An Overview Of Estrogen-Associated Endocrine Disruption In Fishes: Evidence Of Effects On Reproductive And Immune Physiology

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Abstract

Simply and perhaps intuitively defined, endocrine disruption is the abnormal modulation of normal hormonal physiology by exogenous chemicals. In fish, endocrine disruption of the reproductive system has been observed worldwide in numerous species and is known to affect both males and females. Observations of biologically relevant endocrine disruption most commonly occurs near waste water treatment plant outfalls, pulp and paper mills, and areas of high organic loading sometimes associated with agricultural practices. Estrogenic endocrine disrupting chemicals (EEDCs) have received an overwhelmingly disproportionate amount of scientific attention compared to other EDCs in recent years. In male fishes, exposure to EEDCs can lead to the induction of testicular oocytes (intersex), measurable plasma vitellogenin protein, altered sex steroid profiles, abnormal spawning behavior, skewed population sex ratios, and lessened reproductive success. Interestingly, contemporary research purports that EDCs modulate aspects of non-reproductive physiology including immune function. Here we present an overview of endocrine disruption in fishes associated with estrogenic compounds, implications of this phenomenon, and examples of EDC related research findings by our group in the Potomac River Watershed, USA.

Key words: intersex, endocrine disruption, estrogen, hormones, immunology

Introduction

Since the late 1990s there has been an increased awareness and concern throughout the scientific and general population regarding endocrine disrupting chemicals (EDCs). Broadly defined, EDCs are exogenous agents that interfere with the production, release, transport, metabolism, binding action or elimination of endogenous hormones that are produced normally for the maintenance of homeostasis and regulation of developmental processes (Kavlock et al. 1996). Permutations of this definition have been synthesized and adopted by groups worldwide including the European Commission, the National Research Council, and the World Health Organization, which further emphasizes the global scope of this issue. While primary EDC concerns are anthropocentric in nature, the chemicals implicated as endocrine disruptors are virtually ubiquitous and have been identified in aquatic ecosystems world-wide (Kime 1998, Vos et al. 2000, Noaksson et al. 2001, Kolpin et al. 2002, Gong et al. 2003, Goksoyr 2006). They pose unique and poorly defined population risks to aquatic organisms including fish. Concerted efforts to investigate the effects of EDCs on fishes have been in progress for over a decade and this topic has been the subject of numerous reviews (Kime 1998, 1999, Matthiessen 2003, Porte et al. 2006, Tyler et al. 2007, Cheshenko et al. 2008, Rempel and Schlenk 2008, Iwanowicz and Ottinger 2009; Kloas et al. 2009). Due to the fact that EDCs by definition modulate endocrine physiology most research to date has rather specifically focused on physiological aberrations resulting from EDC exposure. Interactions and cross-talk between the endocrine and immune systems are well described in mammalian models; however, the influence of EDCs on immune function is sparsely investigated in fishes (Iwanowicz and Ottinger 2009). The following overview on endocrine disruption in fishes is not intended to be a comprehensive review of

this topic. Rather, the intent is to introduce this subject matter to fish health scientists that primarily focus on infectious diseases and encourage future consideration of disease and immune function in the context of endocrine physiology.

Estrogenic Endocrine Disruptors

Perhaps the best studied groups of EDCs are those that interact with the estrogen recaptor. These chemicals, collectively termed estrogenic endocrine disrupting chemicals (EEDCs), differ in many respects but have structural similarities that facilitate their interaction with the ligand binding domain of estrogen receptor subtypes. The sources of EEDCs vary depending on geographical location, but include both natural and anthropogenic origins (Table 1). Natural sources include biologically derived estrogens excreted by humans, wild animals and livestock. Plants and fungi also produce estrogenic compounds. Animal derived estrogens are most often introduced to aquatic systems via municipal wastewater systems and husbandry runoff (Finlay-Moore et al. 2000, Shappell 2006). While the sources of estro-

Table 1: Cursory list of some estrogenic compounds of anthropogenic or natural sources

