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In vivo effects of 17α-ethinylestradiol, 17β-estradiol and 4-nonylphenol on *insulin-like growth-factor binding proteins* (*igfbps*) in Atlantic salmon

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ABSTRACT

Feminizing endocrine disrupting compounds (EDCs) affect the growth and development of teleost fishes. The major regulator of growth performance, the growth hormone (Gh)/insulin-like growth-factor (Igf) system, is sensitive to estrogenic compounds and mediates certain physiological and potentially behavioral consequences of EDC exposure. Igf binding proteins (Igfbps) are key modulators of Igf activity, but their alteration by EDCs has not been examined. We investigated two life-stages (fry and smolts) of Atlantic salmon (*Salmo salar*), and characterized how the Gh/Igf/Igfbp system responded to waterborne 17α-ethinylestradiol (EE_2), 17β-estradiol (E_2) and 4-nonylphenol (NP). Fry exposed to EE_2 and NP for 21 days had increased hepatic *vitellogenin* (*vtg*) mRNA levels while hepatic *estrogen receptor α* (*erα*), *gh receptor* (*ghr*), *igf1* and *igf2* mRNA levels were decreased. NP-exposed fry had reduced body mass and total length compared to controls. EE_2 and NP reduced hepatic *igfbp1b1*, *-2a*, *-2b1*, *-4*, *-5b2* and *-6b1*, and stimulated *igfbp5a*. In smolts, hepatic *vtg* mRNA levels were induced following 4-day exposures to all three EDCs, while *erα* only responded to EE_2 and E_2. EDC exposures did not affect body mass or fork length; however, EE_2 diminished plasma Gh and Igf1 levels in parallel with reductions in hepatic *ghr* and *igf1*. In smolts, EE_2 and E_2 diminished hepatic *igfbp1b1*, *-4* and *-6b1*, and stimulated *igfbp5a*. There were no signs of compromised ionoregulation in smolts, as indicated by unchanged branchial ion pump/transporter mRNA levels. We conclude that hepatic *igfbps* respond (directly and/or indirectly) to environmental estrogens during two key life-stages of Atlantic salmon, and thus may modulate the growth and development of exposed individuals.

1. Introduction

A confluence of anthropological factors has contributed to the decline of anadromous fish populations in North America. Environmental contaminants, including endocrine disrupting compounds (EDCs), have been correlatively linked to the decline of Atlantic salmon (*Salmo salar*) along the eastern seaboard of North America and may currently undermine efforts to restore this species (Fairchild et al., 1999; Parrish et al., 1998). EDCs commonly occur as estrogens, or estrogen mimics (xenoestrogens), and impact biota at scales ranging from individuals to populations (Blazer et al., 2012; Kidd et al., 2007; Lange et al., 2011). Discharged through municipal wastewater, the natural steroid 17β-estradiol (E_2), and the synthetic estrogen, 17α-ethinylestradiol (EE_2) are two of the most prevalent feminizing compounds in rivers and estuaries (Desbrow et al., 1998). The plasticizer, 4-nonylphenol (NP), used in the production of household and industrial products, and in the past as a surfactant in aerial spraying of pesticides, is also a feminizing compound released through industrial and municipal wastewater (Servos et al., 2003). Fishes with life histories that entail periods of habitation and/or development in rivers and estuaries are especially susceptible to the acute (Purdom et al., 1994), latent (Lerner et al., 2007b) and multigenerational (Bhandari et al., 2015) physiological and/or behavioral effects of EDCs.

The lifecycle of anadromous Atlantic salmon begins in freshwater (FW) streams where hatched larvae reside, develop, and grow through multiple post-larval stages. These early life stages are not well defined

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biologically, but various common names such as alevin, fry, fingerling and parr denote the residential life stages when juveniles defend territories for foraging and growth (Sutterby and Greenhalgh, 2005). The FW phase of the lifecycle that precedes seawater (SW) migration may take 1–7 years depending upon the growth conditions in the environments where juveniles dwell (McCormick et al., 1998). In preparation for SW migration, stream-dwelling 'parr' transform into 'smolts'. Termed 'smolting' or 'smoltification', this transformation includes the development of morphological, physiological, and behavioral phenotypes supportive of survival and growth in marine habitats. When they do migrate downstream as smolts, they are potentially exposed to point-source pollution such as industrial sites and sewage treatment plants as they pass through large rivers and estuaries. In the absence of exogenous influence, endogenous estrogens are low prior to reproductive maturation (and upstream migration) in anadromous salmonids (Patiño and Schreck, 1986). Given that EE_2, E_2 and NP exposures stimulate vitellogenin (yolk protein precursor) production during multiple life stages of Atlantic salmon of both sexes (Arsenault et al., 2004; Duffy et al., 2014), estrogenic compounds can impact physiological systems prior to reproductive maturation, and throughout the lifespan.

Growth hormone (Gh)/insulin-like growth factor (Igf) signaling is essential to the growth and development of vertebrates, including teleost fishes (Duan et al., 2010). Gh can directly stimulate growth by operating as a mitogen via transmembrane receptors that initiate JAK/STAT, PI3K and/or MAPK signaling. Gh indirectly regulates growth by directing the synthesis and secretion of Igfs from liver, muscle and other target tissues (LeRoith et al., 2001). Igfs regulate tissue growth by controlling cellular processes such as differentiation, proliferation, migration, and survival (Castillo et al., 2004; Codina et al., 2008). An extensive suite of binding proteins, appropriately termed Igf binding proteins (Igfbps), affect Igf availability, transport, and receptor binding, and thus play important modulatory roles (Duan et al., 2010; Rajaram et al., 1997).

Salmonids have a greatly expanded *igfbp* gene family due to multiple genome duplication events, with Atlantic salmon expressing at least 19 *igfbp* paralogs (Macqueen et al., 2013). The concerted expression of *igfbps* underlies adaptive responses to environmental conditions, and in particular, the repartitioning of energy when nutrient availability is limited (Shimizu and Dickhoff, 2017). Indeed, Atlantic salmon *igfbp1, -2, -4*, and *-5* transcripts expressed in liver and muscle are highly responsive to fasting and/or re-feeding paradigms (Bower et al., 2008; Breves et al., 2016). While hormones such as Gh, Igf1, thyroid hormones, and cortisol coordinate *igfbp* expression patterns (Breves et al., 2014; Garcia de la serrana et al., 2017; Peterson and Small, 2005; Pierce et al., 2006) there are few studies, especially in salmonids, which describe how estrogens affect *igfbps* (Cleveland and Weber, 2015, 2016). This is an important knowledge gap given that gonadal steroids are known modulators of Gh/Igf signaling in fishes (Filby et al., 2006; Hanson et al., 2012, 2014, 2017; Larsen et al., 2004; Reindl and Sheridan, 2012). Within this context, we investigated whether EDCs can impact hepatic *igfbps* in Atlantic salmon.

In addition to directing somatic growth throughout the lifecycle, the Gh/Igf system is a key driver of parr-smolt transformation (Björnsson, 1997; Hoar and Hoar, 1988; McCormick et al., 1998). The capacity for smolts to effectively osmoregulate upon entry into marine environments depends upon the recruitment of SW-type ionocytes that mediate Na^+ and Cl^- extrusion. These ionocytes rely upon the activities of ion cotransporters and channels, including $Na^+/K^+/2Cl^-$ cotransporter 1 (Nkcc1) and cystic fibrosis transmembrane regulator 1 (Cftr1) (Pelis and McCormick, 2001; Singer et al., 2002). Ion secretion is energized by Na^+/K^+-ATPase (NKA); Atlantic salmon exhibit a "switch" in the relative levels of two *NKA α1* (*nka-α1*) subunit-encoding genes coinciding with smoltification. *nka-α1b* levels are enhanced during smoltification while *nka-α1a* dramatically decreases after SW exposure (McCormick et al., 2013). As Gh/Igf signaling (Pelis and McCormick, 2001; Tipsmark and Madsen, 2009), perhaps in concert with Igfbps (Breves et al., 2017; Fukuda et al., 2015; Shepherd et al., 2005; Shimizu et al., 2003, 2006), supports the acquisition of SW tolerance, we also investigated whether EDC disruption of branchial ionoregulation (Madsen et al., 2004; McCormick et al., 2005) was linked to perturbation of the *igfbp* system.

