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Wastewater treatment plant effluent alters pituitary gland gonadotropin mRNA levels in juvenile coho salmon (*Oncorhynchus kisutch*)

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ABSTRACT

It is well known that endocrine disrupting compounds (EDCs) present in wastewater treatment plant (WWTP) effluents interfere with reproduction in fish, including altered gonad development and induction of vitellogenin (Vtg), a female-specific egg yolk protein precursor produced in the liver. As a result, studies have focused on the effects of EDC exposure on the gonad and liver. However, impacts of environmental EDC exposure at higher levels of the hypothalamic-pituitary-gonad axis are less well understood. The pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) are involved in all aspects of gonad development and are subject to feedback from gonadal steroids making them a likely target of endocrine disruption. In this study, the effects of WWTP effluent exposure on pituitary gonadotropin mRNA expression were investigated to assess the utility of Lh beta-subunit (*lhb*) as a biomarker of estrogen exposure in juvenile coho salmon (*Oncorhynchus kisutch*). First, a controlled 72-h exposure to 17α-ethynylestradiol (EE2) and 17β-trenbolone (TREN) was performed to evaluate the response of juvenile coho salmon to EDC exposure. Second, juvenile coho salmon were exposed to 0, 20 or 100% effluent from eight WWTPs from the Puget Sound, WA region for 72 h. Juvenile coho salmon exposed to 2 and 10 ng EE2 L^{-1} had 17-fold and 215-fold higher *lhb* mRNA levels relative to control fish. Hepatic *vtg* mRNA levels were dramatically increased 6670-fold, but only in response to 10 ng EE2 L^{-1} and Fsh beta-subunit (*fshb*) mRNA levels were not altered by any of the treatments. In the WWTP effluent exposures, *lhb* mRNA levels were significantly elevated in fish exposed to five of the WWTP effluents. In contrast, transcript levels of *vtg* were not affected by any of the WWTP effluent exposures. Mean levels of natural and synthetic estrogens in fish bile were consistent with pituitary *lhb* expression, suggesting that the observed *lhb* induction may be due to estrogenic activity of the WWTP effluents. These results suggest that *lhb* gene expression may be a sensitive index of acute exposure to estrogenic chemicals in juvenile coho salmon. Further work is needed to determine the kinetics and specificity of *lhb* induction to evaluate its utility as a potential indicator of estrogen exposure in immature fish.

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1. Introduction

It is now well established that some chemicals in the environment are capable of disrupting normal endocrine function in humans and wildlife such as fish (Hotchkiss et al., 2008; León-Olea et al., 2014). These endocrine disrupting compounds (EDCs), including certain pharmaceuticals, pesticides, and a variety of industrial

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compounds, can act to mimic or block endogenous hormones by interfering with their synthesis, availability, or action (Crisp et al., 1998). EDC exposure has been associated with reduced fertility (Jobling et al., 2002), sex reversal (Jobling et al., 1998), and reproductive failure (Kidd et al., 2007; Nash et al., 2004) in a variety of aquatic organisms.

Municipal wastewater treatment plant (WWTP) effluents are one of the primary sources of EDCs into the aquatic environment. Fish collected downstream of some WWTPs exhibit symptoms of endocrine disruption and altered reproductive function including reduced gonad size, delayed maturation, and decreased steroidogenesis (Folmar et al., 2001; Vajda et al., 2011, 2008; Woodling et al., 2006). In addition, feminization of male fish has been reported downstream of WWTPs including observations of intersex gonads or reduced primary and secondary male sex characteristics (Jobling et al., 1998; Purdom et al., 1994; Vajda et al., 2011, 2008). Chemical analyses identified natural estrogens [estradiol (E2) and estrone (E1)] and synthetic estrogens [17α-ethynylestradiol (EE2)] as the most likely feminizing agents in wastewater effluents (Desbrow et al., 1998; Rodgers-Gray et al., 2000). In addition, various anthropogenic compounds such as alkylphenols [nonylphenols (NP) and octylphenols (OP)] and bisphenol A (BPA) have also been identified as estrogen receptor (ER) agonists or antagonists and are present in wastewater effluent (Snyder et al., 2001).

Early studies on the effects of WWTP effluent exposure on fish reproduction found elevated expression of vitellogenin (Vtg) in male fish exposed to WWTP effluent (Folmar et al., 1996; Harries et al., 1997; Purdom et al., 1994). Vtg is an egg yolk protein precursor that is induced in maturing female fish in response to rising plasma E2 levels. Vtg synthesis can also be stimulated in male or juvenile fish of both sexes in response to exogenous estrogen exposure. Indeed, Vtg can be increased thousands fold in male fish in response to estrogens making it a widely used biomarker of estrogen exposure (Sumpter and Jobling, 1995; Thorpe et al., 2000). As such, many studies of endocrine disruption or estrogen exposure have focused on the gonad and liver. However, reproduction is controlled by the hypothalamic-pituitary-gonad (HPG) axis and EDCs may exert their effects at higher levels of the HPG axis.

