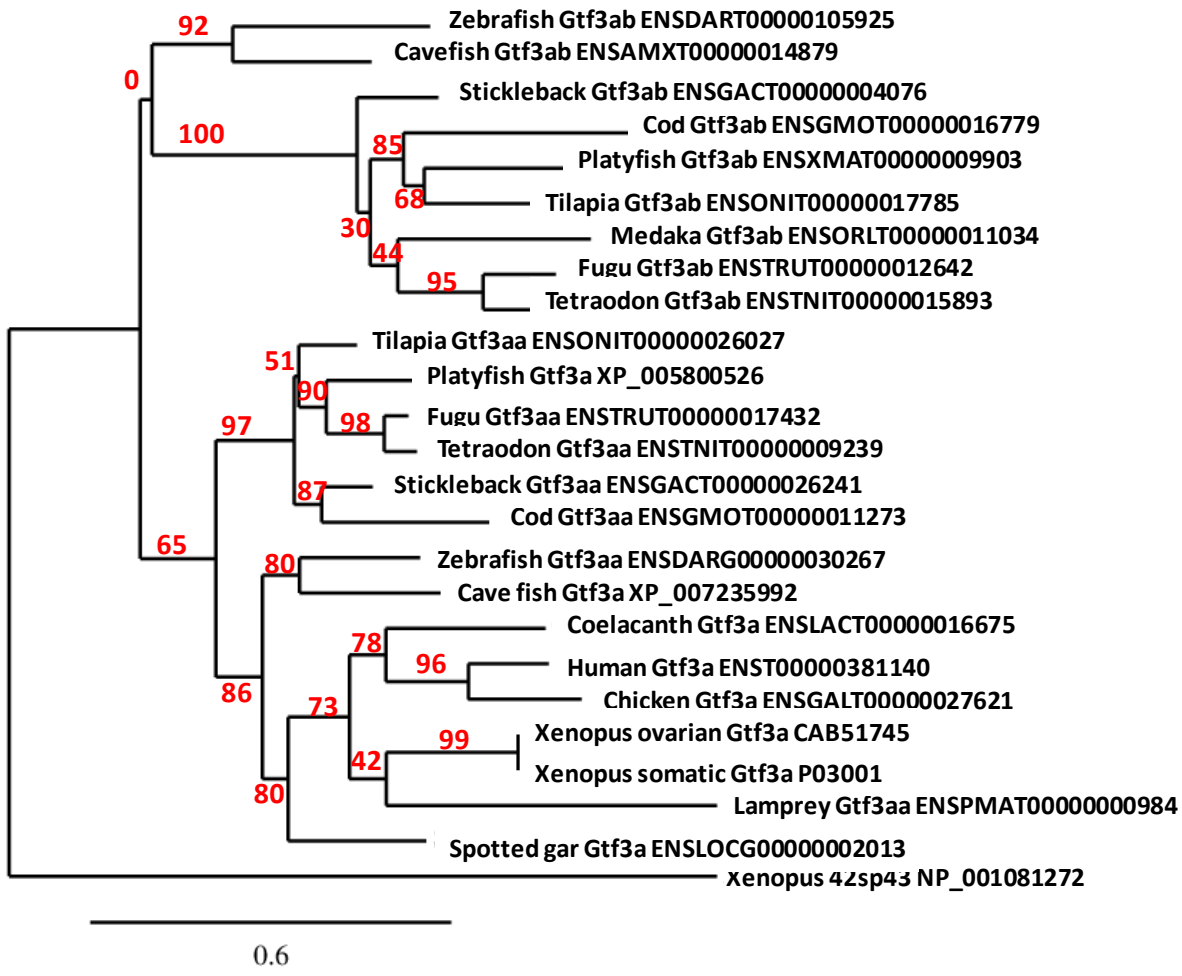
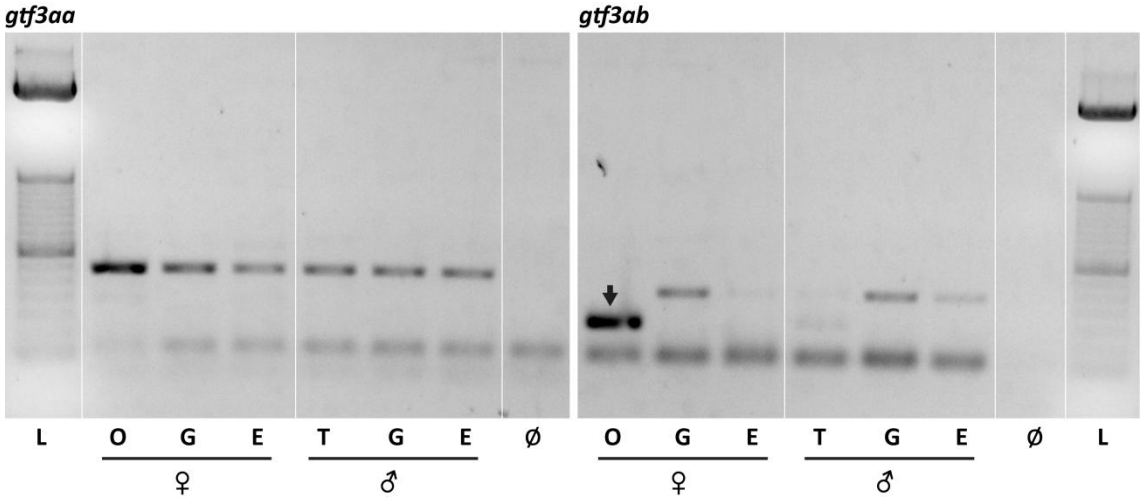
SUPPLEMENTARY MATERIAL



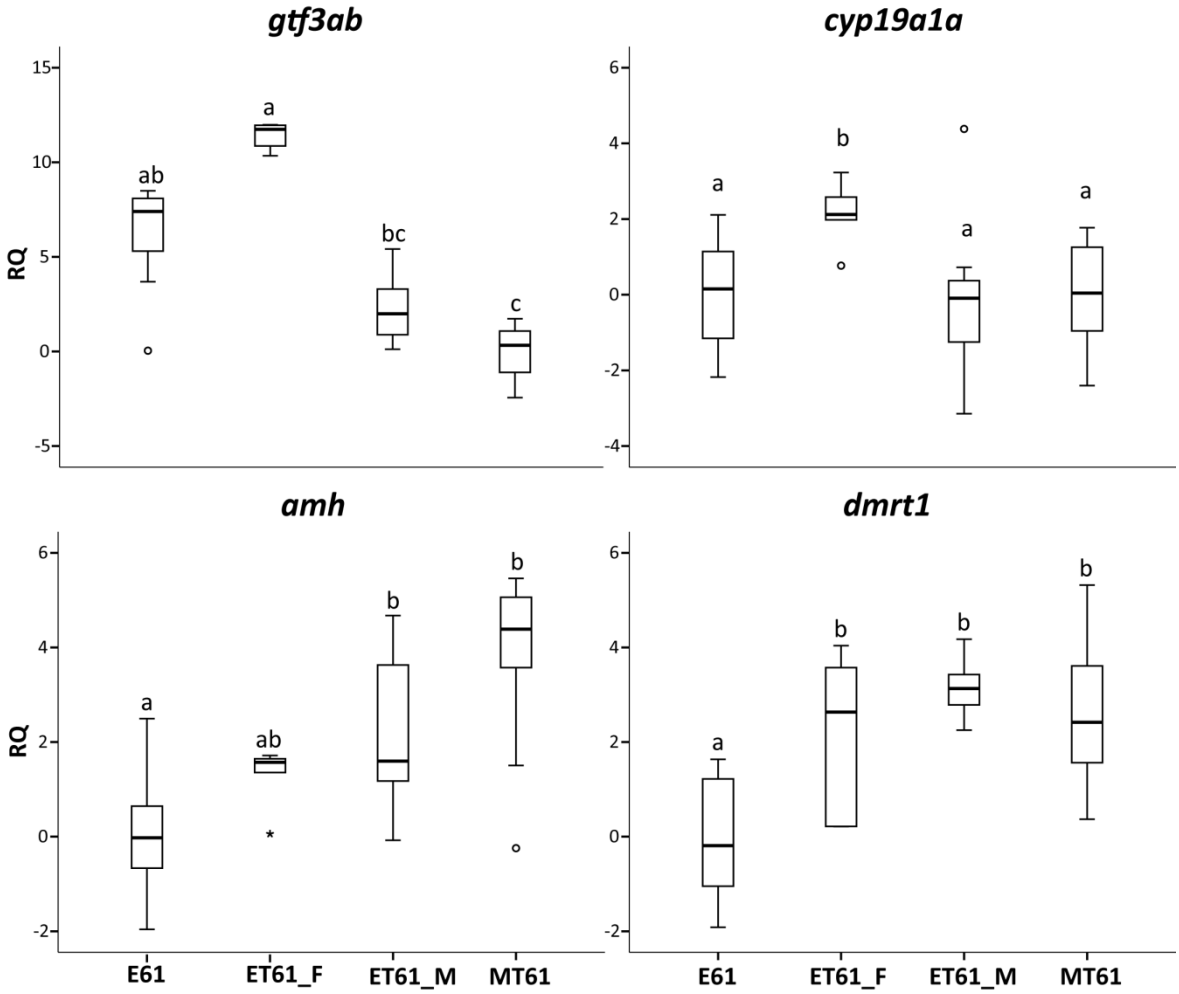
S1 Fig: Experimental design for hormone exposures. A) breeding tanks. B) Embryos exposed in glass Petri dishes from the lay to 5 dpf. C) Set-up of exposure tanks. D) Sketch of exposure tanks with exposure concentrations used. There was a fourth tank with fish in untreated control water.



S2 Fig: Phylogenetic tree showing the relationship between different animal deduced GTF3A protein sequences. Sequences were aligned with MUSCLE (v3.8.31) configured for highest accuracy. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). The default substitution model was selected assuming an estimated proportion of invariant sites (of 0.115) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma=1.392). Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates). The *Xenopus* 42sp43 protein sequence was used as outgroup.



S3 Fig: *gtf3aa* and *gtf3ab* transcript levels in different tilapia tissues. Agarose gel electrophoresis after conventional PCR with cDNA generated from tilapia ovary (O), testis (T), gill (G) and eye (E). Fragments were around 280 nucleotides in length for *gtf3aa* and around 130 for *gtf3ab*. An unspecific amplicon can be observed in the *gtf3ab* gels that does not mask the specific ovarian amplicon (arrow). Ø=no template control; L= Standard 50 bp (Invitrogen).



S4 Fig: Transcript levels of genes related to ovarian (*gtf3ab* and *cyp19a1a*) and testicular (*amh* and *dmrt1*) differentiation in hormone treated zebrafish after 61 days of exposure. The experimental groups after 61 days of exposure were: 17β-estradiol (E61), 17α-methyltestosterone (MT61) and ethanol control group which was separated in female and male (ET61_F vs. ET61_M) considering *gtf3ab* transcription levels. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values (12 individuals per treatment group with 5 individuals in ET61_F and 7 in ET61_M). Different letters indicate significant differences between groups (Kruskal-Wallis, $p<0.05$).

Table S1: Ensembl reference IDs and locations for each of the genes neighbouring *gtf3a* orthologs in animal genomes studied in the synteny analysis.

		Human (*Homo sapiens*)				Frog (*Xenopus tropicalis*)			
Gene symbol	Name	ID	Location	Start-End	Strand	ID	Location	Start-End	Strand
spata13	spermatogenesis associated 13	ENSG00000182957	Chr 13	23.97-24.30 Mbp	+				
c1qtnf9	C1q tumor necrosis factor related protein 9	ENSG00000240654	Chr 13	24.30-24.32 Mbp	+				
mtmr6	myotubularin related protein 6	ENSG00000139505	Chr 13	25.24-25.28 Mbp	-				
nup58	nucleoporin 58KDa	ENSG00000139496	Chr 13	25.30-25.34 Mbp	+				
atp8a2	ATPase. aminophospholiid transporter. class I. type 8A. member 2	ENSG00000132932	Chr 13	25.37-26.02 Mbp	+				
shisa2	shisa family member 2	ENSG00000180730	Chr 13	26.04-26.05 Mbp	-				
rnf6	ring finger protein (C3H2C3 type) 6	ENSG00000127870	Chr 13	26.13-26.22 Mbp	-				
cdk8	cyclin-dependent kinase 8	ENSG00000132964	Chr 13	26.25-26.40 Mbp	+	ENSXETG00000009787	Chr: GL173431.1	3.15-23.14 kbp	+
wasf3(b)	was protein family member 3 (3b)	ENSG00000132970	Chr 13	26.55-26.68 Mbp	+	ENSXETG00000032158	Chr: GL173431.1	72.44-105.24 kbp	+
wasf3a	was protein family member 3a								
grp12	G-protein-coupled receptor 12	ENSG00000132975	Chr 13	26.75-26.76 Mbp	-	ENSXETG00000030348	Chr: GL173431.1	127.68-128.66 kbp	-
usp12(a)	ubiquitin specific peptidase 12 (12a)	ENSG00000152484	Chr 13	27.06-27.17 Mbp	-	(ENSXETG00000009799	Chr: GL173431.1	197.96-211.61kKbp	-
rpl21	ribosomal protein L21	ENSG00000122026	Chr 13	27.25-27.26 Mbp	+	ENSXETG00000009802	Chr: GL173431.1	254.09-258.49 kbp	+
rasl11a	RAS-like family II member A	ENSG00000122035	Chr 13	27.27-27.27 Mbp	+	ENSXETG00000030983	Chr: GL173431.1	262.67-267.33 kbp	+
gtf3a(b)	general transcription factor IIIA (A. b)	ENSG00000122034	Chr 13	27.42-27.43 Mbp	+	ENSXETG00000032930	Chr: GL173431.1	348.26-358.24 kbp	+
gtf3aa	general transcription factor IIIAa								
mtlf3	mitochondrial translational initiation factor 3	ENSG00000122033	Chr 13	27.43-27.45 Mbp	-	ENSXETG00000031401	Chr: GL173431.1	231.37-237.20 kbp	-
lnx2(a)	ligand of numb-protein X2	ENSG00000139517	Chr 13	27.54-27.62 Mbp	-	ENSXETG00000031406	Chr: GL173431.1	392.66-399.80 kbp	-
gsx1	GS homeobox 1	ENSG00000169840	Chr 13	27.79-27.79 Mbp	+				
pdx1	pancreatic and duodenal homeobox 1	ENSG00000139515	Chr 13	27.90-27.92 Mbp	+				
cdx2	caudal type homeobox 2	ENSG00000165556	Chr 13	27.96-27.97 Mbp	-				
urad	ureidoimidazoline (2-oxo-4-hydroxy-4-carboxy-5) decarboxylase	ENSG00000183463	Chr 13	27.97-27.98 Mbp	-				
ftl3	fms-related tyrosine kinase 3	ENSG00000122025	Chr 13	28.00-28.10 Mbp	-				
pan3	PAN2 poly(A) specific ribonuclease subunit	ENSG00000152520	Chr 13	28.13-28.29 Mbp	+				
ftl1	fms-related tyrosine kinase 1	ENSG00000102755	Chr 13	28.30-28.49 Mbp	-				
slc7a1	solute carrier family 7	ENSG00000139514	Chr 13	29.50-29.59 Mbp	-				
lpar2b	lysophosphatidic acid receptor 2b								

		Coelacanth (*Latimeria chalumnae*)				Spotted gar (*Lepisosteus oculatus*)			
Gene symbol	Name	ID	Location	Start-End	strand	ID	Location	Start-End	strand
spata13	spermatogenesis associated 13					ENSLOCG00000004477	Chr: LG3	5.86-5.90 Mbp	+
c1qtnf9	C1q tumor necrosis factor related protein 9					ENSLOCG00000004497	Chr: LG3	5.90-5.91 Mbp	+
mtmr6	myotubularin related protein 6					ENSLOCG00000004016	Chr: LG3	5.20-5.21 Mbp	-
nup58	nucleoporin 58KDa					ENSLOCG00000004040	Chr: LG3	5.22-5.26 Mbp	+
atp8a2	ATPase. aminophospholiid transporter. class I. type 8A. member 2					ENSLOCG00000004080	Chr: LG3	5.25-5.37 Mbp	-
shisa2	shisa family member 2					ENSLOCG00000004070	Chr: LG3	5.24-5.25 Mbp	+
rnf6	ring finger protein (C3H2C3 type) 6								
cdk8	cyclin-dependent kinase 8					ENSLOCG00000004124	Chr: LG3	5.40-5.42 Mbp	+
wasf3(b)	was protein family member 3 (3b)	ENSLACG00000001570	Chr: JH126705.1	22.80-187.07 kbp	+				
wasf3a	was protein family member 3a					ENSLOCG00000004135	Chr: LG3	5.44-5.45 Mbp	+
grp12	G-protein-coupled receptor 12	ENSLACG00000007730	Chr: JH126705.1	294.91-295.91 kbp	-	ENSLOCG00000018189	Chr: LG3	5.46-5.47 Mbp	-
usp12(a)	ubiquitin specific peptidase 12 (12a)	ENSLACG00000011615	Chr: JH126705.1	619.84-714.08 kbp	-	ENSLOCG00000004146	Chr: LG3	5.50-5.51 Mbp	-
rpl21	ribosomal protein L21	ENSLACG00000012678	Chr: JH126705.1	749.90-764.35 kbp	+	ENSLOCG00000004171	Chr: LG3	5.51-5.51 Mbp	+
rasl11a	RAS-like family II member A	ENSLACG00000013026	Chr: JH126705.1	799.02-803.03 kbp	+	ENSLOCG00000004176	Chr: LG3	5.51-5.52 Mbp	+
gtf3a(b)	general transcription factor IIIA (A. b)	ENSLACG00000014594	Chr: JH126705.1	1.05-1.07 Mbp	+				
gtf3aa	general transcription factor IIIAa					ENSLOCG00000004190	Chr: LG3	5.55-5.56 Mbp	+
mtlf3	mitochondrial translational initiation factor 3	ENSLACG00000014694	Chr: JH126705.1	1.07-1.09 Mbp	-	ENSLOCG00000004205	Chr: LG3	5.55-5.55 Mbp	-
lnx2(a)	ligand of numb-protein X2	ENSLACG00000015015	Chr: JH126705.1	1.15-1.21 Mbp	-	ENSLOCG00000004220	Chr: LG3	5.57-5.6 Mbp	-
gsx1	GS homeobox 1	ENSLACG00000016379	Chr: JH126705.1	1.55-1.55 Mbp	+	ENSLOCG00000004253	Chr: LG3	5.64-5.65 Mbp	+
pdx1	pancreatic and duodenal homeobox 1	ENSLACG00000016610	Chr: JH126705.1	1.64-1.67 Mbp	+	ENSLOCG00000004260	Chr: LG3	5.66-5.67 Mbp	+
cdx2	caudal type homeobox 2	ENSLACG00000016728	Chr: JH126705.1	1.70-1.71 Mbp	-	ENSLOCG00000004278	Chr: LG3	5.67-5.68 Mbp	-
urad	ureidoimidazoline (2-oxo-4-hydroxy-4-carboxy-5) decarboxylase	ENSLACG00000022307	Chr: JH126705.1	1.72-1.74 Mbp	-	ENSLOCG00000004296	Chr: LG3	5.68-5.68 Mbp	-
ftl3	fms-related tyrosine kinase 3					ENSLOCG00000004310	Chr: LG3	5.68-5.70 Mbp	-
pan3	PAN2 poly(A) specific ribonuclease subunit	ENSLACG00000017156	Chr: JH126705.1	1.93-207 Mbp	+	ENSLOCG00000004336	Chr: LG3	5.70-5.75 Mbp	+
ftl1	fms-related tyrosine kinase 1	ENSLACG00000017415	Chr: JH126705.1	1.08-2.22 Mbp	-	ENSLOCG00000004379	Chr: LG3	5.73-5.78 Mbp	-
slc7a1	solute carrier family 7								
lpar2b	lysophosphatidic acid receptor 2b								

		Zebrafish (*Danio rerio*)				Tetraodon (*Tetraodon negroviridis*)			
Gene symbol	Name	ID	Location	Start-End	strand	ID	Location	Start-End	strand
spata13	spermatogenesis associated 13	ENSDARG00000062837	Chr: 24	21.71-21.75 Mbp	-	ENSTNIG00000012702	Chr: 6	3.533-5.3537 Mbp	+
c1qtnf9	C1q tumor necrosis factor related protein 9	ENSDARG00000058318	Chr: 24	21.76-21.76 Mbp	-	ENSTNIG00000012701	Chr: 6	3.523-3.525 Mbp	+
mtmr6	myotubularin related protein 6								
nup58	nucleoporin 58KDa								
atp8a2	ATPase. aminophospholiid transporter. class I. type 8A. member 2	ENSDARG00000077492	Chr: 24	21.22-21.35 Mbp	-	ENSTNIG00000012718	Chr: 6	3.75-3.76 Mbp	+
shisa2	shisa family member 2	ENSDARG00000088232	Chr: 24	21.20-21.21 Mbp	+				
rnf6	ring finger protein (C3H2C3 type) 6	ENSDARG00000077955	Chr: 24	21.21-21.36 Mbp	-	ENSTNIG00000012717	Chr: 6	3.741-3.744 Mbp	+
cdk8	cyclin-dependent kinase 8	ENSDARG00000016496	Chr: 24	21.36-21.39 Mbp	+	ENSTNIG00000012716	Chr: 6	3.735-3.739 Mbp	-
wasf3(b)	was protein family member 3 (3b)	ENSDARG00000062948	Chr: 24	21.39-21.43 Mbp	+	ENSTNIG00000012715	Chr: 6	3.72-3.73 Mbp	-
wasf3a	was protein family member 3a	ENSDARG00000059466	Chr: 5	67.32-67.34 Mbp	-				
grp12	G-protein-coupled receptor 12	ENSDARG00000062934	Chr: 24	21.43-21.44 Mbp	-	ENSTNIG00000012714	Chr: 6	3.722-3.723 Mbp	+
usp12(a)	ubiquitin specific peptidase 12 (12a)	ENSDARG00000078109	Chr: 24	21.45-21.47 Mbp	-	ENSTNIG00000012713	Chr: 6	3.717-3.720 Mbp	+
rpl21	ribosomal protein L21	ENSDARG00000010516	Chr: 24	21.47-21.48 Mbp	+	ENSTNIG00000012712	Chr: 6	3.715-3.717 Mbp	-
rasl11a	RAS-like family II member A								
gtf3a(b)	general transcription factor IIIA (A. b)	ENSDARG00000071583	Chr: 24	21.49-21.49 Mbp	+	ENSTNIG00000012711	Chr: 6	3.606-3.608 Mbp	-
gtf3aa	general transcription factor IIIAa	ENSDARG00000030267	Chr: 5	67.30-67.31 Mbp	-	ENSTNIG00000006308	Chr: 18	10.81-10.82 Mbp	-
mtlf3	mitochondrial translational initiation factor 3	ENSDARG00000039649	Chr: 5	67.29-67.30 Mbp	+				
lnx2(a)	ligand of numb-protein X2	ENSDARG00000029177	Chr: 24	21.50-21.52 Mbp	-	ENSTNIG00000012710	Chr: 6	3.59-3.60 Mbp	+
gsx1	GS homeobox 1	ENSDARG00000035735	Chr: 5	67.23-67.23 Mbp	-				
pdx1	pancreatic and duodenal homeobox 1	ENSDARG00000002779	Chr: 24	21.53-21.54 Mbp	+	ENSTNIG00000012708	Chr: 6	3.593-3.596 Mbp	-
cdx2	caudal type homeobox 2								
urad	ureidoimidazoline (2-oxo-4-hydroxy-4-carboxy-5) decarboxylase	ENSDARG00000071579	Chr: 24	21.54-21.54 Mbp	-	ENSTNIG00000012707	Chr: 6	6.592-3.593 Mbp	+
ftl3	fms-related tyrosine kinase 3	ENSDARG00000058503	Chr: 24	21.54-21.56 Mbp	-	ENSTNIG00000012706	Chr: 6	3.589-3.591 Mbp	+
pan3	PAN2 poly(A) specific ribonuclease subunit	ENSDARG00000031637	Chr: 24	21.57-21.60 Mbp	+	ENSTNIG00000012705	Chr: 6	3.579-3.584 Mbp	-
ftl1	fms-related tyrosine kinase 1	ENSDARG00000019371	Chr: 24	21.60-21.66 Mbp	-	ENSTNIG00000012704	Chr: 6	3.563-3.569 Mbp	+
slc7a1	solute carrier family 7	ENSDARG00000099111	Chr: 5	67.13-67.15 Mbp	+				
lpar2b	lysophosphatidic acid receptor 2b								

		Fugu (*Takifugu rubripes*)				Stickleback (*Gasterasteus aculeatus*)			
Gene symbol	Name	ID	Location	Start-End	strand	ID	Location	Start-End	strand
spata13	spermatogenesis associated 13	ENSTRUG00000007318	scaffold_95	264.41-268.09 kbp	-	ENSGACG00000003047	Chr group XXI	7.497-7.501 Mbp	+
c1qtnf9	C1q tumor necrosis factor related protein 9	ENSTRUG00000007579	scaffold_95	276.30-277.51 kbp	-	ENSGACG00000003040	Chr group XXI	7.487-7.488 Mbp	+
mtmr6	myotubularin related protein 6								
nup58	nucleoporin 58KDa								
atp8a2	ATPase. aminophospholiid transporter. class I. type 8A. member 2	ENSTRUG00000001682	scaffold_95	126.81-155.45 kbp	-	ENSGACG00000003172	Chr group XXI	7.637-7.667 Mbp	+
shisa2	shisa family member 2	ENSTRUG00000001619	scaffold_95	119.99-123.89 kbp	+	ENSGACG00000003182	Chr group XXI	7.671-7.675 Mbp	-
rnf6	ring finger protein (C3H2C3 type) 6	ENSTRUG00000003854	scaffold_95	162.46-164.90 kbp	-	ENSGACG00000003167	Chr group XXI	7.624-7.626 Mbp	+
cdk8	cyclin-dependent kinase 8	ENSTRUG00000003969	scaffold_95	166.56-171.76 kbp	+	ENSGACG00000003151	Chr group XXI	7.616-7.622 Mbp	-
wasf3(b)	was protein family member 3 (3b)	ENSTRUG00000004356	scaffold_95	176.55-180.78 kbp	+	ENSGACG00000003147	Chr group XXI	7.605-7.613 Mbp	-
wasf3a	was protein family member 3a								
grp12	G-protein-coupled receptor 12	ENSTRUG00000004806	scaffold_95	185.28-186.30 kbp	-	ENSGACG00000003145	Chr group XXI	7.600-7.601 Mbp	+
usp12(a)	ubiquitin specific peptidase 12 (12a)	ENSTRUG00000004837	scaffold_95	188.71-191.76 kbp	-	ENSGACG00000003114	Chr group XXI	7.593-7.598 Mbp	+
rpl21	ribosomal protein L21	ENSTRUG00000005068	scaffold_95	182.03-194.05 kbp	+	ENSGACG00000003110	Chr group XXI	7.589-7.592 Mbp	-
rasl11a	RAS-like family II member A								
gtf3a(b)	general transcription factor IIIA (A. b)	ENSTRUG00000005241	scaffold_95	197.30-199.09 kbp	+	ENSGACG00000003107	Chr group XXI	7.582-7.584 Mbp	-
gtf3aa	general transcription factor IIIAa	ENSTRUG00000007067	scaffold_206	17.26-18.47 kbp	+	ENSGACG00000019821	Chr group IX	19.645-19.646 Mbp	-
mtlf3	mitochondrial translational initiation factor 3								
lnx2(a)	ligand of numb-protein X2	ENSTRUG00000005500	scaffold_95	199.62-205.19 kbp	-	ENSGACG00000003102	Chr group XXI	7.575-7.582 Mbp	+
gsx1	GS homeobox 1								
pdx1	pancreatic and duodenal homeobox 1	ENSTRUG00000005693	scaffold_95	211.72-213.74 kbp	+	ENSGACG00000003099	Chr group XXI	7.565-7.567 Mbp	-
cdx2	caudal type homeobox 2								
urad	ureidoimidazoline (2-oxo-4-hydroxy-4-carboxy-5) decarboxylase	ENSTRUG00000005741	scaffold_95	214.16-214.76 kbp	-	ENSGACG00000003095	Chr group XXI	7.564-7.564 Mbp	+
ftl3	fms-related tyrosine kinase 3	ENSTRUG00000005748	scaffold_95	215.99-218.01 kbp	-				
pan3	PAN2 poly(A) specific ribonuclease subunit	ENSTRUG00000005777	scaffold_95	222.82-228.50 kbp	+	ENSGACG00000003081	Chr group XXI	7.545-7.552 Mbp	-
ftl1	fms-related tyrosine kinase 1	ENSTRUG00000006184	scaffold_95	231.39-263.24 kbp	-	ENSGACG00000003066	Chr group XXI	7.519-7.540 Mbp	+
slc7a1	solute carrier family 7								
lpar2b	lysophosphatidic acid receptor 2b					ENSGACG00000019874	Chr group IX	19.85-19.86 Mbp	+

