

Investigation of the effects of estrogen on skeletal gene expression during zebrafish larval head development

Ehsan Pashay Ahi, Benjamin S Walker, Christopher S Lassiter, Zophonías O Jónsson

The development of craniofacial skeletal structures requires well-orchestrated tissue interactions controlled by distinct molecular signals. Disruptions in normal function of these molecular signals have been associated with a wide range of craniofacial malformations. A pathway mediated by estrogens is one of those molecular signals that plays role in formation of bone and cartilage including craniofacial skeletogenesis. Studies in zebrafish have shown that while higher concentrations of 17- β estradiol (E_2) cause severe craniofacial defects, treatment with lower concentrations result in subtle changes in head morphology characterized with shorter snout and flatter face. The molecular basis for these morphological changes, particularly the subtle skeletal effects mediated by lower E_2 concentrations, remains unexplored. In the present study we address these effects at a molecular level by quantitative expression analysis of sets of candidate genes in developing heads of zebrafish larvae treated with two different E_2 concentrations. To this end, we first validated three suitable reference genes, *ppia2*, *rpl8* and *tbp*, to permit sensitive quantitative real-time PCR analysis. Next, we profiled the expression of 28 skeletogenesis-associated genes that potentially respond to estrogen signals and play role in craniofacial development. We found E_2 mediated differential expression of genes involved in extracellular matrix (ECM) remodelling, *mmp2/9/13*, *sparc* and *timp2a*, as well as components of skeletogenic pathways, *bmp2a*, *erf*, *ptch1/2*, *rankl*, *rarab* and *sfrp1a*. Furthermore, we identified a co-expressed network of genes, including *cpn1*, *dnajc3*, *esr1*, *lman1*, *rrbp1a*, *ssr1* and *tram1* with a stronger inductive response to a lower dose of E_2 during larval head development.

1 Investigation of the effects of estrogen on skeletal gene 2 expression during zebrafish larval head development

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21

22 Abstract

23 The development of craniofacial skeletal structures requires well-orchestrated tissue interactions
24 controlled by distinct molecular signals. Disruptions in normal function of these molecular
25 signals have been associated with a wide range of craniofacial malformations. A pathway
26 mediated by estrogens is one of those molecular signals that plays role in formation of bone and
27 cartilage including craniofacial skeletogenesis. Studies in zebrafish have shown that while higher
28 concentrations of 17- β estradiol (E_2) cause severe craniofacial defects, treatment with lower
29 concentrations result in subtle changes in head morphology characterized with shorter snout and
30 flatter face. The molecular basis for these morphological changes, particularly the subtle skeletal
31 effects mediated by lower E_2 concentrations, remains unexplored. In the present study we
32 address these effects at a molecular level by quantitative expression analysis of sets of candidate
33 genes in developing heads of zebrafish larvae treated with two different E_2 concentrations. To
34 this end, we first validated three suitable reference genes, *ppia2*, *rpl8* and *tbp*, to permit sensitive

35 quantitative real-time PCR analysis. Next, we profiled the expression of 28 skeletogenesis-
36 associated genes that potentially respond to estrogen signals and play role in craniofacial
37 development. We found E₂ mediated differential expression of genes involved in extracellular
38 matrix (ECM) remodelling, *mmp2/9/13*, *sparc* and *timp2a*, as well as components of
39 skeletogenic pathways, *bmp2a*, *erf*, *ptch1/2*, *rankl*, *rarab* and *sfrp1a*. Furthermore, we identified
40 a co-expressed network of genes, including *cpn1*, *dnajc3*, *esr1*, *lman1*, *rrbp1a*, *ssr1* and *traml*
41 with a stronger inductive response to a lower dose of E₂ during larval head development.

42

43 **Keywords**

44 Craniofacial skeleton; Development; Estrogen; Gene expression; Zebrafish larvae

45

46 **Introduction**

47 Craniofacial development is a critical part of embryogenesis and identification of molecular
48 mechanisms underlying this process is important in gaining a better understanding of
49 morphological diversity in vertebrates (Szabo-Rogers et al., 2010) as well as human health
50 (Oginni & Adenekan, 2012). The viscerocranium in humans is of interest because of oro-facial
51 clefts and associated malformations (Marazita, 2012). The vertebrate craniofacial skeleton,
52 including the viscerocranium, is built from neural-crest derived tissues. Changes in these tissues
53 over evolutionary time have given rise to a wide diversity of facial morphologies among
54 vertebrate species (Trainor, Melton & Manzanares, 2003; Bronner & LeDouarin, 2012).

55 Estrogens, steroid hormones synthesized by aromatase from androgenic precursors, have recently
56 been shown to affect craniofacial development (Fushimi et al., 2009; Cohen et al., 2014).
57 Though estrogens are normally thought of as sex hormones, they affect a variety of tissues
58 including the cardiovascular and skeletal systems (Hall, Couse & Korach, 2001; Allgood et al.,
59 2013; Cohen et al., 2014). Estrogens signal through classical nuclear receptors (ER-alpha and
60 ER-beta) (Jia, Dahlman-Wright & Gustafsson, 2015) and a G-protein coupled receptor, GPR-30
61 (Jenei-Lanzl et al., 2010). These signaling proteins are found in the chondrocytes of many
62 vertebrate species (Tankó et al., 2008) and are present during chondrogenesis (Jenei-Lanzl et al.,
63 2010; Elbaradie et al., 2013). Among vertebrates, teleost fish are highly diversified in
64 craniofacial morphology and estrogen has been shown to greatly affect chondrogenesis in many

65 of the fish species studied so far, including tilapia, fathead minnow, and zebrafish (Ng, Datuin &
66 Bern, 2001; Warner & Jenkins, 2007; Cohen et al., 2014). Furthermore, the teleost zebrafish is a
67 well-developed model system for studying both embryonic development and human disease and
68 it can be useful in understanding the development of the human viscerocranial skeleton as these
69 processes are well-conserved among vertebrates (Kuratani, Matsuo & Aizawa, 1997).

70 Low concentrations of estrogen cause subtle changes in craniofacial morphogenesis during
71 zebrafish larval development (Cohen et al., 2014). These changes are characterized by a shorter
72 snout, flatter face and wider angles of cartilage elements in the viscerocranium (Cohen et al.,
73 2014). Another recent study has also demonstrated that an estrogen mediated signal underlies the
74 evolution of shorter snouts and flatter faces in females of some reptilian species (Sanger et al.,
75 2014). These observations suggest that similar mechanisms might underpin hormone-based
76 phenotypic plasticity and diversity (Dufty, 2002), as well as subtle differences in head/skeletal
77 morphology of dimorphic sexes (Loth & Henneberg, 2001; Fujita et al., 2004; Callewaert et al.,
78 2010). Therefore, studies offering insights into molecular mechanisms rendering the observed
79 phenotypes caused by hormonal changes would be called for.

80 The subtle changes in craniofacial skeletogenesis mediated by low concentrations of Estradiol
81 (E_2) are likely to be a result of differences in level and timing of the expression of
82 skeletogenesis-associated genes during head development (Albertson et al., 2010; Ahi et al.,
83 2014; Gunter, Koppermann & Meyer, 2014; Powder et al., 2015). These morphological changes
84 were only revealed by careful measurements of skeletal elements at zebrafish larval stages
85 (Cohen et al., 2014), therefore the identification of responsible genes might also require precise
86 expression studies in developing heads of zebrafish larvae using a sensitive tool such as
87 quantitative real-time PCR (qPCR) (Bustin, 2000; Kubista et al., 2006). In the present study, we
88 set out to quantitatively assess the effects of estrogen on the expression dynamics of candidate
89 genes which are known as potential targets of estrogen pathway and also involved in craniofacial
90 skeletogenesis in different vertebrate species (Table 1). We hypothesized that these genes may be
91 critical to the estrogen modulation of craniofacial skeletogenesis. We first identified the most
92 stably expressed reference genes in developing heads of zebrafish treated with two doses of
93 estrogen ($2\mu\text{M}$ and $5\mu\text{M}$) across five stages in larval development. Then, we accurately
94 measured small changes in the expression levels of the candidate genes. In addition, we have
95 used available co-expression data from zebrafish to identify a co-expressed network of genes

96 with greater transcriptional response to the lower dose of estrogen (2 μ M) during larval head
97 development.

