Vitellogenin, A Marker of Estrogen Mimicking Contaminants in Fishes: Characterization, Quantification and Interference by Anti-Estrogens

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ABSTRACT

Vitellogenin (Vg), the estrogen inducible protein precursor to egg yolk, serves as an indicator of exposure to estrogen mimicking environmental contaminants. Vg was isolated by size exclusion and ion exchange chromatography from plasma of California halibut (Paralichthys californicus) treated with estrogen. MALDI TOF mass spectrometry (MS) analysis resulted in a molecular mass of 188 kDa. MS/MS de novo sequencing provided evidence that California halibut has more than one form of Vg. Similar analysis on white sturgeon (Acipenser transmontanus) Vg did not reveal adequate evidence to suggest that sturgeon has more than one Vg. The potential of using other MS methods to understand the structure and function of Vg are discussed.

An ELISA for measurement of California halibut plasma Vg was optimized and validated using a commercially available antibody developed for another flatfish species, turbot. Inclusion of overnight preincubation was critical for low detection limits. Increasing the amount of Tween-20 to 0.05% in buffers was most effective for improving recoveries of spiked plasma samples. At the IC50, the average recovery of spiked plasma samples was 104% and the interplate CV was 12%. The working range of the assay was 33-1000 ng/mL, while the detection limit in a plasma sample is 2.2 μg/mL. The response to the model compounds 17β-estradiol and p-nonylphenol show that this is a suitable model for further studies of estrogen mimicking contaminants.

White sturgeon are native to the Sacramento River and subject to agricultural, municipal and industrial waste water effluents that likely contain different classes of endocrine-disrupting contaminants. Reductions in 17β-estradiol-induced vitellogenin levels were observed in white sturgeon co-injected with β-naphthoflavone (BNF, 50
mg/kg), an Ah receptor agonist. The inhibition was maximal when the compounds were injected simultaneously versus prior treatment of fish with BNF. This timing of the effect compared to increases in ethoxyresorufin-\textit{O}-deethylase (EROD) activity suggests that the effect is not directly due to enhanced estrogen metabolism by the Ah receptor-induced enzymes. Results of this study will be relevant for those with monitoring programs who measure vitellogenin, as it is important to understand how Ah (dioxin) receptor active environmental contaminants can influence this endpoint.
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Endocrine disruption

Endocrine disruption refers to the alteration of normal hormone signaling by endogenous compounds, commonly environmental contaminants. It has been a difficult problem in many ways, from understanding the various underlying biological mechanisms to evaluating the overall impact on wildlife reproduction. One of the earliest reports of endocrine disruption was imposex in mollusks, the growth of male sex organs in females, from the use of anti-fouling paint used on ships containing tributyltin (TBT). Almost 30 years later, the exact biological mechanism is still unknown, though TBT is undoubtedly the cause (Oberdorster and McClellan-Green, 2002). Another well known, and poorly understood, incidence of endocrine disruption occurred in the 1980s, when the population of alligators plummeted in lake Apopka, Florida. Surveys found that the remaining population of alligators was primarily female, and that Lake Apopka was polluted with a number of contaminants, including DDT. Although DDT seemed to be the most likely culprit, the story of the disappearing male alligators has been slow to unfold. Initially, it seemed that is was not actually an effect of DDT, but two of its metabolites, $o,p'$-DDE and $p,p'$-DDE, which are weak estrogens in *in vitro* studies. These compounds were thought to have feminized all the male alligators. Later $p,p'$-DDE was found also to be an anti-androgen (Kelce et al., 1995), which might have interrupted normal testosterone signaling during the development of the male alligators. Still, there were the other contaminants, one of which was dicofol, another weak estrogen. Even
now, there is no one explanation for the alligator feminization that is clearly the cause (Guillette et al., 2000). It may well have been a combination of several of the contaminants in the lake, and other environmental factors may have been involved. This Lake Apopka example illustrates well the complex nature of the problem of endocrine disruption.

What is normal?

The incidents of endocrine disruption described above were fairly isolated and these problems have lessened now that TBT and DDT are no longer commonly used. Currently, one of the most concerning symptoms is the widespread observation of fish with both ovarian and testicular tissue in their gonads. Reports of these intersex fish date back many years, however, some observations of abnormal gonads in wildlife may not result from anthropogenic sources. Normal sexuality in fish ranges from gonochorism, having two distinct sexes, to synchronous hermaphroditism, in which one fish has both male and female sexual organs at the same time, to protandrous and protogynous hermaphroditism, in which fish are born male and then undergo a genetically programmed change into female, and the converse (Baroiller et al., 1999). What seems abnormal may in fact be a natural process. Before looking into the effects of contaminants, a basic understanding of sexual development and reproduction of the species in question is needed.

Sex in fish is determined genetically, but the sexual phenotype of fish can change, and the responsible genetic and individual factors vary widely with species. Some fish can change sex based on the sex ratio of their community or the social structure (Baroiller
et al., 1999), while temperature can alter sex in other species (Baroiller et al., 1999; Blazquez et al., 1998; Fishelson, 1970; Patino et al., 1996; Yamamoto, 1999). While genetics is the basis for sex, it is the hormone signaling that initiates formation of male or female specific tissue development. This signaling begins in the brain-hypothalamic-pituitary axis, with the hypothalamus releasing gonadotropin releasing hormone. This signals the release of gondotropins from the anterior potion of the pituitary, including analogs to the mammalian follicle stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins regulate gonad development, steroidogenesis, and ovulation. Steroidogenesis, the production of estrogens and androgens, is regulated in part by positive and negative feedback from the brain and pituitary, and from environmental factors, particularly seasonal changes in light and temperature (Jalabert et al., 2000; Moyle and Cech, 2004). Hormonal signaling is initiated by binding of the hormone to its receptor, such as estrogen binding to the estrogen receptor (ER) or testosterone binding to the androgen receptor (AR).

Types and mechanisms of endocrine disruption

Endocrine disruptors (EDs) encompass any chemical that alters the function of hormones involved in growth, development, and reproduction, including estrogens, androgens, and thyroid hormones. EDs vary in source, from more industrial types, such as surfactants, plasticizers and pesticides, to natural and synthetic hormones and other compounds used in personal care products. Natural estrogens, such as 17β-estradiol (E2) and estrogen (E1), the main synthetic estrogen in hormonal contraceptives, 17α-ethynylestradiol (EE2), and those prescribed for hormone replacement therapy of
menopausal women, logically, all bind to and activate the estrogen receptor. These compounds become environmental contaminants when exceeded in urine, as some pass through sewage treatment. Other compounds can also bind to and activate the estrogen receptor due to similarity in the chemical structure, hence the commonly used term estrogen mimicking contaminants. Often the active moiety is an aromatic ring with a hydroxyl group on a bulky molecule. Some of the most well known environmental estrogen mimics are \( p \)-nonylphenol and bisphenol-A (Sonnenschein and Soto, 1998). Nonylphenol is a degradation product of alkylphenol ethoxylates, which are widely used in industrial surfactants. Bisphenol-A is a component of polycarbonate, a very strong, versatile, and common form of plastic. Bisphenol-A is a less potent estrogen mimic than nonylphenol that is also known to bind to the ER, but bisphenol-A may have other modes of endocrine disrupting effects including anti-androgenic or anti-estrogenic activity (Letcher et al., 2005; Sohoni and Sumpter, 1998). The pesticide dicofol is also a weak ER agonist (Hoekstra et al., 2006) as well as \( o,p' \)-DDT, \( o,p' \)-DDE and \( p,p' \)-DDE, as described above.

Besides estrogen mimics, a number of mechanisms of endocrine disruption have been identified. Androgenic and estrogenic effects can occur through effects on the aromatase enzyme. Aromatase is one of many cytochrome \( P450 \)s that regulate steroid formation from cholesterol and their metabolism. In particular, aromatase mediates the biosynthesis of estrogen from testosterone. TBT, the cause of imposex mentioned above, is thought to be an example of an aromatase inhibitor, which results in an excess of testosterone and produces a penis in the female dog whelk. (Oberdorster and McClellan-Green, 2002). On the other hand, the herbicide atrazine is thought to be an aromatase
inducer, covering more testosterone to estrogen than normal and causing the 
demasculinization of amphibians (Hayes et al., 2006). Otherwise, anti-androgens can 
exert their effects by occupying the androgen receptor without activating it. In doing so, 
they will block normal androgen signaling. This mechanism of anti-androgenic action 
was identified for metabolites of the fungicide vinclozolin (Wong et al., 1995) and \( p,p' \)-
DDE, as described above. Anti-estrogenic effects, can also be produced by from agonists 
of the aryl hydrocarbon receptor (AhR), such as polychorninated dibenzo-\( p \)-dioxins (i.e. 
TCDD), some polychlorinated biphenyls, polyaromatic hydrocarbons, polychlorinated 
dibenzo-furans, and hexachlorobenzene and other compounds (Safe et al., 1998). Two 
additional mechanisms of endocrine disruption have been suggested for triclosan, a very 
commonly used anti-microbial agent. Triclosan is potentially weakly androgenic (Foran 
et al., 2000) and it disrupts thyroid hormone homeostasis (Crofton et al., 2007).

Although the focus of this thesis is on compounds that can alter estrogen 
signaling, all of these mechanisms are important to keep in mind, as well as their basic 
physiology. For example, compounds that actually affect the androgen receptor (anti-
androgens) can be confused with estrogen mimics because they can lead to an overall 
feminizing effect. Additionally, these hormone systems are tied together. Estrogen is 
derived from testosterone, and the level of one affects the other. Another way to create an 
estrogenic or anti-estrogenic effect is to alter one of the many process involved in 
regulating the circulating hormone levels, such as synthesis, metabolism, and the ability 
of the hormones to bind to serum proteins. Estrogen and androgen levels are regulated by 
positive and negative feedback loops in the brain-hypothalamic-pituitary axis, and these
feedback systems can also be affected by contaminants, which would disrupt hormone regulation.

**Occurrence of endocrine disruptors**

Examples of all the types of compounds above have been detected in waterways, many at or near concentrations known to cause effects. A 1999-2000 survey of the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants in 139 U.S. streams detected compounds representing a wide range of residential, industrial, and agricultural origins and uses (Kolpin et al., 2002). Half the samples contained nonylphenol, with average concentration of 0.8 µg/L and a maximum of 40 µg/L. Bisphenol A was in 41% of samples with an average concentration of 0.14 µg/L and a maximum of 12 µg/L. Steroid hormones were less frequent, quantified in about 10% of samples and averaging 9-160 µg/L. Tricolsan was one of the most frequently detected compounds occurring in almost 60% of samples with an average concentration of 0.14 µg/L. Also detected were the PAHs (pyrene and phenanthrene, benzo[a]pyrene, anthracene and more), phthalates, pesticides, and other pharmaceuticals and antibiotics (Kolpin et al., 2002). In the United Kindom (UK), it was found that the concentrations of endocrine disrupting compounds rise and fall depending on the proximity to sewage treatment plant outfalls (Zhou et al., 2007). The compounds and their concentrations upstream, outfall, and downstream ranged as follows: bisphenol A (18.5 - 38 ng/L), 4-nonylphenol (1 - 28.3 ng/L), 4-tert-octylphenol (7 - 27.5 ng/L), E1 (1.1 - 26.5ng/L), and E2 (7 - 22.5 ng/L). The concentration of EE2 was significantly lower (<1 ng/L). Zhou et al. also measured some of the sorption of these lipophilic contaminants to particles in the
water and estimated that between 10 - 29% of endocrine disruptors were associated with aquatic colloids. As colloids are highly abundant in rivers and ocean, they will therefore play a significant role in the environmental behavior and fate of endocrine disrupting contaminants.

Field studies and biomarkers

One of the most common markers used to study endocrine disruption is the protein vitellogenin (Vg). Vitellogenins are large serum proteins synthesized by oviparous vertebrates as yolk precursors. Vg serves as the sole nutrition source for larval fish, and is thus critical to reproduction (Mommsen and Walsh, 1988). Synthesis of Vg is controlled by the hormone estrogen and normally occurs in pre-spawning females. Exogenous estrogen or xenoestrogens can induce synthesis of Vg in males and juveniles, making this protein a useful indicator of environmental contaminants that activate the estrogen receptor signaling pathway (Sumpter and Jobling, 1995).

Beginning about a decade ago, sewage effluents in the UK were found to have estrogenic properties as demonstrated by elevated levels of protein Vg in fish in rivers nearest sewage effluent sources (Harries et al., 1996; Kirby et al., 2004). Later these effects were observed in other European countries, in carp, bream and flatfish (Knudsen et al., 1997; Lavado et al., 2004; Sole et al., 2002; Vethaak et al., 2002), in Japan, in flounder and goby (Hashimoto et al., 2000; Ohkubo et al., 2003), and in catfish and bass in US (Tilton et al., 2002; Todorov et al., 2002). In the UK, Vg levels in males sometimes exceeded those normally found in sexually mature females (Allen et al., 1999). More worrisome has been the observation of intersex fish, mentioned above, which has also
been correlated to the proximity to municipal sewage effluents. This intersex effect is widespread, occurring in white suckers and carp in the US (Folmar et al., 1996; Woodling et al., 2006), in flounder in the UK (Allen et al., 1999; Kirby et al., 2004; Scott et al., 2006), Japan (Hashimoto et al., 2000) and France (Minier et al., 2000), in bream in Germany (Hecker et al., 2002), and in wild Italian cyprinids (Vigano et al., 2001). Not surprisingly males with ovarian tissues in their gonads have reduced fertility. Not only was there a severe reduction in production of milt in the most feminized fish, but the sperm motility, the ability of sperm to successfully fertilize eggs, and the production of viable offspring were also observed to be reduced in intersex fish (Jobling et al., 2002).

Another marker of endocrine disruption is the size of the gonads, as smaller size could indicate retarded development. In a remote Swedish lake in the vicinity of a public refuse dump, evidence was found for endocrine disruption in perch (Perca fluviatilis) and roach (Rutilus rutilus; Noaksson et al., 2001). The most pronounced effect was a 80% reduction in the gonadosomatic index (GSI) for female perch and a corresponding 36% reduction in male perch GSI. The majority of the female perch were in a sexually immature stage, incapable of reproduction.

Changes in plasma levels of steroid hormones can be another indicator of endocrine disruption (Baldigo et al., 2006; Hecker et al., 2007; McMaster et al., 2001; Rickwood et al., 2006). However, steroid levels also depend on temperature, diet, time of day, stage of sexual reproduction, and stress (Moyle and Cech, 2004). Scott et al. (2006) found a correlation between plasma Vg and depressed sex steroids. They argued that the endocrine disrupting contaminants inducing Vg would have a negative effect on the
secretion of gonadotropin from the pituitary, thereby decreasing both estrogen in females and 11-ketotestosterone in males.

In addition to physiological effects, endocrine disruptors have been found to alter mating behaviors. Different fish have complex and critical courting behaviors that accompany spawning (Moyle and Cech, 2004). These are suspected to be some of the most sensitive endpoints, but also the most difficult to study. A significant decrease in the ability of exposed fathead minnows males to acquire and hold a nest site required for reproduction was caused by a nonylphenolethoxylate/octylphenolethoxylate mixture (38 μg/L) modeling the composition of a major metropolitan WWTP effluent (Bistodeau et al., 2006). Similar results were seen with minnows exposed to 2 or 8 ng/L EE2 for 27 days. The ability of these male to compete and acquire territories was impaired. Vg was also induced in the exposed fish relative to controls (Majewski et al., 2002). The behavior of male three-spined stickleback exposed to environmentally relevant concentrations of EE2 decreased the normal aggressive response to other males (Bell, 2001).

**Vitellogenin and a link to long term or population effects**

While Vg is one of the most popular endpoints for endocrine disruption studies, there is no firm link between any level of exogenous Vg induction to an effect at the population level. The induction of Vg itself has not been found to harm males, but is believed to be due to anthropogenic chemicals. Ideally, a relationship could be established between concentrations of elevated Vg and the levels that are detrimental to reproductive function, so Vg could be used to gauge the impact of by contaminants. Correlations have been found between Vg and reproductive parameters. However, Vg
levels typically do not correlate well with the incidence of intersex (Scott et al., 2006). Likewise establishing a link between Vg synthesis and population decline is even more difficult, especially in fish with long life cycles. One of the largest long term studies was a 7 year whole lake experiment conducted in Ontario. A low exposure to synthetic estrogen (5-6 ng/L 17 EE2) nearly eliminated the fathead minnow population. Intersex males, altered oogenesis in females, and production of Vg were also found in fish periodically sampled from the lake (Kidd et al., 2007). Other species in the lake were found with similar effects but no severe effect on populations was observed. However, since the other species examined are longer lived, detection of effects are at the population level may require significantly greater time (Palace et al., 2002).