		Amazon molly (*Poelicia formosa*)				Tilapia (*Oreochromis niloticus*)			
Gene symbol	Name	ID	Location	Start-End	strand	ID	Location	Start-End	strand
spata13	spermatogenesis associated 13	ENSPFOG00000006292	Chr:KI519674.1	560.80-584.84 kbp	+	ENSONIG00000014168	Chr:GL831157.1	597.20-603.35 kbp	-
c1qtnf9	C1q tumor necrosis factor related protein 9	ENSPFOG00000006247	Chr:KI519674.1	556.36-558.47 kbp	+	ENSONIG00000014169	Chr:GL831157.1	613.33-314.65 kbp	-
mtmr6	myotubularin related protein 6								
nup58	nucleoporin 58KDa								
atp8a2	ATPase. aminophospholiid transporter. class I. type 8A. member 2					ENSONIG00000014106	Chr:GL831157.1	324.32-372.98 kbp	-
shisa2	shisa family member 2	ENSPFOG00000009024	Chr:KI519674.1	855.05-863.58 kbp	-	ENSONIG00000014104	Chr:GL831157.1	315.43-320.98 kbp	+
rnf6	ring finger protein (C3H2C3 type) 6	ENSPFOG00000008161	Chr:KI519674.1	780.53-790.59 kbp	+	ENSONIG00000014121	Chr:GL831157.1	373.19-382.70 kbp	-
cdk8	cyclin-dependent kinase 8	ENSPFOG00000008071	Chr:KI519674.1	770.11-781.14 kbp	-	ENSONIG00000014123	Chr:GL831157.1	383.13-391.71 kbp	+
wasf3(b)	was protein family member 3 (3b)	ENSPFOG00000008013	Chr:KI519674.1	753.16-767.98 kbp	-	ENSONIG00000014127	Chr:GL831157.1	394.30-405.01 kbp	+
wasf3a	was protein family member 3a								
grp12	G-protein-coupled receptor 12	ENSPFOG00000020724	Chr:KI519674.1	746.45-747.47 kbp	+	ENSONIG00000020418	Chr:GL831157.1	411.48-412.50 kbp	-
usp12(a)	ubiquitin specific peptidase 12 (12a)	ENSPFOG00000007941	Chr:KI519674.1	732.08-741.16 kbp	+	ENSONIG00000014128	Chr:GL831157.1	415.14-421.51 kbp	-
rpl21	ribosomal protein L21	ENSPFOG00000007900	Chr:KI519674.1	728.87-731.79 kbp	-	ENSONIG00000014130	Chr:GL831157.1	423.26-424.80 kbp	+
rasl11a	RAS-like family II member A								
gtf3a(b)	general transcription factor IIIA (A. b)	ENSPFOG00000007819	Chr:KI519674.1	722.36-724.21 kbp	-	ENSONIG00000014134	Chr:GL831157.1	431.25-434.19 kbp	+
gtf3aa	general transcription factor IIIAa	ENSPFOG00000020753	Chr:KI519954.1	49.41-50.40 kbp	+	ENSONIG00000020701	Chr:GL831280.1	1.625-1.626 Mbp	+
mtlf3	mitochondrial translational initiation factor 3								
lnx2(a)	ligand of numb-protein X2	ENSPFOG00000007692	Chr:KI519674.1	706.28-722.27 kbp	+	ENSONIG00000014132	Chr:GL831157.1	431.24-443.79 kbp	-
gsx1	GS homeobox 1								
pdx1	pancreatic and duodenal homeobox 1	ENSPFOG00000007668	Chr:KI519674.1	697.49-699.44 kbp	-	ENSONIG00000014137	Chr:GL831157.1	452.60-455.36 kbp	+
cdx2	caudal type homeobox 2								
urad	ureidoimidazoline (2-oxo-4-hydroxy-4-carboxy-5) decarboxylase	ENSPFOG00000007646	Chr:KI519674.1	696.28-698.15 kbp	+	ENSONIG00000014138	Chr:GL831157.1	455.98-456.64 kbp	-
ftl3	fms-related tyrosine kinase 3	ENSPFOG00000023223	Chr:KI519674.1	669.52-679.37 kbp	+				
pan3	PAN2 poly(A) specific ribonuclease subunit	ENSPFOG00000007327	Chr:KI519674.1	645.53-665.72 kbp	-	ENSONIG00000014139	Chr:GL831157.1	511.48-531.75 kbp	+
ftl1	fms-related tyrosine kinase 1	ENSPFOG00000006735	Chr:KI519674.1	607.81-643.79 kbp	+	ENSONIG00000014141	Chr:GL831157.1	536.33-574.03 kbp	-
slc7a1	solute carrier family 7								
lpar2b	lysophosphatidic acid receptor 2b	ENSPFOG00000008943	Chr:KI519954.1	370.72-394-85 kbp	-				

		Atlantic cod (*Gadus morhua*)				Platyfish (*Xiphophorus maculatus*)			
Gene symbol	Name	ID	Location	Start-End	strand	ID	Location	Start-End	strand
spata13	spermatogenesis associated 13	ENSGMOG00000015009	scaffold_733	39.91-53.96 kbp	+	ENSXMAG00000009729	Chr:JH556681.1	1.65-1.67 Mbp	+
c1qtnf9	C1q tumor necrosis factor related protein 9	ENSGMOG00000014997	scaffold_733	29.83-31.15 kbp	+	ENSXMAG00000009725	Chr:JH556681.1	1.64-1.65 Mbp	+
mtmr6	myotubularin related protein 6								
nup58	nucleoporin 58KDa								
atp8a2	ATPase. aminophospholiid transporter. class I. type 8A. member 2	ENSGMOG00000015425	scaffold_733	335.73-392.28 kbp	+	ENSXMAG00000009957	Chr:JH556681.1	1.85-1.91 Mbp	+
shisa2	shisa family member 2	ENSGMOG00000015491	scaffold_733	393.51-402.46 kbp	-	ENSXMAG00000010029	Chr:JH556681.1	1.91-1.92 Mbp	-
rnf6	ring finger protein (C3H2C3 type) 6	ENSGMOG00000015404	scaffold_733	318.62-321.22 kbp	+	ENSXMAG00000009954	Chr:JH556681.1	1.84-1.85 Mbp	+
cdk8	cyclin-dependent kinase 8	ENSGMOG00000015383	scaffold_733	308.24-316.28 kbp	-	ENSXMAG00000009914	Chr:JH556681.1	1.82-1.84 Mbp	-
wasf3(b)	was protein family member 3 (3b)	ENSGMOG00000015343	scaffold_733	287.94-297.99 kbp	-	ENSXMAG00000009903	Chr:JH556681.1	1.81-1.82 Mbp	-
wasf3a	was protein family member 3a								
grp12	G-protein-coupled receptor 12					ENSXMAG00000020160	Chr:JH556681.1	1.80-1.80 Mbp	+
usp12(a)	ubiquitin specific peptidase 12 (12a)	ENSGMOG00000015314	scaffold_733	274.64-278.79 kbp	+	ENSXMAG00000009886	Chr:JH556681.1	1.79-1.79 Mbp	+
rpl21	ribosomal protein L21	ENSGMOG00000015298	scaffold_733	270.81-273.29 kbp	-	ENSXMAG00000009876	Chr:JH556681.1	1.78-1.79 Mbp	-
rasl11a	RAS-like family II member A								
gtf3a(b)	general transcription factor IIIA (A. b)	ENSGMOG00000015268	scaffold_733	263.73-266.18 kbp	-	ENSXMAG00000009851	Chr:JH556681.1	1.78-1.78 Mbp	-
gtf3aa	general transcription factor IIIAa	ENSGMOG00000010259	scaffold_4115	7.92-8.96 kbp	-				
mtlf3	mitochondrial translational initiation factor 3								
lnx2(a)	ligand of numb-protein X2	ENSGMOG00000015243	scaffold_733	252.99-263.22 kbp	+	ENSXMAG00000009830	Chr:JH556681.1	1.76-1.77 Mbp	+
gsx1	GS homeobox 1								
pdx1	pancreatic and duodenal homeobox 1	ENSGMOG00000015225	scaffold_733	240.37-242.89 kbp	-				
cdx2	caudal type homeobox 2								
urad	ureidoimidazoline (2-oxo-4-hydroxy-4-carboxy-5) decarboxylase	ENSGMOG00000015217	scaffold_733	237.56-239.90 kbp	+				
ftl3	fms-related tyrosine kinase 3								
pan3	PAN2 poly(A) specific ribonuclease subunit	ENSGMOG00000015169	scaffold_733	184.47-213.98 kbp	-	ENSXMAG00000009821	Chr:JH556681.1	1.74-1.75 Mbp	-
ftl1	fms-related tyrosine kinase 1	ENSGMOG00000015113	scaffold_733	130.02-178.18 kbp	+	ENSXMAG00000009774	Chr:JH556681.1	1.70-1.73 Mbp	+
slc7a1	solute carrier family 7								
lpar2b	lysophosphatidic acid receptor 2b								

		Medaka (*Oryzias latipes*)			
Gene symbol	Name	ID	Location	Start-End	strand
spata13	spermatogenesis associated 13	ENSORLG00000008698	Chr: 20	15.55-15.56 Mbp	+
c1qtnf9	C1q tumor necrosis factor related protein 9	ENSORLG00000008674	Chr: 20	15.54-15.54 Mbp	+
mtmr6	myotubularin related protein 6				
nup58	nucleoporin 58KDa				
atp8a2	ATPase. aminophospholiid transporter. class I. type 8A. member 2	ENSORLG00000009034	Chr: 20	15.76-15.79 Mbp	+
shisa2	shisa family member 2	ENSORLG00000009065	Chr: 20	15.80-15.80 Mbp	-
rnf6	ring finger protein (C3H2C3 type) 6	ENSORLG00000008902	Chr: 20	15.74-15.75 Mbp	+
cdk8	cyclin-dependent kinase 8	ENSORLG00000008885	Chr: 20	15.73-1574 Mbp	-
wasf3(b)	was protein family member 3 (3b)	ENSORLG00000008849	Chr: 20	15.72-15.73 Mbp	-
wasf3a	was protein family member 3a				
grp12	G-protein-coupled receptor 12	ENSORLG00000008826	Chr: 20	15.71-15.71 Mbp	+
usp12(a)	ubiquitin specific peptidase 12 (12a)	ENSORLG00000008821	Chr: 20	15.70-15.71 Mbp	+
rpl21	ribosomal protein L21	ENSORLG00000008802	Chr: 20	15.70-15.70 Mbp	-
rasl11a	RAS-like family II member A				
gtf3a(b)	general transcription factor IIIA (A. b)	ENSORLG00000008788	Chr: 20	15.69-15.69 Mbp	-
gtf3aa	general transcription factor IIIAa				
mtlf3	mitochondrial translational initiation factor 3				
lnx2(a)	ligand of numb-protein X2	ENSORLG00000008783	Chr: 20	15.68-15.69 Mbp	+
gsx1	GS homeobox 1				
pdx1	pancreatic and duodenal homeobox 1	ENSORLG00000008765	Chr: 20	15.67-15.67 Mbp	-
cdx2	caudal type homeobox 2				
urad	ureidoimidazoline (2-oxo-4-hydroxy-4-carboxy-5) decarboxylase	ENSORLG00000008761	Chr: 20	15.67-15.67 Mbp	+
ftl3	fms-related tyrosine kinase 3				
pan3	PAN2 poly(A) specific ribonuclease subunit	ENSORLG00000008758	Chr: 20	15.61-15.63 Mbp	-
ftl1	fms-related tyrosine kinase 1	ENSORLG00000008730	Chr: 20	15.60-15.61 Mbp	+
slc7a1	solute carrier family 7				
lpar2b	lysophosphatidic acid receptor 2b				

Table S2: Zebrafish body length (mm) at days 26 and 61 in the experiment:

Samples (26 dpf)	Lenght (mm)	Samples (61 dpf)	Lenght (mm)
H2O 1.1	11	H2O 1.1	15
H2O 1.2	-	H2O 1.2	10
H2O 1.3	11	H2O 1.3	12
H2O 1.4	8	H2O 1.4	11,5
H2O 1.5	7	H2O 1.5	11
H2O 1.6	7,5	H2O 1.6	11
H2O 2.1	8	H2O 2.1	13,5
H2O 2.2	7,5	H2O 2.2	12
H2O 2.3	7,5	H2O 2.3	11,5
H2O 2.4	7,5	H2O 2.4	11
H2O 2.5	9,5	H2O 2.5	13
H2O 2.6	7,5	H2O 2.6	10
ET 1.1	8	ET 1.1	10,5
ET 1.2	7	ET 1.2	12
ET 1.3	7	ET 1.3	10
ET 1.4	7,5	ET 1.4	11
ET 1.5	8	ET 1.5	13
ET 1.6	9,5	ET 1.6	12
ET 2.1	7,5	ET 2.1	13
ET 2.2	7,5	ET 2.2	12
ET 2.3	7	ET 2.3	11
ET 2.4	8	ET 2.4	12
ET 2.5	7,5	ET 2.5	8
ET 2.6	8	ET 2.6	7,5
E 1.1	7,5	E 1.1	12
E 1.2	8	E 1.2	12
E 1.3	7	E 1.3	11
E 1.4	7,5	E 1.4	12
E 1.5	7	E 1.5	12
E 1.6	7	E 1.6	10
E 2.1	8,5	E 2.1	12
E 2.2	7	E 2.2	10
E 2.3	-	E 2.3	12
E 2.4	6,5	E 2.4	10,5
E 2.5	8,5	E 2.5	9,5
E 2.6	6,5	E 2.6	9
MT 1.1	6,5	MT 1.1	13
MT 1.2	8,5	MT 1.2	9
MT 1.3	7,5	MT 1.3	11
MT 1.4	7,5	MT 1.4	9
MT 1.5	7	MT 1.5	8
MT 1.6	6,5	MT 1.6	7
MT 2.1	7	MT 2.1	11,5
MT 2.2	8	MT 2.2	11,5
MT 2.3	7,5	MT 2.3	11,5
MT 2.4	7	MT 2.4	9
MT 2.5	7	MT 2.5	9,5
MT 2.6	8	MT 2.6	9,5

Table S3. Protein sequences deduced from the cds sequences coding for different *gtf3a* ortholog genes, with length and deduced molecular weight. The sequences presented here were the ones used to produce the phylogenetic tree in Figure S2. Sequence underlined in yellow show the conserved initial sequence in all the oocyte specific Gtf3ab proteins of fish and the *Xenopus* oocytic protein. The sequence in blue shows the last of the C2H2 Zn finger domains of all the Gtf3a-s. In green the conserved transcription activation KRSLAS domain (KRSLAShLsGYPPK), necessary for transcriptional activation of 5S rRNA is shown. In teleostean proteins this is only found in Gtf3aa-s.

NON TELEOSTEAN Gtf3a

> Coelacanth (*Latimeria chalumnae*) Gtf3a ENSLACT00000016675, 298 aa, 34.87 kDa

PKNFICSFEGCDASFNKAWKLDAHLCKHTGEKPFVCDYKGCGKGFTRNYHLTRHQLIHGGEKPFQCPNDGCNAAFSTKSNLKRHTENKHG
NQDAPYVCDFEACGKAFKKHQQLKIHQCEHTNLLPFECDYEGCNKRFPVPSKLRRHKKIHKGYACEKEDCSFVGKTWTEYQKHLKDRHTEKF
ICELCNKTFKRKDFLKQHQKTHDQHREVFRCPHEGCGRTYTTTFNLQSHILSFHEERREYVCRQPGCGKAFAMKWTLNTHSISQDAFCLMY
HLPKKRPPRPKRSLASRLSGYVPPK

> **Spotted gar (*Lepisosteus oculatus*) Gtf3a ENSLOCT00000005013, 373 aa, 43.07 kDa**

MVRSHPCRGFRVVCSARIYGEHVCGKMEGDSVQFSSSSVTSSVIIPLSVFAMGETMGDPVKRFICSFPDCSASFNKAWKLEVHQYKHTGER
PFVCDYEGCGKTFTRSFHLTRHQITHSGEKPFRCPVEGCDEVFPINCSLKRHVARIHEHQGKPYICKYEGCGKSFKKNNQLKSHEYEHTNILPFK
CSFEGCDKRFLIPSKLKRHEKVHRGYPCKEDDCSFVGKNWTEYLKHKNALHQELLQCDQCSRTFKRNRLLQEHQRIHQEGRPILHCPREGCQ
RTYTTPFNLQSHILSFHEEQRPFACPHPGCGKAFAMRQSLQRHGVVHDPEKKKLKIPRPKRSMASRLSGFQPCKDEKKLAQLLQATSLESEEQ
ST

> Chicken (*Gallus gallus*) Gtf3a ENSGALT00000027621, 396 aa, 43.47 kDa

MRAAAASAIAAPGGWDRHVTSLMAVEGAAGSESAPGGGSSSVGGAAVAGGSSDGSAPAPAGSGVGSAPAARSFICSFPGCSATFNKGW
RLDAHLCSHTGARPYVCQYEGCGKSFTRDFHRTRHFLTHSGERPFECTAEGCNQKFGTKSNLKKHVQRKHENQQKLYSCNFEGCGKSFKKH
QQLKVHLCQHTNEPPFKCNQEGCGKNFSTPNSLKRHKKTHEGYACKKENCSYIGKTWTELLKHNKESHTEPIVCTECSKTFKRKDYLKQHKKT
HAAEREVCRCPREGCDRTYTTLFNLQSHILSFHEELKPFSCDHPGCGKVFAMKQSLARHAVHHDPEKKKLKAKRSRPKRSLASRLSGYIPPKT
QPGKDVVVTECKTTDQPTENGIPTVEILTLQ

> Human (*Homo sapiens*) Gtf3a ENST00000381140, 365 aa, 41.50 kDa

LDPPAVVAESVSSLTIADAFIAAGESSAPTPPRPALPRRFICSFPDCSANYSKAWKLDAHLCKHTGERPFVCDYEGCGKAFIRDYHLSRHILTHT
GEKPFVCAANGCDQKFNTKSNLKKHFERKHENQQKQYICSFEDCKKTFKKHQQLKIHQCQHTNEPLFKCTQEGCGKHFASPSKLKRHAKAH
EGYVCQKGCSFVAKTWTELLKHVRETHKEEILCEVCRKTFKRKDYLKQHMKTHAPERDVCRCPREGCGRTYTTVFNLQSHILSFHEESRPFVC
EHAGCGKTFAMKQSLTRHAVVHDPDKKKMKLKVKKSREKRSLASHLSGYIPPKRKQGQGLSLCQNGESPNCVEDKMLSTVAVLTLG

> *Xenopus laevis* Somatic Gtf3a P03001, 366 aa, 40 kDa

MAAKVASTSSEEAEGSLVTEGEMGEKALPVVYKRYICSFADCGAAYNKNWKLQAHLCKHTGEKPFPCKEEGCEKGFTSLHHLTRHSLTHTGE
KNFTCDSDGCDLRFTTKANMKKHFNRFHNIKICVYVCHFENCGKAFKKHNQLKVHQFSHTQQLPYECPHEGCDKRFSLPSRLKRHEKVHAG
YPCKKDDSCSFVGKTWTLYLKHVAECHQDLAVCDVCNRKFRHKDYLRDHQKTHEKERTVYLCPRDGCDRSYTTAFNLRSHIQSFHEEQRPF
VCEHAGCGKCFAMKKSLERHSVVHDPEKRKLKEKCPRPKRSLASRLTGYIPPKSKEKNASVSGTEKTDSLVKNKPSGTETNGSLVLDKLTIQ

> ***Xenopus laevis* Ovarian Gtf3a CAB51745, 344 aa, 38 kDa**

MGEKALPVVYKRYICSFADCGAAYNKNWKLQAHLCKHTGEKPFPCKEEGCEKGFTSLHHLTRHSLTHTGEKNFTCDSDGCDLRFTTKANMK
KHFNRFHNIKICVYVCHFENCGKAFKKHNQLKVHQFSHTQQLPYECPHEGCDKRFSLPSRLKRHEKVHAGYPCKKDDSCSFVGKTWTLYLKH
VAECHQDLAVCDVCNRKFRHKDYLRDHQKTHEKERTVYLCPRDGCDRSYTTAFNLRSHIQSFHEEQRPFVCEHAGCGKCFAMKKSLERHSV
VHDPEKRKLKEKCPRPKRSLASCLTGYIPPKSKEKNASISGTEKTDSLVKNKPSGTETNDSLVLDKLTLQ

TELEOSTEAN Gtf3aa

> Cave fish (*Astyanax mexicanus*) Gtf3a XP_007235992, 450 aa

FRLVVLQCTFEGCGKTFKKNNRLKIHECTHTQLLPYQCSHEGCERRFACPSKQKRHEKVHKGYPCSEEDCSFVGKTWTELLQHRKSHIAKVAC
DQCNRKFTDEWVLKQHQRVHGKERVVFRCPREGCQRSYTTAFNLQNHILSFHQEERAFTCPQPGCGKSFCMRQSLQRHSVVHDPERKKQ
KKPRPKRSLASRLSGYKPSKTRPAENGKPHKSTSSATSKSARSKSGRGQATSQSEPPEDIYDTRTSPELTKIRTNASHELNPTNADITPSSPPRM

EGNETGQSEVTDSDSMIVLILEPLMLNSPAMSQSQPTEMYNTDISQSEPFRSETAMMSQSGPTQLESTMISQVEPLVTSQFESTISQLEPTK
CKNVVISQLEPSKLENTVNSQLESSKFENTVNSQLESSEFENTVNSHLEPSKFENTVNSQLESSKFENIVNSQCDVIIRDG

> Cod (*Gadus morhua*) Gtf3aa ENSGMOT00000011273, 336 aa, 38.72 kDa

MDTKRISERRYICSYPDCDARYNKQWKLDAHLCKHTGIKPFACEQSGCGKAFPSPYHLTRHQLTHSGMKPFPCTAAGCTDTFTTNTNMLRH
FQRQHATDQKKYGCEVAGCGLVFKKNKQLNLHMCEQHTLLPPYQCSFEGCQMRFPCPSKQRRHEKVHNGYPCREEACVFTGKTWTELLK
HRREAHQPVYPCDQCDKVFRKSWMLHQHQAVHADMRVVLKCPRAGCQRSFTTEFNLMSHIKSFHDELRPFACTHEGCGKTFAMKGSLT
RHSVAHDPERRKIPKIRKPRPSRSLASRLSGVNPFKSARKTKELKDNTASGPHSPIKLFPLLQDTTLL

> Fugu (*Takifugu rubripes*) Gtf3aa ENSTRUT00000017432, 339 aa, 39.19 kDa

MEAGSEIQKRYICSFVGCQAAYNKQWKLDAHLCKHTGIKPFTCDRDGCGKSFCSQYHLARHDLSHSGVKPFRCSVDGCEDAFTTNANRDR
HVSRVHSSDRKKYACRWDGCGLEFKKNKQLKAHMCEQHTQLPPYRCTHDGCEMRFAFPSKLKRHEKVHRGYPCSEEGCGFTGKTWTDYL
NHRKEQHRRLLKCDQCSKEFRDSWFLQQHQRVHADTRVVLLCPHQGCGRSFTTVFNLESHIGSFHEELRPWVCTQVGCGKKFTMKQSLH
RHSIVHDPQRKKLKKPRASQSLASKLSGYKETKTVLVKKKKEPESVRRSLQAAETHSSVELLSLLQDTSLQ

> Medaka (*Oryzias latipes*) Gtf3aa XP_004084267, 358 aa

METKTDVHKRYICSFPECSAAYNKQWKLDAHLCKHTGVKPFPCEQSGCGKSFCDRYHLARHELTHTGEKHFVCTIEGCEEAFSTRSNLNRHV
SRKHSQERKTYVCTFDGCGLGFRKNNQLKLHLCEKHTQLPLYACTHEGCEMRFAVPSKLKRHEKVHKGYPCTEEDCTFTGKTWTELLRHKKE
SHQHVVKCEHCSKVFRDSWFLQKHLHVHDETRIVFKCPRDGCDRSYTTTFNLQSHIRSFHEGLRPFACSHSGCGKTFAMRQSLLRHRVVHD
PEKKKQRKPRPKRSLASRLSGYSEAKGTIRKKPKQPKSQADSPKSNQMGSVELVSLLQDAALMCSSAVDAQELANPLTAPLTV

> Platyfish (*Xiphophorus maculates*) Gtf3aa XP_005800526, 358 aa,

METKAEPHRRFFCSFPDCSAAYNRQWKLDAHVCKHTGLRPHSCQHEGCRKSFCTPYHLARHELVHSGERPFRCSVEGCAEAFTTSTNRSRH
VSRAHAREQKKYACAFQGCGQEFKKNKQLRAHMCEQHTQLPPYQCAHDGCQMRFSTPSKLKRHEKVHRGYPCAEEGCPFTGKTWTDLL
KHRKEQHRVVLTCEHCSKVFRDSWFLQQHQRVHADTRVVYKCPREGCDRSYTTMFNLQSHVGSFHENLRPFVCTHDGCGRAFTMKRSLQ
RHSVVHDPEGRKPKKSRPKRSIASRLSGYREAKRVVCEKLPDPKRGLCHDKTEQPGSVVLVSLLQDTSLLCEPTVDTHGLADVMNPPLST

> Stickleback (*Gasterosteus acuelatus*) Gtf3aa ENSGACT00000026241, 334 aa, 37.52 kDa

MESKREPLRRYICSFAGCPAAYNRQWKLDAHLCKHTGVKPHACARCAKSFCTSYHLARHALSHSGEKPFRCPEDGCGEAFTTAANRARHVG
RVHAPGRKRTYACRFEGCALEFRKNKQLKAHVCERHGPPAGHPCTHEGCAMRFAVPSKLRRHEKVHRGYPCADEGCGFTGKTWTEYLRH
RKERHRPVLRCDQCDKSFRDSWFLQQHRRVHSETRVVLRCPRAGCERSFTTAFNLQSHVGSFHEERRPFACAHAGCGKTFAMKQSLRRHS
VAHDPDKKKLAKARPKRSLASRLSGYGGTAAGKPAEESGPPGPVELVSLLQDTSLLCGPAVDTH

> Tetraodon (*Tetarodon nigroviridis*) Gtf3aa ENSTNIT00000009239, 339 aa, 38.84 kDa

MEANTEVSKRYICSFAGCTAAYNKQWKLDAHLCKHTGVKPFACDRDGCGKSFCSRYHLARHDLSHSGAKPFRCSADGCEEAFSTNANRAR
HVSRVHGSDRKTYACRFDGCGLQFRKNKQLRAHMCEQHTQLPPYLCPHEGCGMRFAFPSKLKRHEKVHRGYPCPEEGCGFTGKTWTDYL
NHRKERHRRILKCDQCSKVFRDSWFLQQHQRIHADTRVVLLCPHVGCGRSFTKVFNLESHIGSFHEELRPYVCTHAGCGKKFTMKQSLHRH
SIVHDPQRKKLKTPRASRSLASRLSGYKDTKAVLLKKKREPEPVRGAVQDAEPRTSVELVSLLQDTSLQ

> Tilapia (*Oreochromis niloticus*) Gtf3aa ENSONIT00000026027, 340 aa, 39.78 kDa

METKAESYKRYICSFSGCSAAYNKQWKLDAHLCKHTGVKPYSCERDGCSKAFCSKYHLARHELSHNGEKPFRCTVDGCAEAFTTNANRARHI
GRIHCLEPRKYVCRFEGCGLEFKKNKQLKSHMCEQHTQLPPYQCIYEGCQMRFSFPSKLKRHEKVHRGYPCKEESCVFTGKTWTEYLKHRKE
QHRVTLKCEQCSRVFRDTWFLQQHQRIHSEMRVVLKCPRDTCDRSFTTAFNLQSHISSFHEERRPFVCTHAGCGKTFAMNQSLQRHSVVH
DPQRKKLKKPRAKRSLASRLSGYCETKRVVYKMQTEHAAHRQSSQEKADQPGPFELVSLLQDASLL

> Zebrafish (*Danio rerio*) Gtf3aa ENSDART00000105925, 367 aa, 42.62 kDa

MTMDETNANVDQLGEIFICSYPECHAYYNREWKLQAHLCKHTGERPYKCKYKKCSKSFCTKHHLTRHVLTHTGEKPYRCVEDGCKEGFTTN
SNLQKHISRIHRQETKQYICTFEGCGKAFKKNNQLKTHECTHTQLLPFLCTQEGCGRRFSQRGKLKRHEKVHKGYSCETEGCSFVAKNWAEM
TNHKKVHIVRVQCDQCQKTFRDSWFLKQHQHVHSEERLVFHCPRDGCTRPYTTAFNLQSHILSFHEQQRSFICAHPGCGKAFSMKQSLQR
HGVVHDPEKKQMKPRPKRSLASRLSGYKSKKTRQTKTETSAPQISQSENPTHPQSDNQLHCLSSETLPSSHLEPVKSICSPANPVMHLLEPFL
V

