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Research paper

A game of two? Gene expression analysis of brain (*cyp19a1b*) and gonadal (*cyp19a1a*) aromatase in females of a Neotropical cichlid fish through the parental care period and removal of the offspring

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ABSTRACT

For many species parental behavior is essential for the survival of the offspring. While the ultimate causes of teleost parental behavior have been widely studied, comparatively little is known about its proximate causes. The aim of this study was to analyze the yet unexplored, potential dual role of brain and gonadal aromatases, the enzymes responsible for the conversion of androgens to estrogens in the brains and gonads of teleosts, respectively, on the different stages of the maternal care period of the biparental cichlid *Cichlasoma dimerus*, locally known as *chanchita*. By immunohistochemistry we analyzed the neural distribution of brain aromatase and observed it exclusively within the forebrain, including areas involved in the regulation of parental behavior. We next analyzed the gene expression of brain aromatase in the brain, and gonadal aromatase in the ovary, of female *chanchitas* through the parental care period. To further characterize the physiological environment associated to maternal care, we also evaluated sex steroid levels (17β-estradiol, testosterone and 11-ketotestosterone) and ovarian follicle percentage. The onset of parental behavior specifically downregulated sex steroids synthesis and the rate of ovarian maturation, as denoted by a more than 10-fold decrease in steroid levels and delayed detection of mature follicles in females with offspring, compared to females which eggs were removed. Gene expression levels of both aromatases were independent of maternal care at the evaluated time points, even though they varied during the parental care period.

1. Introduction

Cichlid fish are among the most diverse fish families with more than 1600 species described to date (Froese and Pauly, 2016). All mating systems known to be present in other vertebrate animals can also be found in cichlids (Barlow, 2002). In spite of the great diversity, all cichlids show parental behavior (Barlow, 2002; Kuwamura, 1986). The latter is defined as any behavior performed by the parents that increase offspring survival and growth (Trivers, 1974). In cichlid fishes, different forms of parental care have been described: from substrate guarding to delayed and immediate mouthbrooding, and from monoparental to biparental and cooperative breeding (McKaye, 1984).

Most studies on cichlid parental behavior have focused on its ecological and evolutionary aspects (Goodwin et al., 1998; Oldfield et al., 2015; Santangelo, 2015; Wong and Balshine, 2011), with comparatively fewer analyzing its physiological and neural bases (Birba et al., 2015; O'Connell et al., 2012; Tacon et al., 1996). Neuropeptides such as vasotocin (Ripley and Foran, 2010), and isotocin (O'Connell et al., 2012), neurosteroids (Pradhan et al., 2014), and prolactin (Páll et al., 2004) have all been shown to regulate parental care in fish, including cichlids (Grone et al., 2012; O'Connell et al., 2012; Specker and Kishida, 2000; Tacon et al., 2000).

In teleosts, the enzyme aromatase, responsible for the conversion of C19 androgens to C18 estrogens, exists as two paralogue

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copies: *cyp19a1a* predominantly expressed in the gonads (also called gonadal aromatase), and *cyp19a1b*, mostly expressed in the brain (known as brain aromatase) (Callard and Tchoudakova, 1997). In fish, it has been shown that local estrogen synthesis by Cyp19a1b is particularly prominent in brain areas involved in the regulation of parental care, such as the ventral nucleus of the ventral telencephalon (putative homologue to tetrapod septal formation; O'Connell and Hofmann, 2011), and the preoptic area (POA) (Forlano et al., 2001; Strobl-Mazzulla et al., 2005; Diotel et al., 2010). In turn, Saaristo et al., (2010) described an effect of the synthetic estrogen 17α-ethinylestradiol, on some aspects of parental behavior. Despite the co-localization and potential regulatory effects, no study so far has analyzed the association, if any, between Cyp19a1b and parental care.

In species with the potential for multiple spawning events and with a low reproductive success due to high rates of predation or territory loss, behavioral and physiological plasticity allow to respond rapidly to a continuously changing environment. The coordination of an animal's social and reproductive status with its reproductive physiology is critical at the level of the gonads and gamete production. Gonadal aromatase regulates gametogenesis by local estrogen synthesis, both in males (Schulz and Miura, 2002) and females (Cardinali et al., 2004; Nagahama et al., 1995). On the other hand, gonadal steroids may directly regulate parental behavior by their endocrine action over the brain (Bales and Saltzman, 2016). Accordingly, in some teleosts females the gonads represent the main source of estrogens in the general circulation (Lorenzi et al., 2012, Yaron et al., 1977). Thus, a comprehensive study of the potential role of estrogens on teleosts' behavior, should consider including the analysis of both aromatase paralogues.

Cichlasoma dimerus (Heckel, 1840), locally known as *chanchita*, is a serially monogamous, Neotropical cichlid with biparental care of the eggs and larvae, and multiple spawning events during the reproductive period (see Pandolfi et al., 2009 and Ramallo et al., 2014 for review). Like most American cichlids, *chanchitas* are substrate brooders, and parental behavior begins with the preparation and cleaning of a spawning site, usually a flat surface, and extends all through guarding of the eggs and wigglers, up to around 20 days after larvae began swimming (a detailed description of *chanchitas* parental behaviors and larvae development can be found in Alonso et al., 2011, and Meijide and Guerrero, 2000, respectively). Both female and male *chanchitas* adjust their behavior as the offspring develops from egg to swimming larvae. Different behavioral repertoires are expressed during the egg stage (e.g. fanning the eggs, removing those with fungi), wiggler (e.g. transport of larvae with their mouth to a nest) and swimming larvae stages (e.g. pelvic fin flickering). In this framework we aimed to analyze the expression pattern of brain and gonadal aromatase during the parental care period of female *chanchita*. To better contextualize the gene expression of both aromatases, we also analyzed folliculogenesis and plasma sex steroid concentrations throughout the parental care period.

2. Material and methods

2.1. Animals

We obtained male and female adult specimens of *chanchita* from wild populations in Esteros del Riachuelo (27 °35′S; 58 °45′W; Corrientes, Argentina), using fishing nets. Fish were then transferred to our laboratory in Buenos Aires, Argentina, and housed in community tanks (150 L, 8–10 fish per tank) with artificial aquarium plants and stones, under conditions that tried to mimic their natural reproductive habitat: 25 ± 2 °C and 14:10 h light:dark cycle with full spectrum illumination (Almirón et al., 2008). Every morning we fed fish with fish food sticks (Koi Vibrance Color Enhancer Fish Food – Tetra Brand). A total of 36 females were utilized in this study (average body weight: 26.2 ± 2.7 g; average standard length: 7.3 ± 0.2 cm).