Lake scale studies are not always possible, so model lab fish with a shorter life spans can be used to estimate population effects on a smaller scale. These types of experiments have been used to show that fish produce fewer eggs when exposed to environmentally relevant concentrations of estrogens or nonylphenol. For instance, male fathead minnows (*Pimephales promelas*) exposed to 4.0 ng/L EE2 failed to develop normal secondary sexual characteristics, and after 172 days post hatch, no testicular tissue was observed in any fish. These effects occurred at concentrations lower than those that which significantly induced Vg (16 ng/L), indicating that Vg induction was correlated with developmental and reproductive impairment (Lange et al., 2001). Another life-cycle exposure of fathead minnows to 0.32 ng/L EE2 reduced egg fertilization success and demasculinizes males (Parrott and Blunt, 2005). In the sand goby (*Pomatoschistus minutus, Pallas*), a 7 month exposure to 6 ng/L EE2 impaired male
maturation and reproductive behavior, reduced female fecundity, reduced egg fertility, and ended with a 90% reduction of fertile egg production (Robinson et al., 2003).

Fewer studies on the effects of nonsteroidal estrogen mimics on the reproductive endpoints have been carried out. After a 21-d exposure to 100 µg/L nonylphenol, Japanese medaka (Oryzias latipes) showed reduced egg production and fertility. Hepatic Vg levels were increased significantly in males treated with 10, 50 and 100 µg/L nonylphenol (Ishibashi et al., 2006). In another experiment, mature medaka exposed to measured nonylphenol concentrations of 101 µg/L caused a significant decrease in egg production, and fertility was significantly decreased at 184 µg/L. While induction of ovotestis was observed in male fish at 24.8 µg/L and above, abnormalities in spermatogenesis were found only in those receiving 184 µg/L. Hepatic Vg levels were significantly increased in both sexes receiving nonylphenol treatments of greater than or equal to 50.9 µg/L (Kang et al., 2003). The lower concentrations in both these experiments are probably more environmentally relevant, comparing to the reported concentrations above. However, those analyses also show that in the same water there may also be octylphenols (which have similar estrogenic activities as nonylphenol), plus steroid hormones and other endocrine disrupting contaminants.

**Which contaminants cause the estrogenic symptoms?**

Attempts to find the cause of elevated Vg and intersex in wild fish usually point to the natural estrogens, 17 beta-estradiol (E2) and its metabolite estrone (E1), as major contributors to the observed responses (Houtman et al., 2007; Tan et al., 2007; Vermeirssen et al., 2005). Others report a major role of the main synthetic estrogen
contained in oral contraceptives (EE2) as well. Snyder et al. (2001) reported that E2 and EE2 were the dominant environmental estrogens in water samples from mid-Michigan and Lake Mead, NV. Desbrow et al. (1998) similarly found that the estrogenic chemicals in effluent from sewage treatment works were natural and synthetic steroidal estrogens, and human in origin.

Others suggest that non-steroidal compounds are involved. While still identifying the natural estrogens as the main source of estrogenic activity of effluents, one survey found that androsterone, nonylphenol, bis (2-ethylhexyl)phthalate and an unknown agent(s) were contributing to a lesser extent (Thomas et al., 2001). While nonylphenol may have been a significant contributor in the past, its impact is being ameliorated with restricted discharge limits. Sheahan et al. (2002) observed a decline in estrogenic effects that paralleled the reduction in the concentrations of nonylphenol as well as its mono- and diethoxylates in the UK. In the US an aquatic water quality criteria for nonylphenol was established at 6.6 ug/L, which should protect against endocrine disruption (USEPA, 2006).

Typically the work to identify the causative endocrine disrupting agents employ in vitro means to quantify the biological effects, which are not always the same as that observed in the in vivo situation (Folmar et al., 2002). Metabolism, other biological interactions, pharmacodynamics and pharmacokinetics can modify the in vivo effects. Other factors may cause the in vitro bioassay response to differ from that of a fish in the environment. For example, Vermeirssen et al. (2005) found that Vg in caged fish did not reach (or exceed) Vg levels in feral fish, despite apparent higher levels of 17β-estradiol
equivalents (EEQ) calculated for the caged fish from in vitro testing of the corresponding water samples.

**Cause for concern**

Without much concrete evidence for population level effects, the potential impact of endocrine disruption has been of great concern for several basic reasons. Of foremost importance, hormone signaling occurs at very low concentrations, which suggests that very low concentrations of contaminants could have significant effects. The chemical nature of these compounds, such as high lipid solubility and metabolic persistence are other important factors. Although the synthetic compounds are typically weaker ligands for the estrogen receptor, given their persistence and bioaccumulative potential they may have a greater significance than predicted. Chlorinated compounds, and even synthetic estrogens, are more resistant to chemical and metabolic degradation than the endogens compounds. Another aspect of the chemical nature of many endocrine disruptors is lipophilicity. These compounds tend to accumulate in fatty tissues in organisms and some chemicals can concentrate up food chains. Finally, the nature of the anticipated effects of endocrine disruptors brings another perspective to the evidence we are seeking. Past experience has taught us that serious chronic effects can go on for a long time without being noticed, before a major detriment occurs. For example, without special attention, an effect on behavior that causes 10% decrease in successful reproduction would probably be unnoticed for several generations until the time when a significant population decrease is obvious. Wildlife populations are already decreasing and while it is known that habitat loss is a primary factor, pollution is also a suspected cause. Additionally, in humans,
reduced sperm counts and the high incidence of hormonal related cancer are suspected to be related to exposure to EDs (Sonnenschein and Soto, 1998). The possibility of a widespread chronic adverse effect on reproduction from contaminants is disturbing and justifies careful and continued monitoring.

**California halibut and white sturgeon**

California halibut, *Paralichthys californicus*, are a near-shore flatfish species, more closely related to other left-eyed flounders than the larger Pacific or Atlantic halibut, of the genus *Hippoglossus*. They are found from northern Washington State to Baja, California, including estuaries and bays where they spawn and juveniles remain for about 2 years. Adults are most abundant on soft bottoms in water less than 20 meters deep, and spend most of their lives buried or partially buried in the sediments. Females mature at 4-5 years and males 2-3 years. They can weight as much as 33 kg, reach 1.5 m in length, and live as long as 30 years (Allen, 1990). California halibut are a commercially important fish, with a yearly catch of 1,000 tons and they are recreationally valuable as well, with an annual sport catch of 300,000 fish (Gilbert and Williams, 2002).

Sturgeons belong to the family *Acipenseridae*, and are one of the most ancient ray finned fishes. Sturgeon-like fish have existed for about 200 million years. They hold an important place in evolution as one of the oldest living vertebrates, and a link between fish and terrestrial animals as they are a close relative of tetrapods (Bemis et al., 1997). Sturgeon can live 100 years, weigh over 800 hundred kilograms, and reach 3 meters in length. It takes 10-20 years for various spices to sexually mature and many spawn only one every couple years (Doroshov et al., 1997). There is variation in migration habits,
even within species, from anadromous (migrating from salt water to fresh water to
spawn) to potamodromous (migrating within fresh water only, Bemis and Kynard, 1997).

Sturgeon may be best known for their caviar, but they are also a sport fish and caught for
consumption of their flesh. Most species have significantly declined from historic
numbers in the last 100 years due to over fishing, poaching, habitat destruction, pollution,
and the presence of dams barring their access to spawning sites (Bemis and Findeis,
1994). Nearly all species of sturgeon are either threatened or endangered (Birstein et al.,
1997).

White sturgeon, *Acipenser transmontamus*, inhabit coastal zones, estuaries, and
watersheds in North America from Baja, California to Alaska. While commercial fishing
of white sturgeon was banded in 1919, their numbers have not recovered, probably
because these fish are long lived and slow to mature and reproduce. Surgeon aquaculture
was established in California for production of caviar (Conte et al., 1988; Doroshov,
1985) and to help reduce the harvest of wild sturgeon for this delicacy. The California
Department of Fish and Game recently announced a plan to encourage the recovery of
California white sturgeon because the abundance of legal-sized white sturgeon has
declined to a 50-year low of about 10,000, while the population was estimated at a peak
of about 144,000 in 1998 (CDFG, 2006). Little work has examined the effect of
endocrine disruption on sturgeon. As these are among the oldest vertebrates still in
existence, observations of similar contaminant effects in sturgeon compared to other
modern teleost fish, such as halibut, will attest to the widespread nature of these effects.

These two species are relatively different in their life history and physiology. In
contrast to sturgeon, halibut are a mostly a marine species, they are shorter lived, and
more modern evolutionarily. As adults, halibut are predators, feeding mostly on fish such
as anchovy, while sturgeons remain more opportunistic feeders. A commonality they
share is a propensity to absorb more endocrine disrupting contaminants, as they are
benthic dwelling, with the young mostly feeding on benthic crustaceans. The lipophilicity
of endocrine disruptors can lead the adsorption of these compounds to sediments, hence
the need to monitor bottom dwelling fish. Both species are likely to encounter areas
heavily contaminated by endocrine disruptors. In California, white sturgeon migrate into
San Francisco Bay and up the Sacramento River in fall and late winter for spawning
(Conte et al., 1988). These waters receive effluent from the many industries and
metropolitan sewage treatment plants of this densely populated area, as well as run-off
from the extensively farmed Central Valley, all of which are typical sources of endocrine
disruptors. Similarly, California halibut inhabit developed coastal areas, especially the
estuaries they use for spawning and where the young may be most susceptible to
contaminants. While other fish species have been used for endocrine disruption studies,
for which Vg immunoassay kits are commercially available, none of these are for
relevant for the benthic zone of the California coast.

Research Objectives

The variety of endocrine disruptors, their mechanisms and effects are described
above, and these issues suggest a need to assess the effects of endocrine disruptors in
mixtures. Following that, the goal of this work was to study a mixture of endocrine
disruptors in species that are valuable and relevant to California. Initially, bioassays
needed to be developed for these species. The popular biomarker, Vg, was chosen as a
means to represent the estrogenic potency of compounds. As such, the first experimental chapter describes in the induction and characterization of Vg in halibut and a comparison with sturgeon Vg. The second chapter examines the halibut bioassay in depth from analytical considerations of working with an enzyme-linked immunosorbent assay to appropriate length of bioassay exposures. Finally in the third phase one of the sturgeon bioassays was put to use to examine the effects of a simple mixture of estrogen and a model anti-estrogen. Both species were used in much of the initial phases, so that in the end a comparison of the effects in two very different fish species could be made.

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Chapter 2

Induction and partial characterization of California halibut (*Paralichthys californicus*) vitellogenin and comparison to White sturgeon (*Acipenser transmontanus*).

The main portion of this work on halibut Vg was published as:

Abstract

The egg yolk precursor protein, vitellogenin (Vg), was isolated by size exclusion and ion exchange chromatography from plasma of California halibut treated with estrogen. MALDI TOF mass spectrometry (MS) analysis resulted in a molecular mass of 188 kDa. Protein resolved on the SDS PAGE as a double band of approximately the same mass as determined with MALDI TOF, and the two lower mass bands that were also immunoreactive. Native PAGE and Western blot with an antibody to turbot (Scophthalmus maximus) Vg confirmed the identity of the halibut protein. MS/MS de novo sequencing identified the protein as Vg by matching sequences of tryptic peptides to the known sequences of several other species. Matches were also made to two different forms of Vg in haddock, medaka, and mummichog, providing evidence that California halibut has more than one form of Vg. Similar analysis on white sturgeon Vg did not reveal adequate evidence to suggest that sturgeon has more than one Vg. MALDI TOF and MS/MS de novo sequencing were useful for determining the molecular mass, identification, and exploring the multiplicity of Vg. The potential of using other MS methods to understand the structure and function of Vg are discussed and some comparisons between the analyses of the Vg molecules from the two species are made.
1. Introduction

Vitellogenins (Vgs) are large serum proteins synthesized by oviparous vertebrates as yolk precursors. They have been widely studied for their role in the reproduction of fish (de Vlaming et al., 1980; Mommsen and Walsh, 1988; Tyler and Sumpter, 1990; Tao et al., 1993; Hiramatsu et al., 2002), and as biomarkers for endocrine disrupting xenobiotics.

Vgs are complex glycolipophosphoproteins with molecular mass of 300-640 kDa in teleosts and thought to be a heterodimer or have multiple forms (Tyler and Sumpter, 1996). The multiple forms of Vg have been revealed in at least 17 species of teleosts (Hiramatsu et al., 2006). Vg is synthesized in the liver in response to circulating 17β-estradiol and transported via the circulatory system to the ovaries where it is incorporated into developing oocytes through a receptor-mediated endocytosis (Mommsen and Walsh, 1988). In the egg, Vg undergoes proteolytic cleavage into yolk proteins, the lipid-rich lipovitellins, highly phosphorylated phosvitins, and a β'-component (Hiramatsu et al., 2001; Romano et al., 2004) that are the major source of nutrients for developing embryos. In plasma, Vg binds Ca\(^{2+}\), Mg\(^{2+}\), and K\(^+\) to provide minerals to the developing fish. Additionally, the primary degradation products of Vg were also shown to have a role in regulating the oocyte hydration and buoyancy of teleost eggs (Matsubara et al., 1999).

In female fish, hepatic synthesis of Vg is induced by estrogen produced by the ovarian follicle during the growth of the oocyte (Mommsen and Walsh, 1988; Tyler and Sumpter, 1996). Males and juveniles are also capable of the Vg gene expression, but typically do not have sufficient circulating estrogens to stimulate significant production
of the protein. However, if administered estrogen, males and juveniles will synthesize Vg. This effect is also seen with estrogen-mimicking contaminants, making induction of Vg synthesis in males a useful indicator of endocrine disrupting contaminants (Sumpter and Jobling, 1995; Folmar et al., 2002). Numerous bioassays using vitellogenin induction in males or juveniles have been developed for the study of endocrine disruptors, often employing immunoassays to measure Vg (Heppell et al., 1995; Jones et al., 2000).

California halibut (family Paralichthyidae, sanddabs) are found on the west coast of the United States and Mexico, from Washington State to southern Baja California. They have high commercial and recreational value, and efforts are currently underway to establish a commercial culture (Conklin et al., 2003). One goal of this investigation was to isolate and characterize California halibut Vg for eventual use in an endocrine disruptor bioassay as well as for reproductive studies in developing aquaculture.

In California, white sturgeon inhabit the Sacramento-San Joaquin river system and San Francisco Bay. White sturgeon vitellogenin has already been sequenced (Bidwell and Carlson, 1995), and this information contributes to knowledge of the reproductive biology of sturgeons for aquaculture. Also from an evolutionary point of view, sturgeon Vg is very interesting because it fills a gap in the study of vertebrate vitellogenins between the most primitive vertebrates (silver lamprey) and tetrapods. The phylogenetic difference between halibut and sturgeon also makes for an interesting comparison of their Vg and their responses to contaminants. Knowledge whether or not sturgeon possess two separate forms of Vg would help understand where, evolutionarily, and possibly why a single Vg protein evolved as multiple forms. We also explored the use of mass
spectrometry (MS) as a means for identifying Vg and its potential multiple forms in both species.

2. Materials and Methods

2.1 Animals

Adult male (4 yr, 400-700 g) first generation California halibut from captive stock were obtained from the UC Davis Bodega Marine Laboratory. Fish were maintained in filtered, flow-through sea water (9-15 °C, 34 %o salinity) at the California Department of Fish and Game Marine Pollution Studies Laboratory near Monterey, CA. They were fed a commercial pelleted trout feed (Nelson & Sons, Inc., Murray, UT) at the rate 0.5% w/d. Fish were determined to be all male by gonad histology, likely due to the elevated rearing temperature before metamorphosis, as was observed in a closely related Asian hirame, *Paralichthys olivaceus* (Tabata, 1995; Yamamoto, 1999).