TELEOSTEAN Gtf3ab

> **Cave fish (*Astyanax mexicanus*) Gtf3ab ENSAMXT00000014879, 329 aa, 37.76 kDa**

MGERLKDPNKSFACLYSDCKASFSKSWKLEAHYCKHTGVRPFACDSCTKSFCTRYQLTRHQLSHRGETPHLCSVDGCAEAFSTIGRLKNHVS
RAHEKEQKRYVCNYEGCGKEFCKKRQLKTHHCEHTNELPYECKFEGCGKKYAASKALKKHEKMHNGYPCAEEGCLFKGKTWTEYQAHRKA
EHREILQCGDCKKVFYVAWFLLKHKQFVHSGERRVFKCTKEGCEKTYTTNFNLQNHILSFHEGKRPFICSHAGCGKAFAMEKSLKRHGVAHD
PNKKKMQVNILEKQLTPQKTKRKNVPSKAKASDASALSARLKNVSLNKDASQNNP

> **Cod (*Gadus morhua*) Gtf3ab ENSGMOT00000016779, 299 aa, 34.16 kDa**

MGERIHIKKSFICSFHDCSASFSKSWKLEAHNCKHTGLKPFSCDDCDKNFCTRYQLTRHQLNHSGDRPHKCQADGCGEAFVSQSSMKNHM
DKSHHNEGKPFKCNHQGCGKDFSKRYQLKAHVYEHTKVLPFHCTVTGCTREFPSRGSLDHHKKVHQGYPCEEDGCPFQGKTWSAYQTHK
KEHRVKLPCDKCKKQFNNGRFLLLHKRHVHLGVKKELACPHKCGKSFTRQFHLESHVLLEHEGVRAFGCAFPGCGKRFAMKESLWRHGVV
HDPKRKAVKAKPTSAEGCMLAAKLAKIRGS

> **Fugu (*Takifugu rubripes*) Gtf3ab ENSTRUT00000012642, 327 aa, 38.11 kDa**

MGERLQSQKSFVCTFFDCKAKFSKLWKLEAHLCKHTGLKPFSCESCDKSFCTRYQLTRHELNHSGERPHKCPAEGCPEAFVTHSSMKNHMG
RVHHQRERPYQCDHLGCVKSFNKRNQLKAHQGEHQNVLPFHCSLKGCSREFPTHGKLKHHERVHAGYVCETNACPFEAKTWTEYLKHRK
KHQAKVPCQRCQKLFNNAWFMHQHELRVHFGERRKLLCPKKGCNKEFTHRFNLDSHIQGDHEGKRSFGCAYAGCGKSFVMKESLWRHE
VVHDPAKKKVKKLRPKRNQPWRLALRRKLAAAANQAETSKLAAKLRDTTLERQSSEGNV

> **Medaka (*Oryzias latipes*) Gtf3ab ENSORLT00000011034, 266aa, 31.31 kDa**

MGEKLQSQKTYSCSFSDCKSTFGESWKLEAHMCKHTGLKPFSCGNCDERFCTQHQLTRHELSHSDEKPIMKNHVSNTQELQKKHFNCHM
RVWKEFHKRNQRKLHVSINLCYPFRDCRFPGCAKEFLSNGKRRHHERVHQGYSCNNEVCPFQAKTWTELQKHKKEHEVKVQCGACQKLFS
NKWFLHLHELRVHSGEKKLLSCPREGCDRKFMRRFNLESHVLGEHEGKKPFICAHAGCGKSFAMKESLRRHGAVHDPAKKKLKVKLR

> **Platyfish (*Xiphophorus maculates*) Gtf3ab ENSXMAT00000009903, 318 aa, 36.86 kDa**

MGERLQSQNAYVCSFSDCNATFRKSWKLEAHLCKHTGLKPFSCESCEKSFCARYELTRHERVHSGEKPHTCPMDGCLEAFAKNATMKNHIT
RVHQHQEDRYKCDYEGCGKDFSKKKQLKAHKCEHGEPLAFHCTFNGCGKDFPSREKLKHHEKVHQGYPCSFDLCPTLSKTWTEYLKHRAQ
HREKLVCEKCNRLFNNSWFLRLHELRAHSGEKRYFLCPREGCNRKFTRRVKLESHVLGDHEGKKPFSCAYPGCGKSFALKESLWRHGVVHNP
AKRELKKRKPKKDKPPQVAQEATRSAADDQETGKLAAKLHSTTLE

> **Stickleback (*Gasterosteus aculeatus*) Gtf3ab ENSGACT00000004076, 324 aa, 37.09 kDa**

MGERLQSPKMYICSFSDCKAAFSKSWKLEAHLCKHTGLKPFSCENCDQSFCTRYQLTKHELHHSGEKTHKCPADGCSRVFVTKGSMKNHVA
RAHQQQEKQYPCDHQGCGKGFNKRNQLKAHKCEHLQILPFPCTISGCTKALPTHGKLKRHEKVHRGYQCEQEACPFQGNTWTEYLQHRK
EHKVKVQCGECKKLFNNAWFLHQHGLRVHAGEKPQQQLLCPKEGCGKRFTRPFNLESHVLGDHEGKKPFSCVYAGCGKSFAMKESLWRH
GVVHDVTKKKLKKVPKKKTWVKAVQVRQANQAEANKLAEKLHNATLKDDRGSRSP

> **Tetraodon (*Tetraodon nigroviridis*) Gtf3ab ENSTNIT00000015893, 318 aa, 37.03 kDa**

MGERLQSQKNYVCTFLDCGAKFSKSWKLEAHLCKHTGLKPFSCESCSRSFCTRYQLTRHQLNHSGERPHKCPVEGCPEAFVTHASMKNHM
ARIHRPQEKAYQCDHWGCTKSFNKRNQMKAHQGEHQNLLPFHCSFDGCVREFPTHGKLRHHKRVHAGYVCETDACPFEAQTWTEYLKH
RKKHQDKVPCSRCQKLFSNAWFLHQHELRVHSGEKRKLRCPRKGCNKEFTRHFNLESHLQGEHEGKRPFSCAHAGCGKSFAMKESLWRH
GVVHDPAKRKVQKLRPKRNQPWRLALRRKLAAAANQAEASELAAKLRNTTL

> Tilapia (*Oreochromis niloticus*) Gtf3ab ENSONIT00000017785, 326 aa, 37.97 kDa

MGERLQKTYVCSFSDCKATFSKPWKLDAHLCKHTGLKPFSCESCDKSFCTRYQLTRHELSHSGEKPHKCPADGCSEAFVRNASLKNHIARVH
QQQEKRFQCDHQGCEKDFSKRNQLKAHQCEHQESLPFHCSLTGCTREFLTLKKLKHHEKMHEGYPCETDGCPFQGKTWSDYLKHRKEHK
DKVLCGHCNKLFSNFWFFRLHELRVHSGEKRTFPCPKEGCEKKFTRRFNLESHVLGDHEGKKPFSCAVPGCNKSFAMKESLWRHGVVHDPA
KKKLKKLHPKKNRPMAQRDKQHTFKHMYKTPAAIAGLRLIQLDTALVLQNISIK

> **Zebrafish (*Danio rerio*) Gtf3ab ENSDART00000105925, 318 aa, 37.15 kDa**

MGERIQDPNKHFTCTFADCKATFSKLWKLEVHYCRHTGLKPFACGDCEKTFCTRYQLTRHQLSHSGEKPYLCSVSGCSAAFSTPGSLRNHIA
QVHDNKVRHYVCNYQGCAKEFHKKKQLKTHLCEHTNELSFKCDHEKCNNKFASPKALKRHRKLHEGYPCGEENCNFKGNTWSEYLKHRRT
AHRVNLPCNQCKKVFHKVCFLQMHKKFVHSGERRMFKCTREGCQKSYTRRFNLENHVLDFHEGKRDFTCHFTGCDKAFAMEESLKRHFV
VHMPQKTKPQKPKVKPKRKKTSKAKTSDAAKLSEHLQKVSLTKTPLP

ANNEX 1

Feeding protocol using commercial food (Sera)**:**

- Hatching-15dpf= Micron
- 15-30=microm+artemia
- 31-60=vipan baby+artemia
- 60 untill the end= vipagran baby+artemia

ANNEX 2

Histological processing of samples.

- 70% Ethanol 1 hour
- 96% Ethanol 1 hour
- 96% Ethanol 1 hour
- Absolute Ethanol 1 hour
- Absolute Ethanol 1 hour
- Xylene:Absolute Ethanol 1:1 dilution 1 hour
- Xylene 1 hour
- Xylene 1 hour
- Paraffin (55-60ºC) 2 hours
- Paraffin (55-60ºC) 2 hours
- Paraffin (55-60ºC) 2 hours

Hematoxylin-eosin staining for tissue included in paraffin:

STEP	PROCEDURE	REACTIVE	TIME(MINUTES)
1		xylene	10:00
2		xylene	10:00
3	hydration	absolute alcohol	2:00
4	hydration	absolute alcohol	2:00
5	hydration	alcohol 96º	2:00
6	hydration	alcohol 70º	2:00
7	Wash	water	5:00
8	Staining	hematoxylin	4:00
9	Wash	water	4:00
10	differentiation	acid alcohol	0:10
11	Wash	water	5:00
12	neutralization	Lithium carbonate	0:10
13	Wash	water	1:00
14	staining	eosin	1:30
15	Wash	water	0:01
16	Wash	water	0:30
17	dehydration	alcohol 70º	0:10
18	dehydration	alcohol 96º	0:15
19	dehydration	absolute alcohol	0:15
20	dehydration	absolute alcohol	0:15
21	preparation for mounting	xylene	untill mounting DPX

Chapter 5

Oocyte molecular markers in thicklip grey mullet (*Chelon labrosus*): applications in the identification of female gametogenic stage and in environmental monitoring of intersex condition under exposure to xenoestrogens

Parts of this chapter have been presented at:

28th ESCPB Congress-European Society for Comparative Physiology and Biochemistry. Bilbao, 2012. 5S to 18S rRNA ratio in gonads as an easy and inexpensive index for the identification of sex in a pollution sentinel fish species (*Chelon labrosus*). Rojo-Bartolomé, I; Diaz de Cerio, O; Martos-Bernal, J; Bilbao, E; Ortiz-Zarragoitia, M; Cancio, I**.** ICC, panel

17th INTERNATIONAL SYMPOSIUM ON POLLUTANT RESPONSES IN MARINE ORGANISMS (PRIMO17), Faro (Portugal), 2013. 5S/18S rRNA index in gonads; a powerful method to identify sex, female reproductive stage and xenoestrogenicity in fish. Rojo-Bartolomé I; Díaz de Cerio, O; Bizarro, B; Ortiz-Zarragoitia, M; Cancio I. ICC, panel

NATUR ZIENTZIEN TOPAKETA (UEU2013). Donostia-San Sebastian, 2013. Ingurune kutsatuetan bizi diren arrainen ugalketaren azterketa molekularra. Díaz de Cerio, O Rojo-Bartolomé I; Bizarro, B; Ortiz Zarragoitia, M; Cancio I. CCN, panel

10th INTERNATIONAL SYMPOSIUM ON REPRODUCTIVE PHYSIOLOGY OF FISH, Olhao, (Portugal), 2014. Seasonal dynamics of ribosomal RNA component production and accumulation during oogenesis in fish; cautiously investing in reproduction. Rojo-Bartolomé, I; Diaz de Cerio, O; Cancio, I.ICC, oral

5th INTERNATIONAL WORKSHOP ON THE BIOLOGY OF FISH GAMETES. Ancona, (Italy), 2015. Alteration in molecular markers of oocyte development and intersex condition in mullets impacted by wastewater treatment plant effluents. Valencia, A; Rojo-Bartolomé, I; Bizarro, C; Cancio, I; Ortiz-Zarragoitia, M. ICC, oral

This chapter has been submitted for publication in:

Rojo-Bartolomé, I., Valencia, A., Cancio, I. Transcription of ribogenesis genes in fish gonads: applications in the identification of oogenic stage and in environmental monitoring of intersex condition. Marine Pollution Bulletin.

LABURPENA

Itsasadarretan bizi diren izaki bizidunak konposatu disruptore endokrinoak (EDC) dituzten kutsatzaileen nahaste konplexuen eraginpean bizitzeko arriskua daukate. Xenoestrogenoak emeen hormonaren (estrogenoaren) jarduera imitatzeko gai diren EDC bereziak dira. Organismoengan izan ditzaketen eraginen artean arrain ar eta gazteen feminizazioa da deigarrienetarikoa. Horrela, batzuetan intersex deritzen gonadak, oozitoak garatzen dituen testikuluak, sortzen dira arrain espezie gonokoristikoetan. 5S rRNA oozitoen presentziaren markatzaile molekularra izanik, ikerketa lan honetan 5S/18S rRNA indizea ugalketa ziklo oso batean zehar aplikatu zitzaien Bizkaiko Golkoan kutsatutako guneetan jasotako 296 korrokoien (*Chelon labrosus*) gonadei, jakiteko ea indizea arrainak euren sexuaren arabera, emeak euren ugalketa-fasearen arabera eta intersex banakoak euren intersex-mailaren arabera bereizteko gai ote zen. Horrez gain, *gtf3a*-ren (general transcription factor IIIA) eta *ubtf1*-ren (upstream binding transcription factor 1) transkripzio-mailak neurtu ziren obarioaren garapen-fase desberdinetan. Sexua eta fase gametogenikoa mikroskopioaren bidez ezarri ziren. Histologia analisiek 38 korrokoi intersex identifikatu zituzten, zeinak testikuluan zituzten oozito-kopuruaren arabera sailkatu ziren. Intersex-maila baxua izan zen kasu guztietan eta oozitoak beti prebitelogenesi fasean zeuden. Gonada bakoitzaren RNA totala erauzi eta 5S/18S rRNA indizea kalkulatu zen RNA totalaren gainean eginiko elektroforesiaren bidez eta qPCR bidez ere. 5S/18S rRNA molekularki korrokoien sexua identifikatzeko gai dela ikusi zen, ar eta emeen banaketa puntua 0.4521 izan zelarik. Intersex-maila 5S/18S rRNA-rekin positiboki erlazionatua dagoela ikusi zen ere; zenbat eta oozito gehiago testikuluan, orduan eta 5S/18S rRNA balio altuagoak, korrokoietan intersex-maila identifikatzeko metodo objektibo eta kuantitatibo bat eskainiz. 5S/18S rRNA indizeak obarioaren garapen-fasea identifikatzen du ere, baliorik altuenak oogenesiaren hasieran (5S rRNA-maila altuak) aurkeztu zituelarik eta azken heltzeraino beheranzko joera erakutsiz (18S rRNA-mailen igoera). Gauza bera ikusi zen *gtf3a* transkripzio-mailekin, zeinek 5S rRNA-ren patroia jarraitu zuten. Aldiz, *ubtf1*-en transkripzio-patroia ez zen 18S rRNA-ren antzekoa izan. 45S pre-RNA bitelogenesi aurretik ere asko transkribatzen dela ikusi zen, 18S rRNA-ren maila altuak 45S pre-RNA-ren heltze prozesuaren ondorioa direla iradokiz. Beraz, 5S/18S rRNA indizea era molekular kuantitatiboan arrainen sexua, emeen gametoen fasea eta intersex-maila erraz identifikatzea ahalbidetzen du korrokoietan. Lan honetan sortutako ezagutza beste espezieetan ikusitakoa berretsi eta oogenesian zehar erribogenesian parte hartzen duten mekanismoak ulertzeko abian jartzen gaitu, arrainen ugalketa eta populazioen dinamikan eta ingurumenaren jarraipen ikerketan aplikagarria izan daitekeelarik.

ABSTRACT

Organisms inhabiting estuarine waters can be exposed to complex contaminant cocktails containing endocrine disrupting chemicals (EDCs). Xenoestrogens are EDCs with the ability to mimic female hormone activity causing estrogen-like responses in exposed organisms. One of their best described effects in fish is the feminization of juveniles and males, this sometime leading to the generation of intersex gonads, with oocytes being produced in testis of gonochoristic fish species. Considering that 5S rRNA is a powerful molecular marker of the presence of differentiated oocytes in fish gonads, in the present study a 5S/18S rRNA index was applied in 296 thicklip grey mullets (*Chelon labrosus*) collected from polluted sites in the Southern Bay of Biscay during a complete annual reproductive cycle, to assess whether this index could help to distinguish fish sex, female reproductive stage and intersex severity. In addition, transcription levels of the general transcription factor IIIA (*gtf3a*) and upstream binding transcription factor 1 (*ubtf1*), related to the ribogenesis process and particularly to 5S rRNA and 18S rRNA synthesis and dynamics, were studied in ovaries of females at different gametogenic stages. Sex and gametogenic stage were determined microscopically. Histological analysis resulted in the identification of 38 Intersex individuals that were ranked according to the amount of oocytes in testis. Intersex severity was always low to moderate and oocytes in testis were in previtellogenic stages. Total RNA was extracted from all gonads and 5S/18S rRNA index was calculated in electropherograms obtained from total RNA electorphoresis and after qPCR analysis. The 5S/18S rRNA index molecularly identified the sex in thicklip grey mullets and a threshold value of 0.4521 was established after electropherogram analysis with 100% of the males ranking bellow that threshold. Intersex severity index correlated with 5S/18S rRNA index, with higher values the higher the amount of oocytes in testis thus, providing an easy, quantitative and objective method to identify the severity of intersex condition in male mullets captured during pollution biomonitoring campaigns. The 5S/18S rRNA index also proved reliable in the identification of ovarian developmental stage since values were higher during early oogenesis (high levels of 5S rRNA) and progressively declined towards maturation (increase in 18S rRNA levels). The same happened with the levels of *gtf3a* transcription following the pattern of 5S rRNA. In contrast, *ubtf1* transcription pattern did not resemble that of 18S rRNA. It was observed that 45S pre-RNA was strongly transcribed before vitellogenesis, indicating that 18S rRNA is partially produced at gonad recrudescence through accelerated pre-rRNA maturation. Thus, 5S/18S rRNA index is an easy and quantitative way to identify molecularly the sex, female gametogenic stage, and intersex severity in mullets. The knowledge provided here confirms observations in other fish species and sheds light on the mechanisms governing ribogenesis during fish oogenesis, with possible applications in the study of fish reproduction, population dynamics and environmental monitoring.

INTRODUCTION

Organisms inhabiting estuarine waters can be exposed to complex contaminant cocktails with anthropic origin (Boehm et al., 2011; DellaSala et al., 2013). 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). EDCs include a complex array of substances, with different chemical structures and sources (for detailed information see Tyler et al., 1998; López de Alba & Barceló, 2001; Porte et al., 2006; Casals-Casas & 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). Those compounds in complex mixtures, can interact enhancing their potency and biological activity, and in such 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 et al., 2005). The effects of xenoestrogenic compounds in some aquatic organisms are well known, one of the best described being the feminization of juvenile and male fish (WHO/UNEP, 2012; Tyler & Jobling, 2008; Goksøyr et al., 2003; Goksøyr et al., 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). 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., 2002; Jobling & Tyler 2006; Harris et al., 2011).

Xenoestrogenic effects have been reported also in thicklip grey mullet (*Chelon labrosus*) populations from contaminated estuaries (Ferreira et al., 2004) and intersex gonads have been described in mullets 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., 2016). 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., 2016), both types of scenarios associated to waste water treatment plant effluents and 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 *cyp19a1b* in brain (Bizarro et al., 2014). Thus, thicklip grey mullet is considered an important sentinel species for the biomonitoring of exposure to 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 EDCs exposure (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 (Ortiz-Zarragoitia et al., 2014; Abdel-Moneim et al., 2015).

In this respect, recently, 5S rRNA and transcripts coding for accompanying proteins have been studied in mullets from polluted areas (Diaz de Cerio et al., 2012; Valencia et al.,

2016). The transcription levels of ribogenesis genes enabled the identification of the sex of the studied individuals in a comparative manner 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 fish species. 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, and considering 5S rRNA a molecular marker of the presence of differentiated oocytes in fish gonads (Rojo-Bartolomé et al., 2016), the 5S/18S rRNA index was applied in mullets collected from polluted sites of the Southern Bay of Biscay during a complete annual reproductive cycle to assess whether this could help in the identification of sex, female reproductive stage and intersex severity. 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 and dynamics, were studied in ovaries of females at different developmental stages.

MATERIALS AND METHODS

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: 91 female, 86 male, 38 intersex males and 81 individuals with unknown sex which were not histologically sexed and thus could be males, females or intersex males.

After capture, individuals were immediately anaesthetized in a saturated ethyl 4-aminobenzoate (Fluka, Steinheim, Germany) water bath following the protocol authorised by the ethics commission of the University of the Basque Country (CEBA/152/2010/ORTIZ ZARRAGOITIA). Each fish was sacrificed by decapitation and gonads were removed. A portion of each gonad was embedded in RNA later (Ambion, Life Technologies, Carslbard, 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 (NBF) and transported at 4°C to the laboratory for further processing.

Histological analysis of the gonads

After 24 hours 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), defined as inactive ovary containing perinucleolar oocytes with a very thin ovarian wall. Cortical alveoli (Ca), with oocytes at cortical alveoli stage and some of them starting vitellogenesis (<50%). Vitellogenesis (V), where oocytes appear full of yolk globules and enlarged plasma membrane. Mature/spawning (M), with hydrated oocytes showing coalescence of lipid droplets and very thick oocyte envelope, while the presence of some atretic oocytes begins to become apparent (<%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 intersex individuals, after their identification, the intersex severity index 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).

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

Total RNA was extracted from 50-100 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 quality RNA established at absorbance ratios of 260/280 and 260/230 around 2), was loaded in an Agilent RNA 6000 Nano Kit Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Electropherograms provided by the Bioanalyzer were used to quantify the concentration of the bands corresponding to 5S rRNA and 18S rRNA in each sample using the "Time Corrected Area" of each peak to calculate the 5S/18S rRNA ratio (Bartolomé et al., 2016a). When the presence of one of the rRNAs was below the levels of detection of the machine a 0.1 value was given to each sample instead of 0 (the lowest recordable value was 0.2). The binary logarithm of the ratio was calculated in order to develop a 5S/18S rRNA index that allowed clear visualization of the differences between testes and ovaries.

cDNA synthesis

2 µg of total RNA from each individual gonad were used for cDNA synthesis with the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) using random primers, according to manufacturer's instructions. 5S rRNA is a very small

transcript and random primers may not adequately retrotranscribe such small nucleic acids. For that reason, and for comparative purposes, an additional retrotranscription assay was performed with RNA from ovaries with perinucleolar (Pn) and vitellogenic (V) oocytes using a reverse primer specific for mullet 5S rRNA (5'-AAGCTTACAGCACCTG-3').

PCR analysis in ovaries

Primers to obtain 45S pre-rRNA sequence fragments were designed through alignment (Clustal Omega, http://www.ebi.ac.uk/Tools/msa/clustalo/) of piscine 45S pre-rRNA sequences available in NCBI (http://www.ncbi.nlm.nih.gov/) and searching for highly conserved nucleotide regions. Properties of designed primers were checked employing the IDT OligoAnalyzer Tool (https://eu.idtdna.com/calc/analyzer). Conventional PCRs were run with 0.8 nM of forward 5'-GAGGCCCTGTAATTGGAATGAG-3', reverse1 5'-CAAAGTGCGTTCGAAGTGTCGA-3' or reverse2 5'-AGAGAAGGCGCGAGGACAC-3' primers. The amplification was run with commercial Taq DNA Polymerase, recombinant Kit and 100 mM dNTP Mix (Invitrogen) for 35 cycles in a 2720 Thermal Cycler (Applied Biosystems, Carlsbard, California, USA). PCR procedure was as follows: 94°C for 2 minutes, and 35 cycles with a denaturation step at 94°C for 30 seconds, annealing at 61°C for 30 seconds and elongation at 72°C for 30 seconds. A final step at 72°C was added for 8 minutes. Obtained PCR products were sequenced in the SGIker Sequencing Service of the University of Basque. Once sequenced, fragments were aligned to obtain a consensus sequence and analyzed using CAP3 (http://pbil.univlyon1.fr/cap3.php) and ClustalW2 tools. Sequences obtained have been published in GenBank (KX358060, KX358061).

Specific primers were designed to amplify the fragment ranging from the 18S rRNA 3'-region into the internal transcribed spacer 1 and reaching the 5.8S rRNA 5'-region in cDNA generated from RNA extracted from Pn and V ovaries. This region suffers 45S pre-rRNA internal splicing leading to the generation of the mature 18S rRNA. Thus, the amplification of the sequence in this case is only possible when 18S rRNA and 5.8S rRNA remain together. The fragment was amplified using conventional PCR and employing 0.8 mM of forward 5'-AACCTCAGTGCGTGGCGGA-3' and reverse 5'-TCCACCGCTAAGAGTAGTCATG-3' primers. Amplification was performed as described

above (annealing temperature 61°C). Finally, PCR products were analysed by electrophoresis in ethidium bromide stained agarose gels (1.5%).

Quantitative PCR (qPCR) analyses in ovaries

cDNA obtained by random and specific retrotranscription from ovaries at different developmental stages was quantified in the Synergy HT Multi-Made Microplate Reader (BioTek, Winoosky, USA) by Quant-iT™ OliGreen® ssDNA Assay Kit (Invitrogen, Life Technologies). The quantification was performed in a reaction volume of 100 µl with a theoretical cDNA concentration range of 0.02-0.2 ng/µL, at 485/20 nm excitation and 528/20 nm emission wavelengths. Real PCR input cDNA concentration was calculated using the high-range standard curve according to manufacturer's instructions.

5S and 18S rRNA, *gtf3a* and *ubtf1* transcript levels were quantified (only in ovaries) by SYBR® Green qPCR (Roche, Basel, Switzerland). qPCR was conducted in triplicates using a 7300 Applied Biosystems Thermocycler. The PCR reaction mixtures (20 µl) consisted of 10 µl of 2X SYBR® Green PCR master mix, appropriate concentration of primers diluted in RNase-free water (see Table 2) and 2 µl cDNA template. qPCR procedure was as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles consisting of a denaturation step at 95°C for 15 seconds and an annealing step for 1 minute (temperature for each primer set in Table 2). A dissociation step was added at the end consisting of 15 seconds at 95°C, 1 minute at annealing temperature and a final step at 95°C for 15 seconds.

All amplification results were normalized with the amount of cDNA charged in the qPCR according to Rojo-Bartolomé et al. (2016) corrected in Rojo-Bartolomé et al (2016, in prep.) using an adapted ΔCT formula (RQ) with efficiency correction (E):

$$E = \left[10^{-1/m}\right] - 1$$

m being the slope of the standard curve of the qPCR reaction.

$$RQ = Log_2\left[\frac{(1+E)^{-\Delta CT}}{ng\ cDNA}\right]$$

Where $\Delta CT = CT\ sample - CT\ plate\ internal\ control$

5S vs. 18S rRNA transcription level indexes were calculated using qPCR results obtained for 5S rRNA from cDNA produced using both 5S rRNA specific or random primers.

Table 2: Primer sequences used for the qPCR analysis of the transcription levels of 5S rRNA, 18S rRNA, *gtf3a* and *ubtf1* in ovaries of thicklip grey mullets.

Gene	Forward sequence (5'3')	Reverse sequence (5'3')	Primer concentration (nM)	Sample dilution	Annealing T (°C)
5S rRNA*	CTTACGGCCATACCACCCTG	GTATTCCCAGGCGGTCTCCC	6.25	1/200 & 1/12800[#]	60
18S rRNA	GAGGCCCTGTAATTGGAATGAG	TAAGATACGCTATTGGAGCTGGAA	6.25	1/12800	60
gtf3a	CCAGGAGAAGCGATATAAATGTGA	TCGTGATGCTTCAGTTTTCCATG	12.5	1/400	59
ubtf1	CTCTAAAGCAAAGGTCAGTCCAGA	AATATGAACATGGCTGAGATGGGC	12.5	1/400	60

* Protected under Spanish patent P201130778 and international patent PCT/ES2012/070343.
[#] Dilution for cDNA obtained from 5S rRNA specific retrotranscription.

Statistical analysis

To assess whether female and male individuals could be segregated into two statistically different groups according to their 5S/18S rRNA index the R 3.3.1 (2016) software version was used. To generate the clusters a logistic regression was carried out using the balance between specificity and sensitivity. Then, a maximum Area Under the Curve (AUC) was calculated using the "pROC" package version 1.8 available for R (AUC = 0.996). Finally, the 5S/18S index cutpoint value differentiating males and females was obtained. The statistical analyses of the rest of the results were undertaken using SPSS (SPSS Inc., Chicago, Illinois). Data failed in normality and variance equality after applying the Shapiro-Wilk ($n<30$) test and Levene's test, both at a 0.05 significance level ($p<0.05$). Significant differences between groups were then evaluated using the non-parametric Kruskal-Wallis test. When only two groups were compared (Pn vs. V), data analysis was performed with the non-parametric Mann-Whitney test for two independent samples. In all the cases, significant differences were established at $p<0.05$. In addition, Spearman unilateral correlation analysis was performed to compare the histological intersex

severity index and the 5S/18S rRNA index. Spearman significant differences were established at $p<0.01$.

