EFFECT DIFFERENCES OF ESTROGENIC EXPOSURE BETWEEN AN ENDANGERED SPECIES AND TWO MODEL SPECIES AND ACROSS LIFE STAGES

by

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Zachary G. Jorgenson

Water quality regulations that are currently in place are often based on recommendations from the results of toxicological exposure experiments. These exposures experiments are carried out on a limited number of species, commonly termed model species, at one life stage of the species. These model species act as surrogates for all species in a given environment even though it is often unknown how well the model species exposure effects relate to the species in which they represent. Also, differences in life stage characteristics could impact exposure effects that are observed.

Three experiments were carried out to address these issues observed in common toxicological studies. In the first experiment, an endangered species, the Rio Grande silvery minnow, was exposed to a potent estrogenic compound (17β-ethinylestradiol) at three concentrations (1 ng/L, 5 ng/L and 25 ng/L) at the juvenile stage of development to determine the effects that an estrogenic compound may have on the Rio Grande silvery minnow. Preliminary data suggests that 17β-ethinylestradiol does not caused an effect in the Rio Grande silvery minnow. However, more sensitive biomarkers have yet to be fully analyzed; therefore conclusions as to the effect of estrogenic compounds on the Rio Grande silvery minnow cannot be determined at this time from this experiment. In the second experiment juvenile Rio Grande silvery minnow along with two model species, fathead minnow and bluegill sunfish (also at the juvenile stage of development), were exposed to a natural estrogenic compound (17α-estradiol) at two environmentally relevant concentrations (10 ng/L and 30 ng/L) to determine exposure effect differences between species. Differences

were observed between species using different biomarkers, including survival and whole-body vitellogenin concentrations. Further differences were observed between families Cyprinidae and Centrarchidae. In the third experiment adult and juvenile fathead minnow were exposed to 17α-estradiol at concentrations of 10 ng/L and 30 ng/L over a period of six weeks. A comparison of exposure effects, including results from the second experiment, on different life stages of the fathead minnow demonstrated similar exposure effects between the life stages. Effects were most noticeable at the 10 ng/L treatments for most biomarkers, while a limited set of biomarkers showed a dose-dependent decrease in values with an increase in 17α-estradiol concentrations.

The studies in this thesis demonstrate the limitations that are observed in the current practices for the development of water quality regulations. Current toxicological practices are beneficial for emerging contaminants because of the large database of known exposure effects that different chemicals have on model species that these emerging contaminant effects can be compared against. However, after initial regulations are implemented for the protection of aquatic life to these emerging contaminants, further studies are needed to determine if the regulations in place actually protect all of the species that they are intended to protect. If exposure effects to different chemicals are never determined for some species, we can never truly know if the regulations in place are actually protecting these species, which can be extremely important when dealing with species that are endangered or threatened.

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A HISTORY OF TESTING MODEL SPECIES AND ENDANGERED SPECIES USING MULTI-GENERATION OR PARTIAL GENERATION STUDIES

WATER POLLUTION

Pollution has been an issue for almost as long as the world has been industrialized. The areas most affected are frequently waterways (rivers, lakes, estuaries) and coastal areas (Cundy et al. 2001, Gregory et al. 2001, Jickells et al. 2001). One reason for the prevalence of pollution in these areas is the ability of water to carry toxins away from the point source to other areas over long distances. The most common point sources along waterways include effluents from industrial operations and wastewater treatment plants. The most common non-point source is agricultural runoff. To combat the amount of toxins being discharged into waterways and along the coast in the United States, the federal government introduced the Clean Water Act in 1972 (Federal Water Pollution Control Act 1972). Since 1972, the Clean Water Act has undergone many revisions to include more protection from diverse sources of pollution, such as urban runoff. The act is used to employ "a variety of regulatory and nonregulatory tools to sharply reduce

direct pollutant discharges into waterways, finance municipal wastewater treatment facilities, and manage polluted runoff." (Federal Water Pollution Control Act 1972) It helps to protect not only humans, from contamination in our drinking water, but also the plants and animals that inhabit the water. The act helps protect populations of plants and animals from adverse effects after prolonged or instantaneous exposure to a toxin.

In the past decade, estrogen pollution from the release of natural and synthetic estrogen-like compounds, has become an important area of research in aquatic toxicology (Ankley and Johnson?2004). Natural estrogens that are introduced into the water can come from plants, called phytoestrogens, or animals. Some plants that are common in the human diet that contain high amounts of phytoestrogens are soy (Fukutake et al. 1996) and hops, which is used in making beer (Milligan et al. 1999, Milligan et al. 2002). These phytoestrogens find their way into the aquatic environment primarily through industrial runoff (Lundgren and Novak 2009), such as from pulp mills (Erickson 2001, Kiparissis et al. 2001). Natural estrogens produced by animals, such as estrogen, find their way into the aquatic environment primarily through wastewater treatment plants or agricultural runoff. Synthetic estrogens are mostly introduced through sewage treatment plants, but can also come from agricultural runoff. The input of synthetic estrogens into aquatic ecosystems has dramatically increased over the last few decades, mostly from the increased use of birth control pills/medications by women (Mosher et al. 2004). These synthetic hormones used in birth control pills, predominantly 17α-ethinylestradiol (EE2), are often found at wastewater treatment plant outfalls (Braga et al. 2005, Pauwels et al. 2008). Many studies have begun to investigate how an increase in estrogen pollution can affect fish that live at or near these outfalls (Bjorkblom et al. 2009, Rempel et al. 2006,

Rempel-Hester et al. 2009, Sowers et al. 2009, Woodling et al. 2006). The most widely used biomarker for determining exposures to estrogens by fish is to measure the levels of a specific yolk protein, vitellogenin (vtg), in fish plasma. The vtg concentrations are mostly measured in male fish because males should not be producing large quantities of vtg naturally (Jensen et al. 2001, Watanabe et al. 2007). Even though males do not produce vtg naturally, or at least only in minute amounts, they do retain the ability to produce vtg in their liver. However, because they do not normally produce vtg, males have a hard time removing it from their livers (Schmid et al. 2002). This causes it to accumulate for a long period of time, even after only a short exposure, which can lead to renal failure (Folmar et al. 2001). An increase in vtg concentrations, from EE2 exposure, has been shown to co-occur with population-level effects and individual effects. One such common individual effect is on gonadal morphology (Bjorkblom et al. 2009, Kidd et al 2007, Salierno and Kane 2009), with males often showing female characteristics, such as the production of eggs in the testes. This could have a dramatic effect on the reproductive ability of males in a population. Another way EE2 and other estrogenic compounds could affect fish is on their reproductive behaviors (Bayley et al. 1999, Bjerselius et al. 2001, Schoenfuss et al. 2008). Estrogenic compounds have been shown to have a negative effect on the reproductive behavior of male fathead minnows (*Pimephales promelas*); (Salierno and Kane 2009). These individual effects could lead to a population's inability to sustain itself. And if this species is not represented anywhere else in the world, it would lead to the extinction of an entire species.

Another act that helps in the protection of plants and animals is the Endangered Species Act. The purpose of the Endangered Species Act is to protect "threatened and

endangered plants and animals and the habitats in which they are found" (Endangered Species Act of 1973). This includes any action that may directly impact a species, such as hunting of that species, or any modification to a habitat that will negatively affect a species, such as construction within a critical habitat. The Endangered Species Act also prohibits "taking" of listed species, or any sort of commerce of the species. Certain permits can be obtained to allow an individual or group to take a small amount of individuals from a habitat, such as for research experiments, or to allow a certain amount of commerce of a species. While the Endangered Species Act protects species that are at the most risk of becoming extinct from issues such as poaching and urban development, the regulations (Endangered Species Act of 1973) imposed by the act create difficulties to conduct laboratory studies with these species. These difficulties include the ability of a laboratory to obtain individuals of an endangered species. If they do acquire individuals it usually comes at a high cost, both in time and money. These difficulties have led to a limited number of studies being conducted on endangered species. Consequently, toxicologists have adopted more commonly used species as model species to serve as surrogates for endangered species in toxicity studies.

MODEL SPECIES

The most common model species used are: fathead minnows (Ankley and Johnson 2004, Ankley and Villeneuve 2006, Miles-Richardson et al. 1999, Scholz and Mayer 2008), zebrafish (*Danio rerio*) (Ankley and Johnson 2004, Deng et al. 2010, Scholz and Mayer 2008, Segner 2009, van der Ven 2007), Japanese medaka (*Oryzias*

latipes) (Ankley and Johnson 2004, Broussard and Ennis 2007, Orn et al. 2006, Scholz and Mayer 2008), bluegill sunfish (*Lepomis macrochirus*) (Dutta and Arends 2003, Dutta and Meijer 2003, Maxwell and Dutta 2005, Wang et al. 2008) and rainbow trout (*Oncorhynchus mykiss*) (Fournier et al. 2003, Hollis et al. 2000, Vetillard et al. 2003, Wilson et al 1998). These species of fish are often referred to as model species as they are commonly used in experiments whose results guide laws for protecting all fish species, including endangered species. In contrast, species on the United States Environmental Protection Agencies' (USEPA) listing for endangered species are often delicate and vulnerable, and because of this sensitivity they can be difficult to maintain in a laboratory setting. The model species usually have a wide range of distribution, showing that they can live in a variety of different environmental conditions. However, this should also lead investigators to question how well these model species represent endangered species. If a model species is exposed to a low level of a toxin and it does not show a reaction to the toxin, is that because the toxin is not very toxic to all fish, or because of the model species' tolerance to that toxin? In the case of the former, what would happen to an endangered species if it was exposed to the same low level(s) of that toxin?

In a study performed by Dwyer et al. (2005), seven threatened and endangered species of fish were tested together with fathead minnows and *Ceriodaphnia dubia*. Multiple tests were performed using multiple mixtures of ten different mixtures and effluent samples. The authors found that if toxicity results from both fathead minnows and *C. dubia* were used for regulatory purposes, the listed species would be protected 96% of the time. However, using the IC_{25} (inhibition concentration to 25%), they found that in 21% of the tests the listed species was more sensitive than the fathead minnow. It

was also found that four species were consistently more sensitive than the fathead minnow. These species were the bonytail chub (*Gila elegans*), Cape Fear shiner (*Notropis mekistocholas*), spotfin chub (*Cyprinella monacha*) and shortnose sturgeon (*Acipenser brevirostrum*). The bonytail chub is one of the rarest fish species in the Colorado River, while the cape fear shiner is classified as critically endangered. Results such as these show that if tests are performed on only one species of fish for a toxic chemical to create a management plan the most vulnerable species will not be protected 21% of the time. Also, as shown by the four species that were always more sensitive than the fathead minnow, there are species of fish that may never be protected from certain toxic chemicals if only model species are used for management purposes. Moreover, these species can often be the most critically endangered.

Endangered species can be problematic to obtain for experiments, making it difficult to obtain experimental results to show how differently a listed species' response may be from a model species' response. There have been, however, numerous experiments using the same toxic chemicals but exposed to other, more common species. In one experiment (Orn et al. 2006), zebrafish and Japanese medaka were both exposed to different concentrations of EE2 (Table 1) and 17β-Trenbolone (Tb, Table 2), a known androgen. The results demonstrated that, even though both species are commonly used as model species for aquatic toxicology tests, the two species have very different responses to the same two common pollutants. Zebrafish showed a higher sensitivity to both chemicals than Japanese medaka. At a high concentration of EE2 (100 ng/L), the zebrafish had 100% mortality after 14 days while the Japanese medaka had no mortalities at that time point (Table 1). After 60 days post-hatch exposure to 10 ng/L EE2, the

zebrafish population consisted of 100% females, while Japanese medaka population approximated a 1:1 ratio of males to females (Table 1). Also, at 10 ng/L EE2, zebrafish had a higher concentration (around 8,500 times) of plasma vtg than Japanese medaka (Table 1), while the opposite was observed in the control fish, with the concentration of plasma vtg being higher in Japanese medaka than zebrafish. Exposure to Tb at 50 ng/L resulted in a population of 100% males in zebrafish, while Japanese medaka maintained a 1:1 ratio of males to females (Table 2). In the control for Tb, zebrafish population had a 1:2 ratio of males to females, and Japanese medaka a 1:1 ratio (Table 2).

	100ng/L at 14 Days Post-Hatch	10ng/L at 38 Days Post-Hatch	10ng/L at 60 Days Post-Hatch
Zebrafish	100% Mortality	Vitellogenin levels ≈ 8,500,000 ng vtg/g fish	100% Females
Japanese Medaka	No Mortalities	Vitellogenin levels ≈ 1,000 ng vtg/g fish	51% Males 47% Females 2% Intersex

Table 1. Comparison of exposure effects of EE2 in two commonly used model species. Data summarized from Orn et al. 2006.

	Control at 60 Days Post-Hatch	50ng/L at 60 Days Post-Hatch
Zebrafish	33% Males 67% Females	100% Males
Japanese Medaka	52% Males 48% Females	46% Males 56% Females*

Table 2. Comparison of exposure effects of Tb in two commonly used model species. Data summarized from Orn et al. 2006. "*" represents discrepancy in article.

Other studies have also compared the effects of EE2 on different species of fish. In a study by Ma et al. (2007), the Chinese rare minnow (*Gobiocypris rarus*) and the Japanese medaka were exposed to the same concentrations of EE2. It was found that the Chinese rare minnow showed a ten-fold increase in vtg induction compared to that observed in Japanese medaka. In another study, EE2 was added to a lake in Canadian Experimental Lakes Area over a 3 year period. Population level effects and individual level effects were monitored for 2 years before the exposure, during the 3 years of exposure and for 2 years after exposure. During exposure, EE2 was added three times weekly starting in May and continued for around 20-21 weeks. EE2 could only be added during this time range because of the lake freezing-over during the colder months. A report by Palace et al. (2009) discussed the effects of the exposure on 4 different species of fish in the exposure lake; the fathead minnow, the pearl dace (*Margariscus margarita*), the lake trout (*Salvelinus namaycush*), and the white sucker (*Catostomus commersonii*). The authors found that after 2 years there was a decline in the fathead minnow population, and after the third year there was also a decline in the pearl dace and lake trout populations. The white sucker did not show a population decline. This study demonstrated two points; first that EE2 could possibly affect fish populations, and second that there is a significant difference in the degree that EE2 affects the different species. A report by Kidd et al. (2007) discussed the effects of the exposure on the fathead minnow population and the individual effects to the fathead minnows. The authors found that at the end of the 3 year exposure, the fathead minnow population was almost extinct in the lake, there was intersex in the males, increased concentrations of vtg, and altered oogenesis in females. Some other fish exposed to EE2 in studies include: the blenny,

Lipophrys pholis (Ferreira et al. 2009); the brown trout, *Salmo truttaI* (Korner et al. 2005); the guppy *Poecilia reticulate* (Nielsen and Baatrup 2006); and the three-spined stickleback *Gasterosteus aculeatus* (Bjorkblom et al. 2009).While the blenny and three-spined stickleback showed an increase in vtg concentrations, and the brown trout and three-spined stickleback showed effects in testes structure (including intersex in the brown trout), the guppy showed no effects related to the EE2 exposure.