The aim of the current study was to elucidate the effects of environmental estrogens on key mechanisms of growth and osmoregulation in early and late FW life stages of Atlantic salmon. We adopted the term 'fry' for the ~0.5 g fish in the first experiment and conducted a 21-day trial to model EDC exposure in resident streams. A second experiment was carried out on the late, migratory smolting (or smoltification) stage to model acute (4-day trial) EDC exposure when juveniles migrate downstream prior to entering marine environments (McCormick et al., 1998). We report the effects of EE_2, E_2, and NP on the growth physiology of exposed fry and smolts with a focus on hepatic *igfbps*. We considered patterns within the Gh/Igf/Igfbp system alongside indices of growth and ionoregulatory function, key predictors of the fitness and survival of salmon within these two life stages (McCormick et al., 1998).

2. Materials and methods

2.1. Animals

All fish were the progeny of sea run adults from the Connecticut River stock of Atlantic salmon. Fry exposures were conducted on fish from the U.S. Fish and Wildlife White River National Fish Hatchery. Fish were transported from the hatchery to the USGS Conte Anadromous Fish Research Laboratory (Turners Fall, MA) in early February 2013, and experiments were initiated later in the month. Fry were acclimated to pelleted salmon feed (Zeigler Bros., Garners, PA) in the hatchery and fed daily prior to, and during, the exposure period. For the smolt exposures, parr from the Kensington Atlantic State Fish Hatchery (Kensington, CT) were brought to the laboratory in October 2010 and raised until fish were large enough (> 12 cm fork length) to undergo smoltification in the spring of 2011. Smolts were fed pelleted feed. Natural photoperiod was maintained during rearing and all exposures.

2.2. Experimental design

2.2.1. 21-day treatment of salmon fry with EE_2 and NP

We utilized two common EDCs for long-term exposures: EE_2 at 0.008 and 0.04 nM and NP at 40 and 200 nM. Fry with average body mass (BM) of 0.48 ± 0.01 g and body length (BL; refers to total length) of 39.2 ± 0.2 mm were placed in 30-liter circular tanks at 45 fish/tank (two tanks/treatment including controls) on the morning prior to when exposures were initiated. Fry were fed daily with pelleted feed. Waste and uneaten food were siphoned from the tanks daily.

EE_2 and NP were purchased from Sigma-Aldrich (St. Louis, MO) and solubilized in methanol at a final concentration of < 0.0001% to minimize solvent toxicity (Lerner et al., 2007a). Control treatments received solvent only. Covered 560-liter circular tanks served as head tanks that were filled just prior to the beginning of the exposure period. Head tanks were replenished daily with the appropriate volume of stock compound in methanol mixed with dechlorinated city water, yielding a complete turnover at least 2 times/day and a flow rate of approximately 10.5 l/h. Heat exchangers in each 30-liter tank maintained temperatures at 15 ± 1 °C. At the conclusion of the 21-day exposure period, fry were anesthetized in buffered and neutralized MS-222 (100 mg/l; pH 7.0; Sigma-Aldrich), weighed, and liver was collected and immediately frozen directly on dry ice and stored at −80 °C.

2.2.2. 4-day treatment of smolts with EE_2, E_2 and NP

Smolts (mixed sex) were exposed in three consecutive, 4-day experiments between April 5th and April 19th, 2011, during the predicted

peak of smolting (Duffy et al., 2014). Due to space constraints, one compound at a time (three concentrations) plus a control were executed over a given 4-day period. Smolts of 15.9 ± 1.1 g BM and 32.5 ± 6.3 cm BL were placed in 30-liter circular tanks at 6 fish/tank, two tanks per treatment, on the morning the exposures were initiated. For smolts, BL refers to fork length. Exposures were conducted in a manner consistent with the fry exposures, with the exception that three compounds at three concentrations (EE_2: 0.004, 0.04, and 0.4 nM; E_2: 0.04, 0.4, and 4.0 nM; NP: 4.0, 40, and 400 nM) were tested, each dissolved in methanol as above. The relative estrogenicities of water samples collected during the course of the smolt exposures were determined using the BLYES assay (Ciparis et al., 2012; Sanseverino et al., 2005); the Estrogen Equivalent Quotients of all treatments were reported by Duffy et al. (2014). E_2 was purchased from Sigma-Aldrich. In the wild, smolts entering estuarine environments experience changes in diet composition that may result in prolonged food restriction as they learn to prey on new food items (Andreassen et al., 2001; Stefansson et al., 2003; Renkawitz and Sheehan, 2011); smolts were not fed during the 4-day experimental period to model this scenario. All the smolt experiments were maintained at 15 ± 1 °C.

At the time of sampling, fish were netted and anesthetized in buffered MS-222, and BM and BL measured. Blood was collected from the caudal vasculature by a needle and syringe treated with ammonium heparin within 5 min of the initial netting, separated by centrifugation at 4 °C, and the obtained plasma stored at −80 °C until subsequent analyses. Liver and gill tissues were collected and immediately frozen on dry ice and stored at −80 °C until analyses. No signs of precocious sexual maturation were observed.

2.3. Plasma hormone analyses

Plasma Gh levels were measured by a radioimmunoassay (RIA) validated for Atlantic salmon by Björnsson et al. (1994). Plasma Igf1 levels were measured by a RIA validated for salmonids (Moriyama et al., 1994).

2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues by the TRI Reagent procedure (MRC, Cincinnati, OH) according to the manufacturer's protocols. RNA concentration and purity were assessed by spectrophotometric absorbance (Nanodrop 1000, Thermo Scientific, Wilmington, DE). RNA was DNAse treated (RQ1, Promega Corporation, Madison, WI) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Relative mRNA levels were determined by qRT-PCR using the StepOnePlus real-time PCR system (Life Technologies). We employed previously described primer pairs for *vitellogenin* (*vtg*; Arukwe and Roe, 2008), *estrogen receptor α* (*era*; Luo et al., 2005), *gh receptor* (*ghr*) (Tipsmark and Madsen, 2009), *igf1* and *-2* (Bower et al., 2008), *igfbp1b1*, *-2a*, *-2b1*, *-4*, *-5a*, *-5b2* and *-6b1* (Bower et al., 2008), and *nka-α1a*, *nka-α1b*, *nkcc1* and *cftr1* (Nilsen et al., 2007). Primer sequences and assay efficiencies are reported in Supplementary Table S1. We followed the nomenclature for Atlantic salmon *igfbps* presented by Macqueen et al. (2013). qRT-PCR reactions were setup in a 15 µl final reaction volume with 400 nM of each primer, 1 µl cDNA and 7.5 µl of 2x SYBR Green PCR Master Mix (Life Technologies). The following cycling parameters were employed: 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. After verification that levels did not vary across treatments, *elongation factor 1α* (*ef1α*) levels were used to normalize target genes. Primers for *ef1α* were described previously (Bower et al., 2008). Reference and target gene levels were calculated by the relative quantification method with PCR efficiency correction (Pfaffl, 2001). Standard curves were prepared from serial dilutions of control liver or gill cDNA and included on each plate to calculate the PCR efficiencies for target and normalization genes. Relative mRNA levels are reported as a fold-change from controls.

2.5. Calculations and statistics

Condition factor (CF) was calculated as $CF = (BM \times BL^{-3})*100$.

All group comparisons were performed by one-way ANOVA followed by Dunnett's test, unless otherwise indicated. When necessary (Shapiro-Wilk test), data were log transformed to meet assumptions of normality. Pearson product-moment correlation coefficients were used to assess the relationships between *ghr* and *igf1/2* levels. Sex-specific differences (smolts) were assessed by Student's t-test. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Significance for all tests was set at $P < 0.05$.