RESULTS

5S/18S rRNA index: identification of sex

5S/18S rRNA index was used to identify the sex of thicklip grey mullet individuals studied during a whole annual cycle (Fig 1). 177 histologically sexed individuals (colored dots in Fig 1) were analysed that allowed to distinguish two population clusters based on their 5S/18S rRNA index values with a cutpoint value of 0.4259. Females appeared above this value (pink box), and males below it (blue box). When the 177 histologically sexed individuals were classified using this cluster distribution 95% of females and 100% of males were correctly identified. No male individual showed a 5S/18S rRNA index value higher than the cutpoint and only two males out of 86 did not show negative values (one in February and another one in June). The majority of the females (86 from 91) showed an index value higher than the cutpoint, with the exception of only one female in November and four in January. Sample distribution revealed that while all male individuals displayed a narrow normal distribution with an index peak around -3.0, two populations could be distinguished within females with peaks around 5.0 and 12.0 (S1 Fig).

This sex distribution was used to classify a total of 81 individuals not previously sexed histologically (black lines in Fig 1 and S2B Fig). 35 individuals displayed a 5S/18S rRNA index value above 0.4259 and thus their probability to be females is 100%. Individuals with an index value lower than 0.4259 (46 out of 81) were males with a 94.5% probability. This applies in the case that none of the 81 individuals were intersex.

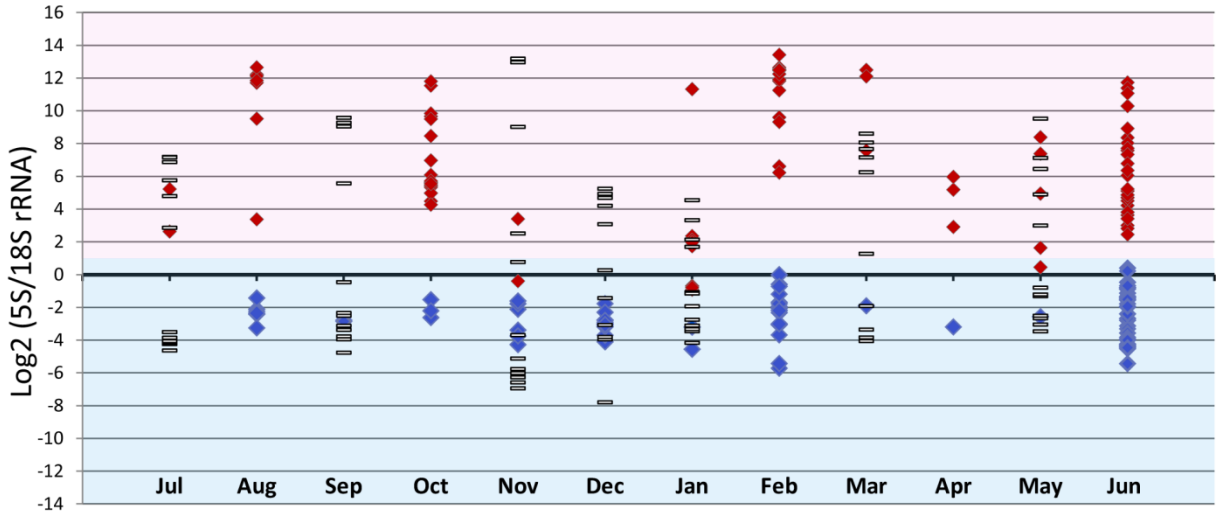


Fig 1: 5S/18S rRNA in gonads of thicklip grey mullets collected during a complete annual cycle. Box in pink groups most of female individuals, and the blue box encompasses 100% of the males. Each red dot corresponds to one histologically identified female individual (n=91) and each blue dot to a male (n=86). Each black line corresponds to an individual whose sex was not identified histologically (n=81).

5S/18S rRNA index and intersex severity

A total of 38 intersex individuals, histologically identified during our samplings in Pasaia, Galindo and Gernika, were analysed in the present study. They all displayed low to moderate intersex severity (severity indexes 1 to 3) according to the ranking methodology used (Jobling et al., 2006; Blazer et al., 2007).

When comparing 5S/18S rRNA index from these intersex individuals and their histologically ranked intersex severity index, a positive correlation (Spearman $p<0.01$) was observed between both indexes (S1 Table). As the amount of oocytes in the testes increased, the 5S/18S rRNA index increased (Fig 2). The lowest 5S/18S rRNA index values were recorded with intersex index values of 1, where testis presented from 1 to 5 oocytes scattered within each testis section, and the highest at intersex index 3, with clusters of 21 to 50 oocytes in each testis section (Jobling et al, 2006). When comparing intersex individuals with males and females, intersex individuals with lower severity indexes did not show significant differences in 5S/18S rRNA index values with males, while individuals with severity index 3 showed 5S/18S rRNA index values similar to females and different from males (Fig 2 and S2A Fig).

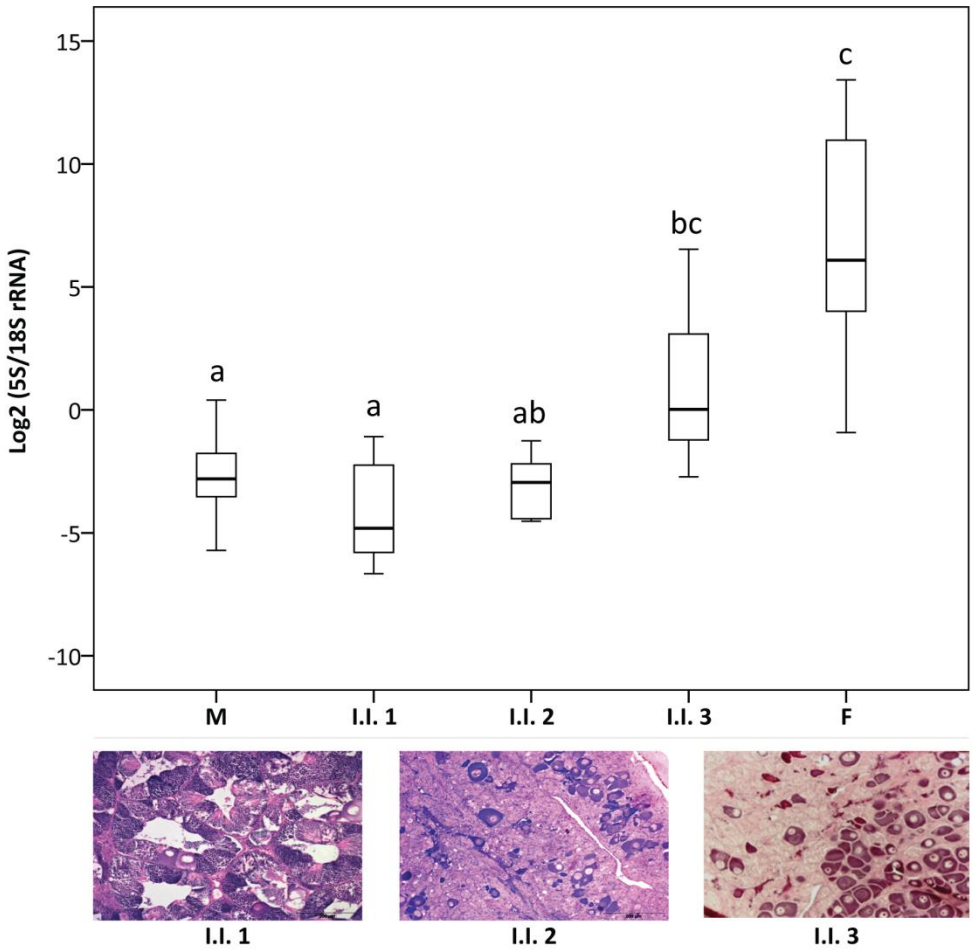


Fig 2: Comparison between 5S/18S rRNA index as calculated from the total RNA electropherograms and histologically calculated intersex severity index in thicklip grey mullet. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between groups (Kruskal-Wallis, $p<0.05$). M: male, I.I: Intersex Index, F: female. The severity ranked from 1 to 3 following the description proposed by Jobling et al. in 2006. Histological micrographs (in all cases scale bars = 200 µm) are representative of all intersex sampled and analysed per group. The total amount of individuals in each group were; 86 in M, 22 in I.I. 1, 9 in I.I. 2,7 in I.I. 3 and 91 in F (Total n=215).

5S/18S rRNA index: identification of female reproductive stage

5S and 18S rRNA relative fluctuations allowed distinguishing ovaries at different developmental stages (Fig 3). At early stages during oogenesis (Pn and Ca) 5S rRNA levels were very high in comparison with 18S rRNA, but as oogenesis advanced (from Pn to V), the relative amount of 5S rRNA decreased and the 18S rRNA relative amount increased in electropherograms. In consequence, the 5S/18S rRNA index that was high

at the beginning of the oogenesis (Pn=6.11 and Ca=7.69), decreased as oogenesis advanced; the lowest index values being recorded at vitellogenesis (V=0.90). Values increased again at regressing stage (R=4.59).

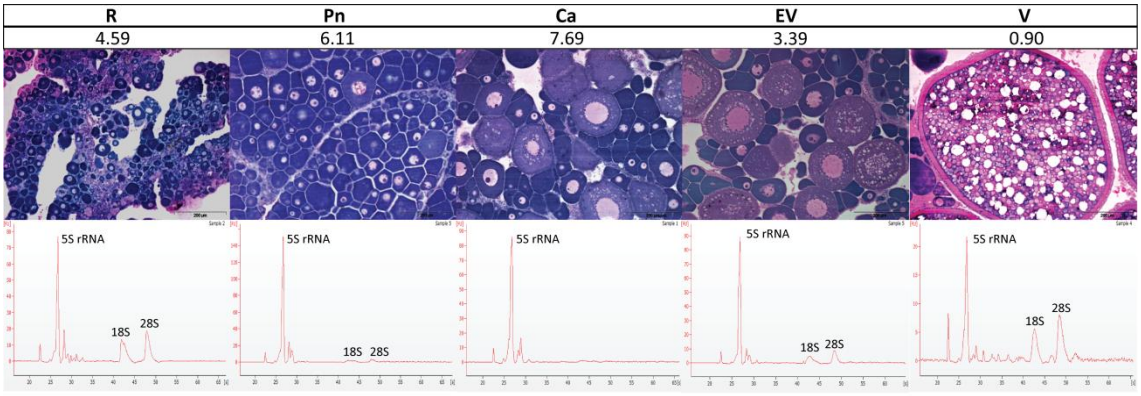


Fig 3: Variations in the relative amount of 5S rRNA, 18S rRNA and 5S/18S index during thicklip grey mullet oogenesis. Representative micrographs of ovaries within each of the developmental stages analysed (Scale bar=200 nm): R: resting, Pn: perinucleolar, Ca: cortical alveoli, EV: early vitellogenesis and V: vitellogenesis. No individuals with mature hydrated ovaries (M) were available for this study as mullets were never sampled at open sea. The value above each micrograph depicts the mean 5S/18S rRNA index value at each stage. In the total RNA electropherograms (representative of individuals in each stage) the peak at 25 seconds corresponds to 5SrRNA and the one at 40-45 seconds corresponds to 18S rRNA. The last peak corresponds to 28S rRNA. [FU]: Fluorescence, [s]: Time in seconds.

To corroborate that we were really measuring 5S and 18S rRNA levels in the electropherograms and not anything else, specific transcription levels were analyzed through qPCR (Fig 4). Comparison of the 5S/18S rRNA indexes obtained from Bioanalyzer electropherograms (Fig 4A) and after qPCR analysis (Fig 4B) showed identical results. But it was noticeable that 5S/18S rRNA index values after qPCR analyses were always negative, this meaning that qPCR analyses always identified higher levels of 18S rRNA than of 5S rRNA, also in Pn ovaries.

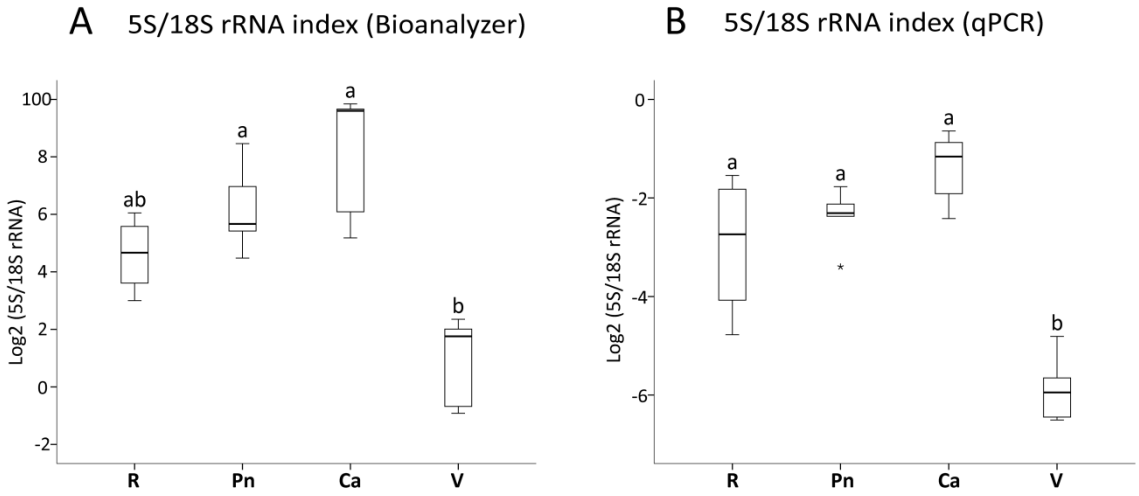


Fig 4: 5S/18S rRNA index in the ovaries of thicklip grey mullets at different gametogenic stages. (A) 5S/18S rRNA index as quantified from total RNA electropherograms. (B) 5S and 18S rRNA index after qPCR analysis of the 5S and 18S rRNA transcript levels in the same samples at different gametogenic stages: R: resting, Pn: perinucleolar, Ca: cortical alveoli and V: vitellogenesis. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between groups (Kruskal-Wallis, $p<0.05$).

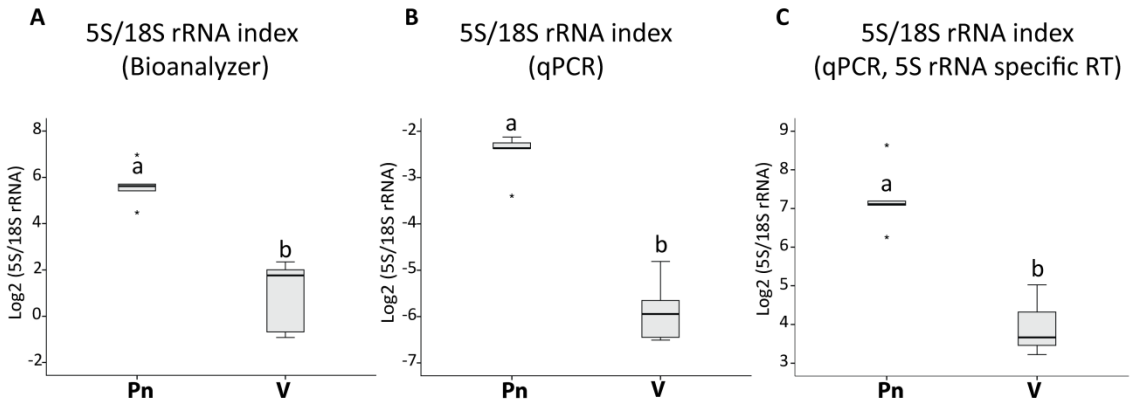


Fig 5: 5S/18S rRNA index after specific 5S rRNA retrotranscription in thicklip grey mullet ovaries. 5S/18S rRNA index values obtained from Bioanalyzer electropherograms of total RNA (A), from qPCR analysis of total cDNA (B) and from 5S rRNA qPCR quantification of cDNA obtained after 5S rRNA specific retrotranscription (C). Pn: perinucleolar stage and V: vitellogenesis stage (n=5 for each group). Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between means (Mann Whitney, $p<0.05$).

In order to clarify this inconsistency with the Bioanalyzer results, 5 Pn ovaries and 5 V ovaries were randomly chosen and retrotranscription was carried out using 5S rRNA specific primers followed by a qPCR analysis of 5S rRNA transcription levels. 5S/18S rRNA index after specific 5S rRNA retrotranscription (Fig 5C) showed the same relative profile obtained with the total cDNA (Pn>V) (Fig 5B), but displaying positive values, as it happened with the index obtained from the electropherograms (Fig 5A).

Transcription levels of genes related with RNA Polymerase I (Pol I) and III (Pol III) activity in ovaries

Regarding the transcription levels of *gtf3a*, related to Pol III activity and 5S rRNA transcription and stockpiling (Fig 6), differences were observed between early previtellogenic (R and Pn) and vitellogenic (V) stages. *gtf3a* was strongly transcribed during previtellogenesis with a significant downregulation at vitellogenesis. The highest transcript levels of the Pol III product, 5S rRNA, appeared later, at Ca stage, decreasing again at V stage. After spawning, at R stage, *gtf3a* was upregulated, showing transcription levels as in Pn. On the contrary, 5S rRNA transcript levels were low at R stage, similar to the levels recorded at V and close also to values at Pn (Fig 6). In the case of *ubtf1* related to Pol I activity and 18S rRNA synthesis (Fig 6), the gene was highly transcribed very early during oogenesis with a maximum at Pn stage, similar to *gtf3a*. Then, the transcription levels decreased to their lowest levels at V and R stages. *ubtf1* did not follow the transcription pattern displayed by 18S rRNA with its highest transcription levels at V stage (Fig 6).

The transcription levels of immature 45S pre-rRNA were measured in Pn and V stage ovaries by conventional PCR results showing that 45S pre-rRNA transcription occurred during both stages (Fig 7). The transcription began early during oogenesis as suggested by the high *ubtf1* transcript levels and 45S pre-rRNA levels maintained constant until vitellogenesis.

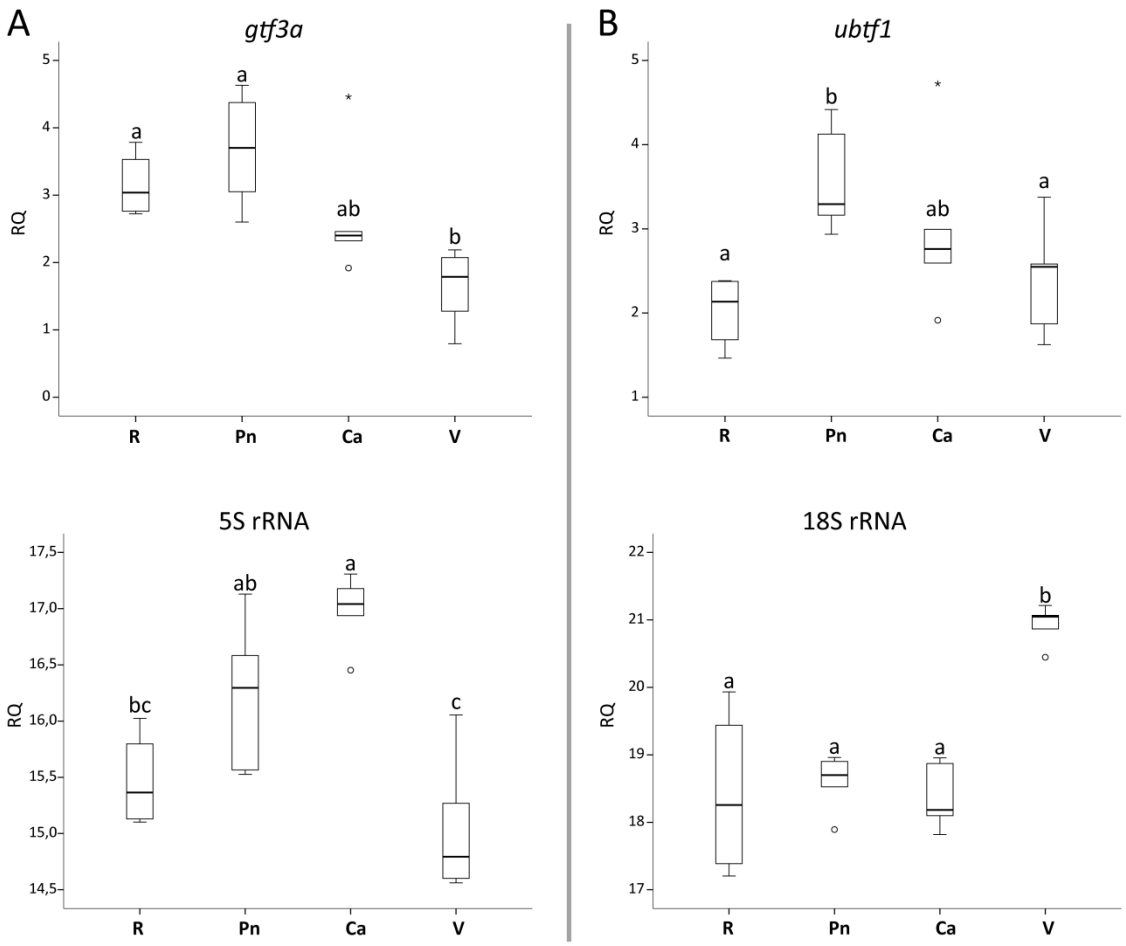


Fig 6: Transcript levels of genes related to RNA Polimerase III (A) and I (B) activity in ovaries of thicklip grey mullets. The developmental stages of the ovaries were: R: resting, Pn: perinucleolar, Ca: cortical alveoli and V: vitellogenesis. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between means (Kruskal-Wallis, $p<0.05$).

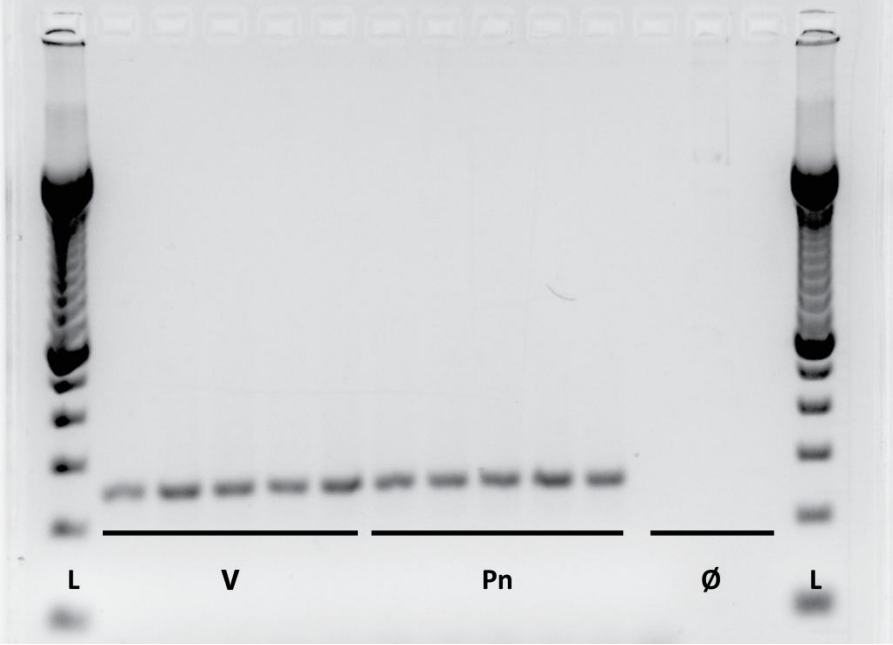


Fig 7: 45S pre-rRNA levels in ovaries with oocytes at vitellogenic (V) and perinucleolar (Pn) stages. Agarose gel electrophoresis of 45S pre-rRNA (fragment around 257 nucleotides in length) after conventional PCR for 35 cycles in ovaries with oocytes at Pn or V stages. Ø = no template control, L = Standard 100 bp (Invitrogen).

DISCUSSION

In the present study the 5S/18S rRNA index was used to molecularly identify the sex in thicklip grey mullets independent of their reproductive stage. In addition, this index provided an easy and unbiased method to identify the severity of intersex condition in males captured in sites with high burdens of xenoestrogenic compounds. Moreover, this methodological approach proved to be reliable and useful to identify the ovarian developmental stage in females due to the high index values (high 5S rRNA) displayed at Pn and Ca stages and the low values (high 18S rRNA) recorded in ovaries at V stage. In relation with this, the transcription levels of *gtf3a*, related with 5S rRNA production and stockpiling, decreased significantly with the onset of vitellogenesis. In contrast, *ubtf1* transcription pattern did not resemble the pattern of 18S rRNA accumulation, which was consequent with the transcription of 45S pre-rRNA already early in oogenesis.

5S/18S rRNA index in fish gonads: identification of sex

When using fish as sentinel organisms in pollution biomonitoring, knowing the sex of each analyzed individual is crucial as many biological parameters differ depending on the sex. It has been demonstrated that exposure to contaminants alters different molecular pathways that can be used as biomarkers of chemical exposure, but such biomarker responses substantially vary between females and males (Williams et al., 2003). Analysing one sex or the other helps to reduce the noise, but for many fish species without clear sexual dimorphism this is something that requires histological skills that are not always in place in molecular laboratories. Moreover, histological sexing is not that easy in some reproductive stages along the reproductive cycle (Diaz de Cerio et al., 2012). In other circumstances, experiments are carried out that do not collect material for histological analysis and the only samples available are those devoted to molecular of biochemical analysis. On the other hand, several studies have described that exposure to EDCs alter the transcription levels of genes related to sex differentiation, lipid metabolism, hatching and ovulation in females and spermiogenesis in testis, which at the same time, hamper the reproductive success in exposed fish populations (Bahamonde et al., 2015a). Thus, the analysis of sex ratios in pollution sentinel fish populations is in itself important to understand the possible effects of exposure to EDCs in the environment.

Previous studies on fish sex identification in our laboratory have demonstrated that 5S rRNA can be used as a molecular marker of the presence of oocytes, due to its high transcription levels and accumulation in ovaries during oogenesis (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2016). This 5S rRNA accumulation in oocytes has been proved in different fish species, e.g. European anchovy (*Engraulis encrasicolus*), hake (*Merluccius merluccius*), megrim (*Lepidorhombus whiffiagonis*) or zebrafish (*Danio rerio*) with only an electrophoresis of the total RNA extracted from gonads (Rojo-Batolomé et al., 2016). Diaz de Cerio and co-authors (2012) used 5S rRNA for the first time to identify sex in thicklip grey mullets, comparing the 5S rRNA transcription pattern of females vs. males. Later, a 5S/18S rRNA index was developed based on the quantification of 5S and 18S rRNA peaks obtained in the

electropherograms provided by the Bioanalyzer RNA nanochips. This index identifies the sex of each individual without the need to compare females vs. males, irrespective of the reproductive period and the fish species studied (Rojo-Bartolomé et al., 2016). Presently, this index has been proved to be useful to sex mullets from different polluted sampling sites (Diaz de Cerio, et al., 2012; Bizarro et al., 2014; Valencia et al., 2016) all along the year and irrespective of their gametogenic stage. A 5S/18S rRNA threshold value (0.4259) has been established for this species above which any individual can be identified as female. This threshold value would allow performing, a preliminary identification of sex from any mullet individual in pollution biomonitoring; easily and without additional cost for transcriptomic studies. In addition, the statistical analysis revealed two groups of individuals within females. The group with the highest 5S/18S rRNA index (peak around 12.0) would presumably belong to females with oocytes at previtellogenic stages while the one with the lowest index (peak around 5.0) would belong to females at V stage.

5S/18S rRNA index and intersex severity

Several studies in different species have described the reduction of reproductive success in intersex fish. Jobling et al. (2002a; 2002b) demonstrated a decrease in the fertility of wild intersex roach (*Rutilus rutilus*) compared to normal males. Kidd et al. (2007) reported a total collapse of a fathead minnow (*Pimephales promelas*) population in which, after exposure to ethinylestradiol the intersex condition was observed; and Harris et al. (2011) demonstrated that intersex roach showed a decrease in reproductive success with the increase in their intersex condition severity. So there is a need to differentiate intersex individuals from females and males and identify the molecular mechanisms associated to the differentiation of oocytes in fish testis in order to better predict the possible effects on affected fish populations (Bahamonde et al., 2015a). Gene expression analysis is becoming a commonly used approach to study the mechanisms of action of pollutants in wild fish (Garcia-Reyero et al., 2008; Ankley et al., 2009 Martyniuk & Denslow, 2012; Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Bahamonde et al., 2015b; Valencia et al., 2016). In this respect, vitellogenin expression in male fish liver has been implemented as a biomarker of fish

exposure to xenoestrogenic compounds (OECD, 2002; WHO/IPCS, 2002; ICES, 2005). However, no statistical correlation has been found between intersex condition and vitellogenin levels in mullet species (Hiramatsu et al., 2006; Bizarro et al., 2014) and specific biomarkers of intersex condition in fish are lacking (Bahamonde et al. 2015b).