Estrogen is not the only pollutant that affects different species of fish differently. In an article by Teather et al. (2006), multiple studies were reviewed to compare different freshwater fishes' responses to a variety of toxic chemicals. The article compared 12 different species of fish (fathead minnows, rainbow trout, bluegill sunfish, guppies (*Poen'lio reticuloto*), common carp (*Cyprinus carpio*), goldfish (*Orrauius auraw*), coho salmon (*Oncorhynchus kisutcb*), largemouth bass (*Microptm salmoidecr*), mosquitofish (*Gambusia ofinis*), mummichogs (*Fundulus heteroclitus*), Japanese medaka and three-spine sticklebacks (*Gasterostrew aculeatus*)) in exposures to 190 different chemicals. The authors found a three-fold difference in sensitivity between the most sensitive fish and the least sensitive fish. The authors also found that the coho salmon and rainbow trout, both of the Salmonidae family, were the most sensitive fish. The carp, goldfish and fathead minnow, all of the Cyprinidae family, were the least sensitive. These groupings by families lead to the possibility that sensitivity to toxic chemicals might be related across the family level. But even if toxic sensitivity can be related across the family level, the difference between families can be a three-fold difference. This difference can be significant in most aquatic environments where there are often multiple families of fish represented.

These differences in sensitivities could be attributed to the fact that the common term of “fish” is a very broad classification. It classifies only to the point of the subphylum Vertebrata. Vertebrata is then branched off into two superclasses: Agnatha and Gnathostomata. Agnatha are jawless vertebrates and Gnathostomata are jawed vertebrates. Fish are represented in both of these superclasses. Having a classification only to the point of subphylum leaves a lot of room for variation between the large numbers of species that will be represented under it. There are approximately 28,000 living species represented in the subphylum Vertebrata. These species are categorized into 515 families in 62 orders (Nelson 2006). The exact definition of a “species” is still a debatable topic. It is almost consistently agreed upon that a species is the basic unit of classification for most schemes. There are two definitions that are most commonly applied to describe species. One is called the evolutionary species concept, which states that a species “is a single lineage of ancestor-descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate.” (Wiley 1981). The other definition is the biological species concept. It states that “a species is a group of interbreeding natural populations that is reproductively (genetically) isolated from other such groups because of the physiological or behavioral barriers.” (Mayr 1997). Each of these concepts has their merits and application, but each also has downfalls. However, between these two definitions a basic understanding of how to perceive species can be attained. A very simplified definition could be each species differs from another species in at least one way. These differences can include, but is not limited to, morphology, physiology or their behavior. With all of the possible differences between species, this allows for a large variation in sensitivity to chemicals.

Some examples of physiological differences that might affect chemical sensitivity are: the presence of glycopeptides that acts as an antifreeze compound to allow fish to live in extremely cold waters (Helfman et al. 1997, p.308); the ability of the bull shark, *Carcharhinus leucas*, to concentrate urea allowing it to inhabit both salt water environments and fresh water environments (Pillans and Franklin 2004); or the lower "rates of enzymatic and metabolic activity" of some deep sea fish compared to their relatives that inhabit the shallow waters (Helfman et al. 1997, p.301). A chemical released into the environment could interact with each of these three species differently. The chemical could have a binding affinity for the glycopeptides found in the cold water fish. Because of the presence of this glycopeptides, this chemical may accumulate to higher concentrations in the fish than if this cold water fish did not have these glycopeptides. The chemical could also have a binding affinity for urea, and with the increase in urea in the bull shark, the concentration of the chemical in the bull shark would also increase. Also, due to a decrease in metabolic activity the deep sea fish may not be able to void the chemical from its system as efficiently as shallow water species.

While physiological factors can affect how a species will react to a chemical, their behavior can dramatically influence how often and to what degree the species is exposed to chemicals. If a species spends most of its life at the bottom of the ocean it will be more susceptible to chemicals that sink to the bottom and accumulate in the sediment. Areas around sewage treatment plants are especially susceptible because of the increase in sediments (Forstner and Wittmann 1981, p. 1-2; Villaescusa-Celaya et al. 2000). This susceptibility can be the consequence of the species either living directly in the sediment, such as most flatfish and invertebrates, or from eating those organisms that are living in

the sediment and bioaccumulating those chemicals (Piraino and Taylor 2009, Wiener et al. 2006). Conversely, species that life closer to the surface will be more susceptible to chemicals that are lighter than water. Oil spills are one of the biggest concerns for these types of species. Species that live in the middle water column would likely be exposed to chemicals as they sink to the bottom, or from chemicals being re-mixed from the sediment back into the water column during upwelling or agitation of the sediment (Lehmann and Myrberg 2008; Open University Course Team 2001).

Depending on the specific environment, there can be numerous species represented across multiple orders (such as in tropical waters in the Indian and Pacific Oceans) or very few species in just one order (such as in Antarctic waters). These differences in environments might be one avenue for scientists to decide if a model species could be used for toxicological testing to protect that area. Because of the basic premise behind classification, the differences between species within a family will be less than species across different families. The same can be applied to species within orders compared to across orders. If there is an environment with low species diversity, and all the species are in the same family, then a model species might be sufficient. But if the environment contains multiple families and orders, then the use of a model species might not be an acceptable approach. Caution still has to be taken when assessing an environment with few species in the same family. Being classified in the same family does not guarantee that the species will respond similarly to different toxins. The classification only implies that there are fewer differences between the species, but it does not specifically specify what those differences are or how much of an impact those differences have on the species.

IMPORTANCE OF EACH SPECIES IN AN ENVIRONMENT- THE SILVERY MINNOW AS A CASE STUDY

All environments, not just aquatic environments, contain not only many different families of organisms, but also different phyla, even kingdoms. Everything exists in relationship with each other, with the loss or decline of one organism having the ability to affect, whether direct or indirect, the health and survival of many other organisms. Most of these relationships are based on the food chain, but they can also be based on other aspects such as symbiosis. These relationships are one of the main reasons why the Endangered Species Act was created, because it was understood that if a species was lost, it could cause a chain reaction detrimental to other species living in the same environment. One popular model that demonstrates the relationships that species share with each other is the relationships between sea otters, sea urchins and kelp forests. It has been shown through multiple studies how a decrease in sea otter populations will lead to a decrease in kelp forest habitats (Estes et al. 2004). Kelp is a common food item for sea urchins, while sea urchins are common prey for sea otters. When the sea otter population declines, it allows a population growth for sea urchins. With the increase of the sea urchin population, they will in turn feed on more kelp, destroying kelp forest habitats. These kelp forests are beneficial not only for animals that feed on them, but also as a habitat for smaller fish to avoid predators and for some invertebrates to find refuge. Through all of these relationships, it is clearly evident how the loss of just one species can impact many other species. The loss of the sea otter in an area could eventually cause the loss of many other species in that environment. This is why each species that has

experienced a significant decline in population size (as compared to its natural population size) or is in danger of becoming extinct requires protection, because the loss of this one species might cause a chain reaction of loss to other species that might not be apparent until it is too late.

One species of fish that was placed under protection by the Endangered Species Act is the Rio Grande Silvery Minnow (RGSM) (*Hybognathus amarus*). The RGSM was once the most populous fish in the Rio Grande basin and had a home range from New Mexico to the Gulf of Mexico through Texas. However, today this species can only be found in around 7% of that range (US Fish & Wildlife Service 2007, Figure 1). It is believed that this decline in population can be attributed to numerous changes that the Rio Grande has experienced in the past century. These include the building of dams and canals (hindering migration movements, Figure 2), the addition of diversions from the river (decreasing water levels and contributing to increased sediment pollution), and a decrease in spring runoff (which helps cue spawning). Being a small fish (maximum length about 89 mm), the RGSM is a food source for larger fish in the river (US Fish & Wildlife Service 2007). With a decrease in the prey, larger fish have had to change their feeding habits and have possibly seen a decrease in their population because of a lack of a sustainable food source if other prey species are not found in large enough numbers. If larger fish were able to find another sustainable food source, it would be safe to assume that the increase in predation on the prey species would cause a decline in that prey species' population. This could result in multiple problems, including being detrimental to a possible second predator species that only feeds on this second prey species, which is now harder to find for this second predator species to feed. Another issue within the

Middle Rio Grande involving the RGSM is the fact that they are an herbivores species that tend to feed on the bottom of the river, predominantly on algae (US Fish & Wildlife Service 2007). Unless another species in the river starts to eat algae, or increases its algae consumption, the amount of algae in the river could begin to increase. This increased growth could result in eutrophication, which would reduce the dissolved oxygen levels within the river.

The RGSM is another good example of how one species, even one as small as the RGSM, can have an effect on many different organisms in its habitat. The case of the RGSM is more severe than that of many other animals. It went from being the most abundant fish with a home range of over 3,000 miles, to being endangered and having a range of less than 150 miles. All of this decrease could be seen within the past 50 years (US Fish and Wildlife Service 2007). That dramatic decline in a primary organism in a habitat in such a short amount of time is a substantial shock to the whole habitat. If the RGSM population does not increase soon, then it may not be long until there are more dramatic alterations to the structure and function of Middle Rio Grande ecosystem. Even after being classified as an endangered species and being the recipient of multiple restoration efforts, the RGSM is still seeing a decline in its population. This may be due to the fact that the RGSM is a pelagic spawner. During breeding season in early spring, RGSM will release their eggs to be fertilized and carried downstream. The concurrence of an increase in water flow from snow melt contributes to the transfer of fertilized eggs downstream. Traditionally, without the presence of dams, as the larvae would mature they would move upstream to reside as adults. With the building of dams and diversions,

this migration is hindered, possibly contributing to the inability of RGSM to repopulate upstream locations.

Although the changes in the geography of the river are detrimental to the RGSM, they may not be the only aspect hurting their population. The increase in chemicals, such as EE2, may be adding to the stress on the population; or the chemicals may possibly be more of a stressor on the population than the geographic changes. If chemicals are a main cause to the decrease in the RGSM population, then efforts such as restocking are insufficient to help the population recover. Regulations may have to be implemented to control the amounts of chemicals of concern that are being added to the river. To determine which chemicals might be detrimental to the RGSM population, chemical analyses need to be performed on the water and sediment in the Middle Rio Grande and exposure experiments need to be performed on the RGSM based on chemicals found frequently or at high concentrations in the river. As shown by previous experiments with EE2 (Bjorkblom et al. 2009, Ferreira et al. 2009, Orn et al. 2006), it does not always require a large amount of a chemical to show a dramatic effect on a species.

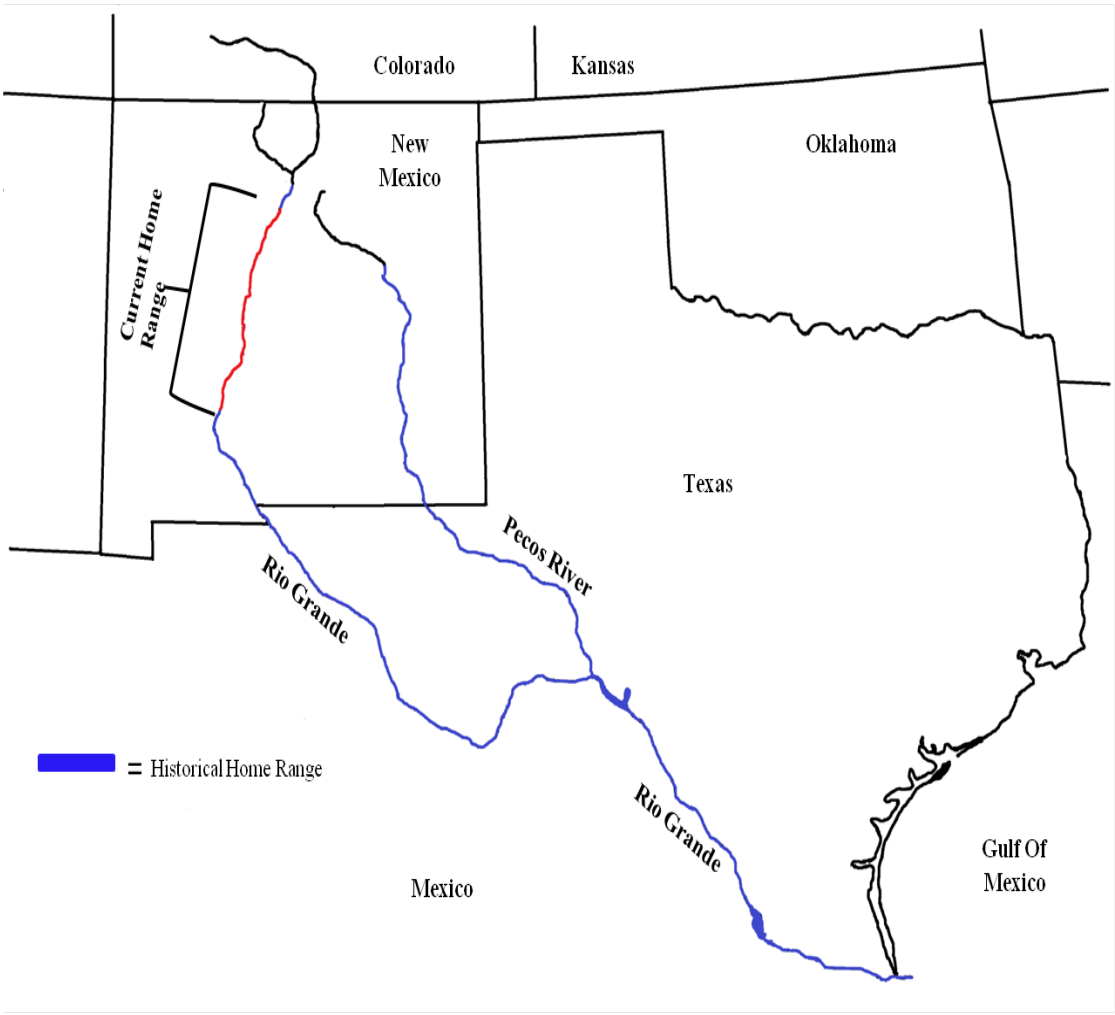

Figure 1. Historical and current home range of the Rio Grande silvery minnow.

