Early-life exposure to 17β-estradiol and 4-nonylphenol impacts the growth hormone/insulin-like growth-factor system and estrogen receptors in Mozambique tilapia, *Oreochromis mossambicus*

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Abstract

It is widely recognized that endocrine disrupting chemicals (EDCs) released into the environment through anthropogenic activities can have short-term impacts on physiological and behavioral processes and/or sustained or delayed long-term developmental effects on aquatic organisms. While numerous studies have characterized the effects of EDCs on temperate fishes, less is known on the effects of EDCs on the growth and reproductive physiology of tropical species. To determine the long-term effects of early-life exposure to common estrogenic chemicals, we exposed Mozambique tilapia (*Oreochromis mossambicus*) yolk-sac fry to 17β-estradiol (E2) and nonylphenol (NP) and subsequently characterized the expression of genes involved in growth and reproduction in adults. Fry were exposed to waterborne E2 (0.1 and 1.0 µg/L) and NP (10 and 100 µg/L) for 21 days. After the exposure period, juveniles were reared for an additional 112 days until males were sampled. Gonadosomatic index was elevated in fish exposed to E2 (0.1 µg/L) while hepatosomatic index was decreased by exposure to NP (100 µg/L). Exposure to E2 (0.1 µg/L) induced hepatic *growth hormone receptor* (*ghr*) mRNA expression. The high concentration of E2 (1.0 µg/L), and both concentrations of NP, increased hepatic *insulin-like growth-factor 1* (*igf1*) expression; E2 and NP did not affect hepatic *igf2* and

pituitary *growth hormone* (*gh*) levels. Both E2 (1.0 µg/L) and NP (10 µg/L) induced hepatic *igf binding protein 1b* (*igfbp1b*) levels while only NP (100 µg/L) induced hepatic *igfbp2b* levels. By contrast, hepatic *igfbp6b* was reduced in fish exposed to E2 (1.0 µg/L). There were no effects of E2 or NP on hepatic *igfbp4* and *igfbp5a* expression. Although the expression of three *vitellogenin* transcripts was not affected, E2 and NP stimulated hepatic *estrogen receptor (erα* and *erβ)* mRNA expression. We conclude that tilapia exposed to E2 and NP as yolk-sac fry exhibit subsequent changes in the endocrine systems that control growth and reproduction during later life stages.

Keywords

Endocrine disruption; Growth; Insulin-like growth-factor binding proteins; Liver; Mozambique tilapia; Pituitary

1. Introduction

Particular compounds released into the environment through anthropogenic activities impact the endocrine systems of vertebrates, including fishes (Colborn et al., 1996). These compounds, known as endocrine disrupting chemicals (EDCs), include hormones, pharmaceuticals, pesticides, plasticizers, and naturally occurring compounds. Fish are employed as indicator species for environmental pollution in aquatic systems because they are among the first animals exposed to waterborne chemicals. The adverse activities of EDCs in fish are known to include impacts on fertility, sexual maturation, somatic growth, and circulating hormone levels. Moreover, EDCs can activate stress responses and induce cellular damage, effects that

may increase the incidence of disease and mortality (Ankley et al., 2009; Bernanke and Kohler, 2009; Bhandari et al., 2015; Breves et al., 2018; Celino et al., 2009; Jones et al., 2000; Lerner et al., 2007a, b).

Many EDCs act as agonists or antagonists of estrogen receptors (Er) (cf. Ankley et al., 2009). Among the most pervasive EDCs in the aquatic environment are 17β-estradiol (E2) and nonylphenol (NP) (Aris et al., 2014; Giger et al., 1984; Xu et al., 2014). E2 is one of the most common feminizing compounds found in sewage effluent discharged into rivers (Desbrow et al., 1998). Nonylphenol ethoxylates (NPEs) are widely used as surfactants in industrial processes and products, including cleaners, detergents, and plastics. As in the case of E2, NPEs are also discharged through domestic and industrial wastewater (Mao et al., 2012; Servos et al., 2003). NPEs are degraded into NP, which persists in the environment (Ahel et al., 1993). Free NP is presumed to be widely distributed in surface waters (Ekelund et al., 1990; Ekelund et al., 1993) with concentrations ranging from approximately 30 to 30,000 ng/L in Guangzhou riverine waters in China, the Seine estuary in France, and the European river basin in Spain (Brix et al., 2010; Cailleaud et al., 2007; Peng et al., 2008). NP accumulates in various aquatic organisms at concentrations ranging from 0.68–160 ng/g tissue weight (Vethaak et al., 2005; Zhou et al., 2019) . NP exerts feminizing effects in mice (Hernandez et al., 2006), reduces fecundity and fertility in Japanese medaka (*Oryzias latipes*) (Ishibashi et al., 2006; Kang et al., 2003), reduces semen volume in rainbow trout (*Oncorhynchus mykiss*) (Lahnsteiner et al., 2005), and diminishes plasma testosterone in male carp (*Cyprinus carpio*) (Amaninejad et al., 2018). Moreover, the presence of NP and NPEs in the environment was linked to a low male:female sex ratio in wild Nile tilapia (*Oreochromis niloticus*) (Chen et al., 2014). Most studies reporting on the effects of E2 and NP on growth and reproduction in fishes have been conducted with

93 temperate species (Harries et al., 2000; Filby et al., 2006; Goetz et al., 2009; Duffy et al., 2014;
94 Breves et al., 2018).

95 Given its importance to worldwide aquaculture (FAO, 2005), the Mozambique tilapia
96 (*Oreochromis mossambicus*) is one of the most thoroughly studied tropical fishes with respect to
97 how environmental conditions impact growth and reproductive endocrinology (Davis et al.,
98 2009a; Davis et al., 2009b; Gaigher and Krause, 1983; Kiilerich et al., 2011; Moorman et al.,
99 2016; Kajimura et al., 2005). Tilapia are widely distributed in tropical areas where they are
100 cultured for human consumption. They inhabit regions where agricultural, municipal, and
101 industrial waters are discharged and are therefore exposed to persistent environmental EDCs
102 based on the contaminants detected in their tissues (Authman et al., 2008; Babu and Ozbay,
103 2013; Hemmatinezhad et al., 2017; Osman et al., 2012).