Cultured juvenile white sturgeon (1 yr, ca 500g each), obtained from Stolz Sea Farm (Sacramento, CA) were maintained in the Meyer Hall Animal Facility (UC Davis.) in a temperature controlled room (19 °C), on a 12 hr photoperiod. Fish were housed in a recirculating system supplied with filtered aerated water with three fish per 200 liter circular fiberglass tank (1m dia. x 0.3 m deep) and fed artificial diets Nelson & Sons Silver Cup Feed: Steelhead Formulation, sinking extruded style (Murray, UT). Water was deionized, salted to 0.5 ppt (Forty Fathoms / Crystal Sea, sea water salt mix), and the quality was monitored daily, including unionized ammonia (< 0.05 ppm), temperature (18-20 °C), and salinity (0.5-1.5ppt). Tanks were cleaned with siphon and 30% water was replaced when needed to maintain water quality.
2.2 Chemicals

The anti-turbot (*Scophthalmus maximus*) vitellogenin polyclonal antibody was purchased from Cayman Chemical Company (Ann Arbor, Michigan). Coconut oil, 17β-estradiol, aprotinin, Bradford reagent, biotinylated goat anti-rabbit antibody, and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO). Reagents, gels, and nitrocellulose membranes used for electrophoresis and blotting were from Bio-Rad Laboratories (Hercules, CA), except for the ECL™ Western Blotting Detection Reagents, including the streptavidin-horseradish peroxidase conjugate and film, that were purchased from Amersham Biosciences GE Heathcare (Piscataway, NJ). The sinapinic acid for MALDI was purchased from Fluka (St. Louis, MO), the Milli-Q water and C18 ZipTips from Millipore (MA), and the trypsin from Promega (Madison, WI).

2.3 Vg Induction

Vg synthesis was induced in anesthetized (MS-222, 100 mg/L) halibut and sturgeon by the intraperitoneal injection of 17β-estradiol (1 mg/kg) in 0.10 mL coconut oil (Roubal et al., 1997). Estradiol was first dissolved in acetone (50 mg/mL) then 5 mL coconut oil was added and stirred to allow most of the acetone to evaporate. Two weeks later blood was drawn from the caudal vasculature with heparin rinsed syringes. Aprotinin was added (2 TIU/mL blood) to inhibit tryptic degradation of plasma proteins, and the blood was centrifuged at 5000 rpm for 5 min. Plasma was collected and stored at \(-80\ ^\circ\text{C}\). Halibut gonads were removed and fixed in buffered formalin to confirm sex by histology. Sturgeon had indifferent gonads and complete sex differentiation at a much
larger size and older age than fish used in this study (Van Eenennaam and Doroshov, 1998) Atomic absorption spectrophotometry was used to measure total plasma calcium from both treated and untreated fish to confirm induction of vitellogenesis (Linares-Casenave et al., 2003).

2.4 Chromatographic separation

Purification of Vg for both species followed the procedure for sturgeon Vg by Linares-Casenave et al. (2003), and was performed in a cold room (5 °C) to reduce Vg proteolysis. Plasma (2 mL) was loaded on a 90 x 2.6 cm Sepharose 6B-100 column and proteins eluted with 0.020 M Tris-HCl (0.4 mL/min, pH 8.0). Putative Vg fractions were combined, dialyzed against DDH₂O (24 h), loaded on a 28 x 2.6 cm DEAE Sephacel ion exchange column, and eluted with a linear salt gradient (0-0.30 M NaCl) in 0.05M Tris-HCl buffer (pH 8.0). Fractions from the one large peak in the ion exchange chromatogram were pooled and dialyzed in DDH₂O, then phosphate buffer saline (pH 7.5, 24 h). Protein concentration was determined using the Bradford Assay.

2.5 SDS PAGE and Western blot analysis

Polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulphate (SDS), was performed according to Laemmli (1970) on a BioRad Mini-PROTEAN II® electrophoresis cell using 4% acrylamide/bis stacking and 5% acrylamide/bis separating gel with 2-mercaptoethanol. Samples were diluted to load approximately 10 µg protein in a 10 µL aliquot. The gel was run at 80 V for 30 min, then 100 V for 70 min, and then stained with Coomassie Brilliant Blue. Proteins were transferred to a nitrocellulose
membrane for Western blot at 100 V for 1 h. The membrane was Ponceau stained for 1.5 min to visualize protein transfer to membrane and air dried overnight. The western blot was initiated by rewetting membrane in blocking solution composed of 2% non-fat dry milk and 2% BSA in tris-buffered saline (TBS) with 0.5% Tween-20 (TTBS) and was incubated for 1 h. The membrane was then probed for Vg during a 1 h incubation with rabbit anti-turbot vitellogenin polyclonal antibody diluted 1:5,000 with blocking mixture at 1:5 in TTBS (BTTBS). Bound antibodies were detected with biotinylated goat anti-rabbit antibody diluted 1:10,000 in BTTBS (1 h), followed by incubation with streptavidin-horseradish peroxidase conjugate diluted 1:10,000 in TBS (20 min). The membrane was covered with a chemiluminescent substrate mixture (ECL™ Western Blotting Detection Reagents), and the manufacturer’s instructions were followed to produce an image on film in a darkroom. Native PAGE and the corresponding Western blot were performed as above, apart from the omission of mercaptoethanol and SDS, and use of a 4-15% gradient BioRad Ready Gel®.

2.6 Protein in-gel digestion

Protein bands were washed thoroughly four times with Milli-Q water, then each band was diced into approx. 1-mm squares and dried in a SpeedVac (Savant, Holbrook, NY). Proteins were reduced and alkylated according to Shevchenko et al. (1996). Briefly, proteins were reduced with 10 mM DTT in 100 mM NH₄HCO₃ (pH 8, 55 °C) for 1 h, then alkylated with 55 mM iodoacetamide in 100 mM NH₄HCO₃ for 45 min in the dark at room temperature. Excess reagent was removed, and gel pieces were washed with 100 mM NH₄HCO₃ and partially dehydrated with acetonitrile; complete dehydration was
then achieved via SpeedVac. Finally, proteins were digested in 50% NH₄HCO₃ containing sequence-grade, modified trypsin at a final concentration range of 10 to 25 ng/μL (37 °C) for 17 h. Peptides were extracted once each with 0.1% trifluoroacetic acid (TFA) and then 5% formic acid-acetonitrile (50:50). The extraction volume was carefully controlled to never exceed 50 μL via Speed-Vac; it was then reduced to 15 μL for mass spectrometry.

2.7 Matrix Assisted Laser Desorption Ionization (MALDI) MS for nominal molecular weight

Samples to be analyzed for nominal molecular weights were exchanged into low salt (<20 mM) buffers, then spotted onto the MALDI target with an equivolume amount of MALDI matrix (Sinapinic acid in 50% ACN/0.1% TFA) and allowed to air dry. Spectra were acquired for 100-200 shots with an accelerating voltage of 25,000 V. Calibration with external standards resulted in typical mass accuracies of 0.1% (ABI Voyager DE, Applied Biosystems, Foster City, CA).

2.8 Peptide Mass Mapping by MALDI - Time of Flight (TOF) MS

Tryptic peptides were analyzed with a Bruker Biflex III MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with a pulsed N₂ laser (337 nm), a delayed extraction ion source and a reflectron. Fractions of tryptic peptides were desalted using C₁₈ ZipTips. Peptides were eluted from the ZipTips with 0.1% TFA-acetonitrile (50:50). The 0.5 μL of the peptide aliquot (eluant) was mixed with an equal volume of matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid
in 0.1% TFA-acetonitrile-50:50) and applied to the target. The mass spectra were acquired in the reflectron mode. Internal mass calibration was performed with two trypsin auto-digestion fragments (842.5 and 2211.1 Da), which typically results in mass accuracies of 50 ppm or better. Measured monoisotopic masses of tryptic peptides were used as inputs to search corresponding organism databases or the NCBInr database (all non-redundant GenBank CDS: translations + PDB + SwissProt + PIR + PRF) using the Mascot search engine with a probability based scoring algorithm (http:www.matrixscience.com). Up to one missed tryptic cleavage was considered in most cases, and a mass accuracy of 50 ppm or lower was used for each search.

2.9 De novo sequencing of peptides by tandem mass spectrometry (MS/MS)

Aliquots of tryptic peptides were cleaned and concentrated using POROS R2 resin (Perspective Biosystems, Framingham, MA) in a microcolumn following the method described in the Protana manual (Protana, Odense, Denmark). The peptide mixture dissolved in 5% formic acid was loaded onto the microcolumn, which was then washed with 5% formic acid. A gold coated nanosprayES capillary (Protana) was aligned in continuation of the microcolumn. Peptides were eluted into the nanoES capillary using 5% formic acid-methanol (50:50). Tryptic peptides were analyzed by a hybrid nanospray/ESI-Quadrupole-TOF-MS and MS/MS in a QSTAR mass spectrometer (Applied Biosystems Inc., Foster City, CA). Peptides in 5% formic acid-methanol (50:50) were sprayed from the gold-coated capillary. QSTAR instrument calibration was performed with a standard peptide mixture to give mass accuracies of 5 ppm or better. Nitrogen was used as the collision gas. Obtained values (un-interpreted MS/MS
fragmentation data) were used for database searches for protein identification using the Mascot search engine. *De Novo* sequencing of peptides was carried out using the QSTAR software (Analyst QS) and confirmed via a manual interpretation of MS/MS spectra. White sturgeon Vg was induced and purified in the same manner as halibut Vg. A tryptic digest and LC-MS/MS was performed on sturgeon vitellogenin, a procedure very similar to the de novo procedure used for halibut, but lacking the manual interpretation.

2.10 *Amino acid analysis*

Purified Vg solution (200 μL) was evaporated to dryness and hydrolyzed with 200 μL of 6 N HCl with 0.1% phenol at 100°C for 24 h, then dried again. Free amino acids were dissolved in Nor Leu buffer, vortexed and centrifuged for 5 min at 13000 g. The supernatant (50 μL) was analyzed with a Hitachi L-8800 (Na-based) amino acid analyzer.

3. *Results*

3.1 *Vg induction and chromatography*

Estradiol treatment of halibut increased plasma calcium levels from 119 ± 29 (controls; mean ± SD) to 490 ± 34 μg/mL, indicating the presence of Vg. Size exclusion chromatography showed a large peak in plasma from treated halibut (Fig. 2.1). Fractions corresponding to this peak were further purified by ion exchange chromatography and eluted as one large peak (not shown). Fractions from this peak were identified as Vg and characterized by PAGE, Western blot, MALDI TOF (for molecular mass only) and amino acid analysis, as described above.
3.2 Gel electrophoresis

SDS PAGE showed the new protein in the plasma of estrogen-treated halibut as two bands at 180 and 160 kDa (Fig. 2.2). In the Western blot, the anti-turbot Vg antibody was immuno-reactive with this new protein, while no proteins in untreated males displayed activity with the antibody. The putative purified Vg preparation contained multiple bands of MW 180, 160, 120, 80 kDa (Fig. 2.2), all of which were immuno-reactive, and the largest two corresponded to the new bands in the plasma from estrogen treated fish. Native PAGE and Western blot (Fig. 2.3) showed 2 bands of immuno-reactive proteins in the plasma from estrogen-treated fish and the same profile in the purified protein sample. There was no protein binding in the plasma from the untreated male.

3.3 Mass spectrometry

The intact protein from halibut produced an \([M + H]^+\) ion at \(m/z\) 188157 and the doubly-charged \([M+ 2H]^{2+}\) at \(m/z\) 93589. Unfortunately, the broadness of the peaks makes it difficult to determine an exact mass (Fig. 2.4). Other smaller ions were observed at \(m/z\) 74372, 27634, and 14477. Each of the digested bands of halibut Vg resulted in about 30 tryptic peptides (data not shown) for Mass Mapping, none of which matched \(m/z\) ratios predicted by digest of Vg sequences available on the Swiss-Prot and TrEMBL databases: rainbow trout (Q92093, O93605, Q9PSX4, Q9W6Z4), medaka (Q8UW61, Q8UW88), European flounder (Q90WQ5), or mummichog (Q98893, Q90508). The De Novo peptide sequencing with MS/MS of California halibut Vg yielded peptide ions that
showed high homology to 17 sequences from several other fish species, including other flatfish, and the mosquito (Table 2.1). Contained in this list of peptide sequences were matches to two different forms of Vg, including haddock Vg A (L/IADIDIDLYPK) and Vg B (LTSALAAQLLTPIK), mummichog Vg I (FFGQEIA/GFANIDK, MVQELALQLYMDK) and Vg II (FIELIQLLR, AEGVQEALLK), and medaka Vg I (L/IADIDIDLYPK, MVQELALQLYMDK) and Vg II (MSVAIVLFE TK, FIELIQLLR, TEGL/LQEALLK).

For white sturgeon vitellogenin, the Mascot search engine provided many peptide sequences covering 49% of the known sequenced portion of sturgeon Vg. There was very little overlap of matching sequences for sturgeon Vg that also matched to Vg of other species (Table 2.3). Only 2 peptides matched the original sturgeon sequence and the Vg of another species. These two peptides matched Vg from a white spotted conger eel, Conger myriaster.

3.4 Amino acid analysis

The amino acid composition of halibut Vg most closely resembled that of the turbot, when compared to Vg of other teleosts reported (2.2). Sturgeon have a comparatively higher serine content as seen from the aa composition of sturgeon Vg, as reported by (Bidwell and Carlson, 1995)

4. Discussion

The protein isolated from California halibut was estrogen-inducible, immuno-reactive to a flatfish Vg antibody protein, had high molecular mass, and exhibited a
calcium binding property. These characteristics identify the protein as Vg. The sequence matching by MS/MS de novo sequencing supported this finding and suggested the presence of at least two different forms of Vg. The molecular mass of 188 kDa found by MALDI TOF and 180 by SDS PAGE are likely the monomeric form of Vg. These values are close to those reported for many other teleosts: 160 kDa for Atlantic halibut (*Hippoglossus hippoglossus*; Norberg, 1995), 180 kDa for seabream (*Sparus aurata*; Mosconi et al., 1998), 180 kDa for sea bass (*Dicentrarchus labrax* L.; Mananos et al., 1994), 170 kDa for barfin flounder *Verasper moseri*; Matsubara et al., 1999) and 184 for brook trout (*Salvelinus fontinalis*; Schafhauser-Smith and Benfey, 2002). Although the masses determined by MS are typically more accurate, it is interesting that native and SDS PAGE showed two distinct bands, while only one was detected with the MS technique. The bands are of different intensity, making it possible that the amount of the smaller band was not sufficient to be detected with MADLI TOF. Alternatively, they could be multiple forms that have nearly the same mass, but varying in posttranslational modification and resolving differently with electrophoresis.

The analysis of California halibut Vg often resembled that of turbot (*Scophthalmus maximus*) as reported by Silversand and Haux (1989). These two species were similar in amino acid analysis and in molecular mass, with the turbot Vg found to be 185 kDa. Native gel electrophoresis was also very similar for the two species, producing two distinct bands, the second of which appeared to be a double band. Silversand and Haux interpreted these bands as the dimeric and monomeric forms, respectively. Or again, these results could suggest different forms of Vg (more than one form of Vg has been found in a variety of marine teleosts, Matsubara et al., 2003).
The lower molecular mass bands seen in SDS PAGE of the purified Vg are likely products of protein degradation since Native PAGE and MALDI TOF of the purified Vg did not indicate the same pattern. The fragments could be the subunits of Vg, or the loss of them, based on the masses of lipovitellin, phosvitin, and the \( \beta' \)-component in other species (Hara and Hirai, 1978; De Vlaming et al., 1980; Hiramatsu et al., 2001; Romano et al., 2004), and the propensity of phosvitin to dissociate from Vg during isolation (De Vlaming et al., 1980; Norberg and Haux, 1985). The MADLI TOF spectrum did also show lower molecular mass peaks, which could be Vg subunits, but their sizes did not match the lower molecular mass bands in the SDS PAGE. In the MALDI TOF spectrum, the peak at 94 kDa is more likely the doubly charged form of the parent Vg ion and the other smaller products could also be formed during the MALDI process.