Persistent Organohalogens	**Synthetic Estrogens**
Dioxins and furans	Chlorotrianisene
Polybrominated biphenyls (PBB)	Dienestrol
Polychlorinated biphenyls (PCB)	Diethylstilbesterol
	Ethinylestradiol
Food Antioxidant	Fosfesterol
Butylated hydroxyanisole (BHA)	Mestranol
Propyl gallate	Polyestradiol phosphate
	Quinestrol
Pesticides	
Aldrin	**Selective Estrogen Receptor Modulators**
Allethrin, d-trans	Afimoxifene
Dichlorodiphenyltrichloroethane (DDT)	Arzoxifene
Dicofol (Kelthane)	Bazedoxifene
Dieldrin	Clomifene
Endosulfan	Femarelle
Fenarimol	Lasofoxifene
Fenvalerate	Ormeloxifene
Chlordecone (Kepone)	Raloxifene
Hexachlorocyclohexane (Lindane)	Tamoxifen
Methoxychlor	Toremifene
Nonachlor, trans	
Permethrin	**Phthalates**
Triadimefon	Butyl benzyl phthalate (BBP)
Toxaphene	Di-n-butyl phthalate (DBP)
	Di-ethylhexyl phthalate (DEHP)
Plasticizers and Other Chemicals	Diethyl phthalate (DEP)
Benzophenone	
Bisphenol A	**Phytoestrogens**
Bisphenol F	Isoflavones
Nonylphenol	Lignans
Octophenol	
Styrene (dimmers/trimers)	**Mycoestrogens**
	Zearalenone

gen may not seem like a significant contribution to the environment, the unnaturally high volumes of these waste constituents in the context of high intensity animal feeding operations must be considered. Phytoestrogens present during algal blooms fueled by eutrophication are also worthy of mention. Synthetic EEDCs include plasticizers, detergents, pharmaceuticals, personal care products, herbicides, pesticides, many of the legacy compounds (e.g. PCBs, DDT, aldrin, dieldrin, etc.) and others. These compounds are introduced to aquatic systems via industrial and sewage discharges, active application and runoff, and atmospheric deposition. While the intrinsic estrogenic potential of many of these anthropogenic chemicals is an unintentional design artifact, some have been specifically tailored to be potent estrogen receptor ligands. For instance, EEDCs such as the active compounds in some contraceptive pills (e.g. ethynylestradiol) are more physiologically potent and stable than endogenous, natural estrogens by intentional pharmaceutical design. Clearly such pharmaceutical compounds are likely to be more persistent in the environment, and they are capable of exerting physiological effects at picomolar concentrations. Selective estrogen receptor modulators (SERMs), which are commonly prescribed for problems such as anovulation, menopause symptoms, bone disturbances (osteoporosis), and breast cancer in humans are have yet to receive great attention as environmental EEDCs, but this will likely change as novel analysis methodologies become available. Of particular note, EEDCs are very rarely present in the environment in singularity. Rather, complex mixtures of these (and other) chemicals are a realistic expectation. The EEDCs in such mixtures affect physiological systems in aquatic biota synergistically, additively, or exert complex agonistic-antagonistic actions of unknown outcome.

Mechanisms of Endocrine Disruption

Based on the broad definition of EEDCs and a general understanding of endocrine networks, the potential mechanisms and pathways available for EEDCs to 'short-circuit' normal endocrine regulation are myriad. Perhaps the best studied mechanism of endocrine disruption is hormone mimicry. In this case, receptors are activated by chemicals structurally similar to the endogenous ligand and bind to that molecule as a functional agonist. Mimicry leads to the inappropriate induction of estrogen responsive genes and synthesis of proteins. During periods of high circulating estrogen concentration, exposure to an EEDC may be of little consequence; however, if exposure occurs during a life-history stage or season when estrogen concentrations are low or undetectable such exposure may have biologically profound consequences. Timing (developmentally and seasonally) of exposure is a critical factor in terms of the biological outcome. Because of the remarkable degree to which endocrine systems are conserved, these structurally diverse EEDCs induce estrogen receptor (ER) mediated gene expression in a wide variety of species (Matthews et al. 2002). Mimicry need not result in inappropriate gene activation to have a significant impact. Endocrine disruption may also occur when a ligand binds the estrogen receptor ligand binding domain without inducing activation. In this case the ligand serves as a functional antagonist and competes for receptor binding sites with endogenous estrogens. Consequently, normal transcription induced by the endogenous ligand is lessened or ablated by the competing disruptor due to reduced receptor availability. Intermediate levels of activation or antagonism are the *modus operandi* exploited by SERMs. Additional mechanisms, which are not necessarily mutually ex-

clusive, include modifying normal hor-mone metabolism (clearance), synthesis or receptor expression (Thibaut and Porte 2004). The net result of the above actions is a disturbance in normal hormone physiology and 'unexpected' pressures on endocrine homeostasis, cell signaling and gene transcription.

