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Effects of endocrine disrupters on the expression of growth hormone and prolactin mRNA in the rainbow trout pituitary

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Abstract

It is now widely accepted that chemical pollutants in the environment can interfere with the endocrine system of animals, thus affecting development and reproduction. Some of these endocrine disrupters (EDs) can have estrogenic or anti estrogenic effects. Most studies to date have focused on the effects of EDs on the reproductive system and sex hormones and only limited information exists on how EDs may affect pituitary gland function. A rainbow trout (Oncorhynchus mykiss) pituitary gland culture system was used for studying the effects of EDs on growth hormone (GH) and prolactin (PRL) mRNA expression. We determined that the pituitary glands actively synthesized and secreted GH and PRL over the experimental time-course. In addition, we found that treatment with 17¥â-estradiol (positive control) increased levels of GH and PRL mRNA, in a concentration-dependent manner. Treatment of pituitary glands with 500 and 1000 nM of a xenoestrogen, o,p¡Ç-DDT (o,p¡Ç-dichlorodiphenyltrichloroethane), resulted in a significant induction of GH and PRL mRNA, with a 20-fold increase for PRL and 3-fold increase for GH following treatment with 1000 nM o,p¡Ç-DDT. Co-incubation of pituitary glands with ICI 182 780 (a selective estrogen receptor antagonist) and o,p¡Ç-DDT resulted in inhibition of PRL mRNA levels; however, the stimulatory effect of DDT on GH mRNA was not seen in this experiment, nor was the inhibitory effect of ICI 182 780 observed with GH mRNA. To the contrary, ICI 182 780 (2.5 nM) had a stimulatory effect on GH mRNA levels. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), which is known to exert antiestrogenic effects, had an estrogenic-like effect that resulted in a concentration-dependant increase in the levels of GH and PRL mRNA. Co-incubation of pituitaries with TCDD and ¥á-napthoflavone (ANF), which is an inhibitor of the aryl hydrocarbon receptor (AhR), caused an inhibition of TCDD-induced PRL mRNA at the higher and lower concentrations, but these effects were less consistent on GH mRNA levels. However, the responses of PRL and GH mRNA to co-incubation with TCDD and ANF, at the various concentrations, were bi-phasic wherein stimulation was seen at the low concentrations and inhibition at the high concentrations. Combined, these results suggest that o,p¡Ç-DDT and TCDD are xenoestrogens and that their effects on the expression of GH and PRL genes in the rainbow trout pituitary are modulated, in part, through the ER and AhR, respectively.

Keywords: Endocrine disruptor; Growth hormone; Prolactin; Pituitary gland organ culture

1. Introduction

Endocrine disrupters (EDs) are pollutants that can act as agonists or antagonists to natural hormones. EDs have been intensively studied for their actions as estrogen mimetics wherein they work by binding to the estrogen receptor, thus affecting estrogen-regulated cellular and reproductive processes (Datson et al., 1997 and Witorsch, 2002). Increasing evidence suggests that aquatic vertebrates, which are widely exposed to EDs, may have altered endocrine physiology and impairment of reproductive processes (Guillette and Gunderson, 2001). For example, juvenile alligators exposed to organochlorine contamination in Lake Apopka (Florida) displayed abnormalities in male and female gonads and altered sex steroid levels (Guillette et al., 1994 and Guillette et al., 1995). In feral fish, male roach, with ovotestis, were found in heavily contaminated rivers in the United Kingdom (Sumpter and Jobling, 1995 and Tyler et al., 1998) and vitellogenin has been detected in male fish in contaminated water bodies in both North America and Europe (Kavanaugh et al., 2004 and Purdom et al., 1994). Vitellogenin is an egg yolk precursor protein normally seen only in female fish and its presence in male fish is used as a biomarker for exposure to estrogenic compounds (Matthiessen and Sumpter, 1998).

DDT (dichlorodiphenyltrichloroethane) is one of the most well studied, estrogenic, environmental pollutants. Since the 1960s, reproductive impairments such as feminization of male birds or eggshell thinning have been linked to exposure to DDT (Fry, 1995 and Fry and Toon, 1981). Most of the estrogenic action of DDT has been attributed to one of the isomers *o,p*¡Ç-DDT which binds the estrogen receptor (ER) and which comprises about 20% of the commercial DDT preparations (Soto et al., 1994). In lower vertebrates, microinjection of medaka eggs with *o,p*¡Ç-DDT was shown to cause sex reversal of genetic males to a female phenotype, with functioning ovaries and were capable of breeding (Edmunds et al., 2000). Furthermore, *o,p*¡Ç-DDT has been shown (in vivo) to induce vitellogenin production in male rainbow trout and tilapia (Edmunds et al., 2000).

In contrast to estrogenic compounds, other environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have tissue-specific antiestrogenic effects in vitro and in vivo. In mice, in utero exposure to TCDD caused decreases in uterine weight, higher incidence of ovarian and mammary tumors, and other reproductive abnormalities (Gray et al., 1995). Carp hepatocytes treated in vitro with TCDD showed decreasing levels of vitellogenin secreted into the culture medium (Smeets et al., 1999), further suggesting an antiestrogenic action.

Endogenous steroid hormones such as 17¥â-estradiol, affect the hypothalamus–pituitary–gonad axis by positive and negative feedback (Arukwe, 2001). The pituitary gland synthesizes and secretes many hormones that regulate organismal physiology directly or indirectly via regulating other endocrine glands (Arukwe, 2001). Growth hormone (GH) and prolactin (PRL) are peptide hormones produced by pituitary gland. These hormones play important roles in controlling growth, osmoregulation, metabolism, reproduction and development in all vertebrates (Björnsson, 1997, Bole-Feysot et al., 1998, Manzon, 2002, McCormick, 2001, Sakamoto et al., 1993, Scanes and Daughaday, 1995 and Scanes and Harvey, 1995).