MALDI TOF Peptide Mass Mapping did not confirm the identity of the protein, although this technique has been used to identify Vg of rainbow trout (Banoub et al., 2003). The method compares only peptide masses, so any slight variation that alters the mass by a few daltons will prevent a match. Banoub et al. (2003) achieved about one third sequence coverage and suggested that non-matching portions in their analysis were due to posttranslational modifications that inhibited predictable fragmentation. A factor in that investigation that may have facilitated matches was that the sequence of Vg for that species had already been determined, whereas the Vg of California halibut has not been sequenced. The extensive post-translational modification of Vg, or perhaps heterogeneity in the sequences of different species, could explain the absence of matches. It seems that Mass Mapping may only be useful for identification of Vg from species that is already in the database.
The MS/MS de novo sequencing provided matches to Vg sequences of other species, confirming the identity of California halibut Vg protein. Banoub et al. (2004) characterized Atlantic salmon (Salmo salar) Vg with both the Peptide Mass Mapping and de novo sequencing techniques, and the latter yielded superior results, matching most of the resulting sequences to known rainbow trout Vg sequences. Cohen et al. (2005) used de novo sequencing and comparison with closely related haddock to show that Atlantic cod has two forms of Vg. California halibut peptide sequences also matched sequences of multiple vitellogenins, Vg A and Vg B in haddock, Vg I and Vg II in mummichog, and Vg I and Vg II in medaka. This suggests that California halibut has more than one form of Vg, although it is not known for certain whether these sequences are located on two separate Vg proteins.

Multiplicity of the teleost Vg was confirmed by cloning and sequencing c-DNAs of different forms (Sawaguchi et al. 2005). It is now accepted that there are several forms of Vg expressed in fish and that these forms are likely to have different functions in oocyte development (Hiramatsu et al., 2006). In fish with marine pelagic eggs, such as haddock, one form functions as the primary source of free amino acids required for the oocyte hydration and egg buoyancy (Matsubara et al., 1999; Reith et al., 2001), which may also be required for the pelagic eggs of California halibut. For the fish producing benthic eggs, such as mummichog, the hydration process has been found to be different. The identified sites of proteolytic degradation in mummichog differed from those in species with pelagic eggs (LaFleur et al., 2005). The responsiveness of the two Vg forms to estrogen has also been found different in tilapia and Japanese goby (Takemura and Kim, 2001; Ohkubo et al., 2003). Different functions and sensitivities to estrogen in the
different forms of Vg are directly relevant to the studies of endocrine disrupting chemicals that use Vg as a biomarker. A full understanding of vitellogenesis is necessary for evaluating the possible effects of environmental contaminants and improving breeding efficiency in aquaculture.

Knowledge of the multiple forms of Vg has been derived mostly from cDNA cloning and biochemical and immunological studies. The MS/MS de novo sequencing is easier than these two approaches, but has the disadvantage in that little of the sequence is provided which is a limiting factor, especially with such a large protein as Vg. Since this study was completed, MS techniques have expanded to include a variety of new approaches that will be useful for Vg characterization. Nano LC-MS/MS is a common tool in proteomics for identifying proteins using database searches and it can also be used for sequencing (Domon and Aebersold, 2006). The extensive coverage of a proteome has been accomplished using this method, in which more than 2,000 proteins were identified in a yeast (de Godoy et al., 2006). The MS analysis can also be applied to proteins in a complex mixture and could be used to track the fate of the multiple Vgs and derived proteins during egg development. Such study has recently been conducted with zebrafish embryos, using 2-D gel electrophoresis coupled with MALDI-TOF/TOF to identify 42 Vg-derived peptides in developing embryo (Tay et al., 2006). Finally, since MS techniques target the protein, there is a great potential to learn about protein function through analysis of post-translational modifications. However, since Vg is such a large and fragile molecule, finding significant modifications may be a challenge with most MS systems, but future refinement of these techniques is likely to aid in the recognition and interpretation of these functional modifications (Mann and Jensen, 2003).
The cDNA of white sturgeon vitellogenin was sequenced by Bidwell and Carlson in 1995 revealed a 186 kDa protein. When compared to chicken, *Xenopus*, and silver lamprey, the 4 sequences could be aligned along the entire length, showing an overall highly conserved structure. The lipovitellin domain, which is involved in binding to the receptor, was most well conserved, and the phosvitin domain, composed of long repeats of serine residues, was the most divergent. Comparison of many species lead to the observation that the phosphoserine content seems to have expanded during evolution, resulting in larger phosphate content of yolk in higher vertebrates (Wahli 1988).

Teleosts, like halibut, have lower serine content, chicken has higher content, while sturgeon and *Xenopus* are in between (Table 2.2).

A little over 10 years ago, when the sturgeon Vg was sequenced, the multiplicity of Vg had yet to be so well described in the literature and a second Vg was not sequenced. However, there was evidence for two Vgs in sturgeon as Bidwell et al., (1991) found two mRNAs that indicated two high-molecular-weight proteins of 180 and 120 kDa. Additionally two proteins of 190 and 210 kDa were evident as characterized by gel electrophoresis (Linares-Casenave et al., 2003). The mass of this Vg and appearance of a double band is also similar to that of California halibut Vg. MS analysis suggested halibut Vg was actually two different proteins. Many fish Vg molecules appear as a double band using gel electrophoresis or western blotting, but it is difficult to say if the two bands represent two halves of a heterodimer, differences in post translational modification, or two different proteins. It was hoped that advances in mass spectrometry techniques may provide a much easier way to determine if the 2 bands of sturgeon Vg were indeed two different forms of the Vg protein, as compared to full sequencing.
Because sturgeons are so remarkable evolutionarily, this question is much more significant compared to the suggestion of multiple forms of Vg in halibut.

No solid evidence was found to a second Vg in white sturgeon. If sturgeon do have more than one form of Vg, one would expect to have peptides that matched to the known sturgeon Vg sequence, as well as, peptides that did not. However all 'new' peptides (Table 2.3) were actually very close to peptides in the previously sequenced sturgeon Vg. Close inspection of the mass spectra found uncertainties in assignment of the amino acid identities in key positions. For instance GILNILQLNLK matched to fathead minnow, while GILNMLQLTIIK matched the sturgeon Vg. There are only three amino acids different, and the residues in question have masses or mass spectra that are difficult to distinguish from each other. It is more likely that the peptides in Table 2.3 were cases of miss-assignment rather than new peptides. One of the few interesting 'new' peptides in Table 2.3 is AEFLQEVIR. This sequence and its position closely matches the underlined portion of this sequence in sturgeon: LHTEGLQEVLMQ. This seems unlikely to be a miss-assignment because this sequence is not preceded or followed by arginine or lysine (R or K). Therefore, trypsin should not cleave this section creating such a peptide. Even so, if this peptide was really a new sequence in sturgeon Vg, this only provides evidence for a small alteration and is not enough to assert that this protein is a separate form.

Overall the nano LC/MS technique worked well to identify the sturgeon Vg by matching it to the known sequence. Fairly good sequence converge was obtained, considering the size of the molecule. Almost half the protein was sequenced which was much greater than that obtained for halibut a few years earlier, due to the rapidly
advancing field of mass spectrometry. However, very little evidence to support a second 
Vg in sturgeon was found, while results from similar methods suggested two forms in 
halibut. Halibut Vg is apparently much more homologous to Vg sequences available in 
the database. This points a to a limit of this mass spectrometry technique, its dependence 
on the database content. This is similar to the difficulty encountered with the mass 
mapping technique for halibut Vg described earlier. The lack of peptide matches to 
sturgeon Vg also underlines how different these fish are from most other fish species.

In conclusion, gel electrophoresis, Western blotting, and amino acid analysis were 
useful in comparing halibut Vg to turbot Vg, as no sequencing has been done for turbot 
Vg to enable sequence matching by MS. Some apparent similarities between the two Vg 
molecules support the use of the turbot antibody in an immunoassay for California 
halibut Vg. MALDI TOF proved to be an easy and rapid method for a more exact 
measurement of the Vg protein mass compared to gel electrophoresis. MS/MS \textit{de novo} 
sequencing appeared to be useful for identifying Vg by comparing with sequences from 
other species. The MS techniques can indicate the presence of multiple forms and aid in 
elucidation of their functions. However, the usefulness of this technique is limited by the 
sequence information available, as seen with sturgeon Vg. Likewise, the Mass Mapping 
technique may be more valuable in analyses where there is a known sequence of Vg 
available for comparison. Further potential applications of MS techniques may include 
identification and quantification of proteins in complex mixtures and analysis of 
posttranslational modifications.
Acknowledgements

Support was provided by the UC Marine Council Coastal Environmental Quality Initiative grant #000659-002. The authors thank Dr. D. Conklin (UC Davis) and Mr. D. Bush (The Cultured Abalone) for generously supplying fish and assistance. We are also very grateful to Mr. B. Phillips (UC Davis) and the Marine Pollution Studies Laboratory staff for fish husbandry, advice, and hospitality. Our sincere thanks to Dr. M. Miller (UC Davis), and to Ms. D. Degroot for her patience during electrophoresis and blotting. Special thanks to Dr. D. Schlenk (UC Riverside) for the initiation of this project, and to Ms. W. Hwang and Ms. M. A. Irwin.
References


Norberg, B., Haux, C., 1985. Induction, isolation and a characterization of the lipid content of plasma vitellogenin from two Salmo species: rainbow trout (Salmo gairdneri) and sea trout (Salmo trutta). Comp. Biochem. Physiol. 81B, 869-76.


Table 2.1. Comparison of MS/MS spectra of California halibut vitellogenin to vitellogenin sequences from other species using the Mascot search engine.

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*a Silversan and Haux, 1989; *b pleuronectes vetulus, Roubal et al., 1997; *c Hara and Hirai, 1987; *d Tyler and Sumpter, 1990; *e Carassius auratus and Xenopus laevis, de Vlaming et al., 1980; *f Sturgeon, Chicken, and Silver lamprey, Bidwell and Carlson, 1995
Table 2.3. White sturgeon Vg sequences matches to Vg from other species

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<th>Species</th>
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<td>(not present not bordered by R or K in sturgeon)</td>
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Species and peptide sequences are results from the Mascot search, except those for 'STURGEON,' which were added for comparison from Bidwell and Carlson (1995). Exact matches in bold. The BLAST search engine (Swiss Institute of Bioinformatics) was used to find approximate position of the residues. Scores are also from mascot search. A score of 50 is generally very reliable.
Fig. 2.1. Size exclusion chromatography of California halibut plasma. Fractions 42-49 were collected for ion exchange chromatography.
Fig. 2.2. SDS-PAGE (A) and subsequent Western blot (B) analysis of untreated male halibut plasma (U), estradiol-treated male plasma (E), and purified Vg (P). Molecular mass markers (MM) and biotinylated markers (BM) are in outer lanes and fractions in labels indicate additional dilution of the same sample.
Fig. 2.3. Native-PAGE (A) and subsequent Western blot (B) analysis of untreated male halibut plasma (U), estradiol-treated male plasma (E), and purified Vg (P). Fractions in labels indicate additional dilution of the same sample.
Fig. 2.4. MALDI TOF MS of California halibut vitellogenin: [M + H]$^+$ and [M + 2H]$^+$ species are observed as broad peaks at 188 and 94 kDa.
Chapter 3

A bioassay for environmental estrogens, measuring vitellogenin in California halibut, *Paralichthys californicus*, by an enzyme-linked immunosorbent assay (ELISA)

To be submitted as: Palumbo, A.J. Koivunen, M., and Tjeerdema, R. S. A bioassay for environmental estrogens, measuring vitellogenin in California halibut, *Paralichthys californicus*, by an enzyme-linked immunosorbent assay (ELISA)
Abstract

Vitellogenin, the estrogen-inducible yolk protein precursor, serves as an indicator of exposure to estrogen mimicking environmental contaminants. An ELISA for the measurement of California halibut plasma vitellogenin was optimized and validated using a commercially-available antibody developed for another flatfish species, turbot. Attempts to enhance assay performance by addition of a biotinylated antibody, polyethylene glycol, and Tween-20, and altering the preincubation step are described. Inclusion of overnight preincubation was critical for low detection limits. Increasing the amount of Tween-20 to 0.05% in buffers was most effective for improving recoveries of spiked plasma samples. At the IC50, the average recovery of spiked plasma samples was 104% and the interplate CV was 12%. The working range of the assay was 33-1000 ng/mL, while the detection limit in a plasma sample is 2.2 µg/mL. Preliminary tests with the model compounds 17β-estradiol and p-nonylphenol show that it is a suitable model for further studies of estrogen mimicking contaminants.
**Introduction**

Vitellogenin (Vg) is an estrogen-inducible protein precursor of egg yolk, produced by female oviparous vertebrates in preparation for spawning. Scientific interest in this molecule has long focused on its role in the natural reproductive cycle, and more recently it is had received attention as a popular biomarker of contaminants that can mimic the effects of estrogen. Because of their ability to alter hormone signaling these compounds, also known as endocrine disruptors, have the potential to impair wildlife reproduction. Indeed, male fish are being found with ovarian tissue in their gonads and elevated levels of plasma Vg, and municipal effluents containing estrogen-mimicking contaminants are thought to be the cause (Purdom et al., 1994; Folmar et al., 1996; Allen et al., 1999).

In the past, plasma Vg was measured indirectly as alkaline-labile phosphate or plasma calcium as these ions bind to Vg and increase proportionately with its content in the blood. Immunoassays were then developed to detect Vg using radiolabeled antibodies, and they now enzyme-labeled antibodies. Current enzyme-linked immunosorbent assays (ELISAs) are performed in 96-well microtiter plates and are valued as quick and easy ways to quantify plasma Vg without the hazards of radio labeled compounds. Many ELISAs for measuring Vg have been standardized and produced in commercial kits. However, one major drawback to Vg immunoassays is that their antibodies do not display a high level of cross-reactivity, and a new assay is usually needed for each species studied.
Although the structure of Vg is a fairly well conserved, past work has shown that assays often are not useful for quantitative measurement of another species (Wheeler et al., 2005). Watts et al. (2003) illustrated this point well by isolating Vg from rainbow trout (Oncorhynchus mykiss), Atlantic salmon (Salmo salar) and greenback flounder (Rhombosolea tapirina) and raising corresponding antibodies. Tests for cross reactivity among the three species found that the anti-sera were highly specific, and that there was little cross reactivity, even between the two salmonid species. Other researchers have had greater success with salmonids (Benfey et al., 1989; Nilsen et al., 1989; Tyler et al., 2002), cyprinids (Tyler et al., 1996; 1999), groupers (Heppell and Sullivan, 1999) and pleuronectids (Lomax et al., 1998). Although these are reports of cross reactivity only, the detection ranges were not described. Tyler et al. (1999; 2002) quantified Vg with heterologous assays and found higher working ranges in assays for the non-native Vg. Interestingly, these studies suggest that using a polyclonal antibody does not increase the potential for cross-reactivity, verses a monoclonal antibody.

The following work was part of an effort to develop a bioassay for estrogen mimics in flatfish along the coast of California. A bottom-dwelling species was desired since many endocrine disruptors are lipophilic, thus tend to remain associated with sediments. Additionally, elevated Vg has been detected in male flatfish species near the Orange County municipal wastewater discharge (Roy et al., 2003). California halibut (Paralichthys californicus) are a flatfish species native to the California coastline. They are a valuable commercial and sport fish, and are now being cultured in California (Conklin et al., 2003). Juveniles may be ideal for correlating adverse effects in fish to
contaminant loads in soil or water, since the young are relatively stationary, remaining in the bays where the adults spawn for a year or more (Allen, 1990).

No existing commercial ELISA kit has been developed for flatfish and available kits were not likely to provide useful detection limits for California halibut Vg. However, there was a commercially available antibody for another flatfish species, turbot (*Scophthalmus maximus*). Research with California halibut Vg in another laboratory (Schlenk et al., 2005), and work to purify halibut Vg (Palumbo et al., 2007), found this antibody to react well in immuno blots. Therefore, it was used in the competitive ELISA described herein.

**Materials and Methods**

*Chemicals and Reagents*

Vg was previously purified and characterized from California halibut as detailed in Palumbo et al. (2007). The anti-Vg antibody used for this assay is a commercially-available polyclonal antibody generated from turbot Vg (Biosense Laboratories, Norway). Bradford reagent, 17β-estradiol, aprotinin, anti-rabbit horseradish peroxidase (HRP) conjugate, biotinylated goat anti-rabbit antibody, polyethylene glycol (PEG; ave. mol. wt. 8,000), and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO). Streptavidin-horseradish peroxidase conjugate was purchased from Amersham Biosciences GE Heathcare (Piscataway, NJ), *p*-nonylphenol was from Fluka Chemika (Switzerland), and MS-222 was from Argent Chemical Laboratories (Redmond, WA).