3. Results

3.1. 21-day treatment of fry with EE_2 and NP: Mortality, growth and hepatic gene expression

Less than 3% mortality was observed in fry during the exposure period, and it did not differ by treatment ($P = 0.43$, one-way ANOVA). BM and BL were significantly lower in the 20 nM and 200 nM NP groups, respectively, than in the controls (Fig. 1A, B). There was no effect of EDC exposure on CF (Fig. 1C). Hepatic *vtg* mRNA levels were markedly stimulated by EE_2 and NP at all tested concentrations

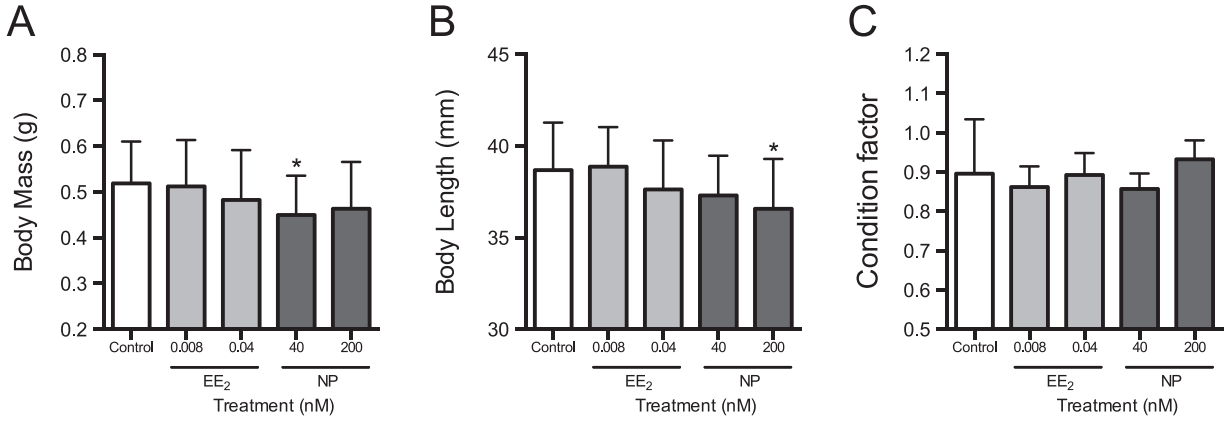

Fig. 1. Body mass (A), body (total) length (B) and condition factor (C) of Atlantic salmon fry after 21-day exposure to water containing 0 (control), 0.008 or 0.04 nM 17α-ethinylestradiol (EE_2) or 40 or 200 nM 4-nonylphenol (NP). Asterisk indicates a significant difference between treatment and control group (One-way ANOVA; Dunnett's test; $P < 0.05$). Means ± SD (n = 22–33).

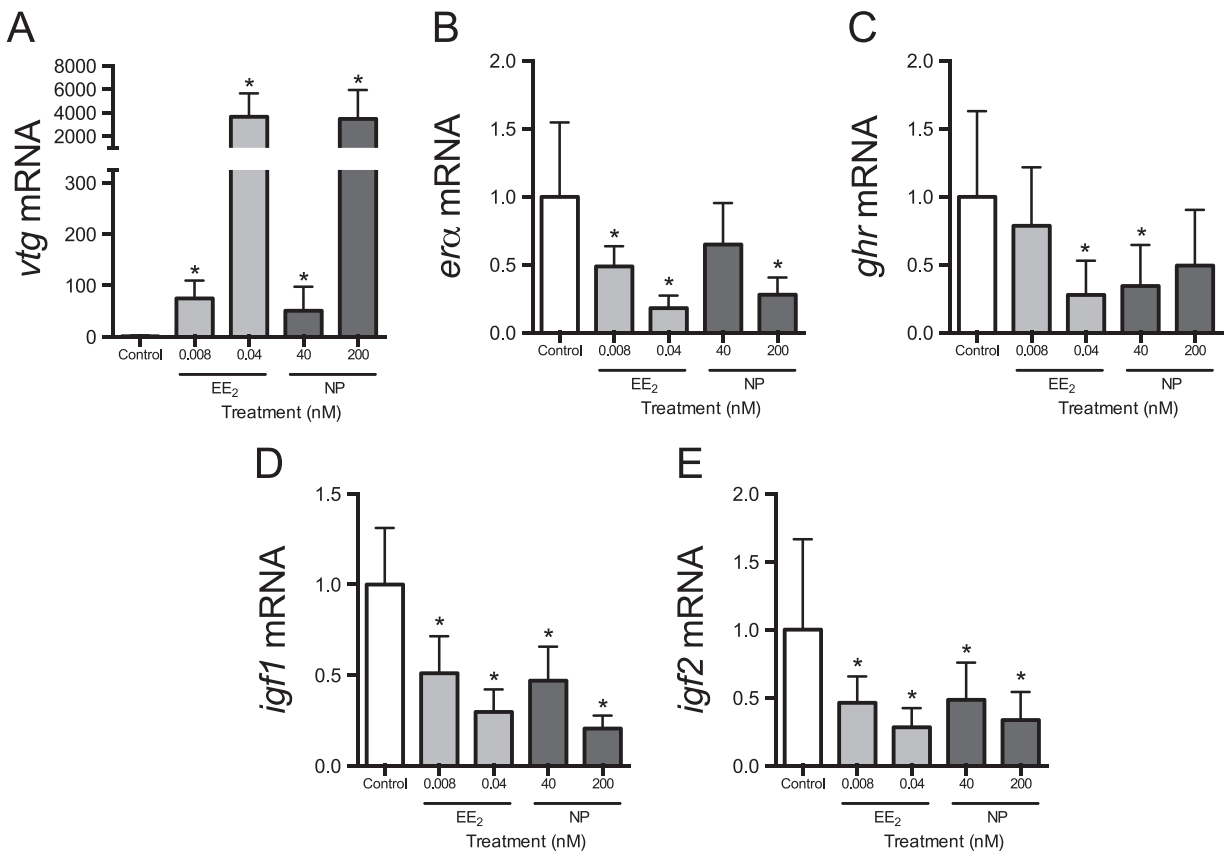

Fig. 2. Hepatic *vtg* (A), *erα* (B), *ghr* (C), *igf1* (D) and *-2* (E) mRNA levels in Atlantic salmon fry after 21-day exposure to water containing 0 (control), 0.008 or 0.04 nM 17α-ethinylestradiol (EE_2) or 40 or 200 nM 4-nonylphenol (NP). mRNA levels are presented as a fold-change from the control group. Asterisk indicates a significant difference between treatment and control group (One-way ANOVA; Dunnett's test; $P < 0.05$). Means ± SD (n = 8–10).

(Fig. 2A). *erα* mRNA levels were diminished following exposures to 0.008 and 0.04 nM EE_2, while only the 200 nM concentration of NP reduced *erα* levels (Fig. 2B). *ghr* mRNA levels were significantly lower than controls following treatment with 0.04 nM EE_2 and 40 nM NP (Fig. 2C). *igf1* and *-2* levels were significantly reduced following all exposures to EE_2 and NP (Fig. 2D, E). *ghr* levels were significantly correlated with *igf1* ($r^2 = 0.44$) and *igf2* levels ($r^2 = 0.38$).

Among the assayed hepatic *igfbp* isoforms, all transcripts, with the exception of *igfbp1b1* and *-5a* were reduced from control levels following 0.008 and 0.04 nM EE_2 treatments (Fig. 3B–D, F, G). Hepatic *igfbp1b1* transcription was suppressed only by 0.04 nM EE_2 (Fig. 3A), *igfbp5a* levels were enhanced > 70-fold (Fig. 3E). Only *igfbp2b1* (Fig. 3C) and *-5b2* (Fig. 3F) were significantly reduced following exposure to 40 nM NP, while 200 nM NP reduced *igfbp1b1*, *-2a*, *-4*, *-5b2* and *-6b1* (Fig. 3A, B, D, F, G). 200 nM NP elicited a > 100-fold increase in *igfbp5a* levels (Fig. 3E).

