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Temperature- and genotype-dependent stress response and activation of the hypothalamus-pituitary-interrenal axis during temperature-induced sex reversal in pejerrey *Odontesthes bonariensis,* a species with genotypic and environmental sex determination

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1 Temperature- and genotype-dependent stress response and activation of the
2 hypothalamus-pituitary-interrenal axis during temperature-induced sex reversal in
3 pejerrey *Odontesthes bonariensis,* a species with genotypic and environmental sex
4 determination

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18 **Abstract**

19 In the pejerrey *Odontesthes bonariensis* (Atheriniformes, Atherinopsidae), exposure to high and
20 low temperatures during the critical period of sex determination (CPSD) induce testicular and ovarian
21 differentiation, respectively, regardless of the presence or not of the sex determining gene *amhy*, which is
22 crucial for testis formation only at intermediate, sexually neutral temperatures. In this study we explored
23 the existence of genotype-specific signaling of Crh (Corticotropin Releasing Hormone) family genes and
24 their associated carrier protein, receptors, and other stress-related genes in response to temperature during
25 the CPSD and the potential involvement of the central nervous system via the hypothalamus-pituitary-
26 interrenal (HPI) axis in sex determination of this species. The Crh family genes *crhb*, *uts1*, *ucn3*, the
27 receptor *crhr1* and the stress-related genes *gr1*, *gr2*, *nr3c2* were transiently upregulated in the heads of
28 pejerrey larvae during the CPSD by high temperature alone or in combination with other factors. Only *crhr2*
29 transcript abundance was not influenced by temperature but independently by time and genotype. In most
30 cases, mRNA abundance was higher in the XX heads compared to that of XY individuals. XX larvae also
31 showed higher whole-body cortisol titers than the XY, downregulation of *cyp19a1a* and upregulation of
32 the testis-related genes *amhy*/*amha* in trunks (gonads) and were 100% masculinized at the high temperature.
33 In contrast, at the low temperature, *crhbp* and *avt* were upregulated in the heads, particularly the former in
34 XY larvae. *cyp19a1a* and *amhy*/*amha* were up- and downregulated, respectively, in the gonads, and fish
35 were 100% feminized. Signaling via the HPI axis was observed simultaneously with the first molecular
36 signs of ongoing sex determination/differentiation in the gonads. Overall, the results strongly suggest a
37 temperature-dependent, genotype-specific regulatory action of the brain involving the Crh family of stress-
38 related genes on the process of environmental sex determination of pejerrey.

39

40 *Key words:* Crh family, stress-related genes, hypothalamus, brain sex differentiation, cortisol, gonadal sex
41 differentiation, environmental sex determination, genotypic sex determination

42

43 **1. Introduction**

44 In fish, genetic and environmental factors may interact to determine gonadal fate (Hattori et al.,
45 2020). Genetic factors establish the genotypic sex at the time of fertilization while environmental factors
46 act later during the critical period of sex determination (CPSD) when the gonad is still bipotential. Strong
47 environmental stimuli transduced as physiological stress (e.g., high temperature, hyper salinity, crowding)
48 can override the genetic predisposition and produce individuals with mismatching genotypic and
49 phenotypic sex. Despite the growing attention that environmental stress on fish sex determination have
50 received in the last decades, their interaction with genetically programmed processes are still elusive.
51 Environmentally induced gonadal sex determination (particularly masculinization) in fish seems to involve
52 a stress reaction and cortisol (Hattori et al., 2020; Strüssmann et al., 2021), epigenesis (e.g., methylation;
53 Piferrer, 2013; Valdivieso et al., 2021), or oxidative stress (Corona-Herrera et al., 2018; Mukai et al., 2022).
54 In the former, which occurs in pejerrey *Odontesthes bonariensis* and the Japanese medaka *Oryzias latipes*,
55 exposure to a stressful stimulus like excessive heat causes a rise in circulating cortisol, leading to a blockade
56 of the ovarian differentiation signaling pathway and activation of genes of the testis-developing cascade
57 (Fernandino et al., 2013; Goikoetxea et al., 2017). This occurs because high circulating cortisol levels lead
58 to the activation of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD), which breakdowns cortisol
59 and at the same time synthetizes 11-ketotestosterone (11-KT) as a by-product (Fernandino et al., 2013).
60 Thus, the mechanism of cortisol-induced masculinization in the gonads is somehow understood, but the
61 upstream regulation of this process in the brain and pituitary has received little attention so far (Fernandino
62 et al., 2012; Hattori et al., 2009). In a recent study in *O. latipes*, Castañeda-Cortés et al. (2019) showed that
63 exposure to heat during early development led to the upregulation of the corticotropin-releasing hormone
64 b (*crhb*) and its receptors *crhr1* and *crhr2* in the brain, causing masculinization of genotypic females. By
65 knocking out the receptors, it was possible to null the masculinizing effect of heat whereas concomitant
66 administration of the downstream effector of the hypothalamus-pituitary-interrenal (HPI) axis, cortisol,
67 rescued the knockout effect, unquestionably implicating the brain in thermal-induced masculinization via
68 the HPI axis (Castañeda-Cortés et al., 2019).

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69 The pejerrey *O. bonariensis* (Atheriniformes, Atherinopsidae) is a fish species in which genotypic
70 sex determination (GSD) coexists with a marked temperature-dependent sex determination (TSD)
71 (Yamamoto et al., 2014). In this species, all-male progenies are obtained at high temperatures (male
72 promoting temperatures, MPT) and all-female progenies at low temperatures (female promoting
73 temperatures, FPT). Intermediate temperatures produce varied proportions of males and females (mixed-
74 sex promoting temperatures, MixPT), reflecting the combined effects of TSD and GSD, whose main player
75 is the Y chromosome-linked Anti-Müllerian-hormone gene (*amhy*) (Yamamoto et al., 2014; Zhang et al.,
76 2018). Earlier studies in this species have pointed out the possibility that gonadal sex determination, and
77 more specifically the effects of temperature, could start in the brain (Miranda et al., 2003; 2001). These
78 studies unveiled the increased abundance of gonadotropin releasing hormone 1 (Gnrh1)-, follicle
79 stimulating hormone (Fsh)-, and luteinizing hormone (Lh)-secreting immunoreactive cells in the brain and
80 pituitary (Miranda et al., 2003; 2001) and the presence of Fsh and Lh receptors in the undifferentiated
81 gonads before the first signs of gonadal differentiation (Shinoda et al., 2010) during the CPSD. Other studies
82 showed that the expression of brain aromatase (*cyp19a1b*) in the head preceded that of gonadal aromatase
83 (*cyp19a1a*) in the trunk of undifferentiated larvae (Karube et al., 2007; Strobl-Mazzulla et al., 2008) and
84 that the brains of pejerrey larvae during the CPSD at a masculinizing temperature had more abundant
85 *cyp19a1b* transcripts than at a feminizing temperature (Strobl-Mazzulla et al., 2008). It has been also shown
86 that Kisspeptin-encoding gene *kiss2* levels increased during the CPSD at MPT conditions (Tovar-
87 Bohórquez et al., 2017). Finally, a stress response and cortisol have been implicated in TSD as well as other
88 forms of ESD of pejerrey (Fernandino et al., 2013, 2012; García-Cruz et al., 2020; Hattori et al 2009) but
89 the molecular players involved in this process and how they are influenced by the genotype and the
90 environment remain unknown.

91 In fish, the stress response is a complex process mediated by neuropeptides, neurotransmitters, and
92 receptors. One group of genes key in the stress response belongs to the so-called Crh (corticotropin releasing
93 hormone) family, formed by the corticotropin-releasing hormone (*crh)*, urotensin 1 (*uts1)* (urocortin 1 in

94 mammals), urocortin 2 (*unc2),* and urocortin 3 (*ucn3)*, neuropeptides that operate through two different G
95 protein-coupled transmembrane receptors: corticotropin-releasing hormone receptor 1 (*crhr1*) and
96 corticotropin-releasing hormone receptor 2 (*crhr2*). The corticotropin-releasing hormone-binding protein
97 (encoded by the *crhbp* gene) also plays an important role in the stress response by sequestering/releasing
98 some of these ligands. Crh and Uts1 bind to Crhr1 and to Crhr2, which in turn provides binding sites for
99 Ucn2 and Ucn3. Thus, Crhr2 is a promiscuous receptor capable of mediating the transduction of multiple
100 signals (Lovejoy et al., 2014; Inda et al., 2017). In addition to Crh family members, arginine vasotocin
101 (Avt) is another neuropeptide that participates in the stress response (Baker et al., 1996; Gesto et al., 2014;
102 Martos-Sitcha et al., 2019). Many of these peptides have central effects, regulating stress and anxiety in
103 different regions of the brain, while others such as *pomc* (proopiomelanocortin) encoding
104 adrenocorticotropic hormone (Acth), expressed in the pituitary are necessary to activate HPI axis and
105 trigger the hormonal (sympathetic) stress response leading to the synthesis and release of cortisol. Finally,
106 the glucocorticoid receptors 1 and 2 (GR1 and GR2, respectively) (*gr1* and *gr2*) and a mineralocorticoid
107 receptor (MR) encoded by the nuclear receptor subfamily 3 group C member 2 (*nr3c2*) are the final targets
108 of cortisol, mediating the physiological actions of this steroid and playing multiple roles in feedback
109 mechanisms and developmental programming (Faught and Vijayan, 2018; Gjerstad et al., 2018; Nesan and
110 Vijayan, 2013).

111 Based on the above evidence for pejerrey and on similar studies in other species such as the
112 European seabass *Dicentrarchus labrax* and the Senegalese sole *Solea senegalensis* (Baroiller et al., 2016;
113 Moles et al., 2007; Guzmán et al., 2009), it is hypothesized that the brain plays a crucial role in the
114 transduction of environmental signals during the sex determination and differentiation in pejerrey. The
115 pejerrey has a different master sex determining gene (*amhy* vs. *dmy*/*dmrt1Y*) and a much more marked TSD
116 than medaka which is operational at environmentally relevant temperatures, and therefore provides an
117 excellent model to corroborate and extend these findings. In this study, we took advantage of the *amhy* gene
118 as a marker of genotypic sex to further explore the involvement of the central nervous system in the sex

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119 determination of this species and the possibility of a genotype-based dimorphism in thermal stress response
120 during the CPSD. First, we analyzed the expression profile of the Crh family genes (*crh*, *uts1*, *ucn2*, *ucn3)*,
121 their receptors *crhr1* and *crhr2*, the carrier protein *crhbp*, the neuropeptide *avt* and the expression of
122 associated genes such as *pomc*, *gr1* and *gr2* as well as *nr3c2* as the targets of cortisol signaling during the
123 CPSD at masculinizing and feminizing temperatures in relation to genotype. We also determined the
124 localization of the mRNAs of selected genes in the brain and analyzed the whole-body cortisol titers in XX
125 and XY larvae during the CPSD to establish a functional link between the HPI axis and the developing
126 gonads. Finally, we compared the temporal expression of the stress-related genes in the brain with that of
127 brain aromatase (*cyp19a1b*) and of molecular markers of sex differentiation in the gonads, including *amhy*,
128 *amha*, and *cyp19a1a*, to establish the hierarchical relationship between the brain and the developing gonads
129 during the CPSD.

130

131 **2. Materials and Methods**

132 *2.1. Source of animals and sampling procedures*

133 All the organisms used in this study were handled and sacrificed according to the Guide for the
134 Care and Use of Laboratory Animals from Tokyo University of Marine Science and Technology
135 (TUMSAT), Japan and the Guide for the Care and Use of Laboratory Animals of the National Research
136 Council (8th edition).

137 Fertilized eggs of pejerrey were obtained by natural spawning from a single-pair cross of an XX
138 female and a XY male of the Yasuda strain kept at the Aquatic Animal Rearing Facilities of TUMSAT,
139 Shinagawa Campus. Eggs were incubated at 17°C until hatching and newly hatched larvae were transferred
140 into 55 L aquaria for rearing at FPT (17°C) and MPT (29°C) for production of all-female and all-male
141 progenies, respectively. All rearing tanks were supplied with dechlorinated tap water (ca. 0.5 L/ hour) with
142 constant aeration. Salinity was maintained at 0.1–0.2% (NaCl) and a constant photoperiod was set to 14 h

light/10 h darkness with a light intensity of about 700 Lx at the surface of the water following previous studies. Larvae were fed with *Artemia nauplii* three times a day for 7 weeks after hatching (wah) and gradually weaned thereafter to a mixture diet of TetraMin® flakes for ornamental fish and a commercial feed for aquaculture (Otohime B1; particle size <0.36 mm, Marubeni Nisshin Feed Co., Tokyo).

Larvae (n=8–12) were sampled at 2, 4, and 6 wah, to cover the CPSD, at MPT and FPT for Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) and *in situ* hybridization (ISH) analyses. Fish were euthanized by a combination of hypothermia in ice-water with an anesthetic overdose of 2-Phenoxyethanol (Fujifilm Wako, Osaka, Japan). The standard length (SL) and body weight (BW) of each fish were recorded to the nearest 0.1 mm and 0.1 mg, respectively. A piece of the tail of each fish was stored in absolute ethanol for sex genotyping (see below). Samples of the head and trunk of each larva were kept in RNA*later*™ solution (Thermo Fisher Scientific, Waltham, MA, USA) in separate vials at -80°C until used. Heads for ISH were fixed in Bouin's fixative solution for 24 hours and transferred to 70% ethanol. All remaining fish were sampled at 20 wah for identification of phenotypic and genotypic sex for determination of sex ratios as described below.

