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Sustained high temperature increases the vitellogenin response to 17α-ethynylestradiol in mummichog (*Fundulus heteroclitus*)

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ABSTRACT

Mummichog (*Fundulus heteroclitus*), an estuarine fish of the western Atlantic, were acclimated to three salinities (0, 16 or 32 ppt) or three temperatures (10, 20 or 26 °C) and exposed to nominal 50 or 250 ng/L 17α-ethynylestradiol (EE2) for 14 days. In a separate experiment, fish were exposed to the same levels of EE2 and were subjected to a 1 h heat shock (20–30 °C) on the 14th day and allowed to recover for 20 h. We were interested in whether or not susceptibility to EE2 exposure, as indicated by increases in vitellogenin (*vtg*) gene expression would change with high and low salinity, warm or cold temperature acclimation or acute heat shock. We also investigated the potential role of heat shock proteins (HSPs) under these conditions. Liver *vtg1* mRNA was significantly induced in male mummichog exposed to 50 and 250 ng/L EE2, but salinity acclimation or acute heat shock did not further affect this induction. Males acclimated to 26 °C and exposed to 250 ng/L EE2 induced 3.5-fold more *vtg1* mRNA than EE2 exposed males acclimated to 10 °C. HSP90 and HSP70 protein were largely unaffected by EE2 exposure. Our findings suggest that mummichog are more susceptible to EE2 under sustained temperature increases that may occur seasonally or with warming of coastal waters.

1. Introduction

Organisms living in coastal marine environments are often subjected to dramatic changes in salinity, temperature and oxygen (Bianchi, 2006). The estuarine killifish or mummichog (*Fundulus heteroclitus*) is an abundant fish species inhabiting coastal marine waters along the Atlantic coast of North America, and thrives over a wide range of water temperature, salinity and dissolved oxygen (Denoncourt et al., 1978; Schulte, 2007). Coastal water temperatures along the Atlantic coast of Canada and northern United States range from −1 to above 15 °C but tidal cycles rapidly increase water temperatures by more than 10 °C (Schulte, 2007). Tidal cycles can also alter water salinity and bring about periodic hypoxia (Smith and Able, 2003). Unfortunately, these fluctuations in environmental conditions are now also coupled with increased contamination from several sources of anthropogenic pollutants (Schulte, 2007) including endocrine disrupting chemicals or EDCs (Sumpter and Jobling, 1995). However, there is little information regarding if and how variable environments may affect the susceptibility of fish to environmental contaminants.

EDCs disrupt the normal hormonal activity that regulates fish growth, development and reproduction by affecting hormone synthesis, storage, release, transport and clearance (Arcand-Hoy and Benson, 1998; Kavlock et al., 1996). A widespread class of EDCs is the estrogen mimics, copying the activity of endogenously produced 17β-estradiol (Arcand-Hoy and Benson, 1998; Jobling et al., 1998). Biological effects of estrogen mimic exposure in fish include the expression of female reproductive proteins in males (Arukwe et al., 1997; MacLatchy et al., 2003), altered behavior (Ward et al., 2006, 2008), reduced egg production (Thorpe et al., 2009) and sex reversal (Jobling et al., 1998). In addition to effects on individual fish, a 7-year study in a Canadian experimental lake showed that chronic estrogen exposure resulted in female-biased sex ratios and reduction in offspring that led to the near collapse of a local fathead minnow (*Pimephales promelas*) population (Kidd et al., 2007).

17α-Ethynylestradiol (EE2) is a potent synthetic estrogen and the main component in female oral contraceptives, entering the aquatic environment through municipal waste discharge (Arcand-Hoy and Benson, 1998; Desbrow et al., 1998). EE2 levels in Canadian sewage treatment plant discharge are in the range of 1–10 ng/L (Ternes et al., 1999) but also have been detected high

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as 176 ng/L in some effluent (Greater Vancouver Regional District, BC, Canada; Campbell et al., 2006). Many key cellular and physiological responses to EE2 have been well described in fish. For example, environmentally relevant levels of EE2 (1–10 ng/L) have been shown to alter normal testosterone and estrogen production in male and female fish (MacLatchy et al., 2003), induce the female egg-yolk precursor protein, vitellogenin (VTG) in male fish (Filby et al., 2007; Martyniuk et al., 2007) and cause intersex (Kidd et al., 2007). Despite the wealth of information on the biological effects of EE2 in fish, most studies on EE2 and other contaminants have been conducted under a single salinity and/or temperature. It is unclear if and how variations in temperature and salinity affect EE2 susceptibility in fish.

There is some recent literature on the effects of EE2 on fish acclimated to different temperatures. Temperature and photoperiod in combination influence *vtg1* mRNA expression in zebrafish (*Danio rerio*) exposed to 10 ng/L EE2 for 21 days (Jin et al., 2010). Fish acclimated to 21 and 30 °C and an extended photoperiod (12–14 h light) induced significantly more *vtg1* mRNA compared to fish kept at 12 °C and shorter photoperiod (10 h light). Körner et al. (2008) found that liver *vtg* mRNA was significantly higher in 19 °C EE2-exposed juvenile brown trout (*Salmo trutta*) compared to 12 °C-exposed fish. It is not known, however, if estuarine fish, with a wider thermal niche and inhabiting more variable environments, respond differently to EE2 exposure at different temperatures. Given that tidal cycles can cause coastal water temperatures to fluctuate ±10 °C within several hours coupled with changes in salinity (21–37 ppt; Todgham et al., 2005, 2006), it is critical that we understand the consequences and mechanisms of action of EE2 exposure in a range of environmental conditions.