Intersex mullets were identified using the relative expression of 5S rRNA as marker of the presence of oocytes in testis by our research group (Diaz de Cerio et al., 2012). Intersex individuals showed 5S rRNA transcript levels in between females and males, although no attention was paid to the severity of the intersex condition in that first study. In this study we propose a new molecular method to rank intersex mullets according to their intersex severity, which could be applied also in other fish species. This methodology is based upon the calculation of the 5S/18S rRNA index. As the severity of the condition increases (more oocytes present in the testis) this index also increases due to the accumulation of 5S rRNA in oocytes. Thus, individuals with low intersex severity display male-like 5S/18S rRNA index values, while higher intersex severity indexes results in 5S/18S rRNA index values similar to those of females. It must be considered, that nearly all of the intersex males found in the Basque coast only display previtellogenic oocytes in their testis, which is the stage at which 5S rRNA transcription levels are at their highest relative to 18S rRNA levels. The high degree of interindividual variability in the 5S/18S rRNA index values within each intersex group probably reflects the sensitivity of the analysis in comparison to a histological ranking. Histological analysis is based on analysis of randomly selected histological sections across a big and heterogeneous organ; the testis. In fact, the severity index used in the present study described by Jobling et al. (in 1998 and updated in 2006) in roach is based on histological observations of 6 testis portions. Other authors have modified this index to their convenience according to the species studied, to the observed amount of oocytes or to the oocyte distribution within the testis (Aerle et al., 2001; Fallet et al., 2003; Blazer et al., 2007; Tanna et al., 2013).

It is advisable to standardize sampling procedures when intersex fish are studied. We strongly recommend using the histological observation to perform a first screening of the samples and to certify intersex condition. Also to identify the developmental stage of the gonad; including the stage of the oocytes present in the testis of intersex

individuals. If only previtellogenic oocytes were present in the testis, as it is often the case, then we recommend using the 5S/18S rRNA index to rank the individuals according to their intersex severity. This provides an easy, unbiased and quantitative way to establish the amount of oocytes in the testis. This molecular approach allows analyzing more tissue than what it is normally analyzed histologically; around 100 mg per sample in each RNA extraction vs. one to nine sections 5 µm in thickness analyzed in histology. Besides, more samples can be analysed simultaneously in less time (around 3h for 24 samples).The use of 5S/18S rRNA index could help to standardize the intersex severity ranking methodology, with reproducible and comparable results among species: more oocytes per testis should result in higher 5S/18S rRNA index. Of course, this index should be tested in other fish species, where high prevalence of intersex condition has been identified, and if possible with availability of the whole range of intersex severities. Roach could be a good candidate species (Jobling et al., 2002a; 2002b; Bahamonde et al., 2013) for such additional studies.

In any case, if histological ranking would be the method of choice, the methodology should be standardized. First the homogeneity of gamete development within the gonad in each studied species should be understood, in order to establish how much tissue should be analyzed. Which portion of the gonads should be analysed: proximal, mid and/or distal? From one or the two lobes? Once this is established, it would seem advisable to ranged intersex severity according to the area coverage of oocytes within the testis. In that case it would be advisable to establish a minimum number of complete tissue sections for microscopical visualization, depending on the homogeneity of the testis (Blazer et al., 2007), leavinga minimum of 10 µm of distance between analysed sections to avoid quantifying the same oocyte twice (Bahamonde et al., 2015a). A stereological or a planimetric approach would help in better estimating the importance of the ovarian tissue per area of testis.

5S/18S rRNA index: identification of female reproductive stage

Kroupova et al. (2011) analyzed in detail the stage-dependent RNA composition in roach ovaries, concluding that during primary growth and early cortical alveoli stages small-size RNAs, which they did not identify as belonging to 5S rRNA, were accumulated. 5S

rRNA quantification has been later shown to provide an easy way to identify the presence of oocytes but also to establish the oogenic stage of ovaries in fish (Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2016). A 5S/18S rRNA index was developed that unequivocally helped in the identification of the oogenic stage in megrim (group-synchronous batch spawner) and European anchovy (asynchronous batch spawner) (Rojo-Bartolomé et al., 2016). The index also proved to be useful to monitor the oogenesis during carp pituitary extract treatment of European eel *Anguilla anguilla* (asynchronous batch spawner) (Rojo-Bartolomé et al., in prep.). Different developmental stages during hormonal treatment were ranked due to the high accumulation of 5S rRNA in comparison to 18S rRNA in previtellogenic oocytes with a progressive relative enrichment in 18S rRNA as oogenesis progressed towards final maturation in eels.

In this work, we used this index in thicklip grey mullet (synchronous spawner) ovaries to identify the maturation stage along oogenesis. Ovaries with oocytes during primary growth and cortical alveoli stage displayed higher index values than those with vitellogenic oocytes, due to the increased transcription of 18S rRNA during ovarian recrudescence. Thus, 5S/18S rRNA index could constitute an interesting approach to study the reproductive stage in females in an objective and quantitative manner, useful in many fish species independent of their ovarian developmental strategy.

Transcription levels of genes related with RNA Polymerase I (Pol I) and III (Pol III) activity

5S rRNA is produced by Pol III in eukaryotic cells, its activity being under the control Gtf3a (Szymanski et al., 2003). Gtf3a is not only a transcription factor but it also binds the 5S rRNA product for its accumulation in the cytoplasm as small ribonucleoprotein particles (7S RNP, Szymanski et al., 2003), and it has been shown to be strongly transcribed in ovaries in contrast to testis in fish (Ortiz-Zarragoitia et al., 2014; Rojo-Bartolomé et al., 2016). Our results hereby demonstrate the accumulation of *gtf3a* transcripts in ovaries of mullets. Moreover, as it occurs in ovaries from megrim and European eel (Rojo-Bartolomé et al., 2016; in prep.), *gtf3a* is strongly transcribed in

previtellogenesis and is down-regulated at vitellogenesis in mullets allowing also the identification of the entry into the secondary stage of oocyte growth.

In contrast to 5S rRNA, 18S rRNA is synthesized by Pol I. Ubtf1 forms an 'enhancesome' in rDNA to enable the access of the Pol I machinery (Bazett-Jones et al., 1994; Reeder et al., 1995). The product of Pol I is the 45S pre-rRNA transcript which suffers 3 main splicing events to form mature 5.8S, 18S and 28S rRNAs (Drygin et al., 2010). Previous qPCR analysis showed no match between the profiles of *ubtf1* and 18S rRNA transcription during eel oogenesis (Rojo-Bartolomé et al., in prep.). The same was observed hereby in mullets and *ubtf1* transcript levels were at their highest during early oogenesis, when, as reported in anuran frogs as *Xenopus*, Pol I activity should be very low (Roger et al., 2002). On the contrary, *ubtf1* was significantly down-regulated during secondary oocyte growth, when Pol I activity should be maximal according to 18S rRNA levels shown here for mullets, and for other fish such as roach (Kroupova et al., 2011), megrim (Rojo-Bartolomé et al., 2016) and eel (Rojo-Bartolomé et al., in prep.). These results reinforce the idea that post-translational events, such as phosphorylation of Ubtf1, are responsible for the activation of the Pol I machinery. This would allow producing 45S pre-rRNA rapidly before meiosis resumption during vitellogenesis (Rojo-Bartolomé et al., 2016; in prep.).

But here it became apparent that 45S pre-rRNA transcription occurs already in ovaries with oocytes at Pn stage. Thus, mature rRNA production could be boosted during vitellogenesis and meiosis resumption, through splicing of accumulated 45S pre-rRNA first at the 18S rRNA-5.8S rRNA site and finally between 5.8S rRNA and 28S rRNA (Drygin et al., 2010). Surprisingly, 45S pre-rRNA transcript levels were similar in ovaries with Pn and V stage oocytes. The strong transcription at Pn stage is consequent with the fact that nucleoli, differentiating characteristic of Pn stage oocytes, are the structural manifestation of Pol I activity and thus 45S pre-rRNA synthesis. It could be hypothesised that the whole process would allow rapid assembly and accumulation of ribosomes in the moment of fertilization (Ortiz-Zarragoitia et al., 2014).

Thus, the 5S/18S rRNA index provides an easy and inexpensive way to distinguish male and female mullets. In addition, this index proved to be a sensible and objective tool to

classify intersex mullets according to the amount of oocytes present in testis (5S/18S rRNA intersex severity index) due to the accumulation of 5S rRNA in the oocytes. Furthermore, the index was useful to identify the ovarian developmental stage in a synchronous spawner (mullets). As it happened in other fish species, the 5S/18S rRNA index decreased with the onset of vitellogenesis, due to the reduction in Pol III activity and the appearance of mature 18S rRNA. In contrast of earlier hypothesis, Pol I is active in fish oocytes early during oogenesis and 45S pre-rRNA is produced in Pn stage oocytes. 45S pre-rRNA then begins cleavage during vitellogenesis together with the additional 45S pre-rRNA that is also transcribed during vitellogenesis. Further studies are needed in different fish species and under different developmental/environmental scenarios, to decipher the mechanisms that govern ribogenesis during oogenesis in females and intersex individuals.

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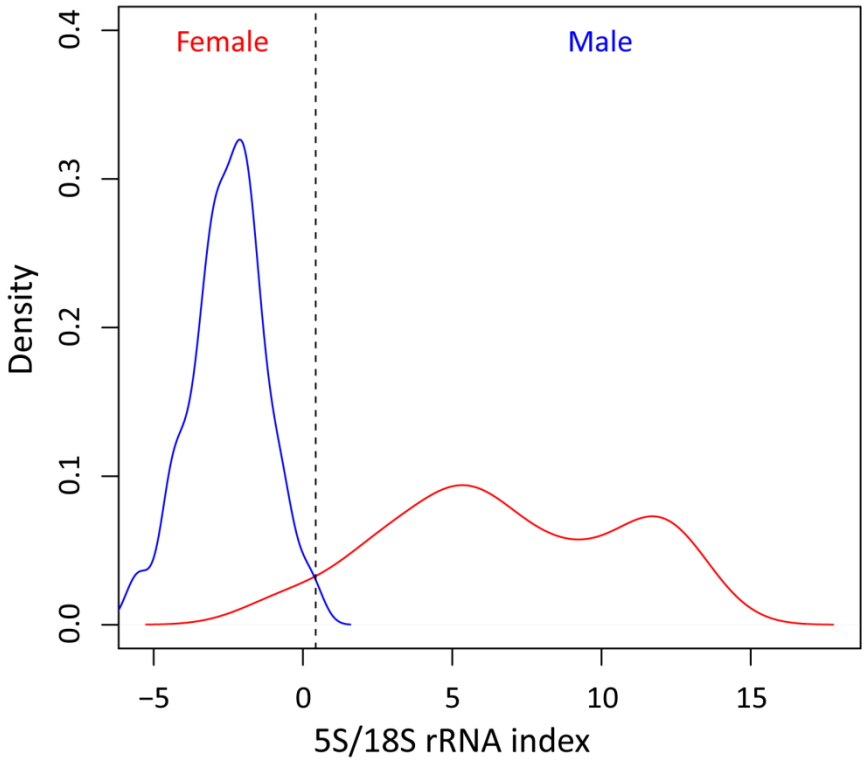
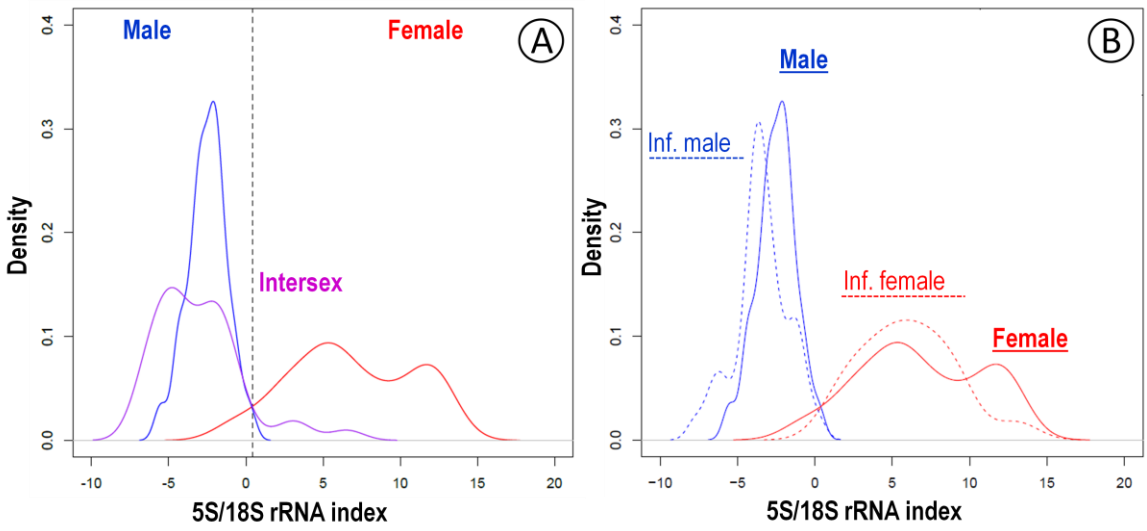
SUPLEMENTARY MATERIAL



S1 Fig: Distribution of previously sexed female and male mullets (n=177) according to their 5S/18S rRNA index value. Vertical line corresponds to 0.4521 cutpoint value (AUC=0.996). The probability to be a female above the cutpoint value is 100%. Below this value the probability to be male is 94.5 %. Males' distribution displayed a narrow normal distribution with an index peak around -3.0. Two populations could be distinguished within females with peaks around 5.0 and 12.0 5S/18S rRNA index value.



S2 Fig: Distribution of (A) inteserx individuals (n=38,) and (B) previously non histologically sexed individuals inferred to be males or females taking into accound the cut point value for both sexes shown in distribution plot in S1 Fig. The distribution of intersex individuals clearly shows that individuals with intersex index 3 resemble females instead of. In plot B the introduced new individuals clearly show a mainly unimodal narrow distribution in males, while the distribution in females is wider with a hint of a bimodal distribution depending on devpomental stage.

S1 Table: Spearman correlation test performed between histologically established intersex severity index and the gonadal 5S/18S rRNA index in mullets.

Correlations

			Intersex I.	5S/18S
Spearman's Rho	Intersex I.	Correlation coefficient	1,000	,638**
		Sig. (unilateral)	.	,000
		N	38	38
	5S/18S	Correlation coefficient	,638**	1,000
		Sig. (unilateral)	,000	.
		N	38	38

**. Significance levels of correlation at 0.01 (unilateral)

Chapter 6

5S rRNA arrainen arrautza-kalitearen markatzaile molekular gisa: erreboilo (*Scophthalmus maximus*) eta lupiaren (*Dicentrarchus labrax*) kasuak

LABURPENA

Arrainen akuikulturan, ugalketa-arrakastaren faktore-mugatzaileetariko bat eskuratutako gametoen kalitatea da. Arrautza-kalitatea era kualitatiboan eta kuantitatiboan estimatzeko irizpide ezberdinak erabili izan diren arren, kalitatearen adierazle hauek askotan arrain-espezie eta hazkuntza-metodo zehatzetan soilik dira erabilgarriak. Azken urteotako ikerketek arrainen ernalketaren aurreko arrautza-kalitatearen erregulazioarekin zerikusia duten mekanismo molekularrak aztertzen jardun dute. Izan ere, oozitoak gene ezberdinak adierazten ditu oogenesiarenn garapan-fase ezberdinetan zehar eta prozesuan sintetizatu eta metatzen dituen molekula berriek kalitate oneko arrautzaren osatze koordinatuan paper garrantzitsua jokatzen dute, horrela, behin ernalduta, enbrioi osasuntsu bat gara daitekeelarik. Gauzak horrela, gene ezberdinen transkribapen-mailak arrautzen kalitatea aztertzeko baliabidea izan daiteke. Lan honek, 5S rRNA-ak kalitate altuko arrautzak identifikatzeko markatzaile molekular posible gisa aztertzea du helburu, horretarako, akuikulturan garrantzitsuak diren bi arrain-espezieekin, erreboiloa (*Scophthalmus maximus*) eta lupia (*Dicentrarchus labrax*), ugalketa-saioak egin ziren, 10 erreboilo emeren arrautzak ar bakar baten espermarekin eta 16 lupi emeren arrautzak 20 arren esperma-nahastearekin ernaldu ziren. Errundako oozitoen RNA totaletik lortutako 5S rRNA eta 5S/18S rRNA-mailak arrautza-kalitatearen parametro tradizionalekin eta bestelako neurketa molekularrekin egon zitezkeen korrelazioak aztertu ziren. Horrela, bi arrain espezieetan, bai 5S rRNA-mailek zein 18S rRNA-mailek erlazio positiboa aurkeztu zuten euren artean. Aldiz, erreboiloan 5S rRNA-mailak eta 5S/18S rRNA-k arrautza-kalitate oneko parametro tradizionalekin (besteak beste, flotagarritasuna eta eklosio-arrakasta) korrelazio positiboak aurkeztu zituzten bitartean, lupian korrelazioak negatiboak izan ziren (arrautzen flotagarritasuna, enbrioien biderakortasuna, obarioarean pH-a, ernalketa-arrakasta, enbrioien biziraupena eta garapena). Nahiz eta 5S rRNA-k arrainen arrautza-kalitatearekin erlazioa duela susma daitekeen, lortutako emaitza kontrajarriek ugalketa saioetan eta laginen prozesamenduan protokolo desberdinak eta desegokiak erabiltzearen isla dira. Etorkizunera begira arrainen arrautza-kalitatearen neurketarako 5S rRNA-ren balioa frogatu ahal izateko, errunaldi luzean zehar kalitate ezberdineko arrautza-kantitate handia eskuratzea ahalbidetzen duen arrain espeziea erabiltzea komeni da. Horrez gain, arrautza guztiak ar bakarraren espermarekin ernaltzea ahalbidetu behar du. Kuantifikazio molekularrak arrautza helduen kopuru jakinetik erauzitako RNA-ren gainean egin behar dira, ernalketa, eklosio eta larben biziraupen arrakastarekin korrelazioak ezarri ahal izateko. Honekin, oozitoetako erribosomen-bitartekarien pilaketak arrautzen kalitatearen adierazleak direla froga daitekeela uste dugu, akuikultura-protokoloen hobekuntzarako baliagarriak izan daitezkeenak.

ABSTRACT

The quality of the gametes is one of the key factors limiting reproductive success in fish aquaculture. In order to estimate the quality of the eggs, both quantitative and qualitative criteria have been traditionally used, but these quality markers are usually only useful for a specific fish species or a particular rearing method. The molecular mechanisms underlying the regulation of the egg quality prior to fertilisation have been the focus of several studies in recent years. In fact, during oogenesis the oocyte expresses different genes depending on the developmental stage, and the new molecules accumulated during this process play a key role in the coordinated growth of the egg, allowing, once fertilisation has occurred, the development of a healthy embryo. Taking this into account, the transcription levels of the different genes involved in oogenesis can be used to study the quality of the eggs. The main objective of this work was to study the use of 5S rRNA as a possible marker of high egg quality. For this purpose, two fish species important for aquaculture, turbot (*Scophthalmus maximus*) and European seabass (*Dicentrarchus labrax*), were used in reproduction experiments. In turbots the eggs of 10 females were fertilised independently with the sperm from a single male, while in sebass eggs from 16 females were fertilised with the pooled sperm from 20 males. Correlations between the 5S rRNA and 5S/18S rRNA levels, obtained from the total RNA in unfertilised oocytes, and traditional egg quality markers were tested. 5S rRNA levels and 5S/18S rRNA index were successfully measured in spawned oocytes, 5S and 18S rRNA levels showing a positive correlation in both species. However, whereas in turbot 5S rRNA levels and the 5S/18S rRNA ratio showed a positive correlation with traditional egg quality markers (buoyancy and hatching success, among others), correlations with egg and embryo quality markers (ovarian pH, egg buoyancy, embryo viability, fertilisation success, embryo survival and development) were negative in the case of the European seabass. The opposing results obtained could be due to the different, experimental set-ups, animal husbandry and sample preparation protocols used with both species. In future experiments, and to prove the value of 5S rRNA to identify the quality of fish oocytes, we recommend selecting a fish species in which enough oocytes can be obtained in a lengthy spawning season where different quality oocytes could be compared, and fertilised with sperm from a single male. Molecular quantifications should be carried out on RNA extracted from a given specific number of fully mature oocytes, stabilising correlations with parameters linked to fertilisation, hatching success and larvae survival. With this approach we believe that accumulation of ribosomal intermediates in oocytes could be use as easily measurable proxy of egg quality to improve husbandry protocols in aquaculture.

SARRERA

Arrainen akuikulturan, ugalketa-arrakastaren faktore-mugatzaileetariko bat eskuratutako gametoen kalitatea da. Gametoen kalitatean oso aldagarriak izan daitezkeen faktoreek eragiten dute. Ikerketa lan askok arrautza (Kjorsvik et al., 1990; Brooks et al., 1997) eta espermaren (Billard et al., 1995) kalitatean eragin dezaketen faktore espezifikoak ikertzen jardun dute eta akuikultura intentsiboa asko garatu den arren oraindik ere eme-ugaltzaile onenak eta kalitate altuko arrautzak bereizteko metodologia estandarizatzetik urrun dago.

Definizioz, arrautza-kalitateari buruz ari garenean, arrautzak ernaldua izateko eta ondorioz sortutako enbrioia modu normalean garatzeko duen gaitasunari egiten diogu erreferentzia (Bobe & Labbé, 2010). Oro har, parentalen ekarpen genetikoa (Colléter et al., 2014), nutrizioa, emearen egoera fisiologikoa eta adina, ugalketa sasoian edo errute garaian dagoen uraren tenperatura eta fotoperiodoa, obulazioa bultzatzeko erabilitako teknika-mota eta momentuan oozitoek duteneko garapen-maila, emeak pairatu dezakeen estresa, errutea eta ernalketa bitartean oozitoen umeldura-maila eta uraren propietate fisiko-kimikoek, zein xenobiotikoekiko esposizioak arrautza-kalitatean eragin dezaketen faktoreak dira (Bobe & Labbé, 2010).

Arrautza-kalitatea era kualitatiboan eta kuantitatiboan estimatzeko irizpide ezberdinak erabili izan dira zuzenean edo zeharka egindako neurketen bitartez. Zuzeneko neurketei dagokienez, ernaldu gabeko oozitoaren hasierako tamaina, morfologia edota kolorazio/gardentasunaren azterketan oinarritzen direnak dira nagusi (Bobe & Labbé, 2010; Żarski et al., 2011). Horrela adibidez, zenbat eta arrautza handiagoa, orduan eta kalitate hobea espero da, hala ere, frogatua dago kalitate ezberdineko arrautzek tamaina eta forma ezberdina aurkeztu dezaketela eta beraz erlazio zuzenik ez dagoela bi baldintzen artean (Bromage et al., 1992; Ciereszko et al., 2009).

Zeharkako neurketek, emearen zeloma eta obarioaren parametro fisiko-kimikoak arrautza-kalitatea aurresateko erabiltzen dute. Kasu honetan, eta erreboiloan (*Scophthalmus maximus*) ikusi denez, obario zein zeloma-fluidoaren pH baxuek arrautza-kalitatearen beherakadarekin erlazionaturik daude (Skaalsvik et al., 2015). Izan ere, pH-

ren beherakada oozitoen apurketaren ondorioz gertatzen dela ikusi da (Dietrich et al., 2007). Hala ere, eta nahiz eta erreboiloan pH-a ernalketa-arrakastarekin erlazionaturik egon, ez da erlazio linealik ezarri pH eta enbrioien biziraupenarekin (Aegerter & Jalabert, 2004). Hala ere, arrautza-kalitatearen parametro adierazle hauek askotan arrain-espezie eta hazkuntza-metodo zehatzetan soilik dira erabilgarriak (Ciereszko et al., 2009).

Arrautza-kalitatea estimatzeko erabiltzen diren parametro doienak "a posteriori" ernalketaren ostean oozitotik sortzen den enbrioiaren arrakastaren neurketan oinarritzen direnak dira. Beraien artean ernalketa-arrakasta bera erabili daitekeen parametro goiztiarrena delarik. Bereziki erabilgarria da oskola gardena duteneko espezieen arrautzak aztertzeko, adibidez erreboiloan. Nahiz eta hazkuntza goiztiarraren arrakastaren estimazio ona izan, parametro honek ez du zertan geroko enbrioein/jubenilen hazkuntza-arrakasta islatu behar (Shields et al., 1997). Izan ere, ernalketa ostean enbrioi-zelulak banatzen hasten dira, orduan zelulen-zatiketa patroi anormalen agerpen-maila erabili daiteke jatorrizko arrautza-kalitatea estimatzeko. Honekin batera ernaldutako arrautzaren flotagarritasuna ere aztertzen da, umeldurarekin erlazionaturik baitago, baina ez batak ez besteak ez dute zertan arrautzaren kalitatea, ezta enbrioiaren bideragarritasuna islatu behar (Brooks et al., 1997; Avery et al., 2009). Gerora garapenaren fase-gako ezberdinetan; hala nola, begi-fasean, eklosioan eta bitelo-sakuaren birxurgaketa fasean, metamorfosian pleuronektidoetan, enbrioiaren biziraupena eta malformazioak azter daitezke, (Bobe & Labbé, 2010).

Azken urteotako ikerketek arrainen ernalketaren aurreko arrautza-kalitatearen erregulazioarekin zerikusia duten mekanismo molekularrak aztertzen jardun dute (Aegerter et al., 2005; Bonnet et al., 2007b; Chapman et al., 2014; Palomino et al., 2014; Ma et al., 2015). Oogenesian zehar oozitoan gertatzen direneko molekulen harrera, sintesia, metaketa eta prozesamenduak kalitate oneko arrautzaren osatze koordinatuan paper garrantzitsua jokatzen dute, horrela, behin ernalduta, enbrioi normal eta osasuntsu bat gara daitekeelarik. Arrautzaren garapena, bitelogenesian zehar gertatzen den bolumenaren handipena barne, luze ikertu izan da (Wallace &Selman, 1981 eta 1985; Brooks et al., 1997; Patino & Sllivan, 2002; Mommsen & Koorsgaard, 2008). Gaur egun, ama-mRNA-k (Bouleau et al., 2014) eta oogenesian zehar oozitoetan pilatutako

molekulek (molekula antioxidatzaileak, gantz-azidoen edukia, mitokondrioen DNA-ren apurketa, transkripto espezifikoak) arrautza-kalitatean duten garrantzia azter daiteke (Tata, 1986; Howley & Ho 2000; Pelegri, 2003; Parma et al., 2015; Schaefer et al., 2016; Sullivan et al., 2016). Ernalketa ostean, ama-faktore horiek zigotoaren transkripzioa aktibatu bitartean enbrioia mantentzen dute eta beraz enbriogenesi goiztiarrean paper garrantzitsua jokatzen dute. Faktore hauei dagokienez, oogenesian zehar duten kokalekua eta pilaketaren araberako funtzioak betetzen dituztela ikusi da (Bally-Cuif et al., 1998; Howley & Ho; 2000; Bouleau et al., 2014) eta kantitate ezberdinean agertzen dira kalitate ezberdineko oozitoetan (Aegerter et al., 2005; Bonnet et al., 2007b).

Horrez gain, oogenesian zehar oozitoek hainbat aldaketa morfologiko, biokimiko eta molekularrak pairatzen dituztela kontsideratuz, obulazioan askatzen oozito helduen kalitatea aldaketa guzti hauen menpean dago (Lubzens et al., 2010; Chapman et al., 2014). Ezaguna da, aldaketa guzti hauen ondorioz transkripzio-profila oogenesian zehar aldatu egiten dela eta fase ezberdinetako oozitoetan gene ezberdinak adierazten direla (Lanes et al., 2013). Gauzak horrela, gene ezberdinen transkribapen-mailak arrautzen kalitatea aztertzeko baliabidea izan daiteke (Bonnet et al., 2007b; Bobe eta Labbé, 2010 ; Mommens et al., 2014; Chapman et al., 2014). Gure ikerketa taldeak 5S rRNA oozitoen agerpenaren markatzaile molekularra dela frogatu izan du (Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2016). RNA erribosomiko hau arrainen oogenesiaren hasierako faseetan era masiboan nukleoan ekoiztu eta zitoplasman pilatzen hasten dela ikusi da. Gerora, bitelogenesian, nukleoloan sintetizatuko diren bestelako RNA erribosomikoekin eta proteinekin batera erribosomak osatzen ditu, enbrioi berrian sintetizatu beharko diren proteina berrien ekoizpenerako erabiliko direlarik (Szymaski et al., 2003). 5S rRNA beraz, arrautza-proteinak sintetizatzeko eta beraz enbrioi osasuntsu eta bideragarri bat lortzeko ezinbesteko molekula gakoa dela susma daiteke. Lan honetan, 5S rRNA-ak kalitate altuko arrautzak identifikatzeko markatzaile molekular posible gisa aztertuko da. Horretarako, akuikulturan garrantzitsuak direneko bi arrain-espezieekin ugalketa-saioak egin dira: erreboiloa (*Scophthalmus maximus*) eta lupia (*Dicentrarchus labrax*).