Studies have shown that the promoter regions of vertebrate GH and PRL genes contain estrogen response elements (ERE) and the expression and release of PRL is modulated by estrogen (Borski et al., 1991, Williams and Wigham, 1994 and Yang et al., 1997b). In addition, secretion of GH from the pituitary has been shown to be modulated, in mammals and fish, by sex steroids especially 17¥â-estradiol (Trudeau et al., 1992, Veldhuis and Bowers, 2003 and Zou et al., 1997). It is therefore plausible that xenoestrogens and antiestrogenic pollutants may also affect pituitary gland function by affecting the synthesis and secretion of GH and PRL. Indeed, recent work has shown that a number of EDs influence pituitary PRL gene expression and release in mammalian pituitary cell lines and in rats (Abraham and Frawley, 1997, Rousseau et al., 2002 and Steinmetz et al., 1997), however, very little is known about the effects of EDs on teleost pituitary function, particularly DDT and TCDD.

Anadromous salmonids undergo a complex process during the parr-smolt transformation that requires changes in physiology, morphology, growth, and metabolism, which enables adaptation to seawater. Many hormonal regulators are involved including gonadal steroids, cortisol, GH, and PRL (Dickhoff, 1993, Hoar, 1988 and McCormick, 1994). GH is known to stimulate seawater adaptability, while PRL inhibits seawater adaptability and changes in the balance between these two hormones can adversely impact osmoregulatory capacity. To address this concern, researchers have begun to examine the effects of steroids and EDs on salmonid physiology, using growth, smoltification, and osmoregulation as study end-points (Arsenault et al., 2004, J©ªrgensen et al., 2004, Madsen et al., 2004 and Moore et al., 2003). Despite promising findings, which indirectly suggest that estrogen and EDs influence these physiological processes, no conclusive links have been made between ED exposure and changes in the pituitary hormones (GH and PRL) that regulate smoltification and seawater tolerance in salmonids.

The regulation of the teleost pituitary is highly analogous to that of higher vertebrates (Harvey, 1993 and Nishioka et al., 1988). Organ culture techniques for the rainbow trout pituitary gland are well established and, therefore, are an excellent model for the analysis of the direct effects of xenobiotics on the pituitary hormone synthesis in salmonids. The goal of this work was to examine the effects of estrogen (positive control), the xenoestrogen *o,p*¡Ç-DDT and the antiestrogen, dioxin (TCDD), on pituitary hormone gene expression. We report the effects of estrogenic and/or antiestrogenic EDs on pituitary GH and PRL mRNA expression in the rainbow trout.

2. Materials and methods

2.1. Animals

Male and female rainbow trout were obtained from the Quinnebaug trout hatchery, Plainfield, CT, USA. Fish used in this study were 12–18 months old with fairly uniform body weights (200–300 g) and were comprised of both sexes. Fish were maintained in the fish culture facility at the University of Connecticut in tanks with partial recirculation of fresh water (12–14 ¡ÆC) under the photoperiod of 12 h light/12 h dark for a minimum of 2 weeks before use. Fish were fed to satiety, once a day, with pelleted trout feed (Melick aqua feeds, Catawissa, PA, USA). Food was with held 24 h before the pituitary glands were removed.

2.2. Reagents and chemicals

Phenol red free minimum essential medium (MEM) and methionine free MEM were obtained from Sigma Chemical (St. Louis, MO). Penicillin-G, streptomycin, 17¥â-estradiol, and protease inhibitor cocktail were also obtained from Sigma Chemical. TCDD, *o,p*¡Ç-DDT, ICI 182 780 and alphanapthoflavone were purchased from Ultra Scientific (North Kingstown, RI). Cytotox One-membrane integrity assay kit was obtained from Promega (Madison, WI), and [^{35}S]-methionine, [^{32}P]-¥ãATP and [^{32}P]-¥ádCTP were purchased from Amersham Biosciences (Piscataway, NJ). Trizol reagent was purchased from Invitrogen (Carlsbad, CA). 17¥â-estradiol was prepared as a 10 mM stock solution in ethanol, and *o,p*¡Ç-DDT, TCDD, ICI 182 780 and ANF were prepared as 10 mM stock solutions in dimethylsulfoxide (DMSO). Stock solutions were diluted to the required concentrations with phenol red free MEM, and the concentrations of ethanol (for 17¥â-estradiol) and DMSO (for the rest of the chemicals) were maintained below 0.01% of the original stocks.

2.3. Pituitary gland culture

The pituitary glands were removed from the fish following decapitation and the glands were cultured in vitro following conditions described by Yada et al. (1991) with modification. Individual pituitary glands were cultured in 96 well plates, in 200 ¥il of phenol red free MEM supplemented with Na-bicarbonate (2.12 g/l), penicillin (100 U/ml) and streptomycin (4 U/ml). The 96 well plates were placed in containers that were gassed with 95% O_2/5% CO_2. The osmolality of the medium was 290 mOsm, which is similar to that of trout plasma. Pituitary gland cultures were incubated at 12–14 ¡ÆC in an orbital shaker and the medium was changed daily. For treatment with 17¥â-estradiol, or other chemicals, pituitary glands were removed from the fish and maintained in culture for 24 h before addition of the test agents. Following the pre-incubation period, media was replaced with media containing the test agents (17¥â-estradiol, *o,p*¡Ç-DDT or TCDD) alone or in combination with estrogen receptor and AhR antagonists, and the glands incubated for an additional 24 h. Antagonists of the estrogen receptor (ICI 182 780) and arylhydrocarbon receptor (AhR) (ANF), were co-incubated with either *o,p*¡Ç-DDT or TCDD. Controls consisted of media with solvent(s) alone. When the pituitary glands were co-incubated with 17¥â-estradiol, and other chemicals, controls contained both ethanol and DMSO. Organ culture medium and pituitaries were harvested and stored at −80 ¡ÆC until assayed. The viability of pituitary glands under the culture conditions was assessed daily for 6 days. Pituitary glands were dissociated with trypsin as described by Chang and Jobin (1994) and the viability of cells assessed by trypan blue exclusion. To examine the integrity of cell structure, pituitary glands were fixed in Bouin¡¯s fixative, embedded in paraffin wax and sectioned into 4 ¥iM thick sections. Tissue sections were stained with eosin and hematoxylin and analyzed.