98

99 **Methods**

100 **Fish husbandry, treatment and sampling**

101 Adult zebrafish were fed a diet of live brine shrimp supplemented with Ziegler zebrafish diet
102 (Pentair) and maintained on a 14/10 day/night cycle. Embryos were raised in E3B (5 mM NaCl,
103 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 0.00025% methylene blue). Embryos were
104 treated with estrogen (17 β -estradiol, E₂, Sigma) dissolved in ethanol and diluted in E3B for a
105 final ethanol concentration of 0.1%. Control fish were treated with 0.1% ethanol with no
106 developmental malformations as described previously (Cohen et al., 2014). For each treatment
107 group (estrogen concentration), zebrafish larva were raised in Petri dishes, and treatment
108 solutions were refreshed daily until the stages indicated (3, 4, 5, 6 and 7 days post fertilization,
109 dpf). Three biological replicates of 30 larva were collected at each time-point (3-7 dpf) and for
110 each treatment group (control, 2 μ M E₂, and 5 μ M E₂) for a total of 90 larva at each time-point
111 and treatment. The fishes were anesthetized with 0.4% tricaine (MS-222, Sigma). Isolated heads
112 (anterior to the yolk sac) were placed into RNAlater (Qiagen) and stored frozen until RNA
113 isolation. Zebrafish experiments were performed under the Roanoke College IRB protocol
114 #14BIO76.

115

116 **RNA isolation and cDNA synthesis**

117 Around 30 heads of zebrafish from each treatment group and larval stage were pooled in TRI
118 Reagent (Sigma) and homogenized with a disposable Kontes Pellet Pestle Cordless Motor tissue
119 grinder (Kimble Kontes). RNA was prepared according to manufacturer's instructions and
120 dissolved in 50 μ l RNase-free water. RNA samples were treated with DNase (New England
121 Biolabs) to remove contaminating DNA. Quantity of the resulting RNA samples was assessed
122 using a NanoDrop ND-1000 UV/Vis-Spectrophotometer (NanoDrop Technologies). The quality
123 of the RNA samples was evaluated by agarose gel electrophoresis and all samples displayed
124 intact 28 S and 18 S rRNA without noticeable high molecular weight genomic DNA
125 contamination. cDNA was prepared from 1000 ng of RNA using the High capacity cDNA

126 Reverse Transcription kit (Applied Biosystems), according to manufacturer's protocol. Several
127 samples without addition of reverse transcriptase (-RT samples) were prepared to confirm the
128 absence of genomic DNA. cDNA was diluted 3 fold in water for further use in quantitative real-
129 time PCR.

130

131 **Gene selection, Primer design and real-time qPCR**

132 In order to validate suitable reference genes for accurate measurement of the transcriptional
133 changes of candidate genes by qPCR, we selected 7 potential reference genes based on published
134 studies in zebrafish (Table S1) (McCurley & Callard, 2008; Pelayo et al., 2012; Schiller et al.,
135 2013), none of which have been validated during development or in zebrafish head. In addition
136 we selected 28 target genes that are known as potential targets of the estrogen pathway in
137 different vertebrate species, and also involved in craniofacial skeletal formation/morphogenesis
138 (Table 1 and Table S1). Finally, we extended our list of candidates by adding more genes
139 showing co-expression with the estrogen receptor *esr1* based on the zebrafish database
140 COXPRESdb (<http://coxpresdb.jp/>) version 6.0 (Obayashi & Kinoshita, 2011). To obtain the
141 maximum number of coexpressed genes with a high degree of reliability, we filtered the genes
142 by setting the mutual rank (MR) to the top-ranked 2000 and the Supportability score of minimum
143 1 (as described by Obayashi and Kinoshita, 2011). This yielded 339 candidate genes, and from
144 them, we selected 11 genes with reported craniofacial expression during zebrafish development
145 according to the ZFIN database (<http://zfin.org>) (Bradford et al., 2011) (Table S1).

146 Locations overlapping exon boundaries of the genes in zebrafish were determined by NCBI
147 Spidey software (www.ncbi.nlm.nih.gov/spidey) and annotated genome sequences in the
148 Ensembl database (http://www.ensembl.org/Danio_rerio). The qPCR Primers were designed on
149 exon boundaries using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA)
150 and checked for self-annealing, hetero-dimers and hairpin structures with OligoAnalyzer 3.1
151 (Integrated DNA Technology) (Table S1).

152 Real-time PCR was performed in 96 well-PCR plates on an ABI 7500 real-time PCR System
153 (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Master Mix (2X) as
154 recommended by the manufacturer (Thermo Fisher Scientific, St Leon-Rot, Germany). Each
155 biological replicate was run in duplicate together with no-template control (NTC) in each run for
156 each gene and experimental set-up per run followed the preferred sample maximization method

157 (Hellemans et al., 2007). The qPCR was run with a 2 min hold at 50°C and a 10 min hot start at
158 95°C followed by the amplification step for 40 cycles of 15 sec denaturation at 95°C and 1 min
159 annealing/extension at 60°C. A dissociation step (60°C – 95°C) was performed at the end of the
160 amplification phase to identify a single, specific product for each primer set (Table S1). Primer
161 efficiency values (E) were calculated with the LinRegPCR v11.0 programme
162 (<http://LinRegPCR.nl>) (Ramakers et al., 2003) analysing the background-corrected fluorescence
163 data from the exponential phase of PCR amplification for each primer-pair and those with E less
164 than 0.9 were discarded and new primers designed (Table S1).

165

166 **Data analysis**

167 To detect the most stably expressed reference genes, three ranking algorithms; BestKeeper
168 (Pfaffl et al., 2004), NormFinder (Andersen, Jensen & Ørntoft, 2004) and geNorm
169 (Vandesompele et al., 2002), were employed. The standard deviation (SD) based on Cq values of
170 the larval stages and treatment groups was calculated by BestKeeper to determine the expression
171 variation for each reference gene. In addition, BestKeeper determines the stability of reference
172 genes based on correlation to other candidates through calculation of BestKeeper index (r).
173 GeNorm measures mean pairwise variation between each gene and other candidates, the
174 expression stability or *M* value, and it excludes the gene with the highest *M* value (least stable)
175 from subsequent analysis in a stepwise manner. Moreover, geNorm determines the optimal
176 number of reference genes through calculation of pairwise variation coefficient ($V_{n/n+1}$)
177 between two sequential normalisation factors (NF_n and NF_{n+1}) and extra reference genes are
178 added until the variation drops below the recommended threshold of 0.15 (Vandesompele et al.,
179 2002). NormFinder identifies the most stable genes (lowest expression stability values) based on
180 analysis of the sample subgroups (stage and treatment group) and estimation of inter- and intra-
181 group variation in expression levels.

182 For the analysis of qPCR data, the difference between Cq values (ΔCq) of the reference genes
183 and the target genes was calculated for each gene; $\Delta Cq_{\text{target}} = Cq_{\text{target}} - Cq_{\text{reference}}$. The
184 geometric mean of Cq values of three best ranked reference genes, *ppia2*, *rpl8* and *tbp* (see the
185 ranking algorithms above), was used as $Cq_{\text{reference}}$ in the ΔCq calculations. All samples were then
186 normalized to the ΔCq value of a calibrator sample to obtain a $\Delta\Delta Cq$ value ($\Delta Cq_{\text{target}} - \Delta Cq_{\text{calibrator}}$).
187 For each primer pair a biological replicate in the control group at 3dpf was selected as

188 the calibrator sample. Relative expression quantities (RQ) were calculated based on the
189 expression level of the calibrator sample ($E^{-\Delta\Delta Cq}$) (Pfaffl, 2001). The RQ values were then
190 transformed to logarithmic base 2 values (or fold differences; FD) for statistical analysis
191 (Bergkvist et al., 2010). A two-way analysis of variance (ANOVA) followed by post hoc Tukey's
192 honest significant difference (HSD) test was implemented for each reference or target gene with
193 larval stages and treatment groups as categorical variables. To assess similarities in expression
194 patterns of the genes Pearson correlation coefficients (r) were calculated for all gene pairs using
195 the data from 3 treatments at 5 larval stages (degree of freedom = 13). R ([http://www.r-](http://www.r-project.org)
196 [project.org](http://www.r-project.org)) was used for all statistical analysis.