*ELISA Development*
The assay and its optimization are based on Linares et al. (2003) and Koivunen et al. (2005). The optimum working concentrations were determined by a checkerboard titration of coating Vg and primary antibody. Vg was diluted in carbonate-bicarbonate buffer (pH 9.6) in three-fold series from 9.0-0.1 μg/mL. Ninety-six well microtiter plates were coated with the Vg solution and incubated overnight (18 h, 4°C). Primary antibody was then diluted in a two-fold series from 1:1,000-1:60,000 and added to a row of the plates for 1 h. Different concentrations of secondary antibody were incubated (1 h) on each half of two plates (1:1,375-1:5,500). Color development and other details of the procedure follow ELISA Procedure (presented below).

Standard curves were obtained using antibody and coating concentrations that gave a maximum absorbance of approximately 1.0, while remaining in the dynamic range (not saturated). Care was taken not to choose concentrations that had signal above background where coating Vg or antibody was absent. The curve that provided a low IC50 without shortening the dynamic range was selected: Vg coating concentration of 3 μg/mL, primary antibody dilution of 1:15,000, secondary antibody dilution of 1:1,375 (Figure 1).

Effect of Preincubation

A standard curve with antibody pre-incubated overnight was assayed on the same plate as two other standard curves with antibody incubated for 1 and 2 h at 40 °C. A fourth curve was also included, made by mixing the reagents directly on the assay plate, being sure to add standard solutions to the plate before the primary antibody.
Effect of Biotin-Avidin Amplification

A biotinylated anti-rabbit antibody was used in place of the HRP-conjugated anti-rabbit antibody, and 20-min incubation with streptavidin conjugated to HRP was incorporated immediately following that step. This assay was optimized separately as described above for the original assay. Similar concentrations were obtained for antigen coating. A comparison of the performance with both systems was done using the primary antibody dilution determined above (1:15,000), and a lower primary antibody concentration obtained from the biotin-avidin optimization (1:60,000).

Effect of PEG and Tween-20

Standard curves were obtained using 0, 1, 2, 4% PEG in the PBS buffer. Another plate was run with standards and plasma spikes with 0 and 1% PEG. The effect of increased Tween-20 content was tested by running standard curves and spikes with and without 0.05% Tween-20 in the standard dilution buffer, while maintaining this concentration of Tween-20 in the antibody buffer.

ELISA Procedure

Ninety-six well microtiter plates with Maxisorb ® surface treatment (NUNC) were coated with 3 μg/mL California halibut Vg diluted in carbonate-bicarbonate buffer (pH 9.6) sealed, and incubated overnight (18 h, 4°C). Anti-turbot Vg antibody and sample or standard were pre-incubated overnight (18 h) at room temperature in another microtiter plate (non-surface treated, Fisher). The standard Vg was serially diluted in phosphate-buffered saline with 0.05% Tween-20, pH 7.4, (PBST) in a range of 24,300 - 0.1 ng/mL
and buffer only, 130 µL per well. Samples were diluted (minimum 1:100) in PBST in microcentrifuge tubes and a 130 µL aliquot was pipetted into triplicate wells. Primary antibody was diluted 1:7,500 in PBST and added in equal volume to the standards and samples on the plate for a final dilution of 1:15,000. The following day, coating antigen was washed from the plate 3-times with 250 µL PBST by an automatic plate washer (Bio-Tek Instruments, Inc., Winooski, VT). Nonspecific binding was blocked by incubating 1% bovine serum albumin and 1% non fat milk in the carbonate-bicarbonate buffer (100 µL, 1 h). After washing, 100 µL of the antibody analyte mixture was pipetted onto the plate. One hour later the sample matrix was washed away, leaving only bound antibody inversely proportional to the amount of analyte. The secondary antibody-HRP conjugate, diluted 1:1,375 in PBST, was added to each well (100 µL, 1 h). Unbound antibody-enzyme conjugate was removed by the last wash. A solution of the chromogen, 3,3',5,5' tetramethylbenzidine and the substrate, 1% hydrogen peroxide in sodium citrate buffer (pH 5.5) was mixed just prior to application. A blue color formed (15-30 min) and the reaction was stopped with 2 M H₂SO₄. The absorbance of the resulting yellow color at 450nm was measured by a microplate reader (Molecular Devices, Menlo Park, CA).

**ELISA Performance**

To determine the proper dilution of samples, the response was measured in plasma samples from untreated males following dilution at 1:10, 1:100, and 1:1000. Similarity of the Vg standard and plasma Vg was examined by serially diluting the Vg standard and a plasma sample from and estrogen-treated male together on one plate.
Parallelism of regression curves of the log-logit transformed data was assessed using analysis of covariance (ANCOVA).

Accuracy and precision were tested by assaying mixtures of untreated male plasma (4 μL) and known amounts (5.7-173 μL) of the Vg standard, diluted with buffer for a 1:100 plasma dilution. Vg concentrations were made targeting the middle and ends of the linear range of the log-logit transformed standard curve. This was repeated on five different days.

**Data Analysis**

A Standard curve was included on every plate. Softmax (Molecular Devices) microtiter software package based on the 4 parameter logistic method of Rodbard (1981) was used to fit curves to the equation $y = \frac{(A-D)}{1+(x/C)^B} + D$, where $y$ is the absorbance, $x$ is analyte concentration, $A$ is $B_{\text{max}}$ (maximum absorbance), $D$ is $B_{\text{min}}$ (minimum absorbance), $C$ is the IC50 (analyte concentration giving 50% inhibition, or midpoint of the standard curve), and $B$ is slope at the IC50. $B/Bo$ is the relative fraction of bound antibody, expressed as percent and calculated as $(y-D)/(A-D)$. All samples were run in triplicate. If the CV of the triplicate measurements was > 20% the sample was assayed again. Multiple dilutions of samples were assayed (1:100, 1:1000, 1:10,000). The dilution with the absorbance closest to the IC50 was used to calculate the final result.

**Animals**

Adult male California halibut, *P. californicus* (two groups: 2 yr old, average weight 145g, length 24cm FL; and 3 yr old, average weight 455g, 35cm FL) obtained
from captive broodstock were obtained from D. Conklin and R. Piedrahita (UC Davis). They were reared outdoors at ambient temperature (9-15 °C) and photoperiod in tanks with filtered, flowing natural sea water at the Marine Pollution Studies Laboratory, Monterey, CA. Fish were fed ad libitum Nelson’s Silver Cup sinking trout chow pellets (Nelson and Son’s, Salt Lake City, UT) using a slow release feeder. Fish were all male, possibly an effect of rearing temperature on sexual differentiation in juveniles as was observed in a closely related Asian hirame, Paralichthys olivaceus (Tabata, 1995; Yamamoto, 1999).

**Halibut Exposures**

Fish were kept in individual aerated 20-L glass aquaria with flowing (0.2 L/min) natural sea water, with the temperature and photoperiod as above. Anesthetized (MS-222 100 mg/L) halibut (2 yr old) were intraperitoneally injected with a range of 17β-estradiol (10 - 3000 µg/kg, 3 fish per treatment), delivered in absolute ethanol (0.5 mL/kg). Fish were not fed during experiments. For the time course, different fish were sampled after 4, 7, and 14 days. Blood was drawn from the caudal vasculature with heparin-rinsed syringes. Aprotinin was added (2 TIU/mL blood) to inhibit tryptic degradation of plasma proteins and the blood was centrifuged at 2500 rpm for 10 min. Plasma aliquots were frozen on dry ice and kept at -80 °C until analysis by ELISA. Fish were euthanized by MS-222 overdose (500 mg/L), and gonads and liver were removed to confirm sex by histology and to calculate the gonadosomatic index (GSI) and the hepatosomatic index (HSI).
Experiments with p-nonylphenol (NP) were conducted similarly to estrogen exposures. Preliminary tests used NP dissolved in ethanol, ranging from 1-250 mg/kg. The final experiment used NP in peanut oil with larger fish (3 yr old). Fish were separated into 6 circular tanks (50 L, 3 m dia.) with 5 or 6 fish each, and sampled seven days of exposure.

Results

Effect of Preincubation and Biotinylated Antibody

The omission or shortening of the preincubation step was found to increase the IC50 by greater than 3 fold compared to overnight preincubation (Table 3.1). This is undesirable as it would indicate a higher detection limit, therefore the overnight preincubation was retained and used in subsequent analysis. The use of streptavidin-HRP conjugate and the biotinylated antibody in place of the secondary antibody HRP conjugate increased the signal produced in standard competitive analyses, enabling the use of a lower concentration of primary antibody. Less primary antibody is generally thought to reduce IC50 and the detection limit. However, the resulting standard curve had very similar IC50 to that of the original procedure using the 2-HRP (Figure 3.2). Use of the biotinylated secondary antibody was unlikely to provide lower detection limits.

Effect of PEG and Tween-20

The addition of PEG reduced the IC50 in the standard curve more than one-half at the highest concentration tested (Table 3.2). The slope of the curve was also lowered, which would extend the dynamic range. PEG increased the recovery of buffer spikes,
overestimating recovery as 137% near the IC50, but recovery of plasma spikes near the IC50 remained low, 42% (Table 3.3).

The increase in Tween-20 decreased the slope of the standard curve from 1.23 to 0.73 and extended the linear range from about 50 – 450 ng/mL to about 35 – 1000 ng/mL (Figure 3.3). The increase in Tween-20 also improved the recovery of spiked plasma samples from 41% near the IC50 to 106%, as seen in comparing final assay performance (Table 3.4) with the original recovery for the assay (Table 3.3, under 0% PEG).

**ELISA Performance**

Plasma dilutions of 1:100 produced very similar absorbance values to a 1:1000 dilution (mean absorbance ± standard deviation of 1:100 dilution: 0.93 ± 0.031 and for the 1:1000: 0.91 ± 0.027), indicating 1:100 was an adequate minimum sample dilution. This choice was verified in the spiked samples, as the 0 ng/mL spikes showed a very low background signal corresponding to 6.9 ± 5.0 ng/mL, which was well below the working range of the assay. No Vg was seen in Western blots of control plasma (CH 2, Figure 2), so this signal was assumed to be background from other plasma proteins.

Purified Vg and plasma Vg responded similarly to the assay (Figure 3.4). An analysis of covariance test for homogeneity of two regressions determined that the slopes of the two resulting curves were not significantly different ($F_{obs} = 0.62, < F_{crit} = 4.21$ for d.f. 1, 27; $p > 0.05$; Figure 3.4).

The working range of the assay (~33 - 1000 ng/mL, 75 - 20% binding) was based on the visual inspection of the linear range of the log-transformed standard curve. The average recovery and CV at 50% binding were 104% and 12%, respectively. At the ends
of the working range, average recovery was 121% and 132%, and average CV was 19% and 24%, as detailed in Table 3.4. Vg concentration above that from a blank (plasma from untreated fish) plus three time the standard deviation should be statically significant, accordingly the detection limit of this assay would be 22 ng/mL. With the necessary dilution of 1:100 to avoid interference from other plasma constituents, the detection limit in a plasma sample would be 2.2 μg/mL.

17β-Estradiol Treatment

The time course showed relatively steady Vg concentrations 4-14 days after injection. 17β-Estradiol induced Vg to near the maximal the response by 4 days, and the response was dose-dependent (Figures 3.5 and 3.6), with no significant production of Vg in control fish.

Nonylphenol Treatment

In preliminary tests, single fish treated with 80, 100, 160, and 250 mg/kg NP died. The last experiment showed a dose-dependent response between 5 and 40 mg/kg (Figure 3.7). The Vg response to NP extended over two orders of magnitude with proportional variability, making the variance unequal across treatment groups and a typical analysis of variance (ANOVA) unsuitable. A log transformation of the Vg data provided a normal distribution (Shapiro-Wilk test, p = 0.08), however, data did not exhibit consistent residual variance across treatment groups (Levene test, p = 0.0013). Therefore, an ANOVA weighted by the reciprocal of the residual variance within groups within each treatment group was used. This weighted ANOVA of log Vg data showed significant
differences (p < 0.001) and a Tukey comparison was used to determine which treatment
groups were significantly different from each other (p < 0.05). All NP treatments,
including the lowest dose of 5 mg/kg, producing an average of 2.9 ng/mL plasma, were
significantly different than the control. No relation was found between NP dose and HSI
or GSI (data not shown).

Discussion

Vg antibodies can be very specific for the molecule from which they were
generated, exhibiting little cross reactivity to Vgs from other species. Although the
structure of Vg is fairly well conserved, it may be the extensive post-translational
modification that creates a great variety of species specific epitopes (Watts et al., 2003).
Watts et al. also suggested that the Vg purification procedure could be of key importance.
Failure to preserve the Vg molecule in its entirety could generate an antibody with high
affinity for only the target species. Efforts have been made to identify a conserved
portion of the protein with which to generate a universal antibody, such as the N-terminus
(Heppell et al., 1995), but a universal assay has yet to be developed.

Since this assay employed a heterologous antibody, which might provide an
inferior detection limit, other means to lower detection limits for this assay were
explored, mainly the use of the avidin-biotin amplification system. Biotin and avidin have
a very specific binding interaction that has been exploited in biochemical analytical
techniques. The small size of these molecules enable an antibody to have multiple biotin
molecules attached to them, thus allowing each to bind many molecules of the avidin–
enzyme conjugate. The result is a greater amount of enzyme present per antibody and
greater signal produced. Streptavidin is often used in place of avidin because avidin has a
greater tendency to bind nonspecifically in biological samples. This amplification has
been used in a variety of immunoassays to enhance the signal and enable detection of
smaller quantities (Tijssen, 1985; Avrameas 1987).

The use of the biotin-avidin amplification system for competitive ELISA has not
been well described in the literature. Only a few references describe a procedure very
similar to the one presented here, using a biotinylated secondary antibody and a
streptavidin-enzyme conjugate in a competitive ELISA. Marx et al. (2001) developed an
ELISA for rainbow trout Vg, using a secondary antibody-HRP conjugate and TMB
detection that had an unsatisfactory detection limit of 250 ng/mL. Adding both an
overnight preincubation and biotin-avidin amplification system improved the detection
limit of the assay to < 5 ng/mL. A highly sensitive assay for papaverine was developed
using the biotin avidin system, however the working range of the ELISA without the
amplification system was not discussed (Yan et al., 2004). While, Kania et al. (2003)
described an ELISA that failed to detect toxic concentrations of the marine algal toxin
domoic acid, using a secondary antibody-HRP conjugate, incorporating the biotin-avidin
system greatly improved their detection limits. However, replacing the secondary
antibody with an alkaline phosphatase antibody conjugate improved the working range as
much as the biotin-avidin system, and was favored because it is simpler. Similarly, in this
ELISA, the signal intensity was increased, but the IC50 was not lowered beyond what
could be obtained with a simpler procedure. The only benefit might be that less primary
antibody would be required. For our assay the primary antibody is readily available, so
the biotin-avidin system provided no significant advantage.
The initial recovery of spiked-plasma samples was poor. This may have been due to plasma interferences, but untreated plasma samples did not exhibit significant nonspecific binding at the minimal sample dilution. Therefore, several options were tested to improve recovery, including additives and eliminating/shortening the preincubation step. Many ELISAs include shorter preincubations that can be done the same day. A long preincubation often can lower the detection limit of an assay by allowing the sample to bind to the antibody before adding it to the plate, where it will compete with the coated antigen for antibody binding. While a long preincubation might lead to a loss of analyte through non-specific binding to plastic containers during the incubation. We observed that, the IC50 was greatly increased with shorter incubation. Since a lower IC50 generally corresponds to a lower detection limit, the overnight preincubation was retained.

The addition of PEG to assay buffers lowered the IC50 in the standard curve, which indicates a lower detection limit. Also the slope of the curve was changed, extending the dynamic range. This polymer has been reported to enhance the antibody-antigen reaction (Hellsing and Richter, 1974; Salonen and Vaheri, 1981; Linares-Casenave, 1993), making immunoassays more rapid and able to detect smaller quantities. It is a non-ionic polymer thought to decrease the solubility of both antigen and antibody by steric exclusion from the polysaccharide network of the PEG molecules (Hellsing, 1972; Rampling, 1974). Upon addition of PEG, the recovery of plasma spikes remained less than 50%, but it greatly increased the results of the buffer spikes, overestimating them at 137%. It appears that PEG would have caused the assay to respond differently to
the Vg standard in buffer solution than to plasma Vg, distorting the accuracy of the assay. For that reason, PEG was not included in this ELISA.

