Hepatic estrogen-responsive genes relating to oogenesis in cutthroat trout (*Oncorhynchus clarki*): the transcriptional induction in primary cultured hepatocytes and the *in vitro* promoter transactivation in responses to estradiol-17β

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Abstract (365 words)

Estradiol-17β (E2) regulates transcription of estrogen-responsive genes *via* estrogen receptors (Esr). In many teleost species, choriogenin (*chg*), vitellogenin (*vtg*) and *esr* genes are transactivated by E2 in the liver. This study aimed i) to compare expression properties of all subtypes of these genes (*chg*: *chgHα*, *chgHβ*, *chgL*; *vtg*: *vtgAs*, *vtgC*; *esr*: *esr1a*, *esr1b*, *esr2a*, *esr2b*) in response to estrogen stimulation, and ii) to confirm how each of four Esr subtypes is involved in the transcriptional regulation of these estrogen-responsive genes in cutthroat trout hepatocytes. In hepatocytes in primary culture, all *chg* and *vtg* subtype mRNA levels, and those of *esr1a*, were increased by E2 treatment (10^{-6} M) at 24 and 72 h post initiation (hpi), but *esr1b*, *esr2a* and *esr2b* mRNA levels were not. Treatment of hepatocytes with various concentrations of E2 (10^{-11} ~ 10^{-6} M) induced dose-dependent increases in the levels of all *chg* and *vtg* subtype mRNAs at 24 and 72 hpi. At both time points, the lowest dose that induced a significant increase in the expression levels of mRNAs (LOEC) for E2 differed among the genes; LOECs were estimated as 10^{-11} M for *chgHα* at 24 hpi, as 10^{-9} M for *vtgC* at 72 hpi, and as 10^{-10} M for other mRNAs at both 24 and 72 hpi. Meanwhile, the levels of *esr1a* mRNA exhibited a dose-dependent increase at 24 and 72 hpi, but the LOEC shifted from 10^{-9} M at 24 hpi to 10^{-7} M at 72 hpi because of a decrease in mRNA levels at treatment groups exposed to high concentrations of E2. All Esr subtypes transactivated *chg*, *vtg* and *esr1a* promoters in the presence of E2 *in vitro*. The activation levels indicated that promoter activity of *chgHα* ≥ *vtgAs* > *chgHβ* > *chgL* ≥ *vtgC* ≥ *esr1a* when mediated by Esr1a, *chgHβ* > *chgHα* > *chgHL* > *vtgAs* ≥ *vtgC* ≥ *esr1a* by Esr1b, *chgHβ* ≥ *chgL* > *chgHα* ≥ *vtgAs* > *vtgC* > *esr1a* by Esr2a, and *chgHβ* ≥ *chgHα* ≥ *vtgAs* > *chgL* ≥ *vtgC* >*esr1a* by Esr2b. Collectively, different Esr subtypes were distinctly different in their ability to transactivate estrogen-responsive target genes, resulting in differential expression of *chg*, *vtg* and *esr1a* genes in the estrogen-exposed hepatocytes.

Keywords: estrogen, estrogen receptor, choriogenin, vitellogenin, transactivation

1. Introduction

Estrogens are steroid hormones that regulate vertebrate reproduction, development, growth and sexual homeostasis (Heldring et al., 2007). Estrogens, estradiol-17β (E2) in most cases, generally act on target cells through a nuclear estrogen receptor (Esr/ESR). The Esr/ESR is a member of the nuclear receptor superfamily of ligand-activated transcription factors, which includes receptors for steroid hormones, for thyroid hormones, for vitamins and for ligands that have yet to be identified (Sladek, 2011). The molecular mechanism underlying E2-Esr/ESR dependent transactivation of its target gene is generally accepted to be as follows: after E2 binds to Esr/ESR in the cell, the complex of E2 and Esr/ESR forms a homodimer that binds to estrogen-responsive elements (ERE) that are present in the promoter regions of targeted genes to induce expression of the target gene. The most typical ERE is composed of two head-to-head GGTCA half sites separated by three nucleotides (5'-GGTCAnnnTGACC-3', Walker et al., 1984).

Circulating E2 regulates expression of hepatic genes that are important for oocyte development in teleosts. As is well known, synthesis of vitellogenin (Vtg), the precursor of yolk protein, is induced by E2 in the liver of oviparous vertebrates. In addition to Vtg, choriogenin (Chg), the precursor of chorion protein, is produced in the livers of many teleosts, including salmonids (Hara et al., 2016). Chg is a glycoprotein belonging to the zona pellucida (ZP) superfamily (Goudet et al., 2008). Both Vtg and Chg are secreted into the blood stream, transported to oocytes, and incorporated in oocytes as yolk proteins and deposited onto oocytes as chorions, respectively. Yolk proteins serve as a source of nutrients for embryonic development and larval growth. Chorions protect the eggs and the embryos from physical and environmental stressors (Grierson and Neville, 1981; Songe et al., 2016).

So far, cDNAs encoding ESR orthologs have been cloned and characterized in

76 various vertebrates, including teleosts. The presence of two forms of ESR, designated as
77 ESR1 and ESR2, has been confirmed in most vertebrates, while most teleosts exhibit at
78 least three distinct subtypes of Esrs, i.e., Esr1 (also designated as Erα), Esr2a (also known
79 as Erγ or Erβ1) and Esr2b (also known as Erβ or Erβ2) (Choi and Habibi, 2003; Halm et
80 al., 2004; Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2004; Nagler et al., 2007).
81 Of the *esr* subtypes, hepatic *esr1* (*esr1a* in salmonids) expression is high in the liver of
82 vitellogenic females (Nagler et al., 2012; Sabo-Attwood et al., 2004) and induced by E2
83 treatment (Boyce-Derricott et al., 2009; Filby and Tyler, 2005; Sabo-Attwood et al., 2004).
84 In rainbow trout (*Oncorhynchus mykiss*), an additional *esr1* subtype (*esr1b*) has been
85 identified (Nagler et al., 2007), considered to be a minor subtype in terms of its mRNA
86 levels in the liver of vitellogenic females and E2-treated fish (Boyce-Derricott et al., 2009;
87 Nagler et al., 2012). So far, the functionality of Esr1b protein (ligand-binding and
88 estrogen-dependent transactivation qualities) has not been confirmed yet. As for Esr2
89 subtypes, recent studies using zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*)
90 have demonstrated their possible involvement in the expression of *vtg* (Griffin et al.,
91 2013; Nelson and Habibi, 2010). Other than these studies, both on cyprinids, functional
92 information on teleost Esr subtypes pertaining to the regulation of hepatic expression of
93 estrogen-responsive genes important for oogenesis (i.e., *chg*, *vtg* and *esr1* etc.) has been
94 quite limited.

95 Widespread multiplicity of *chg*/Chg and *vtg*/Vtg has become evident in teleosts.
96 Chgs are categorized into high (ChgH) and low type (ChgL) based on their molecular
97 weight (Hara et al., 2016). These ChgH and L typically belong to the ZPB and C
98 subfamilies, respectively, based on the unified nomenclature system for the ZP gene
99 family (Goudet et al., 2008). ChgH in salmonids can be further classified into ChgHα and

100 ChgHβ (Fujita et al., 2008; Hyllner et al., 2001; Westerlund et al., 2001). Meanwhile,
101 highly evolved acanthomorph species are likely to express three Vtg subtypes, VtgAa
102 (previously termed VtgA), VtgAb (previously termed VtgB), and VtgC (Hiramatsu et al.,
103 2005). Salmonids, on the other hand, express multiple copies of salmonid-type A Vtg
104 (VtgAs) alongside a VtgC orthologue (Buisine et al., 2002; Mushirobira et al., 2018;
105 Trichet et al., 2000).

106 As described above, E2 regulates the expression of oogenesis-related genes, such as
107 *chg*, *vtg* and *esr* subtypes, in the liver of teleosts during vitellogenesis. However, it is
108 unclear how E2 can differentially regulate the expression profiles of these genes.
109 Resolving this issue leads to further our understanding of oocyte development, and of the
110 molecular mechanisms underlying the transcriptional regulation of multiple genes by E2.
111 To date, many studies have demonstrated responses of *chg*, *vtg* and *esr1* genes to E2
112 stimulation. Because each of these studies used different species and/or did not consider
113 the multiplicity of *chg*, *vtg* and *esr*, it has been difficult to obtain an integrated view of
114 transcriptional regulation of these genes in response to E2 stimulation; it is therefore
115 needed to perform such a study in a single species and include all subtypes of the genes
116 of interest. Recently, the two promoters of *vtgAs* (1 and 2) and one promoter of *vtgC* for
117 cutthroat trout (*Oncorhynchus clarki*) have been cloned and analyzed (Mushirobira et al.,
118 2018).

119 The objective of the present study was *i*) to reveal the properties of estrogen-induced
120 transcription of nine hepatic genes (i.e., *chg*: *chgHα*, *chgHβ*, *chgL*; *vtg*: *vtgAs*, *vtgC*; *esr*:
121 *esr1a*, *esr1b*, *esr2a*, *esr2b*), and *ii*) to evaluate the involvement of each of the four Esr
122 subtypes in the transcriptional regulation of these estrogen-responsive genes, using our
123 model salmonid, the cutthroat trout. To achieve this objective, the present study utilized

124 primary hepatocyte culture to observe and compare the estrogen-induced transcriptional
125 properties of the nine genes. In addition, the functional property of four Esrs in driving
126 the expression of their putative target (*chg*, *vtg* and *esr*) genes was investigated by reporter
127 gene assays using reporter vectors containing promoter regions of the target genes.
128

129 **2. Materials and methods**

130 *2.1. Experimental fish and tissue sampling*

131 Cutthroat trout used in this study were obtained from a breeding stock held in flow-
132 through fresh water under ambient conditions at Nanae Freshwater Laboratory, Field
133 Science Center for Northern Biosphere, Hokkaido University (Nanae, Japan). Fish were
134 reared in outdoor tanks at the Faculty of Fisheries Sciences, Hokkaido University
135 (Hakodate, Japan), receiving a continuous flow of well water under natural photothermal
136 conditions. The fish were anesthetized by 2-phenoxyethanol (Kanto Chemical, Tokyo,
137 Japan) before sampling. All experimental procedures involving live fish followed the
138 policies and guidelines of the Hokkaido University Animal Care and Use Committee.

139 For cloning of *esr* cDNAs (section 2.2), livers were collected from females with the
140 following characteristics: for *esr1b* and *esr2a*, body weight (BW): 355.3 g, total length
141 (TL): 320 mm, gonadosomatic index (GSI: gonad weight/body weight × 100): 5.8; for
142 *esr2b*, BW: 129 g, TL: 222 mm, GSI: 0.3. Tissue samples were immediately immersed
143 in ice-cold RNA later (Thermo Fisher Scientific, Waltham, MA, USA), incubated
144 overnight at 4°C, and stored at −30°C until used for RNA extraction.

145 A two-year-old female cutthroat trout was used in order to clone the promoters of
146 each gene (section 2.3). Blood was collected and immediately mixed with
147 ethylenediaminetetraacetic acid (EDTA) to a final concentration of 17 mM. This whole

148 blood sample was stored at −80°C until used as a source for genomic DNA extraction.

149

150 *2.2. Molecular cloning of cutthroat trout esr1b, esr2a and esr2b*

151 Total RNA was extracted from the liver samples with ISOGEN (Nippon Gene,
152 Tokyo, Japan) according to the manufacturer's instructions. Concentration of total RNA
153 was measured with NanoDrop ND-1000 (Thermo Fisher Scientific). One µg total RNA
154 was reverse-transcribed by PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio,
155 Shiga, Japan), according to the manufacturer's instructions. The resulting cDNA
156 templates were stored at −30°C. Primer sets (Table 1) were designed using sequence data
157 from rainbow trout *esr1b* (NM_001124558), *esr2a* (NM_001124753) and *esr2b*
158 (NM_001124570.1) to amplify the respective open reading frames. PCR for each *esr*
159 subtype was performed using PrimeSTAR® Max DNA Polymerase (Takara Bio) in a
160 volume of 10 µl, which contained 1 µl 1st-strand cDNA, 5 µl PrimeSTAR® Max premix,
161 1 µl of each 1 µM forward and reverse primer and 2 µl nuclease-free water. The PCR
162 amplification was carried out using the following thermal parameters: 40 (*esr1b* and
163 *esr2a*) or 35 (*esr2b*) cycles at 98°C for 10 s, 55°C for 5 s and 72°C for 3 min. The PCR
164 products were separated by electrophoresis on 1% agarose gels, excised from the gels,
165 and purified by GENECLEAN Turbo Kit (MP-Biochemicals, Santa Ana, CA, USA)
166 according to the kit manual. The PCR products were subjected to an A-tailing reaction
167 (Knoche and Kephart, 1999) followed by ligation into pGEM-T Easy Vector (Promega,
168 Madison, WI, USA) according to the manufacturer's instruction. The ligated products
169 were transformed into XL1-Blue competent cells (Stratagene, La Jolla, CA, USA).
170 Recombinant clones were cultured overnight at 37°C on an agar plate containing
171 ampicillin and tetracycline, followed by selection of colonies of interest by blue-white

172 screening. Selected clones were grown in culture medium, and then used to extract and
173 purify plasmid DNA by Wizard Plus SV Minipreps DNA Purification System (Promega).
174 Purified plasmid DNAs were sequenced using BigDye terminator v3.1 Cycle Sequencing
175 Kit (Thermo Fisher Scientific) and a 3130xl Genetic Analyzer (Thermo Fisher Scientific)
176 according to the manufacturer's protocol.
177
178 *2.3. Molecular cloning of chg, vtg and esr1a gene promoters*
179 Genomic DNA was extracted from whole blood as described in Mushirobira et al.
180 (2018). A genome walking library was used for amplification of *chgHβ*, *chgL* and *esr1a*
181 gene promoters. Four genome walking libraries were made from genomic DNA using the
182 GenomeWalker Universal Kit (Takara Bio) according to manufacturer's protocol. Gene
183 specific primers (GSP, sense primer) were designed within the coding region of each gene
184 (Table 1). Primary PCR was carried out after mixing 5 µl of PrimeSTAR Max Premix
185 (Takara Bio), 0.2 µl of adaptor primer (AP, antisense primer) 1, 0.2 µl of 10 µM GSP (for
186 *chgHβ*: chgHβ-GW(Genome Walker)-R1, for *chgL*: chgL-GW-R1, for *esr1a*: esr1a-GW-
187 R1), and 0.2 µl of genomic DNA template; 7 cycles of 98°C for 10 s and of 72°C for 3
188 min were then run, followed by 32 cycles of 98°C for 10 s and of 68°C for 3 min. Nested
189 PCR was carried out using 5 µl of PrimeSTAR Max Premix, 0.2 µl of AP1, 0.2 µl of 10
190 µM GSP-2 (for *chgHβ*: chgHβ-GW-R2, for *chgL*: chgL-GW-R2, for *esr1a*: esr1a-GW-
191 R2), and 0.2 µl of the PCR product from the first round of amplification; after running 5
192 cycles at 98°C for 10 s and at 72°C for 3 min, a further 20 cycles were run at 98°C for 10
193 s and at 68°C for 3 min.
194 Primers (Table 1) for amplification of *chgHα*, *vtgAs* and *vtgC* promoters were
195 designed from rainbow trout whole genome (RefSeq assembly accession:

196 GCF_002163495.1). PCR was carried out as follows: 5 µl of PrimeSTAR Max Premix, 1
197 µl of each 2 µM forward and reverse primer, and 0.2 µl of genomic DNA template were
198 mixed and DNA amplified at 35 cycles of 98°C for 10 s, 55°C for 10 s and 72°C for 3
199 min.

200 The PCR products were ligated into cloning vectors (pGEM-T Easy Vector),
201 transformed into XL1-Blue competent cells, and sequenced as described above.

202

203 *2.4. Computational search for putative transcription factor binding sites*

204 Two online algorithms (ConSite; http://asp.ii.uib.no:8090/cgi-bin/CONSITE/ and
205 NUBIScan; http://www.nubiscan.unibas.ch/) were used for prediction of transcription
206 factor binding sites, including both basal promoter elements and putative EREs. The
207 specifics of ConSite and NUBIScan, were described previously by Sandelin et al. (2004)
208 and Podvinec et al. (2002), respectively.