Experiments were conducted in accordance to international standards on animal welfare, as well as being compliant with institutional (Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) and National (Comité Nacional de Ética en la Ciencia y la Tecnología, MINCyT, Argentina) regulations. All procedures were in compliance with the Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

2.2. Immunohistochemical study

To investigate whether in female *chanchita*s brain aromatase expression co-localized with neural areas involved in the regulation of parental care and reproductive behavior, we performed an immunohistochemical study. We randomly selected four prespawning females, anesthetized them by immersion in a 0.1% benzocaine (ethyl-p-aminobenzoate) solution until opercula movement ceased and euthanized them by decapitation. We carefully removed the brains and then fixed them in Bouin's solution for 18 h at room temperature. Samples were dehydrated and embedded in Paraplast (Fisherbrand, Fisher, Wash). Afterwards, we completely sectioned the brains at 12 μm in the transverse plane and mounted them on gelatin coated slides. We continued with dewaxing of the samples in xylene, followed by rehydration through a graded ethanol series to phosphate buffered saline (PBS, pH 7.4). To block non-specific binding, samples were treated for 30 min with PBS containing 5% nonfat dry milk, and incubation with an anti-aromatase antiserum produced in rabbit, kindly provided by Dr. P.M. Forlano (University of New York, USA), diluted 1:1000. After washing the slides in PBS, we incubated the samples with biotinylated anti-rabbit IgG (Sigma–Aldrich) diluted 1:600 for 45 min at room temperature. Amplification of the signal was achieved by incubating the sections with peroxidase conjugated streptavidin (STRP–HRP) (Dako) diluted 1:800 and visualized with 0.1% 3,30-diaminobenzidine (DAB) in TRIS buffer (pH 7.6) and 0.03% H2O2. Then we lightly counterstained each section with hematoxylin and mounted them in DPX. Samples were examined with a NIKON microphot FX microscope and digitally photographed (Coolpix 4500, Nikon).

Since, unlike mammals where aromatase is mostly expressed in neurons within the adult brain (Cesi et al., 1992), in all teleost studied to date Cyp19a1b is expressed in radial glial cells (Diotel et al., 2010), we performed a double-immunofluorescence using an antibody against a neuronal marker and the anti-aromatase antibody. Whole brains obtained from three randomly selected females were processed as described for the immunohistochemical study up to blocking of non-specific binding, and we then incubated them with a cocktail of primary antibodies: an antibody raised in mouse against a cytoplasmic marker of post-mitotic neurons, acetylated α-tubulin (Sigma-Aldrich), and the anti-teleost specific aromatase antiserum. Antibodies were diluted 1:100 and 1:1000, respectively, and sections were incubated for 16 hs at 22 °C in a humidified chamber. After washing the slides in PBS, we applied a mix of secondary antibodies, an Alexa Fluor 594-conjugated goat anti-mouse IgG (1:100; Jackson Immuno Research Laboratories, Inc.) and a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100; Sigma–Aldrich) for 90 min at 37 °C in the dark, in a humidified chamber. Next we washed the samples in PBS and mounted them with Mounting fluid (Tecnolab S.A.) with 1 μL 4,6-Diamidino-2-phenylidole dichlorhidrate (DAPI) (Sigma-Aldrich). Samples were examined

with a fluorescence microscope (Nikon, Eclipse E600) equipped with a digital camera (Evolution TM VF Fast Cooled Color 12 bits). Antibodies specificity was evaluated by omission of the primary antibodies and no immunolabeling was observed under such conditions.

2.3. Structure of the parental care period and general methodological approach

To analyze female *chanchitas* during the parental care period, we employed a total of 31 fish. As described in Tubert et al. (2012) and Birba et al. (2015), actively reproductive *chanchitas* can be classified within four distinct categories according to the development of their fry: (1) pre-spawning females (PS), which aggressively defend and clean the spawning site along with a territorial male; (2) female with eggs (E), which fan and guard the eggs and remove infected ones; (3) female with hatched larvae (HL), 3 days after fertilization (daf) eggs hatch and the reproductive pair attends the wigglers which are adhered to the substrate by glands on their head (Meijide and Guerrero, 2000); and (4) females with swimming larvae (SL), which starts at 8 daf when the larvae are able to swim, and the pair expands their territory, and groups the larvae protecting them from predators. We obtained territorial PS females from community tanks housing 6–8 *chanchitas*, while E, HL, and SL females were originally PS pairs detected in community tanks and soon after placed (the pair) in physical isolation, where spawning and subsequent parental behavior occurred. Isolated pairs had visual contact, however, with fish from neighboring aquaria. Isolation tanks were 75 L (ambient conditions were identical to those described for community tanks) and contained a flat slab where *chanchita* usually spawns. This design, where reproductive and parental care took place in isolation, allowed us to describe the effect of the offspring on parental physiology without interference from aggressive and territorial behavior, predation of the offspring or any other social interactions which would have occurred in a group scenario. However, detection of pre-spawning behavior was easier in a community set-up, as the territorial pair becomes highly aggressive towards non-territorial *chanchitas* as spawning becomes closer, and reproductive behaviors such as body shaking are more conspicuous in a group environment (personal observation). For these reasons, PS females were obtained directly from community tanks.

When we observed the performance of pre-spawning behavior by a territorial pair in a community tank, or within 24 h of egg laying, egg hatching or swimming of larvae in the isolation aquaria, we removed the female and immediately drew blood for the measurement of sex steroids. We drew blood by puncture of the caudal vein with heparin-coated syringes (needle: 27gauge × 1/2 inch) and collected it in heparin-coated tubes. We controlled for circadian effects by always collecting samples between 14:00 and 15:00 h. Blood was centrifuged at 3000 rpm for 15 min and plasma were collected and stored at −20 °C until assayed.

We then weighed and recorded the total and standard length of each fish and next euthanized them as described above for the immunohistochemical study. Immediately, we dissected the brain and the ovaries. Rapidly, we placed a section of one of the ovaries (either right or left at random) and the forebrain (olfactory bulbs, telencephalic hemispheres, POA, thalamus and hypothalamus) in individual tubes containing cold RNAzol® RT (MRC), and homogenized (Bio-Gen Pro200 tissue homogenizer) them for the analysis of aromatases gene expression. Samples were stored at −20 °C until RNA extraction following the manufacturer's instructions. We then treated the samples with DNase I (Sigma) at 37 °C for 30 min, and started with the synthesis of the first-strand cDNA from 2 μg RNA of each sample using M-MLV(Invitrogen), according to manufacturer's protocol for poly(dT)oligos. The remaining entire ovary from each female was fixed in Bouin's solution and treated for its histological analysis (see below for details).

To more precisely assess the effect of the offspring presence on maternal physiology, and distinguish between the actual effects of the larvae from those resulting from the experimental set up, like isolation time, we performed an experimental removal of the offspring. On a different set of isolated *chanchita* pairs, we removed the eggs soon after spawning, and processed the female as described above, at the time when the eggs would have hatched or the larvae began to swim if the eggs had not been eliminated, 3 daf and 8 daf, respectively. To remove the eggs we extracted the slab with the eggs adhered on it, physically removed them, and returned the clean slab to its original position in the aquaria.

2.4. Hormone analyses

We measured 11-ketotestosterone (11-KT), testosterone (T) and 17β-estradiol (E_2) from plasma samples of PS, E, HL, SL females using commercial enzyme-linked immunosorbent assay (ELISA) kits (11-KT: Cayman Chemical Company, MI, USA; T and E_2: IBL International, Hamburg, Germany). Working dilutions were 1:6 for 11-KT, 1:2 for T and E_2, though some samples had to be diluted between 1:8 to 1:60, as they were above the upper curve limit. Some plasma samples were not enough for the measurement of the three hormones in all the females, resulting in variation in sample size across hormones. In all cases samples were assayed in duplicate and analyses were carried on samples in which the coefficients of variation were below 20%, following the manufacturer's instructions. We employed the ratio E_2:(E_2 + T), as an estimate of overall testosterone metabolization to E_2 (i.e. general aromatase activity), and 11-KT:(11-KT + T) ratio, as an index of the conversion of T to 11-KT. Hormones measurement with ELISA were validated for *chanchita* in previous studies (Alonso et al., 2012; Birba et al., 2015; Morandini et al., 2014; Ramallo et al., 2015).