2.4. Measurement of GH and PRL mRNA levels

Total RNA from each pituitary was extracted according to the acid phenol guanidinium iso-thiocyanate-method (Chomczynski and Sacchi, 1987), or using Trizol reagent (Sigma Chemical, St. Louis, MO). Total RNA was quantified by UV spectrophotometry. Quality of total RNA obtained from individual pituitary glands was determined using formaldehyde agarose gel electrophoresis. Using Northern blots and dot blots, the cDNA probes for GH and PRL mRNA were found to be specific as previously described (Yang et al., 1997a and Yang and Chen, 2003). For quantification of mRNA levels for GH, PRL and 18S rRNA, 4 ¥ig of total RNA from each pituitary gland was blotted onto a pre-wetted nylon membrane using a dot blot apparatus as previously described (Tang et al., 2001, Yang et al., 1997a and Yang and Chen, 2003). For quantification of mRNA levels, different concentrations of GH and PRL cRNA

were also included in the blots to ensure that the signal obtained upon probing was specific and in the linear range of detection.

GH and PRL probes were labeled with ¥á-[^{32}P]-dCTP by the random priming method and hybridized to the blots as previously described (Shamblott and Chen, 1993, Yang et al., 1997a and Yang and Chen, 2003). Membranes were sequentially hybridized, following stripping after each hybridization with GH, PRL and then 18S probes. The membranes were then exposed to a Phosphor Imager screen (BioRad, Hercules CA) and the signal obtained was quantified using Quantity-One software (BioRad, Hercules, CA, version 4.4.1).

2.5. Detection of newly synthesized GH and PRL

Pituitary glands were incubated in methionine free MEM for 2 h, followed by incubation for 24 h in MEM containing 20 ¥ìCi [^{35}S]-methionine (SA 1000 Ci/mmol). The pituitary glands were removed from the incubation medium and homogenized in a lysis buffer (1 ml of PBS containing 0.1% triton X-100 and 1 ¥il of a 100¡¿ solution of protease inhibitor cocktail p8340) (Sigma, St. Louis, MO). The homogenate was centrifuged at 2000*g* for 15 min, and the supernatant and culture medium were stored in −80 ¡ÆC until use. For immunoprecipitation of [^{35}S]-methionine-labeled pituitary hormones, 50 ¥il of a 1:10 dilution of universal GH antiserum or PRL antiserum (Gonzalez-Villasenor and Chen, 1999) was added to 50 ¥il of pituitary homogenate, or medium, and incubated at 4 ¡ÆC overnight in a bi-directional shaker. To this, 6 ¥ìg of protein A–agarose beads were added and incubated for an additional 6 h at 4 ¡ÆC. Following washing with lysis buffer twice, the protein A–agarose beads were re-suspended in SDS–PAGE sample buffer containing ¥â-mercaptoethanol (Laemmli, 1970) and boiled for 5 min. The [^{35}S]-methionine-labeled proteins were resolved by SDS–PAGE (4% stacking gel/12% separating gel). The gels were fixed in a solution of 50% methanol and 10% acetic acid for 1 h and then soaked in Amplify Flurographic Reagent (Amersham Biosciences, Piscataway, NJ) for 15 min before drying. The newly synthesized GH and PRL were visualized by exposing the dried gels to X-ray film (Biomax MR, Kodak) for 4 days at −80 ¡ÆC. The X-ray films were scanned with a table top scanner (Hewlett–Packard ScanJet 3C) and images analyzed using Quantity-One software (version 4.4.1, BioRad, Hercules, CA). The percent release was calculated as [density of bands obtained from medium/density of bands obtained from medium + tissue] ¡¿ 100.

2.6. Cytotoxicity assay

Cytotoxicity was determined by measuring the presence of lactate dehydrogenase (LDH) in the culture medium. Briefly, LDH activity was determined by a fluorimetric, 10 min coupled assay that resulted in the conversion of rezasurin to resorufin by the Cyto Tox-One assay kit supplied by Promega (Madison, WI).

2.7. Statistics

Data presented here are representative of two experimental repeats unless otherwise specified in the figure legend. Data are presented as means ¡¾ standard error of the mean. The data were log or arc sin transformed for homogeneity of variance when necessary. Differences among groups were analyzed by one-way analysis of variance (ANOVA), and effects of time and treatment using two-way analysis of variance (ANOVA). For pair wise comparisons, Fisher¡¯s test for least significant difference (FPLSD) was used for only those groups being compared (Steele and Torrie, 1980), whenever significant differences were obtained following ANOVA. Differences between means were considered significant if $P < 0.05$.

3. Results

3.1. Validation of the pituitary gland culture conditions

To analyze the viability of cells from cultured pituitary glands, the pituitary glands were cultured for 6 days and then dissociated into single cells and the viability of the cells assessed by trypan blue exclusion. About 95–99% of cells did not take up trypan blue. Cytological observation of stained sections of pituitary glands maintained in culture for 1–6 days revealed that different regions of the pituitary gland were clearly distinguishable including regions of GH- and PRL-secreting cells (data not shown). To evaluate whether hormone secretion was retained by the cultured pituitary glands, GH that was released into the media was quantified by radioimmunoassay. The pituitary glands released GH up to the 6 days in culture. The pituitary glands secreted GH continuously for up to a week in serum-free medium (100–300 ng/pituitary/day), but levels decreased over time (data not shown).

To rule out the possibility that the glands were merely releasing stored hormones into the culture medium, newly synthesized GH and PRL in the pituitary gland and culture medium was determined by [^{35}S]-methionine incorporation. Pituitary glands contained newly synthesized GH and PRL from days 1 to 7. On all the days assayed, labeled GH and PRL recovered from the medium was (80%) greater than GH or PRL present in the

pituitary gland, showing that the pituitaries actively stored and released newly synthesized GH and PRL (data not shown).

Specificity of the GH and PRL probes was confirmed by Northern blot hybridization. A single transcript was detected with each of the specific probes for GH, PRL and 18S ribosomal RNA, demonstrating probe specificity (data not shown). For quantitative determination of hybridization signals by RNA dot blot hybridization, a standard curve was established for each experiment using serial dilutions of GH or PRL sense cRNA. The signals obtained from the pituitary RNA samples were determined specific and to be within the linear range of the standard curves (data not shown).