3.2. 4-day treatment of smolts with EE_2, E_2 and NP: Mortality, growth, plasma hormones, and hepatic and branchial gene expression

There were no mortalities during the 4-day EDC exposures. As expected, the 4-day exposure treatments of fasted fish did not significantly affect their BM, BL or CF (data not shown). Plasma Gh (Fig. 4A) and Igf1 (Fig. 4B) levels were similarly reduced in smolts following 4-day exposure to 0.4 nM EE_2. There were no effects of E_2 or NP on plasma Gh and Igf1.

Hepatic *vtg* and *erα* levels were elevated following exposures to EE_2 (0.04 and 0.4 nM) and E_2 (0.4 and 4.0 nM) but not NP (Fig. 5A, B). *ghr* (Fig. 5C) and *igf1* (Fig. 5D) levels were only impacted by EE_2, with significant reductions following exposures to 0.04 and 0.4 nM EE_2. There were no significant differences among the treatment groups regarding *igf2* expression aside from NP at 40 nM inducing expression compared with controls (Fig. 5E). *ghr* levels were significantly correlated with *igf1* ($r^2 = 0.19$) and *igf2* levels ($r^2 = 0.12$).

Hepatic *igfbp1b1* levels were diminished following exposure to 0.04 and 0.4 nM EE_2 and all tested concentrations of E_2 (Fig. 6A). Unlike in fry, in smolts *igfbp2a* levels were not affected by any of the tested compounds (Fig. 6B). *igfbp2b1* was induced following 40 nM NP exposure, but not by EE_2 or E_2 (Fig. 6C). *igfbp4* levels were diminished by 0.4 and 4.0 nM concentrations of EE_2 and E_2, respectively (Fig. 6D). *igfbp5a* levels were induced by 0.04 and 0.4 nM EE_2 and 4.0 nM E_2, whereas there were no effects of NP (Fig. 6E). *igfbp5b2* did not exhibit any responses to any of the tested compounds (Fig. 6F). *igfbp6b1* was diminished by exposure to all tested EE_2 concentrations and by 0.4 and 4.0 nM E_2 (Fig. 6G).

There were no significant effects of 4-day EDC exposures on branchial *nka-α1a*, *nka-α1b*, *nkcc1* and *cftr1* transcript levels (Fig. 7). No significant differences based on sex were found for any morphological or physiological parameters.

4. Discussion

To our knowledge, we provide the first evidence that *in vivo* exposure to EDCs impacts *igfbps* in vertebrates. The two experimental paradigms in the current study allowed us to first assess how long-term exposure of resident (and feeding) FW fry to EDCs impacted growth physiology, and secondly, how EDCs may elicit acute effects on migrating (and fasting) smolts. We observed marked effects of EDCs on plasma hormone and gene expression patterns at various levels of the Gh/Igf/Igfbp system. These endocrine responses indicate that EDCs elicit potentially deleterious consequences upon the growth, reproduction, and survival of exposed individuals. Below, we pay

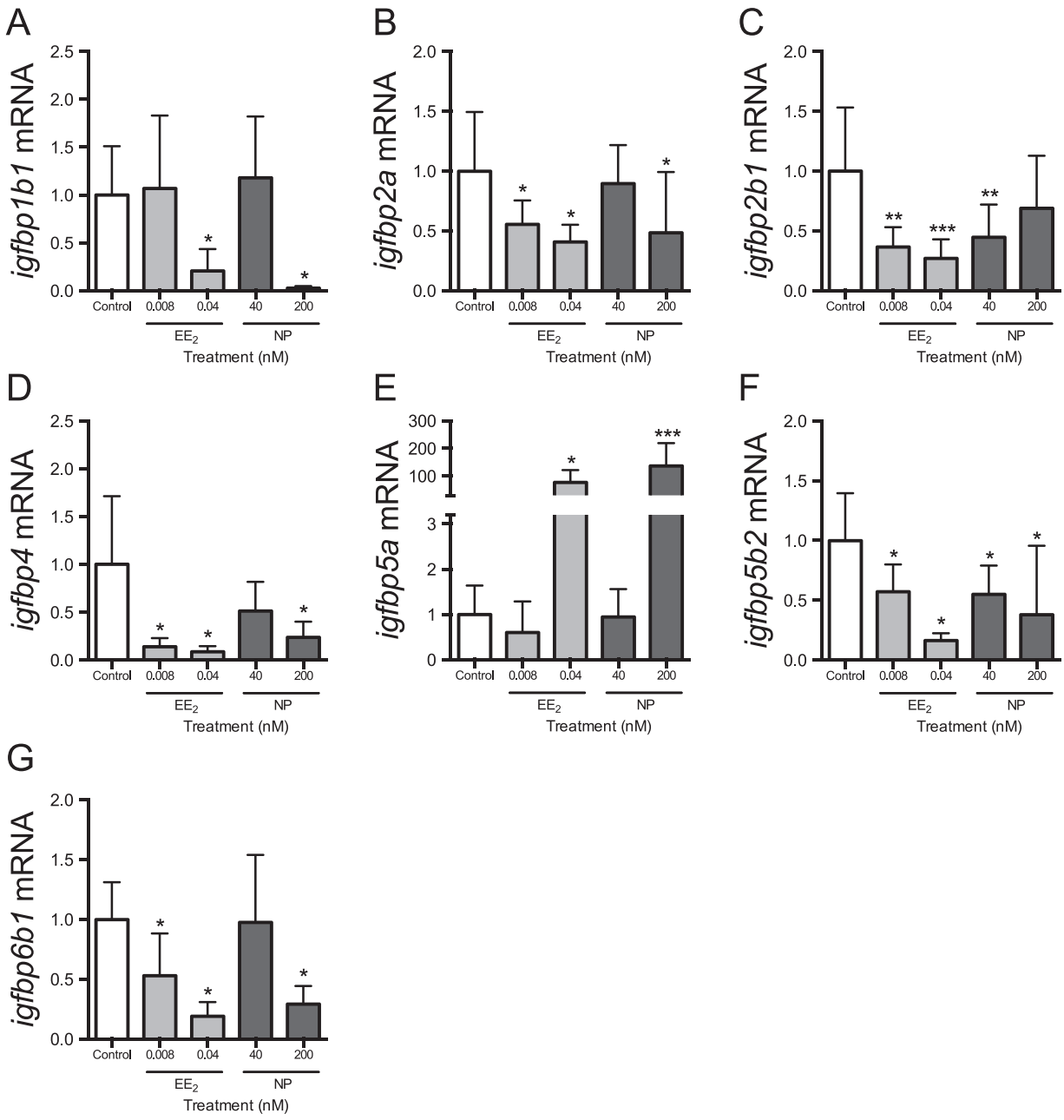

Fig. 3. Hepatic *igfbp1b1* (A), *-2a* (B), *-2b1* (C), *-4* (D), *-5a* (E), *-5b2* (F) and *-6b1* (G) mRNA levels in Atlantic salmon fry after 21-day exposure to water containing 0 (control), 0.008 or 0.04 nM 17α-ethinylestradiol (EE_2) or 40 or 200 nM 4-nonylphenol (NP). mRNA levels are presented as a fold-change from the control group. Asterisk indicates a significant difference between treatment and control group (One-way ANOVA; Dunnett's test; $P < 0.05$). Means ± SD (n = 8–10).

particular attention to whether EDCs directly and/or indirectly (via endocrine intermediaries) modulate the transcription of *igfbp* genes expressed in liver. We discuss the functional consequences of these interactions in light of the current state of knowledge regarding the activities of the members of this expansive gene family.