2.5. Histological analysis of the ovary

We analyzed the follicular composition of the ovaries from female *chanchitas* at the different parental care stages. To do so, we fixed one of the ovaries from each female in Bouin's solution for 18 h at room temperature, and samples were later embedded in Paraplast, sectioned at 5 μm in the transverse plane, and mounted on gelatin-coated slides. Next we treated the sections with Hematoxylin-Eosin staining protocol. The sections were then examined with a Leica DM1000 light microscope at 250X and documented using a computerized image analyzer, Leica DFC295 photographic camera and image capture Leica Application Suite Professional, LASV3.6. We randomly selected three sections from each fish ovary (from the anterior, central and posterior region of the ovary, respectively), and counted the number of follicles with oocytes showing the nucleus. Slices were separated by at least 10 sections to avoid re-counting of the same follicle twice. Follicles were classified according to the criteria given in Coward and Bromage (1998). We analyzed the number of primary follicles (stage I or chromatin nucleolar), secondary follicles (stage II and III or perinucleolar), tertiary follicles (stage IV or cortical alveolar), early vitellogenic follicles (stage V or vitellogenesis; less than 50% of the ooplasm occupied by yolk), late vitellogenic follicles (stage V and VI or maturation; more than 50% of the ooplasm occupied by yolk), mature follicles (stage VII or germinal vesicle migration), atretic and post-ovulatory follicles (Supplementary Fig. 1). As ovaries varied greatly in size, we relativized the follicle count to the total number of follicles counted per section, and averaged the percentages of each follicle type from the three sections per fish.

2.6. Aromatases gene expression analysis

To describe the gene expression pattern of *cyp19a1b* in the forebrain of female *chanchitas* throughout the reproductive and parental care stages, we performed a qPCR using the forebrain first-strand cDNA samples as template and specific primers (qPCR-BrainFORWARD and qPCR-BrainREVERSE in Table 1). Some samples were discarded due to poor RNA quality assessed by the absorbance at 260/280 nm method. The final reaction mix consisted of 5 μL FastStar Universal SyBR green Master (Roche), 1.5 μL forward/reverse primer mix (1 μM), 2.5 μL cDNA template and 1 μL of water (total reaction volume: 10 μL). We performed the qPCR on a StepOnePlusTM Real-Time PCR System (ThermoFisher Scientific), and the reaction progress was monitored by fluorescence detection during each annealing step on duplicate samples. The thermal cycling program was: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 15 s and 72 °C for 20 s, followed by a melting curve to detect possible nonspecific products (temperature range: 95–60 °C, decrease by 0.5 °C each cycle.

For the analysis of *cyp19a1a* expression in the ovary by qPCR, we designed a specific primer pair based on a partial sequence of *chanchitas cyp19a1a* (Genbank accession number: KX260955) (qPCR-OvaryFORWARD and qPCR-OvaryREVERSE in Table 1). The first strand cDNA from the ovaries of females at different stages were employed as templates, and qPCR conditions and data analysis were identical to those described for brain aromatase expression analysis.

LinReg PCR software was used to calculate individual reaction efficiencies and the starting amount per sample (N0) in arbitrary fluorescence units (Ramakers et al., 2003; Ruijter et al., 2009). We then averaged the N0 of duplicate samples and normalized it to the averaged N0 of a housekeeping gene (acidic ribosomal phosphoprotein P0 – ARP) from the same samples (Cánepa et al., 2012).

3. Statistical analyses

All statistical analyses were performed using Statistica 8 (StatSoft®). Differences in follicle percentages, hormone levels and aromatases gene expression between females of the different parental care stages, were analyzed by means of One-way Analysis of Variance (ANOVA) followed by Tukey test. Data sets from females which eggs were removed were compared between them and with data from HL and SL control females. Association analyses between plasma steroid levels and relative gene expression levels were assessed by Pearson's correlation coefficient. Correlations were performed for the control group only and all four stages of the parental care period were included in each analysis. All data sets were checked for normality and homoscedasticity and, if needed, were sin- or log-transformed. In some cases we found data to be outliers (studentized residuals >±2), and they were excluded from the analyses. To avoid the appearance of false positives as a result of multiple hypotheses testing within each of the above mentioned tests, we applied the false discovery rate (FDR) two-stage sharpened method correction (Benjamini et al., 2006), and all presented *p*-values are modified accordingly using the spreadsheet-based software provided in Pike (2011). We set statistical significance at $p < 0.05$.

Table 1
Primer pairs for aromatases expression analyses.

Name	Sequence (5′ → 3′)	Amplicon size (bp)
qPCR-BrainFORWARD	AGAAGCATAAGAGAGCAGCC	144
qPCR-BrainREVERSE	CTCCATGATTCTGAGCGAAG	
qPCR-OvaryFORWARD	GAGTGCCTGTCAATGAGAAG	122
qPCR-OvaryREVERSE	CTTGTGCCTCTGGTGAATC	

4. Results

4.1. Neuroanatomical distribution of brain aromatase gene expression

Aromatase immunoreactive (ir) cell bodies were detected solely at forebrain regions (Fig. 1). The most anterior ir-cells were present lining the border of the medial ventricle at the ventral nucleus of the ventral telencephalon (Vv) (Fig. 1A). Ir-cells were most abundant at the POA forming a dense cellular layer facing the third ventricle (Fig. 1B–F). Some scarce ir-cells were present at the medial division of the dorsal telencephalon (Dm), at the border of the telencephalic ventricle (Fig. 1C). Aromatase ir-cells were also detected at the habenula, thalamus, and various hypothalamic nucleuses (Fig. 1E, F).

Aromatase ir-cells had small, ovoid-shaped bodies, with centrally located spherical nuclei and were exclusively observed at periventricular regions, forming a one or two-cell thick layer lining the borders of the telencephalic ventricles and third ventricle (Fig. 1 inset). In most aromatase ir-cells we were able to distinguish a long, and thin cytoplasmic extension projecting away from the ventricles. Aromatase labeling did not co-localize with the post-mitotic neural marker, acetylated α-tubulin (Fig. 2), indicating that aromatase ir-cells were not of neuronal nature.

4.2. Hormones and parental care behavior

Hormonal levels were greatly affected by egg-laying, as post-spawning females showed a general pattern of lower androgen (T: $p = 0.0003$; 11-KT: $P = 0.006$; Fig. 3A and B, respectively) and E_2 plasma levels ($p = 0.018$; Fig. 3C) compared to pre-spawning females. Removal of the eggs greatly affected *chanchita*'s physiology. In HL females which eggs had been removed, averaged testosterone increased more than sixteen times compared to its control, while in SL females with removed offspring, testosterone was more than eighteen times higher in comparison to females with larvae ($p < 0.0001$, data was log-transformed; Fig. 3A). Changes in E_2 plasma levels followed the same pattern, but were far more drastic, as E_2 rose more than thirty and forty times on HL and SL which eggs had been removed, respectively, compared to controls ($p < 0.0001$; Fig. 3C). Plasma 11-KT concentration was unaffected by offspring removal ($p > 0.05$; Fig. 3B).

Accordingly, the index of overall aromatase activity was lowest during HL ($p = 0.004$), and increased in the egg removal group ($p = 0.001$; Fig. 4A). The index of T conversion to E_2 did not differ between eggs removed and control groups for SL stage (Fig. 4A). On the other hand, the index of T conversion to 11-KT was highest during HL stage ($p = 0.0017$; Fig. 4B), and was greatly reduced after egg subtraction ($p < 0.0001$, data was log-transformed; Fig. 4B).