To determine if pituitary glands maintained in culture for 24 h were responsive to treatment with 17¥â-estradiol (E_2), a concentration–response study was undertaken to examine the effects of different concentrations of E_2 on GH and PRL mRNA expression. As shown in Figs. 1A and B, E_2 induced significant increases in pituitary GH ($P < 0.001$, one-way ANOVA) and PRL mRNA ($P < 0.001$, one-way ANOVA). Specifically, the highest PRL mRNA levels were seen at the 5 nM of E_2 ($P < 0.01$). There was also a significant ($P < 0.01$), 6-fold, increase in GH mRNA levels at this concentration of E_2 (Fig. 1B). In all subsequent experiments, the 2.5 nM E_2 concentration was used for the positive controls as this produced a significant increase in GH and PRL mRNA levels and this dose is within the linear range of the concentration–response curve.

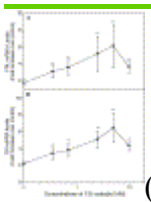
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Fig. 1. Concentration-dependant response of pituitary glands to induction of GH and PRL mRNA by 17¥â-estradiol (E_2). (A) PRL mRNA and (B) GH mRNA in pituitary glands incubated in vitro. The pituitary glands were exposed to E_2 in media for 24 h. Total RNA (4 ¥ìg) from each pituitary gland was dot blotted on to nylon membranes and hybridized with ^{32}P-random-labeled GH and PRL probes and normalized to 18S rRNA. Phosphorimages were analyzed using the Quantity one using software from BioRad. GH and PRL mRNA levels are expressed as fold increase over controls. Data are representative of at least two repeats. Values represent mean ¡¾ SEM ($n = 8$); *$P < 0.05$ and **$P < 0.01$ (ANOVA followed by FPLSD for pair wise comparisons)

represent significant (general *P*-value cut off points) differences from respective controls, at a single time-point.

To assess the responsiveness of pituitary glands to E_2, during several days in culture, the pituitaries were exposed to E_2 every 24 h for 6 days. There were significant effects of E_2 treatment ($P < 0.001$, two-way ANOVA) and incubation time ($P < 0.05$, two-way ANOVA) on PRL mRNA levels, with higher levels occurring at days 1–4 compared with days 5 and 6 (Fig. 2A). There was no significant treatment ¡¿ time interaction on PRL mRNA levels in this experiment. Levels of PRL mRNA, were significantly elevated by E_2 treatment on days 1 ($P < 0.001$, FPLSD), 2 ($P = 0.014$, FPLSD), 3 ($P = 0.02$, FPLSD) and 4 ($P = 0.002$), compared with their time matched controls. Mean control values tended to increase over the time-course, with a significant elevation at day 5 when compared with days 1 ($P = 0.016$, FPLSD), 2 ($P = 0.041$, FPLSD) and 6 ($P = 0.024$, FPLSD). There were significant effects of E_2 treatment ($P < 0.001$, two-way ANOVA) and incubation time ($P = 0.004$, two-way ANOVA) on GH mRNA levels, with higher GH mRNA levels (Fig. 2B) occurring at days 1 ($P = 0.015$, FPLSD), 3 ($P = 0.05$, FPLSD) and 4 ($P = 0.005$, FPLSD), compared with time matched controls. There was no significant ($P = 0.057$) treatment ¡¿ time interaction on GH mRNA levels in this study. In the controls, levels of GH mRNA showed changes with time, with control values on day one being significantly lower than values on days 2 ($P = 0.008$, FPLSD) and 5 ($P = 0.014$, FPLSD) and a decrease with levels on day 6 being lower than levels on day 5 ($P = 0.024$, FPLSD).

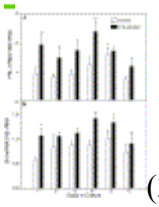
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Fig. 2. Time-course effects of 2.5 nM 17¥â-estradiol (E_2) on GH and PRL mRNA levels in pituitary glands incubated in vitro. (A) Time-course effect of E_2 on PRL mRNA. (B) Time-course effect of E_2 on GH mRNA. Pituitary glands removed from trout were immediately placed in culture medium. After 24 h, the culture medium was replaced with 2.5 nM E_2 in MEM or medium with vehicle alone (for control) and incubated for an additional 24 h. Following this, the glands and culture medium were removed and stored at −80 ¡ÆC for subsequent analyses. The experiment was repeated with pituitary glands maintained in culture from 1 to 7 days. Four micrograms of total RNA was dot

blotted on to nylon membranes and hybridized with GH and PRL probes and normalized to18S rRNA by hybridizing with 18S rRNA oligo probe. Data are representative of at least two repeats. Values are mean ¡¾ SEM values ($n = 8$); *$P < 0.05$ and **$P < 0.01$ represent significant (general P-value cut off points) differences (two-way ANOVA followed by FPLSD for pair wise comparisons) from controls, and ¢Ó$P < 0.05$ represents significant differences between control values at day 1 and days 2–6.

3.2. Effect of *o,p*¡Ç-DDT on Pituitary GH and PRL mRNA Expression

To analyze the effects of EDs on pituitary function, we examined the ability of the estrogenic contaminant *o,p*¡Ç-DDT to influence GH and PRL gene expression. As shown in Figs. 3A and B, both GH ($P < 0.001$; one-way ANOVA) and PRL ($P = 0.003$; one-way ANOVA) mRNA were significantly elevated in a concentration-dependent manner, with the highest levels of PRL ($P < 0.001$, FPLSD) and GH ($P < 0.001$, FPLSD) mRNA expression occurring at a concentration of 1000 nM *o,p*¡Ç-DDT. For subsequent experiments, 750 nM *o,p*¡Ç-DDT was chosen as it was within the linear range of the concentration–response curve and between the two highest concentrations (500 and 1000 mM), where maximal stimulation was seen. When this experiment was repeated, using different fish, PRL and GH mRNA were significantly induced by *o,p*¡Ç-DDT (data not shown); however, while the fold induction of GH and PRL mRNA was lower in these studies, the concentration-dependent trend of increased GH and PRL mRNA levels, to *o,p*¡Ç-DDT treatment, was maintained.