Initially in this ELISA procedure, Tween-20 was not used in both buffers that make up the preincubation mixture, but was only included in the antibody diluent. Addition of 0.05 % Tween-20 to the other buffer, which was used to dilute the Vg standard or samples prior to combining it with the antibody, was most effective in achieving recoveries nearest 100%. Tween-20 is a non-ionic detergent that is commonly used in immunoassays to reduce the background by saturating free binding sites. It has also been found to promote the antibody-antigen reactions, possibly by reducing hydrophobic interactions between the protein and solid support, allowing proteins and antigens to retain their native conformation (Zampieri et al., 2000). The low recoveries may have been due to a non-specific binding interactions between Vg and plasma proteins or the container, which Tween-20 was able to reduce. Use of Tween-20 and PEG were the factors found to have had the greatest affect on the slope of the curve. In this assay, manipulations of the antibody concentrations affected only the magnitude of the assay response, and had little effect on the detection limits and sensitivity (slope). Sugawara et al. (1998) and references within discuss how manipulation of the Tween-20 concentration can increase sensitivity in assays for dioxin and polychlorinated biphenyls. However, the authors ultimately omitted the detergent from their assay because it increased the IC50.

The quantification limits were reasonable for this assay, despite that fact that the antibody was not raised against California halibut Vg. The working range of the assay was ~33-1000 ng/mL, and the detection limit was 22 ng/mL. Compared to several other
Vg ELISAs, this halibut ELISA was comparable to that for bluegill, Lepomis macrochirus (29-2700 ng/mL; Cheek et al., 2004), brown trout, Salmo Trutta (25-500 ng/mL; Sherry et al., 1999), and gag, Mycteroperca microlepis (19-1452 ng/mL; Heppell et al., 1999). However, some assays reportedly can detect significantly lower concentrations, with a minimum detection limits of 1-3 ng/mL for sea bass (Dicentrarchus labrax L., Mananos et al, 1994), artic charr (Salvelinus alpinus, Johnsen et al., 1999) and English sole (Pleuronectes vetulus, Parks et al., 1999), and 10-12 ng/mL for fathead minnow (Pimephales promelas, Lomax et al., 1998) and brook trout (Salvelinus fontinalis, Schafauser-Smith and Benfy, 2002). However, commercially-available Vg ELISA kits from a leading manufacturer have detection limits ranging from 0.1-1.6 ng/mL depending on the species (Biosense Laboratories, Norway). Few heterologous assays in the literature have been developed beyond a test for cross-reactivity. Tyler et al. (2002) used antibody from Atlantic salmon in an assay for rainbow trout. A higher working range was obtained for their heterologous assay (60 - 850 ng/mL), compared to the assay for the native Vg (9 - 70 ng/mL) and our assay.

The precision of Vg ELISAs is often reported for only one point, at or near the IC50, as it is the most reliable portion of the curve. Many other researchers have reported CVs of 8-15% (Cheek et al., 2004; Schafauser-Smith and Benfey, 2002; Johnsen et al., 1999; Heppell et al., 1999; Lomax et al., 1998), and even one less than 5% (Parks et al. 1999). The accuracy and precision of our assay at the IC50 (12%) was comparable to those. Reproducibility or recovery are seldom reported near the detection limits where the assay tends to be less reliable. As expected, Sherry et al. (2002) observed a considerable loss of assay precision at the ends of the working range (from 6.8% CV at
the IC50, to 42.4% and 56.7% at the detection limits) very similar to the pattern of variability of our assay.

In the halibut bioassay, Vg induction by estrogen was relatively stable over the time investigated, indicating that a 4-14 day exposure period would work well for this bioassay. Induction can be seen as soon as 2 days after exposure, depending on the dose (Sun et al., 2003, Parks et al., 1999). In male fish there is no natural sequestration for Vg, allowing it to circulate in the plasma for days to months before being eliminated (Hemmer et al., 2002). This makes correlating plasma Vg concentrations to the location of environmental exposure difficult, as Vg levels in fish could result from contaminant exposure weeks before, when the fish are far from the sampling location.

The dose dependence of Vg on 17β-estradiol and the well known estrogen mimic \(p\)-nonylphenol support the validity of this bioassay for detection of estrogenic endocrine disrupting contaminants. The acute toxicity of NP observed in our study was surprising, since most other studies do not mention mortality occurring at doses close to the levels used to study NP effects on Vg. California halibut and other flatfish may be more sensitive to NP compared to other species (Figure 8). Other studies report no mortality in studies with injected once with similar or higher concentrations for example, \textit{Salmo salar} (Arukwe et al., 1997) and \textit{Cyprinodon variegatus}, (Pait and Nelson, 2003). Some fish tolerated doses almost twice as high as the lethal dose in this study, in either single or multiple injections, including: \textit{Fundulus Heteroclitus}, (Pait and Nelson, 2003), \textit{Cyprinus carpio}, (Casini et al., 2002, injected once a week, for 2 weeks), and \textit{Acipenser transmontanus}, (Chapter 4 of this thesis). When specified, these other studies included use of a similar vehicle (oil), grade and source of NP, and were all exposed via i.p.
injection, save sturgeon, which were injected with NP in an ethanol-saline mixture (Chapter 4, this dissertation). The NP response of another flatfish, flounder (*Platichthys flesus*), which were fairly similar in size (mean weight 300g), was available for comparison, although the dosing scheme was different (Christensen et al., 1999). Males were injected 4 times during 2 weeks. Mortality rates were 61% at the highest dose, 100mg/kg, and 22-33% for NP doses of 25, 50, 75 mg/kg. The more relevant question is whether or not flatfish are more sensitive to NP induction of Vg. This is even more difficult to determine from previous reports because the methods used to quantify changes in Vg varied from indirect plasma alkali-labile phosphorus measurements, to raw absorbance values from the ELISA, to quantitative ELISAs reporting plasma Vg concentrations.

Heterologous antibodies can be used to develop sensitive, accurate, and reproducible Vg ELISAs with low detection limits. As such, commercially-available antibodies for model species can facilitate the development of bioassays for estrogen mimics for local environmentally-relevant species. A sensitive flatfish bioassay was developed for indication of contamination of the California coast by estrogen mimicking compounds. Furthermore, many flatfish species are present in this area. Fish surveys of the Southern California Bight report finding at least twenty-six pleuronectiform (flatfish) species (Allen et al., 1998). This work suggests that monitoring projects that wish to incorporate several of these flatfish species do not need to generate specific antibodies for each one, and that this commercially available antibody may facilitate such efforts.
Acknowledgements

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References


Table 3.1. Inclusion of a Preincubation Step Lowers the Assay IC50

<table>
<thead>
<tr>
<th>Incubation</th>
<th>IC50 ng/mL</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 h 23 °C</td>
<td>178</td>
<td>0.80</td>
</tr>
<tr>
<td>2 h at 40 °C</td>
<td>403</td>
<td>0.84</td>
</tr>
<tr>
<td>1 h at 40 °C</td>
<td>563</td>
<td>0.81</td>
</tr>
<tr>
<td>none</td>
<td>3232</td>
<td>0.95</td>
</tr>
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</table>

Table 3.2. Addition of PEG Lowers the Assay IC50

<table>
<thead>
<tr>
<th>PEG concentration</th>
<th>IC50 ng/mL</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>180</td>
<td>1.0</td>
</tr>
<tr>
<td>1%</td>
<td>120</td>
<td>0.59</td>
</tr>
<tr>
<td>2%</td>
<td>139</td>
<td>0.77</td>
</tr>
<tr>
<td>4%</td>
<td>77</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Table 3.3. Addition of PEG Did Not Improve Spiked Sample Recovery

<table>
<thead>
<tr>
<th>Vg Spike (ng/mL)</th>
<th>% Recovery of Spikes Plasma Samples (n = 3, ± Std. Dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0% PEG</td>
</tr>
<tr>
<td>Buffer Spike</td>
<td>Plasma Spike</td>
</tr>
<tr>
<td>0</td>
<td>ND(11 ± 1.1)</td>
</tr>
<tr>
<td>33</td>
<td>79 ± 18</td>
</tr>
<tr>
<td>100</td>
<td>45 ± 8.0</td>
</tr>
<tr>
<td>300</td>
<td>41 ± 0.90</td>
</tr>
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</table>
Table 3.4. California Halibut Heterologous ELISA Variation and Recovery of Untreated Male Halibut Plasma Spiked with Vg Over 5 Days

<table>
<thead>
<tr>
<th>Vg ng/mL (% B/Bo)</th>
<th>Plasma Sample</th>
<th>Average Recovery (%)*</th>
<th>Interassay CV (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 (20%)</td>
<td>buffer</td>
<td>113</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>139</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>152</td>
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*Coefficients of variation and averages recoveries calculated from analyses run on 5 different days. Letters, a-d, indicate the plasma samples from 4 different fish used. Overall averages were calculated from the 4 plasma spikes only (response form buffer spikes not included).
Figure 3.1. Identification of optimum antibody and coating concentrations. Listed in the legend are the Vg coating concentration (Coat), the primary antibody dilution (1') and the secondary antibody dilution (2'). The curve that provided a low IC50 without shortening the visible dynamic range was selected: Vg coating concentration of 3ug/mL, primary antibody dilution of 1:15,000, secondary antibody dilution of 1:1,375. Each point represents one well of the microtiter plate (n=2). A-D are the 4 parameters of the logistic model, where A is the maximum absorbance, D is the minimum absorbance, C is the IC50 or midpoint of the standard curve, and B is slope at the IC50.
Figure 3.2. Comparison of biotin-avidin system to Ab-HRP conjugate. Listed in the legend are the primary antibody dilution (1') and the dilution of either the secondary antibody conjugated to horseradish peroxidase (2'-HRP) or the secondary antibody conjugated to biotin (2'-biotin). Although the biotinylated antibody enables the use of less primary antibody, a lower IC50 was not obtained; therefore, both systems were likely to provide comparable detection limits. Each point represents one well of the microtiter plate (n=2). A-D are the 4 parameters of the logistic model, where A is the maximum absorbance, D is the minimum absorbance, C is the IC50 or midpoint of the standard curve, and B is slope at the IC50.
Figure 3.3. Effect of increased Tween-20. Listed in the legend is final concentration of Tween-20 in the preincubation mixture as a result of the use of 0.05% Tween-20 or no Tween-20 used in the Vg standard diluent buffer (0.05% Tween was always used in the antibody diluent). The increase in Tween-20 extended the linear range from about 50 – 450 ng/mL to about 35 – 1000 ng/mL. Each point represents one well of the microtiter plate (n=3). A-D are the 4 parameters of the logistic model, where A is the maximum absorbance, D is the minimum absorbance, C is the IC50 or midpoint of the standard curve, and B is slope at the IC50.

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Figure 3.4. Parallel binding of Anti-turbot Vg antibody to plasma Vg and California halibut Vg standard. The similarity in slopes indicates that the assay responds similarly to both the Vg standard and Vg from an estrogen-treated male California halibut plasma sample, which is necessary for accurate quantification. Curves were made by diluting both analytes in a 3-fold serial dilution, after and initial dilution of 1:10 for the Vg standard and 1:100 for the plasma sample. Slope shown to be not significantly different by ANCOVA: $F_{obs} = 0.62$, $< F_{crit} = 4.21$ for d.f. 1, 27; $p > 0.05$. Each point represents the average of 3 wells of the microtiter plate and indicates the relative fraction of bound antibody, calculated as $(\text{raw absorbance} - \text{min absorbance}) / (\text{max absorbance} - \text{minimum absorbance})$. 
Figure 3.5. Estrogen induction of Vg over time in California halibut. Males were i.p. injected with 0 - 1000 µg/kg 17β-estradiol (dose indicated in legend) and plasma was sampled on the day indicated. Each bar represents the mean of 3 fish, ± standard deviation (separate fish were used for each time point).
Figure 3.6. California halibut vitellogenin response to 17β-estradiol. Fish were i.p. injected and blood was sampled after 4 days. Each point represents 3 fish ± standard deviation, except for the last treatment, where n=2.
Figure 3.7. California halibut vitellogenin response to \( p \)-nonylphenol. Fish were i.p. injected and blood was sampled after 7 days. Each point represents 5 fish ± standard deviation, except for the 10 mg/kg treatment, where \( n=6 \). All treatments were significantly different from control \( p < 0.05 \).
Chapter 4

Influence of an anti-estrogen on estrogen-mimicking contaminants in the white sturgeon (*Acipenser transmontanus*)

To be submitted as:

Palumbo AJ, Denison MS, Doroshov SI, Tjeerdema RS. Influence of an anti-estrogen on estrogen-mimicking contaminants in the white sturgeon (*Acipenser transmontanus*).
Abstract

White sturgeon are native to the Sacramento River and subject to agricultural, municipal and industrial waste water effluents that likely contain different classes of endocrine-disrupting contaminants. Reductions in 17β-estradiol-induced vitellogenin levels were observed in white sturgeon co-injected with β-naphthoflavone (BNF, 50 mg/kg), an Ah receptor agonist. The inhibition was maximal when the compounds were injected simultaneously versus prior treatment of fish with BNF. This timing of the effect compared to increases in ethoxyresorufin-O-deethylase (EROD) activity suggests that the effect is not directly due to enhanced estrogen metabolism by the Ah receptor-induced enzymes. Results of this study will be relevant for those with monitoring programs who measure vitellogenin, as it is important to understand how Ah (dioxin) receptor active environmental contaminants can influence this endpoint.
Introduction

About a decade ago sewage effluents were found to have estrogenic properties, as demonstrated by elevated levels of the egg yolk protein vitellogenin in caged fish (Purdom et al. 1994, Harries et al. 1996). Additionally, male fish with ovarian tissue in their gonads have been observed and their occurrence has been correlated to their proximity to municipal sewage effluents. Estrogen-mimicking contaminants present in the effluents are suspected to be responsible for feminization of these fish (Purdom et al. 1994, Folmar et al. 1996, Allen et al. 1999).

These compounds are examples of endocrine disruptors (EDs), a group of contaminants with the potential to interfere with normal species reproduction by altering hormonal signaling. EDs vary in source, from industrial chemicals, such as surfactants, detergents and pesticides, to natural and synthetic hormones, to numerous compounds used in personal care products. Many end up in waterways, often through sewage effluents, where they are likely to have the greatest impact on aquatic wildlife.

Predicting the effects of EDs on wildlife is a complex problem in part because there are a number of mechanisms by which EDs can alter hormone signaling. These chemicals are either agonists (positive regulators) or antagonists (negative regulators) of hormone receptor response, with the focus on the estrogenic and androgenic mechanisms. Accordingly, exposure of an organism to a complex mixture of EDs would produce a response that is the net balance of agonist/antagonistic activities in the mixture, rather than the activity of a single chemical. These mixed exposure scenarios are highly likely and such interactions may confound the identification of EDs in effluents. However, little
effort has been devoted to understanding mixtures endocrine disruptors with various mechanisms of action.

Proper estrogen signaling is required for normal growth, development, and reproduction. These events are regulated in part through ligand binding of estrogen to its receptor (ER). In this ligand-activated form, the ER can bind to a specific DNA sequence, the estrogen response element (ERE), leading to coactivator recruitment and activation of transcription of an adjacent gene. In oviparous vertebrates, one of these genes is responsible for the synthesis of vitellogenin (Vg), a protein precursor of egg yolk normally found in pre-spawning females and whose expression/synthesis is estrogen-inducible. Exogenous estrogens or contaminants with estrogen-like activity can induce synthesis of Vg in males and juveniles, making this protein a useful indicator of chemicals that mimic estrogen (Sumpter and Jobling 1995). Other compounds have been found to affect estrogen signaling, but not by binding to the ER. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related contaminants activate the aryl hydrocarbon receptor (AhR) and anti-estrogenic activity appears to occur though cross talk between the ER and AhR as well as effects on estrogen levels (Safe et al. 1998, Rogers and Denison 2002). Dioxin–like chemicals include planar polychlorinated biphenyls (PCBs), numerous polycyclic aromatic hydrocarbons (PAHs), and polychlorinated dibenzofurans and dioxins, although recent evidence suggests that the AhR can bind and be activated by a structurally diverse array of chemicals (Denison et al. 1999, Denison and Nagy 2003).