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2.2. Identification of genotypic and phenotypic sex in weekly samples and remaining fish at termination

Determination of the genotypic sex of each fish followed the procedures and *amhy* primers described in Yamamoto et al. (2014). Briefly, 1 µL of extracted DNA and 9 µL of PCR mixture were added per reaction tube. The amplification of *amhy* gene was amplified using specific primers (Supplementary Table S1) and performed with 50 ng of DNA and KOD FX Neo kit DNA polymerase (Toyobo, Osaka, Japan). *β-actin* (Supplementary Table S1) was used as control gene. PCR products were electrophoresed in 1% agarose gel and visualized with WSE 5200 Print graph 2M Gel Documentation System (ATTA, Taito, Tokyo). XX and XY genotypes were determined based on the absence or presence of the *amhy* amplification product, respectively.

166 The phenotypic/genotypic sex of each remaining fish at termination was analyzed to confirm the
167 rates of feminization and masculinization at the FPT and MPT. The trunks were fixed in Bouin's fixative,
168 dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin (Paraplast
169 Plus, McCormick Scientific, Richmond, IL) in an automatic tissue processor (HistoCore PEARL, Leica
170 BioSystems, Germany). Specimens were sectioned at 6 µm thickness in a rotatory microtome (Microm HM
171 325, Thermo Scientific, Massachusetts) and stained with hematoxylin-eosin. Histological criteria to identify
172 ovaries or testes were based on Ito et al. (2005).

173 *2.3. Determination of the sequences of the target genes*

174 The sequences of all studied genes were obtained from Next Generation Sequencing (NGS) data
175 from a parallel study (Torres-Martínez, unpublished results). Total RNA from heads and trunks of 2 wah
176 pejerrey larvae was extracted with Trizol® Reagent (Ambion, Carlsbad, California) according to
177 Fernandino et al. (2008). One microgram of total RNA per sample was used for DNA synthesis. Samples
178 were sequenced by DNA Nanoball Sequencing (DNBSEQ™) platform developed by the Beijing Genomics
179 Institute in a DNBSEQ-T7 sequencer. Filtering of the data was performed with the software Trimmomatic
180 (Bolger et al., 2014). Paired reads were further used for The De Novo Assembly of the Transcriptome,
181 which was performed by Trinity v. v2.14.0 (Grabherr et al., 2011). The sequences obtained were analyzed
182 using Genetyx® v.11 (Supplementary Table S2) and the nucleotide and deduced amino acid sequences
183 were deposited in GenBank.

184 *2.4. Quantification of mRNA concentration by Reverse Transcriptase Quantitative Polymerase Chain*
185 *Reaction (RT-qPCR)*

186 Total RNA was isolated from heads and trunks of pejerrey larvae. Samples were transferred to
187 Trizol® Reagent and homogenized in a tissue homogenizer (Precellys 24, Bertin Technologies Montigny-
188 le-Bretonneux, France). RNA extraction, removal of genomic DNA, and cDNA synthesis was carried out
189 as reported previously by Fernandino et al. (2008).

190 Primers for quantification of Crh family, stress-related genes, and *cyp19a1b* in the heads and those
191 used to quantify gonadal markers of sex determination were designed with NCBI Primer Blast Tool
192 (Supplementary Table S1). All amplifications were performed in 15 µL reaction volumes containing 2 µL
193 of first-strand cDNA, 0.3 µL of forward and reverse primers, and 7.4 µL of PowerUp™ SYBR™ Green
194 Master Mix (Thermo Fisher Scientific). For the analysis of *amhy* and *amha*, TaqMan probes (Applied
195 Biosystems, Singapore) were used with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific).
196 Transcript relative quantification was performed by the standard curve method with eight serial plasmid
197 dilution points. Expression levels of the target genes were normalized against the reference gene β-*actin*.
198 Quantification of mRNA abundance was run in duplicate in a Step One Plus Real Time PCR System
199 thermocycler (Applied Biosystems, Singapore).

200 *2.5. Localization of transcripts by chromogenic in situ hybridization*

201 The spatial distribution of genes upregulated in the brain of pejerrey larvae during the CPSD was
202 examined by *in situ* hybridization. RNA probes for the target genes were amplified by RT-PCR using cDNA
203 synthesized from brain of adult pejerrey, using ISH-specific primers to obtain products ranging from 400
204 to 700 bp (Supplementary Table S1). The purified PCR product was ligated into the pGEM-T Easy Vector
205 System (Promega Corporation, Madison, WI). The orientation of inserts (sense and antisense) was
206 determined by linearization with SpeI restriction enzyme (New England Biolabs Inc., US) followed by
207 plasmid electrophoresis in 1% agarose gel. Dixogenin-11-UTP-labeled riboprobes were synthetized with
208 SP6 Polymerase (Roche, Mannheim, Germany) to generate sense and antisense probes. Sense riboprobes
209 were used as a control. Heads of 2 and 4 wah XX pejerrey larvae were dehydrated in ascending
210 concentrations of ethanol, cleared in xylene, and embedded in paraffin (Paraplast Plus, McCormick
211 Scientific, US) and sectioned transversally at 6 µm thickness. All pre- and post-hybridization procedures
212 were performed according to Sarida et al. (2019).

213 *2.6. Cortisol measurements*

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214 Whole-body cortisol titer in larvae was measured using the EIA Cortisol Express Kit (Cayman
215 Chemicals, Ann Arbor, USA). Cortisol extraction was conducted according to a standard procedure used
216 in our laboratory for the extraction of hormones from pejerrey larvae (Hattori et al., 2009). Briefly, single
217 larvae were homogenized in PBS followed by three extractions with diethyl ether and centrifugation at
218 2500 rpm. Homogenates were kept at -80°C during 30 min in order to recover the diethyl ether based-phase.
219 Afterwards, diethyl ether was evaporated under N_2 and the extract reconstituted in 500 µl of EIA buffer.
220 Samples were analyzed in a multimode plate reader SpectraMax iD3 (Molecular Devices, Silicon Valley,
221 California). The recovery rate was estimated by the spike method and found to be superior to 90%. The
222 intra-assay and inter-assay coefficients of variation ranged from 4 to 14%.

223 *2.7. Phylogenetic and statistical analysis*

224 To determine the phylogenetic relationship among the different neuropeptides (ligands) of the Crh
225 family (*crhb*, *uts1*, *ucn2*, and *ucn3*), we first inferred the amino acid sequences using the bioinformatic
226 software Genetyx® 11 (Genetyx Corporation, Tokyo, Japan). Alignment of the deduced peptides was
227 conducted using MUSCLE logarithm. The phylogenetic trees were constructed with the Neighbor Joining
228 Method with 100 bootstraps, using the software Geneious Prime Version 2023.0.4 (Biomatters, New
229 Zealand). For the analysis of the Crh peptides (Crhb, Uts1, Ucn2, Ucn3), the diuretic hormone-like peptide
230 (DHLP) from the tunicate *Ciona intestinalis* was used as an outgroup, based on Grone et al. (2021).
231 Regarding the Crh receptors Crhr1 and Crhr2, we used the diuretic hormone receptor (Dh31R) of the fly
232 fruit *Drosophila melanogaster* (NP_001260951.1). No outgroup was used for the glycoprotein Crhbp, due
233 to the evolutionary conservation of this gene from invertebrates to vertebrates (Westphal and Seasholtz,
234 2006).

235 The RNA transcript levels of each gene were expressed as the mean ± SEM (standard error of the
236 mean) and the statistical analysis was performed using GraphPad Prism Version 9 (GraphPad, San Diego,
237 CA). A Three-Way-ANOVA was used to test the effects of time from hatching, temperature, and genotype
238 as well as their interactions (second and third order interactions) on the mRNA abundance of all the target

239 genes (except *amhy*), followed by Tukey's multiple comparison test. Because *amhy* is present only in XY
240 individuals, only two factors, namely, temperature and time from hatching were considered and *amhy*
241 expression was analyzed by Two-Way ANOVA. Cortisol titers were also analyzed by Two-Way ANOVA
242 followed by a Tukey's post hoc test. In all cases, differences were considered statistically significant at
243 $p<0.05$.

244 **3. Results**

245 *3.1. Phenotypic and genotypic sex ratios at masculinizing and feminizing temperatures*

246 Analysis of phenotypic sex by histology showed that rearing fish at MPT and FPT resulted in 100%
247 masculinization and feminization, respectively (Table 1). The proportions of XX and XY fish were nearly
248 50% at both temperature regimes.

249 *3.2. Structure and phylogenetic analysis of the Crh family members*

250 The deduced amino acid sequences of *crhb*, *uts1*, u*cn2,* and *ucn3* are shown in Supplementary
251 Figure S1. Multiple attempts to clone *crha* using degenerated primers based on conserved nucleotide
252 sequences from several related teleosts species fail to amplify this gene. In addition, no reads of *crha* were
253 detected after the De Novo Assembly of NGS data. The amino acidic sequences of *crhb*, *uts1*, u*cn2,* and
254 *ucn3* consist of a signal peptide of 25, 18, 28, and 23 amino acids in the N-terminus, respectively, and a
255 mature peptide with 41, 41, 38, and 39 amino acids in the C-terminus, respectively. Mature peptides were
256 flanked by dibasic- (*crhb*, *uts1*, and *ucn3*) or monobasic amino acids (*ucn2*) in the N-terminus and by a
257 putative amidation signal in the C-terminus (Supplementary Fig. S1A). The % of identity between Crhb
258 and Ust1 (Crh-related subgroup) and between Ucn2 and Ucn3 (Urocortin subgroup) was similar (53% and
259 55%, respectively). However, the similarity between the members of these two groups was much lower.
260 Compared to Crhb and Uts1, Ucn3 showed only 19% and 22% of similarity, respectively. For Ucn2, the
261 percentage of identity with Crhb and Uts1 was 21 and 19%, respectively (Supplementary Fig. S1B). The
262 phylogenetic analysis of the inferred amino acid sequences showed the existence of two clades within the

263 Crh family, one clade formed by *crhb* and *uts1* and another one formed by *ucn2* and *ucn3* (Supplementary
264 Fig. S2).

265 *3.3. Expression of CRH family and stress-related genes in the heads of pejerrey larvae during the CPSD*

266 A summary of the results of the Three-Way ANOVA conducted on the transcript levels of genes related to
267 the stress response is provided in Table 2. The internal control, ß-actin, used for the normalization of gene
268 expression data did not show statistical variations across treatments (Supplementary Fig. S3). The
269 expression patterns of the different groups of genes analyzed are described below.

270 *3.3.1. Crh family (crhb, uts1, ucn2, ucn3)*

271 The transcript levels of *crhb* in XX larvae peaked at 4 wah to 6 wah in the MPT and at 6 wah in
272 the FPT (Fig. 1A). *crhb* mRNA abundance at 4 wah in MPT was significantly higher than in the same week
273 at FPT. XX larvae generally had higher *crhb* expression than XY regardless of temperature. *crhb* transcripts
274 in XY individuals were similar on all weeks and in both temperature regimes. Temperature, genotype, and
275 time from hatching all had an effect over *crhb* expression (Table 2). The expression of *uts1* did not vary
276 with time after hatching or with genotype (Fig. 1B). However, levels were generally higher in the MPT
277 compared to the FPT and inter-temperature differences were significant for XX larvae at 2 and 4 wah.
278 Temperature and genotype influenced the expression of *uts1,* but most of the variation was explained by
279 temperature (Table 2). Expression of *ucn2* in XX larvae at the MPT was highest at 2 wah and then decreased
280 (Fig. 1C). Neither XX larvae at the FPT nor XY larvae at both temperatures showed any significant changes
281 in *ucn2* transcript levels and there were no significant differences between genotypes. Changes in *ucn2*
282 were largely determined by time from hatching and temperature-genotype interaction (Table 2). *ucn3*
283 transcript levels were statistically higher in XX than XY larvae in the MPT at 2 wah (Fig. 1D). Besides this,
284 there were no other significant differences in relation to time, genotypic sex or temperature. Genotype and
285 the interaction of time from hatching and temperature had the most effect on the mRNA abundance of *ucn3*
286 (Table 2).

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287 *3.3.2 crh receptors*

288 *crhr1* was transiently upregulated at 4 wah in both genotypes at the MPT (Fig. 1E). No other
289 significant differences were observed regarding genotype, time after hatching or temperature regime. Most
290 of the variation in *crh1* expression was explained by time and to a lesser extent by the interaction of
291 temperature with the former (Table 2). *crhr2* expression was significantly higher in XX than in XY larvae
292 in the MPT at 4 wah (Fig. 1F). mRNA levels of *crhr2* did not show any other significant changes with
293 genotype, week, or temperature. Genotype and time from hatching explained most of the variation in *crhr2*
294 expression (Table 2).

295 *3.3.3 Other stress-related genes (crhbp, avt, pomc, gr1, gr2, nr3c2)*

296 *crhbp* was upregulated in XY larvae at 2 wah in the FPT compared to the same genotype at other
297 weeks and to XX larvae at the same week (Fig. 2A). At 2 wah, XY larvae at the FPT also had significantly
298 higher *crhbp* transcript levels than the same genotype at the MPT. The most significant effects on *crhbp*
299 expression were by time from hatching and temperature-genotype interaction (Table 2). *avt* expression in
300 XY larvae at the MPT increased significantly at 6 wah (Fig. 2B). At the FPT, there was also an increase in
301 *avt* mRNA levels between 4 and 6 wah but in XX larvae. Larvae of both genotypes had higher mRNA
302 levels of *avt* at the FPT than those at MPT on almost all weeks. Temperature and time after hatching had
303 the most pronounced effects on the expression profile of *avt* (Table 2). The transcript levels of the Acth
304 precursor *pomc* did not show any consistent trend with time from hatching, temperature, or genotype (Fig.
305 2C) but the statistical analysis indicated a significance effect of the interaction of time and temperature
306 (Table 2). The GR *gr1* and *gr2*, and the MR *nr3c2* showed relatively similar trends including temporary
307 upregulation in the MPT at some time during the CPSD, but differed in which genotype was upregulated.
308 The mRNA abundance at the FPT, on the other hand, generally remained low. *gr1* was upregulated in XY
309 larvae at 4 wah and values were significantly higher also compared to larvae of the same genotype in the
310 FPT (Fig. 2D). Most of the variation in *gr1* expression was explained by the interaction time-temperature
311 and secondarily by temperature (Table 2). *gr2* on the other hand, was upregulated in XX larvae at 2 wah,

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312 with significantly higher levels than the same genotype on other weeks at the MPT and 2 wah in the FPT.
313 The 2 wah XX larvae in the also had significantly higher transcript levels than XY larvae on the same week
314 (Fig. 1E). Variation in the transcripts of *gr2* resulted mainly from the effects of time from hatching,
315 temperature, and their interaction (Table 2). *nr3c2* showed essentially the same pattern as *gr2* (Fig. 1F) but
316 the major source of variation included the effects of temperature and the interactions of time with
317 temperature and genotype (Table 2).

