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ABSTRACT

Natural and synthetic estrogens are contaminants present in aquatic ecosystems. They can have significant consequences on the estrogen-sensitive functions of organisms, including skeletal development and growth of vertebrate larvae. Synthetic polyphenols represent a group of environmental xenoestrogens capable of binding the receptors for the natural hormone estradiol-17β (E2). To better understand how (xeno-)estrogens can affect the skeleton in fish species with high ecological and commercial interest, 16 days post-hatch larvae of the seabass were experimentally exposed for 7 days to E2 and Bisphenol A (BPA), both used at the regulatory concentration of surface water quality (E2: 0.4 ng.L^{-1}, BPA: 1.6 μg.L^{-1}) or at a concentration 100 times higher. Skeletal mineralization levels were evaluated using Alizarin red staining, and expression of several genes playing key roles in growth, skeletogenesis and estrogen signaling pathways was assessed by qPCR. Our results show that E2 exerts an overall negative effect on skeletal mineralization at the environmental concentration of 0.4 ng.L^{-1}, correlated with an increase in the expression of genes associated only with osteoblast bone cells. Both BPA exposures inhibited mineralization with less severe effects and modified bone homeostasis by regulating the expression of gene encoding osteoblasts and osteoclasts markers. Our results demonstrate that environmental E2 exposure inhibits larval growth and has an additional inhibitory effect on skeleton mineralization while both BPA exposures have marginal inhibitory effect on skeletal mineralization. All exposures have significant effects on transcriptional levels of genes involved in the skeletal development of seabass larvae.

1. Introduction

The adverse outcomes of exposure to estrogenic compounds have mainly been evaluated in relation to reproductive system (Alavi et al., 2021; Jobling and Tyler, 2003) but relatively little attention has been given to other estrogen-sensitive physiological systems (Wojnarowski et al., 2022), including organogenesis and development (Cohen et al., 2014; Coumailleau et al., 2015; Gorelick et al., 2014), immunity (Canesi and Fabbri, 2015; Prossnitz and Barton, 2011), osmoregulation (Lerner et al., 2007; McCormick et al., 2005), skeleton development and homeostasis (Farcy et al., 2022; Zuo and Wan, 2017). Fish larval stages might be particularly vulnerable to waterborne estrogenic compound exposure that may interfere with normal estrogen-mediated developmental events (Chin et al., 2018), in particular regarding growth and skeletal development. Estrogen signaling is mostly mediated by specific intracellular receptors, the estrogen receptors (ERs) that are located in the cytoplasm, nucleus or membrane. In the cytoplasm and nucleus, some estrogen receptors act as estrogen-activated transcription factors (ERa and ERb) and modify gene expression. Estrogen bound to membrane ERs such as the G protein-coupled estrogen receptor (GPER), transmit a direct cellular signal (Xu et al., 2019; Zapater et al., 2024). The activity of the nuclear ERs can be modulated by the estrogen-related receptors, ERRs, which constitute a subgroup of orphan receptors with high degree of similarity in their DNA binding domain but that do not bind estrogens (Huss et al., 2015). Overall, the activity of estrogens is a combination of direct (modulated by ERs) and indirect genomic effect on target cells (via other transcription factors) or rapid non-genomic effects initiated by binding to membrane receptors (involving

Abbreviations: BPA, bisphenol A; E2, Estradiol-17β; ER, estrogen receptor; ERR, estrogen-related receptor; dph, days post hatching.

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activation of protein kinase cascades or alterations in the levels of secondary messengers).

Among the natural estrogens, estradiol-17β (E2) is the compound with the highest potency towards ERs (Asnake et al., 2019). Synthetic polyphenols represent a group of ubiquitous environmental xenoestrogens that are related in structure, although somewhat different from the natural hormone E2. Among them, Bisphenol A (BPA), a well-known endocrine disrupting chemical, is a constituent of plastic polycarbonate and epoxy resins, which has been widely used to produce plastic containers, paints, adhesives and aquaculture tanks. With the huge demand of these materials, thousands of tons are released into the environment annually (Careghini et al., 2015). BPA, through its binding to nuclear ERs, membrane ERs and ERRs has estrogenic activity even at concentrations below 1 $ng.L^{-1}$ making it potentially deleterious to living organisms (Faheem and Bhandari, 2021; Kang et al., 2007; Rykowska and Wasiak, 2006). BPA also mediates non-estrogenic effects by interacting with the androgen receptor (AR), corticosteroid receptor (GR and MR), thyroid receptor (TR), peroxisome proliferator-activated receptor (PPAR) and pregnane X receptor (PXR) (Batista-Silva et al., 2022; Liu et al., 2019; Mathieu-Denoncourt et al., 2015; Yuan et al., 2023). Data are available about detrimental effects of elevated concentrations BPA or E2 on development in model species such as zebrafish (Gibert et al., 2011; Lam et al., 2011; Saili et al., 2012; Wang et al., 2013) or rainbow trout (Orrego et al., 2011). These studies reported several embryotoxic effects such as lethal and sublethal abnormalities, delay in time to hatch or decreased hatchability. In contrast, knowledge of the impact of E2 and BPA on larval skeletal development is scarce.

In mammals and other bony vertebrates, the natural estrogen is involved in skeletal development and mineral homeostasis by regulating genes involved in the differentiation and secretory activity of different cell types: chondrocytes, osteoblasts, osteocytes and osteoclasts (Prein and Beier, 2019; Zuo and Wan, 2017). Chondrocytes are key skeletal cells during the embryonic and early post-embryonic development when the skeleton first occurs as cartilaginous units which are then used as a frame for perichondral and endochondral bone growth. Chondrocytes and their matrix are gradually replaced by osteoblasts that synthesize bone. During bone remodeling, osteoclasts resorb the old bone and osteoblasts synthesize new bone. The balance between bone formation and bone resorption is crucial for preserving bone integrity and maintaining mineral homeostasis (Prein and Beier, 2019). In mammals, membrane and cytoplasmic ERs, as well as ERRs, are expressed in all bone cell types. E2 protects the skeleton by promoting differentiation and increasing the lifespan of osteoblasts, inducing bone mineralization, and decreasing the formation, activity, and lifespan of osteoclasts (Prein and Beier, 2019; Zuo and Wan, 2017). Thus, estrogens contribute to the development and maintenance of bone.

The GH/IGF pathway is key mediator of growth in teleosts by regulating body and bone formation. The growth hormone (GH) signaling stimulates Insulin-like growth factors (IGFs) production, that are used as growth markers in farmed fish (Aluru et al., 2010; Björnsson et al., 2002; Chandhini et al., 2021; Hanson et al., 2014; Pérez-Sánchez et al., 2018; Reinecke, 2010; Takagi et al., 1992; Triantaphyllopoulos et al., 2020; Wargelius et al., 2005; Wood et al., 2005). All bone and cartilage cells express GH and IGF1 receptors. GH induces resting chondrocytes to start differentiating and stimulates local IGF1 production, leading to clonal expansion of proliferative chondrocytes. IGF1 also stimulates osteoblastogenesis, promoting collagen secretion and bone matrix mineralization (Frantz and Rabkin, 1965; Juul, 2001; Thompson et al., 1972; Yakar et al., 2018; Yakar and Isaksson, 2016). Estrogen and xenoestrogens exposure were shown to disturb growth and skeletal development during fish larval development (Breves et al., 2018; Celino-Brady et al., 2019; Farcy et al., 2022; Wojnarowski et al., 2022). Previous works in rainbow trout suggest that (xeno)estrogens modulate the GH-IGF system at elevated concentrations, above environmentally realistic concentrations (Hanson et al., 2014). E2 regulates the expression of GH receptor expression via nuclear and membrane estrogen receptors (ERs and GPERs) or by non-genomic effects (JAK/STAT pathway) (Leung et al., 2004). Experimental BPA exposure resulted in growth delay or suppression, and modified expression levels for genes of the GH-IGF axis in zebrafish and rainbow trout (Aluru et al., 2010; Dang et al., 2018).