Very few studies have examined the effect of mixtures of EDs with varying mechanisms in vivo. Vaccaro et al. (2005) found that the dioxin–like PCB 126 had an
inhibitory effect on estrogen-induced Vg synthesis. In contrast, Arukwe et al. (2001) found either an inhibitory or stimulatory effect of the planar PCB 77 on nonylphenol-induced Vg synthesis, that depended on the dose and timing of the experiment. Because of the inexplicability of the latter results, additional in vivo studies are needed to clarify the response of AhR agonists on estrogen-induced Vg synthesis. β-Naphthoflavone (BNF), a non toxic AhR agonist, has been used to examine these effects. BNF has been shown to inhibit Vg synthesis in vitro (Anderson et al. 1996a, Smeets et al. 1999, Navas and Segner 2000, Bemanian et al. 2004), and in vivo studies have demonstrated inhibitory and additive effects in rainbow trout (Oncorhynchus mykiss, Anderson et al., 1996b) and mosquitofish (Gambusia holbrooki. (Aubry et al. 2005). These studies also suggest that BNF inhibits estrogen induced Vg synthesis through a mechanism involving the AhR, but the exact mechanism remains unclear.

White sturgeon (Acipenser transmontanus) are prized for their caviar and meat, as a sport fish, and scientifically as these are evolutionarily, a very primitive species. Unfortunately, their numbers have dramatically declined from historic levels because of over fishing, poaching, pollution, habitat destruction, and the presence of dams barring their access to spawning sites (Bemis and Findeis 1994, Birstein et al. 1997). In California, sturgeon inhabit the Sacramento-San Joaquin River System as well as San Francisco Bay. These waters receive effluents from various industries and metropolitan sewage treatment plants, plus run-off from the extensively farmed Central Valley, all of which are typical sources of EDs. While sturgeon have seldom been the subject of ED studies, a few studies have suggested a link between contaminant levels/exposure and typical signs of endocrine disruption, such as reduced gonad size, gonad abnormalities,
reduced plasma androgens, and elevated ethoxyresorufin-O-deethylase (EROD) activity (Harshbarger et al. 2000, Foster et al. 2001, Moore et al. 2003, Feist and et al. 2006). Moreover, intersex shovelnose sturgeon (Scaphirhynchus platatorynchus) in the Mississippi River were found to have higher levels of organochlorines in their brain–hypothalamic–pituitary complex compared to mature males (Koch et al. 2006). As these fish are among the oldest vertebrates still in existence, observations of similar contaminant effects in sturgeon compared to other modern teleost fish may attest to the widespread nature of ED effects on all fish, and perhaps even all vertebrates.

Here, we seek a better characterization of the interactions of estrogens and their antagonists in white sturgeon in vivo in order to provide guidance to scientists and regulatory agencies on how to consider anti-estrogens when assessing the effects of endocrine disruptors on fish and wildlife.

**Materials and Methods**

*Chemicals and Reagents*

Vg was previously purified from white sturgeon as detailed in Linares et al. (2003). This reference also describes the anti-Vg antibody used for this assay, provided by S. Doroshov (UCD). Bradford reagent, 17β-estradiol (E), β-naphthoflavone (BNF), and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO). The p-nonylphenol (NP) was from Fluka Chemika (Switzerland), and MS-222 (tricaine methane sulfonate) was from Argent Chemical Laboratories (Redmond, WA).
Animals

Cultured juvenile white sturgeon (A. transmontanus), obtained gratis from Stolz Sea Farm (Sacramento, CA), were maintained at the UCD Center for Aquatic Biology and Aquaculture in 2-m diameter tanks supplied with ambient well water (19°C, 0.5%/oo salinity) and fed Nelson & Sons Silver Cup Feed: Steelhead Formulation (sinking extruded style, Murray, UT). Experiments took place over the course of 15 mo. Fish weighed on average, approx. 17 and 45 g (age 3-5 mo) for the E and NP dose-response experiments, 90, 150, and 300 g (age 6-9 mo) for the three repeated 7-day BNF + E experiments, and 500 to 700 g (age 9-17 mo) for all other experiments. Gonads of sturgeon are not fully sexually differentiated at this size and age (Van Eenennaam and Doroshov 1998)

Sturgeon Exposures

Exposures were conducted in the UCD Meyer Hall Animal Facility in a temperature-controlled room (19°C), on a 12-h photoperiod. Fish were housed in recirculating filtered, aerated tanks with three fish per 200-liter circular fiberglass tank (1 m dia. x 0.3 m deep). Water was deionized, salted to 0.5%/oo (Forty Fathoms/Crystal Sea, sea water salt mix), and dissolved oxygen (> 80%), unionized ammonia (< 0.05 ppm), temperature (18-20°C), and salinity (0.5-1.5%/oo) were monitored daily. Tanks were cleaned via siphoning and 30% water changes were performed as needed to maintain water quality.
In all experiments, anesthetized (MS-222, 100 mg/L) sturgeon were injected (i.p.) with compounds using 0.5 mL/kg carrier; they were not fed during experiments. Blood was sampled from the caudal vasculature, just behind the anal fin of anesthetized fish with heparin-rinsed syringes, centrifuged at 2500 rpm for 10 min, and plasma aliquots were frozen on dry ice. Fish were euthanized by overdose of MS-222 (500 mg/L), followed by severing of the notochord. The liver was removed weighed and placed immediately in liquid nitrogen, and blood plasma and hepatic tissues samples were kept at -80°C until analysis.

Seven-day dose-repose relationships were characterized using E and NP (both in absolute ethanol:saline, 70:30). For the BNF time course, fish were injected with 50 mg/kg BNF in peanut oil and different fish were sampled after 1, 2, 3, and 7 days.

The first BNF + E mixture experiment lasted seven days. The six treatments included a vehicle control (ethanol:saline, 60:40), a low dose of E (10 μg/kg), a high dose of E (1 mg/kg), and each of those treatments plus BNF (50 mg/kg in peanut oil). Injections were made on the right side of the abdomen starting with the BNF (or oil carrier for E-only treatments), positioned 2/3 of the way toward the pelvic fin from the pectoral fin in order to avoid hitting organs. E injection immediately followed at about 1-2 cm closer the pelvic fins. This experiment was repeated three times over a period of about three months to obtain nine replicates.

The BNF pretreatment experiment consisted of six treatments and two sampling times. Treatments were: vehicle control (peanut oil and ethanol:saline, 60:40), positive control, 17β-estradiol (E, in ethanol:saline, 60:40, plus peanut oil vehicle control injection), a treatment with E + BNF injected simultaneously, and three treatments with
the BNF administered 1, 2, or 3 days before E. In independently conducted experiments, fish were sampled 1 or 2 days after the E injection, indicated as '1 day post E treatment' and '2 days post E treatment'. As above, these experiments were performed with three fish in each treatment and repeated to achieve 6-9 replicates. In these and later experiments, water quality was maintained under static conditions after injection of compounds to avoid contamination of the recirculation system with E.

A NP + BNF combination experiment was run for two days, with a dose of NP high enough to produce a Vg response in this short time period (50 mg/kg).

**ELISA Procedure**

The optimized procedure for the Vg ELISA was based on Linares-Casenave (1993) and Linares et al. (2003). Ninety-six well microtiter plates with Maxisorb® surface treatment (NUNC) were coated with 3 µg/mL white sturgeon Vg diluted in carbonate-bicarbonate buffer (pH 9.6), sealed, and incubated overnight (18 h, 4°C). Anti-white sturgeon Vg antibody and sample or standard were preincubated overnight (18 h, room temperature). Vg standards, 1.95 - 1000 ng/mL, serially diluted in phosphate-buffered saline with 0.05% Tween-20 (PBST, pH 7.4), and diluted samples (minimum 1:100 in PBST) were pipetted into triplicate wells (100 µL). Primary antibody was diluted 1:60,000 in PBST and added in equal volume to the standards and samples. The following day coating antigen was washed from the plate 5x with 250 µL PBST by a handheld microplate washer, and nonspecific binding was blocked by incubating with 1% bovine serum albumin and 1% non-fat milk in carbonate-bicarbonate buffer (100 µL, 1 h). After washing, 100 µL of the antibody-analyte mixture was pipetted onto the plate.
One hour later, the plate was washed again and bound antibody was detected by an incubation of the secondary antibody-HRP conjugate, diluted 1:5,500 in PBST (100 μL, 1 h), and a final wash followed by incubation of a solution of the chromogen (3,3',5,5' tetramethylbenzidine) and substrate (1% hydrogen peroxide in sodium citrate buffer, pH 5.5). The reaction was terminated with 2M H₂SO₄ and the absorbance at 450 nm was measured in a Tecan Sunrise microplate reader (Phenix Research Products, Hayward, CA).

To analyze data by linear regression, a log-logit transformation (Diamandis and Christopoulous 1996), based on the four-parameter equation of Rodbard (1974), \( y = \frac{(a-d)}{1+(x/c)^b} + d \), was employed. Data were fit to Logit \( r = \ln(c)-b \ln (x) \), wherein \( r = \frac{B}{B_0} \) and logit \( r = \ln \left[ \frac{r}{1-r} \right] \) and \( B/B_0 \) is the relative fraction of bound antibody, calculated as \( \frac{(y-d)}{(a-d)} \). The accuracy and reproducibility of this assay for spiked samples is shown in Table 1.

**Liver Microsome Isolation and EROD Assay**

Livers were defrosted on ice, weighed, minced and homogenized 1:5 (w/v) in ice-cold buffer (0.02M Tris / 1.15% KCl, pH 7.4) using Teflon glass homogenizers (6 strokes). The mixture was centrifuged at 9,000 x g for 20 min at 2°C. A syringe was used to remove the post-mitochondrial supernatant, being careful to avoid the fat layer, and the supernatant was centrifuged at 100,000 x g for 1 h at 4°C. To wash the microsomal pellet, the supernatant was decanted and half the original volume of buffer was added, the microsomal pellet resuspended by it pulling through a syringe and recentrifuged at 100,000 x g as before. The supernatant was again decanted and approx. 1 mL of GET
buffer (0.2 g/mL glycerol, 1 mM EDTA, 0.01 M TrisCl, pH 7.4), was added per g
original tissue. The microsomes were resuspended with a small Teflon glass hand
homogenizer, aliquoted and stored frozen at -80°C until analysis.

Microsomal protein was measured with the Bradford Assay (BioRad) and
solutions were diluted with GET buffer to concentration of 1 mg/mL. Cytochrome P450
(CYP) 1A1/1B1 activities were assessed by measuring EROD activity. Assays were
performed in black clear-bottomed 96-well plates (Costar), using a modification of the
original procedures (Reiners et al. 1990, Besselink et al. 1997, Willett et al. 2001). Each
well contained 40 μg of microsomal protein (in 40 μL GET buffer) and a final
concentration of 3.85 μM 7-ethoxyresorufin, 0.6 mM NADPH, and 0.5% Tween-20 in
0.1 M HEPES buffer (pH 7.8), in 130 μL. The fluorescence wavelengths used were 544
nm and 590 nm for excitation and emission, and the enzyme activates were measured
kinetically every 90 sec for 25 min, measured in a Fluorostar fluorescence plate reader
(BMG Labtechnologies, Germany). The linear portion of the curve was used for
quantification by comparison to a 5.3 to 42.5 pmol resorufin standard curve.

Statistical Analysis

Since the Vg responses often extended over two orders of magnitude with
proportional variability, making the variance unequal across treatment groups, the Vg
data was log transformed before ANOVA. A two-way ANOVA of the data from both the
7-day BNF + E experiment and the pretreatment experiment showed that differences in
the individual experiments (as these both were conducted as three replicate experiments)
Results

To verify the sturgeon bioassay responds to estrogenic compounds sturgeon were injected with control solvents, and a range of 17β-estradiol and p-nonylphenol, and plasma Vg was determined after seven days. E and NP dose dependence increase in sturgeon Vg synthesis are shown in Figures 4.1 and 4.2, respectively. The response to NP plateaus in the highest doses, although the maximal Vg response to NP was one-tenth the amount of Vg from the highest E dose tested. The ELISA used to quantify Vg in the sturgeon bioassay was checked for reproducibility and accuracy. Table 4.1 shows that about 20% variability is typical in this assay.

To examine the effect of the AhR agonist on E induced plasma Vg levels, fish were injected with control solvents, E or E + BNF, and plasma Vg was determined after seven days. The BNF significantly decreased E-induced Vg after simultaneous administration to sturgeon (Figure 4.3A). A Factorial ANOVA on the 4 treatments with BNF + E showed that overall there was a significant effect from the BNF (p = 0.0038). Comparing the high and low doses of E individually to their E + BNF counterpart, BNF significantly reduced Vg in the high E treatment (p = 0.0016), but not as significantly in the low E treatment (p = 0.0518). The BNF treated fish had significantly induced EROD activity (Figure 4.3B) and this activity was unaffected by E concentration. Increased EROD activity indicated that the BNF is activating the AhR and inducing CYP. Upon
dissection, a large portion of the dose of solid BNF was always found in the i.p. cavity of treated animals, indicating that much of the dose was not absorbed.

Because CYP isoforms are known to be capable of decreasing E concentrations via metabolism, CYP activity over time was monitored in fish injected with only BNF. Maximal CYP induction occurred by three days after injection (Figure 4.4). If maximal CYP activity from a BNF injection could be correlated to the inhibition of Vg it would suggest that CYP may be the cause. Therefore the next experiment was designed to target maximal CYP activity by injecting fish with BNF 1-3 days prior to the E. A shorter exposure time to E was also used (1 or 2 days) to allow for less influence of other processes to affect the Vg levels before sampling. In the resulting pretreatment experiment, there was no significant influence of BNF on Vg induction, when sampled 1-day post-E treatment (Figure 4.5A, white bars). In samples taken 2 days post-E treatment, BNF did reduce E-induced Vg by 50% (Figure 4.5A, gray bars) similarly to the seven day experiment (Figure 4.3A). Dunnett's test showed significant differences in the simultaneous injection of E and BNF, 1-day BNF pretreatment and 3-day BNF pretreatment compared to E-only treatment. The significant difference between the E and E + BNF combination treatment was lost in the 2-day pretreatment. BNF pretreatment of 1 to 3 days did not enhance the anti-estrogenic effect of BNF. In a comparison of just the four E treatments with BNF, Tukey pair-wise comparisons did not reveal any significant differences (at p < 0.10). EROD activity in liver from the same experimental animals also showed that maximum enzyme activity was a couple days after BNF injection (Figure 4.5B) and the level of induction paralleled the Vg results observed for the 2-day post-E treatment samples (Figure 4.5A). Since maximal CYP activity did not coincide with
minimal Vg response (maximal Vg inhibition) from fish exposed to BNF + E, these results do not indicate the involvement of AhR induced CYP in the anti-estrogenic effect.

To examine the effect of the AhR agonist on NP induced plasma Vg levels, fish were injected with control solvents, NP or NP and BNF, and plasma Vg determined after 2 days (Figure 4.6). Due to the high variability of the results, no significant effect if either treatment were determined, although the NP treated resulted in higher plasma Vg levels, NP and BNF was not different than NP alone. The lack of effect of NP may also be partly due to the very low levels of Vg induced by NP as compared to E (Figure 4.5A).

Discussion

The dose-dependent increase of white sturgeon Vg to E and NP confirms that these fish respond similarly to other species to E and a well known estrogen mimic. The only other report of the response of sturgeon to an estrogen mimic measured induction of Vg mRNA in Chinese sturgeon (*Acipenser sinensis* Gray) from NP exposure (Zhang et al. 2005). Testing of the Vg ELISA showed that a 20% of variability in the following results may be due to quantification methods. This variability is not unusually for this type of immunoassay, see Chapter 3 of this dissertation for more discussion on Vg ELISAs.

In white sturgeon, the model AhR agonist, BNF, reduced Vg synthesis by about 50% in the seven-day experiment. This same anti-estrogenic effect of BNF was observed after 2 days in the pretreatment experiment. The earliest report of the inhibitory effect of AhR agonists on estrogen-induced Vg synthesis involved rainbow trout liver cells exposed to three different AhR ligands: 2,3,4,7,8-pentachlorodibenzofuran, dioxin, or
BNF (Anderson et al. 1996a). This same study also revealed that BNF did not alter either synthesis of other proteins (e.g. albumin) or general cell function, and was consistent with a mechanism involving the AhR. One alternative mechanism could be the competitive inhibition of estrogen binding to the ER. However, BNF has little affinity for the ER as determined in an estradiol binding assay (Anderson et al. 1996b, Kloas et al. 2000).