209

210 *2.5. Primary culture of hepatocytes*

211 For Experiments 1 and 2 described below, hepatocytes were isolated from one male
212 cutthroat trout for each experiment, using a two-step collagenase perfusion technique as
213 described previously (Klaunig et al., 1985) with modifications; briefly, trout (body weight,
214 BL: 420 ~ 480 g; total length, TL: 314 ~ 410 mm) were anesthetized in 2-phenoxyethanol).
215 The liver was perfused with 50 ml of Ca-free modified Hanks solution (137 mM NaCl,
216 5.4 mM KCl, 0.5 mM NaH_2PO_4, 0.42 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 5 mM glucose,
217 0.5 mM EGTA, 10 mM HEPES, pH 7.4) to remove the blood from the liver. The liver
218 was then perfused with 100 ml of the same solution without EGTA and glucose and with
219 added $CaCl_2$ (5 mM), 0.05% collagenase (Wako, Tokyo, Japan) and 0.005% trypsin

220 inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Perfusion was performed at room
221 temperature using a peristaltic pump (Iwaki, Tokyo, Japan) at a flow rate of 10 ml/min.
222 After perfusion, the hepatocytes were dispersed in ice-cold L-15 medium (Thermo Fisher
223 Scientific) supplemented with 10 mM HEPES (pH 7.4), 1% of an antibiotic + antimycotic
224 solution (final concentration: 100 units/ml penicillin, 100 μg/ml streptomycin sulfate and
225 250 ng/ml amphotericin B; Wako) and 5% fetal bovine serum (FBS, Thermo Fisher
226 Scientific). The cell suspension was filtered through a cell strainer (Ikemoto Rika, Tokyo,
227 Japan) and the filtrate centrifuged at 100 g for 90 s at 4°C. The pellet was resuspended in
228 fresh medium and re-spun at 100 g for 2 min at 4°C; altogether, the cell pellet was washed
229 three times. Cell viability was about 95% as determined by the trypan blue exclusion test.
230 Cells were plated at a density of 3×10^5 cells/well on 24-well Falcon Primaria Multiwell
231 plates (Corning, New York, NY, USA) that were coated with Matrigel (basement
232 membrane matrix, Corning) as described by Schreer et al. (2005). Cells were cultured at
233 15°C in 0.5 ml of L-15 supplemented with 10 mM HEPES (pH 7.4), 1% antibiotic +
234 antimycotic solution, 5% FBS and 10 μg/ml bovine insulin (Sigma-Aldrich). The cultured
235 cells were settled for 24 h in order to adhere to the culture plate.
236
237 *2.6. Hormone treatment*
238 *2.6.1. Experiment 1: effects of continuous treatment with E2*
239 Cells in each well were washed once with 0.5 ml of FBS- and insulin-free L-15.
240 Then, cells were treated ('initiation') with 1 μM E2 or with solvent (ethanol) only; the
241 amount of ethanol in the medium did not exceed 0.1%. Half the volume (0.25 ml) of the
242 culture medium was changed every 24 h. At 0, 24 and 72 h post initiation (hpi), the
243 medium was removed and 200 μl ISOGEN was added to each well. Following 5 min

244 incubation, the samples were stored at −80°C until use. All incubations were run in
245 triplicate.
246
247 *2.6.2. Experiment 2: effects of various doses of E2*
248 Cells were treated with various concentrations of E2 (10^{-11} ~ 10^{-6} M). Treatments
249 and sampling were performed as described in Experiment 1 with the following
250 modifications; culture medium was not changed after treatment, and all incubations were
251 replicated 4 times.
252
253 *2.7. Quantitative real-time PCR (qPCR)*
254 Primers used in qPCR for *chg*s, *vtg*s, *esr1a* and elongation factor 1-α (*ef1-α*;
255 reference gene) were designed in our previous studies (Luo et al., 2013; Mushirobira et
256 al., 2013; Nagata et al., 2018). Primer sets for *esr1b*, *esr2a* and *esr2b* (Table 1) were
257 designed in Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) to cover
258 intron/exon boundaries which were predicted from the rainbow trout genome database
259 (Accession No. GCA_900005705.1). Predicted amplicon sizes for each gene were as
260 follows: *esr1b*: 141 bp, *esr2a*: 111 bp and *esr2b*: 118 bp.
261 Total RNA was extracted from hepatocytes in ISOGEN following manufacturer's
262 instructions. The RNA (200 ng) was reverse-transcribed by SuperScript® IV VILO cDNA
263 Synthesis kit (Thermo Fisher Scientific). Some aliquots of total RNA from all E2-treated
264 groups were pooled, reverse-transcribed and used as an inter-assay control (IAC) to
265 normalize between plates.
266 All qPCR reactions were performed as described in Nagata et al. (2018) using
267 FastStart Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland) and

268 StepOnePlus (Thermo Fisher Scientific) with the following modification. Primers were
269 added to the reaction at a final concentration of 150 nM except for *chgHα* and *esr2b* (50
270 nM). No PCR amplification was observed from no-reverse-transcription control
271 templates. Primer specificity was confirmed by dissociation curve analysis of PCR
272 products. The efficiencies of the standard curves were within the range of 81–104%, with
273 R^2 values > 0.99.
274
275 *2.8. Reporter gene assays*
276 The reporter plasmid containing either one of six promoters (*chgHα*, *chgHβ*, *chgL*,
277 *vtgAs*, *vtgC* and *esr1a*) and the expression plasmid containing either one of four *esr*
278 subtypes (*esr1a*, *esr1b*, *esr2a*, *esr2b*) were co-transfected into HeLa cells, and the
279 transactivation of the reporter gene was induced in the presence or absence of 1 μM E2
280 (10^{-6} M).
281 Construction of plasmid vectors for reporter gene assays was done using In-Fusion
282 HD Cloning Kit (Takara Bio) according to the manufacturer's instructions. Primer sets
283 (Table 1) for In-Fusion cloning were designed using the Primer Design tool for In-
284 Fusion® HD Cloning Kit (http://www.takara-
285 bio.co.jp/infusion_primer/infusion_primer_form.php). The promoter regions (2 kb in
286 size) of *chg*Hα, *chgHβ*, *chgL*, *vtgAs, vtgC* and *esr1a* in pGEM T-Easy vector were
287 subcloned into pGL4.10[*luc2*] Vector (Promega). The open reading frames of esr*1b*, *esr2a*
288 and *esr2b* were subcloned into pcDNA3.1(+) Vector (Thermo Fisher Scientific). The
289 Esr1a expression plasmid, pcDNA3.1-Esr1a, has been described previously (Mushirobira
290 et al., 2018). The subcloned plasmids were purified for transfection using PureLink
291 HiPure Plasmid Filter Midiprep Kit (Thermo Fisher Scientific).

292 Reporter gene assays were carried out as described previously (Mushirobira et al.,
293 2018) with some modifications. HeLa cells were seeded in 24-well Falcon Primaria
294 Multiwell plates at 3×10^4 cells/well in phenol-red free Dulbecco's Modified Eagle
295 Medium (DMEM; Sigma-Aldrich) supplemented with 10% charcoal/dextran treated FBS
296 (Hyclone, Logan, UT, USA). The cells were pre-incubated for 24 h at 37°C under 95%
297 air, 5% CO_2 and 100% humidity. After pre-incubation, the cells were transfected with 400
298 ng of either the promoter-harboring pGL4.10 (pGL4.10-*chgHα*, pGL4.10-*chgHβ*,
299 pGL4.10-*chgL*, pGL4.10-*vtgAs,* pGL4.10-*vtgC* and pGL4.10-*esr1a*) or the empty
300 pGL4.10 (negative promoter-construct control), 200 ng of the *esr*s-harboring pcDNA3.1
301 (pcDNA3.1-*esr1a*, pcDNA3.1-*esr1b*, pcDNA3.1-*esr2a* and pcDNA3.1-*esr2b*), and 100
302 ng of pRL-TK Vector (internal control to normalize for variation in transfection efficiency
303 between wells, Promega) using X-tremeGENE HP DNA Transfection reagent (Roche)
304 according to the manufacturer's instructions. Four h after commencement of transfection
305 at 37°C, the cells were treated with 1 μM E2 or vehicle (ethanol). The amount of ethanol
306 in the medium did not exceed 0.1%. After a further 40 h of incubation at 37°C, the cells
307 were collected to measure luciferase activities using Dual-Luciferase Reporter Gene
308 Assay System (Promega) by the Luminescaner-JNR (ATTO, Tokyo, Japan). All
309 incubations were run in quadruplicate.
310
311 *2.9. Statistics*
312 All data analyses were carried out using JMP Pro 14 Software program (SAS
313 Institute, Cary, NC, USA). Data were analyzed by two-way ANOVA with interaction,
314 using time × dose as factors for the primary hepatocyte cultures, and promoter type ×
315 dose for the reporter gene assay. When significant effects were found ($P < 0.05$),

316 comparisons between groups were conducted by Student's t-test, and multiple
317 comparisons were done with Tukey Kramer HSD test. The difference between groups
318 was considered significant at $P < 0.05$. All the results are expressed as means ± SE. For
319 statistical analyses, samples with values below the detection limit (100 copies/reaction
320 mix) in qPCR were assigned this minimum detectable level.
321
322 **3. Results**
323 *3.1. Molecular cloning of esr1b, esr2a and esr2b*
324 Cutthroat trout *esr1b*, *esr2a* and *esr2b* cDNAs (*esr1b*: 1782 bp, *esr2a*: 2324 bp,
325 *esr2b*: 2354 bp) encoding the cutthroat trout Esr1b, Esr2a and Esr2b were isolated and
326 sequenced (GenBank accession no. *esr1b*: LC577088, *esr2a*: LC577089, *esr2b*:
327 LC577090). The open reading frames of *esr1b*, *esr2a* and *esr2b* encoded 556, 594 and
328 606 amino acids, respectively. The deduced cutthroat trout Esr1b, Esr2a and Esr2b
329 proteins exhibited domain features typical of the estrogen receptor (A/B, C, D, E, F
330 domain). The Esr1b, Esr2a and Esr2b sequences shared high similarity (Esr1b: 99.3%,
331 Esr2a: 99.3% Esr2b: 98.8%) with the homologous sequences of rainbow trout (GenBank
332 Accession No. Esr1b: NP_001118030.1, Esr2a: NP_001118225.1, Esr2b:
333 NP_001118042.1).
334
335 *3.2. Hormone Treatment*
336 *3.2.1. Experiment 1: effects of continuous treatment with E2*
337 In *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA levels, significant interaction
338 effects of time × dose were observed ($P < 0.001$), as well as the significant main effects
339 of time ($P < 0.001$) and dose ($P < 0.001$), respectively. In both *esr2* subtypes, interaction

340 effects of time × dose were not significant while significant main effect of time was
341 observed ($P < 0.01$); main effect of E2 was significant only in *esr2b* ($P < 0.01$).
342 Levels of *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA in E2-treated
343 hepatocytes were significantly higher than those in the corresponding control group, both
344 at 24 hpi and 72 hpi (Fig. 1). In the E2 culture, *chgHβ*, *chgL*, *vtgAs*, *vtgC* and *esr1a* mRNA
345 levels significantly increased from 24 hpi to 72 hpi, while *chgHα* mRNA levels did not.
346 Levels of *esr1b* mRNA were undetectable in all groups at 24 hpi and 72 hpi. Treatment
347 with E2 did not affect mRNA levels of *esr2a* at 24 hpi and 72 hpi when compared to
348 levels in the corresponding control groups. Levels of *esr2b* mRNA in E2 exposure groups
349 were significantly lower than those in the corresponding control groups at 24 and 72 hpi.
350 In cultures supplemented with E2, *esr2b* mRNA levels decreased from 24 to 72 hpi.
351
352 *3.2.2. Experiment 2: effects of various doses of E2*
353 Exposure of hepatocytes to various doses of E2 yielded significant interaction effects
354 of time × dose in all mRNAs ($P < 0.001$: *chgHα*, *chgHβ*, *chgL*, *vtgC*, *esr1a*; $P < 0.01$:
355 *vtgAs*), in addition to the main effect of each factor ($P < 0.001$).
356 In hepatocytes cultured for 24 and 72 h, *chgHα*, *chgHβ*, *chgL*, *vtgAs* and *vtgC*
357 mRNAs levels were upregulated by E2 treatments in a dose-dependent manner (Fig. 2).
358 The lowest doses that induced a significant increase in the expression level (LOEC) for
359 *chg*s and *vtg*s were 10^{-10} M at both sampling points, except for those of *chgHα* at 24 hpi
360 (10^{-11} M) and *vtgC* at 72 hpi (10^{-9} M). The *chgHα*, *chgHβ* and *chgL* mRNAs levels in the
361 high E2 concentration groups (*chgHα*, *chgHβ*, *chgL* and *vtgAs*: 10^{-7}, 10^{-6} M E2; *vtgC*: 10^{-}
362 ^{6} M E2) increased from 24 hpi to 72 hpi; treatment means of all target gene mRNAs,
363 excluding that encoding *vtgAs*, exhibited significant differences. At the 24 hpi sample
364 collection, *esr1a* mRNA levels were upregulated by E2 treatments in a dose-dependent
365 manner and yielded an LOEC *esr1a* of 10^{-9} M E2. Unlike other target genes, *esr1a* mRNA

366 levels in cultures exposed to 10^{-9} ~ 10^{-6} M E2 significantly decreased from 24 hpi to 72
367 hpi; moreover, significant differences between the E2-treated and the control group were
368 found only in high concentration groups (10^{-7}, 10^{-6} M E2) at 72 hpi, and the LOEC was
369 10^{-7} M..
370
371 *3.3. Molecular cloning and sequence analysis of cutthroat trout chgHα, chgHβ, chgL,*
372 *vtgAs, vtgC and esr1a promoters*
373 The *chgHa* DNA promoter sequence consisted of 2851 bp located upstream of the
374 translation initiation site and 113 bp in the transcribed region (Fig. A. 1, Accession No.
375 LC577091). A complete palindromic ERE (GGTCAnnnTGACC) was not identified in
376 the analyzed promoter sequences. One ERE-like palindrome sequence differing from the
377 consensus ERE and six ERE half sites (1/2 ERE: GGTCA or TGACC) were predicted in
378 *chgHα* at the following positions: ERE-like: -198 to -184 (AGATCTatgTGACCT);
379 1/2ERE: -2594 to -2590, -2468 to -2464, -1014 to -1010, -237 to -233, -215 to -211 and
380 -103 to -99. Other putative regulatory elements such as activator protein-1 (AP-1) and
381 specificity protein 1 (Sp1) were predicted at the following positions: AP-1: -2077 to -
382 2070 (AGACTCAC), -1198 to -1191 (ATGATTCA), -1197 to -1190 (TGATTCAT) and -
383 565 to -558 (GTGACTGA); Sp1: -985 to -976 (ACCCTCCCTA) and -69 to -60
384 (ACACACCCCA). The conserved TATA box and CAAT box were found at positions -55
385 to -41 (GTATAAAAGCAGCAA) and -103 to -88 (GTCAGCCAAGGAGGTG),
386 respectively.
387 The *chgHβ* promoter sequence was 2320 bp in length and was followed by the
388 translation initiation site and of DNA transcribed into 101 bp of exon (Fig. A. 2, Accession
389 No. LC577092). No complete palindrome ERE was found in the analyzed promoter

390 sequences. Two ERE-like sequences and eight 1/2 ERE were predicted in *chgHβ* at the
391 following positions: ERE-like: -2246 to -2228 (GGACAcactaagtcTGATC) and -1139 to
392 -1129 (GGTTAcTGACC); 1/2ERE: -2124 to -2120, -1245 to -1241, -1071 to -1067, -
393 1057 to -1053, -260 to -256, -234 to -230, -137 to -133 and -97 to –93. The *chgβ* promoter
394 contained other putative regulatory elements at the following positions: AP-1: -1354 to -
395 1347 (GTGACTGA), -1088 to -1081 (TTACTGAC) and -728 to -721 (GTGACTAA).
396 The TATA box and CAAT box were predicted at -17 to -3 (TTATAAAGGTGGCCG) and
397 -234 to -219 (AGCCTCCAATGACATG), respectively.

398 The *chgL* DNA promoter was 3636 bp in length. The promoter was upstream of the
399 translation initiation site and of 111 bp of transcribable DNA (Fig. A. 3, Accession No.
400 LC577093). No complete palindrome ERE was identified in the analyzed promoter
401 sequences. One ERE-like sequences and seven 1/2 ERE were predicted at the following
402 positions: ERE-like: -703 to -692 (GGTCAtTCACC); 1/2ERE: -3544 to -3540, -2303 to
403 -2299, -2268 to -2264, -1732 to -1728, -1616 to -1612, -1395 to -1391 and -760 to -756.
404 The AP-1 and Sp1 were at the following positions: AP-1: -3333 to -3326 (TTAATCAC),
405 -3179 to -3172 (TTAATCAC), -2538 to -2531 (ATGAGTCA), -2537 to -2530
406 (TGAGTCAC), -2480 to -2473 (ATACTCAC), -1972 to -1965 (GTGAATAA), -1719 to
407 -1712 (TGGGTCAC), -1144 to -1137 (TCAGTCAC), -765 to -758 (TTACTGAC), -703
408 to -696 (GTCATTCA) and -386 to -379 (GTGACACA); Sp1: -3559 to -3550
409 (GAGGGGTGGT), -3514 to -3505 (GAGGCAGTGA), -2109 to -2100
410 (ACACAGCCCC), -1642 to -1633 (GGGGCATGGA), -1517 to -1508 (TCCATGCCTC),
411 -95 to -86 (GGGGGCGGGT) and -94 to -85 (GGGGCGGGTT). The TATA box and
412 CAAT box were found at positions -57 to -43 (TTATAAAACTGGCCA) and -153 to -138
413 (TGTGCCCAATGGGCAG), respectively.