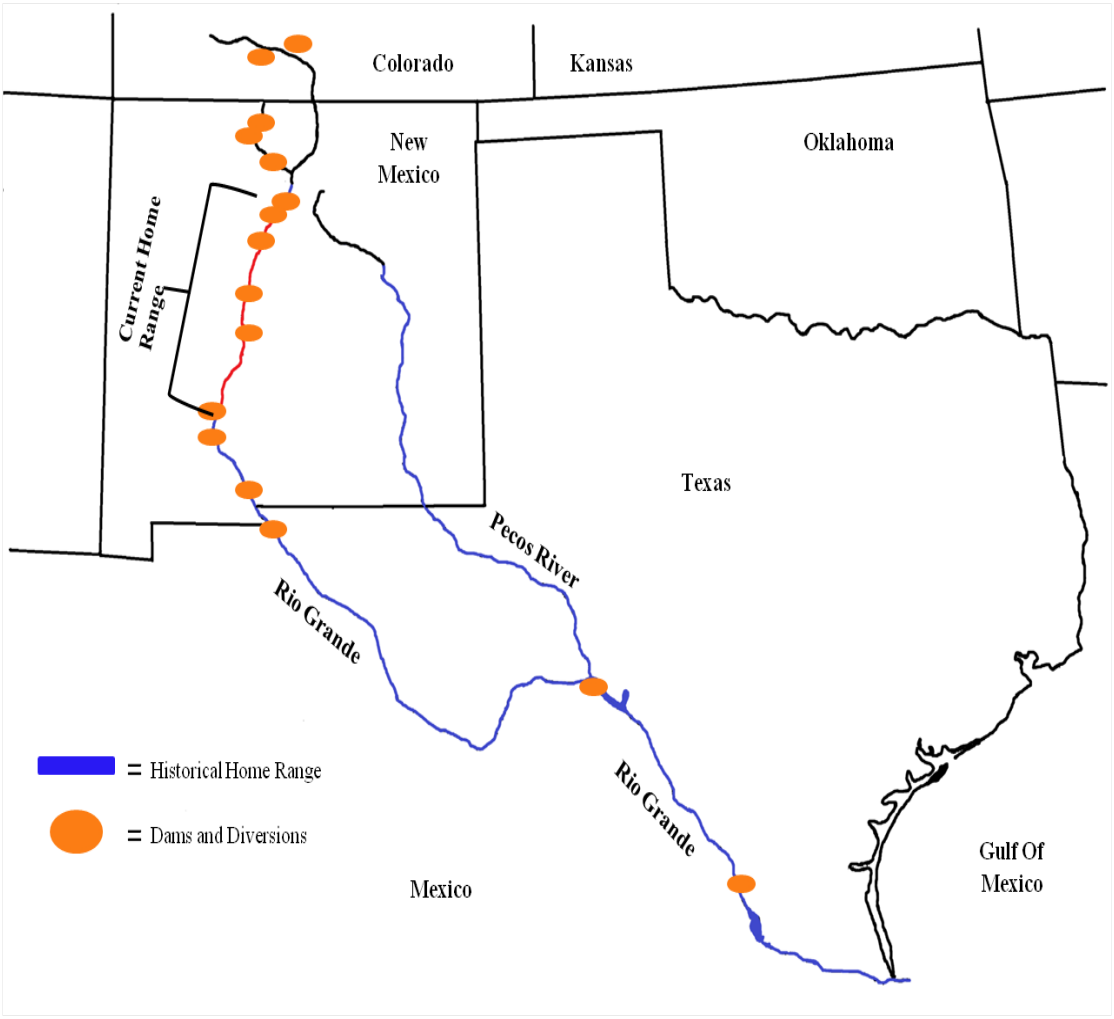

Figure 2. Dams and diversions along the Rio Grande River.

MULTIPLE GENERATION STUDIES

A majority of studies in the aquatic toxicology field look at effects on a species over a limited duration of time during the lifespan of one generation of that species (partial generational studies) (Clotfelter and Rodriguez 2006, Dutta and Arends 2003, Ma et al. 2007, Shved et al. 2008). These studies will examine the effects of a chemical on a specified life stage of the species. There have been some studies that have looked at a chemical's effect on a species over multiple generations (Deng et al. 2010, Kidd et al. 2007, Palace et al. 2009, Sowers et al. 2009), but these studies are greatly outnumbered by the partial generational studies. Partial generational studies can be useful for many toxicological studies to asses if a chemical of concern will cause mortalities in the species being tested. If the chemical does cause mortalities, then it is obvious that the chemical is harmful and needs to be regulated. However, sometimes reactions to chemicals will take longer to manifest and these reactions may not always be apparent as lethality. The reactions may cause a change in the individual that could be detrimental to its ability to reproduce, which in turn could be detrimental to that species' population. Some of the induced effects may be a change in secondary sex characteristics (such as fatpads) (Miles-Richardson et al. 1999), a change in mating behavior (Clotfelter and Rodriguez 2006), or a change in gonadal structure or functionality (Woodling et al. 2006). These changes in an individual might not cause an immediate effect on their population, but instead could affect the future survival of the population.

The main difference between a partial generational study and a multi-generational study is that one looks at short-term consequences while the other looks at long-term consequences. Partial generational studies asses an immediate result from an exposure over a short time. However, this chemical insult is not how a fish will commonly experience exposures to chemicals in a natural setting. The chemicals that are of most concern to aquatic toxicologists are the ones that are introduced many times over an extended period of time ("pseudopersistance"). Chemicals that are chronically introduced into the aquatic environment require testing for the long-term effects on a population. The multi-generational study addresses these long-term effects of chronic exposure. A useful aspect of how multi-generational studies are conducted is that they include most, if not all, of the same analyses that a partial generational study would use. As a means to monitor the changes that occur during the extended study, measurements are taken numerous times throughout the study, which can sometimes be correlated with the same length of time that a partial generational study would have been run. This helps researchers to assess which effects the chemical had on the individual and the population in response to chronic exposure.

A study that demonstrates this chronic exposure approach very well is the exposure lake study reported by Kidd et al. (2007) and Palace et al. (2009). Kidd et al. (2007) recorded the change in population abundance and age ratio of the fathead minnows in two different lakes (an EE2 exposed lake and a reference lake). Besides looking at the start and final population abundances and age ratios, these authors also assessed population sizes in the spring, summer and fall of each year. Along with those observations, the authors also measured the plasma vtg concentrations, gonadal somatic

indices and the condition of the gonads (using common histological techniques). The report by Palace et al. (2009) described most of the same measurements reported by Kidd et al. (2007). Palace et al. (2009) also assessed population abundance and age ratios of four species of fish (fathead minnow, pearl dace, lake trout and white sucker) at the start and end of the study. The authors also took samples and measurements at different points throughout the study. While also assessing vtg concentrations, gonadal somatic indices and the histology of the gonads like in the Kidd et al. (2007) report, Palace et al. (2009) also measured liver somatic indices of the lake trout and white sucker. They were able to collect the liver somatic indices for the lake trout and white sucker because of their large size. The fathead minnow and pearl dace are too small to take liver somatic indices and still have enough of the liver for vtg concentration analyses. For this study, if they had tested these species of fish using a partial generational study they would have assessed all of the same measurements for an individual (vtg concentrations, gonadal somatic and liver somatic indices and gonadal histology), but these measurements would have been taken typically only at one point at the end of the study and the time frame of the study would be equivalent to one of the sampling periods during the multi-generational study.

One major limit to partial generational studies is that they only test a species for a short time (when compared to the species entire life cycle). As many studies on humans have shown, chronic exposure to a variable can show different reactions than if there was only a shortened exposure time, such as with asbestos (Cooke 1924, National Cancer Institute 2009) and heavy metals (Patrick 2006). When, for example, assessing exposure to radiation through tanning, a partial generational study would examine the effects after

one summer of tanning. While exposure over one summer could lead to skin cancer, it more likely would not show any immediate effects during the experimental period and would, therefore, conclude that sun exposure through tanning does not have any effect on humans. But it is well known that chronic sun exposure can induce skin cancer (Engel et al. 1988, Marks et al. 1990). A similar delayed response may occur in animals exposed in their aquatic environment. They may not show a reaction to a chemical initially during a partial generational study, but may show a reaction after a prolonged exposure to the chemical. If the species did show a reaction during the partial generational study, a prolonged exposure might show a greater response.

In a study by Rowe (2003), the early life stage of the sheephead minnow (*Cyprinodon variegates)* did not show a noticeable reaction to the mixture of chemicals that the minnow was being exposed to. However, around day 125, the growth of the fish started to differentiate between the control fish and the test fish. The study did find that the chemicals accumulated in the bodies of the fish over their lives. If this study had only been performed with one life stage, it is highly likely that Rowe (2003) would not have seen any effects of the exposure. The opposite effect was observed in a study by Vogt et al. (2007) that looked at a species of midge (*Chironomus riparius*) and its response to tributyltin (TBT). This species is a small two winged fly, in the same order as mosquitoes, which lays its eggs in an aquatic environment. The authors found that after 11 generations, *C. riparius* seemed to develop a tolerance to TBT. At first, the population exposed to TBT showed a decrease in growth rate, but after a few generations the growth rate started to return to the same levels as the control population and level off. Vogt et al. (2007) also noticed that the number of eggs increased over time in the TBT population. If

a partial generational study had been run instead of the multi-generational study for this experiment, the researchers would have only seen the initial drop in growth and not the recovery that was observed.

The reason Rowe (2003) observed effects at later stages could possibly be because of the differences in the sensitivity among the life stages of the fish. Biological processes differ between life stages, such as larva growing at rates greatly increased compared to adults. These biological differences may lead to differences in how the different stages might interact with a chemical. During the larval stage, the fish are growing at a faster rate than adults (Helfman et al. 1997, p.143), partitioning almost all of their energy into growth. Furthermore, size difference between the larval stage and the adult stage could affect the outcome of an exposure study. Smaller fish have a higher metabolic rate per unit of mass (Helfman et al. 1997, p.62), which could affect their reaction to a chemical. Also, at a smaller size it would not take as large of a quantity of a chemical to show the same response a larger sized fish would show. Just like in humans where someone should not give an adult's amount of a prescribed drug to an infant because that amount would be too strong for the infant. This increase in chemical response during the larval stage as compared to the adult stage has been demonstrated in previous studies, including a study by Soares et al. (2009), where zebrafish were exposed to low levels of EE2 during embryonic development up through eight months of age. The authors measured vtg concentrations in the adult fish and the mortalities in the three different EE2 concentrations and in the control. Soares et al. (2009) observed a dose dependant relationship in the mortalities in the larva from 8-24 hours post-fertilization. After the 24 hour mark, the authors did not observe any more mortalities among that

generation. The adults did not show a dose dependant response in their vtg concentrations, except in the highest concentration. In this study, it was demonstrated that while the adults did not exhibit a strong adverse response to the EE2 exposure (except at high concentrations) the larvae were adversely affected at lower EE2 concentrations. But it may not always be the case that the larval stage is more sensitive. During the adult stage, fish are using most of their energy for reproductive purposes (Helfman et al. 1997, p123), whether it's producing eggs in females or sperm in males. This change in energy expenditure can change how a chemical might affect the individual. A change in chemical sensitivity could also be attributed to a change in the morphology of the fish. A study by Kawabata et al. (1997) looked at the change in sensitivity of pale chub (*Zacco platypus*) to ammonia at different life stages. They found that the adult is in fact the most sensitive life stage in the presence of ammonia. The eggs were the least sensitive to ammonia. The authors attributed this sensitivity difference to the fact that ammonia affects the gills of fish. Adult fish have complete gills, while earlier life stages may be able to regenerate new gills, or they may lack gills altogether. If a partial generational study were performed, the differences in the life stages would greatly affect what researchers would see and what conclusions they could draw from the study.

The biggest factor that partial generational studies do not account for is the effects a chemical can have on the next generation. The chemicals affect the next generation mostly by affecting the larva's ability to survive. The problems with partial generational studies mentioned earlier can sometimes be corrected for by running multiple partial generational studies. But to see the effect a chemical can have on the survival of future generations, a multi-generational study needs to be performed. One famous instance of a

chemical affecting the survivability of the next generation is how dichlorodiphenyltrichloroethane (DDT) affected the avian population, including the brown pelican (*Pelecanus occidentalis*) and bald eagle (*Haliaeetus leucocephalus*), after DDT was used as pesticide. DDT poisoning did not directly kill the birds, but instead affected the integrity of their eggs. DDT would break down to dichlorodiphenyldichloroethylene (DDE), which accumulates in the fat cells of the birds and causes the thinning of the birds' egg shells (Blus et al. 1997). The birds would then crush the egg while attempting to sit on their nest, killing the offspring inside. The shells become thinner in relation to the amount of DDE that was found in the fat cells of the bird. While some chemicals affect the actual egg of the species, others are accumulated in the mother and transferred to the larva directly, such as selenium (Gillespie and Baumann 1986). Foekema et al. (2008) found that 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) would accumulate in the lipids of the fish the parent sole (*Solea solea*), and it would then be transferred from the females to the eggs. The major effect of PCB 126 on fish is a delay in critical development by two weeks. Therefore, the fish would remain in an earlier life stage for a longer period of time. Being arrested in an earlier life stage can be detrimental because the smaller fish did not settle to the bottom and were more susceptible to predation. Through terminating exposure after reaching a certain life stage, Foekema et al. (2008) were able to show that PCB 126 is accumulated in the fish and is not easily removed because of its lipophylic properties. The ability of some chemicals to be transferred from mother to offspring or for a chemical to affect the mother's ability to produce viable offspring demonstrates the need for multi-generational studies to be performed in aquatic toxicology.

However, partial generational studies do have benefits, including that they can be conducted in a much shorter amount of time. If there is interest in how different life stages react differently to a chemical, a partial generational study can also be applicable to allow for all life stages to be tested at the same time, instead of running one generation starting from the earliest life stage all the way through to the adult life stage. A researcher would be able to see the differences in the reactions a lot sooner with a partial generational study. But as was previously mentioned, the partial generational study would still only give a snapshot of all of the possible effects the chemical might have on a species. To have a complete picture of the chemical's possible effects, a multi-generational study would still be needed. After a multi-generational study, a researcher would be able to determine with confidence that their findings on the effects of a chemical on a species would encompass most possible effects.

Chapter II

EFFECTS OF 17α-ETHINYLESTRADIOL ON THE RIO GRANDE SILVERY MINNOW

INTRODUCTION

Protection of our aquatic systems from chemical pollution has been a concern for many years. As a result, the United States enacted the Clean Water Act in 1972. The act is used to employ "a variety of regulatory and nonregulatory tools to sharply reduce direct pollutant discharges into waterways, finance municipal wastewater treatment facilities, and manage polluted runoff" (Federal Water Pollution Control Act 1972). These tools would help "restore and maintain the chemical, physical and biological integrity of the Nation's waters" (Federal Water Pollution Control Act 1972).

One family of chemicals that has seen an increased focus in regulatory efforts are the endocrine active compounds (Ankley and Johnson 2004). These compounds interact with the endocrine system by either mimicking natural hormones or by blocking the function of the natural hormones (Crisp et al. 1998). A set of endocrine active compounds that is of concern are

the estrogenic chemicals. These estrogenic chemicals can be natural, such as 17β-estradiol (E2), or synthetic, such as 17α-ethynylestradiol (EE2). Natural estrogenic compounds are endogenously produced by humans, animals and plants. Synthetic estrogenic compounds come from supplements that are used by humans or used in livestock, but can also come from chemicals found in cleaning products. The effects of exposure to estrogenic chemicals have been widely documented (Bjerselius et al. 2001, Kidd et al 2007, Salierno and Kane 2009). Effects have ranged from morphological, including reduced secondary sexual characteristics (Salierno and Kane 2009), to behavioral (McGee et al. 2009, Shappell et al. 2010), to physiological, including vitellogenin induction in males (Jobling et al. 1998, Orn et al. 2006) to mortality (Kidd et al. 2007). Although the effects of these chemicals have been well documented, the numbers of species that have been tested are limited.