As a consequence, a better understanding of the hormonal influences on growth and skeletogenesis of larval fish is relevant not only to follow the impact of (xeno)estrogen pollution on fish species in their natural environment, but also to help solve one of the most important bottlenecks in reared fish larvae: growth defects and skeletal malformations with severe economic impact on aquaculture. The marine teleost European seabass *Dicentrarchus labrax* was selected for this project owing to its high ecological and commercial value and to the fact that its reproduction and breeding are well mastered in aquaculture. The European seabass has the capacity of migrating from marine to freshwater (e.g., estuaries, lagoons, rivers) at early life stages (larvae, juveniles) to feed (Kelley, 1988). In these transition waters, the concentrations of chemicals are often higher than in open seawater. Indeed, estuarine shallow areas and coastal lagoons are known to receive and concentrate multiple inputs, either from the watershed or coastal areas, including estrogenic contaminants. Estrogenic contamination in aquatic environments is ubiquitous worldwide (Ciślak et al., 2023). Recent survey in European coastal and estuarine waters indicates that E2 and BPA concentrations can reach concentrations up to 1 $\mu g.L^{-1}$ (Ersoy Korkmaz et al., 2020; Zainuddin et al., 2023). Endocrine and pleiotropic effects of BPA have been observed at doses within the $\mu g.L^{-1}$ range (Flint et al., 2012), possibly reflecting the effective concentrations present in the environment.

In a previous work (Farcy et al., 2022), we showed that exposure to E2 and BPA strongly modifies the expression of skeleton-associated genes in early larval stages of *Dicentrarchus labrax* (between 12- and 16-days post-hatching, dph). Although transcriptional overexpression of these genes was significant in larvae exposed to the high concentration of E2 (40 $ng.L^{-1}$) and two concentrations of BPA (1.6 and 160 $\mu g.L^{-1}$), increased mineralization was detected only in larvae exposed to the high concentration of E2, suggesting a different regulatory response to BPA treatment in head skeleton development and remodeling. To further our understanding of the influence of (xeno)estrogens on growth and skeleton development, we here focused on a subsequent larval stage of *Dicentrarchus labrax*, when the vertebrae are mineralizing. Waterborne exposure was performed on 16-dph larvae with E2 and BPA, both used at an environmentally relevant regulatory concentration applicable to surface waters and a 100 times more elevated concentration. After 7 days of exposure, we measured the transcript levels of genes involved in several regulatory pathways: estrogen signaling, skeletogenesis, and GH-IGF1 axis, to identify the potential regulatory effects of E2 and BPA at this stage. The phenotypic effects were evaluated by recording the standard length of the larvae and by evaluating the level of bone mineralization using alizarin red staining.

2. Material and methods

2.1. Experimental design

2.1.1. Animals

Larvae of the European seabass *Dicentrarchus labrax* were obtained from natural reproduction of Mediterranean genitors at the aquaculture farm Les Poissons du Soleil (Balaruc les Bains, France). At the age of 11-dph, larvae were transferred at the Ifremer station of Palavas Les Flots. At this stage, the swim bladder is formed, saving the larvae from high mortality due to manipulation and transfer. Larvae were then raised in 80-L circular tanks under controlled condition (temperature of 17 °C, salinity of 25 ppt, dissolved oxygen above 7.5 $mg.L^{-1}$ and a light/dark photoperiod of 10/14 h) until they reached the appropriate stage for experimentation of 16 dph. According to the seabass development table produced by Darias et al. 2010 and our previous work (Farcy et al.,

2022), the 16-dph stage is before the start of axial skeleton mineralization. Twice a day, the larvae were fed with live AF INVE® artemia nauplii, at a concentration of approximately one nauplius per mL. All the following experiments were conducted in compliance with European Union directives and French law regulating animal experimentation (directive 86/609 and decree 87/848, respectively). The experimental design has been approved by French legal requirements concerning the welfare of laboratory animals (APAFIS permit no. 9045-201701068219555).

2.1.2. Experimental exposure

Stock solutions of 40 mg.L^{-1} E2 (estradiol-17β, ≥98 % purity, Sigma-Aldrich) and 40 g.L^{-1} BPA (bisphenol A, ≥99 % purity, Sigma-Aldrich) were prepared in ethanol (100 %, Fluka) and stored at 4 °C protected from light. For treatments, working solutions were prepared in 25 ppt filtered seawater. The final ethanol concentration was 0.0008 % in all treatment groups and in the solvent control.

To avoid any contamination of the water with BPA from plastic materials, the treatments were carried out in glass containers and were realized on 200 seabass larvae in a final volume of 4 L. After a 24 h period of acclimation in 25 ppt seawater, 16-dph larvae (0.73 ± 0.06 cm) were exposed for 7 days (until 23-dph) to nominal concentrations of 0.4 or 40 ng.L^{-1} for E2 and 1.6 or 160 µg.L^{-1} for BPA, and 0.00008 % ethanol for solvent control. The lowest concentrations for E2 and BPA refer to the regulatory levels applicable to surface water (Water Framework Directive): Environmental Quality Standards (EQS, Directive 2008/105/EC) or PNECaqua when EQS was not available: respectively PNECaqua = 1.6 µg.L^{-1} for BPA (EC, 2008) and provisional EQS = 0.4 ng.L^{-1} for E2 (EC, 2018). After this initial water contamination, E2, BPA and ethanol were delivered continuously at a flow rate of 2 mL/h by a peristaltic pump (8-canals, IPC-N, Ismatec), using a turnover rate of 12 h, through BPA-free pharmed tube tubing (diameter 0.51 mm, PharMed® BPT, Saint-Gobain Performance plastics). Every 48-h, half the water volume was renewed with freshly contaminated water, and the working solutions delivered by the peristaltic pump were freshened. During exposure, larvae were fed daily with live AF INVE® artemia nauplii. Temperature, salinity, dissolved oxygen and ammonia constants were monitored daily.

2.2. Biological analyses

2.2.1. Biometry

After 7 days of treatment, larvae were collected and sacrificed in 100 ppm benzocaine. For each condition, forty larvae were randomly sampled and individually photographed with a camera attached to a binocular magnifier in order to measure their standard length (named SL_{fresh}) and to assess possible developmental abnormalities. Larvae measurements were carried out using the Toupview software. After calibration according to the magnification used, the standard length was measured from the last caudal vertebra to the edge of the upper jaw. Skeleton developmental anomalies (jaw, scoliosis, fusion of vertebrae) and swimming bladder malformation (overinflation or underdevelopment) were assessed.

2.2.2. Alizarin staining and mineralization level determination

At the end of exposure, 31 to 63 larvae per condition were randomly sampled and fixed 48-h at 4 °C in 4 % paraformaldehyde prepared in phosphate buffer saline adjusted at 25 ppt with NaCl, then dehydrated in ethanol and stored at −20 °C. For Alizarin staining, the larvae were gradually rehydrated in ethanol/KOH 0.5× (*v*/v; in sequence 75:25 / 50:50 / 25:75) then in KOH 0.5×. They were subsequently depigmented for 30 min in a 0.3 % hydrogen peroxide solution in the dark, rinsed in KOH 0.5×, then incubated in a 0.01 % Alizarin red S/KOH 0.5× solution overnight to detect the mineralized elements. Stained larvae were transferred in graded series of KOH 0.5×/glycerol (v/v; in sequence 75:25 / 50:50 / 25:75) and then stored and imaged in 100 % glycerol. Staining was carried out simultaneously for all treatments to avoid technical variability of the staining. The standard length of all larvae was measured after staining, herein identified as SL_{aliz}, to test correlation between mineralization and size. Note that the SL_{aliz} measure was always about 5 % shorter than the SL_{fresh} measure due to the fixation and dehydration of the samples in glycerol.