Teleosts from various clades exhibit impaired growth performance when exposed to estrogenic compounds (Aluru et al., 2010; Ashfield et al., 1998; Hanson et al., 2014; Shved et al., 2007, 2008). The 21-day exposure of fry to NP elicited detectable changes in size, with the low and high doses eliciting reductions in BM and BL, respectively (Fig. 1A, B). To initially assess whether individuals elicited physiological responses to the tested EDC concentrations, we assayed two established targets of estrogenic compounds, *vtg* and *erα* (Bowman et al., 2002). In fry, EE_2 and NP induced hepatic *vtg* in association with reductions in *erα* levels (Fig. 2A, B), whereas induction of *vtg* by EE_2 and E_2 in smolts coincided with increased *erα* levels (Fig. 5A, B). Dissimilar *erα* responses to EE_2 seem linked to the duration of treatment. While an increase in *erα* following E_2 further enhances the *vtg* transcriptional response to E_2 (Bowman et al., 2002), the down-regulation of *erα* with prolonged exposure would seemingly act to counteract chronically high levels of estrogenic compounds. The cellular mechanisms of such a negative feedback scenario have not been proposed. Shved et al. (2008) similarly observed that hepatic *erα* was diminished in Nile tilapia (*Oreochromis niloticus*) chronically (> 20 days) exposed to EE_2; this study did not report *vtg* expression patterns. Interestingly, we did not observe an associated drop in *vtg* expression with decreased *erα* levels (Fig. 2A, B).

In the current study, EDCs elicited effects at multiple levels of the Gh/Igf/Igfbp system. At the level of the pituitary, EE_2, NP and/or wastewater treatment plant effluent affected numerous gene transcripts in Atlantic and coho salmon (*Oncorhynchus kisutch)*, particularly genes

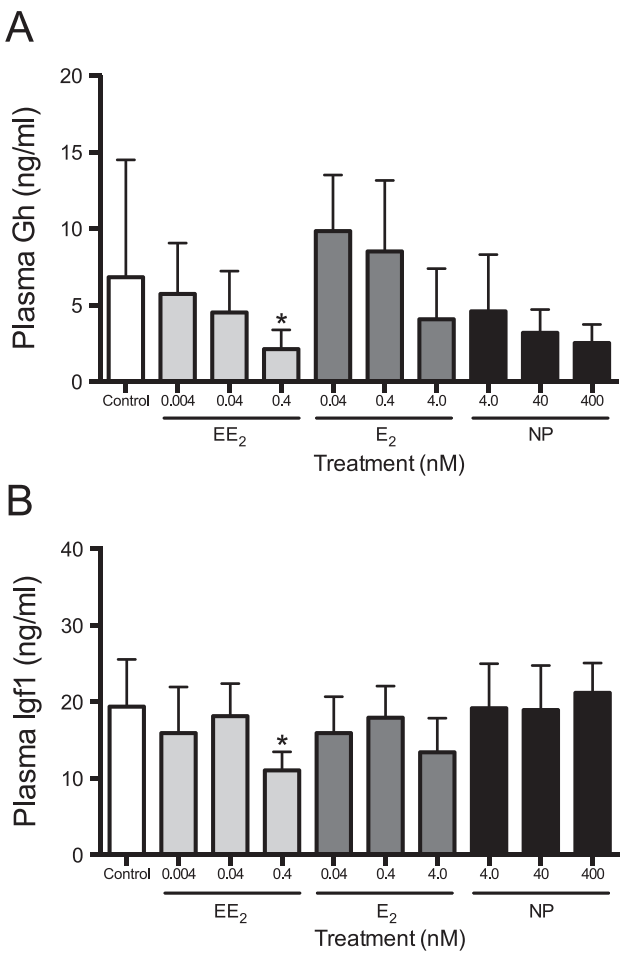

Fig. 4. Plasma Gh (A) and Igf1 (B) levels in Atlantic salmon smolts after 4-day exposure to water containing 0 (control), 0.004, 0.04 or 0.4 nM 17α-ethinylestradiol (EE_2), 0.04, 0.4 or 4.0 nM 17β-estradiol (E_2), or 4.0, 40 or 400 nM 4-nonylphenol (NP). Asterisk indicates a significant difference between treatment and control group (One-way ANOVA; Dunnett's test; $P < 0.05$). Means ± SD (n = 8–14).

encoding pituitary gonadotropins (Harding et al., 2013, 2016; Yadetie and Male, 2002). With both stimulatory and inhibitory effects of estrogenic compounds on *gh* expression and/or Gh secretion previously described (Elango et al., 2006; Holloway and Leatherland, 1997; Shved et al., 2007, 2008; Zou et al., 1997), the smolt experiment provided sufficient plasma to investigate by RIA whether any of the tested EDCs affected plasma Gh. Here, we observed reduced plasma Gh following EE_2 treatment (Fig. 4A). While this pattern may reflect a direct effect of EE_2 on somatotropes expressing estrogen receptors (Kah et al., 1997), the absence of estrogen-response elements in teleost *gh* genes suggests that intermediaries may be necessary (Chen et al., 1994; Filby et al., 2006). Indeed, E_2 is known to impact a complex network of hypothalamic regulators of Gh (Canosa et al., 2007), and EE_2 could operate in a similar fashion. In either case, the effect of EE_2 on Gh levels occurred when endogenous levels are otherwise elevated at the peak of smoltification (Björnsson et al., 1995). In parallel with decreased plasma Gh, we observed attendant reductions in plasma Igf1 and hepatic *igf1* at the highest concentration of EE_2 (Figs. 4B, 5D). In fry, EE_2 and NP similarly reduced hepatic *igf1* (Fig. 2D). The consistent reductions in Igf1/*igf1* in fry and smolts seen here and in a previous study (McCormick et al., 2005) were likely mediated by the combination of decreased plasma Gh and diminished sensitivity of hepatocytes to Gh at the receptor level (Fuentes et al., 2012; Hanson et al., 2014; Pierce et al., 2005). Accordingly, we observed correlations between hepatic *ghr* and *igf1* levels in addition to reduced *ghr* levels following EE_2 and NP exposures (Figs. 2C, 5C). Diminished *ghr* gene expression paralleled reductions in Gh binding capacities following treatment with estrogenic compounds (Hanson et al., 2017; Lerner et al., 2012; Norbeck and Sheridan, 2011). Only a few studies to date have investigated the direct effects of estrogens on *ghr* expression in cultured hepatocytes (Hanson et al., 2014, 2017; Norbeck and Sheridan, 2011). While it is unresolved whether estrogen receptors interact with *ghr* gene promoters (Leung et al., 2004), co-treatment with an estrogen receptor antagonist blocked the inhibition of two *ghrs* by E_2 and NP in trout (Hanson et al., 2017). Given the effects of E_2 on intracellular Ghr signaling (Hanson et al., 2017; Leung et al., 2004), it will be important to identify whether EDCs impact components of JAK/STAT, PI3K, MAPK signaling and/or SOCS-2 in Atlantic salmon.

An important observation made in this study was that EDCs impact hepatic *igf2* levels in fry (Fig. 2E). Growing evidence supports Igf2 as a somatomedin in teleosts (Reindl and Sheridan, 2012). This contrasts with mammalian Igf2, which supports placental and fetal growth, but is not strongly stimulated by Gh during post-natal life (Humbel, 1990). Given that *igf2* was diminished in fry following EE_2 and NP exposures (Fig. 2E), and that *igf2* is both sensitive to E_2 (Davis et al., 2008; Hanson et al., 2014) and stimulates growth (Chen et al., 2000), our findings further underscore that both Igf1 and -2 should be considered when probing the effects of EDCs on teleost growth physiology.

For both fry and smolts, our data indicate a very strong suppression of the Gh/Igf system by environmental estrogens, especially by EE_2. Not only are key hepatic genes of the Gh/Igf system, *ghr*, *igf1*, and *igf2*, all suppressed at both stages, but also in smolts, where such measurements were possible, plasma levels of both Gh and Igf1 were found to be suppressed by high EE_2 levels. At the fry stage, any inhibition of the Gh/Igf system is likely to have consequences for survival, as Gh stimulates appetite, feed intake, foraging activity, dominance and aggression (Jönsson and Björnsson, 2002). These key behavioral traits are essential to sustaining growth during the FW phase of the lifecycle. At the smolt stage, suppression of the Gh/Igf system will, in addition to inhibiting growth, have detrimental impacts on the development of SW tolerance (Madsen et al., 2004; McCormick et al., 2005), a key fitness trait for successful growth and development during the SW phase of the lifecycle.