In female fish, estrogen normally binds to the estrogen receptor (ER). In teleosts, there are three ER isoforms (ERα, ERβa and ERβb), which are encoded by different genes and exhibit distinct tissue-specificity (Greytak and Callard, 2007; Hawkins et al., 2000; Pinto et al., 2009). In rainbow trout (*Oncorhynchus mykiss*), a second ERα isoform has been identified, ERα2 (Nagler et al., 2007). EE2 largely imparts its negative effects by binding to the ER with greater affinity than endogenously produced 17β-estradiol (E2) (Matthews et al., 2000). Once EE2 is bound, the receptor complex binds to specific DNA response elements and initiates the transcription and translation of estrogen responsive genes and proteins, respectively (Green, 1990; Pelissero et al., 1993). However, male and juvenile fish express low levels of the ER and are therefore susceptible to the effects of EE2 exposure (Pelissero et al., 1993). One of the most commonly used markers of EE2 susceptibility in fish is vitellogenin gene or protein induction in males (Filby et al., 2007; Hogan et al., 2010; MacLatchy et al., 2003; Martyniuk et al., 2007). As is the case in mammals (Pratt and Toft, 1997), ERs in fish are thought to be receptive to ligand binding through the chaperoning action of several heat shock proteins (HSPs) including HSP70 and HSP90 (Osborne et al., 2007; Rendell and Currie, 2005; Yang et al., 2010). HSPs are probably best known in fish for their role in thermal protection (Currie, 2011), and we know that the mummichog exhibit a robust heat shock response (Fangue et al., 2006). Furthermore, it has been shown that fish regularly induce HSPs in response to natural fluctuations in the tidal cycle (Todgham et al., 2006). Thus, we reasoned that fish living in variable coastal environments, would induce HSPs on a regular basis and that an increase in HSPs may increase the number of receptive ERs, thus increasing their susceptibility to endocrine disruptors.

The objective of our study was to determine if changes in salinity acclimation, temperature acclimation or acute heat shock affect the susceptibility of the estuarine mummichog to EE2 by examining the induction of *vtg1* mRNA. Based on previous literature, we hypothesized that fish in warmer water would be more susceptible to environmental estrogens. Due to the putative involvement of HSPs in the estrogen-signaling pathway, we were also interested to know if HSP90 and HSP70 expression and/or subcellular localization changed as a result of EE2 exposure. Examining HSP subcellular distribution could give us novel information on whether HSPs are moving in or out of the nucleus and chaperoning proteins regulating vitellogenesis.

2. Materials and methods

We conducted three simultaneous experimental series. Adult male and female mummichog were exposed to either: two concentrations of waterborne EE2 at three different salinities (Series 1), or one concentration of EE2 at two different short-term acclimation temperatures (Series 2). In the third experiment, mummichog were exposed to two concentrations of EE2 prior to an acute 1 h heat shock (Series 3).

2.1. Animal holding

For Series 1 and 2, mummichog were collected by seining in June 2010 from Little Shemogue, NB, Canada (N46°10′75″, W64°04′46″). Mummichog in Series 3 were collected in September 2009 by seining from Trout River, PEI, Canada (N46°25′31″, W64°27′01″). All fish were kept in 250 L stock tanks at the University of New Brunswick, Saint John, NB, Canada. Fish were fed commercial crushed trout pellets daily (ad libitum; Corey Feed Mills, Fredericton, NB, Canada) and maintained under natural photoperiod in 16 parts per thousand (ppt) salinity (pre-filtered Bay of Fundy sea water and dechlorinated City of Saint John water) at 20 °C with a dissolved oxygen (DO) >80%. Minimal mortalities (<5%) occurred in the stock tanks. Each fish was handled according to Canadian Animal Care Protocols (approved by the University of New Brunswick Saint John Animal Care Committee).

2.2. Exposure

2.2.1. Chemicals and exposure method

17α-Ethynylestradiol (98% purity; Sigma–Aldrich, Oakville, ON, Canada) was stored at −20 °C in 100% ethanol at stock concentrations of 1000 and 5000 ng/mL. Fish were exposed in 20 L aquaria to nominal 0, 50 or 250 ng/L EE2 (Series 1 and 3) and 0 or 250 ng/L EE2 for Series 2 under static renewal conditions, with a complete daily renewal in the morning. Concentrated EE2 was diluted in 1 mL of ethanol (0.005% ethanol/tank) to create nominal exposure concentrations of 50 and 250 ng/L EE2. For the 0 ng/L concentrations, an ethanol vehicle control was used (0.005% ethanol/tank).

2.2.2. Series 1 and 2: salinity and temperature acclimation

Prior to the exposure, fish were distributed over five holding tanks and acclimated to 20 °C and different levels of salinity (i.e. 0, 16 or 32 ppt, Series 1) or 16 ppt at different temperature levels (i.e. 10 or 26 °C, Series 2) under a summer photoperiod (16 h:8 h light:dark) for 7 days. Fish from Series 1 (acclimated to 16 ppt) were used as 20 °C-acclimated fish in Series 2. In Series 1, salinity was decreased or increased 3 ppt per day from 16 ppt, and for Series 2, temperature was decreased or increased 2 °C per day from 20 °C to reach the appropriate experimental conditions (i.e. 3–5 days). Mummichog were then randomly allocated to 52 20 L aquaria with three fish/sex/tank, and acclimated to their specific environmental conditions for one week and then exposed to EE2 each day for 14 days under their appropriate temperature/salinity condition.

2.2.3. Series 3: acute heat shock

Mummichog were kept in holding tanks as described in Section 2.1 at 16 ppt, 20 °C and a summer photoperiod (16 h:8 h light:dark) and randomly allocated to 24 20 L aquaria with three fish/sex/tank.