318 *3.4. Localization of crhb, ucn3, crhr1, and crhr2 in the brain of pejerrey larvae during the CPSD*

319 The mRNA distribution of some of the upregulated genes at the MPT (*crhb*, *ucn3*, *crhr1*, *crhr2*) in
320 the diencephalon (in areas related to the neuroendocrine regulation of the pituitary gland) of XX larvae is
321 shown in Fig. 3 and their negative controls (sense probes) are depicted in Supplementary Fig. S4. *crhb* was
322 identified in the medio-basal hypothalamus in the ventral zone of the tuberal hypothalamus, neighboring
323 the pituitary gland (Fig. 3A). Transcripts of *ucn3* were detected in the dorsal hypothalamus, in the upper
324 part of the nucleus posterioris tuberis (Fig. 3B). Analysis of entire head sections with ISH confirmed the
325 absence of mRNA transcripts of *crhb* and *ucn3* in all other tissues including the gills (Supplementary Fig.
326 S5). Expression of *crhr1* was detected in the nucleus recesus lateralis in the dorsal zone of the tuberal
327 hypothalamus (Fig. 3C). *crhr2* was not detected in the diencephalon of pejerrey larvae despite multiple
328 attempts to localize it by ISH (Fig. 3D). However, using the same anti-sense probes, *crhr2* mRNAs of this
329 gene were detected in the in the caudal zone of the periventricular hypothalamus and pituitary gland of 24
330 wah pejerrey juveniles (Supplementary Fig. S6). The distribution of *crhb*, *ucn3*, and *crhr1* mRNAs are
331 summarized in figure 3F.

332 *3.5. Whole-body cortisol titers during the CPSD at the MPT*

333 The changes in cortisol titers are shown in Fig. 4. Cortisol titers were maximal at 2 wah and
334 decreased significantly with time in XY larvae but not in the XX. Cortisol titers were significantly higher
335 in XX than in XY larvae at 4 wah.

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336 *3.6. Expression of cyp19a1b in the heads and of genetic markers of sex differentiation (amha, amhy,*
337 *cyp19a1a) in the trunks of pejerrey larvae during the CPSD*

338 The mRNA abundance of *cyp19a1b* in the heads was significantly higher at the MPT
339 compared to the FPT and in XY larvae compared to XX larvae at 2 wah (Fig. 5). The transcripts
340 levels in the heads of XY larvae at the MPT decreased and then increased between 2 and 6 wah
341 whereas those at the FPT as well as those of XX larvae in both conditions did not change.

342 The results of the Two-Way ANOVA of mRNA abundance for the gonadal markers of sex
343 differentiation are summarized in Table 2. The expression of *amhy* was significantly higher in XY larvae
344 at the MPT compared to the FPT at 2 and 6 wah (Supplementary Fig. S7A). Time and temperature had a
345 significant effect on *amhy* expression (Table 2). *amha* also had higher transcript levels at the MPT compared
346 to the FPT but a significant difference was noted for both genotypes only at 4 wah. Expression of *amha*
347 peaked at 4 wah in XY larvae (Supplementary Fig. S7B). Variation in *amha* transcript levels was explained
348 mainly by time from hatching, temperature, and their interaction (Table 2). The female marker *cyp19a1a*
349 showed low mRNA values at the MPT and moderately high values at the FPT (Supplementary Fig. S7C).
350 The differences between the MPT and the FPT were significant on all weeks for XX larvae but not for XY.
351 At the FPT, XX larvae had higher expression of *cyp19a1a* than XY larvae at 2 and 6 wah. Time, temperature,
352 genotype, and the interactions between genotype and time or temperature explained much of the variation
353 in the expression profiles of *cyp19a1a* (Table 2).

354

355 **4.0. Discussion**

356 The role of stress as a trigger of environmentally induced masculinization in pejerrey is well
357 established (Fernandino et al., 2012; Hattori et al., 2009). Despite the importance of this process for pejerrey
358 and other species in the context of climate change, the integration and transduction of environmental cues
359 by the brain and their interactions with genotypic sex during sex differentiation are poorly understood. Here,

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360 we examined the possibility of a genotype-biased expression of Crh family genes, their receptors, and other
361 stress-related genes in the brain during the CPSD of pejerrey at feminizing and masculinizing temperatures.
362 The overarching hypothesis was that the brain, and more specifically the HPI axis, played a significant,
363 genotypically dimorphic role in the coordination of the process of environmentally induced sex reversal in
364 this species.

365 Of all the CRH family members, Crh is considered the master trigger of the stress response (Inda
366 et al., 2017). Most teleosts have two *crh* paralogs, namely *crha* and *crhb*. The former was restricted to the
367 fish retina, but recent research has shown that both *crha* and *crhb* can be expressed either in retina or in
368 brain (Grone and Maruska, 2015). In this study, *crhb* was identified, but attempts to characterize *crha* with
369 degenerated primers and cDNA (from eyes and brains) or through the analysis of the brain transcriptome
370 failed to identify this gene in pejerrey (Torres-Martínez, unpublished results). We are currently screening
371 the pejerrey genome in a further attempt to locate it, however, it is important to consider that the existence
372 of redundant functions between these two paralogs has led to the loss of *crha* as in some teleost species
373 (Cardoso et al., 2016). The deduced amino acid sequences and the molecular structure of the pre-pro-
374 proteins of the neuropeptide members of the CRH family in pejerrey were like those of *O. latipes* and
375 Burton's Mouthbrooder *Astatotilapia burtoni* (Grone et al., 2021; Hosono et al., 2017). The phylogenetic
376 analysis of *crhb*, *uts1*, *ucn2*, and *ucn3* showed that Crh genes are grouped in two subfamilies with similar %
377 of amino acid identity (around 50%), one formed by *crhb* and *uts1,* and another by *ucn2* and *ucn3*. These
378 two subgroups have been identified in multiple vertebrates and it is consistent with the hypothesis that a
379 single ancestral peptide gene was duplicated giving rise to these two clades (Lovejoy et al., 2013).

380 The high expression of the neuropeptide-encoding genes *crhb*, *ucn3*, and *uts1* in the heads of XX
381 larvae during the CPSD at MPT indicates a genotype-specific stimulatory role of high temperature in the
382 Crh system in the brain during sex differentiation. *uts1* (the homologous of the mammalian *ucn1*) and *ucn3*
383 are genes linked to stress, anxiety, and anorexia in teleosts (Asaba et al., 1998; Grone et al., 2021; Hosono
384 et al., 2017; Sobrido-Cameán et al., 2021; Tang et al., 2019). The peripheral roles of *crhb*, *uts1* and *ucn3* in

385 stressed fish have been investigated with special mention to the former (Flik et al., 2006; Grone et al., 2021;
386 Hosono et al., 2017). In fact, a recent study in adult black porgy *Acanthopagrus schlegelii* localized *crh*
387 expression in the gills after exposure to osmotic stress (Adimoolam et al., 2021). In this study we did not
388 observe hybridization signals for *crhb* or *ucn3* in the gills of pejerrey larvae. Therefore, we presume that
389 the mRNA abundance of these genes determined by RT-qPCR corresponds solely to their expression in the
390 brain. The peripheral roles of *crhb*, *uts1*, and *ucn3* in stressed fish have been investigated with special
391 mention to the former (Flick et al., 2006; Grone et al., 2021; Hosono et al., 2017), but their central actions,
392 particularly those involved in brain reprogramming are comparatively much less understood than in
393 mammals (Maras and Baram, 2012). The selective upregulation of the neuropeptides *crhb*, *ucn3*, and *uts1*
394 in the heads of XX pejerrey larvae is a considerable departure from the pattern seen in *O. latipes* embryos,
395 whereby there were neither profile changes nor genotypic dimorphism in mRNA abundance during heat-
396 induced masculinization (Castañeda-Cortés et al., 2019, 2020).

397 *crhr2* was also upregulated in XX larvae at the MPT, which suggests that this receptor could be
398 mediating the activation of the stress-related- or other neural processes by Crhb, Uts1, and Ucn3 during the
399 CPSD. Crhr2 has been found to mediate sex specific responses in the mouse (Kubat et al., 2013), and its
400 potential role in the triggering of an intensified stress response during the sex determination of XX pejerrey
401 larvae is also supported by its well documented ability to bind all the members of the Crh family (although
402 with different affinities) across vertebrates (Lovejoy et al., 2014). This hypothesis of "one receptor-multiple
403 ligands" is supported by the distribution of *crhb*, *ucn3*, and at least the receptor *crh1* (*crh2* was not detected)
404 in several areas of the diencephalon of pejerrey larvae, including the hypothalamus, suggesting a potential
405 interaction between the different elements of the Crh system. The nature of these interactions remains to be
406 investigated in pejerrey, since studies in mammals have shown that Crh receptors have multiple functions
407 (Sukhareva et al., 2021) or act via different mechanisms depending on their localization and cellular context
408 (Henckens et al., 2016; Inda et al., 2017). We could not determine the expression sites of *crhr2* in the brain
409 of pejerrey larvae. Assays with the same mRNA probe identified this gene in the caudal hypothalamus and

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410 the pituitary of juvenile pejerrey. According to some studies in mammals, *crhr2* is expressed in very specific
411 areas of the brain and its low expression challenges the ability to determine its exact site of action (Hauger
412 et al., 2006; Henckens et al., 2016). We surmise that the transcript levels of *crhr2* are much lower than
413 those of *crhr1* in pejerrey larvae, making its detection difficult. Although the functions of Crhr2 in the brain
414 are still controversial (Henckens et al., 2016; Sukhareva et al., 2021), it is often linked to stress and anxiety
415 in rodents (Henckens et al., 2016; Takahashi, 2001). We hypothesize that both receptors might operate
416 together to trigger a stronger stress response in XX larvae.

417 The upregulation of Crh family genes, particularly *crhb*, and the high cortisol titers at the MPT
418 corroborate the activation of the HPI axis by heat-stress. Surprisingly, *pomc*, the gene encoding for Acth,
419 was not significantly upregulated and yet showed higher values in the heads of XX larvae at the MPT. Other
420 studies that have tried to quantify this gene have failed to find differences at the mRNA level, but variations
421 could be detected at the protein level (Castañeda-Cortés et al., 2019). The higher circulating levels of
422 cortisol in XX larvae coincided with the upregulation of GR and MR in the brain, which further supports
423 the genotype-specific action of cortisol in the brain centers during the CPSD. In addition to Crh family
424 genes, *gr1*, *gr2*, and *nr3c2* also showed higher expression in larvae at the MPT. Of these, *gr1* was
425 upregulated in XY larvae whereas the other two were upregulated in XX larvae, indicating that they act in
426 a genotype-specific manner under thermal stress. Their involvement in negative feedback is improbable,
427 since previous studies in mammals and fish have shown that chronic stress leads to the downregulation, not
428 upregulation of glucocorticoid receptors (Castañeda-Cortés et al., 2020; Herman et al., 2016; Mizoguchi et
429 al., 2003). Indeed, an enhanced expression of cortisol receptors in fish has been linked to cell proliferation
430 and differentiation that are related to neural reorganization in response to stressful stimuli and the
431 preparation of organisms for a much complex, stressful environment (Sadoul et al., 2018; Dunlap et al.,
432 2006).

433 It is interesting that high temperatures caused a rise in whole-body cortisol in both genotypes at the
434 beginning, but these higher levels were maintained throughout the CPSD only in XX larvae. This might

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435 reflect genetic differences in the clearing of cortisol. A similar genotypic dimorphism in cortisol titers was
436 observed in pejerrey larvae reared at different backgrounds colors (García-Cruz et al., 2019), a known
437 source of stressor in other teleost species (Mankiewicz et al., 2013). Interestingly, Kikuchi et al. (2015)
438 reported female-biased resting cortisol levels, supporting the existence of sex-specific predisposition to
439 stress in adult Medaka. Various studies in vertebrates have demonstrated the developmental effects of
440 cortisol in the brain, especially in the programming of the HPI axis (see Best et al., 2017 and references
441 therein) as well as in the determination of sex specific behaviors (Arterbery et al., 2010). Cortisol regulates
442 neurogenesis via the GR and MR in the developing brain of fish and it can alter multiple behaviors later in
443 life (Best et al., 2017; Sopinka et al., 2015). Taken together, this information suggests that the Crh system
444 and cortisol via the *gr2* and *nr3c2* might be involved in the brain differentiation of XX pejerrey larvae
445 during the CPSD. What it is uncertain is if the action of the Crh system and cortisol signaling on the brain
446 of XX larvae lead to adaptative changes that are restricted to the stress response (either at the central level
447 or in the HPI axis) (Best et al., 2017), or if it goes beyond as to induce directly or indirectly sex-specific
448 neural differences that may have effects on reproductive neuroendocrinology and behavior. It is important
449 to note that this study revealed a higher expression of *cyp19a1b* in the brains of larvae exposed to the
450 masculinizing temperature during the CPSD and that XY larvae had comparatively more *cyp19a1b*
451 transcripts than XX larvae. Overexpression of *cyp19a1b* seems to be a hallmark of brain masculinization
452 in pejerrey (Strobl-Mazzulla et al., 2008) and these results clearly show the onset of heat induced
453 masculinization at the brain level. Temperature has been found to induce changes in the brain in species
454 with TSD. For instance, in the leopard gecko *Eublepharis macularius*, incubation of eggs at different
455 temperatures produced sex differences in the hypothalamus (Coomber et al., 1997; Crews et al., 1996).
456 More recently in the Nile tilapia, it was found that heat induced sex reversal caused a reduced number of
457 Avt and Gnrh1 immunoreactive neurons in the preoptic area (Dussenne et al., 2020) and behavioral
458 differences (more aggressivity) in XX neomales compared to normal XY males (Dussenne et al., 2022).
459 This shows that both elevated temperature and genotype can affect brain structure and behavior during sex
460 determination/differentiation.