There is limited information on how steroid hormones regulate Igfbps in both mammals (Rajaram et al., 1997) and fishes (Duan et al., 2010; Reindl and Sheridan, 2012). In this regard, it is particularly noteworthy in the current study that EDCs modulated multiple *igfbp* transcripts in both fry and smolts. Liver is the putative major source of circulating teleost Igfbps (Zhou et al., 2008; Shimizu and Dickhoff, 2017), and similar to other teleosts (Kamei et al., 2008; Pedroso et al., 2009; Shimizu et al., 2011a, b; Zhou et al., 2008), Atlantic salmon exhibit robust expression of *igfbp1* and *-2* paralogs in liver (Macqueen et al., 2013). Among teleost Igfbps, Igfbp1s are best characterized, and play a conserved role in inhibiting somatic growth, development, and glucose metabolism by restricting Igf1 from binding its receptor (Kajimura et al., 2005; Kamei et al., 2008). For example, 22- and 23-kDa Igfbps (putative Igfbp1b paralogs) are important modulators of Igf signaling in response to reduced nutrient availability in chinook (*O. tshawytscha*) and Atlantic salmon (Hevrøy et al., 2011; Shimizu et al., 2005, 2006, 2009). Thus, the decreases in *igfbp1b1* expression following EE_2, E_2 and NP exposures observed in this study (Figs. 3A, 6A) suggest that factors within the Gh/Igf system (hepatic *ghr*, plasma Igf1, and hepatic *igfs*) modulated by EDCs in a fashion generally associated with catabolism may be attenuated (or counterbalanced) by Igfbps. In other words, assuming that decreased *igfbp1b1* corresponded with reductions in hepatic Igfbp1b1 secretion, as shown *in vitro* by Pierce et al. (2006), reduced Igfbp1b1 levels would enhance interactions between plasma Igf1 and its cognate receptor. Furthermore, considering that mammalian Igfbp1 prolongs the half-life of circulating Igf1 (Lewitt et al., 1993), a decrease in Igfbp1b1 levels could also underlie the decreased plasma

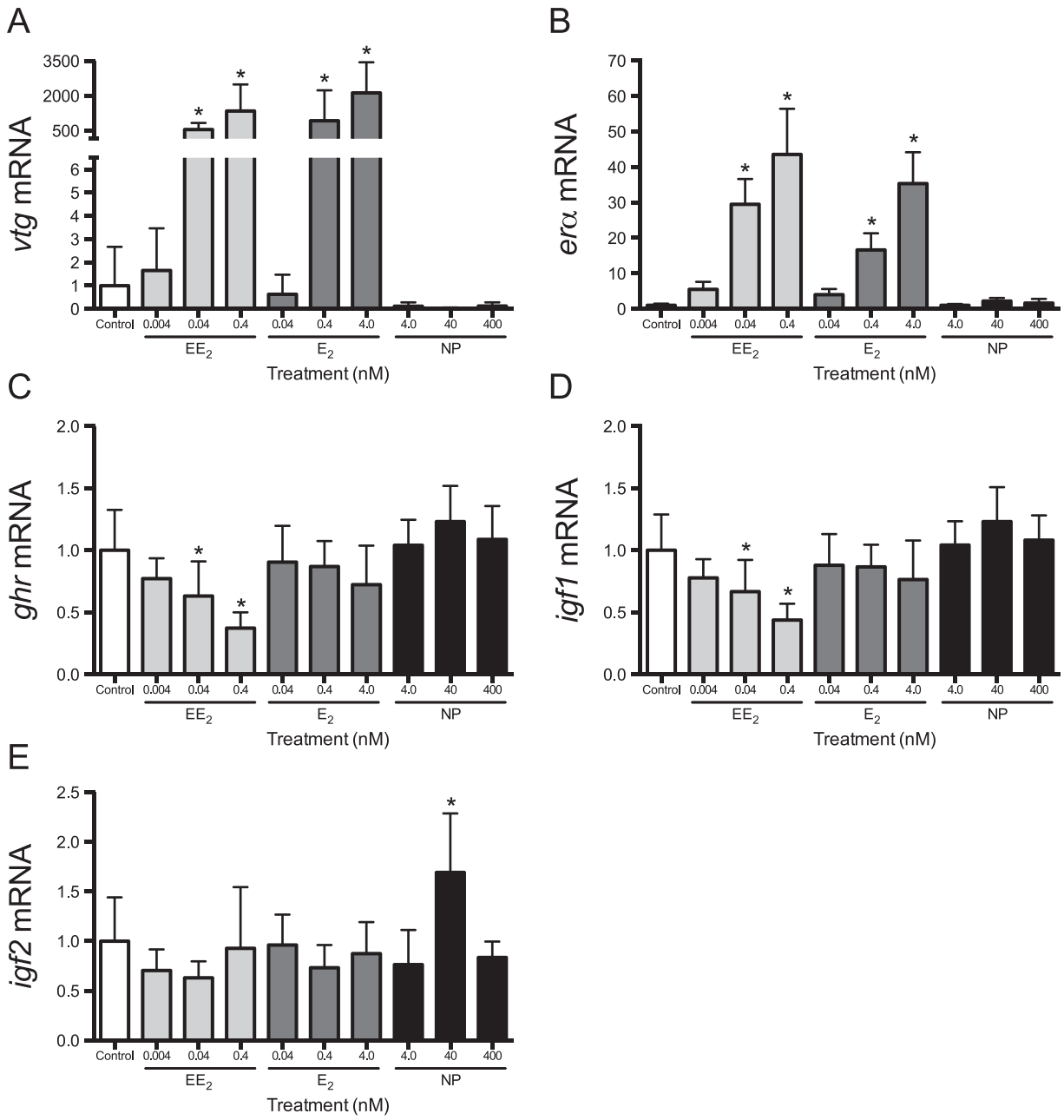

Fig. 5. Hepatic *vtg* (A), *erα* (B), *ghr* (C), *igf1* (D) and *-2* (E) mRNA levels in Atlantic salmon smolts after 4-day exposure to water containing 0 (control), 0.004, 0.04 or 0.4 nM 17α-ethinylestradiol (EE_2), 0.04, 0.4 or 4.0 nM 17β-estradiol (E_2), or 4.0, 40 or 400 nM 4-nonylphenol (NP). mRNA levels are presented as a fold-change from the control group. Asterisk indicates a significant difference between treatment and control group (One-way ANOVA; Dunnett's test; $P < 0.05$). Means ± SD (n = 8–14).

Igf1 seen in the smolts (Fig. 4B). The apparent link between estrogenic compounds and Igfbp1 may be particularly deleterious to salmon when a springtime increase in *igfbp1b1* expression corresponds with smoltification-related changes in body shape and lipid and glycogen utilization (Breves et al., 2017; McCormick and Saunders, 1987; Saunders and Henderson, 1970; Sheridan, 1989). While E_2 similarly inhibited *igfbp1b1* expression (along with hepatic *igf1* and *-2*) in rainbow trout (Cleveland and Weber, 2016; Hultman et al., 2015), E_2 stimulated the release of putative Igfbp1s from striped bass (*Morone saxatilis*) and Mozambique tilapia (*Oreochromis mossambicus)* liver (Fukazawa et al., 1995; Riley et al., 2004), likely reflecting species and/or life stage-dependent responses to estrogenic compounds.