104 The endocrine system of fishes mediates the effects of environmental stimuli, including
105 contaminants, on growth and reproduction. The growth hormone (Gh)/insulin-like growth-factor
106 (Igf) system plays a major role in regulating the growth and development of vertebrates,
107 including teleosts (Duan et al., 2010; Reindl and Sheridan, 2012). Upon binding to the Gh
108 receptor (Ghr), Gh stimulates the release of Igf1 which has growth-promoting actions in target
109 tissues (Butler and Le Roith, 2001; Duan, 1998; Fan et al., 2009; Le Roith et al., 2001; Le Roith
110 and Roberts, 2003). Igfs interact with a family of binding proteins, known as Igf binding proteins
111 (Igfbps), which influence their availability and activities (Duan and Xu, 2005; Duan et al., 2010;
112 Rajaram et al., 1997) and teleost fishes possess an expanded suite of Igfbps (Allard and Duan,
113 2018). Steroid hormone receptors mediate target-tissue responsiveness to the actions of steroid
114 hormones, in addition to compounds that mimic hormone actions (Park et al., 2007; Gross and
115 Yee, 2002). The production of vitellogenin (Vtg), a precursor of egg yolk protein produced by

the liver of female oviparous animals (Denslow, 1999; Hiramatsu et al., 2005), is stimulated by activation of *ers* and Ers (Bowman et al., 2002; Flouriot et al., 1996; Jalabert, 2005; Nelson and Habibi, 2010). These same Ers are the pathway in which estrogenic EDCs interfere with normal estrogen signaling.(Shanle and Xu, 2011). Hence, Vtg/*vtg* and Ers/*ers* are often used as indicators of estrogenic EDC exposure (Jones et al., 2000; Matozzo et al., 2008; Leet et al., 2011; Park et al., 2007). Plasma Vtg has been detected in male white sucker (*Catostomus commersoni*) and rainbow trout inhabiting waters contaminated by sewage effluent (Purdom et al., 1994; Vajda et al., 2008). Estrogenic EDCs such as E2, 17α-ethinylestradiol (EE2), diethylstilbestrol, and NP induce *vtg* expression and plasma Vtg levels in male fish (Davis et al., 2007; Davis et al., 2009b; Folmar et al., 2000; Hemmer et al., 2001). In male tilapia, injection of E2 induces Vtg production while concurrently suppressing the Gh/Igf system (Davis et al., 2007; Davis et al., 2008). Moreover, in juvenile Atlantic salmon, *vtg* transcripts were induced, while hepatic *er*, *ghr*, *igfs*, and several *igfbps* were reduced following waterborne exposure to EE2 and NP (Breves et al., 2018). Filby et al. (2006) previously showed that male fathead minnows (*Pimephales promelas*) exposed to E2 exhibited reduced hepatic *igf1* expression levels. Here, we examined the long-term effects of waterborne exposure to NP and E2 (as positive control) in Mozambique tilapia. We reared tilapia fry in water containing E2 (0.1 and 1.0 µg/L) and NP (10 and 100 µg/L) for 21 days, and then measured the expression of pituitary *gh*, and hepatic *ghr*, *igfs*, *igfbps*, *vtgs*, and *ers* after an additional 112 days of growth and development.

2. Materials and methods

2.1 Animals

Mozambique tilapia yolk-sac fry were obtained from broodstock tanks maintained in dechlorinated city water at the Hawai‘i Institute of Marine Biology (Kāne‘ohe, HI). Fry were initially reared in 7-L conical tanks supplied with filtered dechlorinated city water for 2-3 days. Fry were then distributed to 5-L aerated flow-through tanks (33 fry/tank) supplied by 19-L header tanks and reared for an additional 5-10 days until yolk absorption was ~90% complete. Header and fish-holding tanks were lined with modified polytetrafluoroethylene (MPTFE) (Welch Fluorocarbon, Inc., Dover, NH). Two replicate tanks were used for each treatment and subsequent rearing. Water temperature was maintained at ~26-28 °C under a 12L:12D photoperiod. After yolk absorption, fry were fed crushed trout chow pellets (Skretting, Tooele, UT) twice daily for the remainder of the experiment. Fecal material and uneaten food were siphoned out from the tanks before 60-70% of the water volume was changed daily. All housing and experimental procedures were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee of the University of Hawai‘i.

2.2 Chemical exposures and rearing

Fry (0.029 ± 0.001 g body weight) were exposed to E2 (0.1 and 1.0 µg/L) and NP (10 and 100 µg/L) in fresh water for 21 days. The duration of the exposures was chosen following previous studies aimed at identifying responses to chronic EDC exposures (Woltering, 1984; Lerner et al., 2007a, 2007b). The range of nominal concentrations of E2 used in this study were above those typically found in the environment inasmuch as they were included as a positive control. The concentrations of E2 and NP were based on those employed in previous studies where Atlantic salmon yolk-sac larvae, fry, juvenile, and smolts were subjected to the aqueous exposure of these chemicals; the concentrations of the chemicals in the water have been previously validated (Breves et al., 2018; Duffy et al., 2014; Lerner et al., 2007a, b). E2 and NP

were purchased from Sigma-Aldrich (St. Louis, MO) and Acros Organics (Fair Lawn, NJ). All chemicals were solubilized in ethanol and then added to fresh water at a final concentration of 0.0001% ethanol to minimize solvent toxicity. Control treatments received solvent only. Header tanks were covered and refilled every two days with filtered and dechlorinated city water with or without the experimental chemicals. The flow-rate from the header tanks to the rearing tanks averaged 0.2 L/h. The fry were maintained in 5-L MPTFE-lined tanks until the end of the exposures (see Fig. 1 for the experimental setup) at which time the juveniles were transferred to 19-L aerated flow-through MPTFE-lined containers and reared for an additional 112 days until sampling. MPTFE was used to prevent the leaching of chemicals from the plastic containers used as tanks and header buckets. This approach has been previously used in a similar study employing coho salmon (Harding et al, 2016). Bodyweight (BW) and total length (TL) were measured every two weeks throughout the duration of the experiment.

At the end of the experiment, male tilapia were netted and anesthetized with 2-phenoxyethanol (0.3ml/L; Sigma-Aldrich) and BW and TL were measured. Anesthetized fish were euthanized by rapid decapitation. Testes and liver were removed and weighed for calculation of gonadosomatic index (GSI; (gonad weight/BW)*100) and hepatosomatic index (HSI; (liver weight/BW)*100). Condition factor (CF) was calculated as $CF = (BW/TL^3)*100$. Liver and pituitary were collected, immediately snap-frozen in liquid nitrogen, and stored at -80 °C until RNA extraction.

2.3 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from liver and pituitary using TRI Reagent (MRC, Cincinnati, OH) according to the manufacturer's protocols. The concentration and purity of extracted RNA

were assessed using a NanoDrop (NanoDrop One, Thermo Scientific). Total RNA (100-500 ng) was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The mRNA levels of reference and target genes were determined by the relative quantification method using a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The qRT-PCR reaction mix (15 µL) contained Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 200 nM of forward and reverse primers, and 1 µl cDNA. Dilution of experimental cDNA from liver ranged from 10- to 175-fold. PCR cycling parameters were: 2 minutes at 50 °C, 10 minutes at 95 °C followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. All qRT-PCR primers have been previously described; PCR efficiencies are reported in Table 1. Since hepatic *elongation factor 1α* (*ef1α*), *ß-actin*, and *18s* levels varied across treatments, the geometric mean ($\sqrt[3]{x_1 * x_2 * x_3}$; where x= quantity of each reference gene) of these reference genes was used to normalize target genes in the liver. *ef1α* levels were used to normalize pituitary *gh* expression after verification that *ef1α* expression did not vary across treatments. Data are expressed as a fold-change relative to control.

2.4 Statistical analysis

Group comparisons were performed by one-way ANOVA followed by Fisher's protected LSD test. In order to meet assumptions of normality (assessed by Kolmogorov-Smirnov), individual values were log-transformed when necessary prior to statistical analysis. Pearson correlation coefficients were used to describe the relationships between *gh, ghr, igf1,* and *igf2* mRNA levels. Statistical calculations were performed using Prism 8.0 (GraphPad, La Jolla, CA). Significance for all tests was set at $P < 0.05$.