461 One peculiar aspect of pejerrey (and atheriniforms in general) sex determination is that it allows
462 not only heat-induced masculinization, but also low temperature-induced feminization (Strüssmann et al.,
463 2021). The results of this study show that two neuropeptide-encoding genes, *crhbp* and *avt* were upregulated
464 at the FPT compared to the MPT; the former was upregulated only in XY larvae whereas the latter was
465 upregulated equally in both genotypes. Crhbp has been found to be important for the homeostasis of Crh
466 and Uts1 due to its ability to regulate the availability of these peptides or by modulating the binding
467 capabilities of the Crh receptors (Doyon et al., 2005; Alderman and Bernier, 2007; Kalin, 2018; Ketchesin
468 et al., 2017). Given these known functions of *crhbp*, the lack of any significant differences in the expression
469 of Crh family genes and their receptors between XY larvae at the MPT and FPT in this study, and in view
470 of the inherent propensity of XY larvae to be masculinized (due to the presence of *amhy*), it could be
471 hypothesized that upregulation of *crhbp* at the low temperature is a mechanism to null the effect of Crh
472 family members in the brain to allow feminization. Regarding Avt, it has been shown to inhibit the HPI
473 axis by downregulation of *crh* expression and inhibition of the synthesis of cortisol in the gilthead seabream
474 *Spaurus aurata* (Martos-Sitcha et al., 2019). If *avt* plays similar roles in pejerrey larvae it could regulate
475 negatively the HPI axis at the FPT play an important role in feminization.

476

477 **5.0. Conclusions**

478 This study provides clear evidence of genotype- and temperature- dependent molecular changes in
479 the brain of pejerrey larvae that occur as early as the first signs of the onset of sex determination in the
480 gonads. Heat-stress triggers elevated expression of Crh members (*crhb*, *uts1*, *ucn3*) and stress-related genes
481 (*crhr2*, *gr1*, *gr2*, *nr3c2*) in XX larvae compared to XY larvae that leads to a stronger stress response in the
482 former, including higher cortisol titers during the CPSD. This stronger stress response in XX may be
483 necessary to compensate the lack of the masculinization stimulus provided by *amhy* in XY larvae to
484 successfully override the putative (default) female developmental pathway (see Zhang et al., 2018; Sarida
485 et al., 2019; Strüssmann et al., 2021). Less clear but of equal importance is the finding of upregulation of

486 *crhbp* and *avt* in the brain of larvae at the FPT, particularly the former in XY larvae, as they provide for the
487 first time a working hypothesis to explain the process of low temperature-induced feminization in pejerrey.
488 Studies in mammals have demonstrated a direct function of the sex chromosomes in the sexual differences
489 observed in the brain, which are independent of the organizational/activational effects induced by the
490 gonads (McCarthy, 2010; 2021). Some of these differences have been attributed to a dosage effect of an
491 additional X chromosome in putative females and not to the action of the sex determining gene (Arnold et
492 al., 2004). It is tempting to conclude that a similar dosage-dependent could be behind the high sensitivity
493 of XX pejerrey to environmental sources of stress and this aspect should be examined in future studies. The
494 programming role of Crh family and cortisol on the brain during sensitive life stages is an open avenue for
495 future research on the brain sexual differentiation of fish, which have received little attention compared to
496 mammals and birds (Arnold & McCarthy, 2016).

497

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503

504 **7.0. Declaration of interest**

505 The authors declare no conflict of interest.

506

507 **8.0. Appendix A. Supplementary data.**

508 Supplementary data to this article can be found online at https://doi.XXXX.

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510 **9.0. References**

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Table 1. Genotypic and phenotypic sex ratios of larvae reared at female-promoting (FPT, 17°C) and male-promoting (MPT, 29°C) temperatures at the end of the experiment (18 wah).

Temperature	FPT (17°C)		MPT (29°C)	
Genotype	Phenotype		Phenotype	
	Male	Female	Male	Female
XX	0 (0%)	22 (49%)	11 (52%)	0 (0%)
XY	0 (0%)	23 (51%)	10 (48%)	0 (0%)
Total	0 (0%)	45 (100%)	21 (100%)	0 (0%)

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Table 2. Summary of the results of Three- or Two-Way-ANOVA (in case of *amhy* only) on the effects of the different factors (time after hatching, temperature, and genotype) and their interactions (three factors: third order interaction; two factors: second order interaction) on the transcript levels of CRH family genes, (*crhb*, *uts1*, *ucn2*, *ucn3*), Crh receptors (*crhr1*, *crhr2*), other stress-related genes (*crhbp*, *avt*, *pomc*, *gr1*, *gr2*, *nr3c2*), brain aromatase (*cyp19a1b*), and the markers of sex differentiation (*amhy*, *amha*, *cyp19a1a*) during the critical period of sex determination. Values indicate the percentage of the variation of gene expression explained by each factor or interaction. Significant effects are indicated with an asterisk. N.A. indicates non-applicable cases (such as for *amhy*).

Factors and interactions	% of total variation in gene expression explained by each factor and their interactions															
	CRH family genes				Crh receptors		Other stress-related genes						Brain aromatase	Sex differentiation markers		
	crhb	*uts1*	*ucn2*	*ucn3*	*crhr1*	*crhr2*	*crhbp*	*avt*	*pomc*	*gr1*	*gr2*	*nr3c2*	*cyp19a1b*	*amhy*	*amha*	*cyp19a1a*
Time	16.08*	3.72	36.21*	4.91	43.02*	12.76*	15.38*	24.34*	4.04	5.41	15.04*	0.01	24.90*	11.31*	7.18*	4.76*
Temperature	19.74*	50.32*	0.03	1.25	0.34	0.07	4.35	44.88*	1.10	10.41*	6.42*	15.92*	30.33*	34.55*	32.86*	31.83*
Genotype	12.53*	0.06	2.13	11.99*	0.10	17.75*	0.68	0.42	2.09	0.81	0.52	0.48	2.42*	N.A.	0.29	13.54*
Time-Temperature	8.17*	2.99	4.36	26.85*	9.44*	3.37	2.85	0.66	12.12*	26.08*	10.30*	16.30*	8.11*	7.85	9.7*	0.89
Time-Genotype	2.11	3.39	4.43	2.71	0.09	2.5	4.91	2.13	5.87	3.06	6.96	13.66*	5.92*	N.A.	3.44	2.48*
Temperature-Genotype	0.002	6.33*	12.24*	3.57	0.13	3.58	11.81*	0.17	3.98	0.02	8.14*	4.52	0.59	N.A.	0.49	17.30*
Time-Temperature-Genotype	4.75*	1.14	3.51	1.66	0.36	3.41	4.18	1.39	0.36	3.5	8.18	7.75*	3.26	N.A.	1.50	1.19

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Table S1. List of primers and PCR conditions used for the amplification/quantification of target genes.

GENE	PURPOSE	FORWARD ('5 – '3)	REVERSE ('5 – '3)	SIZE (bp)	PCR CONDITIONS
amhy	Genotyping	TAGTTTCCTACCCCAGTC	CTGTTTTGTGATTTTCCGATGGGTT	504	[1X, 94°C, 2 min] [35X, 98°C, 10 sec; 35X, 60°C ,30 sec; 35X, 68°C, 1 min] [1X, 68°C, 5 min]
amha	Genotyping	ATTACACATTTCAACCAGTTTTGTAGGTG	GTTTAGCATCGTTTCAAGCACTCTG	286	[1X, 94°C, 2 min] [35X, 98°C, 10 sec; 35X, 60°C ,30 sec; 35X, 68°C, 1 min] [1X, 68°C, 5 min]
crhb (SYG)	RT-qPCR	CTCCCCAAACCCAAAACTCC	CCAGTCGGATGAAATACTCCTCTC	102	[1X, 95°C, 5 min] [35X, 95°C, 30 sec; 35X, 60°C ,30 sec; 35X, 72°C, 1 min] [1X, 72°C, 5 min]
ucn2 (SYG)	RT-qPCR	CTTAAATCGCAGCGACGTGT	GTGGGGACATCAAGGGACAG	147	1X, 95°C, 2 min] [35X, 98°C, 10 sec; 35X, 58°C ,30 sec; 35X, 72°C, 1 min] [1X, 72°C, 5 min]
crhr2 (SYG)	RT-qPCR	AGGACCAGTTATTCTTGTGCTCTTG	TGTATTGTTTCAGATGTGGTTGAGG	105	[1X, 95°C, 2 min] [35X, 98°C, 10 sec; 35X, 59°C ,30 sec; 35X, 72°C, 1 min] [1X, 72°C, 5 min]
β-actin (SYG)	RT-qPCR	GCTCGGCTGTGGTGGTCAAGC	GCTCGGCTGTGGTGGTCAAGC	200	[1X, 50°C, 2 min] [1X, 95°C, 10 sec] [40X, 95°C ,10 sec; 40X, 60°C, 30 sec]
crhr1 (SYG)	RT-qPCR	AGGGGAACTACTCCATGTGTAAGG	AGAAGAGGAAGAAGGCGATGAG	139	[1X, 95°C, 2 min] [35X, 98°C, 10 sec; 35X, 60°C ,30 sec; 35X, 72°C, 1 min] [1X, 72°C, 5 min]
ucn3 (SYG)	RT-qPCR	ACAGTATGCTGTCGTCCCTG	CCGGACCCTGACTTCCATTT	134	
crhbp (SYG)	RT-qPCR	TGACAATGTGGACGCTGACT	ACATAGCGCTCGTATGGAGG	149	
avt (SYG)	RT-qPCR	AGGAAAACTACCTGCTCACCC	GGCAGTCAGACTCCACCATAC	149	
gr1	RT-qPCR	CAGCACTTCGGGAGGACAGAG	ATGGTCGTTATTGGGAGGTACAGG	114	[1X, 95°C, 2 min] [35X, 98°C, 10 sec; 35X, 60°C, 30 sec; 35X, 72°C, 1 min] [1X, 72°C, 5 min]
gr2	RT-qPCR	CCAGAACGGGCGAGACCAG	TGTCGTGCTCTCCCATCCTTCG	178	
pomc (SYG)	RT-qPCR	AAAGTCTACGCCTCCAACGG	AGCGGAAGTGCTTCATCTTGT	159	
uts1 (SYG)	RT-qPCR	TCGACGAGGTAGGGAAGTGA	GCACCACGTGTGCGTAAAAT	147	
cyp19a1b (SYG)	RT-qPCR	CCATCTTGATTACTCTGTTGTCTCGTT	CTTGATGCTGTTGAGGTTGCA	71	

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cyp19a1a (SYG)	RT-qPCR	GCGAGCTGTCTGGCTGAGAA	AGGAGCAGCAGCATGAAGAAGA	100	[1X, 95°C, 2 min] [35X, 98°C, 10 sec; 35X, 60°C ,30 sec; 35X, 72°C, 1 min] [1X, 72°C, 5 min]
amhy (TAQ)	RT-qPCR	GCACGTCGGAGGTCGGA Probe: TCGTGCATCGGCAGAG	GAGGTTATGAGGTGCTGAGGAAGTTA	182	[1X, 50°C, 2 min] [1X, 95°C, 20 sec] [40X, 95°C, 3sec; 40X, 63°C, 30 sec]
amha (TAQ)	RT-qPCR	AAACAGCAGCAGGTGAGAGTCA Probe: CCAGTCCACGACCTCCAGGGGGT	TGATGGAGAGAAAAGACTCTTCCG	405	[1X, 50°C, 2 min] [1X, 95°C, 20 sec] [40X, 95°C, 3 sec; 40X, 60°C, 30 sec]
β-actin (TAQ)	RT-qPCR	TCGTGCGCGCACATTAAGGA Probe:CTGTGTTACGTTGCATTGGACTTTGAGCA	GCAGCGTCCCCATTTC	70	[1X, 50°C, 2 min] [1X, 95°C, 20 sec] [40X, 95°C, 3 sec; 40X, 60°C, 30 sec]
crhb	ISH	TGCTTGTTGCCTTCTTACCG	TGACACCAACATTGAAATGGC	683	[1X, 95°C, 2 min] [30X, 95°C, 15 sec; 30X, 60°C ,30 sec; 30X, 72°C, 1 min] [1X, 72°C, 5 min]
ucn2	ISH	AGTGGTGACCGAAAAGTCCT	TCCTTCTGCCTATACGTGCC	397	[1X, 95°C, 2 min] [30X, 98°C, 10 sec; 30X, 57°C ,30 sec; 30X, 72°C, 1 min] [1X, 72°C, 5 min]
ucn3	ISH	GTATGCTGTCGTCCCTGAAGAC	CCGTCCAATCTGCGCCAGTAGA	452	[1X, 95°C, 2 min] [35X, 98°C, 10 sec; 30X, 55°C ,30 sec; 30X, 72°C, 30 sec] [1X, 72°C, 5 min]
crhr1	ISH	GAACCAAACCCTGAGCAACG	AGAAGAGGAAGAAGGCGATGAG	347	[1X, 98°C, 1 min] [30X, 98°C, 15 sec; 30X, 55°C ,30 sec; 30X, 72°C, 1 min] [1X, 72°C, 5 min]
crhr2	ISH	AGGACCAGTTATTCTTGTGCTCTTG	ACTCGAAGAGCATGGTTGTC	345	[1X, 98°C, 1 min] [30X, 98°C, 10 sec; 35X, 69°C ,30 sec; 30X, 72°C, 1 min] [1X, 72°C, 5 min]

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Table S2. Sources of Crhb, Uts1, Ucn3, Ucn2, Crhr1, Crhr2, and Crhbp deduced amino acid sequences for teleosts used for the multiple alignment and construction of phylogenetic trees.