The species that are tested are frequently model species. Model species are used as surrogates for endangered and threatened species in toxicity tests. An endangered species is one that "is in danger of extinction throughout all or a significant portion of its range" (Endangered Species Act of 1973). A threatened species is one that "is likely to become endangered within the foreseeable future" (Endangered Species Act of 1973). The results of the tests on model species are used to help implement regulations that would protect all species in a given environment (Stephan et al. 1985). Traditionally the species that were used in toxicity tests, which would later be considered model species, were chosen because of their ease in maintenance in a laboratory setting. While model species seem to be able to withstand changes in their environment, endangered and threatened species have already shown a vulnerability to changes in their environment. Because of the lack of known exposure data on endangered or threatened species, it is unknown if the model species are in fact reliable surrogates.

The Rio Grande silvery minnow (*Hybognathus amarus*, RGSM) is a species that is currently listed as endangered. The RGSM can be found in an area of the Rio Grande that is considered the middle Rio Grande; an area stretching from the Cochiti Dam to the Elephant Butte Reservoir. The current home range of the RGSM is around 150 miles, which is approximately 7% of the historical home range (US Fish & Wildlife Service 2007, Figure 1). This decrease in home range has occurred over the last 100 years. The RGSM, once a highly abundant species in the Rio Grande River, are now considered one of the most endangered species in the nation. A dramatic decrease of a primary species in such a short amount of time, such as the Rio Grande has seen with the RGSM, would create a substantial shock to the ecosystem. This change in the ecosystem could have dramatic effects on the biological dynamics within the system.

The Rio Grande has seen many changes in the past, including the building of dams and the divergence of the river to other areas for irrigation purposes. These changes are thought to be major causes of the reduction in the RGSM population (US Fish & Wildlife Service 2007).The changes can affect the RGSM's migration movements (through the use of dams), spawning behavior (caused by the decrease in spring runoff after diversions), or survivability (from the increased sediment pollution). Although these changes in the Rio Grande may be attributing to the reduction in the RGSM population, other issues such as the introduction of non-native species and different chemicals may be compounding the effects from the changes in the river. Conservation efforts, such as stocking the river, to this point have not shown evidence of recovery in the RGSM, although this is still being evaluated. This species has continued to see a decline in their population. This supports the idea that diversions and dams alone are not the only cause in the decline of the population. Other changes in the river need to be researched to

develop a conservation plan that will restore the RGSM population within the Rio Grande. With the limited knowledge on the susceptibility of the RGSM to different chemicals, it is impossible to discern the contribution that chemical pollution play in the population decrease. To fully understand what is actually causing the decline, the susceptibility of the RGSM to different chemicals must be assessed.

To address the issue of limited knowledge on the effects of different chemicals on the RGSM, specifically endocrine active compounds, I set out to expose the RGSM to an estrogenic compound and document the reaction to exposure. Fish were exposed to a strong estrogenic compound (17α-ethynylestradiol (EE2)) known to cause endocrine disruption in other species (Shved et al. 2008, Soares et al. 2009, Swapna and Senthilkumaran 2009) at an age near sexual differentiation (juvenile stage) to assess effects on the sex ratios of the fish. Fish were also monitored for physiological and biological endpoints, such as vitellogenin induction, body condition factor (BCF), and mortality. I hypothesized that the RGSM would show a strong reaction to EE2, causing a decline in survival and BCF, and an incline in plasma vtg concentrations.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Experiments were conducted at the U.S. Geological Survey (USGS) Yankton Field Research Station of the Columbia Environmental Research Center and methods are derived from Study Plan 08-Field-05 (Buhl 2009). The 21-day study was performed utilizing 68-L aquaria

filled with 45 L of water. The aquaria were held in two temperature-controlled water baths that were connected to form a continuous unit. The system contained five different water treatments; one control, one carrier control (ethyl alcohol), and three exposure concentrations (see Section Exposure Chemicals); with four replicates per water treatment. The test water used simulated the major water quality characteristics (anions and cations) of the Rio Grande at Isleta, NM (Buhl 2002). The test water was made every two to three days by mixing specific amounts of mineral salts with deionized water. The test water was analyzed for selected water quality characteristics before use in the study (see Section Water Quality). A flow-through glass proportional diluter system was used to create and maintain the five treatments with four replicates. The diluter system was calibrated to deliver one liter of experimental water to each of 20 aquarium every 15 minutes through glass tubing. Water quality was analyzed weekly for selected water quality characteristics (see Section Water Quality). Juvenile Rio Grande silvery minnows were randomly distributed into the aquaria at a density of 75 fish per aquarium. A random sample of 20 fish was collected every seven days from each aquarium. Fish were analyzed for whole-body vitellogenin concentrations, BCF, and histological sex (described below).

EXPOSURE TREATMENTS

Stock solutions were prepared in four identical 500 mL amber bottles using 100% reagent-grade ethyl alcohol (Fisher Biotech) and different concentrations of 17α-ethynylestradiol (EE2, Sigma-Aldrich Co.) to achieve final exposure concentrations of 0, 1, 5 and 25 ng/L EE2. The amber bottles were then covered with aluminum foil. The stock solutions were added to the dilutor system using automated pipetting systems (Hamilton Microlabs 504A dispensers fitted

with 100 µl Hamilton syringes) utilizing Teflon tubing to deliver the stock solutions into the mixing chambers of the dilutor system. The stock solutions were added to the mixing chambers every 15 minutes, initiated by the running of the dilutor system.

EXPOSURE ORGANISMS

Rio Grande silvery minnow eggs were obtained from a fish hatchery (City of Albuquerque Biological Park and Aquarium, Albuquerque, NM) and raised at the Yankton Field Research Station. The fish were incubated in water consistent with the major water quality characteristics of the Rio Grande River at Isleta, NM (Buhl 2000) under constant environmental conditions (a photoperiod representative of Albuquerque, NM (10h 45min light and 13h 15min dark); 25°C temperature). Aquaria were partially submerged in a temperature-controlled water bath, and received supplemental aeration in each aquaria by air stones that received compressed air from an oil-less compressor. The fish were fed live *Artemia* nauplii twice a day and ground Silvery Minnow Starter flake diet (Dexter National Fish Hatchery and Technology Center, Dexter, NM) three times a day. Fish were 153 days old at the onset of the experiment.

ANALYSIS

WATER QUALITY

Water samples were taken twice a week directly from the exposure tanks using dedicated pipettes specific to each tank. One set of samples were analyzed for levels of calcium (USGS

1994a), magnesium (USGS 1994a), alkalinity (USGS 1994b), hardness (USGS 1994c), chlorides (USGS 1994d), ammonia (USGS 1995) and sulfates (USGS 1997a). Samples for ammonia were preserved in 0.4% sulfuric acid and stored under refrigeration until analyzed. The second set of samples was analyzed for EE2 levels in the aquaria by using a solid phase extraction (SPE) (See Section Solid-Phase Extraction). Temperature (USGS 1996) and dissolved oxygen (USGS 1997b) were measured daily in each aquaria. Conductivity (USGS 1994e) and pH (USGS 1997c) were measured in one set of replicates daily, with the replicates being measured on a rotating schedule.

SOLID-PHASE EXTRACTION

Water samples were buffered to a pH of 7.00 (± 0.01) using 0.1 N NaOH and 0.1 N HCl, and then 10 mL of 100% reagent grade methanol was added to the samples. Filter reservoirs (50 mL syringe barrel with 25-mm o.d., 1µm mesh Gelman type A-E glass fiber filter and a 1.0 in. o.d. steel ring) were rinsed with 100% reagent grade methanol and attached to 6.0 mL, reverse-phase, solid-phase extraction (SPE) concentration columns (Agilent Technologies) using PTFE leur-loc SPE column-cap adapters. Filter reservoirs and SPE concentration columns were attached to a Visiprep vacuum filtration system (Supelco, Sigma-Aldrich Co.). The SPE system was washed using the vacuum filtration system with 5 mL of 100% reagent grade methanol and 10 mL of 0.5% methanol solution. Water samples were filtered through designated filter reservoirs with a drip rate of five to six mL per minute, refilling the filter reservoirs as needed, until all of the samples were filtered. The columns were dried by allowing the vacuum system to run an additional five minutes after the water samples had run through the filters and SPE

columns. The columns were then washed with five mL of hexane. After washing, 15 mL screw-capped tubes were placed under the SPE vacuum outlet tubes and the SPE concentration columns were eluted with 10 mL of dichloromethane. The dichloromethane was evaporated using N_2 and a tube warmer. After the dichloromethane had evaporated the tubes were capped, using Teflon®-lined caps, packed in ice in a cooler and shipped to the USGS Columbia Environmental Research Center (CERC),Columbia, MO for completion of the solid-phase extraction. At the CERC, the precipitate was reconstituted with 0.1 mL of methanol and 1.9 mL of distilled water. Samples were stored in a 4°C fridge until assayed by enzyme-linked immunosorbent assay (ELISA) using in-lab standard operating procedures.

BIOLOGICAL ENDPOINTS

On sample days, 20 fish were randomly sampled from each replicate across all four tanks (20 fish from each tank) and were sacrificed using tricaine methanesulfonate (MS222). Whole body weights were measured to the nearest 0.001 g, and total lengths were measured to the nearest 0.5 mm. Biological endpoints, both physiologic and morphologic, were measured to determine if exposure to an estrogenic compound would elicit a response in the Rio Grande silvery minnow. Fulton's body condition factors (BCF) were calculated by dividing the total weight (in g) by the total length (TL, in mm) cubed and multiplying by 100,000 ((Weight, g/(TL, mm^3))*100,000) for each fish (Anderson and Gutreuter 1983).

SEX DETERMINATION

A section of each fish was removed for histological sex determination of each fish. A cut was made directly posterior to the anal pore with another cut approximately five mm anterior to the first cut. The section of fish was placed into a histocassette and preserved in a 10% buffered formalin solution until the tissues could be histologically processed (Appendix A). Slides were analyzed for sex determination, which was concluded through the presence of ovarian or testicular tissue. A lack of ovarian tissue but the presence of reproductive material was identified as female, in an attempt to prevent a Type I error (finding a significant difference when there should be no significant difference) during statistical analysis.

WHOLE-BODY VITELLOGENIN ANALYSIS

The remaining pieces of each fish, after the section for sex determination was removed, were weighed to the nearest 0.001 g and in a tared labeled vial. The vials were placed into a -80ºC freezer and stored until sex was determined. The samples of the fish were placed into a cooler with dry ice and transferred to the CERC facility for whole-body vitellogenin analysis. At CERC, the whole-body fish tissues were thawed, homogenized and then centrifuged. The supernatant was analyzed using a sandwich ELISA as described by Folmar et al. (1996) for vtg concentrations.

STATISTICAL ANALYSIS

All data sets were analyzed for assumption of normality using the Kolmogorov-Smirnov test for normality (PASW Statistics 18, IBM Corporation, Somers, NY; Prism 4.01 statistical package, GraphPad Software Inc., Oxnard, CA). If the data was found to be normally distributed with an equal variance, a one-way ANOVA followed by Bonferroni post-hoc test was used for the analysis. Data not meeting these assumptions were analyzed the Kruskal-Wallis non-parametric ANOVA test **a**nd Dunn's test for multiple comparisons. Sex ratios were tested using Chi-Square analysis, with an expected ratio of 50% males and 50% females (Vajda et al. 2008). A significance level of 95% ($p < 0.05$) was used for all tests.

RESULTS

WATER QUALITY

Water quality results can be found in Buhl et al. (in preparation).

BODY CONDITION FACTOR AND SEX RATIO

At the completion of the exposure period, BCF was significantly increased in the 5 ng/L and 25 ng/L treatments as compared to the other treatments (Figure 3) for females ($p < 0.01$) and for males as compared to the control and solvent control treatments ($p < 0.05$). Males also showed a significant increase in BCF in the 25 ng/L treatment as compared to the 1 ng/L treatment ($p = 0.001$). Both males and females showed similar patterns, as a dose-dependent

increase in BCF with an increase in E2 levels. The 25 ng/L treatment had the highest average BCF (male, 0.807; female, 0.805), followed by the 5 ng/L treatment (male, 0.790; female, 0.802) and 1 ng/L (male, 0.766; female, 0.759). The lowest average BCF was in the control (male, 0.759; female, 0.759), and the second lowest average BCF was in the solvent control (male, 0.756; female, 0.757).

There were no significant differences observed in the ratios of males to females for all five treatments as compared to what was expected. P-values for treatments were found to be: Control = 0.216, Solvent Control = 0.732, 1 ng/L = 0.305, 5 ng/L = 0.311, and 25 ng/L = 0.736.

WHOLE-BODY VITELLOGENIN AND SURVIVAL

Staff at the Columbia Environmental Research Center are currently continuing to develop and analyze a whole body homogenate technique for use with the Rio Grande silvery minnow. Data, including survival, will be published in a future manuscript (Buhl et al. in preparation).

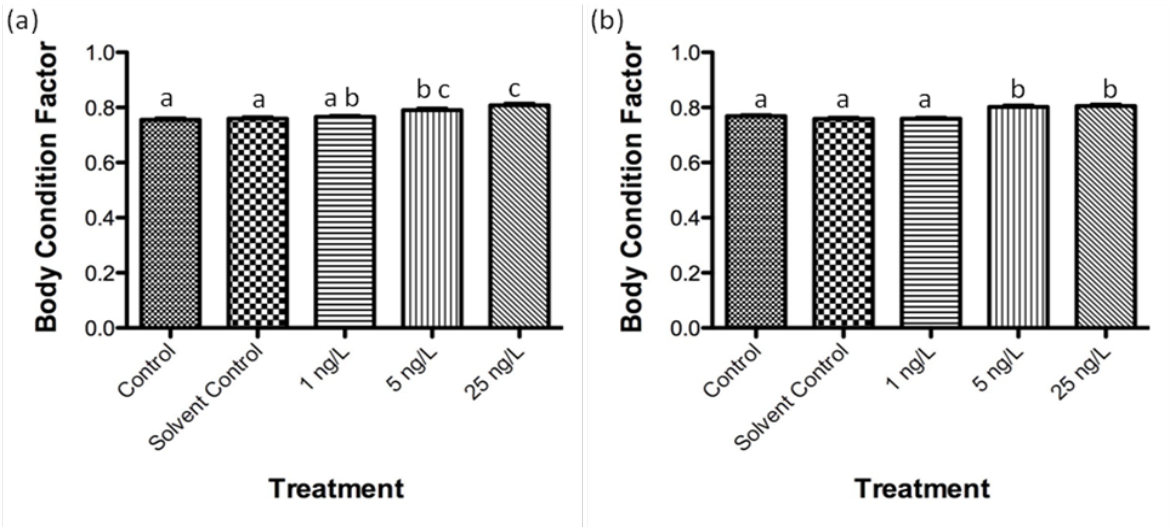

Figure 3. Body condition factors across five different 17α-ethinylestradiol treatments. Mean ± standard error. Letters indicate statistically significant differences between treatments (One-Way ANOVA with Bonferroni post-hoc test, $p < 0.05$). (a) Males; (b) Females.