197

198

199 Results

200

201 *tbp*, *ppia2* and *rpl8* are the most suitable reference genes

202 Real-time quantitative PCR for the 7 reference gene candidates was performed on cDNA
203 generated from zebrafish head homogenates in three treatment groups at five larval stages. The
204 expression levels of the candidates varied from *ppia2*, with the highest expression (lowest Cq)
205 (Fig. 1A), to *tbp* with the lowest expression (highest Cq). Statistical analysis revealed that all of
206 the candidates except *actb1* are stably expressed between the treatment groups (Fig. 1B).
207 However, only *tbp* showed constant expression during the larval stages examined. Two genes,
208 *ppia2* and *rpl8*, were also stably expressed in developing heads of zebrafish larvae except for the
209 first stage (3dpf). Based on these results *tbp* followed by *ppia2* and *rpl8* were found to be the
210 overall most stable reference genes both over time and between the treatment groups. The
211 candidate reference genes were ranked using three algorithms, *i.e.* BestKeeper, geNorm and
212 NormFinder, and based on standard deviation (SD) as described in (Ahi et al., 2013) (Table 2).
213 In all of the analyses three genes; *ppia2*, *rpl8* and *tbp*, were the three highest ranking candidates,
214 however their order varied between the rankings (Table 2). Furthermore, geNorm suggested the
215 use of the three best ranked candidate genes as sufficient for accurate normalisation (Fig. S1).
216 The data reflect the high expression stability of the best ranked candidate genes and suggests the
217 combination of *ppia2*, *rpl8* and *tbp* as a suitable and sufficient normalization factor to accurately

218 quantify small differences in gene expression in developing heads of zebrafish larvae across the
219 E₂ treatment groups.

220

221 **Components of different signalling pathways and skeletogenesis-associated** 222 **genes are affected by estrogen during larval head development**

223 The selected 28 candidate target genes, listed in Table 1, can be classified into distinct functional
224 groups; (I) estrogen receptors with potential involvement in vertebrate craniofacial development
225 (*esrra* and *esr1*); (II) components of hedgehog (Hh) signaling pathway (*ptch1/2* and *shha/b*);
226 (III) potential skeletogenic targets of estrogen pathway with critical roles in viscerocranial
227 development/ morphogenesis (*bmp2a/b*, *opg*, *rankl*, *runx2b* and *sox9b*); (IV) potential targets of
228 estrogen pathway involved in ECM formation and associated with shortened snout
229 morphogenesis in vertebrates (*col2a1a*, *ctsk*, *mmp2/9/13*, *sparc* and *timp2a*); and (V) other
230 potential targets of estrogen pathways with diverse functions which are also involved in
231 viscerocranial skeletogenesis (*alx4*, *dlk1*, *erf*, *ets2*, *pbx1a/b*, *rarab* and *sfrp1a*). The expression
232 levels of all candidates were measured in the three treatment groups during larval head
233 development (Fig. 2-5). We found effects of different E₂ concentrations on the expression of
234 most of the target genes, except *col2a1a* and *pbx1a*, the effects, however, were highly variable
235 among the genes (Fig. 2-5). For instance, while some genes, i.e. *esr1*, *ptch1/2* and *rarab*
236 displayed differential expression between the treatment groups in most of the larval stages, other
237 genes such as *alx4*, *bmp2b*, *ctsk*, *ets2*, *opg*, etc., showed expression differences in only one stage.
238 Among the more highly affected genes, *erf*, *esrra*, *mmp9*, *rankl*, *shha*, *sfrp1a*, *sparc* and *timp2a*
239 were differentially expressed in at least three larval stages (Fig. 2-5). Although significant, most
240 differences in expression levels of the target genes were slight between the treatment groups (RQ
241 < 0.5), except for *esr1* in the last larval stages (Fig. 2). Moreover, for all of the affected genes,
242 except *esr1* and *mmp13*, the different E₂ treatments had mainly repressive effects on
243 transcription. These repressive effects were not, however, increased by higher E₂ concentration
244 particularly at the last two stages when the lower E₂ dose (2μM) repressed expression of many of
245 the genes more than the higher dose. At the last three stages, the expression of *esr1* was induced
246 at highest levels for 2μM treatment groups (Fig. 2). The transcriptional repression by E₂ was also
247 variable between the genes and it was more pronounced for *erf* and *ptch2* showing higher
248 expression in control groups than both E₂ treated groups at three larval stages. Taken together,

249 these results show significant effects of low E₂ concentrations on the expression of a variety of
250 genes involved in skeletogenesis and/or craniofacial development.

251 We calculated the Pearson's correlation coefficient of the expression levels for the target genes
252 over all treatment groups and larval stages and found positive expression correlation between
253 many pairs of target genes (blue shadings in Fig. S2). Some of the genes i.e. *mmp9*, *ptch1*, *rarab*
254 and *timp2a* displayed positive expression correlation with most of the genes whereas others such
255 as *mmp13*, *sfrp1a*, *shhb* and *sparc* showed the least correlated expression. Negatively correlated
256 expression was only seen between *esr1* and *sfrp1a*, and between *shhb* and six genes including
257 *esr1*, *ets2*, *mmp13*, *opg*, *pbx1a* and *spp1* (red shadings in Fig. S2).

258

259 **A co-expressed network of genes shows higher expression induction in lower** 260 **E₂ treatment groups**

261 The stronger transcriptional response of *esr1* to the lower E₂ treatment (Fig. 2) could indicate a
262 distinct regulatory mechanism associated with slight increase in estrogen concentration during
263 zebrafish larval head development. In order to identify additional genes showing similar
264 expression dynamics, we selected 11 candidate genes constructing a co-expression network with
265 *esr1* using co-expression data for zebrafish in COXPRESdb (Obayashi & Kinoshita, 2011)
266 (Table S2). These candidates are also known to have craniofacial skeletal expression during
267 zebrafish development based on data submitted to ZFIN database (<http://zfin.org>) (Bradford et
268 al., 2011). Strikingly, we found stronger inductive effects of the lower E₂ concentration on the
269 expression of six genes, i.e. *cpn1*, *dnajc3*, *lman1*, *rrbp1a*, *ssr1* and *tram1* (Fig. 6). The
270 expression of these six genes followed a similar pattern and their higher expression levels were
271 more pronounced in the last three stages of 2μM treatment groups. Moreover, the gene showing
272 strongest coexpression relationship with *esr1* among the candidates, *rrbp1a*, had shown higher
273 expression levels in the last four stages of 2μM treatment groups (Table S2 and Fig. 6). Finally,
274 we also demonstrated positive expression correlations between the six candidates and *esr1*, but
275 not the rest of the non-differentially expressed genes (blue shadings in Fig. S3).

276

277 **Discussion**

278 Estrogen signaling, through both canonical nuclear estrogen receptors and G-protein coupled
279 receptors, is important in embryonic development (Griffin et al., 2013; Shi et al., 2013).
280 Estrogens can act at autocrine, paracrine, and endocrine distances in the embryo and the adult
281 (Boon, Chow & Simpson, 2010). Aromatase, the enzyme that synthesizes estrogens, is present in
282 the developing brain of many species, including zebrafish (Lassiter & Linney, 2007) and would
283 be a local source of the hormone during head development. In fact, the teleost brain produces
284 relatively high levels of estrogen compared to other vertebrates (Forlano et al., 2001). Estrogens
285 are thus present in the cranium of developing embryos and modulate viscerocranial development
286 (Fushimi et al., 2009; Marquez Hernandez et al., 2011; Cohen et al., 2014). Estrogen signalling
287 has been implicated in the sexual dimorphism of cranial bones in the Anolis lizard (Sanger et al.,
288 2014). Hence, it may play a role in craniofacial morphological divergence among species and
289 within sexes of the same species.

290 A previous attempt to identify mechanisms underlying the effects of estrogen on zebrafish
291 craniofacial development was conducted with a high concentration of 17- β estradiol (10 μ M)
292 giving rise to major disruptions of chondrogenesis followed by severe morphological defects
293 (Fushimi et al., 2009). In the same study, analysis of gene expression after high dose estrogen
294 treatment was limited to a semi-quantitative method (*in situ* hybridization) and a few
295 chondrogenic genes belonging to only one molecular pathway (Fushimi et al., 2009). We
296 hypothesized that many other candidate genes would be involved and hence, in the present study,
297 we sought to quantitatively assess the expression of genes that could play role in the subtle
298 effects of estrogen on the development of the craniofacial skeleton in zebrafish larvae (Cohen et
299 al., 2014). Since our expression analysis depended on accurate qPCR, a prior step of careful
300 validation of reference genes was essential to acquire reliable results (Bustin, 2000; Kubista et
301 al., 2006). An increasing number of stably expressed reference genes have been validated for
302 qPCR studies in a variety of fish species (Ahi et al., 2013; Fuentes et al., 2013; Liu et al., 2014;
303 Altmann et al., 2015; Wang et al., 2015), and also in zebrafish at different developmental stages,
304 body parts/tissues, and treatments (Tang et al., 2007; McCurley & Callard, 2008; Lin et al.,
305 2009; Casadei et al., 2011). There is however a necessity for validation of reference genes
306 depending on the experimental conditions under study. Here, we found three genes, *ppia2*, *rpl8*
307 and *tbp*, to be the most stably expressed candidate genes by all the methods of analysis used
308 (Table 2 and Fig.1) and their combination could ensure robust qPCR data normalisation (Fig.