414 The *vtgAs* promoter sequence (2872 bp) was located upstream of the translation
415 initiation site and 225 bp of the transcribed gene (Fig. A. 4, Accession No. LC577095).
416 Unlike the other tested promoters, *vtgAs* promoter contained the consensus palindrome
417 ERE at position -690 to -678 (GGTCAagcTGACC). Putative sequences of seven ERE-
418 like sequences and six 1/2 ERE were found at the following positions: ERE-like: -2767
419 to -2754 (GGTGAaatcTGACCT), -2758 to -2749 (TGACCTCTCC), -2168 to -2156
420 (GGTCAgttTGTAC), -1027 to -1014 (GGTCAagttTGATG), -612 to -599
421 (GGACAagctTGGAC), -509 to -496 (GATCAtacaTGTAC) and -233 to -224
422 (TGACCTCTCC); 1/2ERE: -2713 to -2709, -2058 to -2054, -2005 to -2001, -1987 to -
423 1983, -1197 to -1193 and -1175 to -1171. The putative AP-1 and Sp1 sites were identified
424 at the following positions: AP-1: -2057 to -2050 (GTCAGTAA), -1760 to -1753
425 (TGAATGAC), -1746 to -1739 (TGTCTCAC), -1582 to -1575 (GTGTGTCA), -1434 to
426 -1427 (GTGAGTGA), -1167 to -1160 (CTGAGTAA), -948 to -941 (TTACTCAT), -830
427 to -823 (TGTCTCAC), -721 to -714 (TGACTGAC) and -274 to -267 (GTGATTCT);
428 Sp1: -2774 to -2765 (GAGGCGAGGT), -2349 to -2340 (GGGGCTGGGA), -2209 to -
429 2200 (ACGGCGTGGT), -2092 to -2083 (GGGGCAGGCA), -1092 to -1083
430 (ACCCTGCCCA), -1009 to -1000 (GGGGGGTGGT) and -201 to -192
431 (GGGGCAGGTT). The predicted TATA box and CAAT box were found at -47 to -33
432 (CTTTAAAAGGCGGAC) and -189 to -174 (CCTAACCTATGGGTGT), respectively.
433 The *vtgC* DNA promoter sequence consisted of 3025 bp located upstream of the
434 translation initiation site and of 39 bp of transcribable DNA (Fig. A. 5, Accession No.
435 LC577096). No complete palindrome ERE was observed in the analyzed promoter
436 sequences. Six ERE-like sequences and seven 1/2 ERE were predicted at the following
437 positions: ERE-like: 1611 to -1601 (GGTCAgAGACC), -1474 to -1461

438 (GTTCAttggTGTCCA), -1324 to -1315 (GAGCAtccTGACC), -586 to -575
439 (GTTCAaaTGCAC), -312 to -309 (GGTCAaagaTGTTG) and -87 to -78
440 (GGTCATGTAC); 1/2ERE: -2193 to -2189, -2087 to -2083, -1273 to -1269, -1118 to -
441 1114, -712 to -708, -660 to -656 and -189 to -185. Other gene regulatory elements were
442 predicted at the following positions: AP-1: -2570 to -2563 (TTACTCAT), -2351 to -2344
443 (ATACTCAC), -1633 to -1626 (ATGAGACA), -1562 to -1555 (GTGAGCAA), -862 to -
444 855 (GTGAGTAA) and -73 to -66 (TTAGTCAT); Sp1: -2221 to -2212 (ATACTGCCCC).
445 The conserved TATA box was identified at position -48 to -34 (TTATAAAACTGGCCA).
446 A total of 6155 bp of sequence upstream of the translation initiation site and a 1041
447 bp transcribable region were isolated and squenced for the *esr1a* DNA promoter (Fig. A.
448 6, Accession No. LC577094). The promoter contained no complete palindrome ERE. The
449 presence of three ERE-like sequences and eight 1/2 ERE was predicted at the following
450 positions: ERE-like: -1107 to -1090 (GAGCAaccgaggcTTGAC), -525 to -511
451 (GGTCAagagtTGTCC) and -79 to 67(TGTCAtgtTGACC); 1/2ERE: -5609 to -5605, -
452 5509 to -5505, -5095 to -5091, -4948 to -4944, -4865 to -4861, -4086 to -4082, -3761 to
453 -3757, -3698 to -3694, -3645 to -3641, -3600 to -3596, -3287 to -3283, -3057 to -3053, -
454 2191 to -2187, -2091 to -2087, -1482 to -1478, -899 to -895, -285 to -281, -100 to -96
455 and -70 to -66. Other putative gene regulatory elements were confirmed at the following
456 positions: AP-1: -5754 to -5747 (GTGAGCCA), -5493 to -5486 (TTACTCAC), -5368 to
457 -5361 (TGTGTCAC), -5050 to -5043 (GTGACTGA), -5049 to -5042 (TGACTGAT), -
458 4213 to -4206 (TGAATGAC), -3815 to -3808 (GTGAGTTA), -3711 to -3704
459 (TGACTCCC), -2213 to -2206 (GTGAGAAA), -1126 to -1119 (TGAATGAC) and -267
460 to -260 (TTAGTCAG); Sp1: -5855 to -5846 (AGGGCAGTGT), -5808 to -5799
461 (GGGGCGGTAT), -4409 to -4400 (ACCCAGCCAG), -3604 to -3595

462 (CAGGGAGGGT), -3329 to -3320 (ACAATGCCTC), -3099 to -3090
463 (GGGGCAGGGG), -2201 to -2192 (AAGGGAGGGT), -2099 to -2090
464 (GGGGCTGTGA) and -396 to -387 (TTGGCGGGAT). The putative TATA box and
465 CAAT box were found at positions, -203 to -189 (CTATGAAAAGGGGGA) and -336 to
466 -321 (AAGGCCCAATGATAGC), respectively.

467 Numbers of ERE, ERE-like, 1/2 ERE, AP-1 and Sp1 sites in *chgHα*, *chgHβ*, *chgL*,
468 *vtgAs*, *vtgC* and *esr1a* promoters are presented in Table 2.

469 The transcriptional response of *esr1b*, *esr2a* and *esr2b* genes to E2 stimulation were
470 weak in salmonids, unlike those of *chg, vtg* and *esr1a* genes described above. In a
471 preliminary analysis that was based on the whole genome database of rainbow trout, the
472 presence of many predicted sites involved in the E2 responsiveness of genes (ERE-like
473 sequences, etc.) was observed in *esr1b*, *esr2a* and *esr2b* promoters, as well as the *chg, vtg*
474 and *esr1a* promoters (supplemental Table: Table A. 1).

475

476 *3.4. Transactivation of estrogen-responsive gene promoters by E2 via Esr1a, Esr1b,*
477 *Esr2a and Esr2b*

478 Significant interaction effects of promoter type × dose were observed in
479 transactivation *via* each Esr subtype ($P < 0.001$: *chgHα*, *chgHβ*, *chgL*, *vtgAs*, *vtgC*, *esr1a*),
480 as well as the main effect of each factor ($P < 0.001$).

481 In all promoter-containing constructs, Esr1a-mediated reporter activity in the
482 presence of E2 was significantly higher than that in the solvent control group (non-E2
483 controls) and the empty-vector control group (Fig. 3). The reporter activities in E2-treated
484 HeLa cells transfected with *chgHα* and *vtgAs* promoters were significantly higher than
485 those in HeLa cells provided with the *chgHβ* promoter. The reporter activities of the

486 remaining gene promoters in E2 culture were significantly lower than those of *chgHβ*
487 promoter, and higher in the order of *chgL* and *esr1a*.

488 All promoters in HeLa cells were significantly transactivated by Esr1b in the
489 presence of E2 compared to the corresponding solvent control groups or empty vector
490 construct. Reporter activity in E2-supplemented cultures was highest for the *chgHβ*
491 promoter, followed, in descending order, by *chgHα, chgL, vtgAs*, *vtgC* and *esr1a*.
492 Significant effects on reporter activity were found among *chgHβ, chgHα, chgL, vtgAs* and
493 *vtgC* promoters in the E2-treated groups. No significant difference was detected between
494 *vtgC* and *esr1a* promoters in E2-treated groups.

495 In the presence of E2, Esr2a significantly transactivated all promoters relative to the
496 solvent control groups and empty-vector groups. Again, as for the Esr1b-expressing HeLa
497 cells, the *chgHβ* promoter showed the highest Esr2a-mediated activity when exposed to
498 E2. The reporter activities in E2-supplemented cultures ranked highest in HeLa cells
499 transfected with *chgHβ*, followed by *chgL*, *chgHα*, *vtgAs*, *vtgC* and *esr1a*. E2-induced
500 reporter activities differed significantly between the following promoter pairs: *chgL* and
501 *chgHα*, *vtgAs* and *vtgC* and *vtgC* and *esr1a*.

502 Esr2b-mediated transactivation of the different target gene promoters followed a
503 pattern essentially the same as seen for Esr1b and Esr2a; thus, the *chgHβ* promoter
504 displayed the strongest, and the *esr1a* promoter the weakest activity when Esr2b was
505 evaluated as receptor for E2. The reporter activities in E2 culture decreased in the order
506 of *chgHβ*, *chgHα*, *vtgAs*, *chgL*, *vtgC* and *esr1a*.

507

508 **4. Discussion**

509 The predicted cutthroat trout Esr1b, Esr2a and Esr2b polypeptide sequences showed

510 high similarities with homologous sequences of rainbow trout (Esr1b: 99.3%, Esr2a:
511 99.3%, Esr2b: 98.8%). Primary structures of cutthroat trout Esr1b, Esr2a and Esr2b
512 exhibited typical functional domains reported for vertebrate ESRs/Esrs and the motifs
513 required for DNA and ligand binding were highly conserved (data not shown). These
514 structural similarities suggest that cutthroat trout Esr1b, Esr2a and Esr2b have the basic
515 functions of vertebrate ESRs/Esrs, such as ligand (estrogen) binding and transactivation
516 of target genes (Davis et al., 2010; Menuet et al., 2002).

517 To reveal the responsiveness to E2 stimulation, effects of continuous treatment with
518 a high E2 dose (10^{-6} M) on expression of *chg*, *vtg* and *esr* genes were examined in
519 Experiment 1. With replenishment of E2 every 24 hpi, the level of *chg*, *vtg* and *esr1a*
520 mRNAs increased by 72 hpi. Levels of these mRNAs were also upregulated by E2
521 treatment *in vivo* in male and immature cutthroat trout (Mushirobira et at., 2018), as were
522 mRNA/protein levels in the liver of other salmonids (Amano et al., 2010; Boyce-Derricott
523 et al., 2009; Hiramatsu et al., 1997; Thomas-Jones et al., 2003; Westerlund et al., 2001).
524 Thus, these results indicate that E2-induction of hepatic expressions of *chg*s, *vtg*s and
525 *esr1a* is a common feature in salmonid species.

526 In Experiment 1, levels of only *esr1a* mRNA were upregulated by E2 treatment –
527 those of the other *esr*s (i.e., esr*1b*, *esr2a* and *esr2b*) were not. In rainbow trout fed with
528 E2-containing pellets for five days, Casanova-Nakayama et al. (2018) confirmed that E2
529 could stimulate hepatic *esr1b* gene expression. Osachoff et al. (2013) confirmed that 7-
530 day exposure to E2 upregulated hepatic *esr1b* mRNA levels in rainbow trout, but that 2-
531 day exposure did not. Similarly, Boyce-Derricott et al. (2009) observed no significant
532 effect of E2 on hepatic *esr1b* mRNA expression in rainbow trout by 24 h following
533 injection. These results suggest that it takes a relatively long time for E2 to upregulate

534 *esr1b* gene expression. Meanwhile, E2 did not show any significant effect on hepatic
535 *esr2a* expression in rainbow trout, regardless of the duration of the exposure period
536 (Boyce-Derricott et al., 2009; Casanova-Nakayama et al., 2018; Cleveland and Weber,
537 2015; Osachoff et al., 2013), which is in keeping with our results on primary cultured
538 hepatocytes of cutthroat trout in this study. As for *esr2b* in rainbow trout, Cleveland and
539 Weber (2015) reported downregulation of the mRNA levels by E2 treatment, similar to
540 the results for cutthroat trout in the present study, but other studies (Boyce-Derricott et
541 al., 2009; Casanova-Nakayama et al., 2018; Osachoff et al., 2013) did not document such
542 a response of *esr2b* mRNA to E2 treatment. Collectively, *esr1a* gene alone appears to
543 exhibit strong and acute upregulation by E2 stimulation, suggesting that *esr1a* is the major
544 transcriptional factor among *esr* subtypes involved in active (i.e., strong and acute)
545 synthesis of hepatic *chg* and *vtg* mRNAs in salmonids.

546 Because expression of six gene transcripts (i.e., *chgHα*, *chgHβ*, *chgL, vtgAs*, *vtgC*
547 and *esr1a*) appeared to be strongly and acutely upregulated by E2 treatments, a follow-
548 up experiment (Experiment 2) sought to examine the effects of E2 dose (10^{-11} ~ 10^{-6} M)
549 on the expression of these six genes. Transcript levels of *chg*s and *vtg*s at 24 and 72 hpi
550 increased in a E2-dose-dependent manner. In addition, *esr1a* mRNA levels in E2-
551 supplemented incubations also showed a dose-dependent increase at 24 hpi. These
552 patterns suggest that the expression of these genes is under strict regulation by E2. The
553 LOECs of *chg* and *vtg* mRNAs for E2 were 10^{-11} M for *chgHα* at 24 hpi and 10^{-9} M for
554 *vtgC* at 72 hpi, which differed from the LOEC (10^{-10} M) for other target gene mRNAs at
555 both time points, indicating that the *chgHα* and *vtgC* exhibit differential sensitivity to E2
556 from the other *chg* and *vtg* subtypes in primary cultured hepatocytes of cutthroat trout.

557 From 24 to 72 hpi, *chgHα*, *chgHβ*, *chgL*, *vtgAs* and *vtgC* mRNA levels increased or

558 were maintained at high values in high-dose E2 cultures, both in Experiments 1 (culture
559 with replacement of medium) and 2 (culture without replacement of medium). Meanwhile,
560 *esr1a* mRNA levels in high-dose E2 incubations increased in Experiment 1 but decreased
561 in Experiment 2 from 24 hpi to 72 hpi. Thus a difference in the way of E2 supplementation
562 possibly caused the opposite *esr1a* response to E2 between the two experiments, similar
563 to a study using primary cultured hepatocytes of rainbow trout (Flouriot et al., 1996). In
564 said study, high *esr1a* mRNA levels were maintained from 24 to 72 h following
565 supplementation with 10^{-6} M E2 and replacement of E2-containing culture medium every
566 24 h, but levels decreased when the medium was not replaced – those findings are in good
567 agreement with the results of Experiment 1 and Experiment 2 in our study on cutthroat
568 trout. It has been shown that hepatocytes of *Xenopus laevis* in primary culture rapidly
569 metabolize E2 in culture medium (Tenniswood et al., 1983). Although such analysis was
570 not performed in the present study, rapid metabolism of E2 in the medium could thus be
571 a cause of the time-dependent decrease of *esr1a* mRNA expression. Adhesion of E2 to
572 the wall of the plate could be another potential cause for this decrease.

573 Numbers of ERE, ERE-like, 1/2 ERE, AP-1 and Sp1 sites in *chgHα*, *chgHβ*, *chgL*,
574 *vtgAs*, *vtgC* and *esr1a* promoters were different, suggesting these promoters have
575 differential transcriptional properties. An ERE consensus sequence was identified only in
576 the *vtgAs* promoter while all promoters had ERE-like and 1/2 ERE sequences. In teleosts,
577 ERE-like and 1/2 ERE sites elicit a significant increase in estrogen-dependent synthesis
578 of reporter protein (Le Drean et al., 1995; Menuet et al., 2004; Teo et al., 1998). Thus,
579 aside from the consensus ERE, ERE-like and 1/2 ERE sites are likely to be responsible
580 for the E2-induced expression of *chg*, *vtg* and *esr1a* genes. This was also supported by
581 the results of this study; cutthroat trout *chg*, *vtg* and *esr1a* promoters were transactivated

582 in the presence of E2 and Esrs. In addition, AP-1 and Sp1 binding sites, which are known
583 to interact with Esr, were predicted in promoters of cutthroat trout *chg*, *vtg* and *esr1a*.
584 These binding sites possibly contribute to the transactivation of *chg*, *vtg* and *esr1a*.
585 *In silico* analysis predicted the binding sites for transcription factors; it is unclear if
586 the predicted sites are functional *in vivo* and *in vitro*. Therefore, binding sites with low or
587 no functionality for E2 responsiveness of genes were possibly identified in *esr1b*, *esr2a*
588 and *esr2b* promoters. To verify the transcriptional response of the targeted genes, *in vitro*
589 experiments, such as promoter assays, will thus be needed.
590 In reporter gene assays with teleost Esrs, several mammalian cell lines (CHO-K1,
591 HepG2, HeLa, HEK-293, CHO, etc.) have been used (Davis et al., 2010; Le Drean et al.,
592 1995; Lee Pow et al., 2016; Menuet et al., 2004; Mushirobira et al., 2018). It has been
593 shown that these cells have endogenous ESRs and other estrogen-related proteins. For
594 example, CHO-K1 expresses functional endogenous Esr2 (Thomas et al., 2003), whereas
595 HepG2 expresses the gene encoding G protein-coupled estrogen receptor 1 (*GPER1*) at
596 high levels (Transcripts Per Kilobase Million: TPM: 15.3, calculated by next-generation
597 sequences) compared to levels in other human cell lines (TPM; HeLa: 0.9, HEK-293: 1.9;
598 see The Human Protein Atlas, https://www.proteinatlas.org/). The GPER has been shown
599 to bind estrogens and initiate subsequent signaling cascades *in vitro* (Langer et al., 2010).
600 Because of high expression of endogenous estrogen receptors, the CHO derivative cells
601 (CHO and CHO-K1) and HepG2 are perhaps not best-suitable for the Esr/ESR-based
602 reporter gene assay. HeLa cells have been widely used for reporter gene assay for Esrs of
603 teleosts (Lee Pow et al., 2016; Menuet et al., 2004; Sumida and Saito, 2008). In addition,
604 HeLa expresses low levels of Esr1 (TPM: 0.1) and Esr2 (TPM: 0.4). Thus, the HeLa cell
605 line was selected for the reporter gene assays in the present study. To eliminate the

606 influence of promoter length on the transfection efficiency, reporter gene assay in this
607 study was performed using 2000 bp promoter regions of all genes.