DISCUSSION

Effects of estrogenic exposure on fathead minnows have been well documented (Ankley and Villeneuve 2006). Effects on the endangered Rio Grande silvery minnow, however, are currently not known (US Fish & Wildlife Service 2007). Currently, protection of the Rio Grande silvery minnow from chemical pollution is achieved through the use of the fathead minnow (*Pimephales promelas*) as a surrogate species. The reliability of the fathead minnow as a surrogate species for the Rio Grande silvery minnow in the face of exposure to organic chemicals, including estrogenic compounds, is not known. This study was carried out to

determine the effects an estrogenic compound, 17α-ethynylestradiol (EE2), would have on the Rio Grande silvery minnow.

Preliminary results suggest that the Rio Grande silvery minnow is a robust species when exposed to a strong estrogenic compound. There was no significant variation from an expected male to female ratio of 50/50. However, I did not expect a change in sex ratios. Sex ratios were also not expected to be affected because the Rio Grande silvery minnow were exposed to EE2 after sexual differentiation. Studies have found that estrogenic exposure does not affect the sex of the fish, including the induction of intersex (Pawlowski et al. 2004). In these studies, although no intersex is observed, the authors did detect an increase in vitellogenin concentration with an increase in estrogenic exposure (Pawlowski et al.2004).

A significant difference was observed in the BCF between treatments, however not in the way that was hypothesized. The Rio Grande silvery minnow showed an increase in BCF with an increase in E2 concentration levels, as opposed to the expected decrease in BCF with an increase in E2 concentration levels. This may be due to the fact that flocculation was observed in the treatment tanks at higher E2 concentrations. This floc may have helped increase the nutrients in the tanks, allowing for more food for the Rio Grande silvery minnows in the higher E2 concentrations. However, having a higher BCF may not always be beneficial. The BCF measures the differences in weight as compared to the length. Therefore, an increase in BCF could also be caused by an increase in body fat, which may not be beneficial to the fish.

Although concentration-dependant effects were observed only observed in BCF, a full conclusion of this study cannot be made at this time as the most sensitive biomarker in this study, vitellogenin induction, has yet to be fully analyzed. Another endpoint, mortality, also has yet to

be analyzed. A dose-dependent effect may still be observed in either endpoint, or it may not be observed. If no dose-dependent response is observed, then it can be concluded that the fathead minnow is most likely an effective surrogate for the Rio Grande silvery minnow for estrogenic pollution because the fathead minnow has shown a response to estrogenic exposure (Hyndman et al 2010, Palace et al. 2009, Salierno and Kane 2009). But a difference in vulnerability raises questions about the differences in responses that might be seen when exposed to other chemicals, with the Rio Grande silvery minnow possibly showing a greater response to a different chemical.

If a dose-dependent response is observed in vitellogenin induction and mortality, it could be concluded that the Rio Grande silvery minnow is vulnerable to estrogenic exposure. In order to determine if the fathead minnow is a satisfactory surrogate, the level of response that the Rio Grande silvery minnow exhibited would need to be compared to the level of response that the fathead minnow exhibits after exposure to EE2. This is often a difficult to determine as endpoints can be measured using different techniques or equipment, or the fact that the experiments are run in different labs with different extraneous variables. For the best opportunity to observe effect differences between species, an exposure would need to be conducted with different species being exposed at the same time in the same facility.

	Control	Solvent Control	1 ng/L	5 ng/L	25 ng/L
Males	**34**	**40**	**43**	**44**	**38**
Females	**45**	**37**	**34**	**35**	**41**
Percent Males	**43.04%**	**51.95%**	**55.84%**	**55.70%**	**48.10%**

Table 3. Number of male and female Rio Grande silvery minnow at the end of the 21 day exposure period. Percent males across five different 17α-ethinylestradiol treatments.

Chapter III

EXPOSURE EFFECTS OF 17β-ESTRADIOL ON AN ENDANGERED SPECIES, THE RIO GRANDE SILVERY MINNOW (*HYBOGRNATHUS AMARUS*), AND TWO MODEL SPECIES, THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*) AND BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS*)

INTRODUCTION

Species commonly used in toxicological studies are termed "model species." These species are regularly used as surrogates for endangered and threatened species. Traditionally, model species have been used because of their ease of maintenance in the laboratory. They are typically more tolerant to fluctuations in changes in water characteristics, such as temperature or pH. An endangered species is one that "is in danger of extinction throughout all or a significant portion of its range" (Endangered Species Act of 1973). A threatened species is one that "is likely to become endangered within the foreseeable future" (Endangered Species Act of 1973). As seen by their decrease in populations, some endangered or threatened species have already shown an increased vulnerability to changes in their environment. They may not be as resilient to changes as model species tend to be.

The results of toxicological tests on model species are typically used to direct the development of conservation regulations (Stephan et al. 1985). The results are used to calculate a Criterion Maximum Concentration and a Criterion Continuous Concentration. The Criterion Maximum Concentration is equal to one half of the Final Acute Value and is meant to protect 95% of the genera in a given area. The Criterion Continuous Concentration is equal to the lowest of the Final Chronic Value, the Final Plant Value and the Final Residue Value. But these calculations are based on assumptions as to how relatable these species are to each other. With the dramatic diversity that can be seen among fish species (Helfman 1997) and the limited knowledge as to how relatable these species are to each other in their reactions to exposures to different chemicals, it has to be questioned as to how well these model species actually represent endangered and threatened species. Dwyer et al. (2005) showed that if fathead minnows (*Pimephales promelas*) and *Ceriodaphnia dubia* were used as surrogates for seven endangered and threatened species, the listed species would be protected 96% of the time. They also found that, when compared to the fathead minnow, no listed species was consistently more or less sensitive. The sensitivity varied within each species in relation to different chemicals. While a species would be protected against one chemical, it may not be protected against another chemical. The same dilemma can be seen when using the Criterion Maximum Concentration. If a chemical was tested using standard toxicity techniques and then applied to calculate the Criterion Maximum Concentration, 95% of the genera in a given area would be protected against that one specific chemical. If another chemical was tested and the Criterion Maximum Concentration was again calculated, the species that make up the 95% that are protected might be different. That would indicate that there are different species which make up the 5% that are not protected per chemical. After a few Criterion Maximum Concentrations are calculated and implemented, it is

very likely that there would not be 95% of the genera protected against all chemicals. With the number of chemicals that can be found in the aquatic environment, there would be a possibility that no species would be protected from all chemicals. Although, special considerations are made if a commercially or recreationally important species is more sensitive than either criterion calculates.

The Rio Grande silvery minnow is one species that is currently classified as endangered. Over the past 100 years, the historical home range (around 2,400 miles) of the Rio Grande silvery minnow has seen a dramatic decrease. Its current home range is around 150 miles, which is less than 7% of the historical home range (US Fish & Wildlife Service 2007). The Rio Grande silvery minnow is now considered one of the most endangered species in the United States despite once being highly abundant in the Rio Grande River. Much of the decrease in home range has been attributed to the building of dams and other diversions along the river (US Fish & Wildlife Service 2007). While the alterations to the habitat could be playing a substantial part to the decrease in habitat range of the Rio Grande silvery minnow, other factors, such as chemical pollution or the introduction of invasive species, could be compounding the effects from habitat alterations. To combat the decrease of species due to habitat alterations, restocking is a commonly used technique for recovery. If other issues, such as chemical pollution, are in fact contributing to the decline of the Rio Grande silvery minnow, restocking may not be an effective technique. Current water quality standards may need to be modified to become more rigorous so that chemical pollution is not compounding habitat alteration effects.

To know if current water quality standards need to be modified, it is necessary to know at what concentrations of specific chemicals can the Rio Grande silvery minnow survive. The surrogate species that is currently being used for the protection of the Rio Grande silvery

minnow is the fathead minnow (US Fish & Wildlife Service 2007). It has been concluded in previous studies that the fathead minnow is a suitable surrogate for the Rio Grande silvery minnow when exposed to inorganic compounds (Buhl 2002). It has not been concluded, however, if the fathead minnow is a suitable surrogate for organic compound exposures. While the home range of the Rio Grande silvery minnow has decreased, the fathead minnow has continued to be observed in all reaches of the Rio Grande River (Carter 1995). Fathead minnow may still be found in all reaches of the Rio Grande River because of their prevalent use as a bait fish, allowing for a possible source of reintroduction into the river system. Although this may be the main rationale, it is possible that the fathead minnow continues to be found in all reaches because they possess a tolerance to chemicals that the Rio Grande silvery minnow does not possess.

A family of chemicals that has received increased attention for regulatory purposes are the endocrine active compounds (Ankley and Johnson 2004). One group of endocrine active compounds is the estrogenic chemicals. These chemicals interact with the natural pathways of estrogenic compounds by either mimicking or blocking the function of the natural compounds. These estrogenic chemicals can be natural, such as estrone and 17β-estradiol, or synthetic, such as 17α-ethynylestradiol. Some of these chemicals can be found at high levels within the river. In 2001, estrone was found at levels as high as 140 nanograms per liter in water sampled below a discharge point from a wastewater treatment plant. These estrogenic chemicals could be a source of tolerance that the fathead minnow possess and the Rio Grande silvery minnow do not.

To address possible differences in tolerance to an organic compound between species of fish, I exposed three species to 17β-estradiol (E2) at environmentally relevant concentrations (in relation to the total estrogenicity found within rivers). An endangered species (Rio Grande

silvery minnow), its surrogate (fathead minnow) and another model species (bluegill sunfish, *Lepomis macrochirus*) were chosen for exposure. Fish were monitored for morphologic, physiologic and behavioral variation between treatments; including survival, body condition factor (BCF), sex ratios, vitellogenin induction, and predator avoidance response. I hypothesized that there were would a difference in all endpoints between species, with the Rio Grande silvery minnow showing the greatest response to E2, showing the greatest decline in survival, BCF, and predator avoidance response while also showing the greatest increase in vitellogenin (in relation to an increase in E2).

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Experiments were conducted at the USGS Yankton Field Research Station of the Columbia Environmental Research Center. The 21-day study was performed utilizing 12 x 58-L aquaria, each of which were separated into two equal exposure areas, and held in a temperature-controlled water bath. Aquaria were randomly assigned for species and treatment. The system contained three different water treatments; one control, and two exposure concentrations (see Section Exposure Chemicals); with two replicates per water treatment per species. The test water used simulated the major water quality characteristics (anions and cations) of the Rio Grande River at Isleta, NM (Buhl 2002). The test water was prepared every two to three days by mixing specific amounts of mineral salts with deionized water. The test water was analyzed prior to use in the study for selected water quality characteristics (see Section Water Quality). A flow-through glass proportional diluter system was used to deliver 500 mL (± 11.5) of water to each

exposure tank every 15 minutes through glass tubing. Water quality was analyzed semi-weekly for selected water quality characteristics (see Section Water Quality). Juvenile fathead minnows, bluegill sunfish and Rio Grande silvery minnows were randomly distributed into the pre-labeled aquaria at a density of 40 fish per exposure area. A subsample of 10 fish was randomly collected every seven days from each exposure aquarium. All species were analyzed for whole-body vitellogenin concentrations, mortality, a body condition index, and the histological sex. Fathead minnow and Rio Grande silvery minnow were also analyzed for predator avoidance behavior. Bluegill sunfish were initially tested for predator avoidance behavior, but showed no reaction to the test and were therefore discontinued for use in this endpoint.

EXPOSURE CHEMICALS

Stock solutions were prepared in two identical 500 mL amber bottles using 100% reagent-grade ethyl alcohol (EMD Chemicals) and different concentrations of 17β-estradiol (E2, Sigma-Aldrich Co.) to achieve final nominal concentrations of 10 and 30 ng/L E2. The amber bottles were then covered with aluminum foil. The stock solutions were added to the dilutor system using automated pipetting systems (Micromedic model 25000 automatic pipette with 200 microliter glass pumps and stainless steel pistons; ICN Micromedic Systems, Horsham, PA) utilizing Teflon tubing to deliver draw the stock solutions into the mixing chambers of the dilutor system. The solutions were injected every 15 minutes, initiated by the running of the dilutor system.

EXPOSURE ORGANISMS

Rio Grande silvery minnow eggs were obtained from a fish hatchery (City of Albuquerque Biological Park and Aquarium, Albuquerque, NM). Fathead minnows were obtained from CERC (Columbia, MO). Bluegill sunfish were obtained from 10,000 Lakes Aquaculture, Inc (Osakis, MN). All species were maintained at the Yankton Field Research Station in a reconstituted water designed to simulate the major water quality characteristics of the Rio Grande River at Isleta, NM (Buhl 2002) at 25 ±2ºC under a constant photoperiod representative of Albuquerque, NM (10h 45min light and 13h 15min dark). The aquaria were partially submerged in a temperature-controlled water bath and received supplemental aeration, utilizing glass pipettes, of compressed air from an oil-less compressor. The fish were fed live *Artemia* nauplii twice a day. Fish were 100-120 days old at the onset of the experiment.

ANALYSIS

WATER QUALITY

Two sets of water samples were collected twice a week from one replicate of each treatment for one species (n=3) for analysis of E2, with species and replicates on a rotating schedule;one set was collected directly from the delivery lines and the other sample from the exposure tanks using dedicated pipettes specific to each tank. The samples were collected in 250-mL polyethylene bottles and stored in freezer until analysis. The concentrations of E2 were measured using an estradiol specific competitive enzyme immunoassay (See Section Estradiol Immunoassay). A second set of water samples were collected semiweekly from one replicate of

each treatment for a given species (n=3) for analysis of calcium (USGS 1994a), magnesium (USGS 1994a), alkalinity (USGS 1994b), and hardness (USGS 1994c). Temperature (USGS 1996) and dissolved oxygen (USGS 1997a) were measured daily in each aquaria. Conductivity (USGS 1994e) and pH (USGS 1997b) were measured in one set of replicates for all species daily, with the replicates being measured on a rotating schedule.