4.3. Ovary morphology through the reproductive and parental care period

The follicle percentage and weight of the ovary varied across the different stages. Pre-spawning females had the highest GSI ($p < 0.0001$; Fig. 5A), three times more than that of either E, HL or SL females. GSI negatively correlated with the proportion of primary follicles ($r = -0.84$, $p = 0.0001$; data not shown), and showed a positive correlation with the percentage of mature follicles ($r = 0.947$, $p = 0.0002$; only ovaries with mature follicles were considered, $n = 7$; data not shown). Within the ovary, the percentage of primary ($p = 0.0002$, Fig. 5B) and secondary follicles ($p = 0.016$; Fig. 5C) were lowest during PS, and with little or non-variation among post-spawning stages. The proportion of early vitellogenic follicles was highest at the HL stage ($p = 0.012$; Fig. 5E). Mature follicles were almost exclusively observed at the PS stage ($p < 0.0001$;

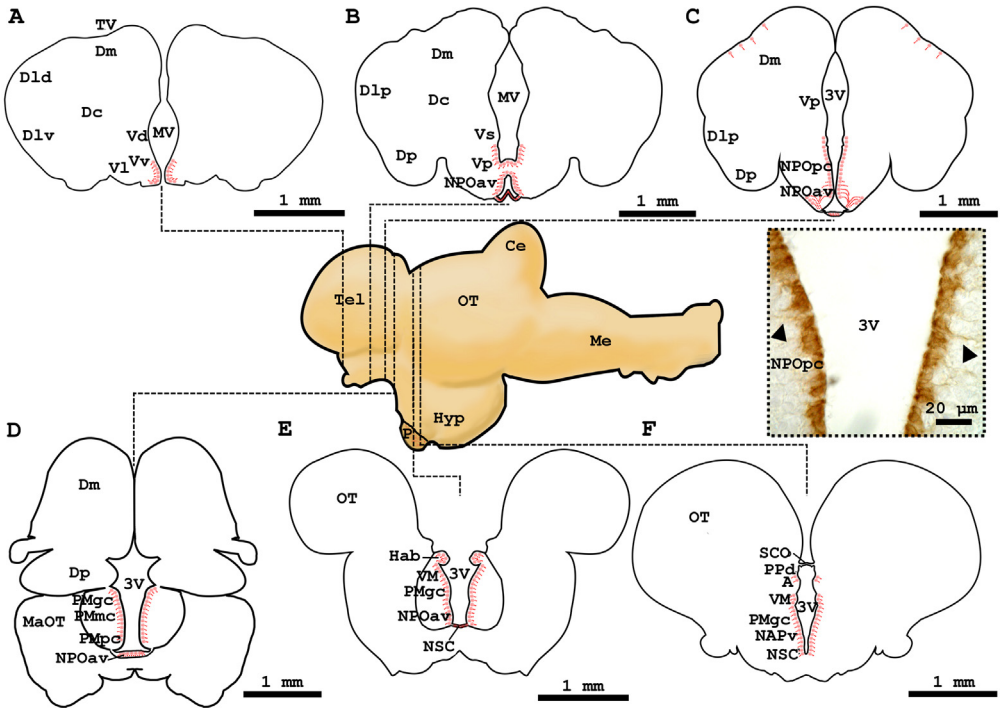

Fig. 1. Neuroanatomical localization of brain aromatase (Cyp19a1b) producing cells within female *chanchitas*' brain. (A–F) Digital drawings of a lateral view of *chanchita*'s brain and transverse brain sections where the location of immunoreactive-cells (ir-cells) is indicated by a red shading, representing ir-cell bodies lined against the border of the ventricles. The dashed lines show the approximate position of each section. Abbreviations: 3V, third ventricle; A, anterior thalamic nucleus; Dc, central part of the dorsal telencephalon; Dld, dorsal division of the lateral part of the dorsal telencephalon; Dlp, posterior division of the lateral part of the dorsal telencephalon; Dlv, ventral division of the lateral part of the dorsal telencephalon; Dm, medial part of the dorsal telencephalon; Dp, posterior part of the dorsal telencephalon; Hab, habenula; MaOT, marginal optic tract; MV, medial ventricle; NAPv, anterior periventricular nucleus; NPOav, anteroventral part of the parvocellular preoptic nucleus; NPOpc, parvocellular part of the parvocellular preoptic nucleus; NSC, suprachiasmatic nucleus; OT, optic tectum; PMgc, gigantocellular part of the magnocellular preoptic nucleus; PMmc, magnocellular part of the magnocellular preoptic nucleus; PMpc, parvocellular part of the magnocellular preoptic nucleus; PPd, dorsal periventricular pretectal nucleus; SCO, subcommissural organ; TM, telencephalic ventricle; Vd, dorsal nucleus of the ventral telencephalon; lateral nucleus of the ventral telencephalon; Vp, postcommissural nucleus of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; Vv, ventral nucleus of the ventral telencephalon. Inset: Immunohistochemical study. Photomicrographs of transverse section of female *Cichlasoma dimerus* brain showing details of aromatase ir-cells, putative radial glial cells, in the NPOpc where a dense group of ir-cells was observed in periventricular regions of the third ventricle. Cell bodies had spherical and ovoid nuclei and showed cytoplasmic projections that stretched deeper into the brain (arrowhead). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

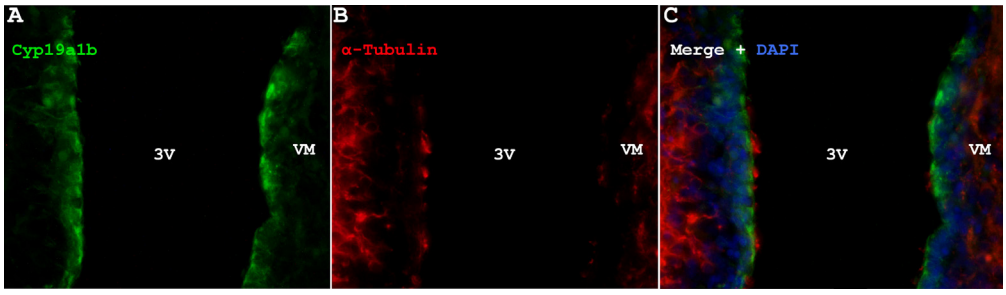

Fig. 2. Double fluorescence detection of brain aromatase (Cyp19a1b; green; A, D) and acetylated-tubulin (α-tubulin; red; B, E) along the ventricular surface (3V) at the ventral medial thalamus (VM) and the medial part of the lateral tuberal hypothalamic nucleus (NLTm). The merged images show the lack of co-localization of brain aromatase and the neural marker (C, F). The nuclear stain DAPI (blue) is also shown. Magnification: 400X. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5G), while post-ovulatory follicles were most abundant right after spawning in the E stage ($p = 0.002$; Fig. 5I, data was sin-transformed). We did not detect any change in the percentage of tertiary, late vitellogenic and atretic follicles between the four parental care stages analyzed.