208 **3. Results**

209

210 *3.1 Effects of E2 and NP on physical characteristics*

211 BW was significantly higher in fish exposed to 10 µg/L NP compared with controls (Fig.
212 2A) whereas TL and CF were unaffected by E2 and NP (Fig. 2B, C). The high concentration of
213 NP (100 µg/L) reduced HSI relative to controls (Fig. 2D). GSI was elevated in fish exposed to
214 E2 at 0.1 µg/L (Fig. 2E).

215

216 *3.2 Effects of E2 and NP on ghr, igf1, igf2, and gh gene expression*

217 Hepatic *ghr* levels were >2-fold higher in fish exposed to E2 (0.1 µg/L) compared with
218 controls; E2 at 1 µg/L and NP at both concentrations did not impact *ghr* levels (Fig. 3A). By
219 contrast, hepatic *igf1* was elevated >2-fold in fish exposed to the high concentration (1 µg/L) of
220 E2 and >1-fold to 2-fold to both concentrations of NP (Fig. 3B). Hepatic *igf2* levels were highly
221 variable and were not impacted by E2 and NP (Fig. 3C). Pituitary *gh* levels in the E2 and NP
222 treatments were not different from control levels (Fig. 3D). Hepatic *ghr* was significantly
223 correlated with *igf1* (r^2=0.33) and *igf2* (r^2=0.22).

224

225 *3.3 Effects of E2 and NP on igfbp gene expression*

226 Exposures to E2 and NP at 1 and 10 µg/L, respectively, induced hepatic *igfbp1b* levels
227 by >2-fold from controls (Fig. 4A). Fish exposed to NP (100 µg/L) exhibited elevated *igfbp2b*
228 levels compared with controls (Fig. 4B). *igfbp4* and *igfbp5a* levels were unaffected by E2 and
229 NP exposures (Fig. 4C, D) whereas *igfbp6b* was the only *igfbp* transcript suppressed by E2 (Fig.
230 4E).

231

232 *3.4 Effects of E2 and NP on vtg and er gene expression*

233 Although all three *vtg* transcripts had a tendency to be elevated in fish exposed to E2, no
234 significant effects of E2 or NP were detected (Fig. 5A-C). On the other hand, *erα* levels were
235 increased following exposures to all tested concentrations of E2 and NP (Fig. 5D); *erβ* levels
236 were stimulated by E2 and NP at 0.1 and 100 µg/L, respectively (Fig. 5E).

237

238 **4. Discussion**

239 The objective of this study was to determine whether Mozambique tilapia exposed to
240 estrogenic chemicals as fry exhibit long-term responses that impact physiological systems
241 underlying growth and reproduction as adults. Mature tilapia exhibit concentration-dependent
242 responses to estrogenic compounds commonly found in sewage effluents, such as E2, o,p'-DDE
243 (dichlorodiphenyl dichloroethene), heptachlor, and NP (Davis et al., 2007; Davis et al., 2008;
244 Davis et al., 2009b). Little is known, however, on whether exposure to estrogenic compounds
245 during early-life stages may impart long-term physiological effects on adult tilapia. To our
246 knowledge, this is the first study demonstrating that early-life exposure to estrogenic EDCs
247 affects the Gh/Igf system and *er* expression in adult tilapia. At very low levels, E2 and NP still
248 elicited physiological responses in exposed individuals.

249 In previous studies, BW was significantly reduced in fry and adult male fish after
250 exposure to estrogenic compounds such as EE2 and NP (Meredith et al., 1999; Breves et al.,
251 2018). In the current study, however, the BW of adults exposed to NP (10 µg/L) as fry was
252 actually greater than controls. This may be attributed to a capacity for the somatotropic axis to
253 compensate for poor growth during early life-stages (Bertram et al., 1993; Chambers et al, 1998;

Gagliano and McCormick, 2007; Segers et al., 2012), in this case when the EDCs were present. Compensatory growth following periods of suppressed growth, such as food restriction, occurs in several teleosts, including striped bass (*Morone saxatilis*), Atlantic halibut (*Hippoglossus hippoglossus*), channel catfish (*Ictalurus punctatus*), rainbow trout, and hybrid tilapia (*O. mossambicus* x *O. niloticus*) (Gaylord and Gatlin 2000; Heide et al., 2006; Montserrat et al., 2007; Picha et al., 2008). In the current study, we observed that HSI was lower in fish exposed to NP (100 µg/L) as fry. Since the liver is a major site for metabolism, detoxification, and vitellogenesis, there are a variety of factors that likely contributed to this response (Roberts, 2012; Asem-Hiablie et al., 2013). HSI is naturally elevated during reproductive periods as a result of increased protein synthesis and Vtg production (Jia et al., 2019). In salmon fry and smolts, E2, EE2, and NP elevated HSI (Lerner et al., 2012; Duffy et al., 2014). While elevations in HSI have been correlated with the occurrence of xenobiotics in polluted zones (Karels et al., 1998; Billiard and Khan, 2003), in some cases, such as in Mozambique tilapia, African catfish (*Clarias gariepinus*), spotted pim (*Pimelodus maculatus*), and Japanese medaka, lower HSIs were observed in fish exposed to sewage effluents and agricultural runoffs (Ma et al., 2005; Asem-Hiablie et al., 2013; Sadekarpawar and Parikh, 2013; Araújo et al., 2018). Further investigation is needed to determine the mechanisms that underlie reductions in HSI following EDCs exposures.

GSI has been extensively used as an indicator of sexual maturation as well as a biomarker for exposure of aquatic organisms to estrogenic EDCs. Several laboratory studies have reported that exposure to estrogenic chemicals inhibits testicular development (Gimeno et al., 1997; Komen et al., 1989; Christiansen et al., 1998). Field studies have also documented a correlation between estrogenic compounds and lower GSI in exposed male fish (Andersson et al., 1988;

277 Harries et al, 1997; Kukkonen et al., 1999; Hassanin et al., 2002). In the current study, we
278 observed that fry exposed to E2 (0.1 µg/L) exhibited elevated GSI as juvenile males. Jobling et
279 al. (1996) found that the inhibitory effects of estrogenic compounds on sexually maturing
280 rainbow trout were not evident in mature or regressing fish. In juvenile male salmon, a relatively
281 low concentration of E2 (2 µg/L) increased GSI (Lerner et al., 2007a). Furthermore, E2 also
282 plays a role in male gonad development. Although high concentrations of E2 could inhibit
283 testicular development in some fishes such as Japanese eel (*Anguilla japonica*) and three-spot
284 wrasse (*Halichoeres trimaculatus*), low concentrations of E2 were found to induce
285 spermatogonial stem cell renewal and spermatogonial proliferation, suggesting a modulatory role
286 of E2 in normal testicular development (Miura et al., 1999, 2003; Kobayashi et al., 2011). These
287 previous studies, therefore, may explain the increase in GSI in fish exposed to the low
288 concentration of E2. The other concentrations of E2 and NP employed in this study were
289 seemingly insufficient to affect GSI, especially given that fish were exposed to these
290 concentrations as fry. Taken together with previous findings, our results suggest that the impact
291 of estrogenic chemicals on GSI is dependent on both concentration and timing of exposure.
292 Our results indicate that the somatotropic axis of adult tilapia was impacted by early-life
293 exposure to the tested EDCs. Both stimulatory and inhibitory effects of estrogenic chemicals on
294 *gh* transcript levels and Gh synthesis were previously reported in teleosts (Elango et al., 2006;
295 Holloway and Leatherland, 1997; Shved et al., 2007, 2008; Zou et al., 1997). In juvenile Atlantic
296 salmon, E2 and NP injection had no effect on pituitary transcript levels of *gh* (Yadetie and Male,
297 2002), a pattern that is consistent with our current observations. Diminished hepatic *ghr* gene
298 expression was associated with reductions in Gh binding capacity, circulating Igf1 levels, and
299 *igf1* expression following exposure to estrogenic compounds in salmonids (Breves et al., 2018;