608 Reporter gene assay with four different trout Esr subtypes revealed that all of them
609 are functional and can transactivate *chg*, *vtg* and *esr1a* promoters in the presence of E2.
610 E2-induced transactivation of *esr1a* promoters mediated by the different Esr subtypes was
611 lowest for *esr1a*. In rainbow trout, reporter assay using *esr1a* cDNA and *esr1a* promoter,
612 E2 treatment increased activities up to 10-fold only in the presence of different Esr
613 subtypes (Le Drean et al., 1995); such weak induction of reporter activities was also seen
614 in zebrafish when using *esr1* cDNA and *esr1* promoter (Menuet et al., 2004). The fold-
615 activation of teleost *esr1* seems generally low, findings that are reinforced for the *esr1a*
616 promoter of cutthroat trout in the present study.

617 Each Esr subtype differentially transactivated *chg*, *vtg* and *esr1a* promoters in the
618 presence of E2. For example, E2-induced transactivation of the promoters through Esr1a
619 was *chgHα* ≥ *vtgAs* > *chgHβ* > *chgL* > *vtgC* > *esr1a*, while that through *esr1b* was *chgHβ*
620 > *chgHα* > *chgL* > *vtgAs* > *vtgC* ≥ *esr1a*. These results suggest that the different Esr
621 subtype vary in their transactivation properties, conceivably explaining the different
622 transcriptional profiles of *chg*, *vtg* and *esr1a* genes in primary cultured hepatocytes. The
623 selectivity of Esr subtypes in transactivation of such E2-responsive genes remains unclear.
624 Further investigations are required to address the differential role of the Esr subtypes in
625 the transactivation of *chg*, *vtg* and *esr1a* genes.

626 In conclusion, the present study documented i) *in vitro* hepatic responses of *chg*, *vtg*
627 and *esr* gene expression to E2 stimulation within a single teleost species, and ii)
628 transactivation properties of *chg*, *vtg* and *esr* promoters by four Esr subtypes. To our
629 knowledge, this is the first report to show the different responsiveness to E2 among

630 hepatic estrogen-responsive genes in a single teleost species alongside the differential
631 roles of Esr subtypes in the transactivation of promoters for those genes. We demonstrated
632 that there were clear differences in E2-induced transactivation of *chg*, *vtg* and *esr1a* gene
633 promoters by four discrete Esr subtypes. Differences in *chg*, *vtg* and *esr1a* gene
634 expression in trout hepatocytes following E2 exposure possibly reflect differential
635 transactivation properties among four Esr subtypes for these gene promoters.
636

637 **Acknowledgements**
638 We are grateful to Dr. M. Lokman (University of Otago, New Zealand) for critical reading
639 of the manuscript. We thank Dr. E. Yamaha, Mr. S. Kimura and Dr. E. Takahashi, Nanae
640 Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido
641 University, for rearing experimental fish in this study. We acknowledge Dr. M. Shimizu,
642 Faculty of Fisheries sciences, Hokkaido University for helpful discussions.
643

644 **Funding**
645 This work was supported in part by the JSPS KAKENHI [grant number 18H02272 to N.
646 H.]
647

648 **Declarations of interest**
649 None
650

651 **Appendix A. Supplementary data**
652

653 **References**

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837 **Figure captions**

838 Fig. 1 Effects of continuous treatment of primary cultured hepatocytes of male cutthroat
839 trout with estradiol-17β (E2, black columns) or a control solvent (C, white columns) on
840 the transcript levels of choriogenin (*chg*: *chgHα*, *chgHβ* and *chgL*), vitellogenin (*vtg*:
841 *vtgAs* and *vtgC*) and estrogen receptor (*esr1a*, *esr1b*, *esr2a* and *esr2b*) subtypes.

842 Hepatocytes from one trout were treated with 10^{-6} M E2. Half of the culture medium was
843 replaced by fresh medium every 24 h post initiation (hpi) of E2 treatment. At 0, 24 and
844 72 hpi, cells were harvested and mRNA levels quantified by quantitative real-time reverse
845 transcription PCR. Columns indicate mean values and vertical lines indicate standard
846 errors. Different letters denote that values are significantly different ($P < 0.05$).
847
848 Fig. 2 Effect of treatment of hepatocytes of male cutthroat trout in primary culture with
849 single doses of estradiol-17β (E2, black columns) or a control solvent (C, white columns)
850 on the transcript levels of choriogenin (*chg*: *chgHα*, *chgHβ* and *chgL*), vitellogenin (*vtg*:
851 *vtgAs* and *vtgC*) and estrogen receptor (*esr1a*). Hepatocytes from one trout were treated
852 with 10^{-11} ~ 10^{-6} M (-11 ~ -6 in horizontal axis) E2 for 72 h without replacement of the
853 medium. At 24 and 72 h post initiation (hpi) of E2 treatment, cells were harvested and
854 mRNA levels quantified by quantitative real-time reverse transcription PCR. Columns
855 indicate mean values and vertical lines indicate standard errors. Different letters denote
856 that values are significantly different ($P < 0.05$). Arrow heads exhibit the lowest dose that
857 induced a significant increase in the expression levels of each mRNA (LOEC) for E2.
858
859 Fig. 3 Fold-induction change in transactivation of choriogenin (*chg*), vitellogenin (*vtg)*
860 and estrogen receptor 1a (*esr1a*) promoters mediated by four Esr subtypes (Esr1a, Esr1b,
861 Esr2a and Esr2b) in the presence or absence of estradiol-17β (E2). Hela cells were co-
862 transfected with two constructs: one *esr* expression vector (*esr1a*, *esr1b, esr2a* or *esr2b*)
863 and one gene promoter (*chgHα*, *chgHβ*, *chgL*, *vtgAs, vtgC* or *esr1a*) in a reporter vector.
864 Reporter vector without a promoter (empty vector, 'vector') was used as negative control.
865 The transfected cells were treated with 1 μM E2 (closed columns) or control solvent (C,
866 open columns). The fold-induction was initially normalized to the luminescence from
867 Renilla luciferase; data were averaged from 4 replicate wells. Fold-activation was
868 represented after setting the empty vector (vehicle control) set to 1. Columns indicate

869 mean values and vertical brackets standard errors. Different letters denote significant
870 differences ($P < 0.05$).

1 **Highlights**

2 • Choriogenin, vitellogenin and estrogen receptor-1a genes were estrogen-responsive

3 • These estrogen responsive genes responded differentially to estradiol-17β (E2)

4 • E2 transactivated these gene promoters in the presence of four estrogen receptors

Table 1 Name, nucleotide sequence, direction, and use of primers

Name	Sequence	Direction	Use
esr1b-F	GCCATCTCACCCCAGAAACT	Forward	Cloning for *esr1b*
esr1b-R	ACTCTCACCTCCACAAATGTCA	Reverse	Cloning for *esr1b*
esr2a-F	CACGGATGGATTGCTACTCC	Forward	Cloning for *esr2a*
esr2a-R	GAACAGTGCTAATGCCTGAAAGG	Reverse	Cloning for *esr2a*
esr2b-F	CACTGATGACAGGCTTGGCAG	Forward	Cloning for *esr2b*
esr2b-R	AATGGTCACAGACACTGATAAAG	Reverse	Cloning for *esr2b*
esr1b-F-qPCR	AGCCTCCCCAGCCAGTCTATC	Forward	qPCR for *esr1b*
esr1b-R-qPCR	TGAGCCTGACCCTGACTCCAC	Reverse	qPCR for *esr1b*
esr2a-F-qPCR	TCCATTGTCTCTGCACCATCG	Forward	qPCR for *esr2a*
esr2a-R-qPCR	TTCCTCAGAGGCTTACTGCTCTC	Reverse	qPCR for *esr2a*
esr2b-F-qPCR	TCCAAACGAGGCCTGTCATTC	Forward	qPCR for *esr2b*
esr2b-R-qPCR	TCTTCATGCTAGAGAGGTGCTG	Reverse	qPCR for *esr2b*
chgHα-promoter-F	ACTCCCCCAACCTTCCTCTT	Forward	Cloning of *chgHα* promoter
chgHα-promoter-R	GTTCTGAGGGGGTTGGTAAGG	Reverse	Cloning of *chgHα* promoter
chgHβ- GW-R1	CCCTGGTTTTTCCAAGTAAATCTGAG	Reverse	Genome walking for *chgHβ*
chgHβ- GW-R2	GAACAGTGCTAATGCCTGAAAGG	Reverse	Genome walking for *chgHβ*
chgL-GW-R1	CTGACGATTGGGTCTGAAGGGTTGCTG	Reverse	Genome walking for *chgL*
chgL-GW-R2	TTTACTGAAGGGTGGCCAATTCTGAG	Reverse	Genome walking for *chgL*
esr1a-GW-R1	CTGACGATTGGGTCTGAAGGGTTGCTG	Reverse	Genome walking for *esr1a*
esr1a-GW-R2	TTTACTGAAGGGTGGCCAATTCTGAG	Reverse	Genome walking for *esr1a*
vtgAs-promoter-1-F	AAGATGCAATTCGTCAGACTTCG	Forward	Cloning of *vtgAs* promoter 1
vtgAs-promoter-1-R	AAAGTTAACAGATTGACTCGCTACA	Reverse	Cloning of *vtgAs* promoter 1
vtgC-promoter-F	ATCAAACCATGCAATAATCTGAGTC	Forward	Cloning of *vtgC*
vtgC-promoter-R	GGCCAAGGCCACAAGGT	Reverse	Cloning of *vtgC*
pcDNA3.1-linealize-F	GTTTAAACCCGCTGATCA	Forward	Subcloning of *esr1b* , *esr2a* and *esr2b*
pcDNA3.1-linealize-R	GCTAGCCAGCTTGGGTCT	Reverse	Subcloning of *esr1b* , *esr2a* and *esr2b*
esr1b-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGTACCCTG	Forward	Subcloning of *esr1b*
esr1b-pGL3.1-infusion-R	TCAGCGGGTTTAAACTCATGGAATGGG	Reverse	Subcloning of *esr1b*
esr2a-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGTCACAAT	Forward	Subcloning of *esr2a*
esr2a-pGL3.1-infusion-R	TCAGCGGGTTTAAACTCACCACTGTCTT	Reverse	Subcloning of *esr2a*
esr2b-pGL3.1-infusion-F	CCCAAGCTGGCTAGCACCATGGCATGTT	Forward	Subcloning of *esr2b*
esr2b-pGL3.1-infusion-R	TCAGCGGGTTTAAACTTACTGAGGTACA	Reverse	Subcloning of *esr2b*

Table 1 (Continued)

pGL4.10-linearize-F	CTGTTGGTAAAGCCACCATGGAAG	Forward	Subcloning of *chgHα*, *chgHβ*, *chgL*
pGL4.10-linearize-R	GCGAGCTCAGGTACCGGC	Reverse	Subcloning of *chgHα*, *chgHβ*, *chgL*
chgHα-pGL4.10-infusion-F	GGTACCTGAGCTCGCGATATTTCTGTGTC	Forward	Subcloning of *chgHα* promoter
chgHα-pGL4.10-infusion-R	TGGCTTTACCAACAGAGACAATCCGAGG	Reverse	Subcloning of *chgHα* promoter
chgHβ-pGL4.10-infusion-F	GGTACCTGAGCTCGCTCATAATGCATCAA	Forward	Subcloning of *chgHβ* promoter
chgHβ-pGL4.10-infusion-R	TGGCTTTACCAACAGAAGAACTAATCCG	Reverse	Subcloning of *chgHβ* promoter
chgL-pGL4.10-infusion-F	GGTACCTGAGCTCGCATCCGGGTTTGCAG	Forward	Subcloning of *chgL* promoter
chgL-pGL4.10-infusion-R	TGGCTTTACCAACAGCAGCAATGTTCAC	Reverse	Subcloning of *chgL* promoter
vtgAs-promoter-1-pGL4.10	GGTACCTGAGCTCGCGGGCAGTCAGAATG	Forward	Subcloning of *vtgAs* promoter 1
vtgAs-promoter-1-pGL4.10	TGGCTTTACCAACAGGGCCAGTGTGATGT	Reverse	Subcloning of *vtgAs* promoter 1
vtgC-promoter-pGL4.10	GGTACCTGAGCTCGCCCGCACCATAGCAC	Forward	Subcloning of *vtgC* promoter
vtgC-promoter-pGL4.10	TGGCTTTACCAACAGGGTGAAATCCAG G	Reverse	Subcloning of *vtgC* promoter
esr1a-pGL4.10-infusion-F	GGTACCTGAGCTCGCCTTCTCCCATTTAAC	Forward	Subcloning of *esr1a* promoter
esr1a-pGL4.10-infusion-R	TGGCTTTACCAACAGAGATTTAAAAAAG	Reverse	Subcloning of *esr1a* promoter

Table 2 The numbers of putative transcription factor binding sites in choriogenin, vitellogenin and estrogen receptor 1a promoters of cutthroat trout

Promoter	Transcription factor binding site				
	ERE	ERE-like	1/2 ERE	Sp1	AP-1
chgH α	–	1	6	2	4
chgH β	–	2	8	–	3
chgL	–	1	7	7	11
vtgAs	1	7	6	7	10
vtgC	–	6	7	1	6
esr1a	–	3	19	9	11

ERE: Estrogen responsive element; ERE-like: Incomplete ERE; 1/2 ERE: ERE half-site; Sp1: Specificity Protein-1;
AP-1: binding sites for activator protein 1

Table A. 1 The numbers of putative transcription factor binding sites in choriogenin, vitellogenin and estrogen receptor 1a promoters of rainbow trout. The 2000 bp promoter sequences upstream of each gene (Accession No., *chgH α*: NM_001124273.1; *chgH β*: NM_001124600.1; *chgL*: NM_001124274.1; *vtgAs*: XM_036969074.1; *vtgC*: XM_021599921.2; *esr1a*: AJ242741; *esr1b*: NM_001124558.1; *esr2a*: NM_001124753.1; *esr2b*: NM_001124570.2) were obtained from whole genome database (Accession No.: GCF_013265735.2). Two online algorithms (ConSite; http://asp.ii.uib.no:8090/cgi-bin/CONSITE/ and NUBIScan; http://www.nubiscan.unibas.ch/) were used for prediction of transcription factor binding sites.

Promoter	Transcription factor binding site				
	ERE	ERE-like	1/2 ERE	Sp1	AP-1
chgH α	-	4	4	-	2
chgH β	-	2	7	-	2
chgL	-	2	4	2	3
vtgAs	1	4	4	2	4
vtgC	-	7	5	-	3
esr1a	-	2	5	1	-
ese1b	-	2	3	-	3
esr2a	-	4	7	1	2
esr2b	-	5	3	2	3

ERE: Estrogen responsive element; ERE-like: Incomplete ERE; 1/2 ERE: ERE half-site; Sp1: Specificity Protein-1; AP-1: binding sites for activator protein 1