Igfbp2 paralogs are major carriers of plasma Igf1 in salmonids (Shimizu and Dickhoff, 2017). The prevailing 40–50 kDa Igfbp in circulation is not Igfbp3, as in mammals, but an Igfbp2 paralog (Shimizu et al., 2011b). In turn, the attenuation of *igfbp2a* and *-2b1* by EDCs in fry (Fig. 3B, C), but not smolts (Fig. 7B, C), would further augment *igfbp1b1* patterns in controlling the degree to which plasma Igfs are bound. Interestingly, plasma Igfbp3 in postmenopausal women is reduced by EE_2 (Kam et al., 2000), thus it seems the link between EE_2 and Igfbp3 in mammals, and EE_2 and NP on *igfbp2s* in Atlantic salmon, is indicative of a functionally conserved link between estrogens and the prevailing plasma Igfbp. As Gh stimulated Igfbp2b/*igfbp2b* levels in coho salmon (Shimizu et al., 2003) and Mozambique tilapia (Breves et al., 2014), the reductions in *igfbp2a* and *-2b1* seen here following EDC exposure could have arisen secondarily from a primary reduction in Gh signaling (via reduced plasma hormone levels and/or hepatic Gh-sensitivity); future *in vitro* work is required to resolve how estrogenic

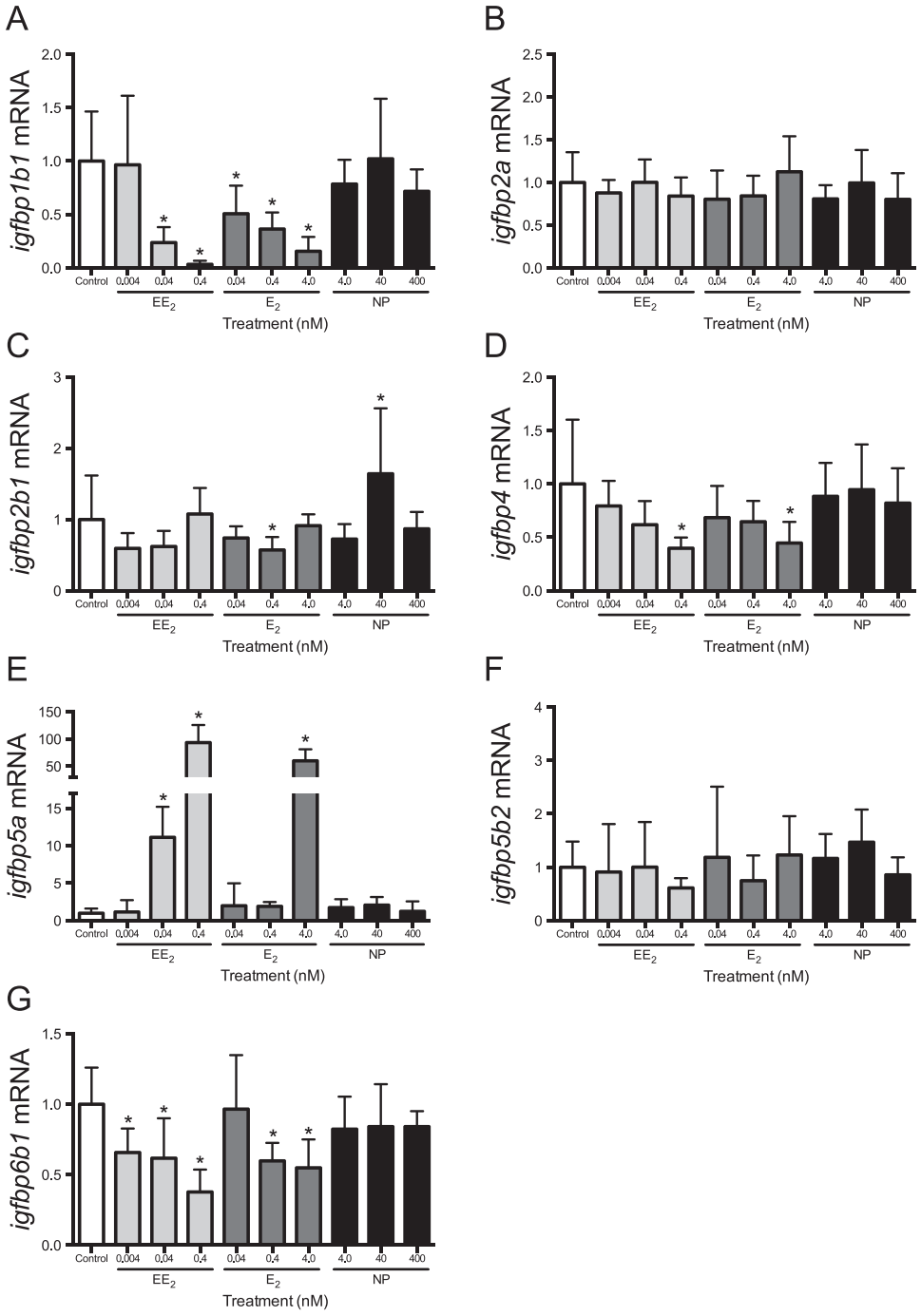

Fig. 6. Hepatic *igfbp1b1* (A), *-2a* (B), *-2b1* (C), *-4* (D), *-5a* (E), *-5b2* (F) and *-6b1* (G) mRNA levels in Atlantic salmon smolts after 4-day exposure to water containing 0 (control), 0.004, 0.04 or 0.4 nM 17α-ethinylestradiol (EE_2), 0.04, 0.4 or 4.0 nM 17β-estradiol (E_2), or 4.0, 40 or 400 nM 4-nonylphenol (NP). mRNA levels are presented as a fold-change from the control group. Asterisk indicates a significant difference between treatment and control group (One-way ANOVA; Dunnett's test; $P < 0.05$). Means ± SD (n = 8–14).

compounds and *igfbp2s* are linked.

Contrasting with *igfbp1* and *-2*, Atlantic salmon *igfbp4*, *-5* and *-6* paralogs exhibit very modest mRNA levels in liver (Macqueen et al., 2013). Thus, rather than contributing to the pool of plasma Igfbps, their translated products likely support autocrine/paracrine activities by governing the degree to which Igfs bind Igf receptors in the tissues where they are produced (Breves et al., 2017; Cleveland and Weber, 2015; Macqueen et al., 2013). Using predicted 3D structures, Macqueen et al. (2013) modeled the interactions between Atlantic salmon Igfs and Igfbps and proposed that Igfbp4 and -5 potentiate Igf signaling, a prediction consistent with enhanced muscle *igfbp4* expression during periods of rapid growth (Bower et al., 2008). In turn, the reductions in *igfbp4* following EDC exposures in both fry and smolts (Figs. 3D, 6D) could thereby reduce Igfs from binding to hepatic Igf receptors that are already in low abundance (Maures et al., 2002). There is no information in teleosts on whether secreted Igfs feedback upon hepatocyte function. Nonetheless, in rat, Igf1 promotes the proliferation of hepatic stellate cells (Skrtic et al., 1997), which are adjacent to hepatocytes and stimulate hepatocyte proliferation via paracrine signaling. Clearly, the physiological link between *igfbp4* and EDCs identified here remains unresolved, and further work should seek to determine the role of *igfbp4* in growth and differentiation of the liver and other tissues.