Given the high level of mineralization in 23-dph larvae and the fact that mineralization level is strongly correlated to size (Farcy et al., 2022), a measurement of the mineralized area (alizarin-stained area in pixels) was performed for the whole body in a lateral view (included head, vertebrae and caudal fin) on 10 individuals of the size class 0.87–0.93 cm, which corresponds to the mode of the SL_{aliz} distribution. The pixel-based quantitation was performed with the GIMP software by trimming the bony parts of the whole animal (Supp data Fig. S1).

2.2.3. mRNA expression analysis

Sixteen to twenty individuals per condition and from the same size class (SL_{fresh} 0.85–1 cm) were used for gene expression analysis. All the procedures for RNA extraction, reverse transcription and quantitative real-time PCR were performed individually on whole larvae, as previously described (Farcy et al., 2022). Briefly, RNA extraction was performed using the Nucleospin® RNA kit (Macherey-Nagel, Germany), quantity and purity of extraction products were verified using a UV spectrophotometer and a Bioanalyzer 2100 (RIN levels were all above 8), respectively. Reverse transcription was performed using 1 µg of RNA using the qScriptTM cDNA SuperMix (Quanta Biosciences™) with the three successive incubation steps (5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C). Real time q-PCR conditions were as follows: 2 min denaturation at 95 °C followed by 35 cycles (95 °C for 30 s, 61 °C for 45 s and 72 °C for 1 min) and a final elongation step at 72 °C for 4 min. All measurements were run in triplicates. Relative mRNA levels were normalized to 18S rRNA levels and expressed as ΔΔCt (Ct, threshold cycle number) using solvent control as a reference for treatment effect comparison (see Farcy et al., 2022 for details).

Several classes of genes were selected as markers for cartilage (chondrocytes) and bone (osteoblasts, osteocytes and osteoclasts). These genes encode structural matrix proteins: *col1a2*, *col2a1a* and *col2a1b*; calcium-interacting matrix proteins: *sparc* and both *bgp1a* and *bgp1b* paralogs; the tissue nonspecific alkaline phosphatase *alp*; some signaling and transcription factors: *ihha* and *ihhb*, *sox9a* and *sox9b*, *runx2* and *sp7*; markers of osteoclast activity: *rankl*, *ctsk* and *acp5*. To study estrogen signaling pathways, we measured mRNA expression of *era*, *erb1* and *erb2*, *gpera* and *gperb*, and the five estrogen-related receptors *erra*, *errb1* and *errb2 errga* and *errgb*. The expression of the *era, gpera* and *errgb* genes was below the limit of detection, therefore these genes are not further discussed, though we cannot rule out that these receptors may play a role. We also investigated the expression of *gh* and *igf1* (Supp data Table S1).

2.3. Statistics

Statistical analyses were performed on GraphPad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA). Differences between groups (treatment versus control) were tested using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test (multiplicity adjusted p-value accounting for familywise error rate due to multiple pairwise comparisons) since data did not fit normality nor homoscedasticity assumptions. The strength and direction of the monotonic relationship between fish standard length and the number of mineralized vertebrae was tested using the nonparametric Spearman correlation test ($p < 0.05$). Nonparametric ANCOVA was performed to compare the effect of treatment on ossification levels and test differences between predicted non-linear regression curves using the r package fANCOVA (0.6–1). Three pairwise comparisons were made for each data set. Therefore, the significance threshold was adjusted to $p < 0.017$ according to Bonferroni correction.

3. Results

3.1. Larval skeleton mineralization in physiological conditions

The levels of mineralization were first investigated at 16 and 23 dph in control rearing conditions using Alizarin red labeling. At 16 dph, the otoliths, cleithrum and jaws were mineralized, as well as the parasphenoid, branchial arches and basioccipital bones (Fig. 1, A and C). At 23 dph, a much greater number of skeletal elements were mineralized, both in the cephalic and the axial skeleton. The mineralized cephalic skeleton extended, including the premaxillae, branchiostegal rays and vomer. Pharyngeal teeth were present on the posterior branchial arches. The mineralized axial skeleton included up to 18 vertebral centra (median number of 12) along the unmineralized notochord, caudal fin rays and fin supports were mineralized together with additional structures (Fig. 1, B and D). Within this cohort, there was a large variation in the group in terms of mineralization, with strong correlation to length parameter (Spearman's p-value <0.0001), supporting the fact that the process of mineralization should be analyzed in relation to the size of the individuals.

3.2. E2 and BPA disrupt morphological aspects of larval growth and mineralization

3.2.1. Biometric characteristics of larvae exposed to E2 and BPA

During exposure, larvae of all five conditions have significantly gained in standard length. The mean growth percentages over the 7 days of exposure were + 21 % for solvent control, +21 % for E2 0.4 ng.L^{-1}, +19 % for E2 40 ng.L^{-1}, + 21 % for BPA 1.6 µg.L^{-1} and + 24 % for BPA 160 µg.L^{-1} (Supp data Table S2). The larvae treated with E2 40 ng.L^{-1} were significantly smaller (0.90 ± 0.06 cm) compared to the larvae treated with BPA 160 µg.L^{-1} (0.95 ± 0.06 cm, Dunn's multiple comparisons test, p = 0.0034), but none of the four treatments significantly differed from the solvent control (Fig. 2, left panel). These results are confirmed by the distribution of larvae by size class (Fig. 2, right panel), where larvae exposed to BPA 160 are more numerous in the size class >1 cm than other treatments.

The mean survival rate between 16 and 23 dph was 52 ± 6 %, depending on the tested modality, which is consistent with the survival rates recorded in aquaculture at these early life stages (Chatain and Dewavrin, 1989). The percentage of individuals exhibiting abnormal swim bladder was 5 % at the beginning of the exposure in the control rearing tank, while it was 15 ± 8 %, at the end of the experiment, depending on the tested modality, with almost only over-inflated swim bladders (Supp data Table S2). Similar rates of swim bladder anomalies are commonly observed in aquaculture in *Dicentrarchus labrax* (Barnabé, 1989; Chatain and Dewavrin, 1989; Miller, 2009). No other major developmental anomalies were detected (fusion of vertebrae, lordosis, craniofacial anomalies). Larvae with abnormal bladder were discarded for subsequent mRNA or Alizarin analyses.