300 Hanson et al., 2017; Lerner et al., 2012; Norbeck and Sheridan, 2011). By contrast, we observed
301 elevations in hepatic *ghr* and *igf1* gene expression in addition to positive correlations between
302 *ghr* and both *igf1* and *igf2*, following exposure to estrogenic compounds. As discussed above,
303 these patterns may be associated with a compensatory growth response. In this instance, the
304 Gh/Igf system is seemingly 'activated' following the withdrawal of estrogenic chemicals. During
305 restricted feeding, for example, the catabolic state preceding compensatory growth is
306 characterized by depressed levels of hepatic *ghr, igf1*, and plasma Igf1 (Gray et al., 1992; Duan
307 et al., 1995; Pierce et al., 2005; Norbeck et al., 2007; Picha et al., 2008). Upon re-feeding, a rapid
308 increase in specific growth rate and hepatic *ghr*, *igf1*, and *igf2* expression occurs (Picha et al.,
309 2008). Although there was no clear inhibition of pituitary *gh* expression, we found a negative
310 correlation between pituitary *gh* and hepatic *igf1,* a possible indication of feedback regulation of
311 Gh by Igf1 (Reinecke, 2010). No correlation was observed, however, between hepatic *igf2* and
312 pituitary *gh*. Moreover, the differing responses by hepatic *igf1* and *igf2* to estrogenic EDCs
313 observed in this study were similar to patterns in male Mozambique tilapia injected with E2
314 (Davis et al., 2008), in which hepatic *igf2* was not affected. In mammals, Igf2 is mainly
315 associated with fetal growth and development (Constancia et al., 2002; Daughaday and Rotwein,
316 1989). In teleosts, however, some studies suggest that Igf2 is also an important factor in adult
317 growth (Pierce et al., 2011; Reindl and Sheridan, 2012). The varying responses of *igf2* to
318 estrogenic compound may be due to differences among species and tissue sensitivity.
319 Igfbps are key modulators of Igf activity (Duan and Xu, 2005). Only a few studies in
320 mammals and fishes have described how steroid hormones regulate Igfbps (Duan et al., 2010;
321 Garcia de la Serrana et al., 2017; Rajaram et al., 1997; Reindl and Sheridan, 2012), and fewer yet
322 have examined the long-term effects of EDC exposure on *igfbps* during early developmental

323 stages (Breves et al., 2018). In teleosts, it is generally accepted that the liver is a major site for
324 Igfbp synthesis and secretion (Shimizu and Dickhoff, 2017; Zhou et al., 2008). In Mozambique
325 tilapia, *igfbp1b*, *-2b*, *-5a*, *-4*, and *-6b* are expressed in the liver (Breves et al., 2014). In
326 vertebrates, Igfbp1 plays a highly conserved role as a negative regulator of somatic growth by
327 restricting Igf1 from binding to its receptor (Kajimura et al., 2005; Kamei et al., 2008).
328 Knockdown of Igfbp1 in zebrafish, for instance, alleviates hypoxia-induced retardation of
329 growth, while its overexpression causes growth and developmental retardation (Kajimura et al.,
330 2005). In chinook (*O. tshawytscha*) and Atlantic salmon, Igfbp1b paralogs are important
331 modulators of Igf signaling in response to nutrient availability (Hevrøy et al., 2011; Shimizu et
332 al., 2005, 2006, 2009). Therefore, the increase in *igfbp1b* transcript levels after E2 and NP
333 exposures may provide a mechanism for EDCs to impact growth. In other words, by enhancing
334 *igfbp1b*, a negative regulator of growth, estrogenic EDCs inhibit somatic growth. Alternatively,
335 the increase in *igf1* mRNA levels (and plasma Igf1) and other factors within the Gh/Igf system
336 following EDC exposures may counterbalance the *igfbp1b* response. Previous studies in mature
337 male Mozambique tilapia (Riley et al., 2004) and striped bass (*Morone saxatilis*) (Fukazawa et
338 al., 1995) reported that E2 stimulated the release of putative Igfbp1s from hepatocytes. In
339 Atlantic salmon fry and smolts, however, estrogenic compounds inhibited *igfbp1b* (Breves et al.,
340 2018). Diets supplemented with E2 also inhibited hepatic *igfbp1b1* expression (along with
341 hepatic *igf1* and *igf2*) in rainbow trout (Cleveland and Weber, 2016). These findings support the
342 notion that responses to estrogenic compounds are both species and life stage-dependent.
343 In salmonids, Igfbp2 paralogs are major carriers of plasma Igf1 (Shimizu and Dickhoff,
344 2017). In mammals and teleosts, varying patterns exist on the regulation of igfbp2 gene
345 expression. For example, overexpression of Igfbp2 in mouse embryos reduces growth rates,

346 which was proposed to be related to a reduction in Igf availability (Hoeflich et al., 1999). On the
347 other hand, in salmonids, circulating Igfbp2 increase in response to Gh (Garcia de la Serrana and
348 Macqueen, 2018; Shimizu et al., 1999; 2003). In tilapia, *igfbp2b* expression increased with an
349 increased plasma Igf1 induced by Gh injection (Breves et al., 2014). Moreover, *igfbp2b*
350 expression was increased by treatment of NP but decreased by treatment of E2 in salmon smolts
351 (Breves et al., 2018). By contrast, in rats, hepatic *igfbp2* expression is induced by E2 (Hoeflich et
352 al., 2014; Ricciarelli et al., 1991). In rainbow trout, *igfbp2* expression in ovarian follicles is also
353 increased by E2 treatment (Kamangar et al., 2006). Hence, the observed increase in *igfbp2b*
354 levels following exposure to NP, and its tendency to increase after E2 exposure may be either
355 associated with the increase in *igf1* levels or modulated by E2 and E2 analogues. Further studies,
356 however, are needed to assess whether there are direct actions of Igf1 and E2 on hepatic *igfbp2b*
357 expression. In Atlantic salmon, *igfbp4*, *-5a*, and *-6b* regulate the binding of Igfs to its receptor in
358 the tissues where they are produced (Breves et al., 2017; Cleveland and Weber, 2015; Macqueen
359 et al., 2013). Unlike patterns observed in Atlantic salmon (Breves et al., 2018), we found no
360 significant effect of estrogenic EDCs on *igfbp4* and *-5a*. *igfbp6*, on the other hand, was
361 significantly decreased following E2 exposure. In teleosts, Igfbp6 inhibits Igf-signaling that
362 supports growth and development (Wang et al., 2009). While the decrease in *igfbp6b* may be a
363 residual effect from the earlier exposure to E2, additional work should address whether E2 and
364 NP act directly on the liver to regulate *igfbp6*.