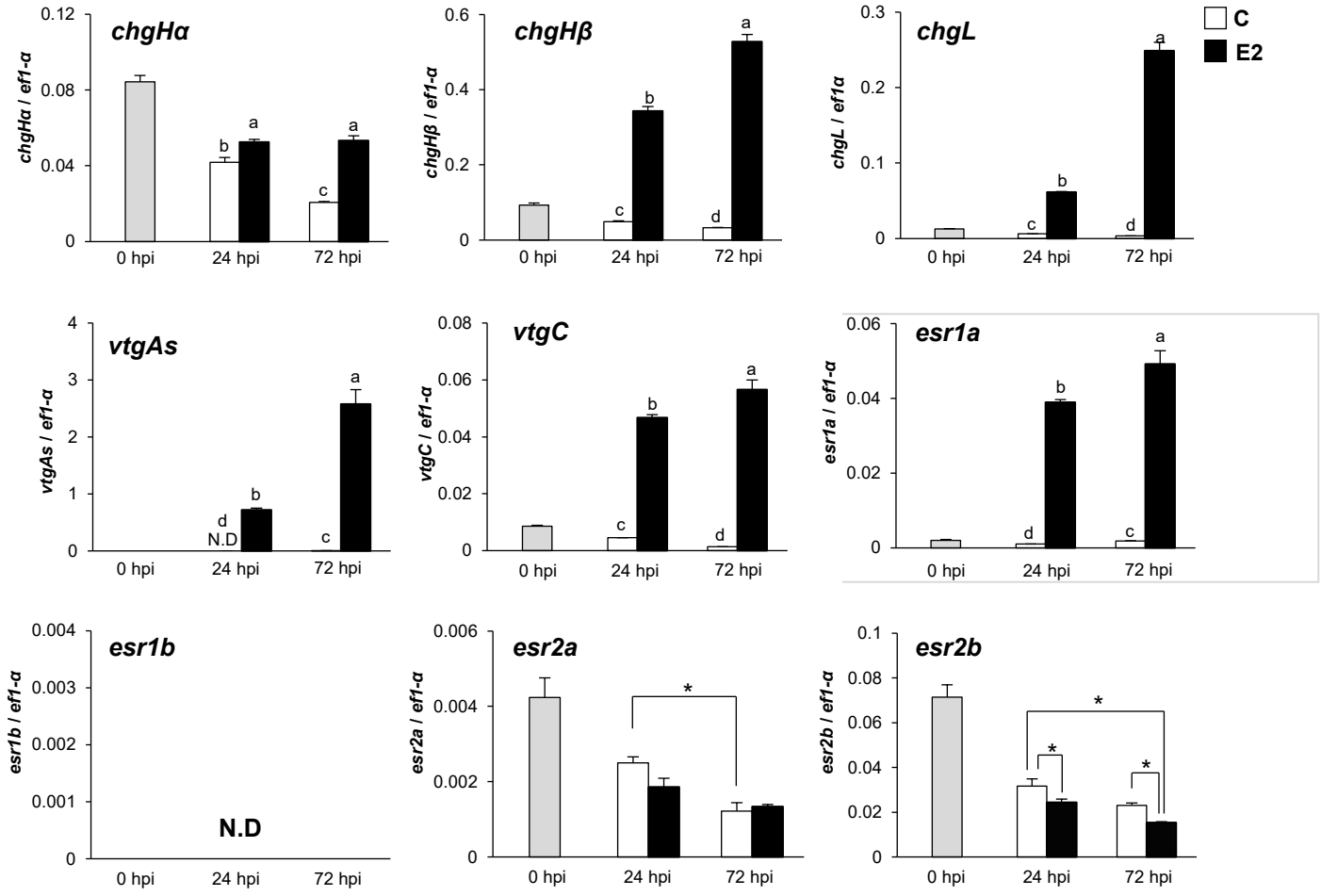
chgHα
chgHα / ef1-α
0.12
0.08
0.04
0
b
a
c
a
0 hpi
24 hpi
72 hpi
chgHβ
chgHβ / ef1-α
0.6
0.4
0.2
0
c
b
d
a
0 hpi
24 hpi
72 hpi
chgL
chgL / ef1α
0.3
0.2
0.1
0
c
b
d
a
0 hpi
24 hpi
72 hpi
C
E2
vtgAs
vtgAs / ef1-α
4
3
2
1
0
d
N.D
b
c
a
0 hpi
24 hpi
72 hpi
vtgC
vtgC / ef1-α
0.08
0.06
0.04
0.02
0
c
b
d
a
0 hpi
24 hpi
72 hpi
esr1a
esr1a / ef1-α
0.06
0.04
0.02
0
d
b
c
a
0 hpi
24 hpi
72 hpi
esr1b
esr1b / ef1-α
0.004
0.003
0.002
0.001
0
N.D
0 hpi
24 hpi
72 hpi
esr2a
esr2a / ef1-α
0.006
0.004
0.002
0
*
0 hpi
24 hpi
72 hpi
esr2b
esr2b / ef1-α
0.1
0.08
0.06
0.04
0.02
0
*
*
*
0 hpi
24 hpi
72 hpi

Fig. 1

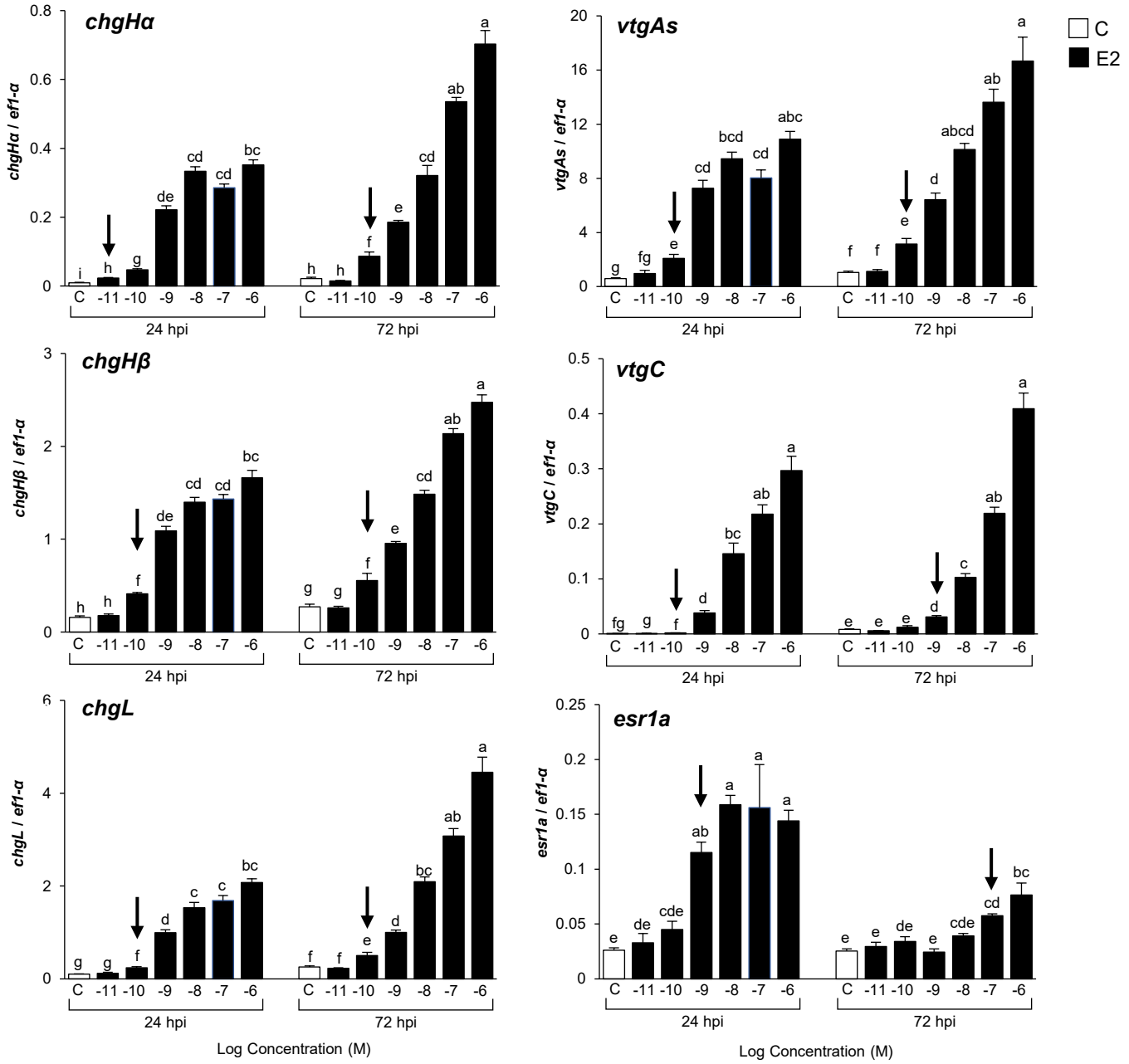
chgHα
chgHα / ef1-α
vtgAs
vtgAs / ef1-α
C
E2
chgHβ
chgHβ / ef1-α
vtgC
vtgC / ef1-α
chgL
chgL / ef1-α
esr1a
esr1a / ef1-α
C -11 -10 -9 -8 -7 -6
24 hpi
72 hpi
Log Concentration (M)

Fig. 2

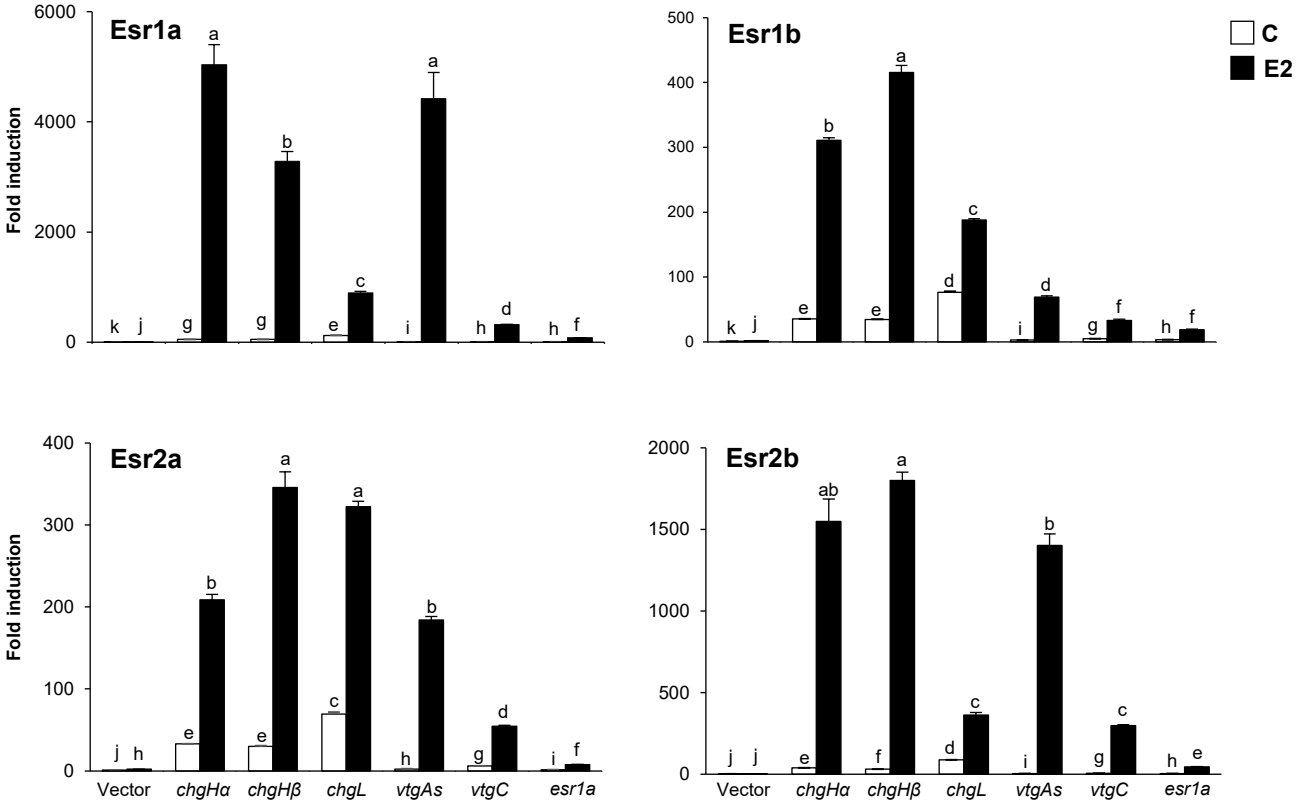
Esr1a
Esr1b
Esr2a
Esr2b
C
E2
Fold induction
Vector
chgHα
chgHβ
chgL
vtgAs
vtgC
esr1a

Fig. 3

```
-2851   ACTCCCCCAACCTTCCTCTTCCTGGAGAGGTGTTTATCTCTGTCGGCGCT

-2801   ATGCATGGAGAAGCCTGGTGGCTGAACAGATTCTGACAACATATCCCGAG

-2751   AGAGCCCTGTTTCTGTGAAAAAGATAATGTTACAATTGTTGATGTCTCTC

-2701   TGGAAGGCCACCCTTGCTTGAATTTCATCTACCTTGTTGTCAAGAGACTG

-2651   GACATTGCCGAGTAGTATACTCGGGAGCGGTGGGCAATGTGCATGTCTAC
              1/2ERE
-2601   GAAGCCTGACCACGAGGCCACTCTGTCTGCCCCTTCTGCGGCGCCGTTAT

-2551   CTTGGGTCGAATTCTGGGATTAGATCCATTGTCCTGGGTGGTGGTGCAAA
                                          1/2ERE
-2501   CAGAGGATTCGCTTTGGGAAAGTCATATTCCTGGTCATAATGTTGACAAG

-2451   TTGACGTCGCTCTTATATTCAATAGTTCTTCCCAGCCGTATGTAATAAGA

-2401   CTTAAGATTTCCTGGGGTAACAAAGTAAGAAATAATTCATTAAGAAAACA

-2351   AAATACTGCATAGTTTCCTAAGGACTCGAAGCGAGCCGACCATCTCTGTC

-2301   GGTGCCATCTCCATAGGAGATCTACACAGGAGTTGCTTACCAAGAAGACA

-2251   GTGAATGTTCCTGAGTGGCCAAGTCAGTTTTAACTTAAATCTACTTTAAA

-2201   AGATATGGCAAGACCTGAAAATGGTTGTCTAGCATTGATCAACAACTAAT

-2151   TTGGCAGAGCTTGAAGAATTTTGAACAGAATAATGGGCAAATTTTGTACA
                                     AP-1
-2101   ATCCAGGTGTAGTAAACTCCAGAAAGACTCACAGCTGTAATCGCTGCCAA

-2051   AGCTACTTCTAAAAATTATTGACTTGGGGGTGTGAATACTTATGTAAATT

-2001   AGATATTTCTGTGTCTTATTTTCAATACATTTGCAAAAATGTCTAAAAAC
```

Fig. A. 1 Putative promoter sequence of the cutthroat trout choriogenin Hα gene including 5'flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from the 5'-end of exon 1, negative numbers representing the 5'-flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein-1; AP-1: activator protein-1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
-1951   ATGTTTCCACTTTGTCATTATGGGGTATTGTGTGTAGATTAATAAAAACT

-1901   GTAACACAACAAAATGTGGAATTAGTCCATGGGTATGAATACTTTCTGAA

-1851   GGCACTGTATGTACTTGTCCCTGCTTACAAATATCTGGGCATCTGGATAG

-1801   ATGAAAAGCTGTCTTTAAAAAAGCATATTGATGAGTTAGTTAAGAATCTG

-1751   AGAATAAAAAATGGGCTTTACTATAGAAATACAGTAAGTCCTGCCTCTCG

-1701   CTAAATAGTAGAAAGCAGATTATTCAGTCGACATTACTATCGGTCCTAGA

-1651   CCATGGCGAAATCATATATATGAACGCAGCTGACACTTCATTAAAGCCGG

-1601   TAGATGCAGGTGATCATAATGCACTGTGCTTTATTACGGGCAACAATTTT

-1551   AATACTCATCACTGCATTTTCTAGTAGAAAGTTGGTTGGTCCTCTTTGAT

-1501   GTCACCTAGGTTGATACATTACTATGTTTTCATTTATAAAGCCATTTTAC

-1451   AAAAAGTCCCACTGTACCTAACATCATTACTGAACTTTTGACATGAGTTA

-1401   CCAAACCTGGTCTCATGAATGGCTAACTCTGGAAATTCCTTTTGTCTCTA

-1351   ATTAGTTAGGTAAATCAGTTTTTTTATGATTGTGTTTTTCTTTCTGCTTG

-1301   CATTTTGTGTTTAGATTTGTGTATTTCCTGTAAAATTATCCCTGATAACA

-1251   TAAAGGTTAAATAGTATAAAAAAGTTTTGTAGGAATGCTGTTATTTTCTT
           AP-1   AP-1
-1201   TGAATGATTCATTTGGTCGTTGGTAAACATTATAGGAAGAACCTACTACT

-1151   GAAAGAGACACGAGAAACCACATTGAACTTTGCAAAAGCCCACCTAAACA

-1101   AGTCTAAATCCTGTGAAAATGTTCTGTGGACCACTGAAACAAAATTAGAG
```

Fig. A. 1 (Continued)

```
                                                  1/2ERE
-1051   CTATTTGGCAATACAGATCAGTGCTATGTTTACAGATGACCAACTGAAGC
                            Sp1
-1001   TTTAAAAGAAAAGAACACCCTCCCTACAATCAAACATGGGGAGGTTTGAT

 -951   AATGCTGTGGGGTTGTTTTGCTTCCTCTGGTACTGAGGACCTTGAACATG

 -901   TGAAAGACGTAGTAGATTATCAATGTGTTTTTGGTAAACTAGTGGGTGTC

 -851   TGTTGAAGGTTGTGGGTCTTCCATCAGGACAAAAACACAAACACACATCA

 -801   ATAAACAACAGGAATGGTTAAAAATGAACACTGGACTGTTCTGGAGCGGC

 -751   CAGCGATGAGTTCAGATCAGAATCCCATCAAAAACCTATGGTGAGATATT

 -701   TTTCACAGATAACTCCAGTACCTGAAAATAAGTACCTGGATGTTCATATG

 -651   TTCTTCAGATTTTGTGTATGAGATCTGAAAACAGTAGTTAGTGGAAGGCA
                                                    AP-1
 -601   CCACTCAAACATTGAAGAATTAGAGCAGTTTGAAGAGTGACTGAAGAGTG

 -551   GGACAAATTGCCATTAGAAAGGTGCAGCAAGCTCATTGATGGCAACAAGA

 -501   AGCATTTTCTGGTGGTTCTGCAATGCTGACAATCCAATAACAACATGTCT

 -451   AGACTGAAGTTTTTTTTAAATGTCAACTTATTTTTGAAGAAATAGGGAAT

 -401   TATTTAAGAAAAGTGAAAGGCAATATATTTATCTATATTAGAAATAGAAT

 -351   ACAATAGATACATTTGTGTCTATTATTATTTATAAAATGTTTACTTCTTG

 -301   AGTGTAGTTCTGCACACCGTGTGTACCTGCAAAATAAACATTTGTTAACA
                    1/2ERE                  1/2ERE
 -251   AATACAATGTTACGGTCACAAAGACCTTCAACTCTGGTCAAAGGCATGTC
             ERE-like
 -201   AGAGATCTATGTGACCTGAACATTGCGTTTGTCAATAAATGTTTGGGACT
```

Fig. A. 1 (Continued)