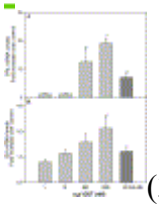 (34K)

Fig. 3. Concentration–response effect of *o,p*-DDT on mRNA levels of (A) PRL mRNA and (B) GH mRNA in pituitary glands incubated in vitro. Pituitary glands removed from trout were immediately placed in culture medium. After 24 h, the culture medium was replaced with the media containing different concentrations of *o,p*¡Ç-DDT. The glands were incubated with media containing *o,p*¡Ç-DDT for an additional 24 h. Following this, the glands and culture medium were removed for analysis. Controls consisted of media with vehicle alone and 2.5 nM of 17¥â-estradiol as a positive control. Total RNA was extracted and dot-blotted on to nylon membranes. GH and PRL

signals were normalized to 18S rRNA levels. Levels of GH and PRL mRNA are represented as fold induction over control and data are representative of at least two repeats. Values are represented as means ¡¾ SEM ($n = 7$); *$P < 0.05$ and ** $P < 0.01$ for significant (general P-value cut off points) increase in mRNA levels over controls (ANOVA followed by FPLSD for pair wise comparisons).

To test whether the stimulatory effect of *o,p*¡Ç-DDT on GH and PRL mRNA levels was mediated through binding with the estrogen receptors, ICI 182 780, a known estrogen receptor antagonist, was co-incubated at various concentrations (1.25, 2.5 and 5.0 nM) with 750 nM *o,p*¡Ç-DDT. As shown in Fig. 4A, there were significant ($P = 0.001$; one-way ANOVA) effects of E_2, *o,p*¡Ç-DDT and ICI 182 780 on PRL mRNA levels. As a positive control, pituitaries were incubated with 2.5 nM E_2, which resulted in a significant ($P < 0.001$; FPLSD) induction of PRL mRNA levels, and the addition of 2.5 nM of ICI showed that the E_2-induced PRL gene expression was also significantly ($P = 0.009$, FPLSD) inhibited by ICI (Fig. 4A). Additionally, incubation of pituitary glands with 750 nM *o,p*¡Ç-DDT resulted in a significant ($P < 0.05$, FPLSD) elevation in PRL mRNA levels, compared with controls. Furthermore, the addition of ICI 182 780 to pituitaries incubated with 750 nM of *o,p*¡Ç-DDT, resulted in a significant decrease in PRL mRNA levels at the ICI 182 780 concentrations of 1.25 nM ($P < 0.05$, FPLSD), 2.5 nM ($P = 0.04$, FPLSD) and 5 nM ($P = 0.04$, FPLSD) used in this study.

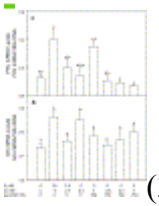 (35K)

Fig. 4. Effect of the estrogen receptor antagonist ICI 182 780 on 17¥â-estradiol (E_2) and *o,p*¡Ç-DDT induced PRL mRNA and GH mRNA levels. (A) Effect of ICI 182 780 on PRL mRNA. (B) Effect of ICI 182 780 on GH mRNA. Following a 24 pre-incubation period, pituitary glands were then co-incubated (media replaced with test agents and solvents for control) with E_2 and ICI 182 780, *o,p*¡Ç-DDT and ICI 182 780 or *o,p*-DDT and ICI 182 780 alone for an additional 24 h. Negative controls consisted of solvent only. Four micrograms of total RNA was dot blotted on to nylon membranes and hybridized with GH and PRL probes against GH and PRL mRNA and normalized to18S rRNA by hybridizing with 18S rRNA oligo probe. Values represent mean ¡¾ SEM ($n = 6$) obtained from one experiment. Groups with different alpha characters (a–e) are

significantly ($P < 0.05$, a general P-value cut off) different from other groups (one-way ANOVA followed by FPLSD for pair wise comparisons).

Pituitary GH mRNA levels were significantly ($P < 0.01$; one-way ANOVA) elevated by E_2 treatment and ICI 182 780 treatment. Specifically, the stimulatory effect of E_2 was significantly ($P < 0.01$, FPLSD) attenuated when ICI 182 780 (1.25 nM) was present (Fig. 4B); however, ICI 182 780 alone significantly ($P < 0.05$, FPLSD) elevated GH mRNA above the solvent controls. Unlike that of PRL, the 750 nM *o,p*¡Ç-DDT treatment was without effect ($P > 0.05$, FPLSD) on GH mRNA levels and co-incubation with ICI 182 780 (1.25-5.0 nM) had no further ($P > 0.05$, FPLSD) effect on GH mRNA expression, although mean levels tended to increase with higher amounts of ICI 182 780 ($P > 0.05$, FPLSD) (Fig. 4B). To rule out the possibility that the inhibition of PRL mRNA by ICI 182 780 was not the consequence of the toxic effect of the compound, the cytotoxicity of ICI 182 780 was assessed by measuring the presence of lactate dehydrogenase (LDH) in the medium. There were no observable cytotoxic effects in all concentrations, and treatments, employed in the study (data not shown).

3.3. Effect of TCDD on GH and PRL gene expression

In contrast to xenoestrogens, TCDD is known to have antiestrogenic effects. The effect of different concentrations of TCDD (0.01–20 nM) on expression of the GH and PRL genes was assessed. Treatment with TCDD significantly altered PRL ($P < 0.001$; one-way ANOVA) and GH ($P < 0.01$; one-way ANOVA) mRNA levels in a concentration-dependent manner. Interestingly, bell shaped concentration–response curves were seen for both PRL and GH, as shown in Figs. 5A and B.