MATERIALA ETA METODOAK

Erreboiloa (*Scophthalmus maximus*) (1. Irudia):

Eme-ugaltzaileen zaintza, arrautzen ernalketa eta larben garapena

Lan honetarako erabili diren 10 erreboilo (*Scophthalmus maximus*) emeak "Galician cluster of aquaculture"-k duen haztegiko eme-ugaltzaileen artean aukeratuak izan ziren (CETGA, Aguiño, Galizia). Emeak 3-6 urte bitartekoak ziren eta 4 kg-tik 10 kg-rako pisua zeukaten. Astean behin edo bitan masaje abdominala egin zitzaien erruterako prest zeuden jakiteko, haztegiko prozedura normala jarraituz eta beraien jarduera komertzialen baitan. Behin errutea burututa, eme bakoitzaren arrautza guztiak 6 urte eta 6.5 kg-tako ar bakarraren espermarekin ernaldu ziren. Ernalketa ostean arrautzen flotagarritasuna aztertu zen. Arrautza hondoratuak zenbatu eta arrautza txar moduan sailkatu ziren. Flotatzeko ahalmena zutenekoak aldiz (arrautza onak), eklosiorako inkubatzen jarri ziren (1. Taula). Eklosio ostean larbak larbarioan inkubatu ziren (batezbesteko tenperatura 18°C) 20-40 dph (edo errute osteko egunak, ingelesezko days post hatching) egunerarte. 40. egunetik aurrera eta hazkuntza osoan zehar larbariotik atera eta beste tanke batean hazi ziren larbak (batezbesteko tenperatura 15°C). 82-84. egunean jubenilak pisatu eta sailkatu ziren, salmenta mugatzen duteneko faktore morfologikoak kontuan hartuta (1. Taula).

1. Taula. Erreboiloaren errute eta enbrioi eta larben hazkuntzan zehar neurtu eta kalkulatutako parametroak.

Arrain-kopurua	Kategoria bakoitzari dagokion arrain-kopuru totala
Amaiera	Kategoria bakoitzean dagoen arrain-kopuru totala/amaierako arrain-kopuru totala*100
Hasiera	Kategoria bakoitzean dagoen arrain-kopuru totala/hasierako larva-kopurua*100
Arrautza-kopuru totala	Errundako arrautza guztiak (1 mL-ko)
Arrautza onak (kopurua)	Flotatzeko ahalmena zuteneko arrautzak
Hasierako larba-kopurua	Eklosio ostean hazkuntzarako aukeratutako larba-kopurua
Biziraupena %	Amaierako arrain biziak/Hasierako larvb-kopurua *100

1. Taula (jarraipena).

Saltzeko onak	Saltzeko onak (arrain-kopurua)	Gaizki migratuak	Gaizki migratuak (Arrain-kopurua)
	Saltzeko onak % (Amaiera)		Gaizki migratuak % (Amaiera)
	Saltzeko onak % (Hasiera)		Gaizki migratuak % (Hasiera)
Txikiak onak	Txikiak onak (arrain-kopurua)	Totala	Arrain-kopuru totala
	Txikiak onak % (Amaiera)		Totala % (Amaiera)
	Txikiak onak % (Hasiera)		Totala % (Hasiera)
Albinoak	Albinoak (arrain-kopurua)	Jubenilen pisua	Batezbesteko pisua
	Albinoak % (Amaiera)		Desbiderazioa
	Albinoak % (Hasiera)	Masaje abdominala	Arrautza-kopuru totala
Albinoak + Malformatuak	Albino eta malformatuak (Arrain-kopurua)		Arrautza onak (kopurua)
	Albino eta malformatuak % (Amaiera)		Eklosionatutako larbak
	Albino eta malformatuak % (Hasiera)	Arrautzak eta larbak	Arrautza onak %
Malformatuak	Malformatuak (Arrain-kopurua)		Eklosionatutako labak/arrautza onak %
	Malformatuak % (Amaiera)		Eklosionatutako larbak/arrautza totalak %
	Malformatuak % (Hasiera)		Biziraupena/arrautza totalak %
Punkiak (begi gaizki migratuak)	Punkiak (Arrain-kopurua)		Hasierako larba-kopurua
	Punkiak % (Amaiera)		Biziraupena %
	Punkiak % (Hasiera)		

RNA, DNA eta proteinen erauzketa, 5S/18S rRNA indizea

RNA totala, DNA eta proteina TRIzol-aren (Life Technologie Carslabad, California, AEB) metodo organikoa erabiliz eta hornitzailearen argibideak jarraituz erauzi zen. Erauzitako RNA 90 µL RNasa/DNasa-rik gabeko uretan disolbatu zen. RNA-kontzentrazioa eta -kalitatea espektrofotometrikoki (Biophotometer, Eppendorf, Hamburgo, Alemania) zehaztu ziren. Kalitate ona 260 nm/280 nm=1.80-2.0 eta 260 nm/230 nm=2.0-2.2 inguruko baliotara mugatu zen. DNA-kantitatea eta -kalitatea ere espektrofotometrikoki neurtu ziren (260 nm/280 nm=1.6-1.8). RNA kantitatea eta kalitatea neurtzeko ere 2100 Bioanalyzer (Agilent Technologies, Santa Clara, AEB) erabili zen. 5S/18S rRNA indizea kalkulatu zen Bioanalyzerraren elektroferogrametan 5S rRNA eta 18S rRNA zituzteneko azalerak neurtuz eta Rojo-Bartolomé et al. (2016) zehazten den prozedura jarraituz.

Proteinen erauzketaren kasuan disolbagarritasuna handitzeko %1 SDS eta urea soluzioan mantendu ziren. Erauzi eta jarraian Lowry et al. (1951) metodologian oinarrituz lagin bakoitzaren proteina kantitatea neurtu zen Bio-Rad DC Protein Assay Kit komertsiala erabilliz (Bio-Rad, Hercules, California, AEB) eta hornitzailearen argibideak jarraituta. DNA eta proteinen datuak honako ratioak kalkulatzeko erabili ziren: 5S rRNA/DNA, Proteina/DNA eta 5S rRNA/Proteina.

Lupia (*Dicentrarchus labrax*) (1. Irudia):

Eme ugaltzaileen zaintza, arrautzen ernalketa eta larben garapena

Lan honetarako 3 kg inguruko 16 lupi (D*icentrarchus labrax*) eme IFREMER haztegian (Palavas-Les-Fots, Frantzia) erruterako prestatu ziren. Lehenengo eta behin oozito post-bitelogenikoak zituzteneko emeak aukeratu ziren. Garapen-fasea identifikatu ahal izateko, oozitoak kateter baten laguntzaz eskuratu ziren. Behin egokiak zireneko emeak aukeratuta, hormona luteinizatzailearen hormona askatzailearen analogoarekin edo LHRHa-rekin (ingelesezko Luteining Hormone Releasing Homorne analog) (Sigma-Aldrich, St Luis, Misuri, AEB) tratatu ziren 10 µg/kg dosian, obulazioa bultzatzeko.

Obulazioaren ostean (3 egunetako hormona-tratamenduaren ostean), arrautzak masaje abdominalaren bidez eskuratu ziren. Eme bakoitzaren arrautza-lagin bat nitrogeno likidoan sartu eta -80°C-tara izoztuta mantendu zen prozedura molekularrekin hasi arte. Bideragarritasunean aitaren eragin posibleak ekiditeko (Saillant et al., 2001), eme bakoitzaren gainontzeko arrautzak 20 arren esperma-nahasketarekin ernaldu ziren, $20*10^5$ espermatozoide/arrautzako proportzioan (Fauvel et al., 1993). Ernalketa itsas ura aktibazio medio moduan erabiliz burutu zen.

Ernalketa ostean 2.5 mL-ko arrautza-bolumena 14°C-tara inkubatu zen 3 orduz (3 hdf, ingelesezko hours post fertilization) inkubatu ziren. 3hdf-an garatzen ari diren enbrioiak 4 zelulatako fasea aurkezten dute eta garatzen ari ez direnen artean ondo bereiz daitezke. Orduan ernaltze-arrakasta kalkulatu zen (2. Taula). %65-ko ernaltze-arrakasta muga moduan jarrita, kalitate baxua eta altuko arrautza-taldeak bereiztu ziren. Ernalketa osteko 3 dpf (ingelesezko days post fertilization) eta 4 dpf-tan garapen-arrakasta eta

eklosioa (5 dpf) kalkulatzeko, arrautza talde bakoitzetik 72 banaka inkubatu ziren Oyen eta kideek proposatutako metodologia jarraituta (1997) (2. Taula).

2. Taula. Lupiaren errute, ernalketa eta enbrioien hazkuntzan zehar neurtu eta kalkulatutako parametroak.

Emea	Emearen adina (hilabetetan)
	Emearen pisua
	Emearen garapen fasea
Indukzioa	Indukzio ur tenperatura
	Indukzio gazitasuna
	Indukzio baldintzak CxH
Errutea	Errundako bolumena (ml)
	Bideragarritasuna %
	Bideragarritasuna% (umeldura)
	Oozito-kopurua (500 µl)
	Obarioaren osmolaritatea
	Obarioaren pH
Ernalketa	Uraren pH
	Obario eta uraren pH (ernalketan)
	Obario eta uraren pH (3minutu)
	Uraren gazitasuna
	Ernalketa-arrakasta % (3hpf)
Inkubazioa	Inkubazio uraren tenperatura
	Inkubazio uraren gazitasuna
Enbrioaia	Biziraupena % (4hpf)
	1 lipido tanta (%)
	2 lipido tanta (%)
	3 lipido tanta (%)
	>3 lipido tanta (%)
	Biziraupena % (4 dpf)
	Flotazio % (4 dpf)
	Hondoraketa %(4 dpf)
	Eklosio-arrakasta %
Larba	Jubenilen luzera (0 dph)
	Jubenilen luzera (7 dph)
	Jubenilen luzera (12 dph)
	Igeriketa puxika (12 dph)

RNA erauzketa, 5S/18S rRNA indizea

RNA erauzketa Tri-reagent erabiliz burutu zen (Sigma-Aldrich). Laginen purutasuna ziurtatzeko Nucleospin RNA II kit komertziala erabiliz purifikatu ziren aurretik Bonnet eta

lagunek amuarrainean erabilitako protokoloa jarraituz (2007a). RNA kantitatea eta kalitatea neurtzeko NanoDrop NP-1000 espektrofotometroa (NanoDrop technologies, Wilmington, Delawere, AEB) eta 2100 Bioanalyzer (Agilent Technologies, Santa Clara, AEB) erabili ziren. 5S/18S rRNA indizea kalkulatu zen Bioanalyzerraren elektroferogrametan aurretik erreboilorako azaldu den prozedura berdina jarraituz.

cDNA-ren sintesia eta qPCR bidezko *gtf3a* genearen transkripzio-mailen neurketa

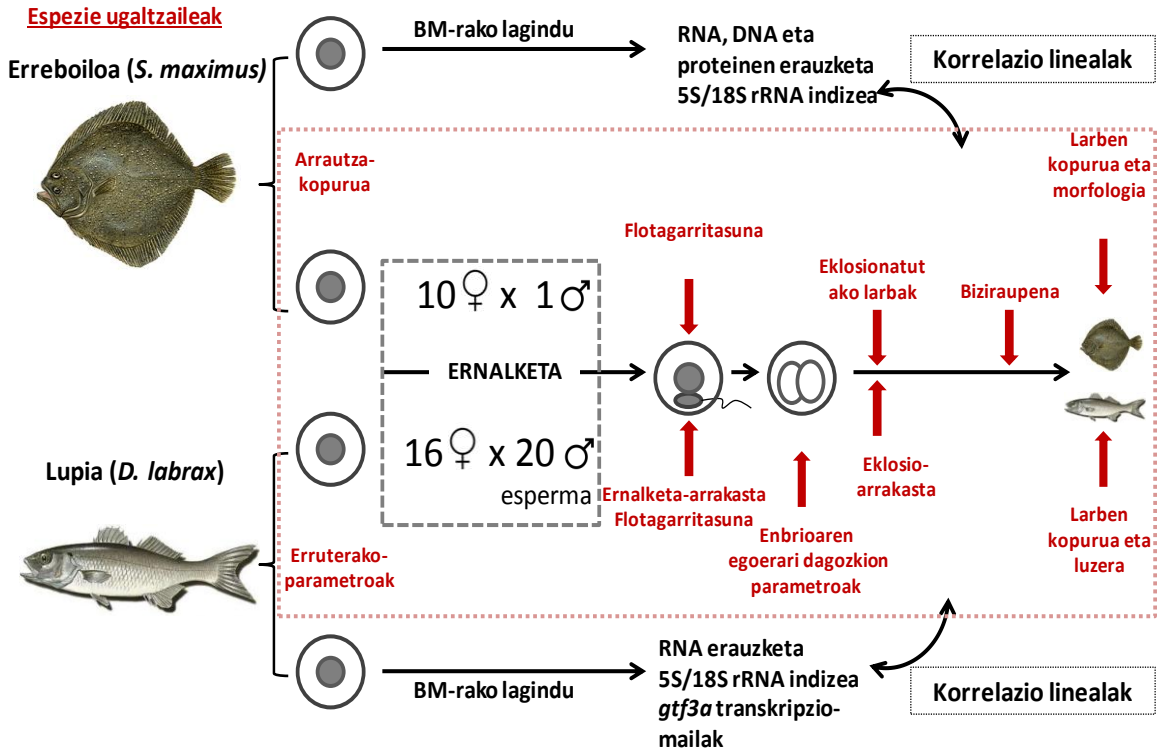
2 µg RNA totaletik hasita cDNA Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, Massachusetts, AEB) erabiliz eta hornitzailearen argibideak jarraituz ekoiztu zen. *gtf3a* genearen transkripzio-maila qPCR bidez kuantifikatu zen, horretarako *gtf3a* sekuentzia NCBI-ko (National Center for Biotechnology Information) GenBank datu basetik eskuratu zen (KC191720). Hasleen sekuentziak: zuzena edo Fw, 5'-TCAGCTCACCAGACATGAGC-3', eta alderantzizkoa edo Rv, 5'-GACAAAGGCCTCAGAGCATC-3'.

gtf3a eta erreferentzia geneen qPCR-ak Go Taq master mixa (Promega, Madison, Wisconsin, AEB) erabiliz egin ziren. 4 µL cDNA lagin kargatu ziren erreakzioaren 10 µL bolumen totalean 5 µg/µL-tara diluituak. Hasleak 1500 pmol/L-tara diluitu erabili ziren eta qPCR-ak Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, California, AEB) termozikladorean burutu ziren baldintzak honakoak zirelarik: 2 minutu 95°C-tan, jarraian 40 ziklotako desnaturalizazioa, 15 segundo 95°C-tan, eta lotura fasea 1 minutu 60°C-tan. Azkenik disoziazio-pausu bat gehitu zen prozesuaren espezifikotasuna aztertzeko. *gtf3a*-ren adierazpen erlatiboa Julien Bobe-n taldeak (INRA Laboratoire de Physiologie et Génomique des Poisson-LPGP, Rennes, Frantzia) neurturiko 6 erreferentzia geneen batezbesteko geometrikoarekin zuzendu zen. Gene hauek beste lan baten baitan arrautzen kalitatea aztertzeko buruturiko "*microarray*"-an neurtuak izan ziren, eta lagin berdinetan adierazpen konstantea izateagatik aukeratuak izan ziren (Bobe et al., argitaratugabea).

Korrelazio linealak eta estatistika erreboilo eta lupi arrautzetan

Bi espezietan lorturiko emaitzak SPSS.22 (SPSS Inc., Microsoft Co.) erabiliz aztertu ziren. Taldeen arteko ezberdintasun estatistikoak ezartzeko Mann-Whitney testa erabili zen. Desberdintasun esangarriak $p<0.05$-an ezarri ziren. Emaitzak aurkezteko kutxa diagramak aukeratu ziren, non kutxa bakoitzak 25 eta 75 pertzentilen arteko datuak, erdiko marrak mediana eta goi eta beheko marrek maximo eta minimoak adierazten dituzten.

Horrez gain, parametro molekularrak eta ugalketa saioan zehar neurturiko parametroen artean erlazioak bilatu nahian korrelazio linealak burutu ziren. Horretarako Spearman korrelazio unilateral ez parametrikoa erabili zen. Desberdintasun esangarriak $p<0.01$ (**) eta $p<0.05$ (*) ezarri ziren.



1. Irudia: Jarraitutako materiala eta metodoen eskema laburtua. Gorriz aztertutako arrautza-kalitatearekin zerikusia izan dezaketen parametro tradizionalak. BM: Biologia molekularra.

EMAITZAK

Markatzaile molekularren eta garapen-faktoreen arteko erlazioak: erreboiloen arrautzen kasu praktikoa

Neurtutako oozitoen 5S rRNA-mailak, 18S rRNA-mailak eta 5S/18S rRNA indizea, zein bestelako parametro molekularrak (DNA, proteinak eta eurekin sortutako ratioak) enbrioi eta jubenilen garapenean zehar neurtutako parametroekin alderatu ziren (3. Taula).

5S rRNA-mailek erlazio positiboa aurkeztu zuten honako parametroekin: arrautza onak %, eklosionatutako larbak/arrautza totalak %, 5S/18S rRNA ratioa, 18S rRNA-maila, 5S rRNA/DNA eta 5S rRNA/proteina. 18S rRNA-mailek aldiz, 5S rRNA-z gain erlazio positiboa aurkeztu zuen arrautza onak %-rekin eta 5S rRNA/proteinarekin, aldiz negatiboki erlazionatuta agertu zen proteina/DNArekin.

Bi rRNA-n arteko ratioak 5S rRNA-rekin, arrautza onak %-rekin, eklosionatutako larbak/ arrautza onak %-rekin, eklosionatutako larbak/arrautza totalak %-rekin, 5S rRNA/DNA eta 5S rRNA/proteina ratioekin erlazio positiboak aurkeztu zituen. RNA totalak eta 5S rRNA/DNA ratioa positiboki erlazionatuta agertu ziren. 5S rRNA/DNA-ak, RNA totalaz gain, 5S rRNA, 5S/18S rRNA eta 5S rRNA/proteina-rekin erlazionatuta zegoen ere. DNA kantitatea halaber, 5S rRNA-rekin, 5S/18S rRNA-rekin, biziraupena/arrautza totalak %-rekin eta 5S rRNA/proteina ratioarekin positiboki erlazionatuta agertu zen. Proteina kantitateak erlazio positiboak erakutzi zituen biziraupena %, saltzeko onak (hasiera), albinoak (hasiera), albino eta malformatu (amaiera), punkiak (hasiera), arrainen kopuru totala hasiera eta proteina/DNA-rekin. Aldiz, proteina/DNA-k saltzeko onak (amaiera) eta punkiak (hasiera)-kin positiboki erlazionatuta agertzeaz gain, negatiboki erlazionatuta zegoen 18S rRNA-mailarekin eta 5S rRNA/proteinarekin. Azkenik 5S rRNA/proteina kantitatea aurretik esan bezala proteina/DNA-rekin negatiboki eta 5S rRNA-mailak, 18S rRNA-maila, 5S/18S rRNA eta 5S rRNA/DNA-rekin positiboki erlazionatuta egoteaz gain, arrautza onak %-arekin, eklosionatutako larbak/arrautza totalak %-rekin ere positiboki erlazionatuta zegoen.

3. Taula: Erreboilo arrautzetan eginiko Spearman korrelazioen emaitza esangarrien taula. (*)-ak p<0.05 eta (**)-k p<0.01 esangarritasun estatistikoa adierazten dute.

S. maximus	5S rRNA	18S rRNA	5S/18S rRNA	Biziraupena %	Saltzeko onak % (Amaiera)	Saltzeko onak % (Hasiera)	Albinoak% (Hasiera)	Albino eta malformatuak % (Amaiera)	Punkiak % (Hasiera)	Totala % (Hasiera)
5S rRNA	1	**.762***	**.905****	-.119	-.024	.143	-.214	-.013	.071	-.119
18S rRNA	**.762***	1	.571	-.524	.524	-.048	-.524	-.457	-.500	-.524
5S/18S rRNA	**.905****	.571	1	-.095	-.333	.048	-.310	.038	.214	-.095
Proteina	-.190	-.595	-.095	**.842****	-.527	**.697***	**.758****	**.575***	**.673***	.842**
DNA	.350	.167	.119	.406	.055	.394	.273	.175	.127	.406
RNA	.357	.048	.476	.055	-.273	.079	.006	.356	.091	.055
5S rRNA/ DNA	**.881****	.619	**.976****	-.237	.140	-.122	-.255	.000	-.109	-.237
Proteina/DNA	-.405	**-.738***	-.167	.479	**-.600***	.297	.479	.459	**.564***	.0479
5S rRNA/ Proteina	**.929****	**.833****	**.810****	-.381	.143	-.095	-.476	-.228	-.167	-.381

3. Taula (jarraipena): Erreboilo arrautzetan eginiko Spearman korrelazioen emaitza esangarrien taula. (*)-ak p<0.05 eta (**)-k p<0.01 esangarritasun estatistikoa adierazten dute.

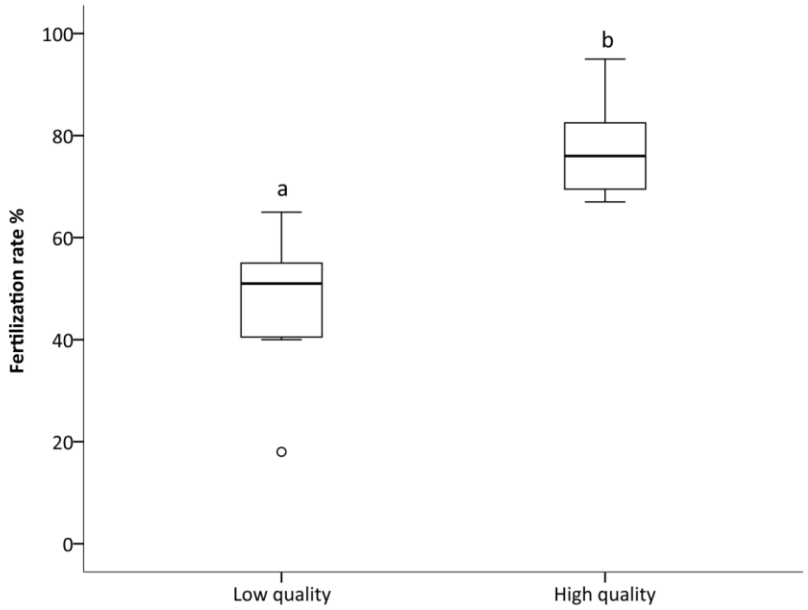
S. maximus	Arrautza onak %	Eklosionatutako labak/arrautza onak %	Eklosionatutako larbak/arrautza totalak %	Biziraupena/arrautza totalak %	Proteina	RNA	5S rRNA/ DNA	Proteina/DNA	5S rRNA/ Proteina
5S rRNA	**.802****	.619	**.881****	.500	-.190	.357	**.881****	-.405	,929**
18S rRNA	**.695***	.095	.548	.024	-.595	.048	.619	**-.738***	,833**
5S/18S rRNA	**.683***	**.762***	**.810****	.500	-.095	.476	.976**	-.167	,810**
Proteina	-.383	-.024	-.176	.333	1	.418	-.134	**,806****	-.452
DNA	.274	.073	.273	**.552***	.079	-.212	-.176	-.503	.452
RNA	.292	.067	.067	-.103	.418	1	**.559***	.467	.190
5S rRNA/ DNA	.457	.381	.365	-.061	-.134	**.559***	1	-.043	,762*
Proteina/ DNA	-.492	-.018	-.297	-.006	**.806****	.467	-.043	1	-,667*
5S rRNA/ Proteina	**.802****	.500	**.810****	.357	-.452	.190	**.762***	**-.667***	1

Lupien arrautza-kalitatea: ernalketa-arrakasta

3 hdf ostean neurtutako ernalketa-arrakasta irispide moduan erabilita eme guztien arrautzak bi arrautza-taldetan banatu ziren: “kalitate baxuko” taldearen ernalketa-arrakasta %18-tik %65-ra zihoan bitartean, kalitate altuko arrautza-taldeak %67-tik %95-rako ernalketa-arrakasta erakutsi zuen (4. Taula). Ernalketa-arrakasta talde bien arteko banaketa-irizpide moduan bi taldeen artean sortarazten zen ezberdintasun estatistikoan oinarritu zen (2. Irudia).

4. Taula: Lupien arrautzen taldekatzea ernalketa-arrakastaren (%) arabera.

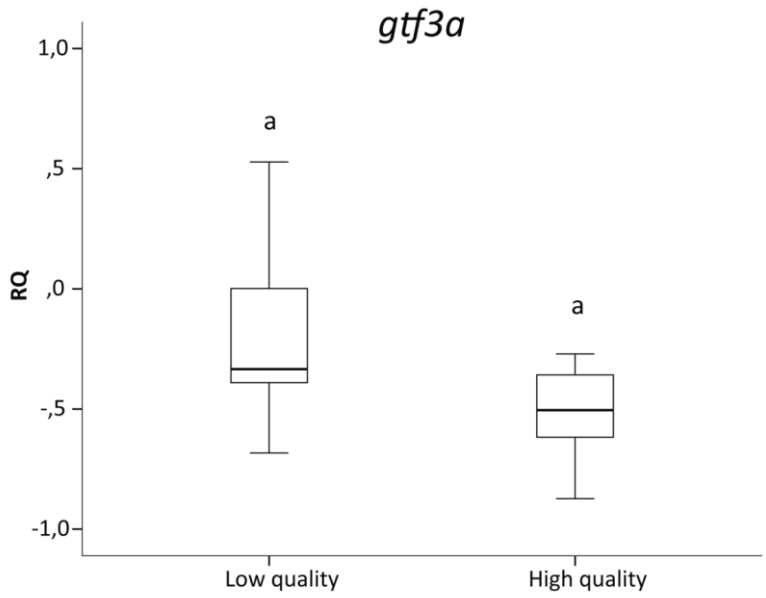
	Ernalketa-arrakastak (%)		Ernalketa-arrakastak (%)
Low quakity (kalitate baxua)	18	High quality (kalitate altua)	67
	40		68
	41		71
	44		71
	44		76
	51		76
	52		89
	65		95



2. Irudia: Kalitate baxu eta altuko arrautzen ernalketa-arrakasta lupietan. Ardatz bertikalean ernalketa-arrakasta %-tan adierazita (ingelesezko Fertilization rate %). Ardatz horizontalean, kalitate baxuko (ingelesez, Low quality) eta kalitate altuko arrautzak (ingelesezko High quality). Letra desberdinek desberdintasun estatistiko esanguratsuak adierazten dituzte talde bien artean ($p<0.05$).

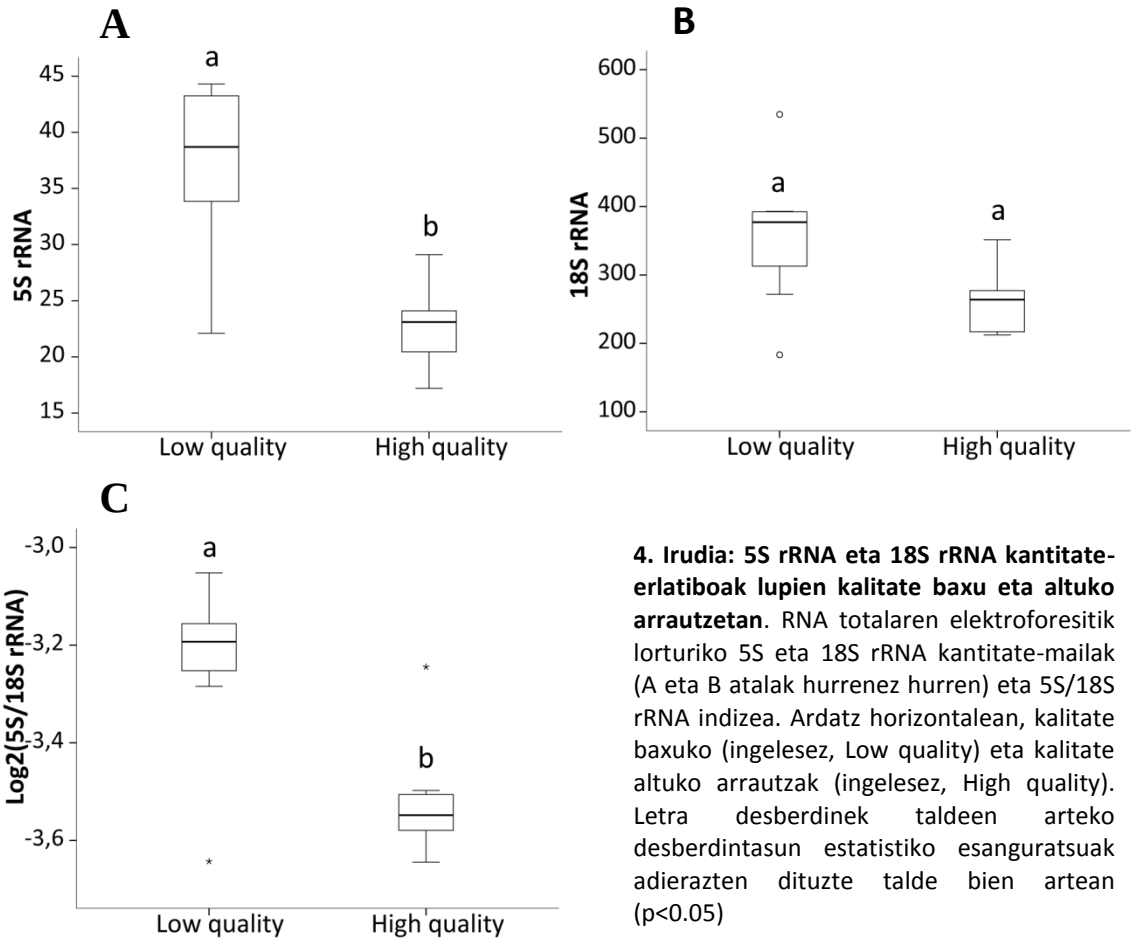
Oozitoen markatzaile molekularrak eta arrautzen kalitatea lupietan

Ernalketa-arrakastan oinarritutako taldekatzea mantenduz *gtf3a*-ren transkripzio-mailak aztertu ziren (3. Irudia). Ez zen desberdintasun esanguratsurik ikusi bi taldeen artean eta orokorrean bi taldeek *gtf3a* genearen transkripzio-maila baxuak aurkeztu zituzten.