365 To assess the effects of the tested EDCs on estrogenic biomarkers in males, we measured
366 hepatic *vtg* and *er* transcripts. While a trend in all *vtg* transcripts was observed, no significant
367 effects of E2 or NP were detected. Alternatively, both *erα* and *erβ* were stimulated by E2 and
368 NP. In previous studies, *vtg* and *er* (α and β) were induced in liver and testis after injection of E2

and other estrogenic compounds in mature male Mozambique tilapia (Davis et al., 2009b). A concurrent increase in *vtg* and *erα* expression was also observed in Atlantic salmon embryos, yolk-sac fry, feeding fry, and smolts in response to E2, EE2, and NP (Duffy et al., 2014; Breves et al., 2018). The lack of effects on *vtg* in the current study may be linked to the time of EDC exposures and life stage. Indeed, it is noteworthy that even after 112 days since E2 and NP exposures, both *ers* were still elevated. This elevation suggests that males may be more sensitive to E2 and similar chemicals after a previous EDC exposure. Increased sensitivity to estrogenic compounds through enhanced expression of *ers* may render males more susceptible to further detrimental effects on their reproductive development.

5. Conclusion

Our current findings indicate that early aqueous exposure to estrogenic EDCs exerts long lasting effects on the somatotropic axis of tilapia, a central mediator of adaptive patterns of growth and development throughout the life cycle in vertebrates. Thus, an improved understanding of how EDCs impact the endocrine systems controlling growth and reproduction attest to the importance of fish as sentinels for assessing the health of the aquatic ecosystem. Moreover, studies such as this one shall be instrumental in optimizing culture practices for tropical fishes in environments where EDCs are pervasive. Nonetheless, future work that include female tilapia is needed to characterize the long-term effects of estrogenic EDCs in both sexes. Moreover, additional analyses, such as histological examination of testicular tissue would further shed light on the long-term effects of early exposure to estrogenic EDCs on testicular development. Future investigations should also seek to determine the effects of these estrogenic

391 chemicals on the indices of reproductive capacity such as spawning efficiency, fertilization
392 success, and viability of embryos.
393
394 **Contributions**
395 F.T.C.B. conducted experiments, conceived and designed experiments, collected and
396 analyzed data, and wrote the manuscript. C.K.P.S. collected data and conducted experiments.
397 J.P.B designed experiments and revised the manuscript. D.T.L. coordinated the study and revised
398 the manuscript. A.P.S. conceived and designed experiments, coordinated the study, and wrote the
399 manuscript. All authors approved the final article.
400
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412 The authors declare no conflicts of interest.
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TABLE 1. List of primers used in qPCR assays.

	Forward primer	Reverse primer	R^2	% efficiency	Accession Number	Reference
	Sequence (5'–3')	Sequence (5'–3')				
18s	GCTACCACATCCAAGGAAGGC	TTCGTCACTACCTCCCCGAGT	0.999	89.1	AF497908	Magdeldin et al., 2007
ef1α	AGCAAGTACTACGTGACCATCATTG	AGTCAGCCTGGGAGGTACCA	0.999	95.1	AB075952	Breves et al., 2010
β-actin	CTCTTCCAGCCTTCCTTCCT	ACAGGTCCTTACGGATGTCG	0.998	96.5	FN673689	Tipsmark et al., 2011
ghr	CACACCTCGATCTGGACATATTACA	CGGTTGGACAATGTCATTAACAA	0.997	96.8	EF452496	Pierce et al., 2007
igf1	CTGCTTCCAAAGCTGTGAGCT	GATCGAGAAATCTTGGGAGTCTTG	0.999	92.3	AF033796	Kajimura et al., 2004
igf2	GCTTTTATTTCAGTAGGCCAACCA	CACAGCTACAGAAAAGACACTCCTCTA	0.997	90.1	AH006117	Davis et al., 2008
igfbp1b	CCTTCCCTTTGATCACCAAG	GTGTGACATGGACCCTGTTG	0.997	90.8	XM_003438121	Breves et al., 2014
igfbp2b	CCGACTTCCCTTTACAGCAG	TCAGTCCCATGCACCTCATA	0.998	92.2	XM_0054504847	Breves et al., 2014
igfbp4	ATCCCCATACCCAACTGTGA	TGATCCACACACCAGCATTT	0.999	85.6	XM_003454633	Breves et al., 2014
igfbp5a	AACTGGACGGGATCATTCAG	GCACTGTTTGCGTTTGAAGA	0.999	106.7	XM_003443250.2	Breves et al., 2014
igfbp6b	TCCTACCTGCAGAGGAAAGC	CGCAGCTCAGAGTGTAGACG	0.975	96.5	XM_003441337	Breves et al., 2014
gh	TTACATCATCAGCCCGATCG	AGATCGACAGCAGCTTCAGGA	0.999	94.3	AF033806	Magdeldin et al., 2007
vtga	GAATGTGAATGGGCTGGAAATAC	TTTGTTTGATCTGGATGTCAGCTT	0.999	90.3	EF408235	Davis et al., 2007
vtgb	AAGTTGCAGACTGGATGAAAGGA	GCGGTACTCGTCTCCGACAT	1.000	97.7	EF408236	Davis et al., 2007
vtgc	GGACCTTGCAGAACCCAAAG	CATCGTTTCTTGCCAGTTCCA	0.998	94.9	EF408237	Davis et al., 2007
erα	GGCTCAGCAGCAGTCAAGAA	TGCCTTGAGGTCCTGAACTG	0.990	87.0	AM284390	Park et al., 2007
erβ	ACCTTCCGGCAGCAGTACAC	TCCAACATCTCCAGCAACAG	0.994	108.0	AM 284391	Park et al., 2007

Figure captions

Fig. 1. Schematic illustration of header and experimental tanks. Nineteen-liter capacity header tanks supplied water either containing solvent only (control), 0.1 µg/L or 1.0 µg/L 17β -estradiol (E2), or 10 µg/L or 100 µg/L nonylphenol (NP) to fish tanks. Both header and fish tanks were lined with modified polytetrafluoroethylene. Black squares = aeration, white rectangles = pipes.

Fig. 2. Body weight (BW) (A), total length (TL) (B), condition factor (CF) (C), hepatosomatic index (HSI) (D), and gonadosomatic index (GSI) (E) of Mozambique tilapia adults 112 days after 21-day exposure as fry to water containing 0 (control), 0.1 µg/L or 10 µg/L 17β-estradiol (E2) or 10 µg/L or 100 µg/L nonylphenol (NP). Values are means ± SEM (n = 5-23; BW, TL, and CF; n = 5-12, HSI and GSI). Asterisk indicates significant difference between treatment and control group (One-way ANOVA; Fisher's protected LSD; $P < 0.05$).