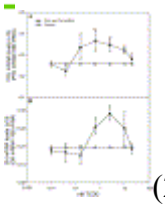
(29K)

Fig. 5. Concentration–response effect of TCDD on levels of PRL and GH mRNA expression in pituitary glands incubated in vitro. (A) Levels of PRL mRNA in TCDD treated samples and controls (B) Levels of GH mRNA in TCDD treated samples and controls. Following a 24-h pre-incubation period, pituitary glands were exposed to different concentrations of TCDD for an additional 24 h. Controls contained vehicle only. Data are representative of at least two repeats. Values represent mean ¡¾ SEM

($n = 8$); $*P < 0.05$, $**P < 0.01$ represent significant (general P-value cut off points) differences between treatment and control values at the respective concentration of TCDD (one-way ANOVA followed by FPLSD for pair wise comparisons).

To determine whether the effects of TCDD on pituitary PRL and GH mRNA expression were mediated via the arylhydrocarbon receptor (AhR), pituitary glands were co-incubated with TCDD (0.03 and 2.5 nM) and alphanapthoflavone (ANF, a known antagonist of the AhR, Merchant et al., 1993) at various concentrations. A 0.03 nM concentration of TCDD, instead of a 0 concentration, was chosen based upon preliminary work (and a limited number of animals) which demonstrated a synergistic effect when low concentrations TCDD were co-incubated with the AhR antagonist (ANF), resulting in an increase in mRNA levels; this was in contrast with the stimulatory effects of higher doses of TCDD (2.5 nM) that could be attenuated with co-incubation with ANF. Additionally, there was no E_2 control, due to the lack of animals; however, the response to E_2 had been consistent from study to study (see Fig. 1, Fig. 2, Fig. 3 and Fig. 4). Consequently, the E_2 and TCDD treatments in this study were designed to be compared with the effects seen with 0.03 nM and 2.5 nM TCDD alone.

In this experiment, there were significant effects of E_2, TCDD, ANF, and their combinations, on GH ($P = 0.009$; one-way ANOVA) and PRL ($P = 0.004$; one-way ANOVA) mRNA levels. An induction of GH ($P = 0.004$, FPLSD) and PRL ($P < 0.001$, FPLSD) mRNA was observed at 2.5 nM TCDD (Figs. 6A and B). Co-incubation of TCDD (2.5 nM) with E_2 (2.5 nM) resulted in a significant ($P < 0.05$, FPLSD) attenuation of the induction caused by TCDD (2.5 nM) with both PRL and GH (except for the 0.03 TCDD ¡¿ 2.5 nM E_2 group) mRNA. In contrast, co-incubation of 2.5 nM E_2 and 0.03 nM TCDD resulted in an increase in mean PRL and GH mRNA levels over control values, however, this increase was not statistically significant. The TCDD-dependent induction of PRL mRNA was significantly ($P < 0.05$) attenuated, in a concentration-dependant manner, upon co-incubation of with ANF, with the greatest decrease occurring at 12.5 ¥iM ANF, which was indistinguishable from control values. At the 0.03 nM concentration of TCDD tested (Fig. 6), where a non significant induction or inhibition of GH and PRL was seen, co-incubation with all three concentrations of ANF showed significant ($P < 0.05$) increases over controls in GH mRNA and increases in mean levels (non-significant) of PRL mRNA (Figs. 6A and B); however, ANF alone (6.25 ¥iM) did not produce any changes in GH or PRL mRNA levels (Figs. 6A and B). The concentrations of ANF or TCDD used in the study did not

exert any significant cytotoxic effect on the pituitary glands as determined by the LDH assay (data not shown).

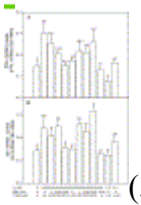
(50K)

Fig. 6. Dose–response, and interaction, of the AhR antagonist, alphanapthoflavone (ANF) and TCDD, on (A) PRL mRNA and (B) GH mRNA in pituitaries cultured in vitro. The gray bars, in each treatment row on the *x*-axis, represent the treatment concentrations immediately to the left of each bar. Following a 24-h pre-incubation period, pituitary glands were incubated (media replaced with test agents or solvents for control) with 2.5 nM TCDD alone or co-incubated with 2.5 nM TCDD and 1.25, 6.25, and 12.5 ¥ìM ANF for an additional 24 h. Similarly, the glands were also treated with 0.03 nM TCDD alone or co-incubated with 1.25, 6.5, and 12.5 ¥ìM ANF. Pituitary glands were also co-incubated with 2.5 nM TCDD and 2.5 nM 17¥â-estradiol or 0.03 nM TCDD and 2.5 nM 17¥â-estradiol. Glands were also cultured in medium containing 6.25 ¥ìM ANF alone or with vehicle alone for control. The treatment procedure was as described earlier and the dot blot hybridization for PRL and GH mRNA was performed as described in the materials and methods section. Values represent mean ¡¾ SEM ($n = 6$) obtained from one experiment. Groups with different alpha characters (a–f) are significantly ($P < 0.05$, general *P*-value cut off) different from other groups (one-way ANOVA followed by FPLSD for pair wise comparisons).

4. Discussion

In this study, we have demonstrated that the two EDs, *o,p*¡Ç-DDT and TCDD, exert a direct effect on the pituitary physiology of an aquatic vertebrate, the rainbow trout. While some differences appear to exist between the mechanisms by which the PRL and GH genes respond to treatment with these EDs, we have shown that the EDs used in this study stimulate PRL and GH mRNA expression in a manner similar to that of 17¥â-estradiol (estradiol), and that these effects are likely mediated via the estrogen and aryl hydrocarbon receptor(s).

Since our study focused on the pituitary hormones, GH and PRL, we validated the functionality of pituitary glands in serum-free culture, and then examined the effects of the *o,p*¡Ç-DDT (estrogenic) and TCDD (antiestrogenic) on the gene transcription of GH and PRL. To analyze the response of cultured pituitary glands to xenoestrogens,

estradiol was used as a positive control. Exposure of pituitary glands to estradiol resulted in a significant increase of mRNA levels for both GH and PRL. PRL gene expression and secretion is known to be regulated by estradiol (Williams and Wigham, 1994) in rainbow trout and in higher vertebrates (Cullen et al., 1993). It is therefore important that PRL mRNA was found to be significantly elevated by 0.5–10 nM estradiol as this verifies that the in vitro system used in this study responds as predicted.