ESTRADIOL IMMUNOASSAY

Water samples were subjected to solid-phase extraction (SPE) for concentration of E2. SPE concentration columns (6.0 mL, Agilent Technologies) were connected to a Visiprep vacuum filtration system (Supelco, Sigma-Aldrich Co.) and SPE column-cap adaptors affixed with Teflon tubing were connected to the SPE concentration column. Teflon tubing was placed into a graduated cylinder filled with 100 mL of methanol (EMD Chmicals). Vacuum filtration system was run until the entirety of methanol was pulled through the tubes and each tube had around 1 mL of methanol remaining. The graduated cyclinder was then filled with 100 mL of distilled water. The vacuum filtration system was again run until the entirety of distilled water had been pulled through the tubes and each tube had around 1 mL remaining. SPE concentration tubes were not allowed to run dry during the methanol and distilled water rinses. Teflon tubing was the placed into the sample water bottles and samples were filtered through designated filter reservoirs with a drip rate of five to six mL per minute. The SPE concentration columns were dried by allowing the vacuum system to run for an additional five minutes after dripping had ceased. Glass 6 mL graduated cylinders were placed into the vacuum chamber for collection of samples. Each SPE concentration column was then washed to remove E2 from the columns with

1 mL methanol and allowed to dry for 30 seconds after methanol drip has ended. This wash was repeated two additional times. SPE column-cap adaptors were removed, and a nitrogen air apparatus was then attached to the vacuum filtration system. Nitrogen was allowed to run for evaporation of methanol until 1 mL of each sample remained. Samples were placed into pre-labeled 2 mL glass vials and stored in a 4°C refrigerator until E2 analysis.

Samples were analyzed for E2 concentrations using an estradiol specific competitive enzyme immunoassay (Cayman Chemicals). Samples were diluted using EIA Buffer to a dilution of 1:5000. Standards were prepared as a seven-step 2.5-fold dilution with a range of 4,000 pg/mL to 6.6 pg/mL, utilizing an Estradiol EIA Standard. A tracer was made by reconstituting the 100 dtn Estradiol AChE Tracer with 6 mL EIA Buffer. An antiserum was made by reconstituting the 100 dtn Estradiol EIA Antiserum with 6 mL EIA Buffer. Solutions were added to the 96-well plate as follows: EIA Buffer was added to the NSB well (100 µL) and B_O well (50 µL); standards and samples were added to wells (50 µL each); tracer was added to the NSB, B_O, standard, and sample wells (50 µL each); and antiserum was added to the B_O and sample wells (50 µL each). The plate was then allowed to incubate for one hour at room temperature. Ellman's Reagent was made by reconstituting 100 dtn with 20 mL UltraPure water. After washing of the plate in an automated plate washer, Ellman's reagent was then added to each well (200 µL). Tracer was also added to the TA well (5 µL). The plate was covered with aluminum foil and allowed to incubate for one hour. After the incubation period, the plate was read at 420 nm on a Multiskan EX (Thermo Electron) plate reader.

BODY CONDITION FACTOR

On sample days, 20 fish were randomly sampled from treatment group (10 fish from each exposure area). Fathead minnow and Rio Grande silvery minnow were subjected to a predator avoidance behavior test (See Section Predator Avoidance Behavior), and then sacrificed using tricaine methanesulfonate (MS222). Bluegill sunfish were immediately sacrificed using MS222. Whole body weights were measured to the nearest 0.001 g, and total lengths were measured to the nearest 0.5 mm. Biological endpoints, both morphologic and physiologic, were measured to determine if exposure to an estrogenic compound would elicit the greatest response in the Rio Grande silvery minnow. Fultons' BCF was calculated by dividing the total weight (in g) by the total length (TL, in mm) cubed and multiplying by 100,000 ((weight, g/(TL, mm^3))*100,000) for each fish.

SEX DETERMINATION

The posterior section of each fish was removed for histological sex determination. A cut was made approximately two to five mm (species dependant) anterior of the anal pore, allowing for gonadal tissue to be present in the anterior region of the fish but not liver tissue. The posterior section of fish was placed into a labeled histocassette and preserved in a 10% buffered formalin solution until the tissues could be histologically processed (Appendix A). Slides were analyzed for sex determination, which was concluded through the presence of ovarian or testicular tissue. A lack of ovarian tissue but the presence of reproductive material was identified as female, in an attempt to prevent a Type I error (finding a significant difference when there should be no significant difference) during statistical analysis.

WHOLE –BODY VITELLOGENIN ANALYSIS

The anterior section of the fish was placed into a tared labeled vial and weighed to the nearest 0.001 g . The vials were placed into a -80 ºC freezer and stored until sex was determined. The samples of male fish were placed into a cooler with wet ice and transferred to St. Cloud State University (SCSU) in St. Cloud, MN for whole-body vitellogenin analysis. At SCSU, fathead minnow and bluegill sunfish were thawed on ice and homogenized using a 1X PBS solution (0.075M, pH 7.5) in a 1:1 body section weight to PBS solution volume. Rio Grande silvery minnow were homogenized using a 1X PBS solution in a 1:0.5 body section weight to PBS solution volume. Upon homogenization, all samples were centrifuged at 13,000 revolutions per minute for 15 minutes. Supernatant was separated into a labeled vial and placed into a -80 ºC freezer and stored until analyzed using a competition antibody-capture ELISA.

Microtiter plates were coated (except for one microtiter well per assay plate) using purified fathead minnow vitellogenin for fathead minnow and Rio Grande silvery minnow assays, and purified bluegill sunfish vitellogenin for bluegill sunfish assays; both at a 1:4000 dilution with coating buffer (0.35M sodium bicarbonate, 0.15M sodium carbonate, pH 9.6). One well was coated with coating buffer and PBS+BSA buffer (1X PBS, pH 7.5 + 1% BSA) at a 1:1 dilution. Plates were allowed to incubate at room temperature for at least one hour. Plasma samples were thawed and diluted in PBS+BSA buffer to dilutions of 1:50, 1:250 and 1:1000 (plasma to buffer) for fathead minnows and bluegill sunfish. Rio Grande silvery minnow plasma samples were thawed and diluted in PBS-BSA buffer to dilutions of 1:10, 1:25 and 1:50 (plasma to buffer). Standard curve dilutions for fathead minnows and Rio Grande silvery minnows were

prepared as seven-step twofold serial dilutions with a range of 4.8 ug/mL to 0.0375ug/mL by diluting purified fathead minnow vitellogenin in PBS-BSA buffer. Standard curve dilutions for bluegill sunfish were prepared as seven-step twofold serial dilutions with a range of 8 ug/mL to 0.0625 ug/mL by diluting purified bluegill sunfish vitellogenin in PBS-BSA buffer. Internal standards were also included at 1:50 and 1:100 dilutions of control fathead minnow vitellogenin for fathead minnows and Rio Grande silvery minnows; and control bluegill sunfish vitellogenin for bluegill sunfish. A maximum binding control, a true-blank control and a BSA-coated well control were also included on each plate. A primary antibody solution was then prepared by diluting a polyclonal anti-fathead minnow vitellogenin antiserum (courtesy of Gerald LeBlanc, NC State University), for fathead minnows and Rio Grande silvery minnows, or a polyclonal anti-bluegill sunfish vitellogenin antiserum (provided by Gerald LeBlanc, NC State University), for bluegill sunfish, with PBS-BSA buffer to a concentration of 1:10,000 (species specific antibody to PBS-BSA buffer). The primary anti-body was then added to each sample dilution, the standard curve dilutions, the maximum binding control and the BSA-coated well control at a 1:1 volume to volume ratio. All dilutions were allowed to incubate at room temperature for a minimum of one hour, and not exceeding one and one-half hours.

Following washing of the plates with a wash buffer (1%PBS, 0.1% Tween-40) in an automated plate washer, sample dilutions, standard curve dilutions, internal standards and controls were added to plates and allowed to incubate at room temperature for a minimum of one hour, and not exceeding one and one-half hours. Plates were washed again with a wash buffer in an automated plate washer, followed by coating of a secondary antibody solution. The secondary antibody solution was prepared by dilution a horseradish peroxidase labeled anti-rabbit IgG (Sigma, St. Louis, USA) in PBS-BSA buffer to a dilution of 1:10,000 (antibody to PBS-BSA

buffer). Plates were again allowed to incubate at room temperature for at least one hour, and not exceeding one and one-half hours. Following the incubation period, plates were washed with a wash buffer in an automated plate washer, and TMB substrate (Sigma, St. Louis, USA) was subsequently added to the plates and allowed to incubate for 15 to 20 minutes. Plates were then read at 620nm on a Multiskan EX (Thermo Electron) plate reader.

PREDATOR AVOIDANCE BEHAVIOR

At each sampling day 20 random fish from each treatment (10 from each replicate) were placed into 8-L plastic bins containing control water. Fish were randomly chosen from the bins and subjected to a predator avoidance behavior test (Appendix B). Each fish was subjected to the predator avoidance behavior test. After which the fish was euthanized (placed in lethal solution of MS-222), measured for TL, weighed, and dissected as described above. Control treatments for Rio Grande silvery minnows and fathead minnows were tested before testing fish from 10 ng/L treatments. Both species in the 10 ng/L treatments were tested before either species from the 30 ng/L treatments.

STATISTICAL ANALYSIS

All data sets were analyzed for assumption of normality using the Kolmogorov-Smirnov test for normality (PASW Statistics 18, IBM Corporation, Somers, NY; Prism 4.01 statistical package, GraphPad Software Inc., Oxnard, CA).. One-way ANOVA followed by Bonferroni post-hoc test was used to analyze data that met standards of normality. Kruskal-Wallis analysis

followed by Dunn's post-hoc test was used to analyze data that did not meet standards of normality. Sex ratios were tested using Chi-Square analysis. An assumed sex ratio of 50% males and 50% females was used for analysis (Vajda et al. 2008). Survival was compared using a Chi-Square analysis with Yates correction for low sample sizes. A significance level of 95% ($p < 0.05$) was used for all tests.

RESULTS

WATER QUALITY

Water characteristics remained stable throughout the experiment. Mean (±SD) water characteristics during the exposures were: temperature = 24.2°C ± 1.7; dissolved oxygen = 6.87 mg/L ± 1.74; pH = 8.13 ± 0.97; conductivity = 469 µS ± 72; calcium = 41.7 mg/L ± 0.8; magnesium = 7.35 mg/L ± 0.40; alkalinity = 127 mg/L $CaCO_3$ ± 3; and hardness = 134.3 mg/L $CaCO_3$ ± 3.7. Concentrations of E2 in the exposure water are in the process of being analyzed.

BODY CONDITION FACTOR AND SURVIVAL

At the end of the exposure period, BCF was not significant between treatments for male fathead minnows (Figure 4). For female fathead minnows, significance was found between the control and 30 ng/L treatments ($p = 0.044$), while no other significance was observed. A significant difference in BCF was observed between treatments for Rio Grande silvery minnow (Figure 5) males ($p = 0.003$), with the 10 ng/L treatment having the highest BCF, but not for

females ($p = 0.478$). A significant difference in BCF was observed between treatments for bluegill sunfish (Figure 6) males ($p = 0.007$) and females ($p = 0.003$), with the 30 ng/L treatment having the lowest BCF.

Rio Grande silvery minnow showed a significant decline in survival (Table 4) at day 14 and day 21 in the 30 ng/L treatment as compared to the control and 10 ng/L treatments ($p < 0.001$ for both days). No other significance was observed between treatments for the Rio Grande silvery minnow. On day 21, the fathead minnow showed a significant decline in survival in the 30 ng/L treatment ($p < 0.001$) as compared to the control and 10 ng/L treatments. No other significance was observed between treatments for the fathead minnow. There were no significant differences observed between treatments on any sampling date for the bluegill sunfish.

On day 14 in the 30 ng/L treatments, the Rio Grande silvery minnow showed a significant decline in survival as compared to the fathead minnow ($p = 0.007$) and bluegill sunfish ($p < 0.001$). The fathead minnow and bluegill sunfish did not differ significantly on day 14 in the 30 ng/L treatment ($p = 0.127$). On day 21 in the 30 ng/L treatments, the Rio Grande silvery minnow and fathead minnow showed a significant decline in survival as compared to the bluegill sunfish ($p < 0.001$ for both). However, the Rio Grande silvery minnow and fathead minnow did not differ significantly ($p = 0.370$) in survival.

SEX RATIO

Sex ratios, as compared to an expected value of 50%, showed significance in a few instances, although there was no dose-dependent pattern (Tables 5, 6, and 7). Rio Grande silvery

minnow showed a significantly higher male sex ratio on day 14 and day 21 in the 10 ng/L treatment ($p = 0.018$ and $p = 0.025$, respectively). Significance was not observed for any other data point for the Rio Grande silvery minnow (Table 5). Bluegill sunfish showed a significantly higher male sex ratio on day 14 in the control treatment ($p = 0.046$), but on day 21 in the control treatment there was a significantly lower male sex ratio observed ($p = 0.025$). Significance was not observed for any other data point for the bluegill sunfish (Table 6). Significant differences were not observed for any data point for the fathead minnow (Table 7).

WHOLE-BODY VITELLOGENIN CONCENTRATIONS

A species response difference in whole-body vtg concentrations was observed at the completion of the exposure experiment. Fathead minnow showed no significant difference in vtg concentrations between treatments (Figure 7) for males ($p = 0.441$) and females ($p = 0.542$). Male Rio Grande silvery minnow, however, did show a significant increase in vtg concentrations between treatments ($p = 0.022$, Figure 8). The difference was seen between the control treatment and 10 ng/L treatment, with fish in the 10 ng/L treatment having higher vtg concentrations than the controls. While female Rio Grande silvery minnow did not show a significant difference between treatments ($p = 0.064$), the p-value was significantly less than that of males or females of the fathead minnow.

Although not statistically significant, the male fathead minnow did show an increase in whole-body vtg in the 10 ng/L treatment (Figure 7), while the female fathead minnow exhibited the largest concentration in the control treatment (Figure 8). Male Rio Grande silvery minnow did see an increased vitellogenin concentration in the 30 ng/L concentration as compared to the

control treatment (Figure 9), however not at a significant level. Female Rio Grande silvery minnow exhibited a dose-dependent increase in whole-body vitellogenin at a non-significant level (Figure 8).