Ovaries from females which eggs had been removed presented an increased GSI at the SL stage, more than twice when compared to controls ($p = 0.001$; Fig. 5A). Early follicular phases like primary follicles ($p = 0.016$; Fig. 5B) and tertiary follicles ($p = 0.009$; Fig. 5D), were less represented at the HL stage from the egg removed group when compared the HL control group. Likewise, the percentage of secondary follicles was lower in SL when eggs were eliminated compared to SL control ($p = 0.008$; Fig. 5C). On the other hand, late follicular phases like late vitellogenic ($p < 0.0001$; Fig. 5F) and mature follicles ($p < 0.0001$; Fig. 5G) were found at higher percentages at the HL and SL stages, respectively, from the egg removed group compared to controls.

4.4. Expression of both aromatase genes through the reproductive and parental care period

The gene expression of brain aromatase, *cyp19a1b*, measured in forebrain homogenates was highest at the PS stage, and gradually decreased until reaching a minimum at HL and SL stages

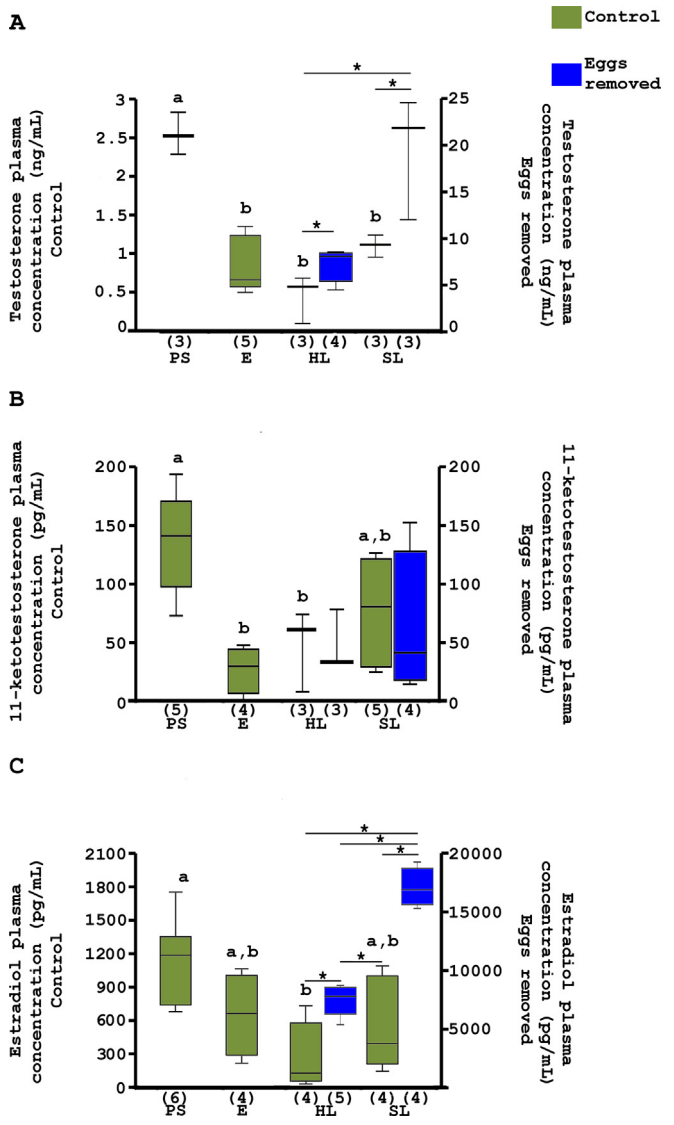

Fig. 3. Sex steroids plasma levels at the reproductive and parental care stages. Testosterone (A), 11-ketotestosterone (11-KT; B) and 17-βEstradiol (C) plasma levels measured in pre-spawning female (PS), female with eggs (E), female with hatched larvae (HL), and female with swimming larvae (SL). Different letters indicate significant differences between control treatments. Asterisks denote significant differences within egg removed treatments and/or with their time-appropriate controls. Sample size (n) is shown between parentheses. 11-KT data was log-transformed for the analysis of the effect of egg removal.

($p = 0.001$; Fig. 6A, data was sin-transformed). At the ovary, gonadal aromatase, *cyp19a1a*, expression was maximum during the eggs caring stage, and the lowest values were observed at the HL stage ($p = 0.002$; Fig. 6B). Brain and ovary aromatase gene expression were not correlated (data not shown), and neither were they affected by the removal of the eggs (Fig. 6A and B, data sets for the analysis of female with eggs only was log-transformed).

Brain aromatase gene expression positively correlated with E_2 ($r = 0.79$, $p = 0.003$; Fig. 7A) and T ($r = 0.96$, $p = 0.002$; Fig. 7B) plasma levels. No correlation was observed between gonadal aromatase relative gene expression levels and E_2 (Fig. 7C) and T (Fig. 7D) circulating levels. The index of conversion of T to E2 did not correlate with the expression of *cyp19a1b* (Fig. 7E), however, it positively correlated with *cyp19a1a* expression at the ovary ($r = 0.91$, $p = 0.01$; Fig. 7F). We did not observe any correlation between brain or gonadal aromatase gene expression and the percentage of any of the follicular types analyzed.

5. Discussion

In this work we analyzed the physiological environment associated to distinct stages of maternal care in the substrate brooder *chanchita*, with emphasis on the gene expression of brain and gonadal aromatases. As the fry developed we observed changes in the mother's brain and ovary gene expression, plasma sex steroids concentration and ovarian follicle relative numbers. Removal of the eggs caused a surge in reproductive hormones and accelerated oogenesis.

In female *chanchitas* the morphology and cellular localization of aromatase ir-cells corresponded to that of radial glial cells (Bentivoglio and Mazzarello,1999; Pellegrini et al., 2007; Rakic, 1990; Strobl-Mazzulla et al., 2010), adding to the wealth of data that shows the exclusive expression of Cyp19a1b on radial glial cells in the teleost brain (Diotel et al., 2010; Forlano et al., 2001; Strobl-Mazzulla et al., 2010; Xing et al., 2014). Even though we did not confirm the glial nature of ir-cells by using a specific cell markers, the fact that we did not observe double-staining with the neuronal marker, makes it highly probable that Cyp19a1b positive cells are glial cells. Besides, the localization of ir-cells on the ventricles borders, and their morphology, particularly the small cell bodies with a long cytoplasmic projection, which spreads beyond the periventricular region, are all characteristics of radial glial cells (Gregory et al., 1988; Stevenson and Yoon, 1982).

Cells immunoreactive to aromatase in *chanchitas* brain were observed exclusively in the forebrain. Particularly, the Vv (homologue to part of the tetrapod lateral septum; O'Connell and Hofmann, 2011) and POA (homologue to the mammalian paraventricular and supraoptic hypothalamic nuclei, and preoptic area; O'Connell and Hofmann, 2011) showed the highest density of ir-cells along with the hypothalamus. These regions have been linked to affiliative and parental behavior across vertebrates (Buntin et al., 2006, Curley et al., 2012; O'Connell et al., 2012) and thus represent putative regulatory targets by local estrogen synthesis. In rats, oxytocin receptor density at the lateral septum correlated with the frequency of maternal behavior, and estrogen treatment significantly increased local oxytocin receptor synthesis in highly active mothers (Champagne et al., 2001). Accordingly, evidence has shown that brain steroid synthesis (neurosteroids) can regulate parental behavior in fish (Pradhan et al., 2014). Thus, given the distribution of brain aromatase synthesis within brain areas involved in the regulation of parental behavior, we wondered if in *chanchita* the parental phases associated to offspring development could be accompanied by changes in brain aromatase gene expression.