3. Irudia: *gtf3a*-ren transkripzio-maila erlatiboak lupien kalitate baxu eta altuko arrautzetan. RQ: kantitate erlatiboa (ingelesezko relative quantification). Ardatz horizontalean, kalitate baxuko (ingelesez, Low quality) eta kalitate altuko arrautzak (ingelesez, High quality). Letrek desberdintasun estatistiko esanguratsurik ez zegoela adierazten dute ($p<0.05$).

RNA erribosomikoei zegokienez, 18S rRNA-k (4B. Irudia) ez zuen taldeen arteko ezberdintasunik erakutsi. 5S rRNA-k (4A. Irudia) eta bi RNA-mailen artean sorturiko 5S/18S rRNA indizeak (4C. Irudia) balio esanguratsuki altuagoak aurkeztu zituen kalitate baxuko arrautzen taldean.



4. Irudia: 5S rRNA eta 18S rRNA kantitate-erlatiboak lupien kalitate baxu eta altuko arrautzetan. RNA totalaren elektroforesitik lorturiko 5S eta 18S rRNA kantitate-mailak (A eta B atalak hurrenez hurren) eta 5S/18S rRNA indizea. Ardatz horizontalean, kalitate baxuko (ingelesez, Low quality) eta kalitate altuko arrautzak (ingelesez, High quality). Letra desberdinek taldeen arteko desberdintasun estatistiko esanguratsuak adierazten dituzte talde bien artean ($p<0.05$)

Markatzaile molekularren eta garapen-faktoreen arteko erlazioak: lupien arrautzen kasu praktikoa

Ernalketa-arrakastan oinarritutako arrautzen sailkapenak bi talde bereizgarri sortu zituen arren, eta nahiz eta sortutako bi taldeak estatistikoki desberdinak izan, bi taldeen arteko muga arbitrarioki ezarri zeni. Hori dela eta, laginak taldekatu gabe aztertzea erabaki zen. Horretarako, eta erreboiloan egindakoa jarraituz, neurtutako parametro guztien arteko erlazioak korrelazio linealen bidez aztertu ziren (5. Taula).

5. Taula: Lupi arrautzetan eginiko Spearman korrelazioen emaitza esangarrien taula. (*)-ak p<0.05 eta (**)-k p<0.01 esangarritasun estatistikoa adierazten dute.

D. labrax	Emearen pisua	Indukzio uraren tenperatura	Indukzio gazitasuna	Indukzio baldintzak CxH	Bideragarritasuna %	Bideragarritasuna % (umeldura)	Obarioaren osmolaritatea	Ur pH
5S/18S rRNA	.291	.198	-.198	.332	**-.729****	**.729****	-.077	-.198
5S rRNA	**.479***	**.602****	**-.469***	**.674****	**-.521***	**.521***	**-.514***	-,712**
18S rRNA	.379	**.563***	**-.478***	**.641****	-.282	.282	**-.556***	-,704**
gtf3a	-.130	-.059	.083	.010	-.421	.421	.063	.037

D. labrax	Obarioaren pH	Obario eta ur pH (ernalketan)	Obario eta ur pH (3 min)	Ur gazitasuna	Ernalketa-arrakasta % (3 hpf)	3 lipido tanta	Biziraupena % (4 dpf)	Inkubazio ur tenperatura
5S/18S rRNA	**-.721****	**-.461***	**-.618****	-.184	**-.591****	**-.445***	**-.669****	.056
5S rRNA	-.285	-.062	-.115	**-.551***	**-.741****	-.299	-.309	**.572***
18S rRNA	.074	.278	.260	**-.534***	**-.538***	-.174	-.163	**.484***
gtf3a	-.387	-,517*	-,504*	.090	-.400	-.352	-.183	-.047

D. labrax	Flotazio (4 dpf)	Hondoraketa (4 dpf)	Jubenilen luzera (0 dph)	Jubenilen luzera (7 dph)	Jubenilen luzera (12 dph)	Igeriketa puxika (12 dph)	5S rRNA	18S rRNA
5S/18S rRNA	**-.712****	**.712****	**-.529***	-.239	-.357	-.350	.415	.079
5S rRNA	-.365	.365	**-.480***	**-.527***	**-.471***	**-.617****	1	**.912****
18S rRNA	-.038	.038	**-.461***	**-.600****	**-.440***	**-.590****	**.912****	1
gtf3a	**-.431***	**.431***	.129	.246	.163	.233	0	-.238

Kasu honetan 5S rRNA emeen pisua, indukzio ur tenperatura, indukzio baldintzak (CxH), umeldura %, inkubaziorako erabilitako uraren tenperatura eta 18S rRNA-mailarekin positiboki erlazionatuta agertu zen. Aldiz indukzio uraren gazitasuna, bideragarritasuna %, obarioaren osmolaritatea, uraren pHa eta gazitasuna, ernalketa-arrakasta %, jubenilen luzera (0, 7, 12 dph) eta igeriketa puxikaren agerpenarekin negatiboki erlazionatuta agertu zen. 18S rRNA-mailek bere aldetik ere, 5S rRNA-mailak, indukzio uraren tenperatura, indukzio baldintzak (CxH) eta indukzio tenperaturarekin positiboki erlazionatuta zegoen eta negatiboki ur gazitasunarekin, jubenilen luzerarekin (0, 7, 12 dph) era igeriketa-puxikaren agerpenarekin (12 dph).

Bi rRNA erribosomikoak konbinatzean 5S/18S rRNA-ratioa negatiboki erlazionatuta agertu zen, bideragarritasun %-rekin eta beraz positiboki umeldura %-rekin, negatiboki obarioaren pH-rekin eta obario zein uraren pH-rekin ernalketa garaian zein 3 minutu ostean, ernalketa-arrakasta %-rekin, 3 lipido tanta aurkezten zituzten arrautzen kopuruarekin, biziraupenarekin (4 dpf) eta flotazio %-rekin (beraz kontrakoa den hondoraketa %-rekin positiboki erlazionatuta zegoen). *gtf3a* transkripto-mailek ere obario eta ernalketa garaiko eta 3 minutu osteko uraren pH-aren balioekin, zein flotazioarekin (hondoratzearen aurkakoa) erlazio negatiboak aurkeztu zituen.

EZTABAIDA

Ikerketa lan honetan akuikulturan oso garrantzitsuak diren erreboilo eta lupien ugalketa eta hazkuntzan zehar neurtutako parametro eta arrautza-kalitatearen markatzaileak izan zitezkeen molekulen kantitateen artean korrelazio linealak ezarri dira. Horrez gain, lupiaren kasuan, ernalketa-arrakasta irizpide moduan erabilita, arrautzak bi talde desberdinetan sailkatu ziren, kalitate altuko eta baxuko arrautza-taldeak hain zuzen ere eta arrautza horietan neurtutako *gtf3a*, 5S rRNA, 18S rRNA transkripto-mailen eta 5S/18S rRNA indizearen balioak taldekatze horren arabera aztertu ziren. Azken honi dagokionez, 5S rRNA-mailen eta 5S/18S rRNA indizearen kasuan, bi kalitate desberdineko taldeen arteko ezberdintasun esangarriak ikusi ziren.

Neurtutako parametro desberdinen arteko korrelazio linealei dagokienez, bai erreboiloen zein lupien oozitoen 5S rRNA eta 18S rRNA-mailek era positiboan estuki

erlazionatuta daudela ikusi da. Honek sentsua dauka bi RNA-k erribosomen parte baitira eta erribosoma bakoitzak molekula horietako bakarra dauka. Nahiz eta 5S rRNA oogenesi hasieran ekoiztu eta oozitoaren zitoplasman pilatu, bitelogenesiaren hasierarekin 18S rRNA ekoizten hasten da amaieran , oozitoa ernalduan, erribosomak ekoiztu ahal izateko sintesi berririk behar eman gabe (Rojo-Bartolomé et al., 2016).

5S rRNA-mailari eta 5S/18S rRNA indizeari dagokienez, erreboiloan erlazio positiboa aurkezten dute arrautza-kalitatea onarekin bat datozen parametro tradizionalekin, hala nola, arrautza onen kopuruarekin, hau da, arrautzen flotagarritasunarekin edota eklosio-arrakastarekin (errundako arrautza totaletik abiatuta zein flotagarriak ziren arrautza onetatik abiatuta ere). Bestalde, lupien arrautzetan lortutako emaitzen arabera ere, 5S rRNA-mailak eta 5S/18S rRNA indizeak kalitate ona islatzen duten parametro tradizionalekin negatiboki erlazionatuta agertzen dira, besteak beste, arrautzen flotagarritasuna eta bideragarritasunarekin, obarioaren pH-rekin, ernalketa-arrakastarekin, enbrioien biziraupenarekin eta jubenilen hazkuntzarekin.

Beraz, bi espezieekin egindako ugalketa saioek kontrako emaitzak eman dituzte. Kontrako emaitza hauentzako arrazoi metodologiko posible ugari daude. Hasteko, erreboiloarekin egindako ugalketa saioan arrautzak lagintzerako orduan, ez zen arrautza-kopurua kontuan hartu eta nahiz eta 5S rRNA/DNA ratioak 5S rRNA-ren mailekin erlazio positiboa eduki, eta beraz, RNA erauzketarako zelula-kopuru berdina erabili izan dela iradoki, ezinezkoa da hau benetan jakitea. Gainera, masajearen bidez oozitoan eskuratzen direnean gertatu oi den bezala, laginetan obario-fluidoa eta odola ere bazegoen. Hauek, RNAlater-arekin batera, oozitoetatik banaezina zen zuntz-matrize sortzen zuten zeina ezinbestean RNA erauzketarako hartu egin zen. Oozitoaz aparte bestelako zeluletatik RNA erauztea, RNA horren jatorri zelularra hein batean ezezaguna izatea dakar. Ugalketa-saioan zehar eginiko neurketei dagokienez, arrautza-kalitatearekin erlazio estua duten parametro garrantzitsuak falta dira, hala nola, obario fluidoaren pH, zeina umeldura estimatzeko erabili daitekeen (Fauvel et al., 1993) eta erreboilo arrautzetan kalitatea eta ernalketa arrakastarekin erlazionaturik dagoela ikusi den (Aegerter & Jaleber, 2004), tenperatura edo gazitasuna. Horrez gain, ez ziren kontuan hartu ernalketa-arrakasta (lupietan parametro zentraltzat hartu dena) eta jubenilen hazkuntza eta biziraupena fase-gako ezberdinetan (begi-fasean, eklosioan eta

bitelo-sakuaren birxurgaketa fasean, metamorfosian pleuronektidoetan), nahiz eta hauek gametoen kalitatea aztertzeko garrantzia duten parametroak izan (Bobe & Labbé, 2010).

Lupiaren kasuan, lagin bakoitzetik RNA erauzketarako arrautza-kopuru berdina hartu zen, laginak euren artean konparagarriak izateko. Hala ere, erauzitako RNA birritan purifikatu egin zen, ondorioz litekeena da RNA molekula ezberdinen proportzioa aldatu izana eta euren arten 5S rRNA molekulen kantitate handi bat galdu izana ere. 5S rRNAren tamaina oso txikia da, 120 bp (Szymansky et al., 2003; Diaz de Cerio et al., 2012) eta zutabeen bitarteko erauzketa prozesuetaan, zein ondoren egindako purifikazio pausuteangaltzeko joera dago. Horrez gain, lupietan ernalketa 20 ar ezberdinen esperma-nahasketarekin egin zen (Saillant et al., 2001), jatorri desberdineko espermak erabiltzerakoan kontrolatu ezin deneko aldakortasuna gehitzen delarik. Ugal-arrakastarako bi gametoen kalitate eta ekarri somatikoa eta genetikoa dira garrantzitsuak, oozitoarena eta espermarena.

Sekuentziazio masiboak eskaintzen duen aukera aprobetxatuz asko izan dira oozitoen kalitatearekin erlazionatutako markatzaile molekularrak deskribatzen saiatu direnak. Adibidez, Bonnet eta kideek (2007b) proliferazioaren aurkako funtzioa duen *phb2* (Prohibitina b2) txaperonaren oozitoko mRNA-mailak ostadar amuarrainaren (*Oncorhynchus mykiss*) enbrioien garapenaren arrakastarekin negatiboki erlazionatuta zeudela ikusi zuten. *npm2* nukleoplasmina ere enbrioiaren garapen-arrakasta bermatzeko ezinbesteko ama-genea dela ikusi da ostadar amuarrain zein zebra arrainean (Aegerter et al., 2005; Bouleau et al., 2014). Folikuluen garapenaz arduratzen den *bmp15* (ingelesezko bone morphogenetic protein 15) eta obulazio garaian garatugabeko oozitoen askapena ekiditeaz arduratzen den *gdf9* (ingelesezko growth differentiation factor 9) ere prebitelogenesian eta errundako arrautzetan kalitatearen adierazle gisa identifikatu dira *Seriola lalandi* arrain espeziean (Palomino et al., 2014). Horiez gain, estresaren aurreko erantzuna, heriotza-zelularra, DNA-kaltea, ATP-sorrera, seinale-transdukzio eta transkripzioaren erregulazioarekin erlazionatutako miRNA-en (mikro RNA) adierazpen-mailak obulazio osteko oozitoen umelduraren eraginez eraldatu egiten direla ikusi da ere ostadar amuarrainetan (Ma et al., 2015). Chapman eta kideek

ere (2014) marradun lupian (*Morone saxatilis*) arrautzen kalitatea eta geroko enbrioien garapen-arrakasta aurresaten zuten gene-bidezidorrak identifikatu zituzten. 26S-ubikitina proteosoma, COP9 (Constitutive photomorphogenesis 9 signalosome) eta ziklo zelularraren kontrolean diharduten geneak dira antza enbrioiaren garapenaren disfuntzioekin erlazionaturik daudenak. Are gehiago, arrautza-kalitatea gene askoren menpe egon daitekeela ondorioztatu zuten, hau da, gene-multzo baten transkripzio-mailen aldaketa txikiak direla kalitatearen arduradunak eta ez gene bakar baten transkripzio-mailen aldaketak.

Gauzak horrela, bi espezieetan eta markatzaile erribosomikoekin lortutako emaitzak ez dira euren artean konparagarriak, lupian arrautzen jarraipen sakona egin den bitartean erreboiloan parametro garrantzitsu asko falta direlako eta aldiz, erreboiloan ernalketa ar bakar baten espermarekin egin den bitartean, lupian aldakortasuna emenda dezaketen ar ezberdinen esperma-nahastea erabili baita. Gainera, laginketak zein biologia molekularrerako jarraituriko pausuak gure eskutatik kanpo egon dira kasu bietan eta ez dira era estandar batean egin eta beraz lortutako 5S rRNA-, 18S rRNA-mailen eta 5S/18S rRNA indizearen balioak ezin dira espezieen artean konparatu. Beraz, lan honen egileek bereziki gomendatzen dute, arrautzen kalitatea aztertzeko esperimentu bat egiterakoan metodologia finko bat jarraitzea baliozko emaitza konparagarriak lortzeko. Besteak beste, honako hauek dira gure gomendioak baldintza esperimentalak finkatzerako garaian:

Lehenengo eta behin akuikulturan erabilia den espeziea maneiagarria aukeratzea gomendagarria da, lupia edo erreboiloa bezala, zeinetan hazkuntza-protokoloak estandarizatuta dauden, etxekotzeak gametoen kalitate galera sortu baitezake (Bobe & Labbé, 2010). Gatibutasunean ugal-arrakastan aldakortasun handia dueneko espeziea 5S rRNA oozitoen kalitatearen adierazle moduan duen balioa frogatzeko egokiena izan daitekeen arren, esan bezala hazkuntza-protokoloak estandarizatuak dituen espeziea aukeratzea da garrantzitsuena. Egokiena ugal-ziklo bakoitzean behin baino gehiagotan erruten duen espezie bat aukeratzea litzateke banako beraren gainean ugalketa-saio ezberdinak egiteko aukera ematen baitu. Horrela, baldintza egokietan hazitako emeetatik oozitoak eskuratu eta gero, animaliak estres egoerapean jarri (elikadura murriztu, ur tenperatura bortizki aldatu, bakterio batekiko esposatzea) (Schreck et al.,

2001) eta errutea bultza daiteke. Eragindako egoera desfaboragarri horretan kalitate txarragoko oozitoak jasoko dira. Banako beretik kalitate ezberdineko oozito-multzoak edukita, erruteen artean bideragarritasunaren edo molekula ezberdinen, 5S rRNA kasu, metatzeko gaitasunaren arteko konparaketak egin daitezke.

Prozedura erraztearren eta ugal-arrakastan aldakortasun handia dueneko espezie bat aukeratzen bada, zeinetan indibiduo bakoitzetik errute bakarra lortuko den, oozitoen garapen sinkronikoa duen espeziea aukera daiteke. Horrela, oozito guztiak heldutasunera batera helduko dira eta errazagoa izango da, obulazioa bultzatzea egin behar izatekotan, garapenaren une egokia aukeratzea. Gainera, multzoka erruten duen espezie sinkronikoa ere aukera daiteke, zeinetan obulazio-zikloak GnRH agonista sintetikoen (GnRHa) bidez bultza daitezkeen, besteak beste lupi zurian (*M.chrysops*), bi errute hiru egunetan eta barramundian (*Lates calcarifer*), 5 errute bi astetan, frogatu den bezala (Mylonas et al., 2010). Hala ere, ikerketaren helburuaren arabera eta batez ere, egoera optimo vs. desfaboragarriaren arteko konparaketa egin nahi bada, espezie asinkronikoak erabilgarriak izan daitezke, zebra arraina kasu, beti ere kontuan izanda obulazio garaian ahalik eta arrautza heldu gehien lortzeko tratamenduak aplikatu beharko direla, hala nola, aipatutako GnRHa-k, guruin-pituitarioaren erauzkina edo LHRHa (Alvariño et al., 1992; Zohar & Mylonas, 2001; Mylonas et al., 2010. Honekin lotuta, kontuan hartu behar da emeen obulazio naturala erabiltzean arrautzen biziraupena bultzatutako obulazioaren ondoren lortzen dena baino altuago izaten dela; lupian esaterako %75-ko eklosio-arrakasta lortzen da obulazio naturalarekin eta %50-a bultzatutako obulazioaren ostean (Fornies et al., 2001). Arrautza-oskola gardena edukitzeak ere prozedura errazten du, ernalketa arrakasta eta enbrioiaren garapenean gerta daitezkeen zatiketa zelular okerrak zein malformazioak erraz ikusiko dira horrela.

Arrautzak, espezieak ahalbidetzen badu, masaje abdominalarekin jaso behar dira (Mylonas et al., 2010). Errutean arrautzaz gain zelula somatikoak eta bestelako jarioak askatzen badira, komenigarria da urarekin arrautzak garbitzea. Horretarako nahikoa da arrautzak prezipitatu-ontzi edo Petri-plaka batean uraz garbitzea. Momentu honetan ere eta ernalketaren ostean egin beharrean, ernaldu gabeko arrautza pelagikoen kasuan, flotatzeko ahalmena duten arrautzak vs. arrautza-hondoratuak azter daitezke, *a priori*

arrautza onak (flotatzen dutenak) eta txarrak (hondoratzen direnak) bereizi ahal izateko. Beste aukera bat da luparen bidez garapenaren adierazle diren tamaina eta forma dokumentatzea. Hori egin ostean, arrautza-kopurua zenbatu behar da. Ernaldu beharreko arrautzak aukeratuta ernalketa ahalik eta azkarren ar bakar baten espermarekin burutu aukeratutako espezieari dagokion ernalketa-protokoloa jarraituz. Horrela, aitak enbrioiaren bideragarritasunean eta hazkuntzarako ahalmenean izan dezakeen eragina arbuiagarria izango da. Kontuan hartu ernalketa atzeratzeak arrautzen kalitatea gutxitu dezakeen umeldura agertzea eragin dezakeela eta beraz ernalketa-saio guztiak errun eta denbora berdinera burutu behar direla ahal den heinean.

Ahal izatekotan gameto-kopuru handia eta errute-maiztasun altua dueneko espeziea aukeratu. Espezieak ar bakar batetik esperma nahikoa lortzeko aukera eman behar du edo behintzat lortutako esperma kriopreserbatzeko aukera eman behar du (Kopeika et al., 2007), gero ernalketa-saioan eme guztien arrautzak ernaltzeko aukera emango duena. Ernalketa-arrakasta neurtu behar da, enbrioian zatiketa zelularra ematen ari den begiratuz. Horretarako adibidez, jada zatitzen ari diren enbrioiak ikusgarri direneko 4-zelulatako zatiketa fase goiztiarretik aurrera enbrioi ernalduak oozito ernaldu gabeetatik bereiz daitezke. Hazkuntza arrautzaren barnean gertatu bitartean, zelula zatiketak eta malformazioak dokumentatu egunean behin edo fase-gakoei dagozkien uneetan lupan garapenaren eboluzioa begiratuz. Geroxeago, eklosio-arrakasta eta larben tamaina aztertu garapen fase-gakoetan (Bobe & Labbé, 2010). Prozesu osoan zehar gomendagarria da animalien hilkortasun-tasa dokumentatzea, inkubazioan zehar eta garapen-fase desberdinetan hiltzen diren animaliak kontuan hartuz. Bestetik, eta saioa behin baino gehiagotan errepikatu nahi izatekotan, emaitza konparagarriak lortzeko garapen-protokolo estandarizatuaren baitan tenperatura, fotoperiodoa eta elikaduran egon zitezkeen aldaketak dokumentatu behar dira, aldaketa hauek eta sortu zitezkeen bestelako ezbeharrek emaitzetan izan ditzaketen eraginak kontuan hartu ahal izateko.

Azterketa molekularrak burutzeko, errutea gertatu eta segituan eme bakoitzetik arrautza-kopuru berdina duten hiru lagin (hiru erreplika tekniko) hartzea gomeni da erauzketa prozesuan zehar sortu zitezkeen akats teknikoak ekidin ahal izateko. Segituan arrautzak RNAlater-ik gabe -80°C-tara edo homogenizazio medioan 4°C-tara gorde. RNA arrautzen 100 mg-tik erauziko da, jakinik zenbat arrautzek osatzen duten pisu hori.

Erauzitako RNA-kopurua eta berarengandik eskuratutako cDNA arrautza bakoitzeko kopuru gisa kontsideratuko da. RNA totala erauzi ostean, laginak oso zikin badaude (DNA kutsadurarekin edo bestelako konposatu organikoekin) purifikazio-pausu bat burutu daiteke. Garrantzitsua da erabilitako purifikazio metodoa eta batez ere gehiegizko purifikaziorik ez egitea, laginetan egon daitekeen 5S rRNA, molekula txikia izanik, gal ez dadin (Masotti & Preckel, 2006). Gainontzeko 5S eta 18S rRNA-ri dagozkien prozedura molekularrak lan honetan materiala eta metodoen atalean azaldu diren moduan egin daitezke emaitza konparagarriak lortu ahal izateko arrainen arrautza-kalitatearen adierazle molekular erabilgarri bat topatzeko bidean. qPCR bidezko *gtf3a*-ren neurketa soilik oozito prebitelogenikoak eskuragarri egon daitezkeen kasuetan neurtzea merezi du. Espezie asinkroniko bat erabiltzekotan garapen ezberdinetako oozitoak banatu (Iwamatsu et al., 1976) eta prebitelogenesian daudenetan *gtf3a*-ren transkripzio-mailak neur daitezke. Horiek oozito helduetan dagoen 5S rRNA eta 18S rRNA-mailekin eta arrautzen eta enbrioien bideragarritasunarekin aldera daitezke. Horrez gain, errundako arrautzetan azpi-unitate erribosomiko osoen kantitatea ere neur daiteke, RNA erribosomikoen eta amaierako erribosoma-kopuruaren arteko erlazioak kalitatean izan dezakeen eragina aztertu ahal izateko.

Gametoen kalitatearen markatzaile objektibo eta goiztiar baten lorpena arrain industrian zein ingurune azterketetan abantaila ikaragarria suposatuko luke. Zehazki etorkizunean, arrautzen kalitatearen adierazle objektibo bat topatzeak, akuikulturan erabiltzen diren hazkuntzarako metodologia ezberdinek arrautza-kalitatean izan dezaketen eragina aztertu ahalko litzateke, besteak beste, ugaltzaileen elikadura, fotoperiodoa edo obulazioa bultzatzeko tekniken eragina ikusi ahalko litzateke (Bromage et al., 1992; Brooks et al., 1997; Bobe eta Labbé, 2010). Horrez gain, arrautzen kalitatea jakinda hasieratik kalitate horren araberako sailkapena egitea ahalbidetuko luke, txarrak direneko arrautzak edo eme ugaltzaileak (beti kalitate txarreko oozitoak ematen dituztenak) gainontzeko hazkuntza prozesuetatik baztertuz eta horrela enbrioien hazkuntza-arrakasta altuago bat ziurtatuz (Żarski et al., 2011; Żarski et al., 2012).

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Horrez gain eskerrak eman nahi dizkiogu “Galician cluster of aquaculture”-ari (CETGA, Aguiño, Galizia) erreboiloekin ugalketa saioa aurrera eramateagatik.

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SUMMARY AND GENERAL DISCUSSION

Sex determination/differentiation in teleost fish species is a poorly understood and plastic process that has become a hot issue of research with implications in basic and applied science in fields, such as basic developmental biology, evolutionary biology, aquaculture, fisheries research, fish stock management, environmental biology or ecotoxicology.

The estimation of maturity and sex of fish stocks in European waters is a requirement of the EU Data Collection Framework as part of the policy to improve fisheries management (http://ec.europa.eu/fisheries/cfp/fishing_rules/data_collection /index_en.htm). It is necessary to understand the natural population dynamics of fish stocks, and specific fish life history characteristics so as to set harvest policies in the sense of maximizing exploitation while protecting the spawning potential of the stocks (Korta, 2010; Prager et al., 2003). This is not easy to establish for some fish species, especially when oceanographic campaigns do not coincide with their spawning seasons (Diaz de Cerio et al., 2012). Moreover, in the last decades pollution and most specifically exposure to reproductive EDCs has been observed in coastal and offshore fish populations (Jobling et al., 1998; Boehm et al., 2011; DellaSala et al., 2013). It is known that the mode of action of some of these pollutants is sex specific (Williams et al., 2003), being the feminization of juvenile and male individuals, resulting in some cases in the onset of the intersex condition, the most studied effect of the exposure to xenoestrogens. Despite having been deeply studied, the intersex condition needs to be identified histologically, while the molecular mechanisms governing the formation of oocytes within spermatic follicles remain partially unknown. Several molecules have been proposed as biomarkers of EDC exposure, but none of them has so far been able to identify intersex condition specifically and unequivocally (Bahamonde et al., 2013).

In order to validate the qPCR analysis normalization method used throughout the present manuscript, and that is based on the exact quantification through fluorescence of cDNA amount amplified in each fish gonad qPCR analysis (chapter 1), we tested the transcription level variability of four commonly used reference genes (*actb*, *ef-1-α*, *gapdh* and 18S rRNA) during the oogenesis in thicklip grey mullet (*Chelon labrosus*) ovaries. We compared such variability with that shown applying the cDNA normalization method. Different normalization methods or approaches (using a single reference gene,

the geometric mean of all analysed genes and the cDNA amount) were compared to study the transcription levels along oogenesis of target genes associated with gonad steroidogenesis; *star*, *cyp19a1a* and *cyp11b*. Our results suggest that normalizing methods based on the combination of different reference gene transcription levels, and/or cDNA alone, were the best approaches as the variability of single normalizing gene, specially *gapdh* and 18S rRNA, is very strong along oocyte differentiation in fish, as it has already been reported by other authors (De Santis et al., 2011; Libus & Štorchová, 2006; Mittelholzer et al., 2007; Filby & Tyler, 2007;).