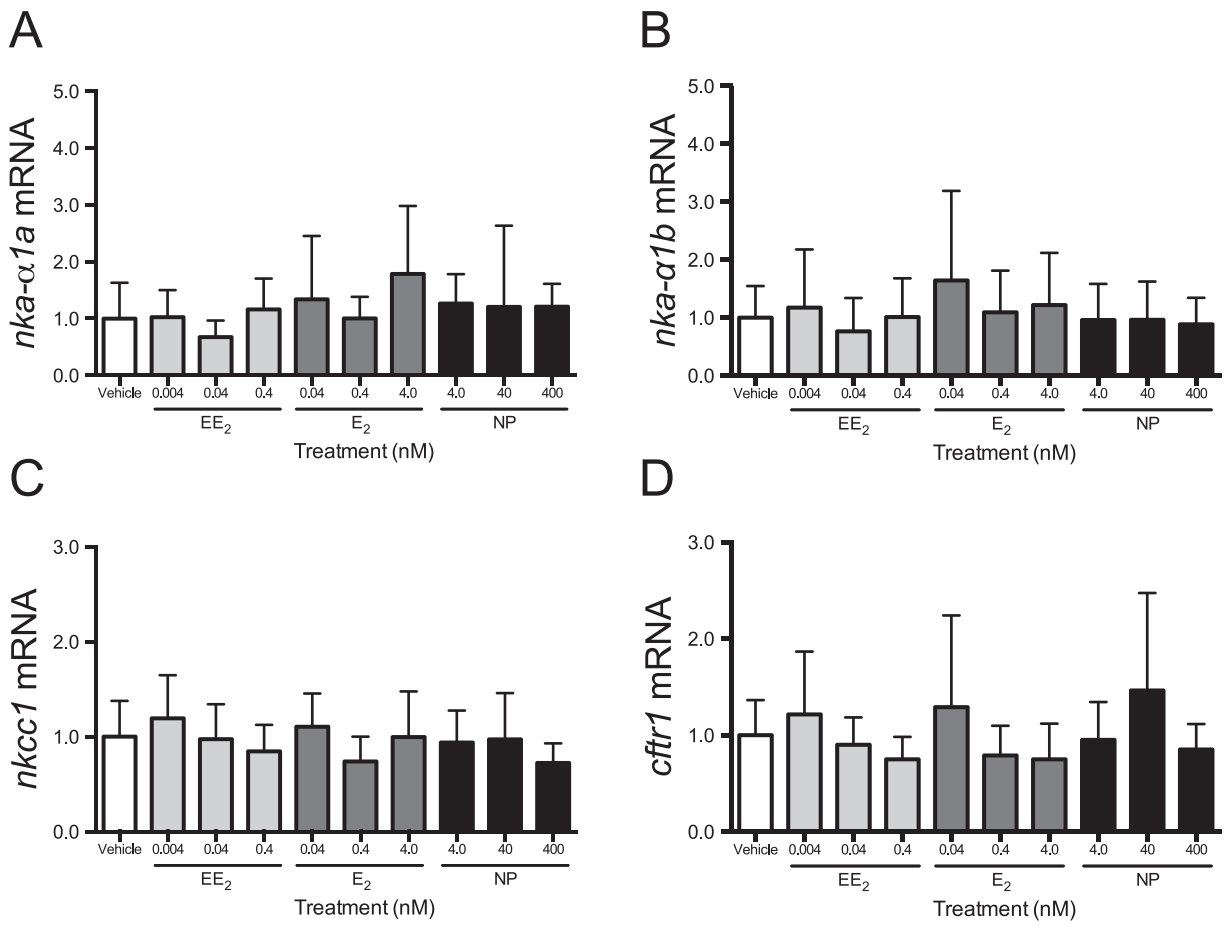

Fig. 7. Branchial *nka-α1a* (A), *nka-α1b* (B), *nkcc1* (C) and *cftr1* (D) mRNA levels in Atlantic salmon smolts after 4-day exposure to water containing 0 (control), 0.004, 0.04 or 0.4 nM 17α-ethinylestradiol (EE_2), 0.04, 0.4 or 4.0 nM 17β-estradiol (E_2), or 4.0, 40 or 400 nM 4-nonylphenol (NP). mRNA levels are presented as a fold-change from the control group. Means ± SD (n = 8–14).

While little is known about its functions in liver, Igfbp5 is one of the major Igfbps expressed in teleost muscle where it mediates anabolic processes (Duan et al., 2010). Accordingly, Igfbp5 was not detected in salmonid plasma (Shimizu and Dickhoff, 2017). In the current study, *igfbp5* paralogs exhibited distinct responses to the tested EDCs; *igfbp5a* was the only *igfbp* transcript induced by EDCs in this study (Figs. 3E, 6E), whereas *igfbp5b2* was diminished in fry, but not smolts, following EDC exposures (Figs. 3F, 6F). *igfbp5b1* was similarly down regulated by E_2 injection in trout (Cleveland and Weber, 2016). These disparate responses by *igfbp5a* and *-5b1/2* are noteworthy given that certain Igfbps exert ligand-independent activities. Among the Igfbp5s in zebrafish (*Danio rerio*), only Igfbp5b, and not Igfbp5a, exhibited ligand-independent transactivational activity (Dai et al., 2010). This distinction between Igfbp5a and -5b paralogs, coupled with their different transcriptional responses to EDCs, provides further indication that sub-functionalization occurred following the duplication of salmonid Igfbp5s (Garcia de la serrana et al., 2017). Salmonid Igfbp5s are highly appropriate to facilitate the further exploration of functional divergence within teleost Igfbps given their disparate responses to not only estrogenic compounds (and other gonadal steroids), but also to corticosteroids (Garcia de la serrana et al., 2017).

Finally, *igfbp6b1* was sensitive to all of the tested compounds (Figs. 3G and 6G). Teleost Igfbp6s attenuate Igf activities that support growth and development, as revealed in embryonic zebrafish (Wang et al., 2009). In mammals, Igfbp6 preferentially binds Igf2 versus Igf1 (Gallicchio et al., 2001). Thus, reductions in *igfbp6b1* could act to enhance Igf2 bioavailability, a pattern that is similar to *igfbp1b1* responses to EDCs (Figs. 3A and 6A).

Knowing that Gh/Igf/Igfbp signaling mediates the development of SW tolerance during parr-smolt transformation (Björnsson, 1997; Breves et al., 2017), and that plasma Gh and Igf1 have been shown to be affected by estrogenic compounds (Madsen et al., 1997, 2004; McCormick et al., 2005; Lerner et al., 2012), we hypothesized that branchial ionocytes (in smolts) would be impacted by exposures to EDCs. However, we did not observe effects of EE_2, E_2, or NP on transcripts that encode essential sub-cellular components of ionocytes, namely *nka-α1a, nka-α1b, nkcc1* and *cftr1* (Fig. 7). Similarly, we did not observe an effect of the tested compounds on branchial *ghr*, *igf1* or *-2* (data not shown). Previous disruptions of smolt development by E_2 and NP via plasma Igf1 levels followed 21- and 7-day exposures, respectively (McCormick et al., 2005; Lerner et al., 2007a). Thus, the absence of changes in branchial gene expression in the present study indicates that effects on the Gh/Igf/Igfbp system require additional time (i.e., greater than the 4 days used in the present study) to manifest changes in the gill. We previously reported that multiple *igfbps* expressed in the gill responded to SW transfer (Breves et al., 2017); thus, a SW challenge paradigm may be a better context to detect acute effects of EDCs on branchial physiology and thus a valuable approach for future studies. Nonetheless, the strong effects of EDCs on the Gh/Igf/Igfbp system in FW smolts suggest that exposures to estrogenic compounds have potentially severe consequences for subsequent survival, growth and reproductive success in SW.

5. Conclusions

The impacts of anthropological factors on the Igfbp system

identified here provide new insight into how EDCs affect the physiological systems of Atlantic salmon during their vulnerable FW life stages. Given that salmonid life-history transitions are interconnected with somatic growth patterns (McCormick and Saunders, 1987) and changes in diet composition (Andreassen et al., 2001; Renkawitz and Sheehan, 2011), Atlantic salmon constitute an important model system to further characterize the impacts of EDCs on growth, the Gh/Igf axis and Igfbps. The *in vivo* approach taken in this study facilitated the characterization of multiple levels of the Gh/Igf/Igfbp system; nonetheless, it limited our capacity to deduce the tissue/cellular-level mechanisms underlying the responses to EDCs. Future work should employ hepatocyte culture (Pierce et al., 2010; Norbeck and Sheridan, 2011) to discern whether EDCs modulate *igfbps* in a direct fashion. Furthermore, as Igfbps regulate extrahepatic Igf signaling (paracrine/autocrine actions) (Azizi et al., 2016; Bower and Johnston, 2010; Castillo et al., 2004; Codina et al., 2008; Eppler et al., 2007), future investigations should also seek to reveal effects of EDCs on muscle *igf1*, *igf2* and *igfbps* (Garcia de la serrana et al., 2017).

Contributions

J.P.B. conceived and designed experiments, collected and analyzed qPCR data, and drafted the manuscript. T.A.D. conceived, designed and conducted experiments. I.E.E. collected and analyzed plasma hormone data. B.T.B. analyzed plasma hormone data and coordinated the study. S.D.M. conceived and designed experiments and coordinated the study. All authors contributed to revising manuscript drafts and approved the final article.

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Role of the funding sources

The funding sources had no involvement in study design, the collection, analysis and interpretation of data, in the writing of the manuscript, or in the decision to submit the article for publication.

Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2018.07.018.

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