The pituitary gonadotropins (Gths), follicle stimulating hormone (Fsh) and luteinizing hormone (Lh), are heterodimeric glycoprotein hormones composed of a common alpha subunit and a hormone-specific beta subunit. The Gths are involved in all aspects of gonad development and function including steroidogenesis, gametogenesis, final gamete maturation, and gamete release (Levavi-Sivan et al., 2010; Swanson et al., 2003). The Gths are synthesized and released in response to a variety of factors from the brain, primarily gonadotropin-releasing hormone (GnRH) released from the hypothalamic neurons that directly innervate the fish pituitary gland. In addition, the Gths are regulated by positive and negative feedback from the gonad via steroid hormones and other gonadal peptides. For example, when immature trout or salmon are treated with estrogen or aromatizable androgens, pituitary and plasma Fsh levels decrease while pituitary Lh beta subunit (*lhb*) mRNA levels and pituitary Lh content increase (Breton et al., 1997; Dickey and Swanson, 1998; Saligaut et al., 1998), suggesting estrogens play an important role in regulating both gonadotropins. In support of this, estrogen response elements (EREs) have been identified in the *lhb* (Le Dréan et al., 1995; Liu et al., 1995; Sohn et al., 1999; Xiong et al., 1994) and Fsh beta subunit (*fshb*) (Rosenfeld et al., 2001; Sohn et al., 1998; Vischer, 2003) gene promoters of various fish species. Therefore, it is possible that Gths may be susceptible to endocrine disruption by estrogenic contaminants such as EE2 or WWTP effluent.

In salmonids, pituitary *fshb* and *lhb* mRNA and plasma Fsh and Lh expression profiles are well characterized (Breton et al., 1998; Campbell et al., 2006; Gomez et al., 1999; Prat et al., 1996; Swanson et al., 1991). In male and female coho salmon, pituitary *fshb* mRNA levels, pituitary Fsh content, and plasma Fsh levels begin to increase about one year prior to spawning (Campbell et al., 2006). In contrast, *lhb* mRNA levels and pituitary Lh content increase during late gametogenesis and final gamete maturation in response to increasing levels of estradiol or aromatizable androgens (Breton et al., 1998; Gomez et al., 1999; Prat et al., 1996; Swanson et al., 1991 Gomez et al., 1999; Prat et al., 1996; Swanson et al., 1991). However, similar to the case of Vtg, increased expression of *lhb* mRNA and accumulation of Lh protein content in the pituitary of immature fish can be induced in response to estrogen treatment. Studies have shown that mRNA levels for *lhb* are induced in response to EE2 or other estrogenic contaminants (Harding et al., 2013; Harris et al., 2001; Johns et al., 2009; Maeng et al., 2005; Rhee et al., 2010; Yadetie and Male, 2002). Using high-throughput sequencing and RNA-Seq, we previously demonstrated that waterborne exposure of previtellogenic coho salmon to 12 ng L^{-1} EE2 for up to 6 weeks had widespread effects on the pituitary transcriptome and dramatically altered Gth mRNA levels. At 6 weeks, *lhb* was induced 395-fold and was the most significantly altered transcript, while *fshb* was downregulated −3.5 fold (Harding et al., 2013). Alterations in plasma Gth levels have also been observed in response to EDC exposure (Brown et al., 2007; Golshan et al., 2014; Harris et al., 2001). Female rainbow trout (*Oncorhynchus mykiss*) exposed to 4-nonylphenol at 0.7, 8.3, or 85.6 μg L^{-1} for 18 weeks during early secondary oocyte growth showed reduced *fshb* mRNA levels, pituitary Fsh content and plasma Fsh levels and increased plasma Lh and Vtg (Harris et al., 2001). These findings suggest that Gths may be sensitive targets of EDC exposure and may be involved in inhibited gonad growth and altered reproduction associated with endocrine disruption (Filby et al., 2006; Harris et al., 2001).

The aim of this study was to examine the effects of WWTP effluents on pituitary Gths in 14–18 month old juvenile coho salmon (*Oncorhynchus kisutch*) and to evaluate the utility of *lhb* as a potential biomarker of estrogen exposure. Based on the strong induction of *lhb* mRNA levels in response to EE2 and other ER agonists, we hypothesized that *lhb* mRNA levels would be increased in response to WWTP effluents containing estrogenic chemicals. Juvenile coho salmon were selected for this study because: 1) coho salmon are ecologically relevant species in the Pacific Northwest region of North America; 2) presmolts or smolts (14–18 month old, early gametogenesis) have low to non-detectable basal expression of hepatic *vtg* and pituitary *lhb* in both male and female fish at this stage; 3) primary oocyte growth and early stages of spermatogenesis occur at this life history stage and may be affected by contaminants in WWTP effluent; 4) the low body size (<50 g body mass) at this stage makes them more practicable for waterborne exposure studies; and 5) salmon presmolts and smolts are residing in or migrating through urban waterways and potentially exposed to EDCs during these stages. To evaluate the response of juvenile coho salmon to EDC exposure, we first conducted a controlled 72-h exposure to EE2 and 17β-trenbolone (TREN; a synthetic androgen used in cattle production). TREN and EE2 were selected as a model androgen and estrogen, respectively. Second, we exposed coho salmon to 100% effluent, 20% effluent, or control water for 72 h. Effluent from eight WWTPs were selected to include a range of treatment processes (secondary and tertiary), which vary in their removal efficiency of steroid estrogens. In addition to pituitary Gth mRNA levels, we measured hepatic *vtg* mRNA levels as a positive control of estrogen exposure. Where possible, chemical analyses on exposure effluents and bile from exposed fish were conducted to quantify exposure to a variety of contaminants with estrogenic activity. Analysis of several selective serotonin reuptake inhibitors (SSRIs) in effluents was also performed because of high occurrence in WWTP effluent and in Puget Sound estuary waters (Hedgespeth