Pituitary GH mRNA was induced upon treatment with estradiol and a maximum increase of 6-fold over controls for GH mRNA was seen with the 5 nM dose of estradiol. In the case of GH, many lines of evidence suggest that gonadal steroids regulate it. In mammals and teleosts, patterns in GH levels and secretion are sexually dimorphic (Harvey and Daughaday, 1995 and Riley et al., 2002). In fish, high levels of GH are seen during ovulation and spawning. Gonadal steroids can modulate GH by affecting hypothalamic releasing hormones, or via receptor/post receptor mediated mechanisms at the level of the pituitary (Melamed et al., 1998). Moreover putative estrogen response elements have been identified in the promoter region of the trout GH gene (Melamed et al., 1998 and Yang et al., 1997b), and gonadal steroids have been shown to stimulate GH release in cyprinids, tilapia and salmonids (Holloway and Leatherland, 1997, Melamed et al., 1995, Shepherd et al., in press and Zou et al., 1997). Nevertheless, the significance of involvement of estrogen in regulating GH gene transcription is not clear.

While our results agree with the aforementioned studies, Yatedie and Male (2002) were unable to demonstrate differences in PRL or GH mRNA levels in juvenile Atlantic salmon (*Salmo salar*) exposed to estradiol or the xenoestrogen, nonylphenol. In the latter study, the lack of a stimulatory effect of estradiol on pituitary PRL and GH is not consistent with current findings reported in this study, nor with previously reported stimulatory effects of estradiol on the teleost pituitary gland (Borski et al., 1991, Melamed et al., 1995, Trudeau et al., 1992 and Zou et al., 1997); however, these differences may be related to experimental differences (route of exposure or dose) or species, sex and developmental differences. It is evident that a conclusive link has not been demonstrated between ED exposure and alteration in the pituitary hormones (GH and PRL) that control salmonid growth and osmoregulation. Consequently, this should be an area of intense focus and investigation.

It is well known that *o,p¡*Ç-DDT acts as a xenoestrogen, although its potency is much lower than that of estradiol. Our data support this as *o,p¡*Ç-DDT caused increases in GH and PRL mRNA at concentrations that were about 500-fold higher than that of estradiol. To establish that *o,p¡*Ç-DDT is working through the estrogenic pathway, we examined

the effects of the estrogen receptor antagonist, ICI 182 780, and found that *o,p*¡Ç-DDT-induced PRL mRNA levels were blocked by co-incubation with ICI 182 780 at increasing concentrations. In this same experiment, it was also found that GH mRNA levels were induced by estradiol (2.5 nM) and that this induction was significantly reduced following the addition of ICI 182 780 (2.5 nM); however, levels of GH mRNA were also significantly induced by ICI 182 780 alone (2.5 nM). As for the stimulatory effect of ICI 182 780 (2.5 nM) on GH mRNA levels, it is interesting to contrast the lack of a stimulatory effect of ICI 192 780 on GH mRNA levels when co-incubated with *o,p*¡Ç-DDT, compared with controls. Another observation from this experiment is that the intermediate concentration (750 nM) of *o,p*¡Ç-DDT used in this experiment did not stimulate GH mRNA levels, unlike the concentration–response study where *o,p*¡Ç-DDT (500 and 1000 nM) did stimulate GH mRNA levels. There is no certain explanation as to why the 750 nM concentration of *o,p*¡Ç-DDT did not stimulate GH mRNA in this instance, however, this difference could be related to the size, condition and sex of the animals used in the different experiments which were also conducted at different times of the year. Additionally, while the stimulatory effect of ICI 182 780 on GH mRNA is difficult to explain at this time, some possible mechanisms are discussed below.

Although *o,p*¡Ç-DDT is known to act as a xenoestrogen by binding to the estrogen receptor (Soto et al., 1994 and Vom Saal et al., 1995), its mechanisms of action are not well understood. For example, in the rat uterus, *o,p*¡Ç-DDT has been known to cause a gene expression pattern that is different from that of estradiol (Diel et al., 2000). Studies using a rat pituitary cell line (MtT/S) have revealed that while estradiol could stimulate GH promoter activity, even in the absence of estrogen response elements, it still required the presence of the pit-1binding site suggesting that the ER might induce GH transcription indirectly via ER/Pit-1 interactions (Iwasaki et al., 2004). Alternatively, work on the rainbow trout ER revealed that proteolysis of the ER protein, bound to nonylphenol, resulted in a differential confirmation compared to that bound to estradiol (Madigou et al., 2001). Therefore, xenoestrogens may alter ER conformation, compared to that seen with estradiol, due to differential binding (proteolysis), recruitment of co-activators, changes in ER subunit (¥á and ¥â) distribution and interactions, and ER-DNA (response element) interactions (Margeat et al., 2003). GH mRNA induction by estradiol requires a number of interrelated, and independent, interactions between ER and Pit signaling, as well as other pathways involving transcriptional and translational (proteolysis) modification, or tissue-specific uptake and metabolism of ICI 182 780 (which may be a partial agonist to the ER) which may account for the stimulatory effects of ICI on GH mRNA levels via one or more of these mechanisms (Dipippo and Powers, 1997, Madigou et al., 2001 and Margeat et al., 2003).

To contrast the effects of estrogenic and antiestrogenic xenobiotics, pituitary glands were treated with tetrachlorodibenzo-*p*-dioxin (TCDD), a xenobiotic with antiestrogenic effects. Most of TCDD¡¯s biological effects have been ascribed to its ability to bind the aryl hydrocarbon receptor (AhR), a receptor that is known to bind a wide range of halogenated aromatic hydrocarbons (Fernandez-Salguero et al., 1995). In our studies, TCDD caused an increase in GH and PRL mRNA levels similar to the xenoestrogen, *o,p*¡Ç-DDT. Though most reports have focused on the antiestrogenic effects of TCDD, these actions are tissue-specific and its antagonistic effects have been shown to include varying mechanisms involving estrogen signaling: (1) TCDD induces cytochrome p450 enzymes (CYP1A1 and CYP1B1) that increase the metabolism of estradiol leading to depletion of intracellular hormone levels; (2) TCDD decreases the levels of expression of various estradiol-inducible genes by blocking or disrupting the binding of estrogen receptor ¥á to the estrogen response element, and binding sites for the transcription factors Sp1 and AP-1 which are adjacent to, or overlapping with, AhR-binding sites (Kietz et al., 2004); and (3) TCDD reduces the level of estrogen receptor ¥á protein by increasing proteosome-mediated degradation (Safe and Wormke, 2003).