When we analyzed the hormonal environment associated to the distinct stages of the parental care, we found that E_2, T and 11-KT levels decreased and remained low (or with intermediate values) during the caring of fry, compared to PS stage. A similar pattern was observed in T and 11-KT plasma levels of male *chanchitas* kept in isolated pairs throughout the parental care period (Birba et al., 2015). Lower androgen levels in the presence of eggs and larvae in both sexes, compared to PS stage, might suggest a negative effect of androgens on parental care, irrespectively of its effect on egg production in females.

In female *chanchitas*, the lower sex steroids levels observed after spawning appeared to be regulated, at least in part, by the presence of the offspring, as removal of the eggs caused a strong endocrine response. Plasma concentrations of E_2 and T augmented by at least one order of magnitude compared to control levels after removal of the eggs, even surpassing PS values (*t*-test: E_2: $p < 0.001$; T: $p = 0.0083$; data were log-transformed). The index of T conversion to E_2 was approximately 0.5 during HL and SL stages of females which eggs had been removed, which indicates similar plasma concentrations for both steroids. On the contrary,

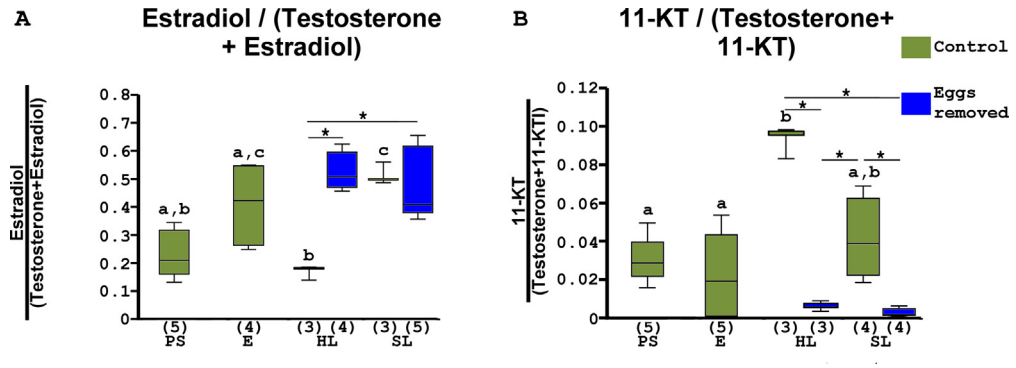

Fig. 4. Indexes of testosterone metabolization to 17-βEstradiol (A) or 11-ketotestosterone (B) at the reproductive and parental care stages: Pre-spawning female (PS), female with eggs (E), female with hatched larvae (HL), and female with swimming larvae (SL). Different letters indicate significant differences between control treatments. Asterisks denote significant differences within egg removed treatments and/or with their time-appropriate controls. Sample size (n) is shown between parentheses. Data from the index of conversion of T to 11-KT for the analysis of the effect of egg removal and from the index of metabolization of T to E_2, were log-transformed.

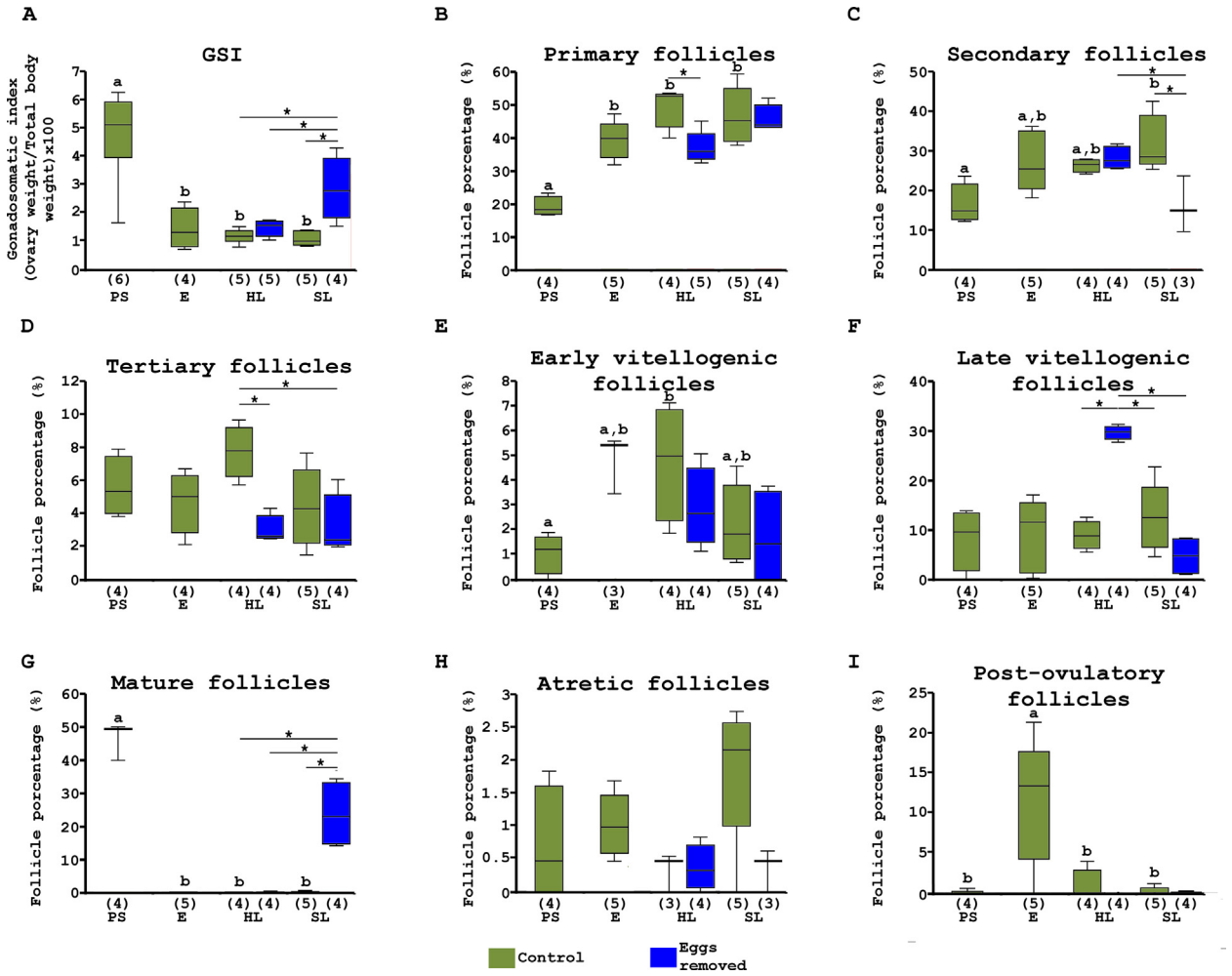

Fig. 5. Gonadosomatic index (GSI) and follicular composition of the ovaries through the reproductive and parental care stages: Pre-spawning female (PS), female with eggs (E), female with hatched larvae (HL), and female with swimming larvae (SL). Different letters indicate significant differences between control treatments. Asterisks denote significant differences within egg removed treatments and/or with their time-appropriate controls. Sample size (n) is shown between parentheses. Data from post-ovulatory follicles was sin-transformed for the analysis of eggs removed and female with eggs.