From studies using cultured mammalian cells, evidence exists for several mechanisms of cross talk between the AhR and the ER that could explain such anti-estrogenic effects: 1) AhR ligands induce CYP isoforms, some of which metabolize estrogen to an ER-inactive form; 2) inhibition of the estrogen-ER complex binding to its DNA recognition site, the ERE (Kharat and Saatcioglu 1996); 3) down regulation of ER via a repressor site in the promoter region of the ER gene (White and Gasiewicz 1993); 4) enhanced degradation of ER through proteasome activation (Wormke et al. 2003), or a ubiquitin ligase complex, (Ohtake et al. 2007); 5) inhibition of an ER coactivator (Shao et al. 2004); or 6) competition between the AhR and the ER for binding to limited levels of nuclear coactivators (Reen et al. 2002) and others. Other evidence shows that new proteins made in response to AhR-dependent gene expression were found to be critical, as a general protein synthesis inhibitor was shown to prevent the anti-estrogenic effects of the AhR ligand (Rogers and Denison 2002). Thus there appears to be many mechanisms by which AhR activation can lead to reduction in E-dependent gene expression and cellular responses.

CYPs are not thought to have a major role in the anti-estrogenic effect as evidenced by the BNF pretreatment experiment. CYP1A1, 1A2, and 1B1 are known to metabolize 17 β-estradiol in humans and animals (Tsuchiya et al. 2005), which would
decrease the amount of available estrogen to produce biological effects. In the initial experiment BNF and E were injected simultaneously. As such, E was injected when there was low activity of AhR-inducible related CYPs. If these enzymes were primarily responsible for the mechanism, then injecting E when enzyme levels where higher (2-3 days post BNF treatment) should increase the metabolism of E and result in even lower levels of induced Vg. However, the reverse was observed. When BNF was injected prior to E, plasma Vg increased to levels near that resulting from E alone. This suggests that AhR inducible CYPs do not appear to be a major player in the anti-estrogenic effects of AhR agonists in sturgeon.

Other studies with fish or fish tissues have also suggested an anti-estrogenic mechanism of AhR agonists with little involvement of CYP dependent metabolism. Using rainbow trout cultured hepatocytes (Navas and Segner 2000) and carp hepatocytes (Cyprinus carpio, Smeets et al., 1999), it was found that anti-estrogenic effects of TCDD, BNF, PAHs and PCB 126 occurred at higher chemical concentrations than needed for CYP1A induction. In a similar study to ours, Aubry et al. (2005) exposed mosquito fish to 0.1 μg/L 17β-ethynylestradiol and BNF in a waterborne static-renewal exposure. A clear reduction of plasma Vg was observed at 4.0 μg/L, but not 1.0 μg/L, BNF. The authors commented that metabolism was unlikely to be the mechanism because E produces a rapid response, before the CYPs would have much effect.

Other studies suggest it is the ER that may be affected. A reduction of ER α-mRNA was found in cultured salmon (Salmo salar) hepatocytes by TCDD (Bemanian et al. 2004). ER expression is controlled by an auto-regulation loop (Castles et al. 1997), so
changes at the protein level or in the concentration of estrogens, could effect ER mRNA expression. An *in vivo* study with BNF showed similar inhibition of E (0.50 mg/kg) induced Vg in rainbow trout with 25 and 50 mg/kg BNF. Interestingly, in this study, when the E was increased to 5 mg/kg or when BNF was reduced to 12.5 mg/kg, the Vg induction response was increased compared to the exposure to E alone (Anderson et al. 1996b). This is the only study that reports a pro-estrogenic behavior of BNF. These authors also found that BNF cotreatment decreased the availability of estrogen binding sites in the low-E treatment and a trend toward increased binding sites with a high-E treatment, which corresponded to the pro- and anti-estrogenic effects they observed. The mechanism responsible for the differential effect on estrogen binding sites remains to be determined. Some studies suggest that whether or not an AhR agonist potentiates or inhibits the effects of estrogen depends on the dose, timing, or other experimental conditions (Mortensen and Arukwe 2007).

Considering possible mechanisms, it is a bit of a mystery as to why the effect seems to be lost when BNF is injected two days before E (Figure 4.5A), and the variably greatly increased in the last treatments, obscuring a trend. This cannot be contributed entirely to the lower number of replicates for those experiments. The variability in the individual experiments of 3 fish increased from an average of 20% standard deviation (range 11-30%) in the E, E + BNF and E + BNF-1 treatment groups, to 22 and 41% standard deviation in the two experiments that were pooled to make up the E + BNF-2 treatment, to 56 and 72% standard deviation in the two experiments that were pooled to make up the E + BNF-3 treatment group. Additional replicates were unlikely to resolve a trend in the last treatments.
Another curious result is that no effect was seen from the anti-estrogen in the 1-day pretreatment experiment. Vg was accumulating very quickly, from about 50 μg/mL to 550 μg/mL between the day-1 sampling and day-2 sampling experiments. Slight differences in sampling timing and the high synthesis rate at day 1 could have obscured a difference in the results. No effect from BNF was observed in the NP experiment either. Since NP acts by the same mechanism as estradiol one would expect reduction of Vg after combining NP and BNF. However, the result was high variability and no sign of a trend of inhibition by BNF. Again this may be due to high variability in the initial rate of Vg synthesis. Since NP is much less potent than E, a longer experiment may have better captured the effect.

A better understanding of the mechanism by which anti-estrogenic action occurs may help integrate the affect of dioxin-like contaminants into field studies. This is particularly important for investigations that relate residue analysis with estrogenic responses in animals. Anti-estrogens may be confounding these analyses, especially in areas heavily contaminated with AhR ligands.

While anti-estrogens may complicate the assessment of estrogen-mimicking contaminants, the implications of possible inhibition of endogenous estrogen-regulation of Vg synthesis by AhR agonists in wild sturgeon may be more severe. If vitellogenesis was slowed, such contaminants could delay female spawning, placing it out-of-sync males or proper environmental conditions. Since the anti-estrogens seem to affect estrogen signaling in general, other important estrogen-regulated events and behaviors could be altered. Fewer habitats are suitable for spawning, so even small chemical disturbances could be significant in species that spawn only once every couple of years.
Additionally Koch et al. (2006), who related intersex sturgeon to contaminants loads, suggested that sturgeon may be more susceptible to EDs because of their long life span. It takes 1.5 - 2 years for most species to sexually differentiate and 10-20 years to mature (Doroshov et al. 1997). Accordingly, sexual differentiation in sturgeon is vulnerable to the influence of ED contaminants for a much longer time period when compared to other species.

Indeed, other studies have reported that AhR ligands can affect reproductive fitness in fish. Chronic dietary exposure to dioxin was found to decrease egg production, spawning success, serum estradiol concentrations, and serum Vg in zebrafish (Heiden et al. 2006). A significant decrease in plasma 17β-estradiol was found in flounders (Platichthys flesus) exposed to PAHs (Monteiro et al. 2000). Aroclor 1254 and benzo(a)pyrene both have been found to reduce plasma Vg levels in rainbow trout, (Chen et al., 1986) and croaker (Micropogonias undulatus,(Chen et al. 1986, Thomas 1989). Moreover, reproductive effects have been recorded in wild fish living in waters receiving pulp mill effluent since the mid to late 1980s, including reduced spawning events, fewer eggs produced, ovipositor development in males, and development of male secondary sex characteristics in females (McMaster et al. 2006, Parrott et al. 2006, Rickwood et al. 2006)

Vg is a popular and valuable marker of estrogen mimicking contaminants in fish. Reduction of Vg by competing contaminants with anti-estrogenic properties is an important consideration for those using Vg for biomonitoring purposes. We are not implying that the reduction of Vg signifies less of a threat to wildlife. Animals exposed to both types of agents, but exhibiting no estrogenic response would still suffer the effects
of the anti-estrogen. Chronic suppression of estrogen signaling by AhR-acting agents could loom as a larger problem than that of the estrogen mimics and a harder issue to study.

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Feist and et al. 2006. Evidence of detrimental effects of environmental contaminants on growth and reproductive physiology of white sturgeon in impounded areas of the Columbia River (vol 113, pg 1675, 2005). Environmental Health Perspectives 114:A90.


reproductively immature white sturgeon (Acipenser transmontanus) from the Columbia River, USA. Archives of Environmental Contamination and Toxicology 41:182-191.


Table 4.1. White Sturgeon Vg ELISA Variation and Recovery of Vg from Spiked Sturgeon Plasma Samples

<table>
<thead>
<tr>
<th>Vg Spike (ng/mL)</th>
<th>Plasma Sample</th>
<th>Average Result (ng/mL)</th>
<th>Percent Standard Deviation</th>
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<tr>
<td>0</td>
<td>A</td>
<td>6.00</td>
<td>17</td>
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<td></td>
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<td>8.67</td>
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<td>C</td>
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<td>D</td>
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<td>A</td>
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<td>Ave. % recovery</td>
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</table>

*Coefficients of variation and averages recoveries calculated from analyses run on 3 different days. Letters, A-D, indicate the plasma samples from 4 different fish used. Overall averages were calculated from the 4 plasma spikes only (response form buffer spikes not included).
Figure 4.1
Vitellogenin response of white sturgeon to estrogen. Fish were i.p. injected with 17β-estradiol and blood was sampled seven days later. Each bar represents the mean of three fish ± standard deviation. Letters indicate Tukey grouping (p < 0.05) from ANOVA of log plasma Vg.

Figure 4.2
Vitellogenin response of white sturgeon to p-nonylphenol. Fish were i.p. injected and blood was sampled seven days later. Each bar represents the mean of three fish ± standard deviation. Letters indicate Tukey grouping (p < 0.05) from ANOVA of log plasma Vg.
BNF reduced 17β-estradiol (E) induced Vg (A) and induces EROD activity (B). Fish were simultaneously i.p. injected with both compounds (or vehicle control) and blood was sampled seven days later. Dose in mg/kg is indicated after compound abbreviation in x axis. Each bar represents the mean of nine fish ± standard deviation. In A: while a factorial ANOVA of all 4 treatments of E or BNF + E showed a significant effect of BNF p = 0.0038, ANOVA was also used to assess the effects in treatment pairs as noted in the figure. In B: asterisks (*) indicate difference from control (p < 0.05).
Figure 4.4
Time course of enzyme activity after BNF treatment. Fish were i.p. injected with 50 mg/kg BNF and blood was sampled after the indicated duration. Each bar represents the mean of 3 fish ± standard deviation. Letters indicate Tukey grouping (p < 0.05)
Figure 4.5
Effect of pretreatment of BNF on estrogen (E) induced Vg (A) and enzyme activity (B). Fish were i.p. injected with 17β-estradiol and BNF (or vehicle control). The number of days BNF was administered prior to the estrogen is indicated in the x axis. Fish were sampled one or two days after estrogen treatment as indicated by legend. Each bar represents the mean ± standard deviation, where n = 9, 8, 9, 6, 6, 6. IN A: statistically significant difference between the estrogen only treatment and any of the last four treatments is indicated by an asterisk (*, p < 0.05) and 'a' indicates statistical difference from control (p < 0.05). In B: Letters indicate Tukey grouping (p < 0.05) and asterisks indicate difference from control (p < 0.05).
Figure 4.6
The lack of effect of BNF on NP-induced Vg. Fish were simultaneously i.p. injected with both compounds and blood was sampled two days later. Each bar represents the mean of 6 fish ± standard deviation.
Chapter 5

Overall Conclusions

Both California halibut and white sturgeon are valuable species of the California coast. For this project, they were subject to many similar analysis and procedures, although not simultaneously, so some comparisons have yet to be made. Overall, the Vg molecules were similar between the species and they both express monomers close to 180 kDa, probably more than one form. The ELISAs were quite comparable too, despite the fact that for the halibut assay a commercial heterologous antibody was used, and the antibody for sturgeon Vg was generated specifically against this species. As expected, the detection limit for the homologous (sturgeon) assay was lower, but only slightly, 10 ng/mL vs. 22 ng/mL (or 1 μg/mL vs. 2.2 μg/mL in plasma).

The largest difference between these two species could be seen in the Vg response to nonylphenol. In California halibut, doses ≥80 mg/kg were lethal. The final dose response was performed with doses of 5-40 mg/kg. For sturgeon the dose response range was 3-243 mg/kg, with no signs of toxicity at the high doses, although there was a decrease in Vg production at the highest dose. Additionally, the maximum plasma Vg concentration obtained was significantly lower compared to that for the estrogen (30 vs. 700 μg/mL). Taken together these results suggest that some toxicity was likely in sturgeon also (Figure 3.8). The overall Vg produced from NP was less in sturgeon compared to halibut (30 μg/mL plasma vs. 300 μg/mL, Figure 4.2 and 3.7). However, the response to estrogen was very similar between the two species. From a seven a day exposure to 1 mg/kg and 10 μg/kg 17β-estradiol, the halibut and sturgeon plasma Vg
responses, respectively, were about 770 μg/mL and 700 μg/mL to the high dose and 9 μg/mL and 3 μg/mL to the low dose. The difference in NP response may not simply be due to species variation since several factors were different in the studies, such as the size, age, and state of sexual maturity, which is unavoidable with organisms that have such different life histories.

The results of the last experimental chapter are the most significant in this dissertation. As described in Chapter 4, AhR ligands can interfere with Vg responses. Since Vg is used as an indicator of estrogen mimicking contaminants, AhR ligands, may be confusing monitoring and interpretation of field studies of these contaminants. The interference of AhR ligands in the fish Vg response could explain differences in results of \textit{in vitro} and \textit{in vivo} assessment of the estrogenic potential of effluents, such as the example noted in the introduction in which the Vg response in the fish was lower than that predicted by the \textit{in vitro} assay. The \textit{in vitro} assay used, the yeast estrogen screen (YES), assay will not pick up anti-estrogenic effects from AhR ligands because the yeast cells contain no AhR. This work suggests that when using a fish bioassay however, AhR ligands would diminish the estrogenic response.

The main indicator of endocrine disruption in this work was the biomarker Vg, and there remains no firm correlation between Vg and impairment in reproduction or the incidence of intersex. The confounding effects of endocrine disruptors with different mechanisms as described in the last chapter could be obscuring some of these connections. Additionally, although changes in protein synthesis is more of short term effect, Vg will circulate for a maximum of a couple months in males. However, the intersex condition may reflect exposure during recent months or years ago during sexual
differentiation. It may be that exposure of juveniles during sexual differentiation causes intersex. It may not be appropriate to expect a correlation between these Vg and intersex and neither of these endpoints should be dismissed because they do not compare well.

One might consider that the anti-estrogenic effects of AhR ligands as a protective response, delaying reproduction until a time when the animal is free of contaminants. Since there is little field evidence of interference of AhR ligands in Vg responses and because AhR ligands reduce Vg, it may seem like these compounds are a minor concern compared to estrogen mimics or even somehow beneficial as they seem to reduce toxicity. An aphorism by Hippocrates, presents another perspective: "When two illnesses arrive at the same time, the stronger silences the weaker". Considering disease to be interchangeable with contaminant effects, it would suggest that the anti-estrogenic effects of AhR ligands may of greater consequence than the feminization by estrogen mimics, by stifling normal estrogen signaling. Even slight estrogen suppression could be very problematic as small changes in estrogen levels (i.e. ER signaling) could cause changes in development or the reproductive cycle, which might impair the reproductive success of wildlife. The nature of this effect makes it very difficult to notice or study.

Returning to the Lake Apopka example described in the introduction, we can see why endocrine disruption is a hard problem to address. There are a variety of chemicals present as a complex mixture coupled with many targets, mechanisms and interactions between chemicals that are not well understood. Complicating the problem further is our limited understanding of the natural reproductive process in all these species. These issues highlight the continuing need for basic science to establish an understanding of
reproductive development and mechanisms of hormone signaling, the interactive effects of chemicals and continued monitoring for toxicity.

Endocrine disruption is one small portion of the negative impacts of the modern use of chemicals. With so many chemicals and the many possibilities of different effects to each species, there is an increasing burden on the environmental and human health, and the regulatory agencies that try to protect us, including the public financing of these agencies. Eventually, a time will come when the benefit of introducing so many new chemicals will be less than the cost to evaluate all possible effects, regulate their use, and remediate unanticipated effects.