PREDATOR AVOIDANCE BEHAVIOR

Larval fathead minnow showed a significant decrease in latency period between control and 10 ng/L and between control and 30 ng/L (Figure 9). Larval Rio Grande silvery minnow also showed a dose-dependent decrease in latency period, however not at a significant level (Figure 11). Significance was not observed between treatments for fathead minnows or Rio Grande silvery minnows in velocity or total escape response. Velocity was observed at the lowest speed in the control treatment for both species (Figure 12), while total escape response was observed at the highest speed in the 10 ng/L treatment for both species (Figure 13)

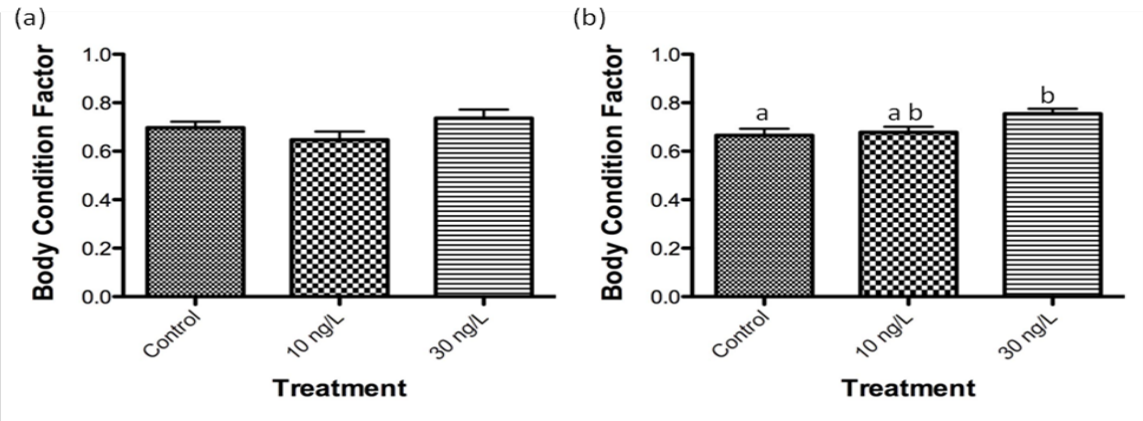

Figure 4. Body condition factors of fathead minnow across three different 17β-estradiol treatments. Mean ± standard error. Letters indicate statistically significant differences between treatments (One-Way ANOVA with Bonferroni post-hoc test, $p < 0.05$). (a) Males; (b) Females

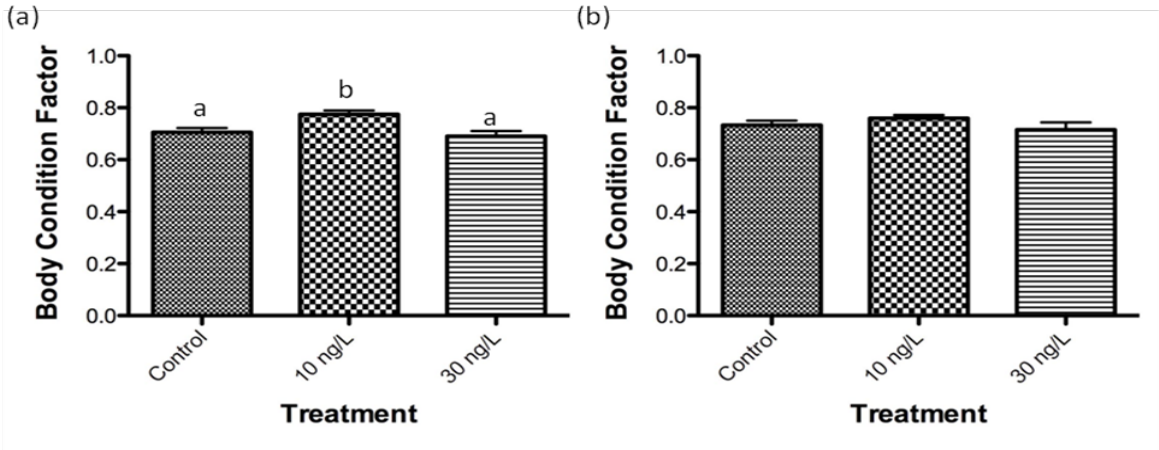

Figure 5. Body condition factors of Rio Grande silvery minnow across three different 17β-estradiol treatments. Mean ± standard error. Letters indicate statistically significant differences between treatments (Kruskal-Wallis with Dunn's post-hoc test, $p < 0.05$). (a) Males; (b) Females

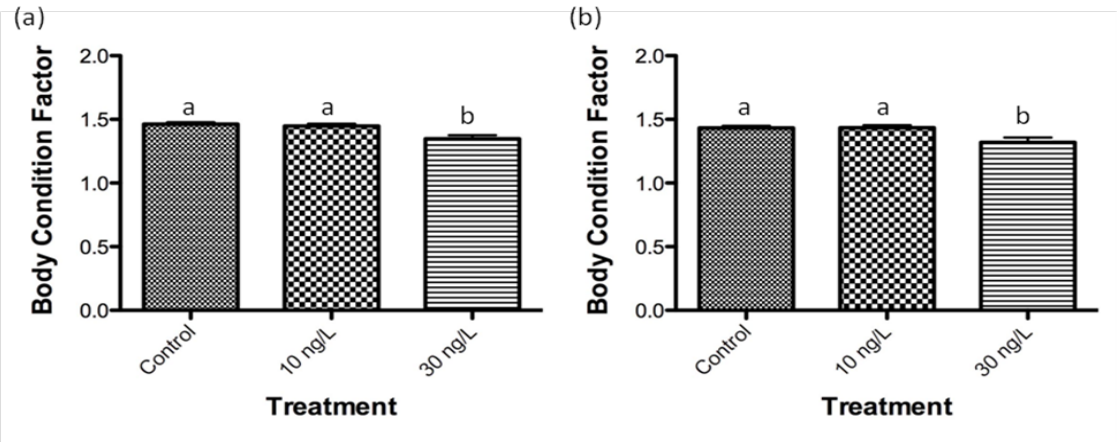

Figure 6. Body condition factors of bluegill sunfish across three different 17β-estradiol treatments. Mean ± standard error. Letters indicate statistically significant differences between treatments (One-Way ANOVA with Bonferroni post-hoc test, $p < 0.05$). (a) Males; (b) Females

	Silvery Minnow			Fathead Minnow			Bluegill Sunfish		
	Day 7	Day 14	Day21	Day 7	Day 14	Day21	Day 7	Day 14	Day21
Control	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Low	100.0%	100.0%	97.5%	100.0%	100.0%	100.0%	100.0%	100.0%	97.5%
High	98.8%	74.0%[a]	61.1%[c]	100.0%	93.3%[b]	46.7%[c]	100.0%	100.0%[b]	100.0%[d]

Table 4: Percent survival of three species at three sample periods across three different 17β-estradiol treatments. Red boxes indicate statistical significance within species (Chi-Square with Yates Correction, $p < 0.05$) and letters represent statistically significant differences between species (Chi-Square with Yates Correction, $p < 0.05$).

	0		7		14		21	
	Percent Males	P-Value	Percent Males	P-Value	Percent Males	P-Value	Percent Males	P-Value
Control	60.00%	0.371	60.00%	0.371	72.22%	0.059	55.00%	0.655
10 ng/L			40.00%	0.371	77.78%*	0.018*	75.00%*	0.025*
30 ng/L			60.00%	0.371	64.71%	0.225	52.94%	0.808

Table 5. Rio Grande silvery minnow percent males and p-value. "*" denotes significance (Chi Square with Yates Correction, $p < 0.05$).

	0		7		14		21	
	Percent Males	P-Value	Percent Males	P-Value	Percent Males	P-Value	Percent Males	P-Value
Control	30.00%	0.074	68.42%	0.108	75.00%*	0.046*	25.00%*	0.025*
10 ng/L			64.71%	0.225	68.75%	0.134	35.00%	0.180
30 ng/L			45.00%	0.655	42.11%	0.491	47.37%	0.819

Table 6. Bluegill sunfish percent males and p-value. "*" denotes significance (Chi Square with Yates Correction, $p < 0.05$).

	0		7		14		21	
	Percent Males	P-Value	Percent Males	P-Value	Percent Males	P-Value	Percent Males	P-Value
Control	61.11%	0.346	68.42%	0.108	37.50%	0.317	60.00%	0.371
10 ng/L			30.00%	0.073	47.37%	0.819	66.67%	0.157
30 ng/L			68.42%	0.108	52.94%	0.808	46.67%	0.796

Table 7. Fathead minnow percent males and p-value.

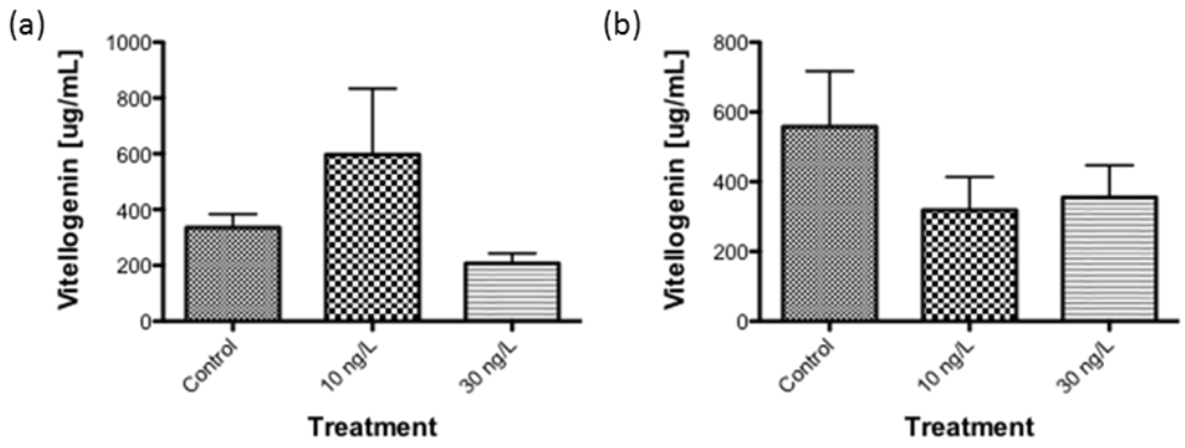

Figure 7. Whole-body vitellogenin concentrations of fathead minnow across three 17β-estradiol treatments. Mean ± standard error. (a) Males; (b) Females

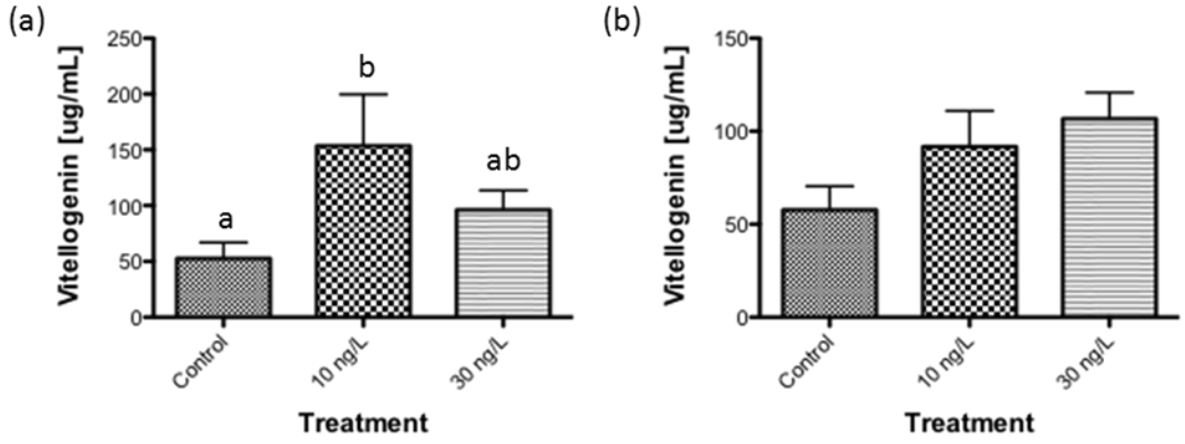

Figure 8. Whole-body vitellogenin concentrations of Rio Grande silvery minnow across three 17β-estradiol treatments. Mean ± standard error. Letters indicate statistically significant differences between treatments (Kruskal-Wallis with Dunn's post-hoc test, $p < 0.05$). (a) Males; (b) Females

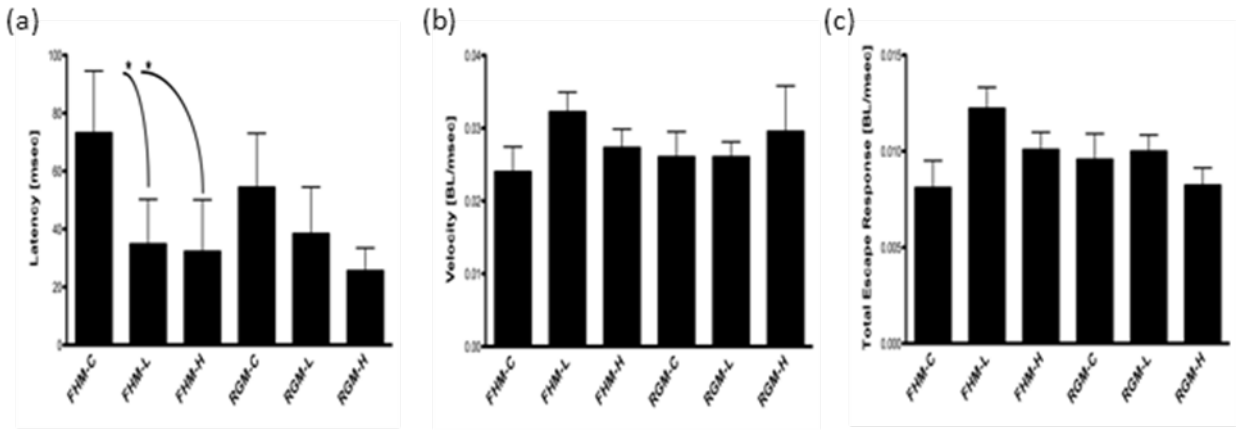

Figure 9. Predator avoidance behavior of two species across three 17β-estradiol treatments. Mean ± standard error. "*" represents statistical significance (Kruskal-Wallis with Dunn's post-hoc test, $p < 0.05$). (a) Latency; (b) Velocity; (c) Total Escape Response. Appreviations represent: FHM, fathead minnow; RGSM, Rio Grande silvery minnow; C, Control; L, 10 ng/L; H, 30 ng/L.

DISCUSSION

Protection of the Rio Grande silvery minnow from chemical pollution is currently achieved through the use of a surrogate species, the fathead minnow (US Fish & Wildlife Service 2007). It has been found that the fathead minnow is a suitable surrogate for the Rio Grande silvery minnow against inorganic chemical pollution (Buhl 2002). However, it is not known if the fathead minnow continues to be a suitable surrogate again organic pollution, including estrogenic compounds. Dwyer et al. (2005) demonstrated a difference in exposure effects between different species of fish, including the fathead minnow. Species showed differences in sensitivity between different chemicals, and when compared to the fathead minnow varied

between being more sensitive and less sensitive that the fathead minnow depending on the chemical. This variation in sensitivity as compared to the fathead minnow could possibly be happening in the Rio Grande silvery minnow. Although the Rio Grande silvery minnow is not more sensitive to inorganic chemicals than the fathead minnow, they may be more sensitive to organic chemicals. To test this issue, I exposed fathead minnow and Rio Grande silvery minnow concurrently to 17β-estradiol (E2) for 21 days to determine if there would be an effect difference between species. I also exposed bluegill sunfish, another model species, to determine if there would be an effect difference in a species that is in another family of fish which is also commonly used in toxicological studies.