11-KT plasma levels remained unchanged compared to controls after elimination of the eggs, which suggests that its concentration in the general circulation was not associated to the regulation of parental care or ovary development. The index of conversion of T to 11-KT was maximal during HL stage, however, this did not let to an increase in 11-KT concentration. This could result from a decrease in T synthesis without change in the rate of its conversion to 11-KT. Synthesis of 11-KT was greatly reduced after elimination of the eggs compared to controls, probably due to the increased estrogen synthesis, leaving less T available for its oxidation into 11-KT. The shift towards an augmented E_2 synthesis probably mediates the acceleration observed in the rate of vitellogenesis in

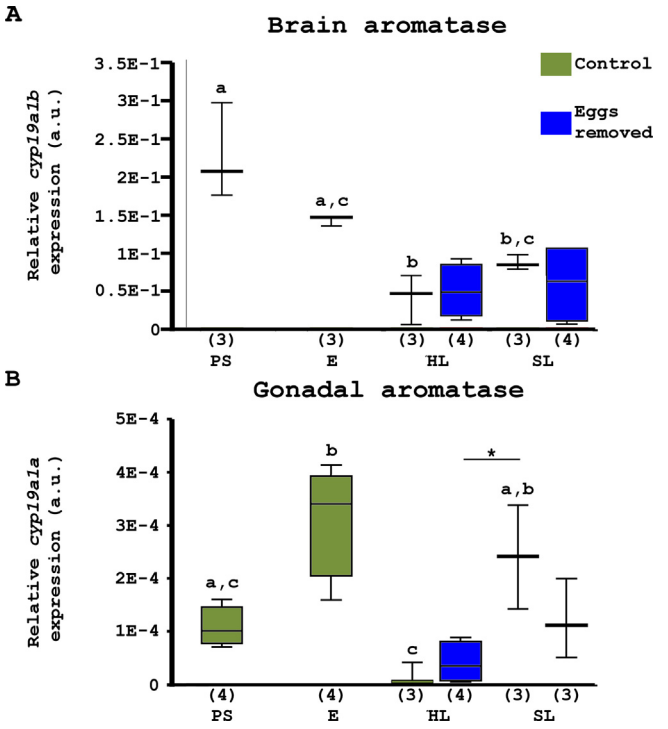

Fig. 6. Aromatases gene expression through the reproductive and parental care stages. Relative expression levels of brain aromatase, *cyp19a1b* (A) and gonadal aromatase, *cyp19a1a* (B) in forebrain and ovaries, respectively, of pre-spawning female (PS), female with eggs (E), female with hatched larvae (HL), and female with swimming larvae (SL). Different letters indicate significant differences between control treatments. Asterisks denote significant differences within egg removed treatments and/or with their time-appropriate controls. Sample size (n) is shown between parentheses.

the ovary through the well-established effect of E_2 on vitellogenin synthesis (Van Der Kraak, 2009). The males and females of isolated pairs were rarely aggressive towards each other after removal of the eggs, thus changes in plasma T and E_2 levels cannot be explained by the occurrence of aggression. However, we cannot rule out an effect of the stress caused by removing the eggs from the aquariums, which might have induced, for example, an increase in adrenal androgen synthesis.

Overall, these results pointed to a negative effect of the offspring over sex steroids synthesis. One may argue that the decrease in sex steroids observed might be the result of isolating the pair, in contrast to the higher levels present in group living PS *chanchitas*. However, while the social environment definitely affects *chanchitas* hormone levels (Alonso et al., 2011; Ramallo et al., 2015), and its isolation has been shown to result in a decrease in E_2 and 11-KT plasma concentration (Morandini et al., 2015), we believe the drop in sex steroids levels is mostly the result of the presence of the eggs and larvae, since: (a) removal of the eggs resulted in an increase of systemic T and E_2 specifically even in isolation; (b) a similar decrease in sex steroids at the onset of parental care has been reported in other species like *Sarotherodon melanotheron* (Specker and Kishida, 2000), *Acanthochromis polyacanthus* (Pankhurst et al., 1999) and *Porichthys notatus* (Knapp et al., 1999); (c) Varela et al. (2017) performed a detailed study of *chanchita*'s oogenesis and hormonal profiles during the parental care period in a more natural, group setting, and also described a drop in sex steroids levels after spawning. The inhibitory effect of the fry on maternal sex hormones quite possibly slows down follicular development at the ovaries, allowing energy resources to be allocated elsewhere, like the performance of behaviors that are absent previous to spawning (e.g. building of the nest, transporting and grouping the larvae) that occurs simultaneously with territorial aggression during the parental care period.

At the level of the ovaries PS females presented the largest ovaries relative to body size as indicated by the highest GSI. Accordingly, GSI negatively correlated with the percentage of primary follicles, and showed a positive association with the percentage of mature follicles, which suggests that in female *chanchitas* GSI is a good index of ovary maturity. The percentages of primary and mature follicles were lower and higher, respectively, in the PS stage compared to the other stages of the reproductive and parental care period. With the exception of post-ovulatory and mature follicles, all remaining states of folliculogenesis were present simultaneously at the four stages of parental care, highlighting the asynchronous nature of *chanchita*'s ovary. This pattern of ovarian composition is in agreement with that described for other species of cichlids like *Oreochromis niloticus* (Tacon et al., 1996) and *Tilapia zillii* (Coward and Bromage, 1998), and a previous study on *chachita* (Tubert et al., 2012). We did not find any association between sex steroids and the percentage of any of the follicular types analyzed, yet, Varela et al. (2017) observed a positive correlation between E_2 and late vitellogenic oocytes. Accordingly, in many teleost species, E_2 plasma levels correlated with vitellogenesis (Corriero et al., 2004; Kobayashi et al., 1988; Matsuyama et al., 1994). However, in maternal *chanchitas* up to 8 daf (the maternal period usually extends up to around 20 daf; Meijide and Guerrero, 2000), we observed a small increase in the percentage of early vitellogenic follicles (maximum percentage: 4.8% ± 1.2 at HL stage), thus it is quite possible that at the time of sampling ovaries were still quite early into vitellogenesis.

In line with the sex steroids surge after egg removal, ovary maturation was accelerated as we observed an increased percentage of late vitellogenic and mature follicles as early as 3 and 8 days post spawning, respectively. The augmented rate in folliculogenesis is believed to result from elimination of parental cost, and thus allow shorter time intervals between spawning events. The presence of the offspring represents a cost on parental breeding frequency as described for a number of teleost, where egg removal reduced the interval till next spawning (Kuwamura, 1986; Smith, 1993; Smith and Haley, 1987).

The expression of gonadal aromatase gene in the ovaries also varied across the parental care period, with maximal levels during E and SL stages. Surprisingly, we did not observe a correlation between gonadal aromatase gene expression and E_2 systemic plasma levels. This suggest that the ovaries would not be the main source of E_2 measured in systemic circulation, at least during the immediate pre-spawning period and the 8 days that followed, when vitellogenesis was reduced. Gonadal aromatase activity and its gene expression have been found to correlate only during vitellogenesis (Chang et al., 1997), and in *Carassius auratus*, *Opsanus tau* (Pasmanik and Callard, 1985), and *Epinephelus akaara* (Li et al., 2007), aromatase activity in the brain is far greater than that from the ovary, consequently it most likely represents the main source of estrogens, providing an equal concentration of T, the substrate of aromatase, in both organs. However, the expression of gonadal aromatase positively correlated with the index of T conversion to E_2 in female *chanchita*, which points at the ovary as the main source of systemic T aromatization. The elimination of the eggs did not affect gonadal aromatase gene expression, as levels did not differ from controls. This fact is contrast with the large increase in E_2 plasma levels, and suggests that (a) gonadal aromatase activity or protein synthesis were up-regulated without any change in aromatase expression, (b) changes in aromatase expression preceded sampling time and peaked rapidly after egg-removal or (c) the ovaries are note the only source of E_2 during this period.