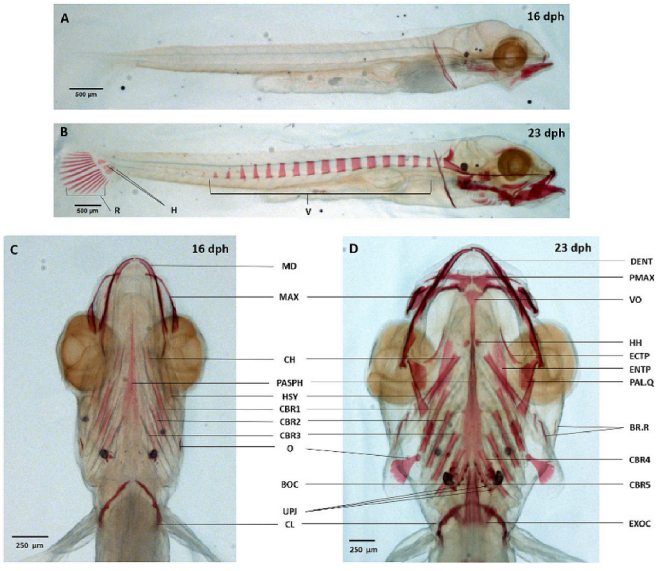

Fig. 1. Lateral (A) and ventral (C) views of a European seabass larvae *Dicentrarchus labrax* at 16 dph (SL_{aliz}: 7.2 mm) and lateral (B) and ventral (D) views of a *Dicentrarchus labrax* larvae at 23 dph (SL_{aliz}: 8.9 mm). H: hypural, R: caudal fin rays, V: vertebrae, BOC: basioccipital, BR.R: branchiostegal ray, CBR 1–5: ceratobranchial 1–5, CH: ceratohyal, CL: cleithrum, DENT: dentaries, ECTP: ectopterygoid, ENTP: entopterygoid, EXOC: exoccipital, H: hypural, HH: hypohyal, HSY: hyosymplectic, MAX: maxillary, MD: mandibular, O: opercular, PAL.Q: palato-quadrate, PASPH: parasphenoid, PMAX: premaxillary, R: caudal fin rays, UPJ: upper pharyngeal jaw, V: vertebrae, VO: Vomer. Bone nomenclature was assigned according to Gluckmann et al., 1999; Kužir et al., 2004).

3.2.2. Phenotypic effects of the E2 and BPA exposures on mineralization

3.2.2.1. The number of mineralized vertebrae is decreased only in larvae exposed to 0.4 ng.L^{-1} E2. In all treatment conditions, including the solvent control, the number of mineralized vertebrae in 23-dph larvae varied from 1 to 19 (Fig. 3). The number of mineralized vertebrae was positively correlated to the standard length for every treatment with Spearman's correlation coefficient values ranging between 0.81 and 0.88 (p < 0.0001). Larvae exposed to the lowest dose of E2 tested (E2 0.4 ng.L^{-1}) exhibited less mineralized vertebrae compared to the solvent control (nonparametric ANCOVA, p < 0.01, Fig. 3A), regardless of the size of the individual. Larvae exposed to E2 0.4 ng.L^{-1} also displayed less mineralized vertebrae compared to those exposed to E2 40 ng.L^{-1} (nonparametric ANCOVA, p < 0.01, Fig. 3A). Conversely, no significant difference in the number of mineralized vertebrae was observed between larvae exposed to E2 40 ng.L^{-1}, BPA 1.6 µg.L^{-1} and 160 µg.L^{-1}, compared to the solvent control (Fig. 3).

3.2.2.2. E2 and BPA decreased the overall level of mineralization. A pixel-based quantitation of bone surface was performed for the whole body (as the sum of head, vertebrae and caudal fin on a lateral view) in individuals chosen in the mean of size class distribution (n = 10; SL_{Aliz}: 0.87–0.93 cm, Fig. 4, Supp data Fig. S1). This analysis confirms that E2 0.4 ng.L^{-1} treatment induced an overall lower bone mineralization compared to the solvent control with −24 % bone surface in whole body (Dunn's multiple comparisons test, p = 0.0016), −17 % bone surface in head (p = 0.0071), −33 % bone surface in vertebrae (p = 0.0005) and −34 % bone surface in caudal fin (p = 0.0004) (Fig. 4). A 14 % decrease in head mineralization was observed in larvae exposed to BPA 160 µg.L^{-1} (p = 0.0498) (Fig. 4B). The caudal fin was 23 % less mineralized in larvae exposed to BPA 1.6 µg.L^{-1} compared to the solvent control (p = 0.0343; Fig. 4D).

3.3. E2 and BPA exposures disrupt molecular aspects of larval mineralization

After 7 days of treatment, in 23-dph larvae, the transcriptional levels of four genes (*col2a1a, ihha, ihhb, alp*) were not impacted by any treatment compared to the solvent control (Supp data Table S3, Supp data Fig. S2). Conversely, four genes encoding osteoblast markers were upregulated in the E2 treatment at 0.4 and 40 ng.L^{-1} concentrations: *bgp1a* (1.6 and 1.7-fold increase, respectively), *bgp1b* (2.5 and 2.8-fold increase, respectively) and *sp7* (1.7-fold increase for both concentrations) and at E2 0.4 ng.L^{-1} only for *sparc* (1.8-fold increase). Similar upregulation was observed in response to the BPA treatments: larvae exposed to BPA 1.6 µg.L^{-1} showed increased regulation of *bgp1a* and *sparc*, those exposed to BPA 160 µg.L^{-1} showed increased regulation of *sp7*, and both BPA concentrations induced increased regulation of *bgp1b*.

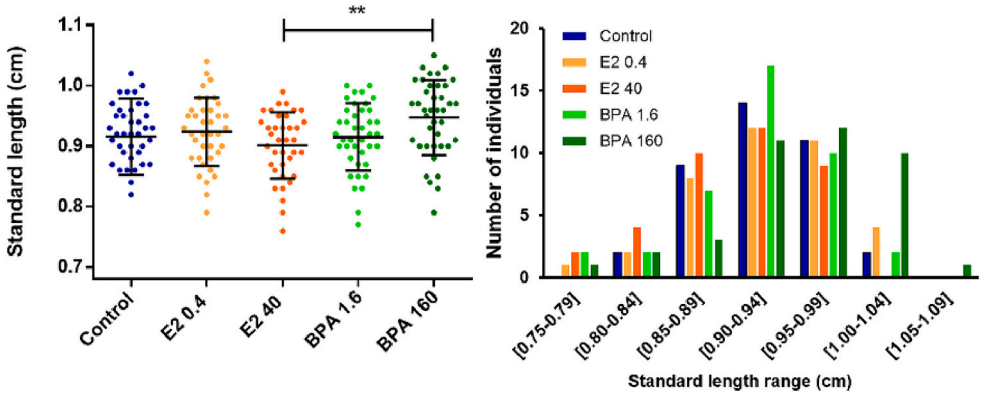

Fig. 2. Standard length (SL_{fresh}, mean +/− standard deviation; left panel) and size class distribution of 23-dph larvae after 7 days of treatment (right panel). Treatments corresponded to ethanol 0.0008 % for solvent control (Control), E2 0.4 and 40 ng.L^{-1} (E2 0.4 and E2 40) and BPA 1.6 and 160 µg.L^{-1} (BPA 1.6 and BPA 160). For each experiment, difference was tested using the non-parametric multiple comparison test Kruskal-Wallis, followed by Dunn's post-hoc test (** $p < 0.01$).