Species	Common name	Accession number						
		Crhb/corticoliberin	Uts1	Ucn3	Ucn2	Crhr1	Crhr2	Crhbp
Odontesthes bonariensis	Pejerrey	OQ597213	OR428262	OQ597212	OR428264	OR428261	OR428263	OQ717011
Oryzias latipes	Japanese medaka	NP_001121990.1	BAX76685.1	BAG16732.1	NP_001121991.1	XP_023805350.1	XP_004079462.2	XP_004074550.1
Danio rerio	Zebrafish	NP_001007380.1	NP_001025351.1	NP_001076423.1	N.A.	XP_696346.3	NP_001107116.1	NP_001003459.1
Haplochromis burtoni	Burton's mouthbrooder	NP_001274333.1	ENSHBUT00000028458.1	XP_014190624.1	ENSHBUT00000000058.1	NP_001273229.1	NP_001273242.1	NP_001273237.1
Clupea harengus	Atlantic herring	XP_012671613.1	XP_031437849.1	XP_012679926.1	XP_012696518.1	XP_031423298.1	XP_042559641.1	XP_031426424.1
Lepisosteus oculatus	Spotted gar	XP_006633965.1	XP_015199644.1	XP_015207759.1	LG5_51350408	XP_015217722.1	XP_015209811.1	XP_006626779.1

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Figure 1

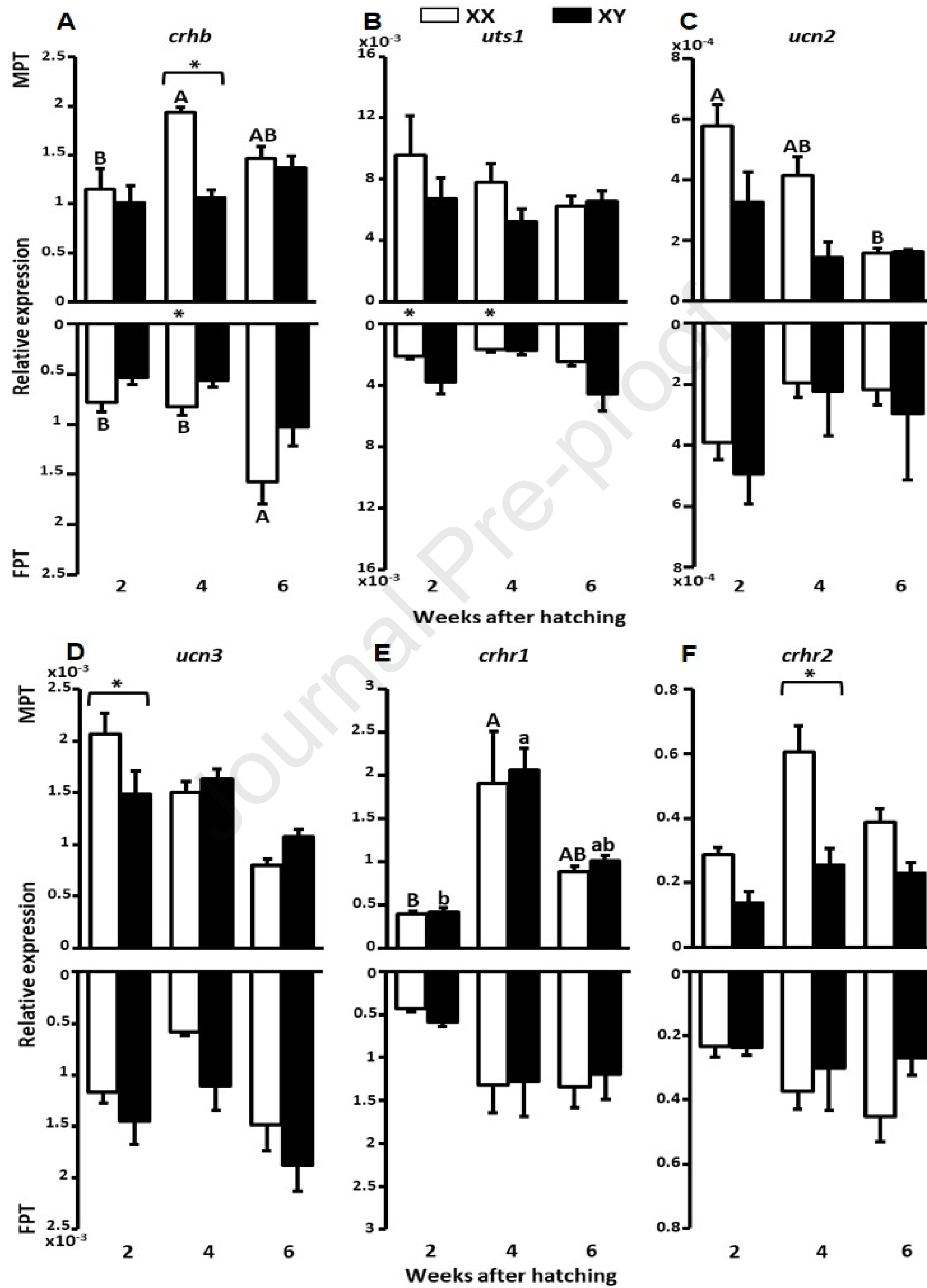

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Figure 1

Developmental changes in the relative abundance of mRNAs of Crh family genes (*crhb*, *uts1*, *ucn2*, *ucn3*) and their receptors (*chr1*, *chr2*) in the heads of pejerrey larvae in relation to genotype and temperature during the critical period of sex determination. XX and XY samples are depicted as white and black bars, respectively. Asterisks connecting white and black bars indicate a significant difference in mRNA abundance between genotypes for a given week and those between upper (MPT) and lower (FPT) panels of the same gene indicate a significant difference between thermal regimes for the same genotype and week. Bars within each panel (same genotype and temperature) with different upper (XX) and lower (XY) case letters are significantly different. For clarity, only comparisons that include statistically significant differences are indicated by asterisks or letters. Statistical significance is assumed at $p<0.05$ (Three-Way ANOVA followed by Tukey's multiple comparison test).

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Figure 2

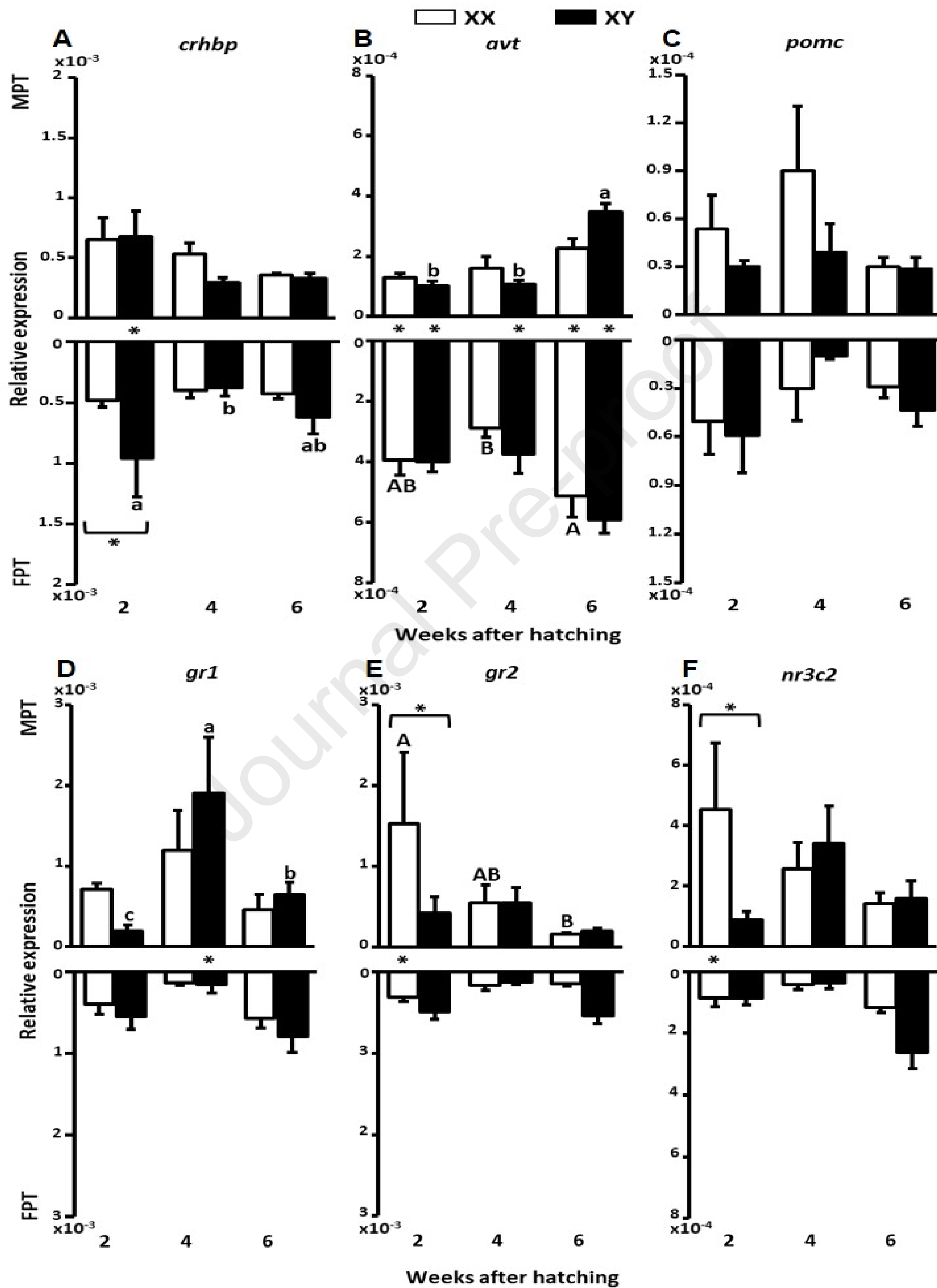

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Figure 2

Developmental changes in the relative abundance of mRNA of several stress-related genes (*crhbp*, *avt*, *pomc*, *gr1*, *gr2*, *nr3c2*) in the heads of pejerrey larvae in relation to genotype and temperature during the critical period of sex determination. XX and XY samples are depicted as white and black bars, respectively. Asterisks connecting white and black bars indicate a significant difference in gene expression between genotypes for a given week and those between upper (MPT) and lower (FPT) panels of the same gene indicate significant differences between thermal regimes for the same genotype and week. Bars within each panel (same genotype and temperature) with different upper (XX) and lower (XY) case letters are significantly different. For clarity, only comparisons that include statistically significant differences are indicated by asterisks or letters. Statistical significance is assumed at $p<0.05$ (Three-Way ANOVA followed by Tukey's multiple comparison test).