309 S1). We next selected candidate genes that are shown to be potential estrogen pathway targets,
310 and at the same time, differential regulation of many of them is associated with morphological
311 changes resembling shortened snout in different vertebrates (many found in mammalian species)
312 (see publications referenced in Table 1). The underlying mechanisms by which these candidate
313 genes could affect skeletogenesis are different from each other. For instance, genes like *bmp2a/b*,
314 *runkl*, *runx2b* and *sox9b* are major factors in differentiation of skeletal cells and some others
315 such as *col2a1a*, *ctsk*, *mmp2/9/13*, *spp1* and *sparc* are critical for the formation of ECM in
316 craniofacial skeletal structures (see Table 1).

317 The treatments with the two different doses of E₂ (2 and 5 μM) resulted in differential expression
318 of many of the candidates during the zebrafish larval head development (Fig. 2-5). Consistent
319 with a previous study in zebrafish using higher E₂ concentration (10 μM) (Fushimi et al., 2009),
320 we also found significant down-regulation of *ptch1* and *ptch2* in the heads of fish receiving
321 lower dose estrogen treatments during larval development. These two genes are the receptors
322 (and the upstream mediators) of the hedgehog (Hh) signaling pathway which plays a crucial role
323 in developmental patterning and skeletal morphogenesis (Eberhart et al., 2006; Swartz et al.,
324 2012). Interestingly, slight changes in expression of *ptch1* were shown to be associated with
325 subtle craniofacial skeletal divergence (shorter snout and flatter face) in cichlid fish (Roberts et
326 al., 2011; Hu & Albertson, 2014). In addition, we found a strong positive expression correlation
327 between *ptch1* and *ptch2* (Fig. S2), indicating potential estrogen mediated co-regulation of the
328 two Hh receptors. In the above mentioned study of high dose E₂ treatment, the upstream
329 activators of the Hh pathway, sonic hedgehog genes, *shha* and *shhb* (*twhh*), did not show
330 significant changes in expression (Fushimi et al., 2009). However, this could be due to technical
331 limitations such as the use of a semi-quantitative method that is unable to reveal small
332 differences in gene expression (Fushimi et al., 2009). In this study we found small and yet
333 significant down-regulation of *shha*, but not *shhb*, in E₂ treated groups, as well as positive co-
334 expression of only *shha* with the two Hh receptors. An important role of the *shh* in craniofacial
335 skeletogenesis through activation of Hh signalling has been described (Hu & Helms, 1999), but it
336 is not clear whether estrogen directly regulates its expression during development.

337 Extracellular matrix remodelling is a critical process in the developmental program of bone and
338 cartilage differentiation and morphogenesis (Werb & Chin, 1998). The spatio-temporal
339 expression of genes encoding matrix metalloproteinases and their tissue inhibitors plays a pivotal

340 role in orchestrating the ECM remodelling process (Werb & Chin, 1998; Page-McCaw, Ewald &
341 Werb, 2007). Moreover, many ECM remodelling genes are downstream targets of pathways
342 mediated by nuclear receptors, including estrogen signalling (Cox & Helvering, 2006; Heldring
343 et al., 2007; Ganesan et al., 2008). The selected ECM remodelling factors (*mmp2/9/13*, *timp2a*
344 and *sparc*) were all reported to be regulated by estrogen signalling (Lehane et al., 1999; Tüshaus
345 et al., 2003; Marin-Castaño et al., 2003; Lu et al., 2006; Nilsson, Garvin & Dabrosin, 2007; Lam
346 et al., 2009; Wang & Ma, 2012) and play role in craniofacial skeletal morphogenesis (Dew et al.,
347 2000; Renn et al., 2006; Hillegass et al., 2007a,b; Mosig et al., 2007; Rotllant et al., 2008; Letra
348 et al., 2012; Ahi et al., 2014). Our results revealed slight but significant effects of the estrogen
349 treatments on expression of the selected ECM remodelling genes during larval head development
350 (Fig. 4). It is interesting to note that previous investigations have shown association between
351 differential expression of these genes and craniofacial phenotypes with flatter face and shorter
352 snout (Hillegass et al., 2007a,b; Ahi et al., 2014). The mechanism by which estrogen regulates
353 the expression of ECM remodelling genes is not well understood. The estrogen dependent
354 regulation might be exerted through interaction between estrogen-receptors and transcription
355 factors that regulate ECM remodelling genes such as members of Ap-1 complex and ETS factors
356 (Lu et al., 2006; Ahi et al., 2014; Cao et al., 2015). The binding motifs for Ap-1 and ETS
357 transcription factors are present in the promoters of many ECM remodelling genes across
358 vertebrate species (Ahi et al., 2014). Additionally, we found the expression of *erf*, an ETS
359 repressor and estrogen target (Sgouras et al., 1995), to be down-regulated in both E₂ treated
360 groups at three larval stages. Remarkably, a recent study showed that small reduction in
361 expression of *erf* causes complex craniosynostosis with shortened snout in both human and mice
362 (Frasor et al., 2003; Twigg et al., 2013). The same study also demonstrated regulatory elements
363 containing Ap-1, ETS and Runx motifs as preferential *erf* binding sites (Twigg et al., 2013).
364 Taken together, the results of the present and previous studies suggest potential estrogen
365 mediated regulation of ECM remodelling genes possibly through interaction with other
366 transcription factors.

367 The E₂ treatments caused small and variable repressive effects on expression of other selected
368 target genes (Fig. 2-5). The genes, *bmp2a* and *rankl*, are well characterized skeletogenic markers
369 (Nie, Luukko & Kettunen, 2006; Hu, Colnot & Marcucio, 2008; Lézot et al., 2015) and their
370 regulation by estrogen signalling has been reported in other vertebrate species (Bord et al., 2003;

371 Zhou et al., 2003). We also found components of retinoic acid and Wnt/ β -catenin signalling
372 pathways, *rarab* and *sfrp1a*, to be transcriptionally affected by E₂ treatment indicating the
373 potential crosstalk of these pathways with estrogen signalling during larval head development
374 (Lohnes et al., 1994; O’Lone et al., 2004; Trevant et al., 2008; Yokota et al., 2008).

375 In addition to skeletogenic genes, we were interested in investigating the effects of different
376 doses of E₂ on the expression of estrogen receptors. Therefore, we assessed the expression of two
377 estrogen receptors, *esrra* and *esr1*, that could mediate estrogen signal during the development of
378 skeletal tissues (Bonnelye & Aubin, 2005; Bonnelye et al., 2007; Auld et al., 2012). While the E₂
379 treatments had small and variable repressive effects on expression of *esrra*, the increased
380 expression of *esr1* was observed in both E₂ treated groups. Strikingly, the lower E₂ concentration
381 (2 μ M) resulted in higher induction of *esr1* expression. This suggests that the distinct effects of
382 lower doses of estrogen on craniofacial skeletogenesis, described by Cohen et al., 2014, might be
383 mediated by *esr1*, however further functional studies are required to demonstrate such a
384 mechanism. To identify genes sharing regulatory mechanisms in response to slight increases in
385 estrogen levels, we further explored the expression of 11 genes constructing a co-expression
386 network with *esr1* (Table S2 and Fig. 6). These candidate genes were selected by using a
387 vertebrate co-expression database (Obayashi & Kinoshita, 2011) which we have successfully
388 used for identification of gene networks associated with subtle craniofacial morphological
389 divergence in another teleost (Ahi et al., 2014, 2015). Our results indicate higher transcriptional
390 induction of six genes, i.e. *cpn1*, *dnajc3*, *lman1*, *rrbp1a*, *ssr1* and *tram1* in the lower (2 μ M), than
391 the moderate (5 μ M) treatment groups, during craniofacial development. The genes also showed
392 positive expression correlation with *esr1* suggesting a common regulatory mechanism mediated
393 by estrogen during head development. Among them only *dnajc3*, a gene encoding protein kinase
394 inhibitor P58 (P58^{IPK}), has been shown to be involved in skeletogenesis through regulation of a
395 cytokine-dependent cartilage degradation (Gilbert et al., 2014). Although all of the six genes
396 have recorded developmental expression patterns in zebrafish craniofacial elements based on
397 data in the ZFIN zebrafish database (Thisse et al., 2001, 2004), their roles in craniofacial
398 morphogenesis have yet to be investigated.