In addition, in the aquaculture industry, it has become common practice to breed monosex populations (Devlin & Nagahama, 2002) as a consequence of the differential growth rate of both sexes, depending on the fish species higher for the males (for instance tilapia) or for the females (for instance rainbow trout). Also in aquaculture, when natural spawning is obtained with difficulty and it cannot be regulated controlling simple husbandry conditions, such as photoperiod or temperature, it is common to induce ovulation/spermiation artificially through hormonal treatments. This must be carried out when the oocytes/sperm are mature in the ripe gonad, something that cannot be achieved in species such as, for instance, the freshwater eels (see chapter 3) in order to ensure success in fertilization. This can be a difficult task and requires the periodical manipulation of reproducing individuals, increasing stress.

Making use of the results obtained by our group in 2012, that demonstrated for the first time the usefulness of genes coding for 5S rRNA and accompanying proteins in the identification of sex and intersex individuals in thicklip grey mullet (*Chelon labrosus*) (Diaz de Cerio et al., 2012), in the present work we have tried to validate these transcripts (belonging in general terms to the process of ribosome production) as sex and female developmental stage markers in different commercial and developmental biology/aquaculture/ecotoxicology model fish species. In addition, we have tried to gain some knowledge regarding the hormonal (gonadotropins, steroid hormones) control of ribogenesis during fish oogenesis and spermatogenesis.

Taking advantage of the differential gene transcription occurring during fish sex differentiation and gametogenesis (Manousaki et al., 2014) and the differential

accumulation of small molecules that occurs in oocytes during early oogenesis (Mazabraud et al., 1975; Denis & Wegnez, 1977; Allison et al., 1995; Kroupova et al., 2011; Espigares et al., 2015), 5S rRNA and the *gtf3a* transcription levels were tested as molecular markers in the identification of sex and gametogenic stage in different economically-relevant fish species from the Bay of Biscay (chapter 2). Gonads of 9 fish species with asynchronous gonad development (Atlantic mackerel, Atlantic-chub mackerel, horse mackerel, blue whiting, bogue, European anchovy, European hake, European pilchard and megrim), were histologically sexed and 5S and 18S rRNA concentrations were quantified by capillary electrophoresis. A 5S/18S rRNA index was developed and the data obtained assessed in the analysis of sex differentiation in all these fish species. Degenerate primers were used to clone and sequence *gtf3a* fragments in 7 of the studied species. 5S rRNA, 5S/18S rRNA index and *gtf3a* transcript levels distinguished ovaries from testis in all of studied species as it had already been published in mullets that display a synchronous gonad development (Diaz de Cerio et al., 2012). In addition, 5S/18S rRNA index values in females were always in their highest when females were captured in early phases of oogenesis, coinciding with 5S rRNA accumulation in oocytes during previtellogenesis (Kroupova et al., 2011; Mittelholzer et al., 2007). Later and along vitellogenesis, and beginning with the apparition of cortical alveoli oocytes, the index values decreased significantly, this meaning that 18S and 28S rRNA accumulation was activated. In megrim and European anchovy, where gonads in different oogenesis stages were obtained, the 5S/18S rRNA index identified the different gametogenic stages unambiguously and quantitatively.

We then studied the hormonally triggered oogenesis in the European eel (*Anguilla anguilla*) to try to understand the mechanisms controlling the production of ribosomal building blocks during artificially induced oocyte maturation (chapter 3). We hypothesised that changes in the transcription levels of genes associated with ribosomal biogenesis could be used to monitor oocyte differentiation, growth and maturation during hormonally induced oogenesis. For that, eels from the Valencia Albufera Lagoon were treated with weekly injections of carp pituitary extract for 15 weeks. Ovaries were processed for histological staging of oogenesis and for RNA extraction. Levels of 5S and 18S rRNA were quantified after total RNA electrophoretic analysis and the 5S/18S rRNA

index calculated. Then, transcription levels of *gtf3a*, 18S rRNA, *ubtf1*, *42sp43*, *rpL5* and *rpL11* were quantified by qPCR. Our results suggest that 5S rRNA and ribogenesis related genes were highly transcribed in ovaries with previtellogenic oocytes. As oocytes differentiated, 5S/18S rRNA index and transcription levels of genes related to 5S rRNA production, accumulation and nucleo-cytoplasmic transport, such as *gtf3a*, *42sp43*, *rpl5* and *rpl11* decreased. On the contrary, entry into vitellogenesis was accompanied by an up-regulation of 18S rRNA in oocytes (Kroupova et al., 2011) although *ubtf1*, necessary for RNA polymerase I activity and 45S pre-rRNA transcription (Bazzett-Jones et al., 1994; Reeder et al., 1995), showed the same transcription profile as 5S rRNA regulating genes. Females that did not respond to the treatment, maintaining perinucleolar oocytes after 12 pituitary extract injections, showed 5S/18S rRNA index values similar to untreated females and failed to express the largest rRNA molecules. We prove that the transcriptional dynamics of ribosomal genes provide useful molecular tools to characterise the process of artificially induced ovarian development in European eels, providing evidence that ribogenesis is molecularly regulated by the hormonally modulated oocyte differentiation process.

Then, and in order to understand the pattern and control of *gtf3a* transcription during sex differentiation and gametogenesis in fish we exposed just-fertilised zebrafish (*Danio rerio*) eggs to 100 ng/L 17β-estradiol and 17α-methyltestosterone during 61 dpf to obtain two monosex populations; one fully feminised and another one fully masculinised (chapter 4). Fish genomes available in Ensembl were analysed, identifying two *gtf3a* paralog genes consequence of the teleost specific third genome duplication event; *gtf3aa* and *gtf3ab*. A sub-functionalisation of both genes followed gene duplication and while *gtf3aa* was ubiquitously expressed, *gtf3ab* was only transcribed in ovaries. Possibly *gtf3ab* has lost the capacity to regulate 5S rRNA transcription and has been kept in oocytes as a protein that exclusively binds 5S rRNA for stockpiling in the cytoplasm. The existence of duplicated genes in relation to reproductive endocrinology and sex differentiation control has been extensively documented in many fish species (Maugars & Dufour, 2015). Nearly undetectable levels of *gtf3ab* transcription were recorded in 26 dpf hormonally treated zebrafish, which did not present differentiated gonads yet. At day 61 fully differentiated ovaries and testes were identified, feminised zebrafish

showing high transcription levels of ovarian *gtf3ab* (mRNA extracted from whole body), while masculinised zebrafish only showed transcription of *gtf3aa*. The transcription of g*tf3ab* in females coincided with the transcription of ovarian *cyp19a1a* and it was opposite to that of male markers *amh* and *dmrt1*. We further demonstrated in normal developing zebrafish embryos that maternal inherited *gtf3ab* transcripts disappear after 8 hpf, and that embryonic *gtf3aa* transcription begins at that time with the activation of zygotic genome. *gtf3ab* transcription thus begins only at the onset of oogenesis, functioning as an effective early marker of oocyte production. We can thus conclude that *gtf3ab* transcription at 61 dpf (17β-estradiol treatment group and control group females) is a consequence of oocyte differentiation and not a result of estrogen exposure, as the first 26 days of 17β-estradiol exposure in our experiment did not result in *gtf3ab* transcription.

Considering that 5S rRNA is a powerful molecular marker of the presence of differentiated oocytes in fish gonads and that its transcription levels served to identify intersex mullet individuals (Diaz de Cerio et al., 2012), and in order to assess whether we could quantitatively and unambiguously distinguish fish sex, female reproductive stage and intersex severity, the 5S/18S rRNA index was applied in 296 thicklip grey mullets (*Chelon labrosus*) collected from polluted sites in the Southern Bay of Biscay during a complete annual reproductive cycle (chapter 5). In addition, transcription levels of *gtf3a* and *ubtf1*, related to 5S rRNA and 18S rRNA synthesis respectively, were studied in ovaries at different stages of oogenesis. Using histological techniques 38 Intersex individuals were identified among the sampled fish and the intersex severity was ranked according to the amount of oocytes present in each testis (Jobling et al., 2006). The 5S/18S rRNA index molecularly identified male and female thicklip grey mullets and an index threshold value of 0.4521 was established, with 100% of the males ranking bellow that threshold. In addition, our results suggested that the histologically established intersex severity index (Intersex severity index 1 to 7, Jobling et al., 2006) correlated with the 5S/18S rRNA index. The highest 5S/18S rRNA index coincided with the individuals showing the higher amount of oocytes in testis (in the present study highest identified intersex severity index = 3), providing an easy, quantitative and objective method to identify the severity of intersex condition in male mullets captured during

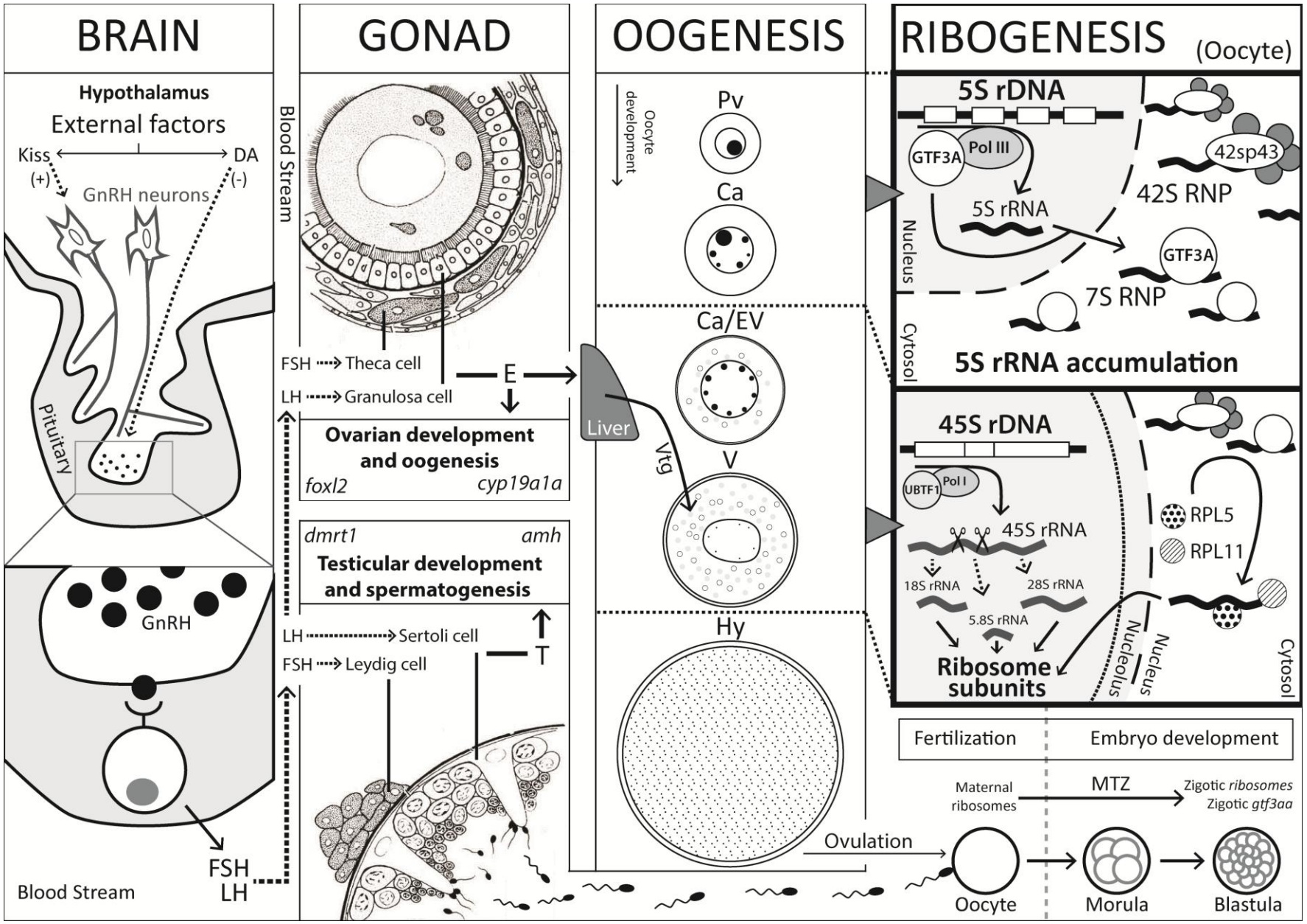
pollution biomonitoring campaigns. The 5S/18S rRNA index also proved reliable in the identification of mullet ovarian developmental stage since, as it happened in other teleost species studied (chapter 2 and 3), values were at their highest during early oogenesis and progressively declined along vitellogenesis towards maturation. The *gtf3a* transcription levels followed the pattern of 5S rRNA production. In contrast, as it happened in European eels (chapter 3) *ubtf1* transcription pattern did not resemble that of 18S rRNA. It was observed that 45S pre-RNA was strongly transcribed before vitellogenesis, indicating that the high amount of 18S rRNA accumulating at secondary oocyte growth is partially produced through accelerated pre-rRNA maturation. In conclusion, it can be said that 5S/18S rRNA index is an easy and quantitative way to identify molecularly the sex, female gametogenic stage and intersex severity in thicklip grey mullets living in polluted estuaries. The knowledge provided here confirms observations in other teleost fish species and sheds light on the mechanisms governing germ cell ribogenesis during fish gametogenesis; most specifically during oogenesis.

As we believed that 5S rRNA amount in spawned fish oocytes could help in the identification of the best quality eggs and thus the best reproductive females, for the improvement of reproductive protocols in aquiculture we carried out two independent assays in two of the most common breed species, turbot (*Scophthalmus maximus*) and seabass (*Dicentrarchus labrax*) (chapter 6). Our aim was to relate the 5S/18S rRNA index calculated on total RNA extracted from spawned oocytes, hand stripped in both cases (after a 3 days treatment with LHRH in the case of sea bass), with the different traditionally used egg quality parameters and the survival rate of the offspring obtained from each egg batch. Our assumption was based on the fact that oocytes accumulate maternal RNA molecules, mainly rRNAs, (Davidson, 1986) during their development and that these molecules must ensure survival among other things by providing protein synthesis during the first embryogenesis stages (Song, 2005). In both turbot and seabass oocytes, 5S rRNA and 18S rRNA levels were positively correlated. Both 5S rRNA levels and 5S/18S rRNA index, showed positive correlations in turbot with traditional good quality egg parameters, such as egg buoyancy and hatching success, while in seabass this correlation with egg quality parameters (*i.e.* buoyancy, viability, pH, fertilization rate, embryo survival and development) was negative. Thus, opposite results were

obtained in both species in relation to 5S rRNA as a molecular marker of oocyte quality, caused probably by the different experimental setups employed in both cases. Future approaches to validate the usefulness of ribosomal markers as good quality markers of fish eggs will need to focus in a set of easily tractable fish species in which husbandry protocols are standardised and could be modified to obtain eggs of different quality from the same reproducing females in subsequent spawns.

All together, the knowledge obtained in the present PhD study, based on the analysis of 5S rRNA an *gtf3a* accumulation dynamics in fish oocytes allows to set a straightforward procedure for the sexing of teleost fish species and for the identification of the maturity stage of females, regardless of their reproductive strategy and the way the oogenesis was induced (naturally vs hormone induction). Moreover, developed 5S/18S rRNA index also proved to be an unambiguous and quantitative method to establish the intersex severity in mullets collected from polluted areas in the Southern Bay of Biscay. In addition, the different mechanisms governing ribogenesis during oogenesis in fish were elucidated reinforcing our proposal to use a normalization method based on cDNA quantification in studies aiming to assess transcriptional gene regulation in very dynamic tissues, such as the fish gonads. We also described that in teleosts and after the third genome duplication event the *gtf3a* gene appears duplicated with the sub-functionalisation of *gtf3ab* paralog for its expression in oocytes in relation with 5S rRNA accumulation in oocytes. Meanwhile *gtf3aa* is expressed in all the tissues. Moreover, we now know that *gtf3ab* transcription is a consequence of oocyte differentiation and not directly directed by estrogen action (either physiologically or environmental exposure). These observation and the methodological approaches developed could have multiple applications in the study of fish reproduction, fecundity, population dynamics, environmental monitoring and fish biology in general. In the future, such markers should be studied deeper as putative indicators of egg quality in fish.

Schematic overview of the main topics and conclusions obtained in the present work



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CONCLUSIONS AND THESIS

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1- In the analysis of gene transcription profiles through qPCR along oogenesis, the normalization method using cDNA amount quantified through fluorescent methods is more accurate for the study of ovaries of thicklip grey mullet (*Chelon labrosus*) than normalization using the usual approach with the quantification of individual normalizing genes (*act*, *elf-1-α*, *gapdh* or 18S rRNA). Transcription profiles are so variable along fish oogenesis that there is no adequate normalizing gene.

2- 5S rRNA is highly transcribed in ovaries (oocytes) in all tested teleost fish species; European anchovy (*Engraulis encrasicolus*) and hake (*Merluccius merluccius*), Atlantic, chub and horse mackerel (*Scomber scombrus*, *Scomber japonicus*, *Trachurus trachurus*), blue whiting (*Micromesistius poutassou*), megrim (*Lepidorhombus whiffiagonis*), pilchard (*Sardina pilchardus*), bogue (*Boops boops*), European eel (*Anguilla anguilla*) and zebrafish (*Danio rerio*), irrespective of their reproduction strategy (synchronous vs asynchronous; single spawner, batch spawner, group batch spawner) and their stage along oogenesis.

3- A 5S/18S rRNA index has been developed that allows sexing all teleost fish species studied and, together with the quantification of *gtf3a* transcription levels, allows the identification of the different stages of ovary development along the reproductive cycle as proved in megrim (*Lepidorhombus whiffiagonis*), anchovy (*Engraulis encrasicolus*) and thicklip grey mullet (*Chelon labrosus*).

4- The cytoplasmic accumulation of 5S rRNA in previtellogenic oocytes early in oogenesis and the posterior relative increase in 18S rRNA along vitellogenesis and oocyte final maturation allows quantitative analysis of ovarian development during maturation triggered in European eel (*Anguilla anguilla*) through weekly injections of carp pituitary extract. 5S/18S rRNA allows the identification of females that do not respond to hormonal treatment, such females showing index values similar to females with previtellogenic oocytes. A general negative trend is observed along oocyte growth in transcription levels related to ribogenesis, with the exception of 18S rRNA which transcription levels increase as oocytes differentiate.

5- The RNA Integrity Number (RIN) is not useful to analyse the quality of total RNA extracted from teleost ovaries, especially those with previtellogenic stage oocytes, due to the prevailing presence of 5S rRNA and nearly non-existent relative presence of 18S and 28S rRNA.

6- Two *gtf3a* paralog genes are present in the genomes of all teleost fish species studied, emerged through the third whole genome duplication event occurred in teleosts. A sub-functionalisation of both genes has resulted in *gtf3aa* to be transcribed in all somatic tissues and in both gonads, while *gtf3ab* is nearly exclusively transcribed in ovaries, where it is responsible of the massive accumulation of 5S rRNA in the cytoplasm of oocytes, as demonstrated in zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*).

7- High transcription levels of *gtf3ab* in feminised zebrafish (*Danio rerio*) after 61 days of 17β-estradiol treatment post-fertilisation, opposite to the lack of transcription identified in 17α-methyltestosterone masculinised fish, demonstrates ovarian specific *gtf3ab* transcription associated to the sex differentiation process. *gtf3ab* transcription is a consequence of oocyte differentiation (females in control and in 17β-estradiol treatment groups at day 61) and not a direct effect of the estrogenic treatment as transcription was not observable at day 26 in the 17β-estradiol treated group in which ovary had not yet been developed.

8- 5S/18S rRNA index quantitatively identifies intersex individuals according to the severity of their intersex condition in thicklip grey mullets (*Chelon labrosus*) due to the massive accumulation of 5S rRNA in previtellogenic oocytes. As the amount of oocytes within the testis increases (intersex severity increase from 1 to 3) the 5S/18S rRNA index also increases giving a quantitative and unambiguous account of the severity of the intersex condition.

9- Contrary to observations in amphibians, 45S pre-rRNA synthesis is active in ovaries during early oogenesis in teleosts, and massive production of 18S and 28S rRNA occurring during secondary oocyte growth and entry into vitellogenesis occurs partly due to maturation of this 45S pre-rRNA.

10- 5S rRNA and 18S rRNA levels are positively correlated in turbot (*Scophthalmus maximus*) and seabass (*Dicentrarchus labrax*) eggs. 5S rRNA levels and 5S/18S

rRNA index in turbot are positively correlated with traditional good quality egg parameters, while in seabass correlations are negative. This discrepancy is probably caused by the use of different methodologies suggesting the need for future experimental set-ups with fish species and egg attention protocols that allow a non-biased quantification of accumulated ribosomal intermediates.

THESIS

Ribogenesis related 5S rRNA and *gtf3ab* transcripts levels are specific molecular markers of the presence of oocytes and thus should be considered sex and intersex molecular markers in teleost fish. Ribosome production is positively regulated during the process of oocyte differentiation and not as a direct consequence of exposure to estradiol. This process is initiated by a massive production and accumulation of 5S rRNA in previtellogenic oocytes, which is then followed by the accumulation of RNA polymerase I derived products that starts at the beginning of vitellogenesis or secondary oocyte growth. Thus, 5S rRNA and 18S rRNA relative level in ovaries functions as a marker for the identification of oocyte development (growth stage) in fish. These observations and tools developed through them could have multiple applications in the study of fish reproduction and fecundity, in the research of fish stock dynamics and management, and in environmental health assessment but, more specifically in the monitoring of environmental exposure to xenoestrogens for the identification of intersex condition and its severity in fish.

ONDORIOAK ETA TESIA

ONDORIOAK ETA TESIA

1- Oogenesian zehar qPCR bidezko geneen transkripzio-profilen azterketarako, fluoreszentzia bidez neurturiko cDNA-kantitatearen bidezko normalizazio banakako erreferentzia-geneak (*actb, elf-1-α*, *gapdh* edo 18S rRNA) erabiltzea baino metodo egokiagoa da korrokoien (*Chelon labrosus*) obarioak aztertzeko, erreferentzia-gene horien transkripzio-mailek oogenesian zehar aurkezten duten aldakortasun altua dela eta.

2- 5S rRNA kantitate handian transkribatzen da obarioetan (oozitoetan) ikertutako arrain teleosteo espezie guztietan; antxoan (*Engraulis encrasicolus*), legatzan (*Merluccius merluccius*), berdel (*Scomber scombrus*), estornino (*Scomber japonicus*), txitxarroan (*Trachurus trachurus*), lirioan (*Micromesistius poutassou*), gailoan (*Lepidorhombus whiffiagonis*), sardinan (*Sardina pilchardus*), bogan (*Boops boops*), aingira europarrean (*Anguilla anguilla*) eta zebra arrainean (*Danio rerio*), euren ugal-estrategiarekiko (espezie sinkronikoa vs. asinkronikoa; errutera bakarrekoa, errute sortaduna edo taldekako errute sortaduna) eta garapen-fasearekiko era independente batean.

3- Garatutako 5S/18S rRNA indizeak ikertutako arrain teleosteo espezie guztiak sexatzea ahalbidetu du eta *gtf3a*-ren transkripzio-mailekin batera, ugal-ziklo osoan zehar obarioaren garapen-faseak identifikatzea ahalbidetzen du, gailoan (*Lepidorhombus whiffiagonis*), antxoan (*Engraulis encrasicolus*) eta korrokoian (*Chelon labrosus*) frogatu den bezala.

4- 5S rRNA-ren metaketa zitoplasmatikoak oozito prebitelogenikoetan, eta bitelogenesian zehar oozitoaren heltzeraino ematen den 18S rRNA kantitate-erlatiboaren emendioak, aingira europarrean (*Anguilla anguilla*), karpa pituitario erauzkinarekin bultzaturiko heltze prozesuan, obarioaren garapen-egoeraren jarraipen kuantitatiboa egitea ahalbidetzen dute. 5S/18S rRNA-k hormona-tratamenduari erantzun ez duten emeak identifikatzea ahalbidetzen du ere, eme horien indizea oozito prebitelogenikoak dituzten emeen antzekoa izanik. Oozitoaren garapenean zehar joera negatibo orokorra ikus daiteke erribogenesiarekin erlazionatutako geneen transkripzio-mailetan, 18S rRNA

salbuespena delarik, zeinaren transkripzio-mailek gora egiten duten oozitoak hazten diren heinean.

5- RNA-integritatearen adierazlea den zenbakia (RIN) ez da erabilgarria teleosteoen obarioetatik erauzitako RNA totalaren kalitatea neurtzeko, batez ere oozito prebitelogenikoetatik erauzitako RNA-ren kasuan, zeinetan 5S rRNA kantitate handietan pilatzen den 18S eta 28S rRNA-rekin alderatuta.

6- Bi *gtf3a* gene paralogo daude ikertutako arrain teleosteo espezieen genomatan, zeinak teleosteoen genomaren hirugarren bikoizketaren ondorioz sortu ziren. Bi geneen azpi-funtzionalizazioak, ehun guztietan transkribatzen den *gtf3aa* genean eta soilik obarioetan transkribatu eta 5S rRNA-aren metaketa zitoplasmikoaren eragilea den *gtf3ab* genea sortarazi ditu, zebra arrainean (*Danio rerio*) eta tilapian (*Oreochromis niloticus*) frogatu den bezala.

7- *gtf3ab*-k sexu-desberdintzapenarekin erlazionaturiko eta obarioarekiko espezifikoa den transkripzioa dauka, ernalketa ostean 61 egunez 17β-estradiolarekin trataturiko zebra arrain (*Danio rerio*) feminizatuetan neurtutako transkripzio-maila altuek eta 17α-metiltestosteronaz maskulinizaturikoetan neurtutako maila baxuek adierazten dutenez. *gtf3ab*-ren transkripzioa oozitoaren desberdintzapenaren ondorioa da eta ez estrogeno-tratamenduaren ondorio zuzena.

8- Oozito prebitelogenikoetan ematen den 5S rRNA-ren pilaketa masiboa dela eta, 5S/18S rRNA indizeak korrokoi (*Chelon labrosus*) intersex banakoak intersex-mailaren arabera sailkatzen ditu era kuantitatiboan. Testikuluan oozitoen kopurua emendatu ahala (intersex-maila 1-tik 3-ra), 5S/18S rRNA indizeak ere gora egiten du intersex egoeraren maila ezberdinak era objektibo eta kuantitatiboan identifikatuz.

9- Anfibioetan ikusi denaren aurka, 45S pre-rRNA-ren sintesia teleosteoen oogenesian goiz aktibatzen da, eta 18S eta 28S rRNA-en ekoizpen masiboa oozitoaren hazkunde sekundarioarekin eta bitelogenesiaren hasierarekin ematen da, hein batean, aurretik sintetizaturiko 45S pre-rRNA-ren heltze prozesuari esker.

10- 5S rRNA eta 18S rRNA mailek euren arteko korrelazio positiboa dute erreboilo (*Scophthalmus maximus*) eta lupien (*Dicentrarchus labrax*) arrautzetan. 5S rRNA-

mailak eta 5S/18S rRNA indizeak erreboiloan arrautza-kalitatearen parametro tradizionalekin korrelazio positiboak aurkezten dituzten bitartean, lupian era negatiboan korrelatuak daude. Desberdintasun hau metodologia ezberdinen erabileraren ondorioa dela dirudi eta etorkizunean prozedura esperimentalean kontu handiagoa jarri behar dela aditzera ematen du, zeinak erribosomen bitartekariak errundako oozito helduetan kuantifikatzea ahalbidetuko duen.

TESIA

Erribogenesiarekin erlazionatutako 5S rRNA eta *gtf3ab*-ren transkripto-mailak oozitoen presentziaren markatzaile molekular espezifikoak dira eta beraz sexua eta intersex egoeraren markatzaileak kontsideratu beharko lirateke arrain teleosteoetan. Erribosomen ekoizpena oozitoen desberdintzapen-prozesuan zehar positiboki erregulatua dago eta ez da estradiolaren esposizioaren ondorio zuzena, ekoizpen hau 5S rRNA-ren sintesia eta pilaketa masiboarekin hasten delarik oozito prebitelogenikoetan. Honi jarraituz, RNA polimerasa I-ren produktuen pilaketa ematen da bitelogenesia edo oozitoaren hazkunde sekundarioaren hasierarekin batera. Beraz, 5S rRNA eta 18S rRNA-maila erlatiboek arrainen obarioetan, oozitoen garapenarenaren (hazkunde-fasea) markatzaile gisa jarduten dute. Behaketa hauek eta eurei esker garatutako tresnek erabilera ugari izan ditzakete arrainen ugalketaren eta ernalketaren ikerketan, arrain erreserben dinamikaren azterketan eta kudeaketan, ingurumenaren osasun-ikerketan, eta zehazki xenoestrogenoek kutsaturiko inguruneen jarraipenean ager daitezkeen intersex banakoen identifikazioan eta intersex egoeraren mailen ezarpenean.

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Other publications:

Valencia, A., **Rojo-Bartolomé, I.,** Bizarro, C., Cancio, I., Ortiz-Zarragoitia, M. (2016). Alteration in molecular markers of oocyte development and intersex condition in mullets impacted by wastewater treatment plant effluents. *Gen. Comp. Endocrinol.* http://dx.doi.org/10.1016/j.ygcen.2016.06.017.

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