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Figure 3

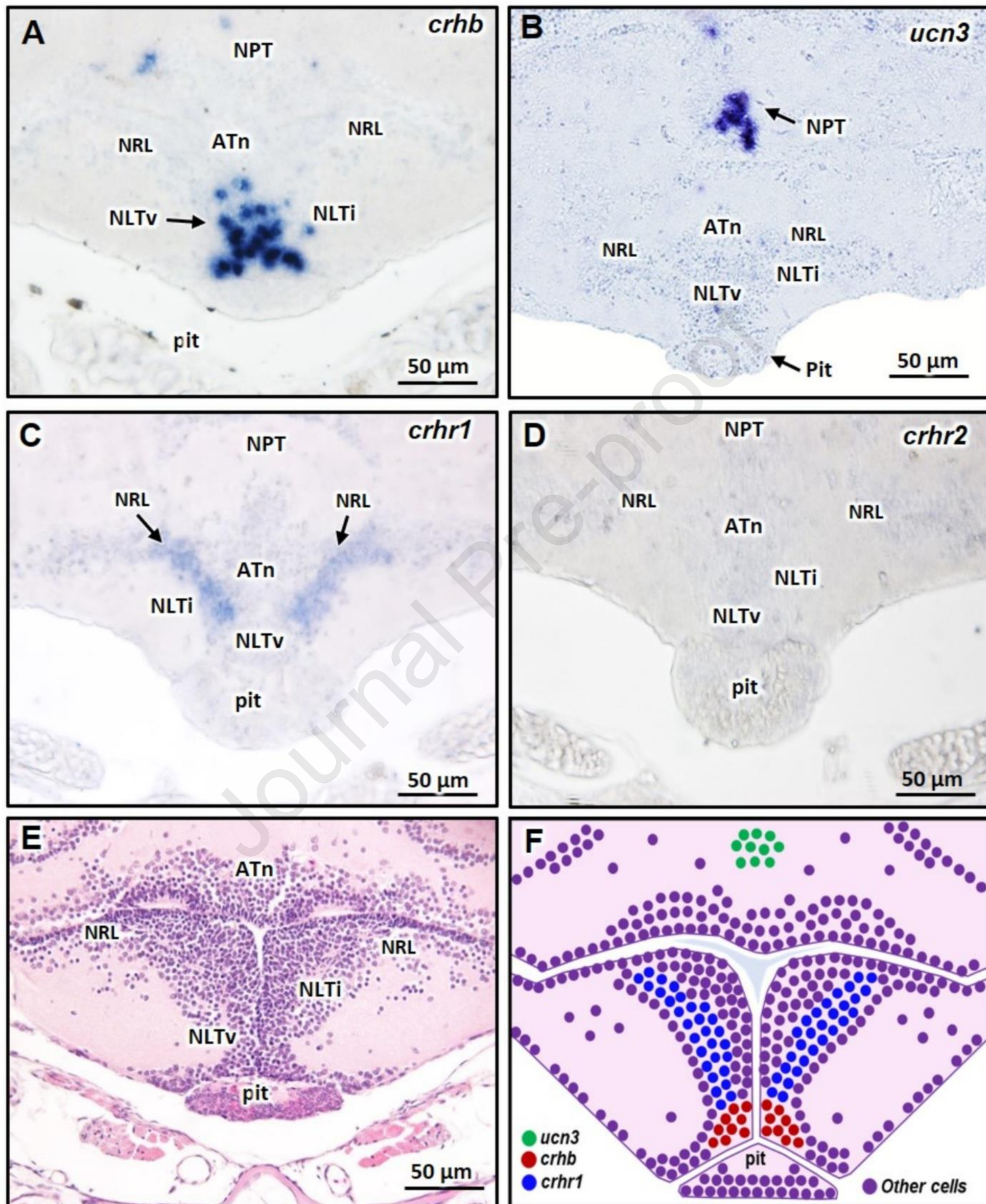

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Figure 3

Localization of mRNAs of Crh family genes by *in situ* hybridization in the hypothalamus of 2- and 4-week-old XX pejerrey larvae reared at the MPT. A: *crhb* mRNA was detected in the mediobasal hypothalamus, in the ventral zone of the tuberal hypothalamus (NLTv), close to the pituitary (pit). B: *ucn3* was localized in the dorsal hypothalamus, in the upper part of the nucleus posterioris tuberis (NPT). C: *crhr1* was localized in the nucleus recesus lateralis (NRL) in the dorsal zone of the tuberal hypothalamus. D: *crhr2* mRNA was not detected. E: Transversal section of the tuberal hypothalamus and pituitary stained with hematoxylin-eosin. F: Illustration of the spatial distribution of the ligands *ucn3* and *crhb* and the receptor *crhr1* in the tuberal hypothalamus of pejerrey larvae during thermal-induced masculinization. NLTi; nucleus lateralis tuberis pars intermedia. ATn; anterior tuberal nucleus.

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Figure 4

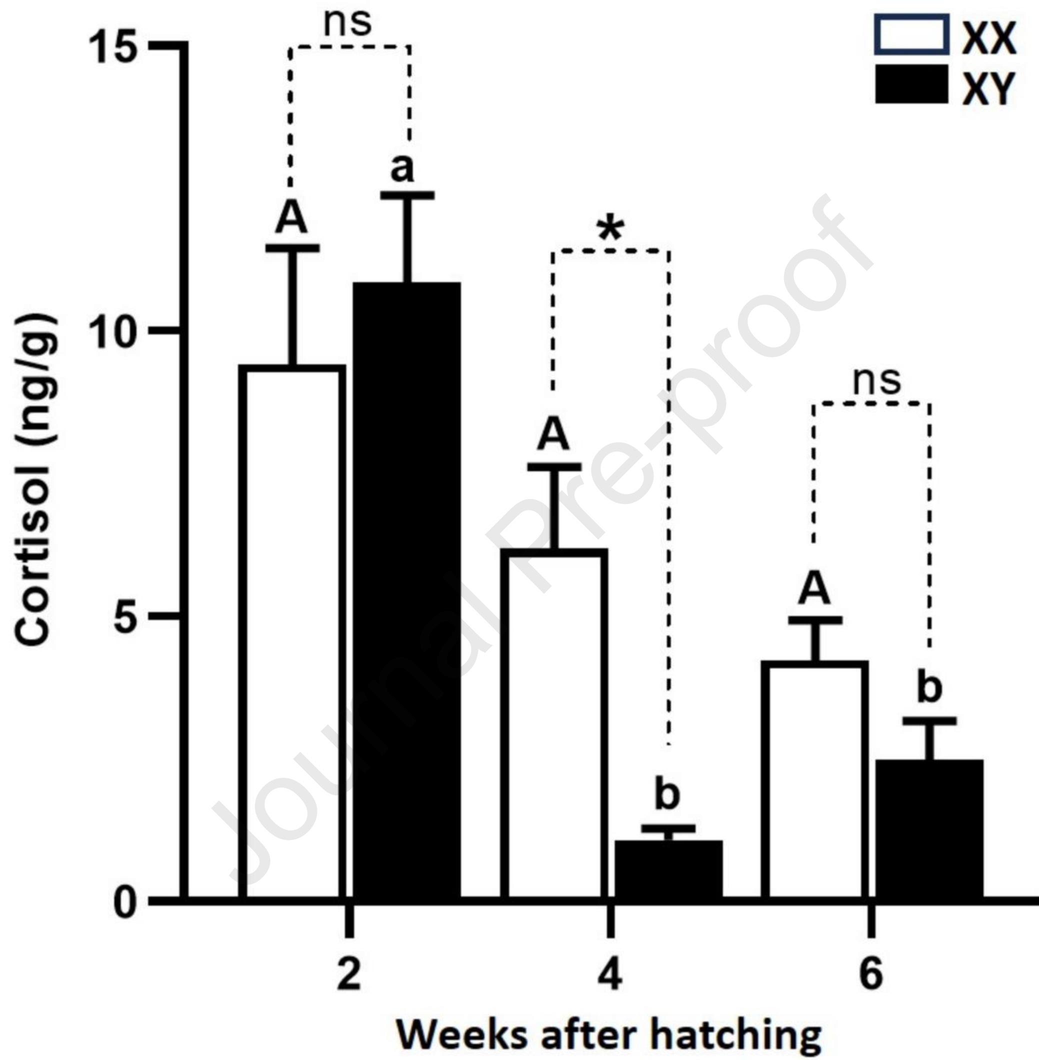

Figure 4

Whole-body cortisol levels of XX and XY pejerrey larvae during the critical period of sex determination at the male-promoting temperature (29ºC). Statistical differences between XX and XY individuals are indicated by asterisks. Bars of the same genotype with different upper (XX) and lower (XY) case letters are significantly different. Statistical significance is assumed at $p<0.05$ (Two-way ANOVA followed by Tukey's multiple comparison test).

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Figure 5

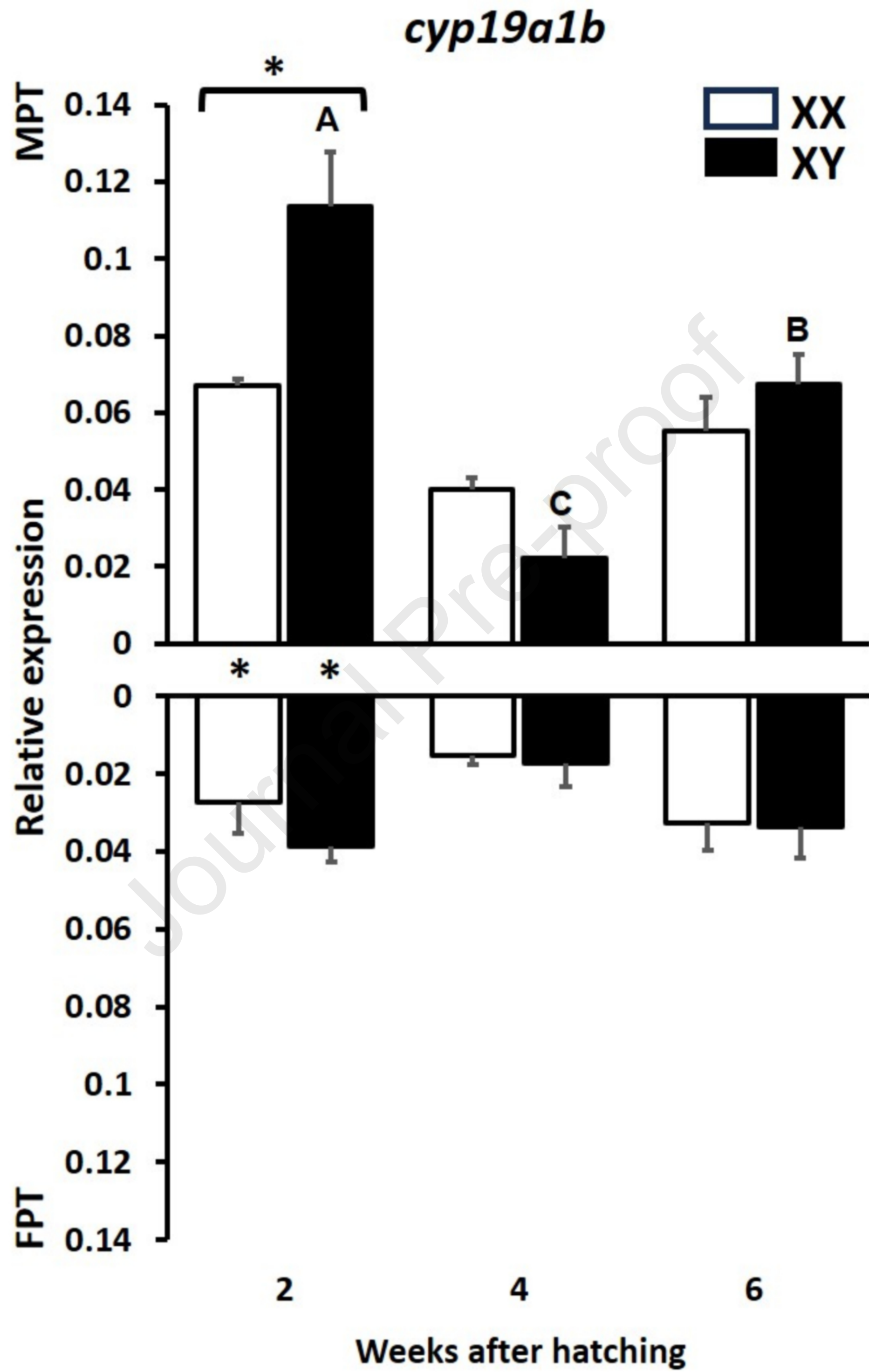

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Figure 5

Developmental changes in the relative abundance of mRNAs of brain aromatase (*cyp19a1b*) in the heads of pejerrey larvae in relation to genotype and temperature during the critical period of sex determination. XX and XY samples are depicted as white and black bars, respectively. Asterisks connecting white and black bars indicate a significant difference in mRNA abundance between genotypes for a given week and those between upper (MPT) and lower (FPT) panels of the same gene indicate a significant difference between thermal regimes for the same genotype and week. Bars within each panel (same genotype and temperature) with different upper (XX) and lower (XY) case letters are significantly different. For clarity, only comparisons that include statistically significant differences are indicated by asterisks or letters. Statistical significance is assumed at $p<0.05$ (Three-Way ANOVA followed by Tukey's multiple comparison test).

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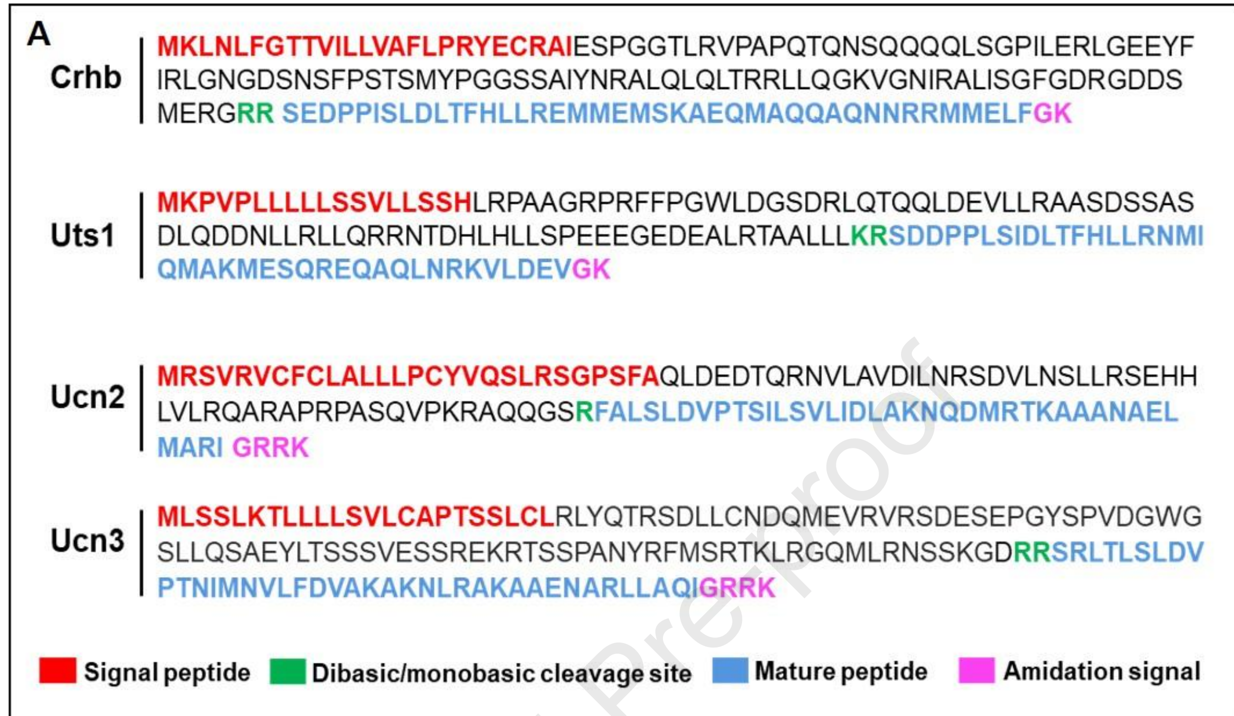
Figure S1



B

1. Crhbp	S E D P P I S L D L T F H L L R E M M E M S K A E Q M A Q Q A Q N N R R M M E L F
2. Uts1	S D D P P L S I D L T F H L L R N M I Q M A K M E S Q R E Q A Q L N R K V L D E V
3. Ucn2	- - - F A L S L D V P T S I L S V L I D L A K N Q D M R T K A A A N A E L M A R I
4. Ucn3	- S R L T L S L D V P T N I M N V L F D V A K A K N L R A K A A E N A R L L A Q I

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Figure S1

Molecular characterization of the neuropeptides from the CRH family. A: Deduced amino acid sequences of pejerrey Crhb, Uts1, Ucn2, and Ucn3 pre-pro-proteins with their multiple domains. B: Alignment of the mature peptides of the members of the CRH family. Crhb and Uts1 mature peptides consisted of a sequence of 41 amino acids long, while Ucn2, and Ucn3 consisted of of 38 and 39 amino acids long, respectively. The conserved amino acids among all the neuropeptides appear on a black background.