EEDCs, Biomarkers, and Reproduction

The effects of EEDCs on aquatic biota are well documented as this tends to be the most intensively studied aspect of endocrine disruption in fishes. These effects manifest at differing levels of biological significance depending on numerous factors including the duration and frequency of exposure, the specific exposure chemical(s), age and developmental stage, season, and sex. Exposure to EEDCs is associated with a number of documented effects that span the gamut of biological organization (molecular, cellular, organ, individual and population) and ecological significance (Ankley et al. 2009). Many of these measureable effects are commonly used as biomarkers of EEDC exposure. For instance, the phospholipoglycoprotein vitellogenin (Vtg), which is an egg yolk precursor in oviparous organisms (including most fishes), is usually only present in minute or undetectable quantities in males. Exposure to EEDCs leads to the induction of hepatic *Vtg* gene and protein expression in male fish. While the biological significance of such induction in male fish is unclear (although kidney pathology has been associated with elevated *Vtg*), circulating *Vtg* in male fishes is a well-accepted biomarker of EEDC exposure (Sumpter and Jobling 1995, Van der Ven et al. 2007). The observation of intersex (such as testicular oocytes) is also commonly used as a biomarker of EEDC exposure (Krisfalusi and Nagler 2000, Nolan et al. 2001, van Aerle et al. 2001, Kavanagh et al. 2004, Blazer et al. 2007). In general, EEDC exposure in fishes is most often associated with the inappropriate induction of estrogen sensitive genes, abnormal development of reproductive organs or gametes, and temporal shifts in sexual maturation and spawning behavior (Arukwe et al. 2000, Jobling et al. 2002a, Kavanagh et al. 2004, Nash et al. 2004, Gunnarsson et al. 2007, Schoenfuss et al. 2008). Perhaps of greatest biological significance in regards to reproduction, EEDC exposure is known to reduce fertility and lead to population collapse (Jobling et al. 2002b, Nash et al. 2004, Kidd et al. 2007).

Estrogens, EEDCs and Immunity

Exposure to estrogen or EDCs is also known to modulate immune responses of fish (Hoeger et al. 2005, Liney et al. 2006, , Filby et al. 2007, Iwanowicz and Ottinger 2009). Likewise EEDC's are known to modulate immune responses in higher vertebrates (Ahmed 2000, Ndebele et al. 2004, Inadera 2006). Documented experiments designed to specifically investigate the effects of estradiol on immune responses of fish are limited. None the less, we cannot discount these limited experiments. Many of the speculated effects of estradiol on fish immune function are based primarily on (but not limited to) observations of modulated immune function or humoral parameters during seasons of increased circulating estradiol. However, there is experimental evidence that further demonstrates functional immunomodulation. The *in vitro* proliferative response of goldfish primary PBLs induced by phorbol myristate acetate (PMA) and the calcium ionophore A23187 is suppressed in a dose dependent manner following *in vitro* estradiol exposure (Wang and Belosevic 1995). Additionally, *in vivo* exposure to estradiol has been shown to modulate the immune response to the hemoflagellate, *Trypanosoma danilewski* (Wang

and Belosevic 1994). Other salient research has revealed effects on chemotaxis and phagocytosis but not on nitric oxide production or the generation of superoxide (Wang and Belosevic 1994). Yamaguchi et al. (2001) obtained similar results when they used physiological concentrations of *in vitro* administered estradiol (0.1, 1, 10, 100 or 1000 nM) and primary leucocytes from carp. Recent work by Robertson et al. (2009) has also demonstrated that *in vivo* exposure to 17β-estradiol ablates the expression of the induced hepcidin-2 response in centrarchids. The modulatory effects of estradiol on complement activity, serum peroxidase activity and IgM in sea bream have also been shown (Cuesta et al. 2007). A list of immune-associated molecules modulated by estrogen in mammals is presented in Table 2.

Table 2: Immune system-associated molecules modulated by estrogens (modified from Iwanowicz and Ottinger, 2009).

Cytokines and cytokine receptors	**Cytokines and cytokine receptors**	**Other immune related molecules**
IL-1β	IL-8	B7-1, B7-2
IL-2 IL-2R		CD40, CD40L
IL-4	MCP-1	
IL-6[d]	MIP-1ß	CTLA-4
IL-7	MIP-2	VCAM-1
IL-10	MIP3α	ICAM-1
IL-12	MIP3β	P-selectin
IL-13	CINC-1, CINC-2β, CINC-3	VEGF
IL-15		ECF
IL-18	CCR1, CCR2, CCR5	
	CXC10, CXC11	
TNFα		Metaloprotease 9
IFNγ	LT-β	B-Defensin
TGFβ		Complement C3
		Hepcidin
APC and granulocyte associated	**Apoptosis/ cell death**	
MHC-II	Fas/ FasL	GATA-3
iNOS	TRAIL	FOXP3
nNOS	bcl-2	RANTES
	shp-1	
Myeloperoxidase		
Elastase		
Superoxide		