399

400 **Conclusions**

401 In this study we quantitatively assessed the effects of two doses of estrogen (2 μ M and 5 μ M) on
402 gene expression during zebrafish larval head development. We performed a highly sensitive and
403 specific qPCR analysis and carefully validated reference genes. We assessed the expression of a
404 selected set of genes involved in craniofacial skeletal development as well as genes coexpressed
405 with *esr1*, an estrogen receptor showing stronger inductive response to 2 μ M than 5 μ M estrogen
406 concentration. The results implicate estrogen in the expressional regulation of genes belonging to
407 distinct signalling pathways such as hedgehog and retinoic acid pathways, as well as genes
408 involved in ECM remodelling during craniofacial development. Furthermore, estrogen mediated
409 transcriptional changes in a few tested major skeletogenic factors (e.g. *bmp2a* and *rankl*), and a
410 transcription factor, *erf*, with a demonstrated role in the formation of a shortened snout
411 phenotype in human and mouse. Finally, we identified a gene network showing positive
412 expression correlation with *esr1* and higher induction in response to treatment with 2 μ M than
413 with 5 μ M estrogen. This could suggest a co-regulated module of genes mediating the effects of
414 low doses of estrogen during craniofacial development which required to be further investigated
415 at functional level.

416

417

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422

423 **Authors' contributions**

424 EPA designed and performed gene expression experiments, conducted data analysis and drafted
425 the manuscript. CSL and BSW performed literature studies, experimental design for larval
426 treatments, tissues dissections and sampling. BSW set up and conducted zebrafish laboratory
427 work. ZOJ supervised data analysis and interpretation. EPA, CSL and ZOJ designed the study
428 and acquired funding. All authors co-wrote, read and approved the final manuscript.

429

430 **Competing interests**

431 The authors have declared that no competing interests exist.

432

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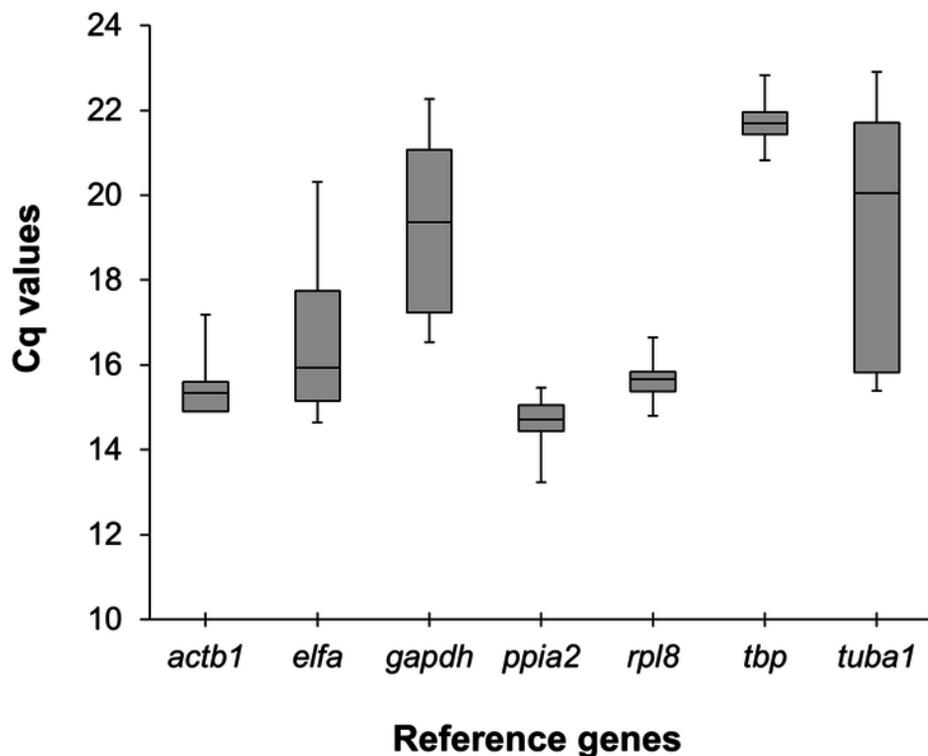
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1

Expression analysis of candidate reference genes in developing heads of zebrafish larvae across control and E₂ treated groups.

(A) Expression profiles of candidate reference genes in raw Cq values for all samples (3 treatments for each of 5 larval stages and with 3 biological replicates). The middle line denotes the median and boxes indicate the 25/75 percentiles. **(B)** Expression differences of candidate reference genes in the head of zebrafish during the larval development and three E₂ treatment groups. Fold changes in expression calculated from the qPCR data, were subjected to ANOVA and Tukey's HSD analysis to test the expression differences amongst three treatment groups (control, 2μM and 5μM) and across five larval stages (3 to 7dpf). White boxes represent low expression, while black boxes represent high expression. Two or more steps of shade differences in the boxes represent significantly different expression between the samples (alpha = 0.05). NS = not significant.

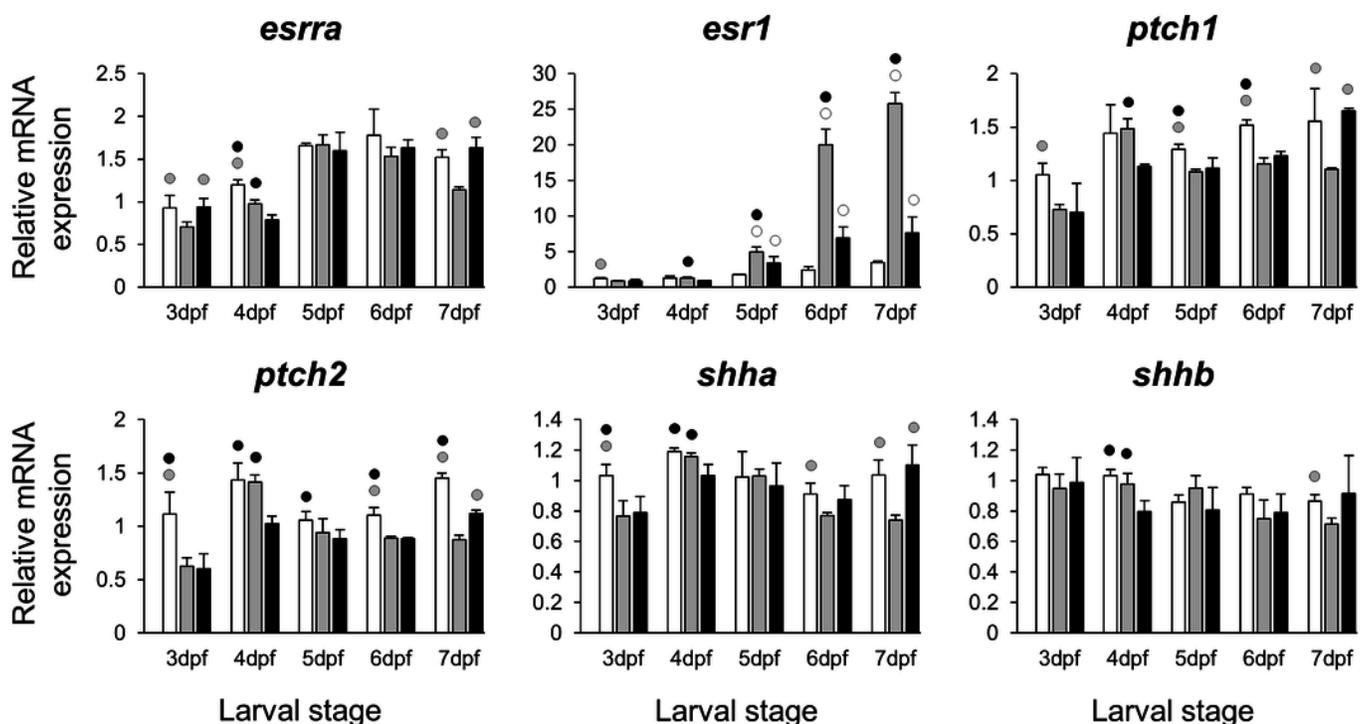
A**B**

Gene	ANOVA treatments	HSD treatments			ANOVA larval stage	HSD larval stage				
		Ctl	2 μ M	5 μ M		3dpf	4dpf	5dpf	6dpf	7dpf
<i>actb1</i>	$P = 0.039$	Ctl	2 μ M	5 μ M	$P = 9.77e-08$	3dpf	4dpf	5dpf	6dpf	7dpf
<i>elfa</i>	$P = 0.758$	NS			$P = 3.44e-06$	3dpf	4dpf	5dpf	6dpf	7dpf
<i>gapdh</i>	$P = 0.437$	NS			$P = 0.002$	3dpf	4dpf	5dpf	6dpf	7dpf
<i>ppia2</i>	$P = 0.64$	NS			$P = 0.001$	3dpf	4dpf	5dpf	6dpf	7dpf
<i>rpl8</i>	$P = 0.084$	NS			$P = 2.04e-05$	3dpf	4dpf	5dpf	6dpf	7dpf
<i>tbp</i>	$P = 0.532$	NS			$P = 0.752$	NS				
<i>tuba1</i>	$P = 0.502$	NS			$P = 1.4e-13$	3dpf	4dpf	5dpf	6dpf	7dpf

2

Expression differences of two estrogen receptors and components of hedgehog signaling pathway in developing heads of zebrafish larvae across control and E₂ treated groups.