```
                                                  1/2ERE
-151   TCAGAGTGTGGAAAGCCTTGATTCCTCCGAACTGGATGTGAATGATTGGT
          CAAT box                          Sp1
-101   CAGCCAAGGAGGTGGGGCTTTTCAACAGTTCAACACACCCCACTATGTAT
        TATA box
 -51   AAAAGCAGCAACAAGTGGCACAGTGAGCCTTGTGGGTTCCTCGGATTGTC
                            Exon1
  -1   TCTTGTTCTGAATCCATGGCCTTGCAGTGGAGTGTTGTTTGTCTCGTAGC

  50   AGTGGCCATGCTTGGCTGTCTGTGTGACGCTCAATTGAAGTGGCCTTACC

 100   AACCCCCTCAGAAC
```

Fig. A. 1 (Continued)

```
-2320   ATCACGATCGTGAAATACGTTGCGCACATTGGCACTCAAACAAAATAATA
                                   ERE-like
-2270   TCCCACATCGCAGGTGGGCAAAAGGACACACTAAGTCTGATCCCCAATTA

-2220   GAGGTAACGATCATCAGCTGCCTCCAATTGGGAACCATGCACACACACAC
                                                   1/2ERE
-2170   ACCAACATAGAAATATAATACCTAGAAACCCCCCTAGTCACGCCCTGACC

-2120   TAAAACACAGGGCGTGACATGGTGTGAATACTGTCCTAACTTATTCTGCA

-2070   AGTGCTGGATTTGCTAGTTAACGTATATGGTTTGATAACTAATGAACTCT

-2020   GTAAATGTCTATCTTTTACCTCATAATGCATCAATTTGACATCAATTTAT

-1970   GTTGCAACCAAATAATATATGGATGTGGCTGTAAATACTGCATCATCATT

-1920   CTCCAATGGAAAGAGTCATTGGAAAAAAGAGTCATCTGTGCTATATTTTG

-1870   GAAATGTAGTTTAGTGTACAATGCACTACTTACCTGGGTTGTATATAACA

-1820   ATATAATCCACTCATTATTTAAATTACAGTAATATATAAATAATTTCAAT

-1770   TAGAGAGCCAGGCAAAACTGTATGATTTGACAAGTCACATAGAAAAGGTG

-1720   ATCTTGTACAATGTTGCATCGGGCAGAAATGGCATACAACAACATGCTAT

-1670   GTTTTCACAGTAGCCACCTGCTTTACAATACACCTATTTCTGGTTCTGTC

-1620   CTATATATGTAGTGTATACGGCAGGGGTTCCCAAACTGTTTTTTTGGCCG

-1570   CAACCCCATTTTGAAATTAAAATGTTTTTGTAATCCCGCCATGTGAAAAA

-1520   TGTTATGTTATAAACGGCCAATGTTTACTTTTTTTATTTGGGCTATGACA

-1470   GTTTATTACAAATCACTTTGACAGTACCTTTGAAAGTATTTCAATCTGAT
```

Fig. A. 2 Putative promoter sequence of the cutthroat trout choriogenin Hβ gene including 5'flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from 5'-end of exon 1, with negative numbers representing the 5'flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
                                AP-1
-1420   GGAAGTATGATTTTAAGTGACTGAAATGCATCAGAACATTTTGAGTGCTC

-1370   TTGATGATGATCTTTTCCCCCCAGTCTGATTTAGTACCTGGTCTTGTCCA

-1320   TGAGTCCTGTGTGGCTTGTAGAGGGAAACAGAAGACACATACGTATTCCT
                                  1/2ERE
-1270   GAGAGTATTATCTTTCAATGATTGGGTCATAATATTTTGTAGCTTAAACG

-1220   GTTTAAAAGATAGAGCCACATTTGTAGGAAGAAAACAGAAACCGCTCTGT
                                       ERE-like AP-1
-1170   TTATTTCAAACACATCTAGCGGCAACCAGAGGTTACTGACCTAATGTCGG

-1120   TTCTATCAACCAAGCTCAATTTGATACATTTGATTTCAGTTTCTCTCGTG
        1/2ERE      1/2ERE
-1070   ACCCCCACATCGGGTCACAACCCCTAGTTTGGGACACGATGGTATAAGGA

-1020   TAATGAAACTGGAAGACTGACATATTTGTCAACATACTAACAACCTGCCG

 -970   TTTGACACAAGTTATTTTAAAAAGTGTGCATGTCAAGTTATAGTCAAATA

 -920   CTGTATAAAAATGATGTCTACTATTCTTTCTATTCAGAACTTCAAAAATA

 -870   TGTTTTGAAATGTGTATCCTGTCATTTATTCTATTGGATTAAATGGTAGT
                                                     AP-1
 -820   TAAAAGGACTAAATGGTGAGCCTATCATTATCTACTGTAGCGGTGACTAA

 -770   TCAAAAGATGCACATGGTATTTCAAGGAAAACTTTGATTTTGCATAAACG

 -720   ACTAAATGTGGAGGTGTGTGAAACCCCAATGAATGTACAATCAAAGCTTC

 -670   TGAACATAAGATAGGGGACATTAGAAGTGTTATCAGTTTAGAGTTTTTTA

 -620   CTTAGAGTTTTGAAAATATACCACTTCTGAAAAATGTGCCAAAGTGGTTG

 -570   AAAAAACAGTATATACAACCCAGGTATTTCAGAAATGCATTCTACACTAC
```

Fig. A. 2 (Continued)

```
-520   ACTATACACTGAGTATACAAAACATTAGAACACCTTGCTACTATTGAGTT

-470   GCACCCTCTTTTGCCCTCAGAAAAGAAAAAAACTGTAATATATACAAAAT

-420   TGTCTAATTATCTATTTATAATGCGTAAATCATTTTGAATTGTTTCTGAC

-370   TATCAAAATAAATATATAAATAAATATATATGTATATTGTCACATACAAC

-320   AACTAGGAGCAAATTAGATACTGACAGAACTGGCAAAGCCTCCAATGACA
               1/2ERE                      1/2ERE
-270   TGACGTGTCGGTCACAAAGACCTTTCTCAAGGCCATGACCGAAATGTTGG

-220   ATGTTCGCAAATGTTACACAACTGTTTTGAATAGTGTGCTACTAAAGGAA
                       CAAT box        1/2ERE
-170   CATTAGCCAGGGCATTTGATTGGCTAAAACTTTGACCCCTCAGAAAAGGA
         1/2ERE
-120   ACGGTCAGATAAAGAGGGGGCGGATTTCCAATAAGTAAACAACGCTCCAT
            TATA box
 -70   CTGTTATAAAGGTGGCCGCAATTCGTTCATCCTCATCACAGCATCCAGTG
                                        Exon 1
 -20   AACATTGCGGATTAGTTCTTGTAGCGAAGCCATTGCGATGAAGTGGAGTG

 +81   CAGTTTGTCTAGTGGCAGTGGCCACGCTTGGCTGGCTGTGTGATGCTCAG

+131   ATTTACTTGGAAAAACCAGGG
```

Fig. A. 2 (Continued)

```
-3636   ACTATAGGGCACGCGTGGTCGACGCGCCCGGGCTGGTCCTGTTCACAGAA
                                     Sp1       1/2ERE
-3586   GTGTTTTAGGGAGCGTGTGACAGTGATGAGGGGTGGTCGTTTGACCGCAG
                                Sp1
-3536   ACCCATTACGGATGCAGGCAATGAGGCAGTGATCGCTGAGATCTTGGTTG

-3486   AAAACAGCAGAGGTGTATTTGGAGGGCGAGTTAGTTAGGATGATATCTAT

-3436   GAGGGTGCCCGTGTTTACGGATTTGGTGTTGTACCTGGTAGGTTCATTGA

-3386   TAATTTGTGTGAGATTGAGGGCATTAAGCTTAGATTGTAGGATGGCTGGG
             AP-1
-3336   GTGTTAATCACAACTTGGAACTACCCATCCCGGATCCAGGAGAATTGTCA

-3286   TCAACTACACTAATTAGCATAGCGCAACGGCCAAATAATCTTACTAGAAA

-3236   ATATTAATATTCATGAAATCACAAGTGAAATATAATGAAACACATTTTAG
                 AP-1
-3186   CCTTTTGTTAATCACCCTGTAGTCTCAGATTTTGAAATGATGCTTTACAG

-3136   CCAAAGCAAGACAAGCGTTTGTGTCAGTTTATCGATAGCCTAGCATAGCA

-3086   TTATGTCCAGCTAGCAGCAGGAAGCTCTGTCACAAAAATCAGAAAAGCAA

-3036   TCAAATTAACCGTTTACCTTTGATGATCTTCGGATGTTTTCACTGACGAG

-2986   ACTCCCAGTTAGACAGCAAATGTTCCTTTTGTTCCATAAAGATTATTTTT

-2936   ATACCCAAAATACCTCAGTTTGTTTGTCACGTTATGTTGAGAAATCTACC

-2886   GGAAATAGCTGTCACAACAACACCAAAAAAAATAATCTAATTATATCAAT

-2836   AATATCGACAGAAACATTGCAAACGTTTTTTTATAATCAATCCTCAAGGT

-2786   GTTTTTCAAGTATCTATTCGATAATATATCAACCGGGACAATTGGCTTTT
```

Fig. A. 3 Putative promoter sequence of the cutthroat trout choriogenin L gene including 5'flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from 5'-end of exon 1, with negative numbers representing the 5'flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
-2736   CAGTAGGACCGAGAGGAAAAATGGCTACCTTTGTCTTTTACGCAATAATC

-2686   ACTCTGAGAGCGCTCATTCTTCAACATAAAGGCGTGAAACTACGTCTAAA

-2636   GGCTGTAGACACCTTAGGGAATACGTAGAAAAAGGAATCTGGTTGATATC
                                                     AP-1
-2586   CCTTTCAATGGCCAATAGGGATGCATAGGAACACAACGGTTTCAAAATAT
            AP-1
-2536   GAGTCACTTCCTGATTGGATTTTTCTTAGGCTTTCGCCTGCAATATCAGT
                AP-1
-2486   TATGTTATACTCACAGACAATATTTTGACAGTTTTGGAAACTTTAGAGTG

-2436   TTTTCTATCCTAAGCTGTCAATTATATGCATATTCTAGCATCTGGTCCTG

-2386   AGAAATAGGCTGTTTACTTTGGGAACGTTATTTTTCCAAAAATAAAAATA
                                        1/2ERE
-2336   GTGCCCCCTAGCTTCAAGCATGTCCCAGTTTAGGTCACCTAGTAGCACGA
                         1/2ERE
-2286   GCTCATAAGATAGATGGGGTCAATCAATTCACATATGATGTCCAGGGCAC

-2236   TTGTTTCTGGAAATAAATAGACTTGTTTCTGGAAAGGTGAATTTTTAGAA

-2186   GTAGAATCTCGAATTGTTTTGGTGCAGACCGGGATGGTAAGACAGAATTT
                                        Sp1
-2136   TGCAGGCTATCTCTGCAGAAGATTACAACACAGCCCCTTTGGCAGTTCTA

-2086   TCTTGTCGGAAAATGTTATAGTTAGGGATGGAAATTTCAGGGTTTTTGGT

-2036   GGTTTTCCTAAGCCAGGATTCAGACGTGGCTAAGACATCCGGGTTTGCAG
                         AP-1
-1986   AATATGCTAAACCAGTGAATAAAGCAAACTTAGGGAGTAGGCTTCTAATG

-1936   TTAACATGCATGAAACCAAGGCTTTTACGGTTACAGAAGTCAACAAATGA

-1886   GAGCACCTGGGGAGAAGGAGTAGAGCTGGGCACTGCAGGTCCTGGATTAA
```

Fig. A. 3 (Continued)

```
-1836   CCTCTACATCACCAGAGGAACAAAGGAGAAGTAGGACTTGTGGTTTCTAG

-1786   CTAACATTACATCAATCAAAGTATTAGAGCATGTCGTGAAGCAAAACTCA
           1/2ERE      AP-1 1/2ERE
-1736   AGTGGTCAAAACCATCTTGGGTCACCGGGAGGAGCGGGTTTGCAAAACAC
                                                     Sp1
-1686   CTTCCCAATATTGAGTTTTGCCCTCAGAACAGCCTCAATTCGTCGGGGCA

-1636   TGGACTCTTCAAGGTGTCAAAAGCGTTCCACAGGGATGCTGGCCCAAGTT

-1586   GGCTCGATGTCAAAGGCACTTCAATATTTTGCTTTGTCCATTCACACTCT
                                Sp1
-1536   GAATGGCACAGATACACAATCCATGCCTCAATTGTCTCAAGGCTTAACAA

-1486   TCCTTCTTTAACCTGTATCCTCCTCTTTATCTACACGGATTGAAGTCAAT

-1436   TTAATAAGTGACATCAGTAAGGGGTTGAAGATTTCACCTGGATTCACCTG
        1/2ERE
-1386   GTCAGTCTTTGTCATGGAAAGAGCAGGTGTTCTTAATGTTTTGTACACTC

-1336   AGTGTATATTTTCTTTATACAGACTAGTAAAAGATAAGTGAAAATTGACA

-1286   AATTATCCAATGGATTATGATAATTTTCTGGTAAAAAAAAGCTTGGGTAG

-1236   TCTGCCTGAGAATTGAGGGTAGTTTGCACCCTCTATTTGTATTTGCTCCA
                                                   AP-1
-1186   TGGCTCAGATGGTTAAATGGAGACAAGGCTGTTTGGCTTATCTCAGTCAC

-1136   GAAGAGGAATACATTTTCATCTGTTTCTAATCAATAAACAATGCCATTCT

-1086   GGTGGTTGGTAACATTCAAATAAAACCTAAACCTGAAATGAAACGTAGGC

-1036   TGAATTTAAAAAAAAAATTGGGTATGATGATGACATTTTTTAAATACATT

 -986   TTTTATTTAACGAGGCATAATCCTGTGGTTTAAATTTAACCCTCAAAACA
```

Fig. A. 3 (Continued)

```
-936   ACAGCTTAAGTTGATTACTTTTTGCAAATCCAATGTATTTTCCACATAGA

-886   CTCCATGTCTCAATATGTTGACAAATTACATTGAAATAACGTTGATTCAA

-836   CCAGTTTGTGCCCAATGGGCAGCCTCCTGTGTTGATCTTGTGTGTATTCG
                            AP-1  1/2ERE
-786   ACTGTGGCCTTGACAGAGGTCTTACTGACCGACACGCAGCATGTCAATAA
                                     ERE-like AP-1
-736   ATGTCAATTTTGCAAGTATACACAGTGTGAGAGGTCATTCACCCAGGCAG

-686   TCTGCTTCGCGTTCACTTAATCTACCCCCATGTGAAAGTAACATGACTTC

-636   AGGTGTGGAAATATCTATTTTTCCATTCAATAAATACATCATCTCAAATT

-586   ATTGCAACCAGAAGTTTCTCTTTAAGAATGAATGAAAGGTTAGTGCATTT

-536   ATTTAATCTAATCATAATATAGACTTGTATAAGTTCATATATAATATATC

-486   ATAATTACACTATATGTTATTTTTGCAAAACGCTATATACTGTATCCACC

-436   TTACAAATAAGAGTTCTCTTACAGAAAAATATGTGGAAAGACGATCAAAT
         AP-1
-386   GTGACACATAAGTAACATTTTAATATATTACTATTGAAAAAATACATTTG

-336   TGGCATCTATATATCAAGCAAATGTAGCAAACACACATATAAAGGTACCC

-286   AAAAGCATTCATTTCTGATGAAAAAGCGTTTTTTTAACAAAAAAATTCTT

-236   GAAACTTTGATAATATAACATAACATTTTTGGTATTAAGAAGTTGCTTTG
                                            CAAT box
-186   AACAGTCTGCTATTAAAGGACCATCAGCCACAGCATTTGATTGGCTAAAA
                                                  Sp1
-136   CCTTGACTCCTGAGAGCTAATAGGGAATGTTCAAATGAGGAGGGGGCGGG
        Sp1                                TATA box
 -86   TTTCCAATAAGTAAACAACGCTCAATCCGTTATAAAACTGGCCACATCTC
```

Fig. A. 3 (Continued)

```
                                                Exon 1
-36    CTTCATCCTCTTCACAGCATCCAGTGAACATTGCTGATCAATTCTTATTG

+15    TGAAGCCATGGCGATGAAGTGGAGTGTAGTTTGTCTCGTGGCAGTGGCCA

+65    TGCTTGGCTGTCTGTGTGTTGCTCAGAATTGGCCACCCTTCAGTAAA
```

Fig. A. 3 (Continued)

```
-2872   AAGATGCAATTCGTCAGACTTCGGAGACTCTCTGGAAATCTCGGGTAACC
                                                     Sp1
-2822   TCGGATTCTCTAGGAAACGGAGACGTCAGAGATTTCCGGGATGCCTCAGA
            ERE-like   ERE-like
-2772   GGCGAGGTAAAATCTGACCTCTCCTTCCTACGTTACCGTAGCCACCCATC
                1/2ERE
-2722   GATCCAGAAGGTCAAGGCGATGAGCAAGTCGAGATCTGTTGGTAGTTCCC