944

945 Fig. 3. Hepatic *ghr* (A), *igf1* (B), *igf2* (C), and pituitary *gh* (D) mRNA levels in Mozambique
946 tilapia adults 112 days after 21-day exposure as fry to water containing 0 (control), 0.1 µg/L or
947 10 µg/L 17β-estradiol (E2) or 10 µg/L or 100 µg/L nonylphenol (NP). mRNA levels are
948 presented as fold-change relative to the control group. Values are means ± SEM (n = 5-12).
949 Asterisk indicates significant difference between treatment and control group (One-way
950 ANOVA; Fisher's protected LSD; $P < 0.05$).

951

952 Fig. 4. Hepatic *igfbp1b* (A), *igfbp2b* (B), *igfbp4* (C), *igfbp5a* (D), and *igfbp6b* (E) mRNA levels
953 in Mozambique tilapia adults 112 days after 21-day exposure as fry to water containing 0
954 (control), 0.1 µg/L or 10 µg/L 17β-estradiol (E2) or 10 µg/L or 100 µg/L nonylphenol (NP).
955 mRNA levels are presented as fold-change relative to the control group. Values are means ±
956 SEM (n = 5-12). Asterisk indicates significant difference between treatment and control group
957 (One-way ANOVA; Fisher's protected LSD; $P < 0.05$).

958

959 Fig. 5. Hepatic *vtga* (A), *vtgb* (B), *vtgc* (C), *erα* (D), and *erβ* (E) mRNA levels in Mozambique
960 tilapia adults 112 days after 21-day exposure as fry to water containing 0 (control), 0.1 µg/L or
961 10 µg/L 17β-estradiol (E2) or 10 µg/L or 100 µg/L nonylphenol (NP). mRNA levels are
962 presented as fold-change relative to the control group. Values are means ± SEM (n = 5-12).
963 Asterisk indicates significant difference between treatment and control group (One-way
964 ANOVA; Fisher's protected LSD; $P < 0.05$).

Figure 1 (2-column fitting image)

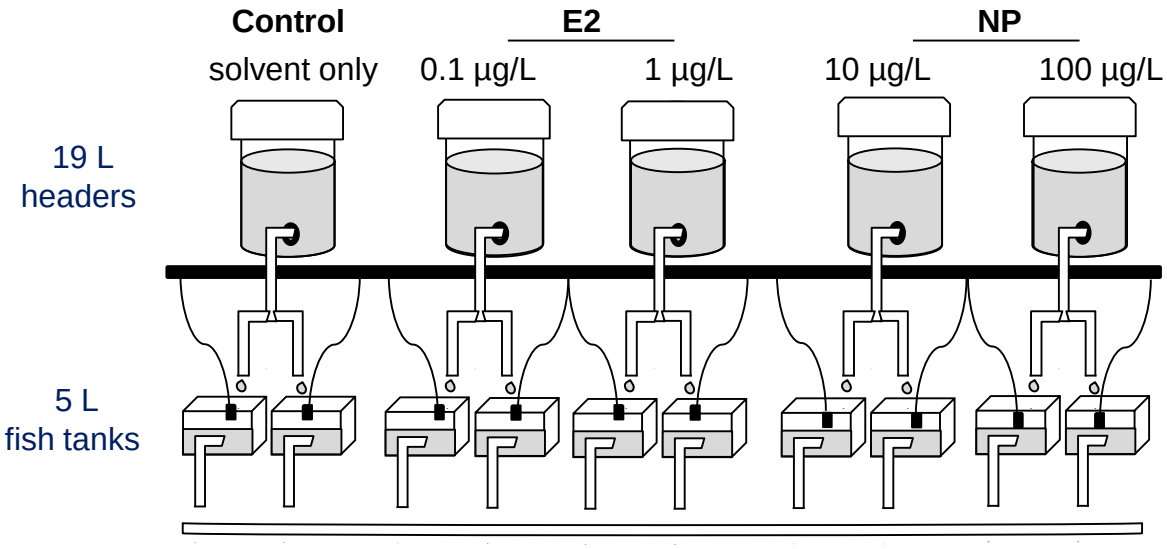

Figure 2 (2-column fitting image)

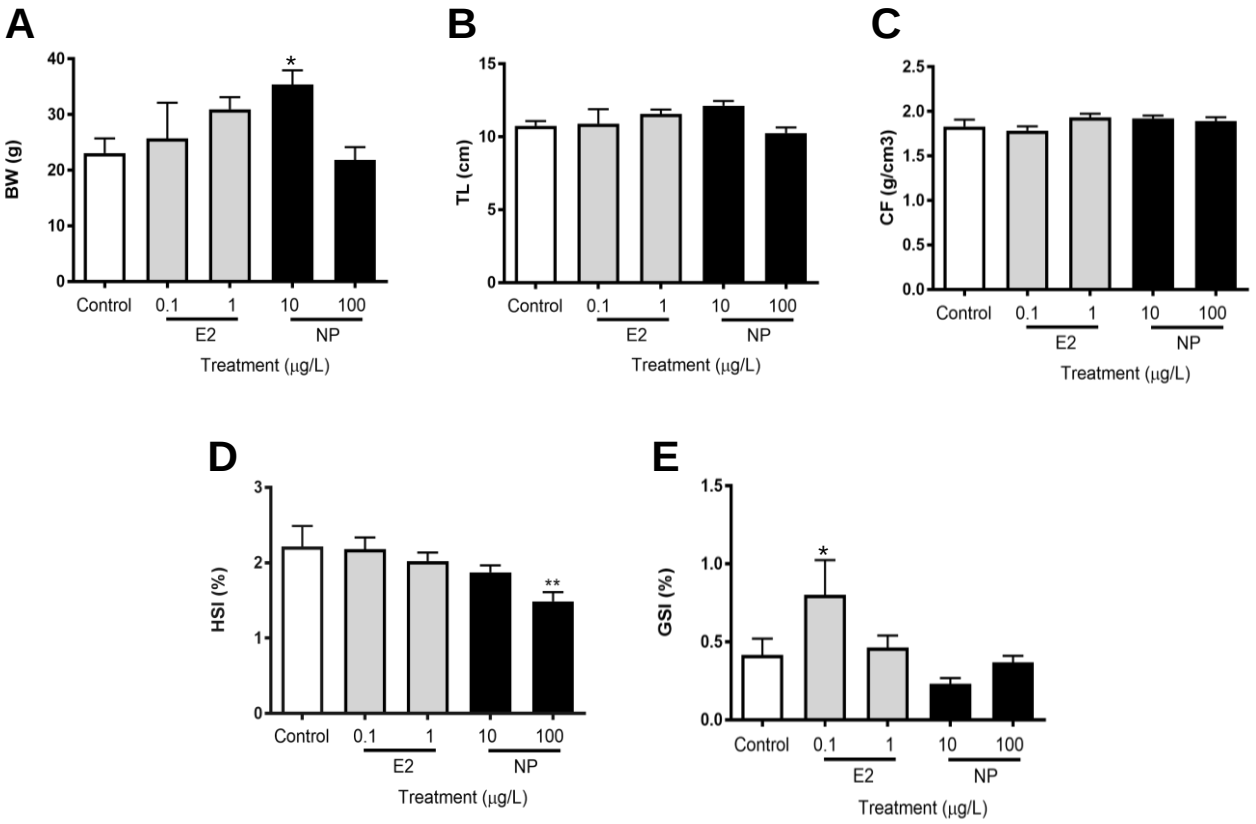

Figure 3 (2-column fitting image)

