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WORKING PAPER

Intersex Fish in the Sudbury, Assabet, and Concord Rivers

By Susan Beede, December 29, 2014

In recent decades, scientists have discovered that the presence of human hormones and hormone-mimicking chemicals in rivers, lakes, and streams can affect the endocrine systems of fish. These chemicals can feminize male fish, masculinize female fish, impact reproduction, and potentially affect the population's ability to survive. This working paper attempts to summarize the local findings of a large federal study titled: "Evidence of estrogenic endocrine disruption in smallmouth and largemouth bass inhabiting Northeast U.S. National Wildlife Refuge Waters: a reconnaissance study" (Iwanowicz et al., 2013). Other studies of endocrine disrupting chemicals in the Assabet River were carried out in 2009-13 by the U.S. Environmental Protection Agency (EPA) in collaboration with the U.S. Geological Survey (USGS), OARS, and UMass Amherst. These will be discussed in future Working Papers.

Background

Considerable evidence exists that reproduction in freshwater fish is being compromised by compounds present in municipal wastewater discharges (Blazer et al., 2007; Hinck et al., 2009; Jobling et al., 1998; Jobling and Tyler, 2003; Kidd et al. 2007, Sumpter and Johnson, 2008). One group of contaminants of particular interest is the estrogens and estrogen mimics, as they can feminize male fish at very low concentrations . For example, feminized male fish may produce egg yolk proteins called vitellogenin, and in the more severe cases, develop eggs in their testes called testicular oocytes, a condition called "intersex" (Jobling et al., 1998). In general, the term "intersex" describes the simultaneous occurrence of male and female reproductive tissues in the testes or ovaries of fish that would normally develop and remain fixed as male or female throughout their lives (Bahamonde et al., 2013). Feminized male fish, whether they produce vitellogenin, have intersex testes, or exhibit other symptoms of feminization such as abnormal spawning behavior, are often found downstream of municipal wastewater discharges, pulp and paper mills, intensive animal feeding operations, and are more common in heavily populated watersheds (Jobling et al., 1998; Blazer et al., 2007; Desforges et al., 2010).

These wastewaters contain numerous potent estrogen mimics, but perhaps of greatest concern are the natural estrogens (e.g., estradiol) and synthetic estrogens (e.g., 17 alpha-ethynylestradiol from the birth control pill) that women excrete (Desbrow et al., 1998; Sumpter and Johnson, 2008). Although treatment can remove estrogens from the effluents (e.g. Kirk et al., 2002; Bringolf et al., 2003) several rivers in the U.S. contain feminized male fish (Hinck et al., 2009). Lab and field studies indicate that the release of estrogens likely explains many of the impacts in wild fish living downstream (e.g., Länge et al., 2001; Parrott et al., 2005; Caldwell et al., 2012; Young et al., 2004). In a now-famous seven-year study in Ontario, Canada, scientists caused the resident population of fathead minnows to collapse by adding low doses of synthetic estrogen (17 alpha-ethynylestradiol--the main ingredient in birth control pills) to a whole lake (Kidd et al., 2007). The lake's top predator, lake trout, began to starve as the minnow population crashed. Happily, the fathead minnow population recovered when the estrogen additions were stopped.

These phenomena are symptoms of *endocrine disruption*, i.e., the disruption of an animal's natural hormonal system by external estrogens and estrogen-mimickers. This is a serious concern to scientists and wildlife managers because they have the potential to harm reproduction of entire fish populations.

What is Endocrine Disruption?
Low-level exposure to some contaminants can disrupt animal reproduction and development by modulating, mimicking, or interfering with normal hormonal function. These contaminants include hormones created in the body, synthetic hormones (such as those manufactured for birth control or menopausal supplement), and industrial/commercial compounds that have some hormonal function (such as alkylphenols, pesticides, pharmaceuticals, and phthalates).

Survey of fish and water quality in the Assabet, Sudbury and Concord Rivers
In September 2013, the USGS and U.S. Fish & Wildlife Service published a study of nineteen National Wildlife Refuges (NWRs) in the northeastern United States examining smallmouth and largemouth bass for symptoms of endocrine disruption (Iwanowicz et al., 2013). The research was carried out in 2008-10. The study area included the Great Meadows National Wildlife Refuge in Concord, Wayland, and Sudbury, Mass., and the Assabet River National Wildlife Refuge in Stow and Maynard, Mass.