At the forebrain, the expression of brain aromatase was highest during PS stage, intermediate at E stage, and lowest in the presence

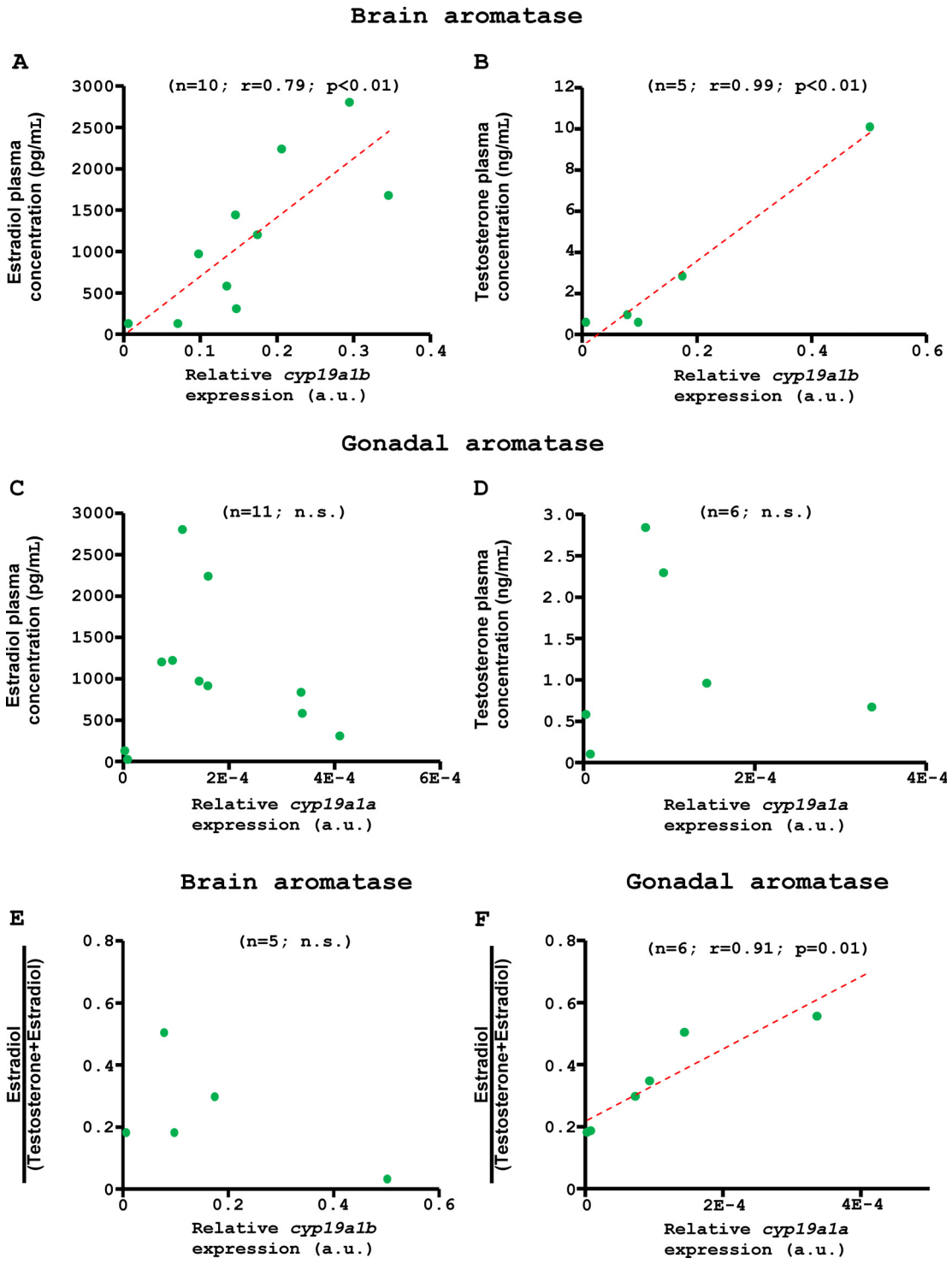

Fig. 7. Relationship between brain aromatase, *cyp19a1b*, (A), (B), (E) and gonadal aromatase, *cyp19a1a*, (C), (D), (F), relative expression with estradiol and testosterone plasma levels, and the index of testosterone aromatization, respectively. Sample size (n), Pearson's correlation coefficient (r), and *p*-value are shown between parentheses. Statistically non-significant correlations are noted as n.s.

of larvae (HL and SL). Removal of the eggs did not alter brain aromatase gene expression, suggesting it might not be involved in the regulation of parental behavior in these stages, and with the fish pair kept in isolation. However, we should mention that by analyzing whole forebrain samples, we analyzed the sum of all individual brain aromatase regulatory niches, which might counterbalance each other, resulting in an apparent lack of effect of the offspring on brain aromatase expression, even though it might not be the case if studied at a local level, particularly at the Vv and POA. Additionally, regulatory changes – if any – might be occurring at the level of enzyme synthesis and activity. For example, in male mice, parental behavior was associated to a specific increase in aromatase activity at the medial POA (Trainor et al., 2003). The absence of correlation between the expressions of both aromatases points to somewhat independently regulated systems.

The higher brain aromatase gene expression at PS stage and it downregulation with the onset of maternal behavior, may be in line with the decrease observed for sex steroids and folliculogenesis. On

one hand, PS fish were obtained from community aquaria, and thus we cannot rule out an effect of the social environment on brain aromatase gene expression, which has been linked to aggressive behavior in territorial male cichlids (Huffman et al., 2013). On the other hand, brain aromatase gene from various studied teleost, including those from several cichlids (Böhne et al., 2013), present an estrogen response elements (EREs) in its promoter region (Diotel et al., 2010). In fact, the high *cyp19a1b* expression levels in the brain are thought to result from its regulation through a feedback loop of estrogens (Callard et al. 2001; Diotel et al. 2010). Brain aromatase gene expression may also be subject to up-regulation by androgens after their aromatization to estrogens (Mouriec et al., 2009). In female *chanchita*, we found that brain aromatase gene expression positively correlated with E_2 and T plasma levels, thus a decrease in sex steroids is likely to drag brain aromatase behind. However, the fact that brain aromatase expression was unaltered after eggs removal, denotes a possible change in brain estrogen sensitivity, with a decreased response to the elevated E_2 and T plasma levels, highlighting its independent regulation.

6. Conclusions

In female *chanchitas* the enzyme brain aromatase is strongly expressed in brain areas associated with the regulation of parental behavior across vertebrates. The onset of maternal behavior was accompanied by a decrease in brain aromatase forebrain gene expression levels, and a specific down-regulation of sex steroids plasma levels and the rate of folliculogenesis, as exposed by the effect of removing the offspring. The elimination of the cost of parental care by removal of the eggs, resulted in a rapid and drastic investment in ovarian follicle maturation, highlighting the physiological plasticity of the multiple-spanwner *chanchita*, as it probably prepares for a future reproductive event. We did not, however, find any evidence which suggests a role of forebrain brain aromatase and ovarian gonadal aromatase gene expression on the regulation of maternal behavior in *chanchita.* Nonetheless, we cannot rule out the occurrence of local regulatory effects by confined changes in brain aromatase expression, and changes in peptide synthesis and/or activity of both aromatases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2017.08.009.

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