While most EEDC-associated reports primarily involve reproductive and developmental effects, a number of immunologically-associated effects have been documented during recent years. For example, the alkyl-phenol 4-nonylphenol (NP) is an estrogen mimic and is perhaps one of the best known EEDCs. NP is known to inhibit LPS-induced NO and TNFα production which is attributed to an ER dependent inhibition of NF-κB transactivation. This response is not associated with ERE directed transcription (You et al. 2002). Others have shown immunological effects induced by other alky-phenols. For instance, p-n-nonylphenol sup-presses T_{h1} development and enhances T_{h2} development. Exposure to p-n-octylphenol elicits similar effects, while NP and p-t-octylphenol have weaker effects. Interest-ingly using the same *in vitro* systems, exclusive treatment with estradiol by itself fails to affect T_{h1}/T_{h2} development (Iwata et al. 2004). Another EEDC, bisphenol A (BPA) has been shown to affect non-specific immune defenses against non-pathogenic *Escherichia coli* (Sugita-Konshi et al. 2003).

In fish, NP and BPA affect the normal function of carp anterior kidney phagocytes at nanomolar concentrations *in vitro*. Specifically, NP and BPA exposure leads to an increased production of superoxide anions and a decrease in phagocytic activity (Gushiken 2002). Expression profiling has demonstrated modulatory effects of NP on members of the complement cascade (Ruggeri et al. 2008). Pthalates have also been shown to negatively impact phagocytic cells of common carp (Watanuki et al. 2003). In mam-mals NP has been shown to inhibit LPS-induced NO and TNFα production which is attributed to an ER dependent inhibition of NF-κB transactivation. This response is not associated with ERE directed transcription (You et al. 2002). Other evidence suggests that p-n-NP suppresses T_{h1} development and enhances T_{h2} development. Reviews on this topic include Jansson and Holmdahl (1998), Druckmann (2001), Lang (2004), Obendorf and Patchev (2004), Cutolo et al. (2005), and Grimaldi et al. (2005). Interestingly, and of particular significance, early life-stage exposure to the EDCs o,p-DDE and Aroclor 1254 are known to induce long-term immunomodulation in salmonids (Milston et al. 2003, Iwanowicz et al. 2005). Thus, in addition to transient effects on immune function, exposure to contaminants and EEDCs during critical developmental windows may permanently affect normal lifelong immune responses.

Fish Kills, Intersex and EEDC

During 2003, an investigation was initiated to discern the putative cause(s) of adult smallmouth bass (*Micropterus dolomieu*) deaths in the South Branch of the Potomac River, USA. A high prevalence of intersex, which histologically presented as testicular oocytes, was observed (Blazer et al. 2007). In an attempt to define the regional prevalence of intersex, subsequent sampling was conducted, the results of which confirmed that intersex is prevalent in male smallmouth bass in the Potomac River Basin. Interestingly, locations in the Shenandoah River with a high prevalence of intersex were also the sites of frequent, significant fish kills (Ripley et al. 2008). Intersex has been identified in male smallmouth bass collected from out-of-basin sites, but the frequency and severity are much lower than those observed in the Shenandoah and Potomac Rivers (Blazer et al. 2007). Attempts to identify specific EEDCs in this drainage using polar organic chemical integrative samplers (POCIS) have been successful. Additionally, estrogenic activity of water sample extracts using a biolumin-

escent yeast estrogen screen (BLYES) has confirmed biological estrogenic activity (Alvarez et al. 2009). Although natural estrogens (17β-estra-diol, 17α-ethynylestra-diol, estrone and estriol) have been identified in water samples at concentrations as high as 8.1 ng/L from the watershed, it appears that the influence of wastewater treatment plants does not singularly explain the incidence of intersex. Additionally, estrogenic activity of water extract samples as high as 79 ng/L E2 EEQ determined by the yeast estrogen screen assay have been reported (Alvarez et al. 2008, 2009, Iwanowicz et al. 2009). A variety of chemicals and sex steroids associated with agricultural land use likely explain some of the observations in the watershed. There does appear to be a correlation between land use and the prevalence of intersex based on simple analyses; however, more intensive analyses are in progress using a cumulative dataset to more conclusively identify associations (Blazer et al. 2007). Another point of interest in the watershed is the coincidence of a high prevalence of intersex and spring fish kills at many of the sites investigated. While not experimentally confirmed, it is possible that the EEDCs and other chemicals identified in the watershed (some that are likely associated with intersex) adversely impact the immune system and disease resistance at these sites.

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