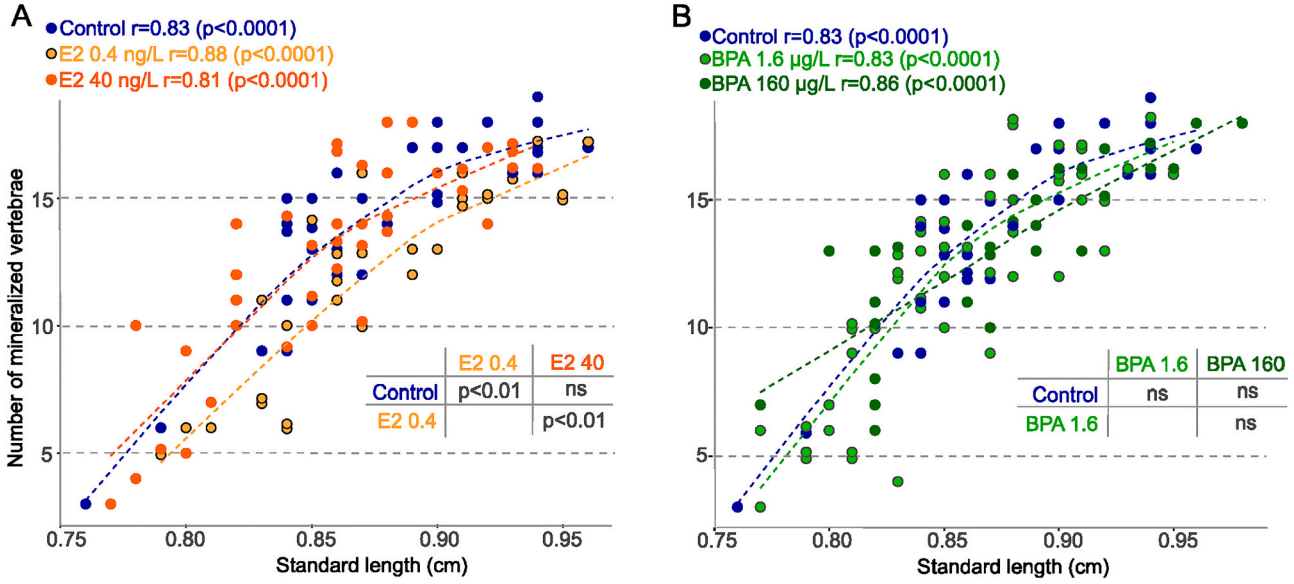

Fig. 3. Relationship between the standard length (SL_{aliz}) and the number of mineralized vertebrae in 23-dph individuals exposed for 7 days at nominal concentrations of 0.4 and 40 ng.L^{-1} E2 (E2 0.4 and E2 40, panel A), 1.6 and 160 µg.L-1 BPA (BPA 1.6 and BPA 160, panel B) and ethanol 0.0008 % (*v*/v) as a solvent control (Control, panels A and B). A non-parametric Spearman's correlation coefficient r was calculated to evaluate the strength and the direction of the monotonic relationship. A nonparametric analysis of covariance was used to test the difference between two treatments (p-value is given in the tables, ns: not significant according to the Bonferroni adjusted $p < 0.017$ threshold).

The fold changes were comparable between BPA and E2 treatments, except in the case of *bgp1b* where a 3.7-fold increase was observed in response to BPA 1.6 µg.L^{-1}. This suggests that these four osteoblast marker genes are transcriptionally sensitive to estrogen signals either by E2 or BPA. Only one gene of the osteoclast markers, *acp5*, had its expression 1.5 to 1.9-fold increased respectively in BPA 160 µg.L^{-1} and in E2 40 ng.L^{-1} treated larvae (Fig. 5 and Supp data Table S3). In response to BPA 160 µg.L^{-1} only, seven other genes (*sox9a* and *sox9b*, *col2a1b*, *col1a2*, *runx2*, *rankl* and *ctsk*) exhibited 2.3 to 3.5-fold decreased mRNA levels compared to solvent control (Fig. 5 and Supp data Table S3). These downregulated genes are chondrocyte markers (*col2a1b*, *sox9a* and *sox9b*), osteoblast markers (*col1a2*, *runx2*) and osteoclast markers (*rankl*, *ctsk*). Even if other parameters such as differential regulation kinetics are probably at stake, these results highlight that BPA may exert antagonistic effects on mineralization-related genes with transcriptional stimulatory and inhibitory effects depending on the gene.

3.4. Estrogen signaling and GH-IGF1 axis

Regarding estrogenic signaling related genes, expression of both *erb1* and *erb2* paralogs were up-regulated 2.3 and 2.2-fold, respectively, and *errb2* was 1.6-fold down-regulated only by E2 40 ng.L^{-1} (Fig. 6). The expression of *errb1* and *errga* was not impacted by any of the treatments compared to the control (Supp data Table S3 and Supp data Fig. S2), while *erra* was 1.4 to 1.6-fold overexpressed at both concentrations of E2 and BPA (Fig. 6 and Supp data Table S3).

Regarding the GH-IGF1 axis, a 1.9 to 3.1-fold increase of mRNA expression of *gh* was observed after E2 and BPA treatments, at all tested concentrations. The same increased expression pattern of 2.5 to 2.9 fold was measured for *igf1* expression after BPA treatment at both concentrations and after E2 treatment at the highest tested concentration of 40 ng.L^{-1} only (Fig. 6 and Supp data Table S3).

4. Discussion

In this work, we focused on a specific time window of the early larval

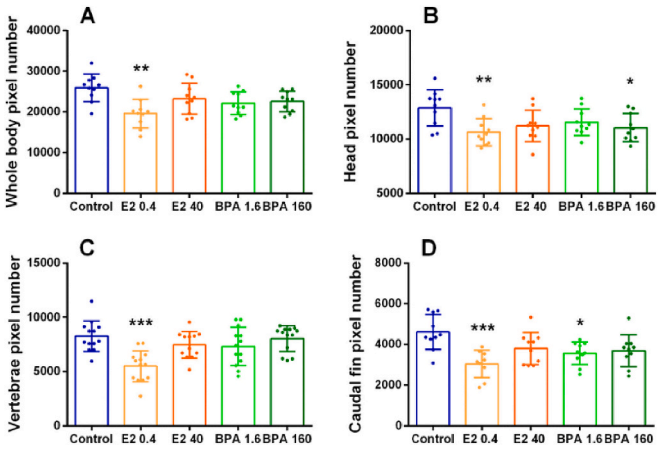

Fig. 4. Bone surface in (A) whole body, (B) head, (C) vertebrae and (D) caudal fin using pixel-based quantitation of Alizarin Red staining in individuals from the size range SL_{aliz} 0.87–0.93 cm (n = 10, Kruskal Wallis followed by Dunn's post-hoc test). Black stars indicate significant differences compared to the solvent control (multiplicity adjusted p-value: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$). E2 0.4 and E2 40: 0.4 and 40 ng.L^{-1} E2, respectively; BPA 1.6 and BPA 160: 1.6 and 160 µg.L^{-1} BPA, respectively; Control: ethanol 0.0008 % (*v*/v) as a solvent control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

development of the seabass: between 16-dph and 23 dph. Over only 7 days, our cohort of larvae growing in natural conditions showed a mean growth of 20 % of their standard length and a rather active process of mineralization in several aspects of their skeleton, both in endochondral and intramembranous bones. The exposure to waterborne E2 and BPA was made over this 7-day period at two concentrations, the lowest representing the upper-limit to environmentally accepted dose, and a 100 times higher dose representing acute exposure. Based on conventional competitive receptor-binding assays, BPA is considered as a weak xenoestrogen since its binding affinity to the nuclear ERs is 10–3 to about 10–4 lower than that of E2 (Cosnefroy et al., 2009; Prossnitz and Barton, 2011; Quesada et al., 2002). This reduced affinity of BPA for ERs implies that higher concentrations of the substance are required to activate the ERs. In natural water, concentrations of BPA are indeed generally 2 to 3 orders of magnitude higher than E2 concentrations (Heemken et al., 2001; Wang et al., 2012a, 2012b; Ying et al., 2009). The concentrations tested in this study are 4000 times higher for BPA than for E2 (low: 0.4 ng.L^{-1} vs 1.6 µg.L^{-1}; high: 40 ng.L^{-1} vs 160 µg.L^{-1}), assuming that this difference in concentration may compensate for the difference in affinity for ERs. In addition, BPA is able to bind a diversity of receptors such as orphan ERRs, androgen receptors, thyroid hormone receptor, glucocorticoid receptor, and PPARγ (Batista-Silva et al., 2022; Liu et al., 2019; MacKay and Abizaid, 2018; Mathieu-Denoncourt et al., 2015; Yuan et al., 2023), all of them having a role in the metabolism of cartilage and bone (Gouveia et al., 2018; Zuo and Wan, 2017), suggesting E2 and BPA may induce signaling on different sets of regulatory pathways. However, BPA at low concentrations, is reported to bind almost exclusively to the estrogen receptors, but may also bind to other receptors at higher concentrations (Batista-Silva et al., 2022). This suggests that BPA binding to different receptors could be an explanation for the differences in the responses between the concentrations tested.