The National Wildlife Refuge study's researchers looked primarily for three signs of endocrine disruption in the bass: intersex testes or ovaries, measureable concentrations of an egg yolk protein called vitellogenin in the blood plasma of male fish (not usually found the male fish), and changes in the weight of testes or ovaries relative to the total body weight (the Gonadosomatic Index; GSI). Researchers also evaluated the concentration of hormones and hormone-like chemicals in rivers, ponds, and reservoirs within or near the refuges. The purpose of this "reconnaissance" study was to identify problems that deserve follow-up, rather than to produce definitive results.

The researchers captured largemouth bass and collected water samples from five sites within our watershed (Figure 1): the Assabet River adjacent to the Assabet River refuge (ASR1); the Sudbury River (GRM1), Concord River (GRM2), and Heard Pond (GRM3) within the Great Meadows refuge; and the Nichols Reservoir (ASR2) in Westborough, which is the headwaters of the Assabet River and the study control site. The fish were collected by electroshocking at all sampling locations.

Fish Findings
The study found intersex male largemouth bass in the Assabet, Sudbury and Concord Rivers at the refuge sites, but not in Heard Pond or the Nichols Reservoir. The percentage of intersex male largemouth bass captured at the river sites ranged from 9% to 75% and averaged 17% across the five sites (Table 1). Intersex was defined in this study as the presence of one or more immature eggs in the testes of the males, or the presence of immature sperm cells in the ovaries of the female fish. No intersex females were found in this study. This was not surprising, as the study scientists have yet to find intersex female smallmouth or largemouth bass in their research (Dr. Vicki Blazer, pers. comm., 10/28/14).

Seventy-five percent of the male largemouth bass from the Sudbury River site (GRM1) were found to be intersex. This was the second highest percentage of intersex largemouth bass among all 14 northeastern national wildlife refuges where largemouth bass were captured and analyzed. The sample size, however, was quite small. Additional research should be done to confirm this finding. The Assabet River site (ASR1) had the ninth highest percentage, 33%, intersex males in this group (Table 1).

Figure 1: Sudbury, Assabet, and Concord Watershed, Massachusetts US F&W Sampling Locations (approximate)