-2672   GGGCTGCTAGCTTATCTTTAACGTCCTCCGATAATCCATGCAGGAACGTG

-2622   GCGAACAGCGATTCCGGGTTCCAGGCACTCTCAGCCACAAATGTACGGAA

-2572   ATCCACTGCATATGTCCAACGGACATCAGCGTGGTGAGGTACGCTTTCTT

-2522   CGAGCAGTCCGAGGGAAAGGAGGAGGGCTGCAGCTCGAAGATGAGGGAAT

-2472   ACTGAGCGAGAAACGCCTGACAGGTTCCCGACTTTCCAGCGAAGCATTCC

-2422   AGAGGAGGTAAGCAGGGTTCTCAGGAAGCCGGGGTGGCCGGGAGAGACGC
                                  Sp1
-2372   GCTGCTGACAGTCGGGTTACTGAGGGGCTGGGAAGTTATATTCGTGGTAG

-2322   GCTGCCTAACAGACAACCCACGGAATTGCTCCAGCAATGTATCCAATGCA

-2272   AAACCTTCCATAAGACCACGAAGCAACTCCTCGTGCCTTCCAATGGTGGC
                      Sp1
-2222   TCCTTGGGAGAAGACGGCGTGGTGGAGCTGGTCCAAGTCTGCTGCGTCCG
              ERE-like
-2172   TCATGGTCAGTTTGTACTATCACGACTCAGGATAAGACCCAGATGGAGTT
                                            Sp1
-2122   CAAAATATCAAATGTATATTTACAAAACAGGGGGCAGGCAAACGACAGGT
                     1/2ERE  AP-1
-2072   CCAGGGCAGGCAGAGGTCAGTAATCCAGAGCAGAGTCCGAGAGGTACAGA
                        1/2ERE             1/2ERE
-2022   ACGGCAGGCAGGCTCAGGGTCAGGGCAGTCAGAATGGTCAAAACCGGGAA
```

Fig. A. 4 Putative promoter sequence of the cutthroat trout vitellogenin As gene including 5'flanking region, exon 1, intron 1 and exon 2. The exons are indicated by open boxes. Nucleotides are numbered from 5'-end of exon 1, intron 1 and exon 2, with negative numbers representing the 5'flanking region. Estrogen responsive element (ERE), ERE-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
-1972   AACAGGGACTAGAGTGAAAACAGGAGTACGTGAAAACCACTAGTAGGCTT

-1922   GACGAGACAAGACGAACTGGTAACAGACAAACAGAGAACACAGGTATAAA

-1872   TGCACCAGGGATAATGGGGAAGATGGGCGACACCTGGAAGGGGGTAGAGA

-1822   CAAGCACAAAGACAGGAGAAACAGATCAGGGTGTGACGGATAGGGTATTT
                     AP-1           AP-1
-1772   ATGGATGTGTTATGAATGACTGAAGGTGTCTCACTTCAAACAAAGTATTA

-1722   CAGTATTACATGAGGTGTCAATAAATAGTTTTTTAATATTCTGCTAATTT

-1672   ATGAAGGTTCTCTCATGATCCACAGGTTATTGTAGGGTATGGGAGATATT
                                                   AP-1
-1622   TAAATGGAGAGATGGACCTCCAAAGCTTGTGTTTGGGTGTGTGTGTCAGA

-1572   GCCAAGATGATTTGGAGTAGTCCAACCTAAGACGACCTCACCAGGTATTC

-1522   CTTTTCTTTTCCCATGCTGGAGACCAGGGCTTGTTCACAACCTGTTATAA
                                                 AP-1
-1472   CGGTGCCAACATTTTGTGGCTGATCTTTCAGCGGGGGAGTGAGTGAATGT

-1422   GTCAAAGACCTGACTGGGCCCAGCACTGCACGGTATGGCTCGTTATGTTG

-1372   TTGTGTGTGGGTTTGTGTACTGAGGAACATGTAACTTGGTTGCAGTAAAA

-1322   GCCACTTTCAATTTCCTTATGTTGGGAGGTTTCATCAGGGTAAGTGTTTC

-1272   TTGGTGTAAAGTCGTCCCGAACACAGCACATATCAGCCTGGGATATCAGC
                                  1/2ERE              1/2ERE
-1222   CATTCATAGTTAGCCTTAGTTGGAGTGACCATAGAATTCTATGGGAGTGA
                AP-1
-1172   CCTTACTGAGTAAAGTATTTGCCATCATTCCCCCTGGTCGTCACTAGTTA
                                                Sp1
-1122   ACGCTGCCACAAAGTCATAATTATGGCTAAACCCTGCCCATTTCCACAAT
```

Fig. A. 4 (Continued)

```
                                                   ERE-like
-1072   TTCTCTTCTAGAAAATAGATTTTAAATCTAACCCTAACTAATGAAGGTCA
                         Sp1
-1022   AGTTTGATGCGTTGGGGGGTGGTATGTGAAAAGTGCTGTAATTTCTAAAT
                                  AP-1
 -972   GGTTTACTCAAATTTCTAAACGGTTTACTCATTATGGATGAAAATACCCT

 -922   CAGATTAAAGCTGACAGTCTGCACTTTAACCTCCGTCATAGCATCATTTC
                                                     AP-1
 -872   AAATCAAAATTGCTGGAGTACAGAGCCAAAAACAACAACAAATGTCTCAC

 -822   TGTCCAATTTTTTTTTTTAAATTGACACATTAATATTTCAGTTGAATTTA

 -772   ACATCTGGATCGAACAACAAACCTGTTGTAGGCCTGCATATTCAGTGTCA
          AP-1                                ERE
 -722   TTGACTGACAAGATGGCAGAGTCTTATCTCTAGGTCAAGCTGACCATTGT

 -672   TGACACATGGTTGGTTCATCTGAATTACAAATACTTGTTCAAGGACTTCA
                     ERE-like
 -622   ATAGTGTTGAGGACAAGCTTGGACCAATGGCTGATCCATGTTCAAGTGCC

 -572   AAAACTCGTTGGGTTTTGACATTACTAGTATTCTAATTTATCACATTCTC
                           ERE-like
 -522   TAACAAATTAGAATCGATCATACATGTACTGATCCAAACCTAGACAATAT

 -472   ATTTTATTTAGCATTGTGTTGCTTCATTAATTTAGGCACCAACTGCCTAC

 -422   TTATCTCTACTGGGTTCTACTTCTAAAGTACAAGGGCCTTTCTGTACATT

 -372   TGTATTTAGTGGTTAGATAAATAGAGAAAAATAACTAATGTAGTATTTAA

 -322   AGAAAAATGAAGGTCGTAGTATACATAACTTGTTGGATAAAACAACACGT
         AP-1                                     ERE-like
 -272   GATTCTCCAGATAATTTTCATTAAAGAACTGACTTAGATGGACATTGATC
                                  Sp1
 -222   TGTAAAGGGCTAAATGGCAGTGGGGCAGGTTAACCTAACCTATGGGTGTA
```

Fig. A. 4 (Continued)

```
-172   TGTGGCCACCTCAGAACTAGTGATAACCCACTCAACAGGCTTTATTATGA

-122   GCACATACTAATCTCAACTTATGTAACGTTAGTTAATTATATATTTTGTT

 -72   ACGATTGTTTTGTCGACAGAACATTTCTTTAAAAGGCGGACTGGGACTGT
                                   Exon 1
 -22   TGTTCCTCACATCACACTGGCCATGAGAGCAGTAGTACTTGCACTGACTC
                                Intron 1
  29   TAGCCCTTGTGGGTAAGTACAGTTTTTCTGTCTTATTCTAGTGCAGATAT

  79   TATAACTAACTTCATGTAAATATAACTAAGTTATGTAAATATAGAAATGT

 129   ACAATTCTGTATCTATGACATTTTTTGAAAAACATTCAGTAACGTATTGT
                                    Exon 2
 179   GACAATAATTTTTGTTTTATCTTGTAGCGAGTCAATCTGTTAACTTT
```

Fig. A. 4 (Continued)

```
-3025   ATCAAACCATGCAATAATCTGAGTCGGCGCTCAGAGCCCAATCAAGACAC

-2975   AAATATATCTGCCATATTATGCAGTCAACAAAAGTCATAAATAGCATAAT

-2925   AAATATTCACTTACCTTTGCTGATCTTCGTCGGAATGCACTCCCAGGAAT

-2875   CCCACTTCCACAAGAAATTTTGTTTTGTTCGGTAATGTCCATCATTTATG

-2825   TCCAAATAATTACTTTTGTTAGCGCATTTGGTAAACAAATCAAGTCACGA

-2775   AGTGCGTTCACTAAAAGCAGACGAAATGTCAAAAAGTTCCGTAACAGTCA

-2725   GTAGAAACATGTCAAACGATTTATTGAATCAATCTTTAGGATGTTTTTAA

-2675   CATAAATCTTCAATAATATTCCAACCGGAGAATTCCATTGTCTTCAGAAG

-2625   TGTGATGGAACAGAGCTCCCTCTCATGTGAACGCGCATGGTTCACATGGC
              AP-1
-2575   AGACCTTACTCATTCCCTTCTCCTTCGGCCCCACTTCACAGTAGAATCAT

-2525   CAGACAAGGTTCTCAAGACTGTTGACATCTAGCGGAAGCCTTAGGAAGTG

-2475   CAACATTAATATATCCCACTGTATCGTCAATTGGAGCTCAGTTGAAAATC

-2425   AACCAACCTCAGATTTCCCAGATTTCCTGGTTGGATTTTTTCTCAGGTT
                                  AP-1
-2375   TTTGCCTGCCGTATGAGTTCTGTTATACTCACAGGCATCATTCAAACAGT

-2325   TTTAGAAACTTCAGAGTGTTTTCTATCCAAATCTACTTATAATATGCTTC

-2275   ATGGCTTTATAGCAGGCCATTTACTCTGGGCATGCTTTTCATCCGGACGT
              Sp1                            1/2ERE
-2225   GAAAATACTGCCCCCTACCCCAAAGTTAAACAGGTCAACATCACAAAGCC

-2175   GCAGGGCCAGACAGATTACCAGGACGTGTACTCAAAGCATGTACGCACCA
```

Fig. A. 5 Putative promoter sequence of the cutthroat trout vitellogenin C gene including 5'flanking region and exon 1. The exons are indicated by open boxes. Nucleotides are numbered from 5'-end of exon 1, with negative numbers representing the 5'flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by

```
                                                        1/2ERE
-2125   ACTGGCATGCGTATTCACTGAGATTTTTAACCTCTCGCTGACCGAGTCTG

-2075   CAATACGTACAGGTTTAAGCAGACCACCATAGTCCCTGTGGCTAAGGAAG

-2025   CGAAGGTAACCTACCAAAATAATTACCGCACCATAGCACTCACGTATGTA

-1975   GCCATGAAGTGCTTTGAAAAGCTGATCATGGATCATATCAATACCATTAT

-1925   CCCAGAAACCCCAGACCCAGCGTTCAACACCATAGGGCCCACAAAGCTAG

-1875   TCACTAAGCTAAGTACCATGGAACTAAACACCTCCCTCTGCAACTGGATC

-1825   CTGGATTTCCTGACGGGACACCCCCAGGTGGTAAGGGTAGGCAACAACAC

-1775   GTCTGCCACGCTGATCCTCAACTGTGGGGACCTCAGGGGTGTGTACTTAG

-1725   TCCCCTCCTGTACTCCCTGATCACCCATGACTGCGCGGCCAAACACGACT
                                                     AP-1
-1675   CAAACACCATCATTAAGTTGGTAAACCTGATCACCAACAATGATGAGACA
                        ERE-like
-1625   GCTTATAGGGAGGAGGTCAGAGACCTGGCAGTGTGGTGCCAGGACAACAA
                       AP-1
-1575   CCTCTTCCTCAATGTGAGCAAGACAAATGAGCTTATTGTGGACTACAGGA

-1525   AAAGGCAGGCTGAACATGCCCCCATTAACATTGACGGGGATGTAGTTTCA
           ERE-like
-1475   AGTTCATTGGTGTCCACATCTCCAACAAACGATCACAGTTGAAACACACC

-1425   AAGACAGTCGTAAAGAGAGCACGACACCACCACCTCAGGAGACTGAAAAG

-1375   ATTTGACATAGGTTCCCAGATCCTCAAAACCTTCTACAGCTGCAACATCG
           ERE-like
-1325   AGAGCATCCTGACCGGTTGCATCACTGCCTGGTATGGCAACTGCTCGGCA
         1/2ERE
-1275   TCTGACCATAAGGCGTTACAGAGGGTAGTGCGTACGGCTCTGCTACCACA
```

Fig. A. 5 (Continued)

```
-1225   CAGCAAGTGGTACCAGAGCGCCAAGTCTTGGACCAAAAGGCTCCTTAACA

-1175   GCTTCTACCCCCAAGACTGCTTAACAATTAAACAAATGGCCACTGGACTA
              1/2ERE
-1125   TTTACATTGACCCCCCTCCATTTGTTTTGTACACTGCTGTTACTCTATGT

-1075   TTATTATCTACGCATAGTCACTTCACCCCCACCTACATGTATAAATTACC

-1025   TCGACTAACCTGTACCCCCGCATATTGACTCGGTACCGGTACCCCTTGTA

 -975   TATAGGCTCATTATTGTTATTTAATTTTGTTACTTTTTATTTATTTTTTA

 -925   CTTTAGTTTATTTGGTAAATATTTTCTTAACTCTTCTTCAACTGCACTGT
                      AP-1
 -875   TGGTTAAGGGCTTGTGAGTAAGCATGTAAGGTCTACACTTGTTGTATTTG

 -825   GTGCATGTGACAAATAGAGTTTGATTTGATTTGATTTTGACTGTCAGTTG

 -775   AGTAACTGTAGCCCGTCTGTTGCTCTGCAAAATTTGTGTCAGTCCTTTAT
                     1/2ERE
 -725   CCTCTTTCATCAATGACCCGTTTTCGACAACTGGCCTGAAATTGTCTGGA
                       1/2ERE
 -675   TGTCCTTTGGATGGTTGACCATTCTTGATACAAAGAGGAAACTGTTGAGC
                                                ERE-like
 -625   GTGAAAAAACGCTGCACCTGGTATCTACCAGCATACCCTGTTCAAATGCA

 -575   CTTAAATATTTAGTCGTACCCATCCACCCTCTGAATTGCACACAAGCACA

 -525   ATCCATGTCTCAATTTTATCAAGGCTTAAAAAGCCTCCTTTAAACCTGTC

 -475   TCCTCCCCTTCATTACACTGATTGAAGTGGAATTAATATCAATAAGGGAT

 -425   CATAGCTTTCACCTGGTTAGTCTATGTCATGGAAAGCACAGGTGTTCCTA

 -375   ATGTTGTGTACATCCTGTGAATATATTATTTGTGCAAGCAGCACACTTTG
```

Fig. A. 5 (Continued)

```
                        ERE-like
-325   TGCATTCAAATAAGGTCAAAGATGTTGCATTTTAAACAATTCTTTGTAAT

-275   GTGAGTTTCACAAAGCACTGATTATTAGTGTCCATTTTAGATCACTGTTT
                                            1/2ERE
-225   ATTTTTCCTGAAATGTCCTCCTTTTCCATGTCATACTGACCACTGTCAAC

-175   GTCACTGGTTACACTCGTCTGACATACCTTTGCCCCCTTGTTACTTTATG
                                              ERE-like
-125   CCCCTCATTGATGCCAAAGATGTGCTGTGCAGACACTGGGTCATGTACTC
          AP-1                          TATA box
 -75   GTTTAGTCATTGAGAACACAGCATTGCATATAAAAGGAAACGTTGAGCTG
                                          Exon1
 -25   CAGGATCCCACTCCTGGATTTCACCATGTGGGGGTTCCTTCTTTGTCACC

  25   TTGTGGCCTTGGCC
```

Fig. A. 5 (Continued)

```
-6155   CCTCGGCAAACTTAATGATGGTGTTGGAGTTAAGCTTAGCCATTCAGGTA

-6105   TGGGTGAGCAGGGAGTTCAGGAGGGGACTGAGCATGCACCCCTGAGGGGC

-6055   CCCTGTGTTGAGGATCAGCGAGGCAGATGTGTTGTTACCTACCCTTACCA

-6005   CCTGGGGGCGGCCCATCAGGAAGTCCACGATCCAGTTGCAGTGGGAGGTG

-5955   TTTAGTCCCAGGGTTCTTAGCTTGATGATGAGCTTTGAGGGTACTATGGT

-5905   GTTGAATGCTGAGCTGCAGTCAATGAATAGCAAAAGGTGTTCCTTTTGAA
            Sp1
-5855   AGGGCAGTGTGGAGTGCAATAGAGATTGCAACATCTGTGGATCTGTTGGG
          Sp1
-5805   GCGGTATGCAAATTGGAGTGGGTCTAGGGTTTCTGGGATAATGGTGTTGA
           AP-1
-5755   TGTGAGCCATTATCAGCCTTTCAAAGCACTTCATGGCTACATATGTGAGT

-5705   TCTACGGGTTGGTAGTCATTTAAGCGTTGTTACCTTGGTGTTCTTGGGCA
                                                   1/2ERE
-5655   CAGTTCTATGGTGGTCTACTTGAAACATGTTGGTGTTACAGACTCGGTCA

-5605   GGGACAGGTTGAAAACGTCAGTGAAGACACTCGCCAATTGCATGCTCAGA
                                                   1/2ERE
-5555   GTACAAGTTCTGGTAATCCGTCTGGCCCTCTGGCCTTGTGAATGTTGACC
                      AP-1
-5505   TATTTAAAGGTCTTACTCACATCGGCTATGGAGAGCATGATCATACAGTC

-5455   GTTCGGAACAGCTGATGCTCTCATGCATGCTACAGTGTTGCTTGCCTCAA
                                                 AP-1
-5405   AGCAAGCATGGAAGTTATTTAGCTTGTCTGGTAGGCTTGTGTCACTGGGC

-5355   AGCTCGTGGCTGTGCTTCCCTTTTGTAGTCCGTAATAGTTTGCAGGCCCT

-5305   GCCACATCCAATGAGCGTCGGAGCCGGTGTAGTACAATTCAATCTCAGTC
```