We investigated the effect of these exposures on the transcriptional level of genes involved in the mediation of estrogenic pathways after 7 days of exposure. Only the elevated concentration of E2 (40 ng.L^{-1}) modified the expression of the nuclear estrogen receptors, by upregulating the transcription of *erb1* and *erb2*, therefore potentially generating a feedback disturbance of the signal by modifying the quantity of receptors. There is a known dynamic expression discrepancy existing between GPERs and nuclear ERs as shown by Pinto et al. (2016) in the scale of seabass, where *gperb* was significantly induced by E2 treatment after 1-day by not after 5-days. GPERs can mediate rapid non-genomic effects, such as the activation of specific enzymes (Pinto et al., 2014). While genomic actions can take hours to days, non-genomic effects occur in seconds or minutes. This could explain the absence of response of *gperb* after 7 days of exposure in this study.

Although E2 is not a ligand for ERRs (Tanida, 2022), E2 can exert a transcriptional effect on ERRs. Overall, the influence of E2 and BPA on *err* transcription was weak, unlike what was observed at an earlier larval stage of seabass in Farcy et al. (2022). However, *erra* expression was increased of about 1,5 times whatever the concentration of E2 and BPA treatment. In mammals, ERRa plays a major role in bone homeostasis by modulating osteoblast differentiation and osteoclast migration (Auld et al., 2012; Feng et al., 2022; Kim et al., 2015), and has an inhibitory effect on *runx2* transcription (Kammerer et al., 2013). Here, we show downregulation of *runx2* expression at 23 dph only after exposure to 160 µg.L^{-1} BPA, but there is an overall activation of the runx2 regulatory pathway with activation of both *bgp* as well as *sp7* and *sparc*. These opposite effects of BPA highlight the multiplicity of concurrent signaling pathways reacting to (xeno)estrogens. BPA treatment had no effect on estrogen(−related) receptor expression apart from that of *erra*. Conversely, *errb2* was downregulated by E2 40 ng.L^{-1} exposure only. Unlike ERRa and ERRg, ERRb is not known for its involvement in bone development either in mammals nor fish. ERRb has a role in the maintenance of pluripotency in mouse embryonic cells (Crevet and Vanacker, 2020; Festuccia et al., 2018) and embryos from *errb−/−* mice have been shown to exhibit growth retardation (Luo et al., 1997).

Since mRNA levels were performed on total body RNA extracts, potential organ-specific effects of E2 or BPA exposure were not detectable. Despite this limitation, we evaluated the impact of E2 and BPA exposure on two aspects of larval development at the phenotypic and transcriptional levels: (i) growth through the body size and mRNA levels of key genes of the GH/IGF pathway and (ii) skeletal mineralization through evaluation of mineralization level (calcium deposition) and mRNA levels of key genes involved in cartilage and bone cell activity and differentiation.

4.1. *E2 and BPA exposure both upregulate gh/igf1 expression but have opposite phenotypical effect on larval growth*

Previous works in fish suggest that environmental (xeno)estrogens modulate the GH-IGF system at elevated concentrations (Aluru et al., 2010; Dang et al., 2018; Hanson et al., 2014; Celino-Brady et al., 2019). Using acute exposure of rainbow trout eggs to 30 and 100 mg.L^{-1} BPA for 3 h prior to fertilization in order to mimic accumulation of the compound by maternal transfer, Aluru et al. (2010) showed a delayed hatching, yolk reabsorption and growth of larvae of about 7 days compared to controls. Growth suppression persisted in juvenile fish and the level of whole-body GH content was significantly higher in BPA treated groups compared to controls at 65 dpf. According to Dang et al. (2018), a 96 h-exposure to 2.2 µM and 22 µM BPA (corresponding to about 500 and 5000 µg.L^{-1}) significantly up-regulated the mRNA expressions of *gh* and down-regulated the mRNA expressions of *igf1* in zebrafish embryos, associated with a decrease of body length at all tested concentrations (50, 500 and 5000 µg.L^{-1}). In the present study, both E2 and BPA molecules had a comparable up regulatory effect on the transcript levels of the genes encoding for the GH and for IGF1 at all tested concentrations, except for *igf1* expression in fish exposed to E2 0.4 ng.L^{-1}, that did not differ from the control. This confirms that environmental (xeno)estrogens can interfere with the GH-IGF axis in larvae of the European seabass. To fully address the effect of E2 and BPA on the GH-IGF system in the larval seabass, other members of this complex network, such as *igfbp*, *igf2*, *igf- and gh- receptors*, might be monitored, and potential organ-specific response might help disentangling the regulatory effects of these molecules. This may help to