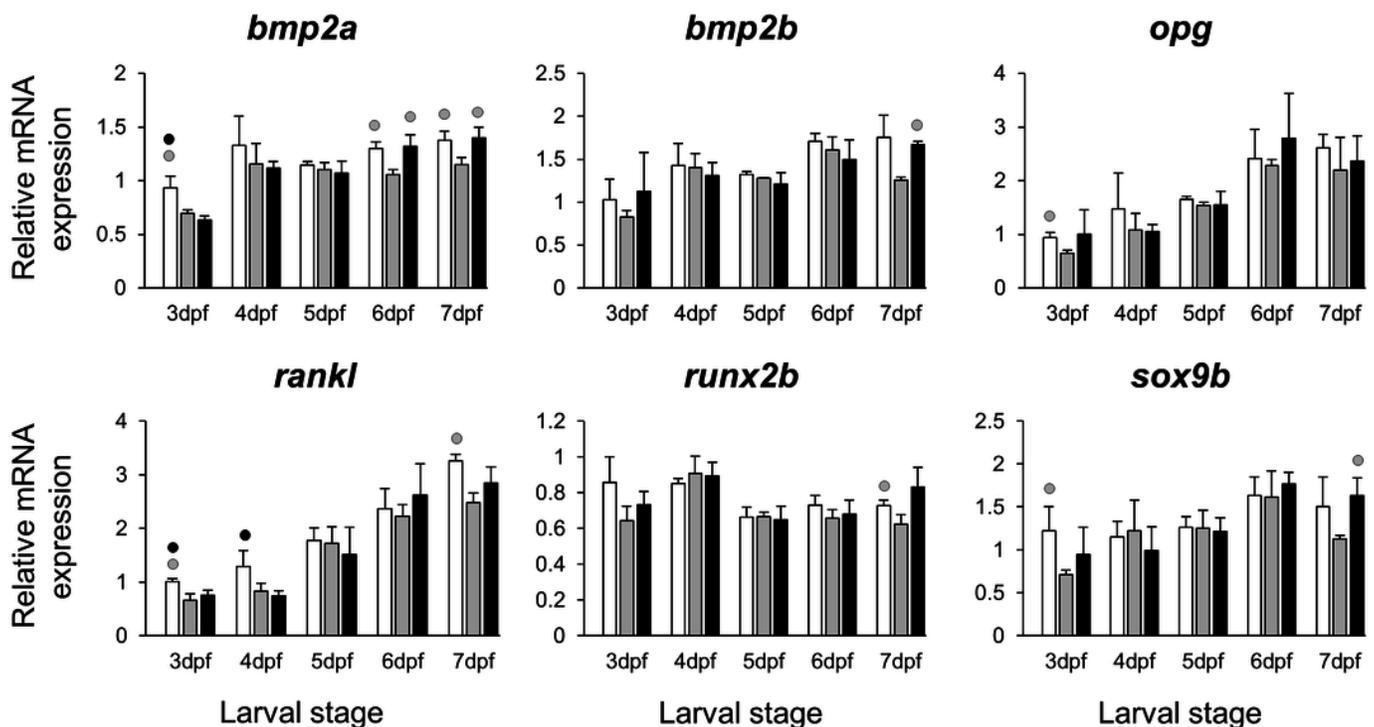
Expression of *esrra*, *esr1*, *ptch1*, *ptch2*, *shha* and *shhb* was examined with qPCR and normalised using three highest ranked reference genes (*ppia2*, *rpl8* and *tbp*). For analysis of relative expression levels for each target gene a replicate of the control group at 3dpf was set to one. The white, grey, and black bars in each graph represent expression levels for control, 2 μ M E₂ treated and 5 μ M E₂ treated groups respectively. Statistical differences of each treatment group versus the others are shown in white, grey, and black circles representing higher expressed than control, 2 μ M E₂ treated and 5 μ M E₂ treated groups respectively (P < 0.05). Error bars represent standard deviation calculated from three biological replicates. Each biological replicate is from a homogenate of 30 heads.



3

Expression differences of six potential skeletogenic targets of estrogen pathway in developing heads of zebrafish larvae across control and E₂ treated groups.

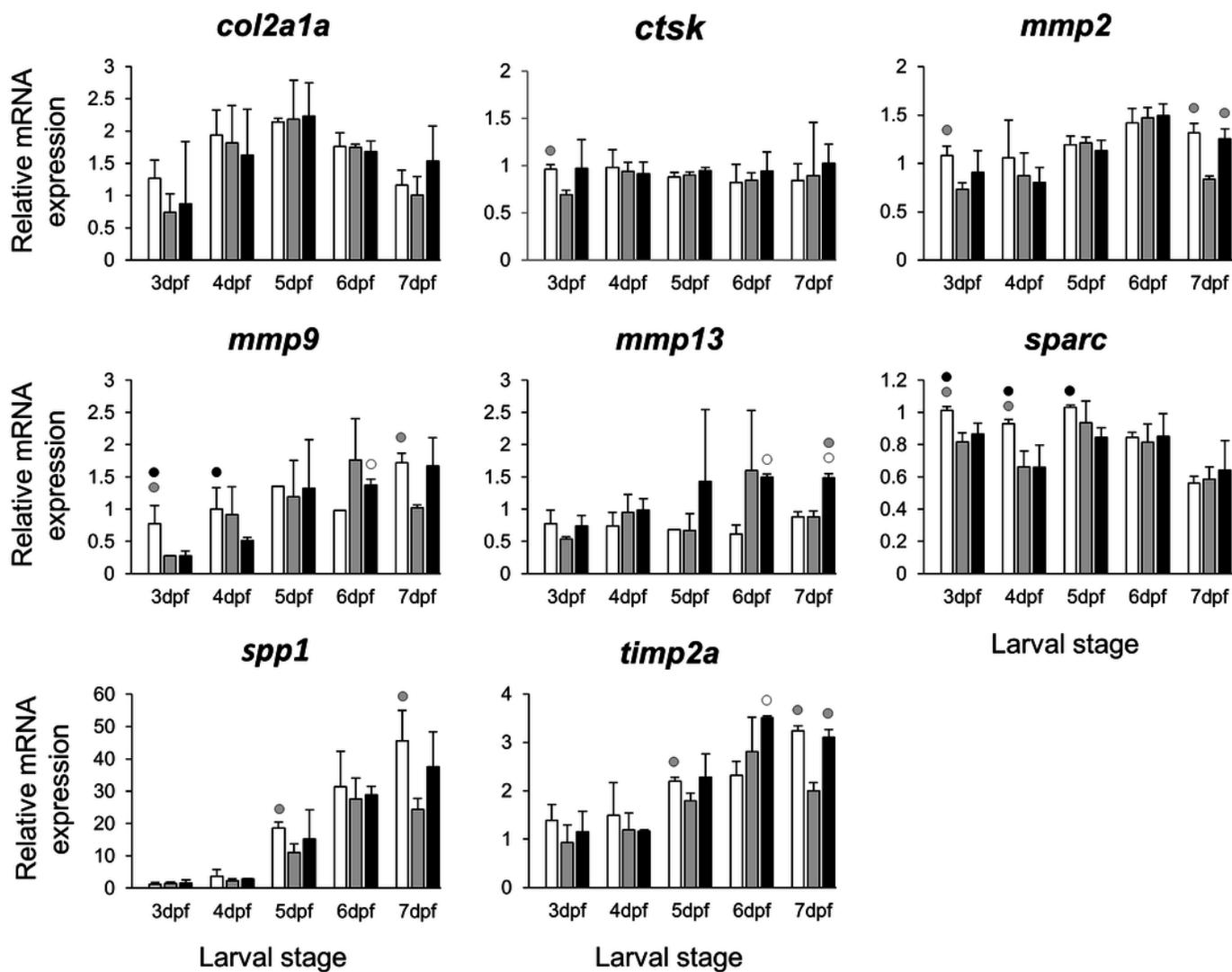
Expression of *bmp2a*, *bmp2b*, *opg*, *rankl*, *runx2b* and *sox9b* was examined with qPCR and normalised using three highest ranked reference genes (*ppia2*, *rpl8* and *tbp*). For analysis of relative expression levels for each target gene a replicate of the control group at 3dpf was set to one. The white, grey, and black bars in each graph represent expression levels for control, 2 μ M E₂ treated and 5 μ M E₂ treated groups respectively. Statistical differences of each treatment group versus the others are shown in white, grey, and black circles representing higher expressed than control, 2 μ M E₂ treated and 5 μ M E₂ treated groups respectively (P < 0.05). Error bars represent standard deviation calculated from three biological replicates. Each biological replicate was made from a homogenate of 30 heads.