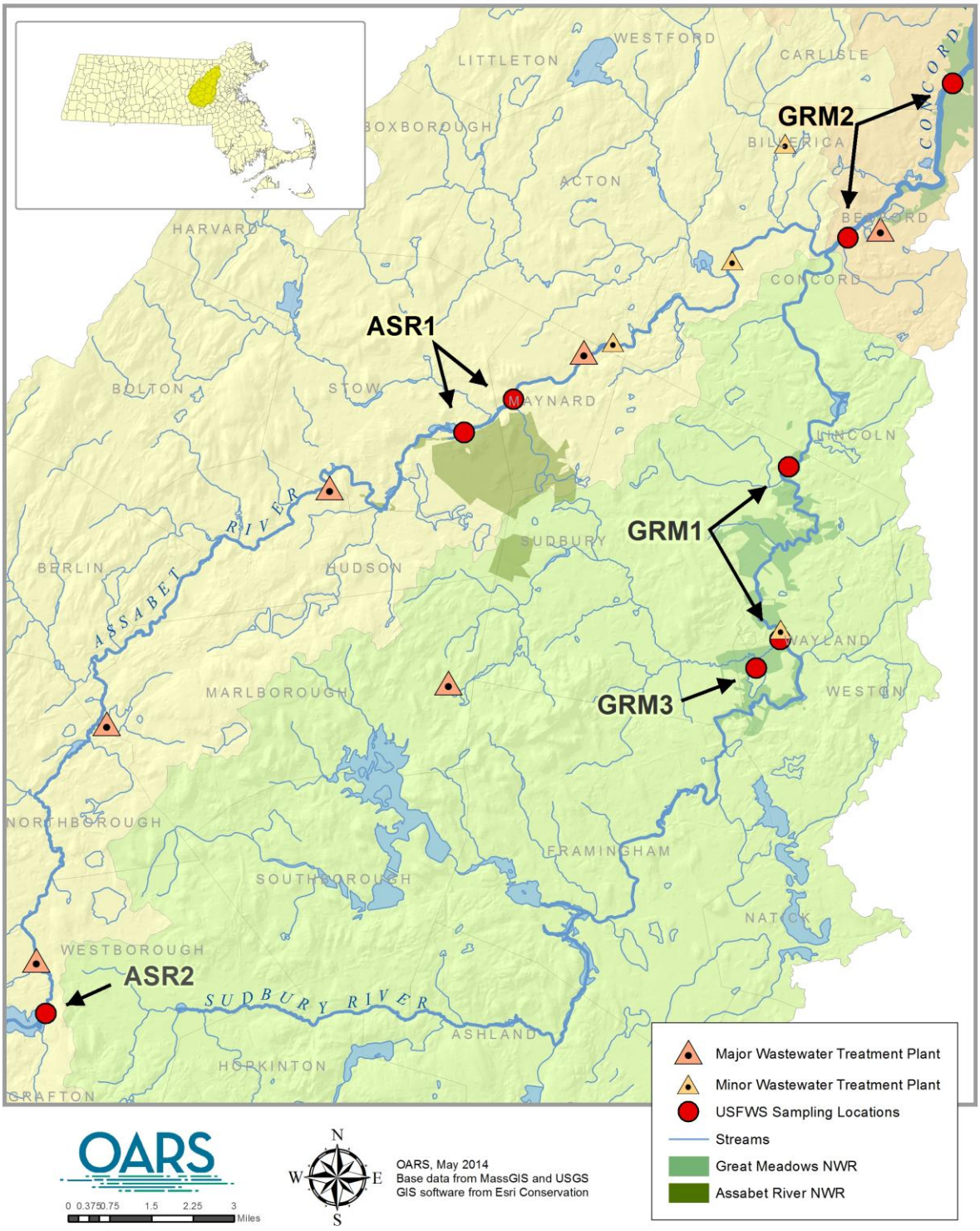

Table 1: Largemouth Bass (LMB) Data Summary for Assabet, Sudbury and Concord Watershed Sites

Sample Site	Date of Sampling	Waterbody	Natl. Wildlife Refuge	Town(s)	Total # male LMB caught	Total # female LMB caught	# intersex male LMB	Percent intersex males	# intersex female LMB	# male LMB with detectable Vitellogenin	Estrogenic Activity Detected	Glucocorticoid Rec-eptor Translocation	Androgen Receptor Translocation
ASR1	8/31/10	Assabet River	Assabet River	Maynard & Stow	9	11	3	**33%**	0	0	**YES ***	Not tested	Not tested
ASR2	9/1/10	A-1 Reservoir (control site)	(headwaters of the Assabet River)	Westborough	10	10	0	0%	0	0	NO	Not tested	Not tested
GRM1	10/2/08	Sudbury River	Great Meadows	Sudbury & Wayland	4	8	3	**75%**	0	0	NO	NO	**YES**
GRM2	10/3/08	Concord River	Great Meadows	Concord, Carlisle & Bedford	11	11	1	**9%**	0	0	NO	NO	**YES**
GRM3	8/30/10	Heard Pond (near Sudbury River)	Great Meadows	Wayland	7	13	0	0%	0	0	NO	Not tested	Not tested

**above the proposed predicted-no-effect concentration of 1 part per trillion EEQ (not a regulatory limit).*

Intersex severity

The study also ranked the severity of intersex features, using a "severity index" based on the number of immature eggs and their distribution (whether single or clustered together) in the testes of the male fish. (For images of testicular oocytes see: Hinck et al., 2009, p. 64, Figure 2).

Scientists made this determination by removing and preserving one of the fish's two testes, slicing it into 5 sections, and viewing the 5 sections under a microscope. Intersex severity was scored by ranking each section of each testis on a scale of 0 – 4, where "0" means no immature eggs present, "1" means one immature egg present, "2" means more than one immature egg is present but the eggs are not clustered, "3" means there is a cluster of immature eggs, and "4" means multiple clusters of more mature eggs or more than five eggs in a cluster. The intersex severity score for each fish is the average of its five section scores. The intersex scores for the individual largemouth bass captured in the Assabet, Sudbury and Concord Rivers are not presented in the report and are not yet available to the public; however, the study report does contain a bar graph in Figure 6 (p. 22) that plots intersex severity scores for each site. The site scores are an average of all the intersex severity section scores of *all* the male largemouth bass captured at that site, including the non-intersex males (who have scores of "0"). For a detailed description of the method see (Blazer et al., 2007). Also, see Bahamonde et al., 2013, for an overview of intersex severity assessment methods.