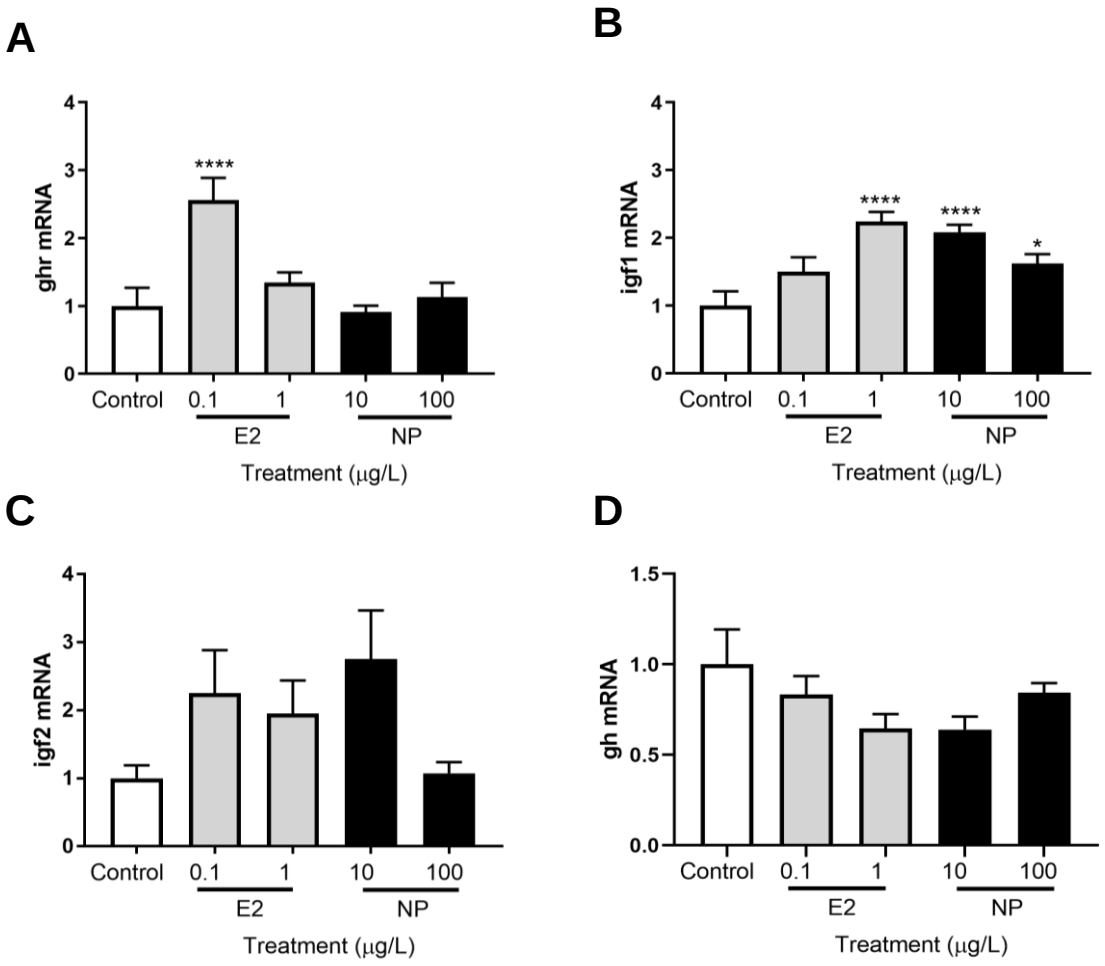

Figure 4 (2-column fitting image)

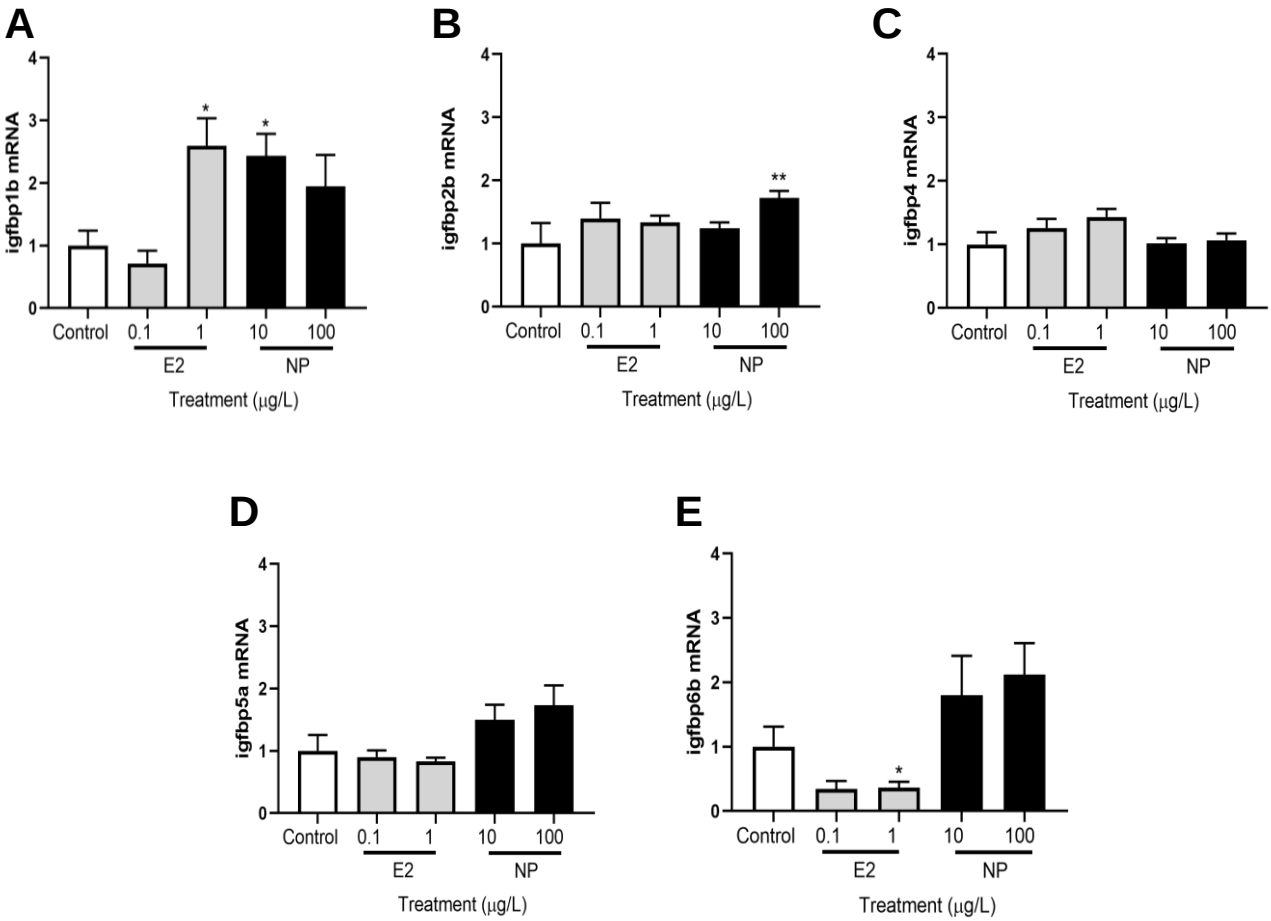

Figure 5 (2-column fitting image)