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Figure S2

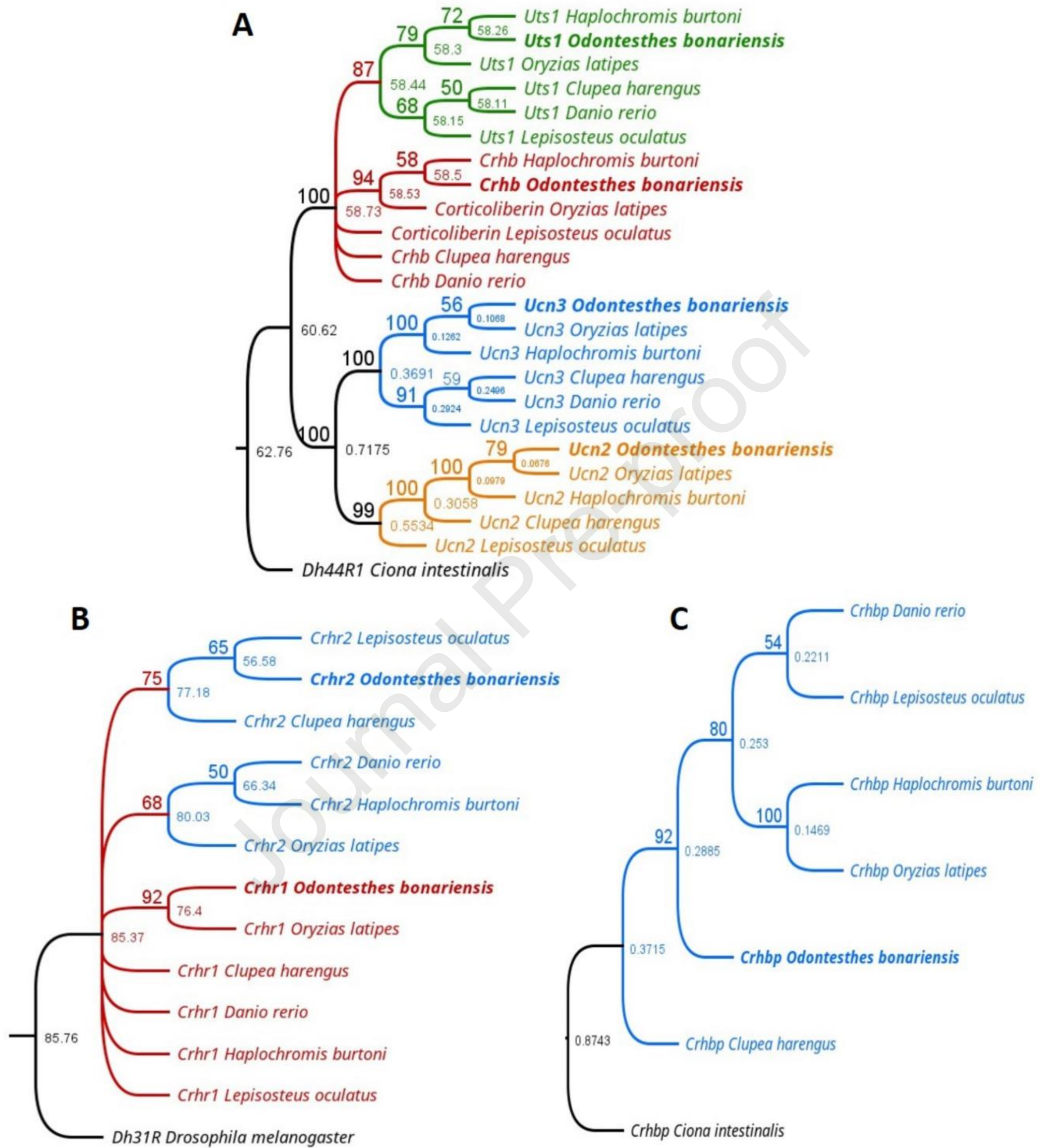

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Figure S2

Phylogenetic trees of teleost CRH family members (Crhb, Uts1, Ucn2, Ucn3), Crh receptors and Crhbp carrier protein based on predicted protein sequences. A: Two groups were identified, one formed by Crhb and Uts1 and another one formed by Ucn2 and Ucn3. The diuretic hormone-like peptide DHLP of *Ciona intestinalis* was used as an outgroup. B: Both Crhr1 and Crhr2 receptors share a common root and appear to be evolutionary conserved. The diuretic hormone receptor (Dh31R) of the fly fruit *Drosophila melanogaster* was used as outgroup. C: The transporter glycoprotein Crhbp of pejerrey showed divergence with that of *Clupea harengus* and other teleosts. Due to the evolutionary conservation of Crhbp from invertebrates to vertebrates, no outgroup was used.

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Figure S3

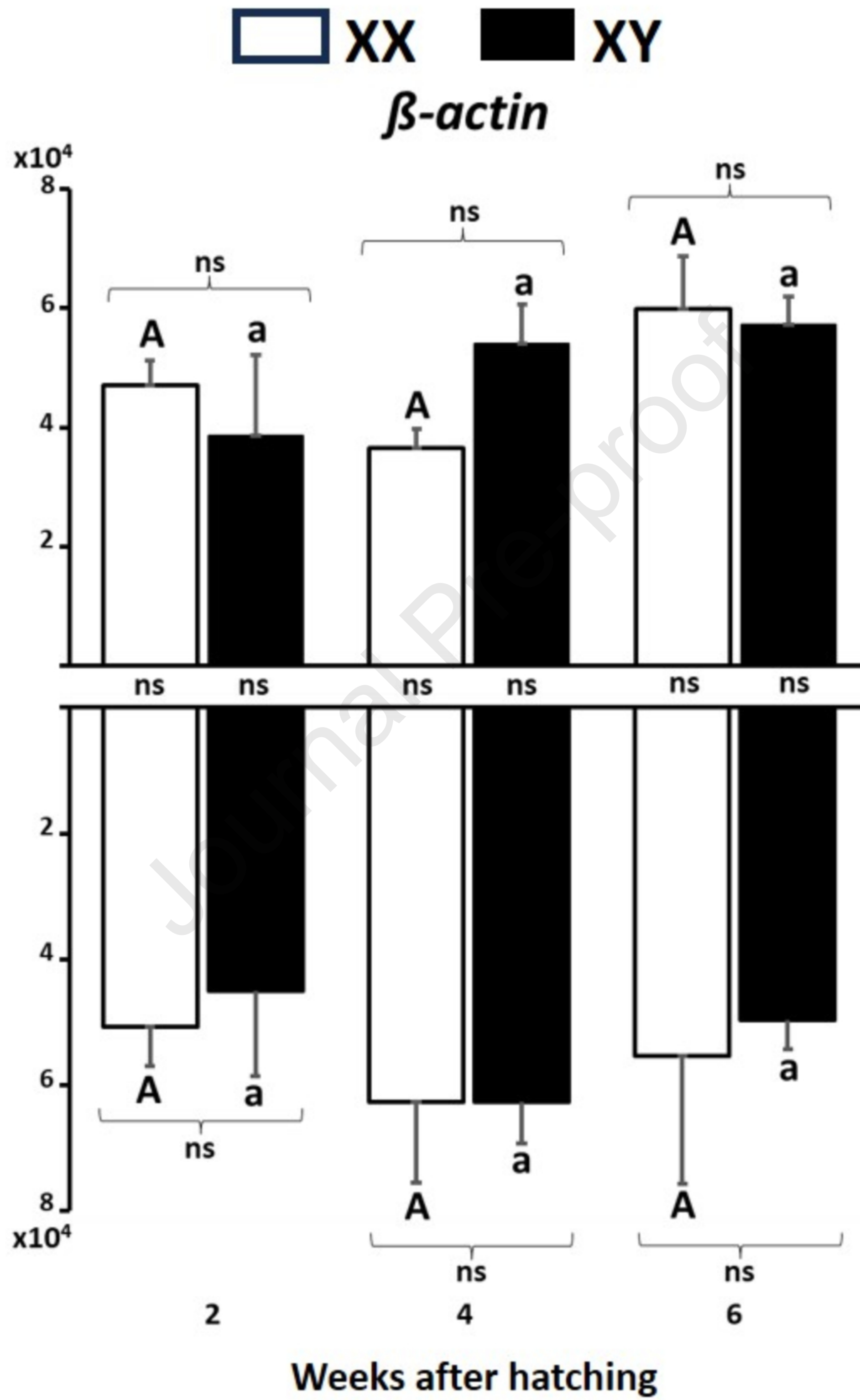

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Figure S3

mRNA abundance of ß-actin in the heads of XX and XY larvae throughout the critical period of sex determination at Male- (MPT) and Female-Promoting Temperatures (FPT). XX and XY samples are depicted as white and black bars, respectively. No statistical differences were found between/among any of the compared groups, which makes ß-actin a reliable gene for expression normalization. Labels (ns) connecting white and black bars indicate a lack of statistical difference in mRNA abundance between genotypes for a given week and those between upper (MPT) and lower (FPT) panels of the same gene indicate no differences between thermal regimes for the same genotype and week. Bars within each panel (same genotype and temperature) with same upper (XX) and lower (XY) case letters are not significantly different. Statistical significance is assumed at $p<0.05$ (Three-Way ANOVA followed by Tukey's multiple comparison test).

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Figure S4

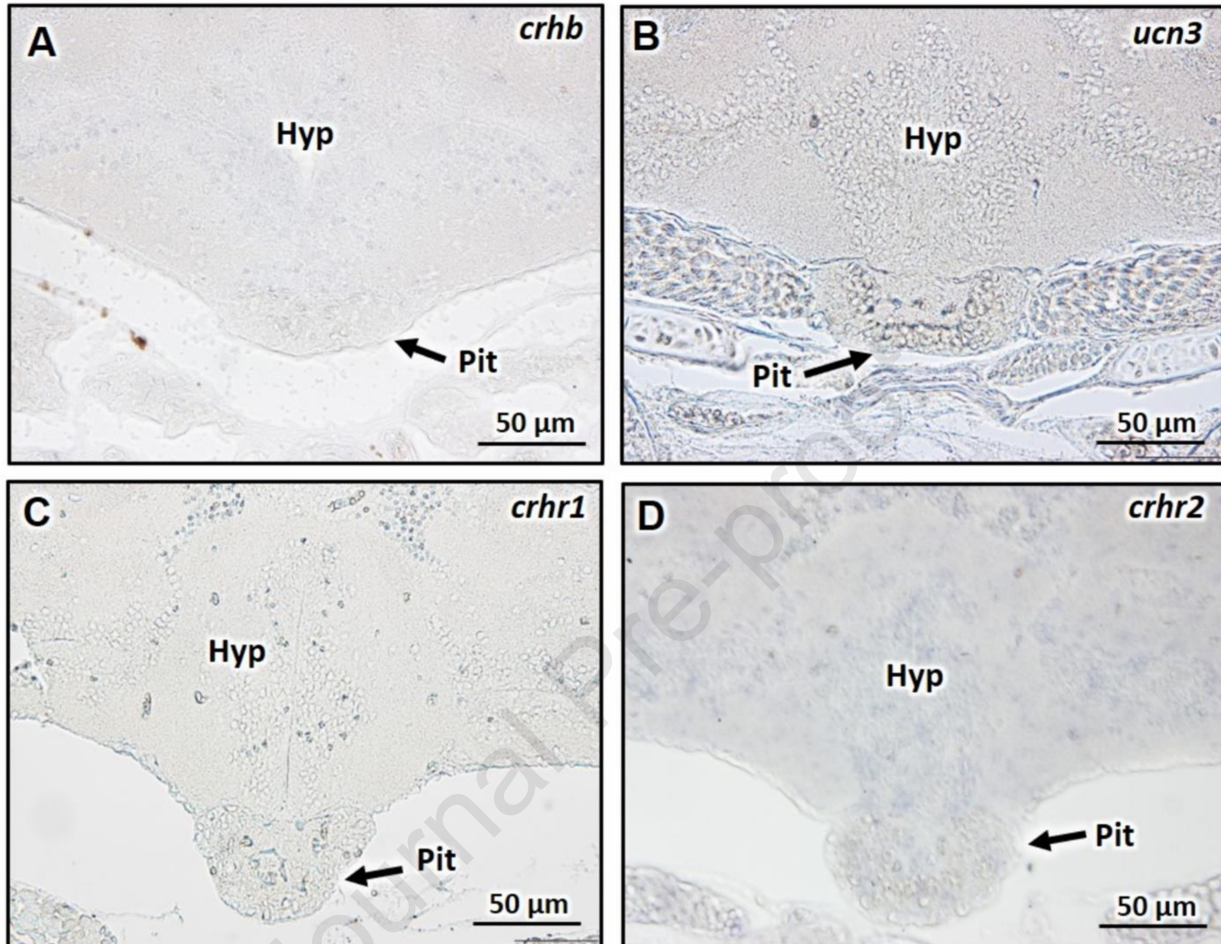

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Figure S4

Brain sections targeted with mRNA sense probes againts *crhb*, *ucn3*, *crhr1*, and *crhr2* showing no hybridization signals. The corresponding brain sections labeled with antisense probes are showed in Figure 3. Hypothalamus (Hyp), pituitary (Pit).