Based on these scores, the study report found generally low intersex severity among the intersex males caught in the Assabet, Sudbury and Concord Rivers. Of these three river sites, the male largemouth bass at the Assabet River site adjacent to the Assabet River refuge (ASR1) had the highest intersex severity score of the three sites, followed by the Sudbury River site (GRM1) in Great Meadows refuge. In physical terms, these scores mean that scientists found at least one immature egg in the sectioned testis of each intersex male fish collected from the Assabet, Sudbury, and Concord River sites. The largemouth bass at the Assabet River site (ASR1) had the highest intersex severity score of the three river sites, followed by the Sudbury River site (GRM1). The intersex severity of the male largemouth bass captured at the other 12 refuges (where largemouth were sampled) was also ranked "low," with the exception of a lake in Virginia.

The study also found a statistically significant difference in the intersex severity observed at the upstream Sudbury River site (GRM1) and the downstream Concord River site (GRM2). Differences between the Assabet River site adjacent to the Assabet River Wildlife Refuge (ASR1) and the A-1 Impoundment control site (ASR2), where no intersex fish were found, were almost statistically significant.

Vitellogenin (Egg Yolk Proteins)

In addition to measuring the incidence and severity of intersex among the male largemouth bass, scientists tested for but did not detect vitellogenin in the blood plasma of the 41 male largemouth bass captured at the 5 sites. The report notes that "[r]elationships between vitellogenin and the incidence of intersex are not necessarily expected. Intersex is presumed to be induced during early developmental stages while the induction of vitellogenin in male fishes can be the result of transient or prolonged exposure to an estrogen, and is less dependent on life stage." (Iwanowicz et al., 2013, p. 9).

Gonadosomatic Index (GSI)

Scientists use the GSI as an indicator of the reproductive health of fish. It is the proportion of a fish's body weight comprised by the testes or ovaries. According to one of the study authors, while GSI is not the most sensitive endpoint, when fish are collected at the same time at upstream and downstream sites it can be used, along with other endpoints such as vitellogenin and gonad histology, to determine if there are delays in sexual maturation, reduced fertility and general health (Dr. Vicki Blazer, pers. comm.,10/3/14). No GSI findings for the five Assabet, Sudbury and Concord watershed sites were presented in the study report.

Water Quality Findings
Water samples were also collected at the study sites to test for estrogens and for the presence (although not quantity) of androgens, such as testosterone, and glucocorticoids.

Estrogens
Estrogens are the primary female sex hormones synthesized by all vertebrates and include estradiol, estriol and estrone. They are both naturally produced and are manufactured for use in oral contraceptives and hormone replacement therapy. They are excreted from the human body and are present in wastewater, which is discharged to our rivers. Previous studies have shown that estrogens can induce intersex in some fish species and harm the ability of fish and other animals to reproduce (Jobling et al., 2002; Kavanagh et al., 2004; Kidd et al., 2007; Krisfalusi et al., 2000; Nash et al., 2004).

Estrogens readily diffuse across the cell membrane. Once inside the cell, they bind to and activate the expression of many genes. In humans, estrogen hormones play an essential role in the growth and development of female secondary sexual characteristics, regulation of the menstrual cycle and the reproductive system. Exposure to estrogen (specifically, 17 beta-estradiol) concentrations that exceed 1-10 parts-per-trillion (1-10 ng/L) can cause intersex in some species of male fish (Caldwell et al., 2012; Young et al., 2004). Although no government agency has yet established an estrogen concentration that is safe for fish, known as a "no effects concentration," many studies have set a level of around 1 part-per-trillion (ppt) as the highest estrogen concentration that will not adversely affect fish (Dr. Vicki Blazer, pers. comm.,10/3/13) That said, fish species vary greatly in their sensitivity to estrogens, so further research is needed.

For this study, scientists measured the total estrogenicity, also known as the Exhibited Estrogen Equivalent (EEQ), using a bioluminescent assay (BLYES). The water sample is incubated with yeast cells that produce bioluminescence when exposed to estrogens. The luminescence is measured and compared to a standard curve of estradiol concentrations to quantify the total estrogenicity of the water sample, i.e., the total of estradiol, ethinylestradiol, estrone, and any synthetic estrogen-mimickers such as bisphenol A (BPA). Each of the five sites in the Assabet, Sudbury and Concord watersheds were tested.