Fig. A. 6 Putative promoter sequence of the cutthroat trout estrogen receptor 1a gene including 5'flanking region and exon 1, intron 1 and exon 2 . The exons are indicated by open boxes. Nucleotides are numbered from 5'-end of exon 1, with negative numbers representing the 5'flanking region. Estrogen responsive element (ERE)-like, and 1/2ERE are indicated by vermillion boxes. Other transcription factor binding sites (Sp1: Specificity Protein 1; AP-1: activator protein 1; GATA: GATA transcription factor; CAAT box; TATA box) are indicated by each color box.

```
-5255   CTGTATTGACGCTTTGCCTGTTTGATGGTTCATCAGAGGACATAGCGGGA

-5205   TTTCTTATGTCCGGGTTAGAGTCCCGCTCCTTGAAAGCGGCAGCTCTAGC

-5155   CTTTAGCTCAGTGGGGAGGTTGCCTGTAATCCATGACTGCTGTTGGAGTA
                1/2ERE
-5105   TGTATGTACGGTCACTGTGGGGACGACGTCATCAATGCACTTATTGGCGA
             AP-1  AP-1
-5055   AGCCAGTGACTGATGTGGTGTACTCCTTAATGCATTCGGAAGAATCCCGG

-5005   AACATATTCCAGTCTGTGCTAGCAAAACAGTCCTGTAGCTTAACATCTGT
             1/2ERE
-4955   GTCAACTGACCACTTCCTTATTGAGCAAGTCACTGGTACTTCCTGCTTTA
                                                 1/2ERE
-4905   GTTTTTGCTTGTAAGCAGGAATCAGGAGGATAGCGTTATGGTCAGATTTG

-4855   TCAAATGGAGGGCGATGGAGAGCTTTGTACACGTCTCTGTGTGTGGAGTA

-4805   AAGGTCGTCTAGAGTTTTTTTCCCCCTCTGGCTGCACATTTAACAAGCT

-4755   GCTAGAAATGAGGTAAAACTGATTTAAGTTTCCCTGTATTAAAGTCCCCG

-4705   GCCACTAGGAGCGCCGCCTCTGGATTAGCATTTTCTTGTTTGCTTATGGC

-4655   AGAATACAGCTCATTGAGAGCCGTCTTAGTGCCAGCATCGGTTTGTAGTG

-4605   GTAAATAGACAGCTACGAAAAATATAGATGTAATTATCTTGGTAAATAGT

-4555   GTGGTCTACAGCTTATCATGAGATACTTTACCTCTGGACTTCCTTAATAT

-4505   TAGATTTCATGCATCAGCTGTTATTTTCAAATAGACACAGACCGCCACCC

-4455   CTTGTCTTACCGGAGGCAGCTGTTCTATCTTGCCGATGGACAGAAAACCC
         Sp1
-4405   AGCCAGTTATCCATGTTGTTGTTCAGCCACGACTAAGTGAAACATAAGAT
```

Fig. A. 6 (Continued)

```
-4355   ATTACATTTGTCCTTTTGGTAGCATAGTCTTGATCGGAATTTGTTATCCA

-4305   TTGATTGCACGTTGGCTAATAGTACTGATGGTAGAGCAATCCTTACAAGG
                                                   AP-1
-4255   CACCCCAACCTACGTCCCCGATATCTCCGTCTCTTCTTCATGTGAATGAC

-4205   GGGGATGTGGGCCTTGTCGGGTGTCCGAAGTAAATCCTTTGCGTCGACTC

-4155   GTTAATGAAAAAGTCTTCGTCCAGTTCGAAGTGTGTAGTCACTGTCCTGA
                         1/2ERE
-4105   TATCCAGAAGCTCTTTTCGGTCAGAGGCGGTGGCAGAAACATTATGTATA

-4055   AAATAAGTTCAAAATAACGCGAAACACGCACAATAGTACAATTGGTTAGG

-4005   AGCCCGTAAAACGGCAGCCATCTCCTCCGGCGCCATCTCACAGGTGAAGC

-3955   TGTGCAAACTTCAGGGGTTTATTATAAGAGGCCAGCTAAATGTTGATTGT

-3905   TGAAAATGAAAGTGAAGTCGCATTACCAAAGATAATATGATGTTATGGCT
                                                  AP-1
-3855   GTTTTGGCAATTAATCCCAAATGTTACGATCACAGTTTCTGTGAGTTAAT
                                                   1/2ERE
-3805   GTCTCTGTTCCTGCTCTTAGGTGTCTCTGTGGGGTAACAGGTGTGACCTG
                                                     AP-1
-3755   TCCATCTCTGCTGGCATGGAGAACTCTCAGAAGGCCAGTCCCATTGACTC
              1/2ERE
-3705   CCTGTCTGACCTGAAGTCTTTTATCCTGGTGGACGACTCCAACATGGTGT
                  1/2ERE
-3655   GGTCTGCTCTGACCTCTGCCCAGAGACCAGGGGAGGATGGCAAAATCACC
             Sp1                                     1/2ERE
-3605   CCAGGGAGGGTGGACATCGTCCTGGACAACGCTGGCTTCGAGCTGGTCAC

-3555   AGACCTGGTCCTCGCTAACTTCCTGGTGTCCGCTGGTCTGGCCAGGGAGG

-3505   TCCGCTTCCATGGCAAGTCCATCCCCTGGTTTGTCTCCGACGTCACCTCG
```

Fig. A. 6 (Continued)

```
-3455   CACGACTTCCAGTGGACCATCCGCCAGACCCTGGCGGCCAATCACAAGTG

-3405   GATGTCCAAGAGCGGTGTCCAGTGGCAGAGCTACGTACGGGAGGGCGTGT
                                        Sp1
-3355   GGTCCTATCACGACCACCCGTTCTGGACAATGCCTCATGAGTACTGTGAC
                          1/2ERE
-3305   ATGGCGGGCGACGCGCCTGACCTCTACGCGACACTGCAGGGAGCCGACCT

-3255   GATTCTGTTTAAAGGAGACCTGAACTACAGGAAGCTGACTGGGGACAGGG

-3205   AGTGGGATCACATGGTGCCGTTTGATAGGGCACTGAGGGGATTCGGCCCA

-3155   GCTCCCCTGTGCAGTCTGAGGACTCTGAAGGCTAACATCCAGGTGGGGCT
                 Sp1                                    1/2ERE
-3105   CCAGCCGGGGCAGGGGGAGAACCTCAACACCCAGGACCCCAGCTGGATGA

-3055   CCAGCGGGAAGTACGCTGTGGTCCAGTTCTCCAGCCCTCACAGGGAACAG

-3005   TAGACCTGTCTGGGACAGGAAATGACGTCAATATGTTCATAATAAGTGGC

-2955   GTCATCATAATCAGAGGAAGTAAGGGCGTGAGCAGGGCCAGTGTACTTGT

-2905   AACCATATGCCAGGTTAGAGGTGTAGTTTGTTTTGTCGTAGAACTACCTA

-2855   ATGTATTTAAATGAGGAGAGTGGTTTAAAGGAGGATGATAGAGGATACTT

-2805   TAAAAAGAGGAGGGTAGTTAAATAGGGAGTGGCTGCAGATCCGACTTCTC

-2755   TGGACCCGTCGTCCCGACAGCTAAAGGTTCACAGCTTCTGTGAATGTGTG

-2705   GTAATCTCTACTTTACACACCATCTCTGTTCTATTTGTTCAGCTGCCCAG

-2655   AGAAGAAGCTACTCTGGCGAAAGGTGCTTGTTTAATAAAGTTAGATCAAA

-2605   CTCGAATCATTGTAAGATTACATAAAGATAATATTCTATAGAACCAAATA
```

Fig. A. 6 (Continued)

```
-2555   AAATAGCATAGTATAACATTACTGTATGACAAAAAACACCTAATTTCTTA

-2505   TATTTTAGGATGATTGTGCTCATCGTCACATTTTTCTCTCATGCAGTCTA

-2455   AACTCAACAGTGCACGCCACTCAGCAAAGGTATGCTACAGTTTAATACCA

-2405   TCTGCAAAAAAATGTGCTAAGGTACATAACTAATTCAAAACACTGTAGCC

-2355   TACTTCAAAACAACACTGTAGCCTACACTCAATAATATTTAAATGCATAT

-2305   GCCCATGAAACTGATTGCAAATGGTTGGCGTGCAGATTCTGCATGTATGT
                                                  AP-1
-2255   TTCACCGGTAAAACTTGGAAGAATTATCATTCACGATTGACAGTGAGAAA
             Sp1      1/2ERE
-2205   TGCGAAGGGAGGGTGACCAGAAGTTTGGGTGTGCGTAGAGTTAAAGAAAC

-2155   ACATGCACAGAACGTGAGTGGGATCTTCAGCTCTGGGGAAAGGGATCCAG
              Sp1     1/2ERE
-2105   TGGGATGGGGCTGTGACCTCCGGATCCCCAGCCTCTGCCATTTAAATAAT

-2055   GATCAACTGCTGGTTAGCAATCCATCCTGGCATAACTACAAAACAATGAC

-2005   GTTTACTTCTCCCATTTAACAAATGTGTTGACGGTTGTTTACCATCATTC

-1955   AGTACTAGGGTCGGGCTCTACCTCGATTCCAGTTCAAATCAGTTAATTTA

-1905   GAAACATTGAAATTCCAAGTCATGAATTGAAACATGCTTATCTGATCTTA

-1855   AGTGACTTTTTTCCAGACATATTATGTAATTTATGTAATGTTTTTGTCTC

-1805   CCCAACAGCGACTACACAAAATATAGATTTGAGACACATTTCATTCATTA

-1755   TCAGAGGCAATAAGATGCAAGGTGCAATTTGAACATTTACATTTTAAGTT

-1705   GGCCTGTCGTAAATAGTTTAGAATTGTTATTTTAAGTTGAGCCCTTATGT
```

Fig. A. 6 (Continued)

```
-1655   AACACCCAAAGCCAGCGGCTATTTTTTAGCCATTTATTTTGCATGAGCTT

-1605   GGCAAGACATTACAGAGTTAACTTGTGGTTGCTGGTATTTTCTGGGTCTT

-1555   AATCAACGTCATCAGTTTGTCTTGTCAATTTATTGCATGCGTTTTTGTTT
                                1/2ERE
-1505   GCATACATCGGGGTTTGCACAGTGACCACAGTCCCGGAGTTTACATTTTC

-1455   ATAGTTAAATACCTGAAGTTGAACGACAAGGTAATGTTCCCACTCTGGGA

-1405   AACTATAGATAAATCATAGTCTTTGGGGCAGTGCAATCACACGTACAAGG

-1355   TGTGTGTTGTTAAACACCTGTCTATGATGTATTTCTGTTTGTTTGATAGT

-1305   ACAAAATGTTATTTCCGACTCATTAGAATGCCTCTGTAACCAGTGCAGGT

-1255   GCCGTTGTGATCCTAAATGACTGGATTGTTCTCTTTGCAGAAATCTGGAA

-1205   CAAAAATTGTTATATTATGATGGACTTGGTTTAAATAAAGAACTTCAATG
                                          AP-1
-1155   ACTGGTCTTATCTCATACTGTTGTATAAATGAATGACATTAAGAAAAGAG
           ERE-like
-1105   CAACCGAGGCTTGACCTAGAAAGATGATTCCCTTTCACTCATTCTCCCCA

-1055   TACTCTTATTACGCTCTTCAATTTATCCTGGGAACATGTATAAAAAATAG

-1005   AGTTATAATCAGACTGCTTGTTTAGCACTTGACATCACATGTCTGAATCT

 -955   GGGCTGTTTACTGAGTGAAGAGACGTTGTAGAGAATGGAGCTTCTCTAGG
            1/2ERE
 -905   CAGCAGGTCAAACAGGAGGCCATTTTCTCCCAGTTTGCAGAGATAGAGGT

 -855   TAATGAACCATCACTTTAATAAGCAAAAAGTTAACTTTCCCCCGTAAAAT

 -805   CCAGCTGTCAATTTGTCAAGAAAGTTTGGAAAAATCAGAATCCCCAGTAT
```

Fig. A. 6 (Continued)

```
-755   GATCTCACTGAATTGTTTGTGGAAATGAAGACACTCATCTTCATACTGTA

-705   ATATGGGGAGCTGATCTGTCCATTTTTATGTACTGAGAATTGTTGCACAC

-655   GTTGAGGACTGGAAATACTAGTAGTCTTAAATTAGTAATAACAGTGTTGT

-605   GCTAGGCACACCCTGCATTTCAACAAGTGACACCGTTATTCCTTTAGAGA
                                        ERE-like
-555   AACAGAGTTATCTTCCTGTGTAGGTTGAAGGTCAAGAGTTGTCCTTGTAC

-505   GCCCTTGAAACTTACCTAAAGGAATGGGGAAGACATTAAATGACTTTTCA

-455   AACATTGGTTGAATTTCATCATGATGCAGCATGTACTTGAAACCCCTTTG
                  Sp1
-405   TTTGCTGCTTTGGCGGGATAATAGTTTATATTTGATCAGTGAATATCGTT
                              CAAT box
-355   GATAAGGTATTCCTCTTGAAAGGCCCAATGATAGCAAGACAATGTACTTT
                        1/2ERE                AP-1
-305   AAATTAGTAGCTATTCCTTGGTCACAGTAGAGAGTGATTTAGTCAGTGTA

-255   GTTTGGTGATCTTACTGCAATGCTGTATTCTGTTTTCCTGAATAGATTTA

-205   GGCTATGAAAAGGGGGAACATGTAAGAACATGCATTGCCTGATGTCTGAA

-155   TCCTCTCATCTTTTCCCCACTGGGAGTTACATAATGTTGGCATCAGAAGG
          1/2ERE                      ERE-like
-105   GTGTTGACCTCACATACTGTTTGCTGTGTCATGTTGACCTGCTCTAGAGA
                                                TATA box
 -55   TACACTATCAATATCGATCCGGCTGCGTTCATTTGTTTCTACCTTTTTTA
                 Exon1
  -5   AATCTCTTTTTTTCTCATTGTGATGCGAAGCCAGATCTCAGAAAGAGGGA

 +46   TGAGAGCGAGAGGACAGGGAGAAAGAGGAACCACTCAACACAACAATGCT

 +96   CATGATTAGACCCAAAGAGCTGAATATTGATCCATAACGTCATTAAGGTA
```

Fig. A. 6 (Continued)

```
                    Intron1
+146   AGTACTGTATATATGACGTCTTTGTGTGTATTTCATGTAATGGGTTCCTA

+196   TTTTATTGCTTCCTTCTCGTGGAATTGTTTTGTATAATTTTTCCATATTT

+246   GCACATAAATCCACTGATAGACACATTTAATAATGATTTAACTTCTTAGA

+296   AGAGTGAGTCAGAAAGGAGTGATATTCTGGGCCACGGCAGTACATGTAAC

+346   TAAATGACTGATAAGCATTCAGAGAGGAAGTCACACCAGGGTGTAACTCT

+396   CCTTGGAGCCTAGCAGCGTCCTCTAAAAGCTTTTCAGGGAAGAGAAAAAT

+446   AAGCTGCATTTTAGAAAAAAATGTAAGGGGACAGGGGAGTTGTTGGAGTG

+496   GTTGCCTCTTTCTTTCGTTCTCTCACTCTCCTTCTCTCTTGCTCTCTATC

+546   TCGAAATATCTGTCACTTGTTGTCCTTTCCTCCACCCCTCTCTCCCACAG

+596   CAGTGTCGATAGTTAAGGGAAAAGAGAAAAGCTCTGCCTCTCAAAAATAA

+646   GTGGGAGGGAGGAATGTTTTTGGGCTGTCTCCAGGCGGAACCTGGGAGGG

+696   AAAGAGAGAGCAAGGAGGGACGAGAAAAGAGAGAGAGAGAACCTAGTGAA

+746   TGCCTCTTCCCTTCCTCTTCCCAACAGCCAGTATTGAGTTGCTTAGCACG

+796   GGCTGTTAAGGAAGAAACAGAGCAAGAGAGGGACGAGAGAAAAGAGAGAG

+846   AGAAGACAGAACAGAGCCCTTCTCCCCTCCCACCCCTTAGTGAGCCAGTC
                                                   1/2ERE
+896   TAAACCAAGCTGCTTGTCACTGCTGTTGTTCTGTGAATGTGATGCTGGTC

+946   AGACAGTCCCATACGCAGATTTCCAAACCTCTCGGAGCTCCTCTCAGATC
                    Exon2
+996   CCGAACGACCCTGGAGAGCCACGTCATCTCCACCCCAAAACTCTCACCAC
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Fig. A. 6 (Continued)

+1046 AGCAGCCGACCACCCCCAACAGCAACATGTACCCTGAGGAGACACGCGGA

+1096 GGTGGTGGGGCGGCCGCCTTTAACTACCTGGACGGAGGGTATGACTACAC

+1146 AGCCCCTGCCCAA

Fig. A. 6 (Continued)