4

Expression differences of eight potential targets of estrogen pathway involved in skeletal ECM formation examined during zebrafish larval head development across control and E₂ treated groups.

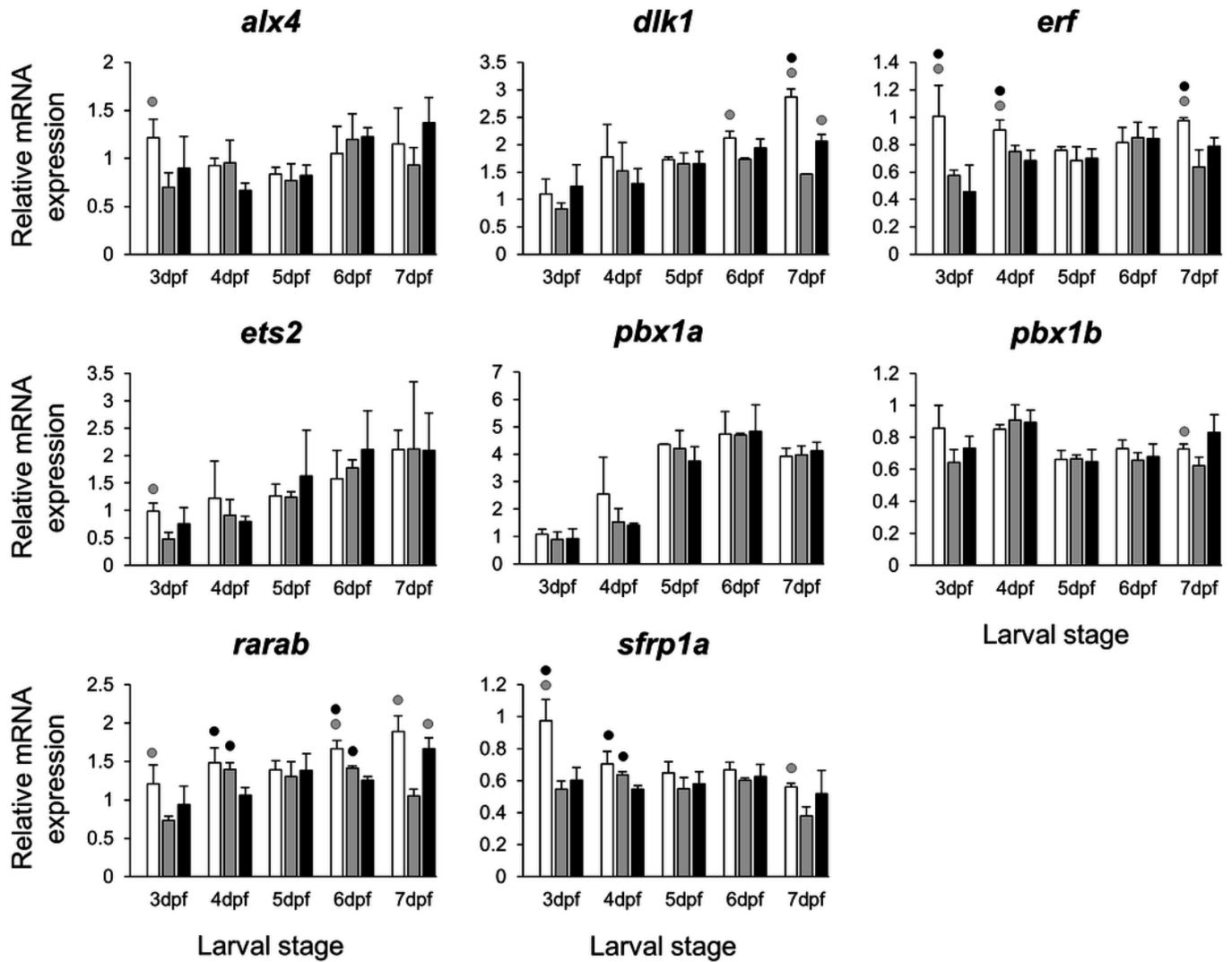
Expression of *col2a1a*, *ctsk*, *mmp2*, *mmp9*, *mmp13*, *sparc*, *spp1* and *timp2* was examined with qPCR and normalised using three highest ranked reference genes (*ppia2*, *rpl8* and *tbp*). For analysis of relative expression levels for each target gene a replicate of the control group at 3dpf was set to one. The white, grey, and black bars in each graph represent expression levels for control, 2µM E₂ treated and 5µM E₂ treated groups respectively. Statistical differences of each treatment group versus the others are shown in white, grey, and black circles representing higher expressed than control, 2µM E₂ treated and 5µM E₂ treated groups respectively (P < 0.05). Error bars represent standard deviation calculated from three biological replicates. Each biological replicate was made from a homogenate of 30 heads.



5

Expression differences of eight other potential targets of estrogen pathway involved in jaw skeletal elongation examined during zebrafish larval head development across control and E₂ treated groups.

Expression of *alx4*, *dlx1*, *erf*, *ets2*, *pbx1a*, *pbx1b*, *rarab* and *sfrp1a* was examined with qPCR and normalised using three best ranked reference genes (*ppia2*, *rpl8* and *tbp*). For analysis of relative expression levels for each target gene a replicate of the control group at 3dpf was set to one. The white, grey, and black bars in each graph represent expression levels for control, 2µM E₂ treated and 5µM E₂ treated groups respectively. Statistical differences of each treatment group versus the others are shown in white, grey, and black circles representing higher expressed than control, 2µM E₂ treated and 5µM E₂ treated groups respectively (P < 0.05). Error bars represent standard deviation calculated from three biological replicates. Each biological replicate was made from a homogenate of 30 heads.



6

Expression differences of *esr1* coexpressed genes in developing heads of zebrafish larvae across control and E₂ treated groups.

Expression levels of eleven candidate genes coexpressed with *esr1*, based on data from COXPRESdb in zebrafish, were examined with qPCR and normalised using three best ranked reference genes (*ppia2*, *rpl8* and *tbp*). For analysis of relative expression levels for each target gene a replicate of the control group at 3dpf was set to one. The white, grey, and black bars in each graph represent expression levels for control, 2µM E₂ treated and 5µM E₂ treated groups respectively. Statistical differences of each treatment group versus the others are shown in white, grey, and black circles representing higher expressed than in control, 2µM E₂ treated and 5µM E₂ treated groups respectively (P < 0.05). Error bars represent standard deviation calculated from three biological replicates. Each biological replicate is based on a homogenate of 30 heads.