An EEQ of 2.2 ppt was measured in the water sample taken from the Assabet River sites along the Assabet River refuge. This was the highest concentration of estrogens (EEQ) measured in the 19 northeastern refuges, with the exception of water samples collected in the discharge zone of a wastewater treatment plant. Moreover, 2.2 ppt is twice the estimated "no effects" EEQ concentration of 1 ppt. By contrast, no estrogens or "estrogenic activity" were detected at sites ASR2, GRM1, GRM2 and GRM3.

Androgens (such as Testosterone)
Androgen is the broad term for any compound, usually a steroid hormone that stimulates or controls the development and maintenance of male characteristics in vertebrates. The primary and most well-known androgen is testosterone.

Collaborators at the National Cancer Institute at the National Institutes of Health in Maryland tested water samples from the Sudbury River (GRM1) and Concord River Refuge sites (GRM2) for androgenicity using a line of genetically-engineered breast cancer cells from mice. Specifically, the protein molecules on the surface of these cells where androgens bind to the cell – called androgen receptors – have a green fluorescent protein molecule bound to them. The fluorescence can be observed under a microscope when illuminated with blue light. When cells are exposed to the water samples containing androgens, these androgens bind to the androgen receptor molecules, which then move from the cell surface into the body of the cell – a process called translocation – taking the fluorescent green proteins with them. The presence of fluorescence inside the cell walls then indicates that the cell has been exposed to androgens. The test, however, does not quantify the concentration of androgens. Only water samples from the Sudbury River

(GRM1) and Concord River (GRM2) sites were tested for the presence of androgens. Both sites tested positive for the presence of androgens.

Glucocorticoids

Glucocorticoids are steroids that reduce inflammation throughout the body. The anti-inflammatory properties of glucocorticoids make them highly prescribed pharmaceuticals that can enter local waterways in treated sewage. Recent studies suggest that certain concentrations of synthetic glucocorticoids may harm fish (Kugathas et al., 2011; Stavreva et al., 2012). The same protein translocation assay used to detect the presence of androgens was also used to detect glucocorticoids in the water samples taken from the Sudbury River (GRM1) and Concord River (GRM2) sites. Both sites tested negative for the presence of glucocorticoids.

See Table 1 for results of estrogenic assays and translocation assays for androgen receptor (AR) and glucocorticoid receptor (GR).

Discussion

The study made two significant findings about the Assabet, Sudbury and Concord Rivers. First, there *are* intersex male largemouth bass in all three rivers. Among the three rivers, the Sudbury River site in Sudbury and Wayland within the Great Meadows National Wildlife Refuge had the highest percentage of intersex males (75%) but with a very small sample size (four fish). A sample size of approximately 12 males would be needed to make statistically significance comparisons of intersex prevalence among sites (Dr. Vicki Blazer, pers. comm., 10/3/14).

Second, the study found that the Assabet River adjacent to the Assabet River National Wildlife Refuge had the highest concentration of estrogens (EEQ) measured in the 19 refuges, with the exception of water samples collected in the discharge zone of a wastewater treatment plant. The concentration of 2.2 ppt EEQ *exceeded* the estimated "predicted no effects concentration." Concentrations above 1 ppt can cause intersex in males, and other forms of endocrine disruption.

This study raises the possibility that wastewater is inducing the intersex condition in largemouth bass in the Assabet, Sudbury and Concord Rivers, but there may be other causes and contributing factors. More research is needed to determine causality.

Next Steps

The US Fish & Wildlife study was a reconnaissance level survey of fish and water quality in 19 National Wildlife Refuges in the northeastern United States intended to prioritize refuges for comprehensive follow-up studies. Both the Great Meadows and Assabet River refuges made the list of the seven priority refuges needing comprehensive follow-up. Among these seven refuges, the study ranked the Assabet River refuge as a *moderate* priority and the Great Meadows refuge as a *low* priority for follow-up. At this time no additional fieldwork or analysis is planned by the U.S. Fish & Wildlife Service. We await the completion of the EPA study that focused on water quality and endocrine disruption in fish in the Assabet River.

This kind of research is relatively new and assisted by improved though often expensive techniques that permit scientists to detect and measure extremely low concentrations of pollutants. It is important that the public and private sectors continue this research so that the causes and impacts of endocrine disruption can be better understood and any necessary actions taken.

* * *

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*A note to readers. The majority of these publications must be purchased online or obtained through university libraries. This situation makes it expensive for anyone who wishes to research this topic but who lacks library privileges at a university. Not surprisingly, the most recent articles are the most difficult to obtain without paying a significant fee.

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