TCDD has been shown to have estrogenic effects, resulting in endometriosis in mammals, where the main stimulus for cell proliferation in the endometrium is estradiol (Rier et al., 1993). Although previous studies have revealed the antiestrogenic effect of TCDD in estrogen responsive tissues (Safe, 2001), this may be the first report to reveal effects similar to that of estradiol on pituitary hormones GH and PRL. The molecular mechanisms of TCDD¡¯s antiestrogenic, and sometimes estrogenic effects, has been recently revealed in the MCF-7 and Ishikawa breast cancer cell lines. Here, cross-talk between estrogen receptors and AhR signaling appeared to be partly involved in the estrogenic actions of TCDD (Ohtake et al., 2003). Specifically, when estrogen was absent, the dioxin-activated AhR (aryl hydrocarbon receptor)–Arnt complex associates with the estrogen receptors ¥á and ¥â in the nucleus, leading to activation of transcription and estrogenic effects. However, when estradiol is present, the AhR activation represses estrogen receptor function, thus inhibiting the expression of estrogen responsive genes (Ohtake et al., 2003). Although not measured directly in this study, the data obtained in our studies are in agreement with the above mechanism. While we lacked an estradiol only control, due to experimental limitations, co-incubation of TCDD and estradiol attenuated the induction of PRL and GH mRNA caused by TCDD alone. With this in mind, one could speculate that a similar mechanism may occur in the pituitary, where TCDD, in the absence of estradiol, may cause elevations in GH and PRL mRNA levels.

To analyze the role of the AhR in the modulation of GH and PRL mRNA by TCDD, we tested the effects of the AhR antagonist alphanapthoflavone (ANF), on TCDD-induced GH and PRL mRNA expression. We found that the AhR appears to be involved with mediating the effects of TCDD on both PRL and GH mRNA expression in the rainbow trout pituitary. Here, co-incubation of TCDD (only the 2.5 nM concentration) with ANF (only at the 12.5 ¥ìM concentration of ANF for GH and all concentrations for PRL) attenuated the estrogen-like response (increased PRL mRNA) of the trout pituitary to TCDD exposure. Additionally, while ANF (6.25 ¥ìM concentration) alone did not affect GH or PRL mRNA levels in vitro, the combination of ANF, with the low dose of TCDD (0.03 nM), had a stimulatory effect on PRL and GH mRNA levels. Unlike the clear concentration-dependent attenuation of TCDD-induced increases in PRL mRNA levels, by ANF, the effect of TCDD on GH mRNA expression was less clear and lacked a concentration–response relationship. However, the effect of TCDD (2.5 nM concentration) on GH mRNA was attenuated by the 12.5 ¥ìM ANF concentration. One possible explanation for these unusual findings is that the threshold model of dose responses used in classical toxicology cannot adequately account for such responses, whereas the hormone-like biphasic dose–response model (seen in this study), termed ¡°hormesis¡±, which is characterized by low-dose stimulation and a high-dose inhibition, is now acknowledged as paradigm that more accurately models biological dose–response phenomena (Calabrese and Baldwin, 2003a and Calabrese and Baldwin, 2003b). In this study, co-incubation of TCDD, with the AhR antagonist ANF, revealed a biphasic response to ANF, when the 0.03 and 2.5 nM concentrations of TCDD (which induced GH and PRL) were compared. The data obtained here, especially with TCDD and the TCDD concentration–response effects on GH and PRL mRNA levels, further underscores the need to consider low dose effects when undertaking endocrine disrupter and toxicology studies.

When we consider that the pituitary hormones GH, PRL and somatolactin (SL), and their intermediaries (insulin-like growth factors I and II), control teleost osmoregulation (McCormick, 2001 and Sakamoto et al., 1993), growth (Duan, 1997, McCormick et al., 1992 and Shepherd et al., in press), reproduction (Bhandari et al., 2003, Björnsson, 1997, Gomez et al., 1999, Planas et al., 1992 and Rubin and Specker, 1992) and immune function (Sakai, 1999, Yada et al., 2001, Yada et al., 2004 and Yada et al., 1999), there is no doubt that EDs can seriously impair some or all of these systems, through their effects on pituitary function. A plausible example of this can entail alterations in the release of PRL at an inappropriate time in the life-history of a euryhaline teleost. Specifically, the most conserved function of PRL, in teleosts, is its sodium-retaining (hyperosmoregulatory) actions that facilitate adaptation to, and

survival in, the freshwater environment (Manzon, 2002). Consequently, the sodium-retaining actions of PRL are antagonistic to the seawater-adapting (hypo-osmoregulation) actions of GH (Madsen and Bern, 1992 and Sakamoto et al., 1997) and to seawater survival. Given the stimulatory effects that estrogens, and estrogenic EDs, have on pituitary PRL release, and the antagonistic actions of PRL on hypo-osmoregulatory processes, one would predict that the stimulation of PRL in a migrating salmonid smolt could be deleterious to this, important, transitory developmental period. Indeed, recent studies on salmonids indicate such a mechanism may be involved in delayed smoltification and migration or impaired hypo-osmoregulatory ability (Arsenault et al., 2004, Madsen et al., 2004 and Mancera et al., 2004).

In conclusion, our studies showed that cultured pituitary glands retained functionality in serum-free culture and were responsive to estradiol and EDs. Expression levels of pituitary two pituitary hormones, GH and PRL, were modulated by two persistent xenobiotics, *o,p*¡Ç-DDT being estrogenic and TCDD which has been typically antiestrogenic, but demonstrating some estrogen-like actions in this model system.

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