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Figure S5

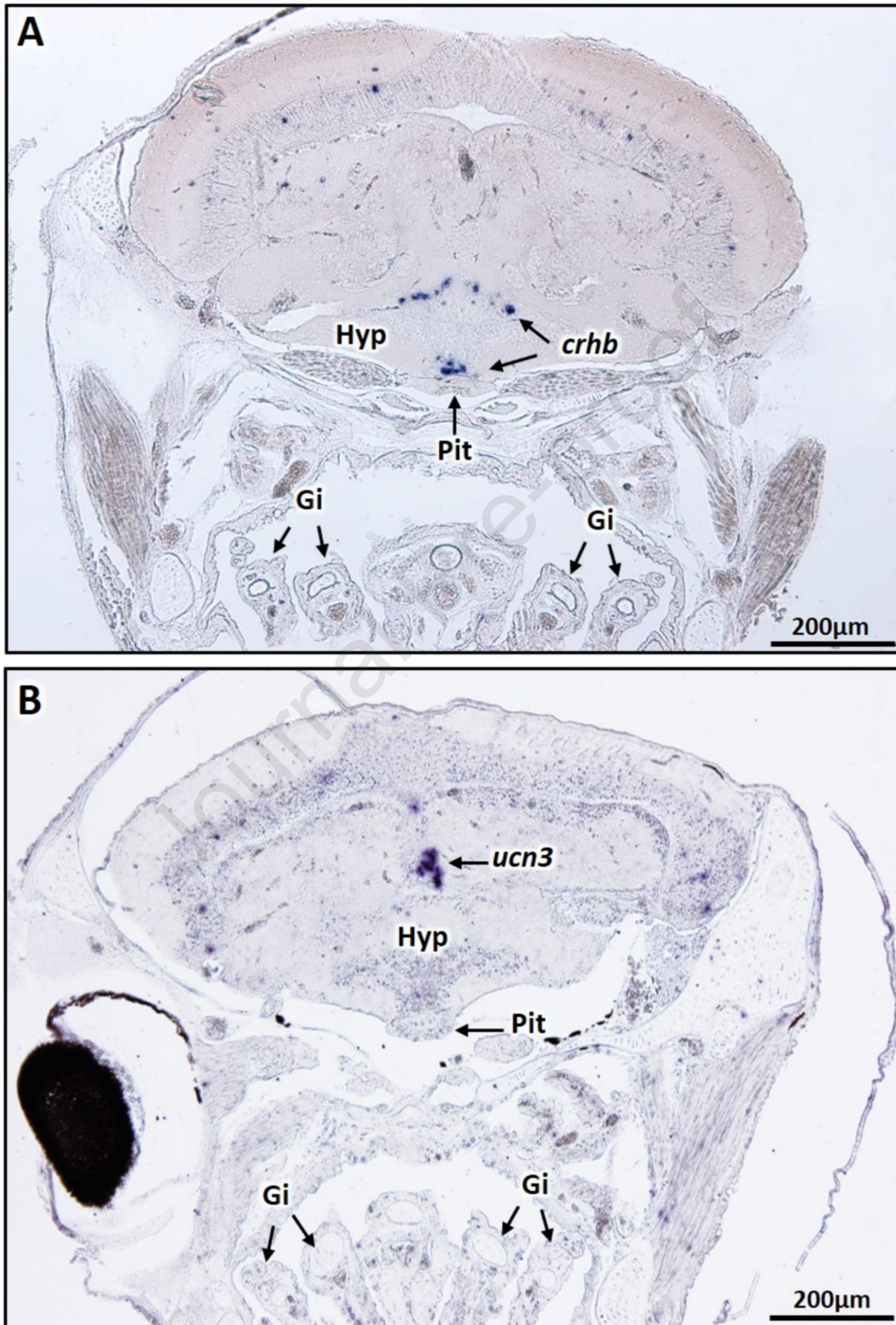

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Figure S5

Localization of *crhb* (A) and *ucn3* (B) by *in situ* hybridization in the heads of pejerrey larvae during the critical period of sex determination. The transcripts of these genes were detected in the brain but not in other tissues or organs in the heads. Neither *crhb* nor *ucn3* were detected in gills. Hypothalamus (Hyp), Pituitary (Pit), Gills (Gi).

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Figure S6

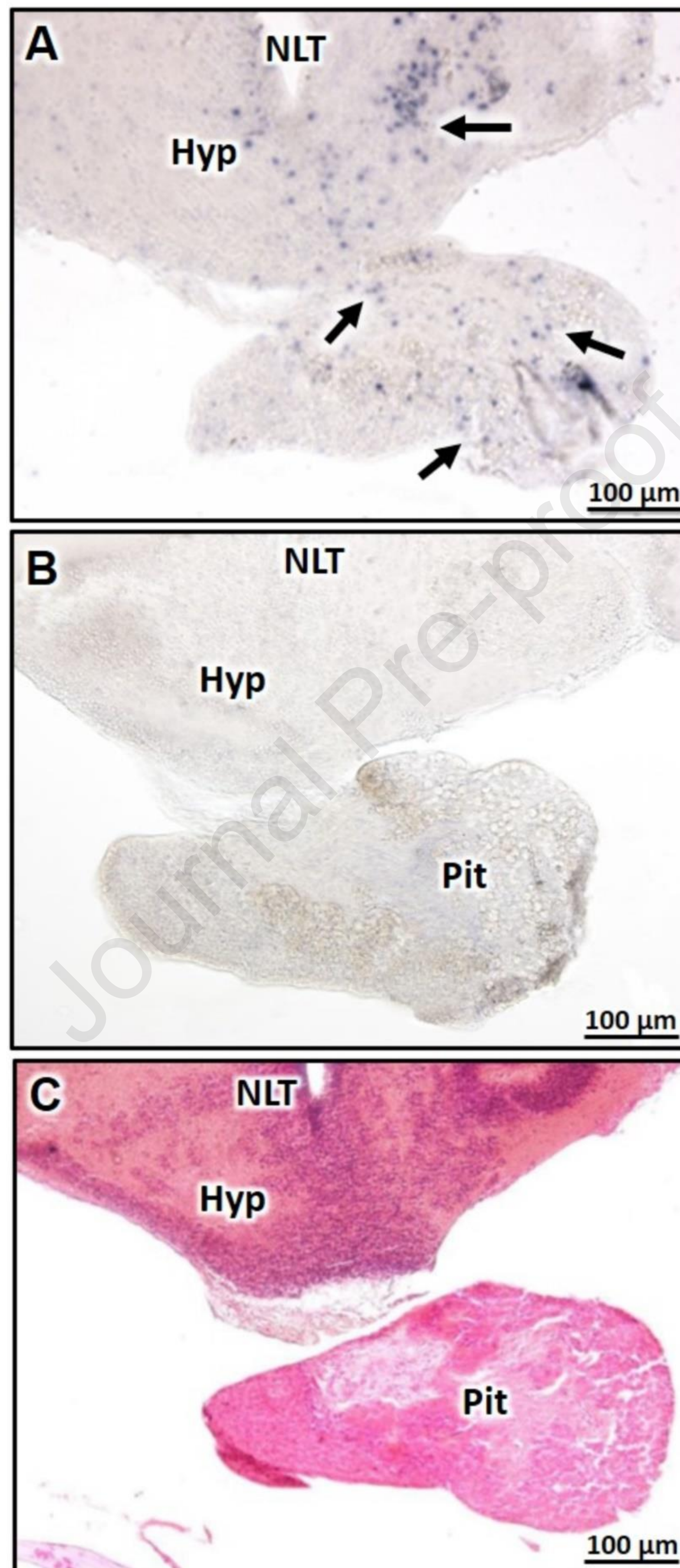

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Figure S6

mRNA abundance of *crhr2* in the brain of 24 week-old juvenile pejerrey. A: *crhr2* mRNAs were detected in the hypothalamus (Hyp) and pituitary (Pit) using the same anti-sense probe that could not detect transcripts in larvae (for a comparison see Figure 3D). The receptor was identified in dispersed or aggregates of *crhr2*-positive cells in the neural tissue (black arrows) and pituitary (Pit). B. Sense probes (negative control) did not show hybridization signal for *crhr2*. C: Histological section of the pituitary of a 24 week-old pejerrey, stained with Hematoxylin-Eosin.

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Figure S7

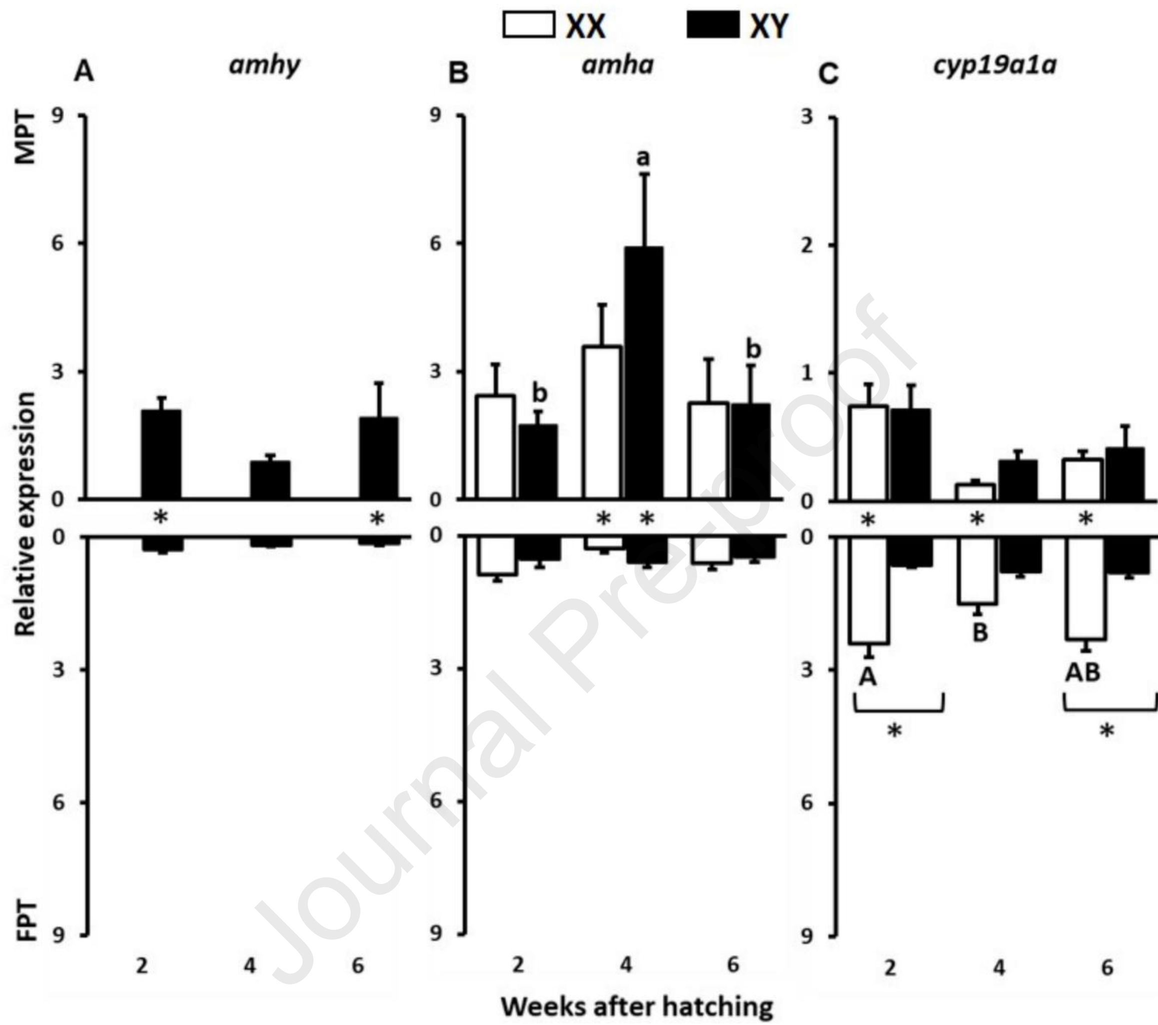

Figure S7

Developmental changes in the relative abundance of mRNAs of sex differentiation markers (*amhy*, *amha*, *cyp19a1a*) in relation to genotype and temperature during the critical period of sex determination. XX and XY larvae are depicted as white and black bars, respectively. Asterisks connecting white and black bars indicate a significant difference in gene expression between genotypes for any given week and those between upper (MPT) and lower (FPT) panels of the same gene indicate significant differences between thermal regimes for the same genotype and week. Bars within each panel (same gene and temperature) with different upper (XX) and lower (XY) case letters are significantly different. For clarity, only comparisons that include statistically significant differences are indicated by asterisks or letters. Statistical significance is assumed at $p<0.05$ (*amhy*: Two-way ANOVA followed by Tukey's multiple comparison test; *amha* and *cyp19a1a*: Three-way ANOVA followed by Tukey's multiple comparison test).

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Highlights

- XX larvae are more sensitive to heat stress than XY larvae
- Heat stress induces masculinization through the Crh system, HPI axis and brain aromatase
- The CRH family and cortisol may be involved in brain reprograming during sex reversal
- A dosage effect on the XX genotype may underly an exacerbated response to stress
- The brain influences gonadal sex determination/differentiation in pejerrey

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Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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