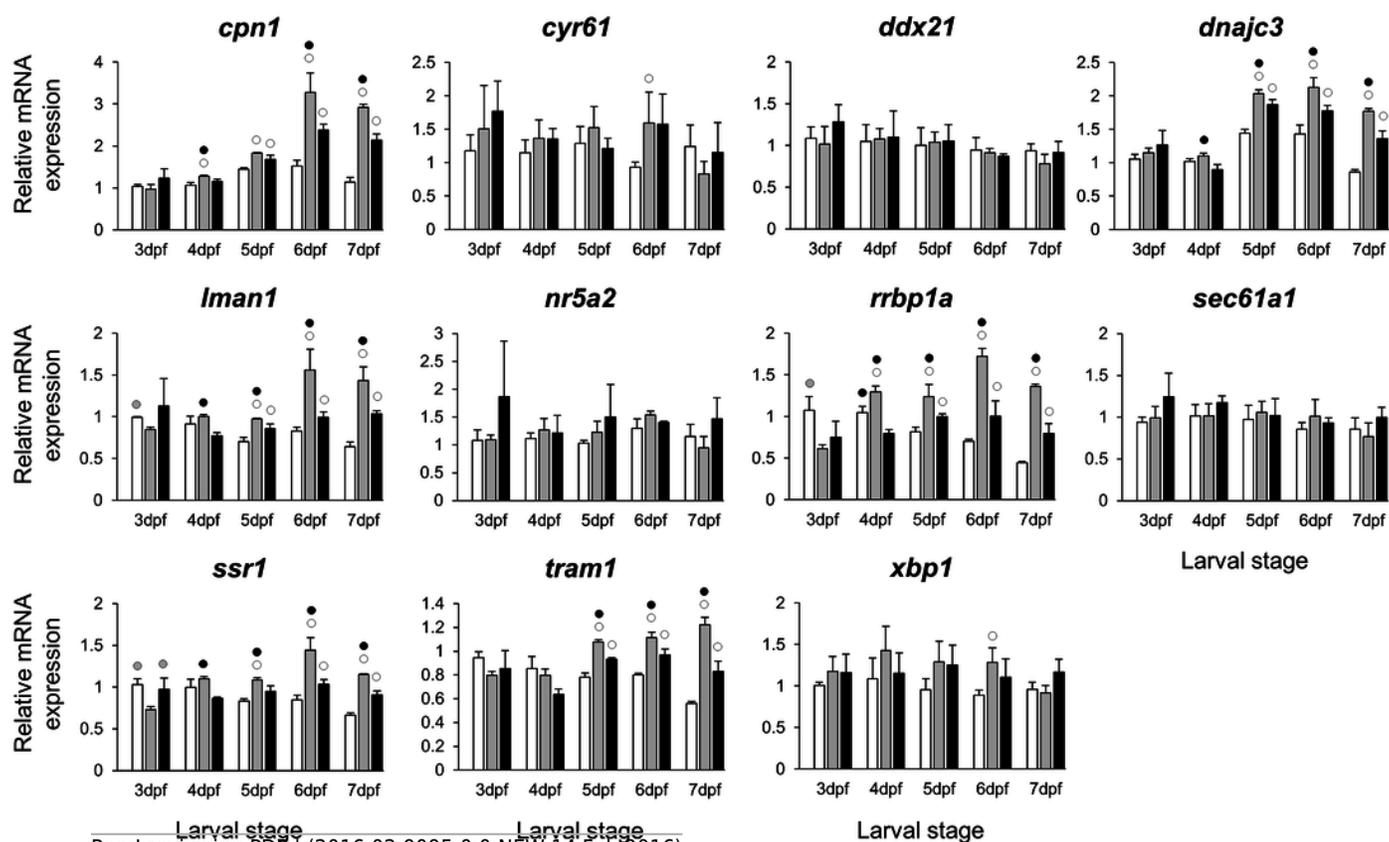


Table 1 (on next page)

Selected putative estrogen-regulated candidate genes, and available literature indicating their role in craniofacial development/skeletal formation in zebrafish or other vertebrates.

Gene Symbol	Related Function	Viscerocranial expression during zebrafish development	Potential Estrogen Responsive *	Craniofacial skeletogenesis		References
				Shortened snout **	Other effects	
<i>alx4</i>	Patterning and development of craniofacial skeleton	+	+	+	+	(Qu et al., 1999; Joshi, Chang & Hamel, 2006; Lours-Calet et al., 2014)
<i>bmp2</i> (a/b)	Induction of bone and cartilage formation	+	+	?	+	(Thisse et al., 2001, 2004; Zhou et al., 2003; Nie, Luukko & Kettunen, 2006; Hu, Colnot & Marcucio, 2008; Yamamoto, Saatcioglu & Matsuda, 2013)
<i>col2a1a</i>	Extracellular matrix formation in cartilaginous tissues	+	+	+	+	(Maddox et al.; Eames et al., 2010; Maneix et al., 2014)
<i>ctsk</i>	Bone remodelling and resorption	+	+	+	+	(Thisse et al., 2004; Troen, 2006; Petrey et al., 2012; Ahi et al., 2014)
<i>dlk1</i>	Differentiation of skeletal cells	?	+	+	+	(Abdallah et al., 2011)
<i>erf</i>	Regulation of cellular senescence	?	+	+	+	(Frasor et al., 2003; Twigg et al., 2013)
<i>esrra</i>	Regulation of estrogen mediated pathway	+	+	?	+	(Bonnelye & Aubin, 2005; Bonnelye et al., 2007; Auld et al., 2012)
<i>esr1</i>	A ligand-activated receptor for estrogen	?	+	?	+	(O'Lone et al., 2004; Syed et al., 2005)
<i>ets2</i>	Regulation of developmental genes and apoptosis	?	+	+	+	(Sumarsono et al., 1996; Deblois et al., 2009; Ahi et al., 2014)
<i>mmp</i> (2/9/13)	Extracellular matrix formation and signal transduction	+	+	+	+	(Breckon et al., 1999; Tüshaus et al., 2003; Marin-Castaño et al., 2003; Lu et al., 2006; Hillegass et al., 2007a,b; Mosig et al., 2007; Nilsson, Garvin & Dabrosin, 2007)
<i>opg</i>	Negative regulation of bone resorption	?	+	?	+	(Bord et al., 2003)(Whyte et al., 2002)
<i>pbx1</i> (a/b)	co-ordination of chondrocyte proliferation and differentiation	+	+	+	+	(Selleri et al., 2001; Thisse, C., and Thisse, 2005; Magnani et al., 2011)
<i>ptch</i> (1/2)	Receptors for hedgehog signalling pathway	+	+	+	+	(Fushimi et al., 2009; Roberts et al., 2011)
<i>rankl</i>	Osteoclast differentiation and activation	?	+	?	+	(Bord et al., 2003; Lézot et al., 2015)
<i>rarb</i>	A receptor for retinoic acid signalling pathway	+	+	+	+	(Lohnes et al., 1994; O'Lone et al., 2004; Linville et al., 2009)
<i>runx2b</i>	Osteoblast differentiation and skeletal morphogenesis	+	+	+	+	(Sears et al.; McCarthy et al., 2003; Flores et al., 2006)
<i>sfrp1a</i>	A soluble modulator of Wnt signalling pathway	?	+	+	+	(Sato et al., 2006; Trevant et al., 2008; Yokota et al., 2008; Fukuhara et al., 2013; Ahi et al., 2014)

<i>shha</i>	Activators of hedgehog signalling pathway	+	?	?	+	(Hu & Helms, 1999; Swartz et al., 2012)
<i>sox9b</i>	Chondrocyte differentiation	+	+	?	+	(Yan et al., 2005; Bonnelye et al., 2007; Lee & Saint-Jeannet, 2011)
<i>sparc</i>	Extracellular matrix synthesis and regulation of cell growth	+	+	+	+	(Lehane et al., 1999; Renn et al., 2006; Rotllant et al., 2008)
<i>spp1</i>	Attachment of osteoclasts to ECM in bone	+	+	+	+	(Craig & Denhardt, 1991; Vanacker et al., 1998; Venkatesh et al., 2014)
<i>timp2a</i>	Inhibition of mmmps and regulation of tissue homeostasis	?	+	+	?	(Dew et al., 2000; Lam et al., 2009; Letra et al., 2012; Wang & Ma, 2012; Ahi et al., 2014)

* The estrogen responsiveness indicates either transcriptional regulation or transactivation and the related information are mainly obtained from different model vertebrates, such as human and mouse, than teleost fishes.

** The shortened snout indicates the skeletal effects resulted from decrease in the length or changes in morphology of viscerocranial skeletal elements in different vertebrate species. This could bear a resemblance to estrogen mediated shorter snout and flatter face phenotype in zebrafish.

1

2

Table 2 (on next page)

Ranking and statistical analyses of candidate reference genes using BestKeeper, geNorm and NormFinder.

1

BestKeeper				geNorm		NormFinder	
Ranking	r	Ranking	SD	Ranking	M	Ranking	SV
<i>rpl8</i>	0.908	<i>tbp</i>	0.294	<i>ppia2</i>	0.111	<i>rpl8</i>	0.137
<i>tbp</i>	0.863	<i>rpl8</i>	0.343	<i>rpl8</i>	0.125	<i>ppia2</i>	0.154
<i>ppia2</i>	0.862	<i>ppia2</i>	0.350	<i>tbp</i>	0.133	<i>tbp</i>	0.157
<i>actb1</i>	0.687	<i>actb1</i>	0.396	<i>actb1</i>	0.26	<i>actb1</i>	0.287
<i>efl1a</i>	0.331	<i>efl1a</i>	1.358	<i>efl1a</i>	0.739	<i>efl1a</i>	1.128
<i>tubal</i>	0.201	<i>gapdh</i>	1.690	<i>gapdh</i>	1.084	<i>gapdh</i>	1.382
<i>gapdh</i>	0.148	<i>tubal</i>	2.773	<i>tubal</i>	1.482	<i>tubal</i>	2.088

2

- 3 Abbreviations: SD = Standard deviation, r = Pearson product-moment correlation coefficient, SV
 4 = stability value, M = M value of stability.