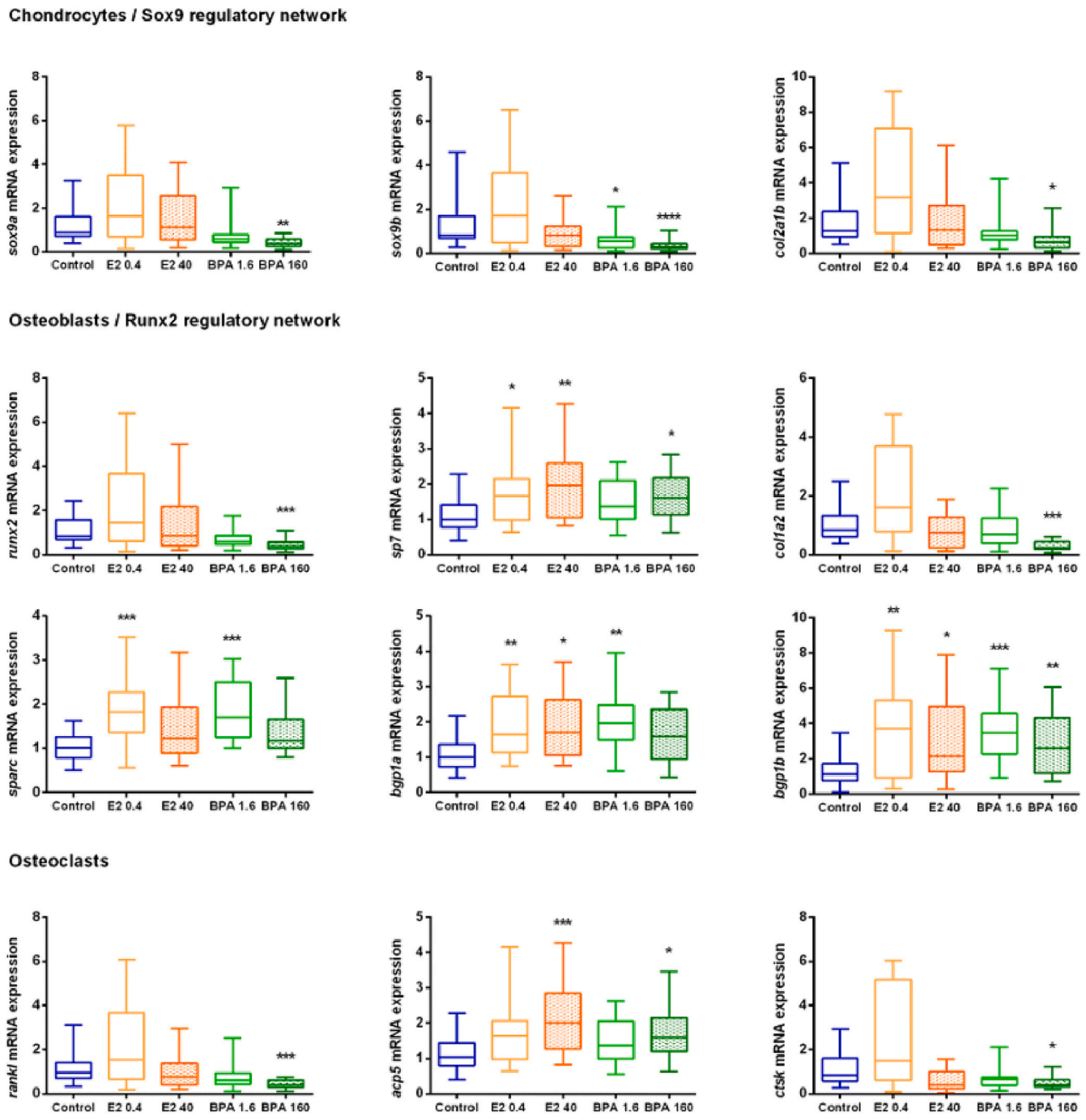

Fig. 5. Relative mRNA expression of mineralization-related genes in 23-dph individuals exposed for 7 days at nominal concentrations of 0.4 and 40 ng.L^{-1} E2 (E2 0.4 and E2 40), 1.6 and 160 µg.L^{-1} BPA (BPA 1.6 and BPA 160) and ethanol 0.0008 % (*v*/v) as a solvent control (Control). Relative mRNA levels are expressed as ΔΔCp with solvent control as a reference (n = 16 to 20 individuals). Sox9 and Runx2 regulatory networks were named according to Gómez-Picos and Eames (2015). In boxplots, hinges indicate first and third quartiles; whiskers indicate the min and max values, and horizontal lines indicate the median. Between control and treatments was tested using the non-parametric multiple comparison test Kruskal-Wallis, followed by Dunn's post-hoc test. Black stars indicate significant differences compared to the solvent control (multiplicity adjusted p-value: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

explain the opposite effects of E2 and BPA on larval growth, observed at the highest tested concentration only (40 ng.L^{-1} and 160 µg.L^{-1}, respectively): BPA-exposed larvae were longer than E2-exposed larvae. This result suggests that interference with the somatotropic axis by E2 and BPA may result in differently modified signaling, involving other pathways.

Previous studies regarding the effect of E2 on fish growth at early life stages are scarce and showed a decrease of post-embryonic growth at 10 µg.L^{-1} E2 (Hanson et al., 2014). When E2 is used in aquaculture to induce sex reversal for economic reasons (Hoga et al., 2018), it has a significant impact on fish growth but this is due to sexual dimorphism and E2 treatments are generally carried out at elevated doses in advanced juvenile stages. For instance, in *Dicentrarchus labrax*, using 12.5 mg.kg^{-1} E2 for 60 days, females show 30 to 50 % higher growth rates and they mature later than males do (Gorshkov et al., 2004). Regarding BPA and similar conditions of exposure at early developmental stages, existing results are rather inconsistent. Some data highlight a negative impact of BPA on the growth of larval and juvenile

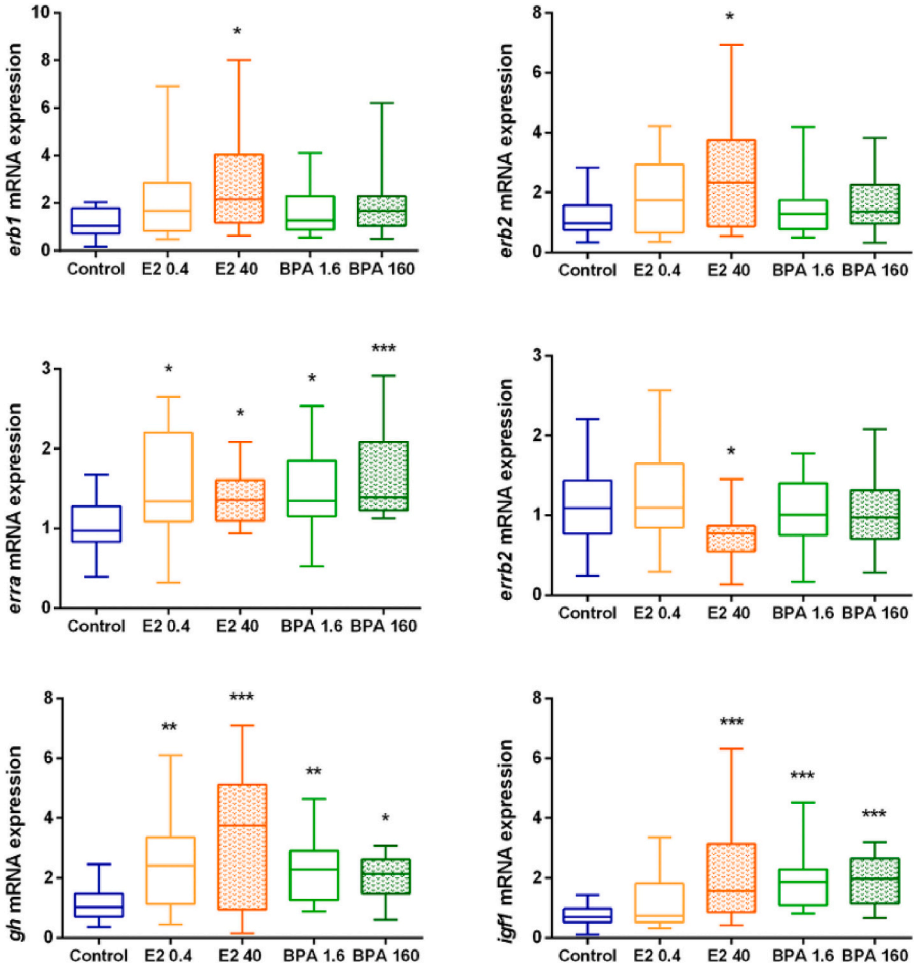

Fig. 6. Relative mRNA expression of nuclear estrogen receptor genes (*erb1*, *erb2*), estrogen-related receptor genes (*erra*, *errb2*) and GH-IGF1 axis related genes (*gh*, *igf1*) in 23-dph individuals exposed for 7 days at nominal concentrations of 0.4 and 40 ng.L^{-1} E2 (E2 0.4 and E2 40), 1.6 and 160 µg.L^{-1} BPA (BPA 1.6 and BPA 160) and ethanol 0.0008 % (v/v) as a solvent control (Control). Relative mRNA levels are expressed as ΔΔCp with solvent control as a reference (n = 16 to 20 individuals). In boxplots, hinges indicate first and third quartiles; whiskers indicate the min and max values, and horizontal lines indicate the median. Differences between control and treatments was tested using the non-parametric multiple comparison test Kruskal-Wallis, followed by Dunn's post-hoc test. Black stars indicate significant differences compared to the solvent control (multiplicity adjusted p-value: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$).

stages after prolonged exposure. Total length was reduced in Japanese medaka with BPA at the highest tested concentration of 1820 µg.L^{-1} from fertilized eggs to 60 dph (Yokota et al., 2000). But Huang et al. (2021) showed an increase of body length in zebrafish larvae exposed to 0.1 µM BPA (corresponding to about 23 µg.L^{-1}) from 0 to 120 hpf. In the same study, there was no change in body length at the lowest tested concentrations (0.038 and 0.5 µM, i.e. 8.74 µg.L^{-1} and 115 µg.L^{-1} respectively) and at the highest tested 1 µM concentrations of BPA, suggesting non-monotonic effects (Huang et al., 2021). Finally in the fathead minnow, a prolonged exposure from egg stage to 25 to 26 dph at 0.1 to 1000 µg.L^{-1} nominal concentrations of BPA did not exert any significant effect on fish length (Warner and Jenkins, 2007). Overall, the discrepancies found may be due to differences in concentrations used, differences in the duration of exposure, and/or non-monotonic effects characteristic of endocrine compounds (Li et al., 2020; Vandenberg et al., 2012). They may also highlight the diversity of phenotypic answers to exposure that may depend on the species, the developmental stage, or the physiological state of an individual.