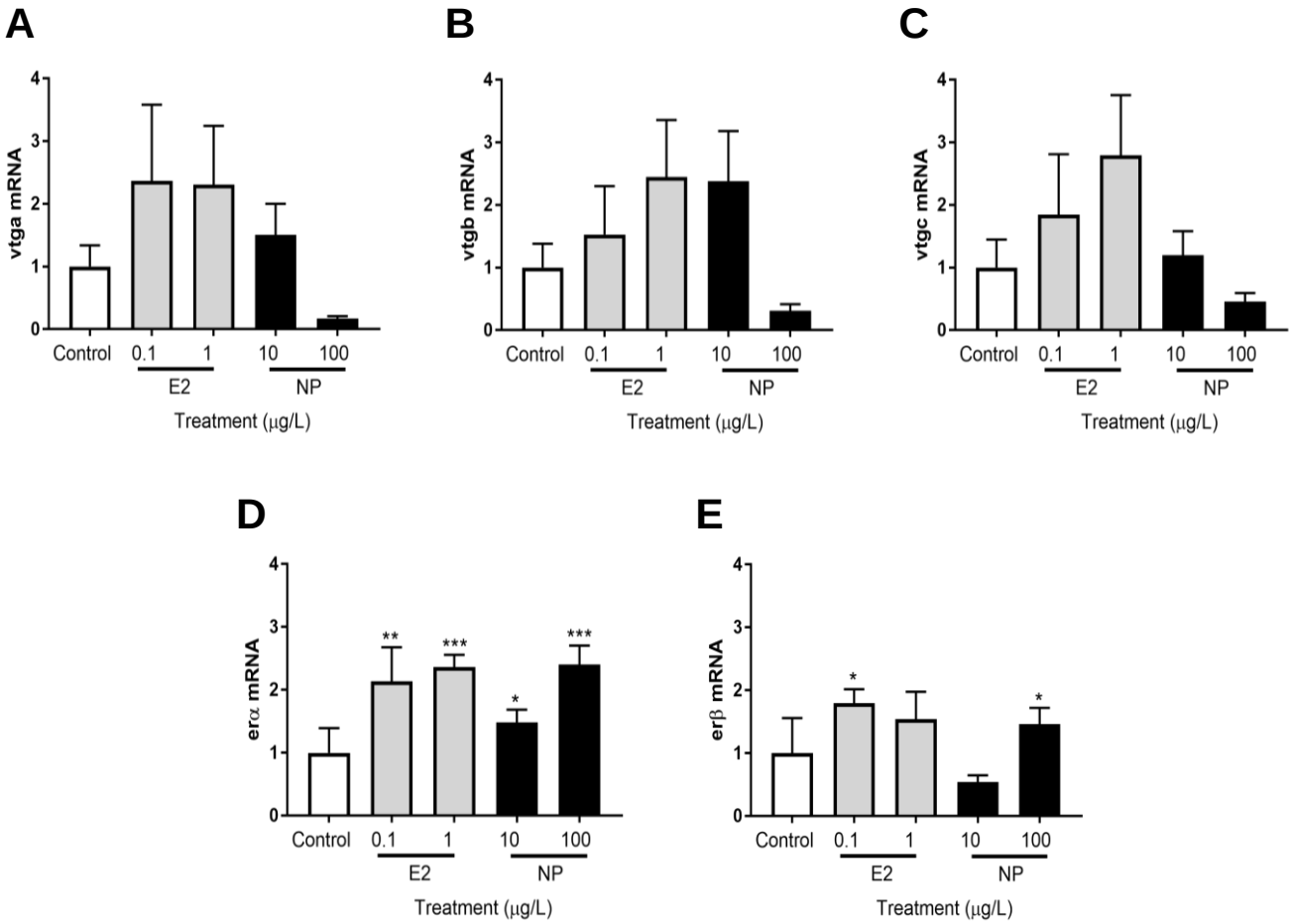

Table 1. Lis of primers used in qPCR assays.

Gene	Forward primer Sequence (5'-3')	Reverse primer Sequence (5'-3')	R^2	% efficiency	Accession Number	Reference
18s	GCTACCACATCCAAGGAAGGC	TTCGTCACTACCTCCCCGAGT	0.999	89.1	AF497908	Magdeldin et al., 2007
ef1α	AGCAAGTACTACGTGACCATCATTG	AGTCAGCCTGGGAGGTACCA	0.999	95.1	AB075952	Breves et al., 2010
β-actin	CTCTTCCAGCCTTCCTTCCT	ACAGGTCCTTACGGATGTCG	0.998	96.5	FN673689	Tipsmark et al., 2011
ghr	CACACCTCGATCTGGACATATTACA	CGGTTGGACAATGTCATTAACAA	0.997	96.8	EF452496	Pierce et al., 2007
igf1	CTGCTTCCAAAGCTGTGAGCT	GATCGAGAAATCTTGGGAGTCTTG	0.999	92.3	AF033796	Kajimura et al., 2004
igf2	GCTTTTATTTCAGTAGGCCAACCA	CACAGCTACAGAAAAGACACTCCTCTA	0.997	90.1	AH006117	Davis et al., 2008
igfbp1b	CCTTCCCTTTGATCACCAAG	GTGTGACATGGACCCTGTTG	0.997	90.8	XM_003438121	Breves et al., 2014
igfbp2b	CCGACTTCCCTTTACAGCAG	TCAGTCCCATGCACCTCATA	0.998	92.2	XM_0054504847	Breves et al., 2014
igfbp4	ATCCCCATACCCAACTGTGA	TGATCCACACACCAGCATTT	0.999	85.6	XM_003454633	Breves et al., 2014
igfbp5a	AACTGGACGGGATCATTCAG	GCACTGTTTGCGTTTGAAGA	0.999	106.7	XM_003443250.2	Breves et al., 2014
igfbp6b	TCCTACCTGCAGAGGAAAGC	CGCAGCTCAGAGTGTAGACG	0.975	96.5	XM_003441337	Breves et al., 2014
gh	TTACATCATCAGCCCGATCG	AGATCGACAGCAGCTTCAGGA	0.999	94.3	AF033806	Magdeldin et al., 2007
vtga	GAATGTGAATGGGCTGGAAATAC	TTTGTTTGATCTGGATGTCAGCTT	0.999	90.3	EF408235	Davis et al., 2007
vtgb	AAGTTGCAGACTGGATGAAAGGA	GCGGTACTCGTCTCCGACAT	1.000	97.7	EF408236	Davis et al., 2007
vtgc	GGACCTTGCAGAACCCAAAG	CATCGTTTCTTGCCAGTTCCA	0.998	94.9	EF408237	Davis et al., 2007
erα	GGCTCAGCAGCAGTCAAGAA	TGCCTTGAGGTCCTGAACTG	0.990	87.0	AM284390	Park et al., 2007
erβ	ACCTTCCGGCAGCAGTACAC	TCCAACATCTCCAGCAACAG	0.994	108.0	AM 284391	Park et al., 2007