4.2. E2 and BPA exposure have different regulatory effects on skeletal development genes but both impair larval skeleton mineralization

Exposures to E2 and BPA at both low and high concentrations had a significant stimulatory effect on the transcription of a combination of genes encoding osteoblast markers: *sp7*, *bgp1a, bgp1b* and *sparc* (Gómez-Picos and Eames, 2015). This effect appears to be common result of both BPA and E2 exposure at this developmental stage (16–23 dph, a phase of active skeletal mineralization), while similar exposure on earlier larvae led to no significant upregulation of *sp7* by either BPA or E2, and no upregulation of *sparc* in E2 exposures (between 10 and 16 dph, a time when cephalic skeletal mineralization is just initiated; Farcy et al., 2022). In contrast, only the BPA exposures led to a modification of gene expression linked to the chondrocyte lineage, and the number of regulated genes is higher at the most elevated concentration: the 1.6 µg.L^{-1} BPA exposure only downregulated *sox9b* expression, while the 160 µg.L^{-1} BPA exposure led to the transcriptional downregulation of *sox9a*, *sox9b* and *col2a1*. These results also strongly contrast with those obtained in the earlier larval stage where *sox9* genes and *col2a1* genes all were upregulated after an exposure to higher concentrations of both E2 and BPA (Farcy et al., 2022). Finally, 160 µg.L^{-1} BPA and 40 ng.L^{-1} E2 exposures both modified expression of genes linked to the osteoclast activity: both upregulated the expression of *acp5* and the BPA exposure downregulated *rankl* and *ctsk*, also in opposition with results obtained at the preceding larval stage where only *ctsk* was upregulated in E2 and BPA exposures (Farcy et al., 2022).

The combination of our previous results at an earlier developmental

stage (Farcy et al., 2022) with this new study highlights the great variation of effects of (xeno)estrogens depending on the developmental stage of exposure, i.e. at the time of head mineralization compared to that of axial skeletal mineralization. Different stages of cell differentiation, or different proportions between stem versus differentiated cells may explain these opposite outcomes (e.g. upregulation versus downregulation of chondrocyte genes by BPA) when different developmental stages are compared. In addition, comparing effects at different developmental stages also highlights how BPA and E2 exposures may have similar or opposite outcomes in different life stage situations (e.g. *ctsk* upregulation by E2 and BPA at 16 dph as shown in Farcy et al., 2022, versus downregulation by BPA only at 23 dph in this study), precluding any general nor simple description of one effect of (xeno)estrogens on development. In particular, the interplay between the modification of several signaling cascades involved in growth or mineralization may lead to opposite outcomes in exposures to E2 or BPA. Although the effect of IGF signaling on skeletal development was poorly addressed in teleost (Vieira et al., 2013), studies in mammals gave evidence that IGF signaling in bone cells is essential for the maintenance of the mineralization processes at a normal level, in particular in regulating the osteoblast/osteoclast precursor balance (Zhang et al., 2002). Exogenous administration of low doses of IGF1 increased markers of osteoblastic activity (*runx2*) and reduced bone resorption (*rankl*) (Guerra-Menéndez et al., 2013). A better understanding of the interplay between the GH/IGF pathway and the skeletal cell differentiation pathways are still needed in teleost fishes, in order to better apprehend the actual effect of (xeno)estrogen on these developmental processes.

In this study, we identified phenotypic effects after 7 days of treatment for the environmentally relevant E2 and BPA concentrations and the highest concentration of BPA. A very strong effect of 0.4 ng.L^{-1} E2 exposure was observed on skeleton mineralization with a significant decrease in the number of mineralized vertebrae (Fig. 3), and significantly less bone surface area in the whole body, head, vertebrae and caudal fin on a restricted size class (Fig. 4). The effect of both BPA exposures was weaker and only significant in the comparison of head bone surface for BPA 160 µg.L^{-1} exposure, and of caudal fin bone surface for BPA 1.6 µg.L^{-1} exposure. Two main points are highlighted through these results. The first one is that despite divergent effects on gene regulation by BPA and E2 exposures, the observed phenotypic effects on mineralization go in a similar direction, the inhibition of mineral deposit in the developing skeleton. This result contrasts with previously published data where only exposure to 40 ng.L^{-1} E2 in seabass larvae led to an increase of head mineralization (Farcy et al., 2022), while exposure to 1.6 and 160 µg.L^{-1} BPA or 0.4 ng.L^{-1} E2 had no detectable phenotypic effect on mineralization. Very few published data discuss skeletal defects after (xeno)estrogen exposure at environmentally relevant concentrations: in the fathead minnow, a prolonged exposure from egg stage to 25 to 26 dph at 0.1 to 1000 µg.L^{-1} nominal concentrations of BPA did not exert any vertebral malformations or impair skeletal development (Warner and Jenkins, 2007). The second main highlight of these results is the fact that the strongest phenotypic effect was observed with E2 at its environmentally relevant regulatory concentration (0.4 ng.L^{-1}) although the transcriptional effects at this concentration were less marked than that observed with E2 40 ng.L^{-1} or with BPA.

5. Concluding remarks

The lowest available PNECaqua for E2 is 0.4 ng.L^{-1} (European Commission. Joint Research Centre. Institute for Environment and Sustainability, 2012; Kunz et al., 2015) and E2 is still in the first Watch List of the Water Framework Directive (WFD, EU Directive 2000/60/EC, EU Implementing Decision 2015/495) (Simon et al., 2022). Our work underlines that steroid hormones or xenoestrogens can be biologically active at low concentrations and over very short exposure times, with specific developmental windows, where active mineralization of the axial skeleton occurs, being particularly sensitive to estrogen exposure. This observation calls for more studies regarding the phenotypic effects of (xeno)estrogens in ecotoxicology, not to be restricted to gene expression measures, in order to generate a more biologically-relevant body of data to improve our understanding of estrogens at environmentally relevant concentrations.

Ethics statement

The experimental design has been approved by French legal requirements concerning the welfare of laboratory animals (APAFIS permit no. 9045-201701068219555). Handling of seabass and all the experiments were conducted in compliance with European Union directives and French law regulating animal experimentation (directive 86/609 and decree 87/848, respectively).

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CRediT authorship contribution statement

Camille Martinand-Mari: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Melanie Debiais-Thibaud:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Eric Potier:** Visualization, Formal analysis. **Eric Gasset:** Methodology, Conceptualization. **Gilbert Dutto:** Methodology, Conceptualization. **Nicolas Leurs:** Methodology, Formal analysis. **Stéphane Lallement:** Methodology. **Emilie Farcy:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2024.109921.

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