Survival results indicate that the Rio Grande silvery minnow is the most sensitive species of the three in this study. They showed a significant decline in survival at a sample period earlier than the other species. The bluegill sunfish appear to be the most tolerant species in this experiment. The only mortality that was observed in this species was at day 21 in the 10 ng/L treatment. By day 21, both the Rio Grande silvery minnow and fathead minnow, both of the family Cyprinidae, showed a significant decline in survival in the 30 ng/L treatment, which was not observed in the bluegill sunfish, of the family Centrarchidae. At day 21, the survival did not differ between the Rio Grande silvery minnow and fathead minnow. This indicates a possible phylogenetic relationship in response to E2. It was interesting that while the bluegill sunfish did not show a decline in survival, they did show a significant decrease in BCF at the 30 ng/L treatment. This could be an indication that the bluegill sunfish is more tolerant to E2 than both cyprinids, even though their BCF were lower than those for the cyprinids. This could also be a factor of time. If the study were to continue for a longer exposure period, a reduction in survival might have also been seen in the bluegill sunfish at a later date. It was also interesting to note

that the male Rio Grande silvery minnow showed a significant increase in BCF in the 10 ng/L as compared to the control and 30 ng/L treatments, while the male fathead minnows did not show a significant difference between treatments.

Differences between Rio Grande silvery minnow and fathead minnow were also observed for whole-body vtg concentrations (Figures 7, 8). The male Rio Grande silvery minnow in the 10 ng/L treatment showed a significant increase in whole-body vtg compared to the controls. While it is noteworthy that there was not a complete dose-dependent response in the males, the whole-body vtg concentrations in fish exposed to 30 ng/L treatment were higher than in the control treatment. The observation of the highest vtg concentrations in fish at the 10 ng/L treatment is not surprising. Other studies have shown that the greatest response to estradiol exposure can be observed in lower estradiol levels than in higher estradiol levels (Hyndman et al. 2010, Panter et al. 1998). This may be due to the limited estrogen receptors available for binding of estrogenic compounds. At the 10 ng/L level all receptors may be utilized, while at the 30 ng/L level the estrogenic compounds could be competing for receptor sites, thus reducing the observed effects within the fish. While not significant, the male fathead minnows also showed this response, with fish in the 10 ng/L treatment showing the highest concentrations of vtg. Another interesting consideration is the p-value difference between the species. Although not significant, the female Rio Grande silvery minnow had a low p-value ($p = 0.064$). Both p-values for the fathead minnow were considerably higher than either p-value for the Rio Grande silvery minnow. It is also important to note that whole-body homogenate techniques had to be altered for each species. Differences in amounts of buffer used and dilutions used were needed to account for the wide variation in total vitellogenin concentrations, as fathead minnows had concentrations around five times higher than Rio Grande silvery minnows. However, for the Rio Grande silvery minnow

and fathead minnow, this could be due to the fact the fathead minnow specific antibody was used for detection of vitellogenin. As was pointed out by Tyler et al. (1996), the absolute vitellogenin concentrations may be different from the observed concentrations because it is a measure of how much Rio Grande silvery minnow vitellogenin will react with fathead minnow specific antibody. If a Rio Grande silvery minnow specific antibody were used, the absolute amounts may be different. However, the results can still be used for quantification and comparison reasons within the same species (Tyler et al. 1996).

While significant differences were observed in sex ratios, these results are used cautiously for explanations of exposure effects. The reason for this is that sex ratios were unknown at the start of the experiment, and sampling was done randomly at each date with small sample sizes. This caution is supported by the observed sex ratios in the control treatment of the bluegill sunfish at days 14 and 21. At day 14, a higher than expected male sex ratio was observed, however at day 21 a lower than expected male sex ratio was observed. In the estradiol treatment groups, no significant differences were observed for any sample date. The Rio Grande silvery minnow also showed an opposite result as what was expected, with a significant increase in male sex ratios in the 10 ng/L treatment group for both day 14 and 21. These variable results in sex ratios lead me to believe that observed differences in sex ratios may be attributable to the small sample size. I also did not expect to see differences in sex ratios because the species were exposed after sexual differentiation had occurred. Studies have found that estrogenic exposure does not affect the sex of the fish, including the induction of intersex (Pawlowski et al. 2004).

Predator escape behavior was observed in similar exposure responses between Rio Grande silvery minnow and fathead minnow. Both species showed a decrease in latency period with an increase in estradiol exposure, although only the fathead minnow showed a significant

decrease. Similar effects between species were also observed with respect to velocity and total escape response. Velocity was the slowest in the control treatment for both species, and total escape response was the highest in the 10 ng/L treatment for both species.

The purpose of this exposure experiment was to determine possible exposure effect differences between species, with the Rio Grande silvery minnow possibly showing the greatest response to exposure. While the results do not completely indicate that the Rio Grande silvery minnow is the most sensitive species in all biomarkers, they do show a difference in responses between the species. The Rio Grande silvery minnow showed that it will respond the earliest and most severe to estrogenic exposure in survival and vitellogenin production. While survival is more obvious as a cause for concern, vitellogenin should also be cause for concern. Increased vitellogenin production has been previously correlated with population level effects over time (Palace et al. 2009). My study indicates that further investigation is needed into actual exposure responses of the Rio Grande silvery minnow and the use of fathead minnow as a surrogate species. My study also contributes to the increasing amount of data indicating that while the use of surrogate species and phylogentic relationships considerations can be beneficial for initial implementation of water quality regulations, continued research into the most vulnerable species is needed to make sure all species are in fact protected.

Chapter IV

EXPOSURE EFFECTS OF 17β-ESTRADIOL ON TWO DIFFERENT LIFE STAGES OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

INTRODUCTION

Research in aquatic toxicology is commonly performed as short-term, partial generational studies. In partial generational studies, organisms are exposed to a chemical(s) for a period that is only a fraction of its complete life cycle. A frequently used exposure period is 21 days, but actually exposure periods can range from 96 hours up to any period of time that would not consist of a full life cycle of the organism. The duration of a complete life cycle will be species specific. An endpoint of partial generational studies is often mortality. These short-term exposures are meant to determine the lethal effects in an organism. Because of lethality being a popular endpoint, concentrations of chemicals in studies can often be seen in levels that are higher than is naturally observed.

A benefit of partial generational studies is that they show results in a short time. Multi-generational studies take a great deal longer to see results. This is important especially for chemicals that have been recently discovered in the environment. Results from these studies can be used to implement regulations more quickly, helping to protect organisms in the environment much more quickly. However, upon completion of partial generational studies for initial protection of organisms, studies that test the effects of chemicals over a complete life cycle, or

over multiple generations, of organisms are needed. These chronic, or multi-generational, studies will show the effects of a chemical(s) on an organism after more environmentally relevant exposure periods. Most chemicals in the environment do not persist for a short period of time and then disappear. A majority of chemicals will be constantly re-introduced into the environment from point and non-point sources. These constant re-introductions will cause organisms to be exposed to a chemical over their entire life cycle and into generations after if the chemical is not regulated.

Effects of chemicals may not be observed after a short term exposure. Some effects are seen only after prolonged exposure on an organism or a population. These effects on the organism may not be lethal to the organism itself, but it may cause a change in the organism that may have population level effects. In a multi-generational exposure to an estrogenic compound (17α-ethynylestradiol, EE2) in two lakes in Canada (Kidd et al. 2007, Palace et al. 2009), the researchers were able to show non-lethal effects at the organismal level but population-level effects for that organism's community. It was shown that fathead minnows had an increase in vitellogenin production (a yolk protein precursor) upon exposure to EE2 (Kidd et al. 2007). An increase in vitellogenin alone does have lethal effects in the organism, although the metabolic needs for producing this protein in a greater abundance could cause a reduction in fitness in other areas of the fish due to a reduction in energy available for other metabolic activities. While EE2 did not have lethal effects on the fathead minnow, it did cause a decrease in the population in subsequent years as a result of prolonged exposure (Palace et al. 2009). By the second year of exposure, the fathead minnow population showed a significant decrease. The effect of dichlorodiphenyltrichloroethane (DDT) on multiple avian species is another example of non-lethal effects that can affect subsequent generations. The DDT did not kill the adult, but instead

affected the integrity of the eggshell (Blus et al. 1997). The eggs were unable to withstand the weight of the adult when the adult would sit on the nest, crushing the egg and killing the developing embryo. This decrease in survivorship of the next generation had detrimental effects on the populations of those avian species that were affected.

While the Canadian lake study showed the long term consequence of non-lethal effects that can be observed in partial generational studies, other studies have shown that exposure effects may take longer to develop than the typical exposure period of a partial generational study. Delayed effects to exposures have been documented in many families of animals; including in fish (Rowe 2003), insects (Vogt et al. 2007), and even humans (Marks et al. 1990, Patrick 2006). These delayed effects could be lethal or non-lethal. Without the use of multi-generational studies, effects may never be documented and the resultant chemicals may never be looked at as detrimental.

Another constraining factor of most partial generational studies is that the exposures take place during one life stage of the organism. Chemicals could affect different life stages in diverse ways. The size difference between larva, juveniles, and adults is one major difference between life stages. This size difference could cause major differences in responses to exposures to chemicals. In humans this can be most clearly observed in responses to medication concentrations between adults and infants. Medications are available for adults and infants. If an infant were given an adult dosage of a medication, it could potentially be lethal to the infant. However, that same dosage in an adult could be beneficial. This dilemma of response differences between life stages can, however, be solved for by conducting multiple partial generational studies. But multiple partial generational studies would not record effects carried over from one

life stage to the next. Being exposed to a chemical as a larva, such as an estrogenic compound, could compromise the maturation process, affecting ensuing life stages.

To address the issue of response differences between different life stages, I exposed fathead minnow (*Pimephales promelas*) as adults and as larva to environmentally relevant concentrations of 17β-estradiol (E2). Fish were exposed for 6 weeks and monitored for biologic, physiologic and behavioral responses between treatments; including a BCF, plasma vitellogenin concentration, hepatosomatic index, gonadosomatic index, secondary sex characteristics, developmental stage, liver vacuolization, and predator avoidance response. I hypothesized that there will be a difference in response to the treatments between the two life stages, with the larval fathead minnows showing the greatest sensitivity to estrogenic exposure. These results are from the first year of a three year multi-generational study that will be continued in ensuing years.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The experiment was conducted at the USGS Upper Midwest Environmental Sciences Center in La Crosse, WI. The six week exposure was performed utilizing 1,136-liter exposure tanks placed in three 0.004 hectare ponds to limit temperature fluctuations in the tanks. The experiment was conducted outdoors in a cage with netting covering the outside to limit abiotic and biotic non-treatment factors, such as predation. Tanks were randomly assigned for exposure treatment and age of fish. The system contained three water treatments; a control, and two E2 exposure concentrations (see Section Exposure Chemicals); and two age treatments; larvae and

adults. There were five replicates for each adult water treatment, and four replicates for each larval water treatment. A continuous flow dilutor system was used, adding 42 L of water to each tank every hour. Water quality was analyzed semi-weekly for selected water quality characteristics (see Section Water Quality). Adult fathead minnow were randomly distributed to the pre-designated tanks so that each tank contained 10 males and 10 females. Larval fathead minnow were randomly distributed into the pre-designated tanks at a density of 100 larvae per tank. At the end of the exposure period, adult fathead minnow were analyzed for biologic and physiologic endpoints. Larval fathead minnow were analyzed for a behavior endpoint.

EXPOSURE CHEMICALS

Stock solutions were prepared in three identical 500-mL amber bottles using 100% reagent-grade ethyl alcohol (EMD Chemicals) and different concentrations of 17β-estradiol (E2, Sigma-Aldrich Co.) to achieve final nominal concentrations of 10 and 30 ng/L E2. Four mL treatment spikes were then drawn from the stock solutions and pipetted into 2-mL Eppendorf microcentrifuge safe-lock tubes (Sigma-Aldrich, Inc.) and stored at 4°C until use. For both treatments, exposure solutions were prepared semi-weekly by mixing the 2-ml treatment spikes in 10 L of deionized water in 18.9-L glass carboys. The carboys were spray painted black prior to use in experiment. After addition of E2 spikes to the water, carboys were gently agitated for ten seconds, the top of the carboys were covered with aluminum foil and placed in a temperature controlled water bath. A stainless steel tube was used to draw the E2 exposure solutions directly into a headbox mixing chambers. Water from the mixing chambers was then delivered pumped to the exposure tanks at a rate of 1.73 mL/min.

EXPOSURE ORGANISMS

Fathead minnows were reared and maintained at the USGS Upper Midwest Environmental Sciences Center in La Crosse, WI. Larvae were reared indoors at a temperature of 13°C. Adults were maintained in outdoor culture facilities and subject to seasonal temperatures. Prior to use in the exposure experiment, adults were transferred indoors where they were acclimated to 25°C by increasing the temperature at a rate of 3°C/day. Photoperiod for indoor culture tanks was 14 hours light and 10 hours dark. Adult and larval fathead minnows were then transported to the outdoor tanks for the exposure experiment.

ANALYSIS

WATER QUALITY

Water samples were taken semi-weekly and analyzed for E2 levels. Samples were collected using 125 mL amber glass containers and were taken from each exposure tank on each sample date. Samples were stored on ice and delivered to the Minnesota Water Science Center (MWSC) in Mounds View, MN. At MWSC, water samples were analyzed utilizing a 17β-estradiol competitive, magnetic particle ELISA kit (Abraxis, Warminster, PA). Water was also monitored for temperature, pH, and dissolved oxygen.

BODY CONDITION FACTOR

At the completion of the exposure, adult fathead minnow were removed from the tanks, placed into pre-labeled transport tubes (PVC tubing with mesh netting at each end of the tube), keeping tanks separated, and placed into coolers filled with control water. Water in the coolers received supplementary aeration utilizing battery powered aerators (Frabill Inc.). Coolers were then transported to St. Cloud State University. Within 24 hours of transport to St. Cloud State University, fish were sacrificed using tricaine methanesulfonate (MS222). Body weights were then measured to the nearest 0.001 g. Standard and total lengths were measured to the nearest 0.5 mm. BCF was calculated by dividing the total weight by the total length cubed and multiplying by 10,000 $((TW/(TL^3))*10,000)$ for each fish.

PLASMA VITELLOGENIN ANALYSIS

Once the fish were deeply anesthetized, the tail of the fish was severed at a point immediately posterior of the anal pore and, utilizing heparinized micro hematocrit tubes, blood was harvested from the anterior portion of the fish. Blood was immediately centrifuged at 5,000 rpm for five minutes to isolate the plasma. Plasma was collected, placed into pre-labeled 0.5 mL Eppendorf microcentrifuge safe-lock tubes (Sigma-Aldrich, Inc.) and stored in a -80° freezer until vitellogenin analysis using a competition antibody-capture ELISA.

Microtiter plates were coated (except for one microtiter well per assay plate) using purified fathead minnow vitellogenin at a 1:4000 dilution with coating buffer (0.35M sodium bicarbonate, 0.15M sodium carbonate, pH 9.6). One well was coated with coating buffer and

PBS+BSA buffer (1X PBS, pH 7.5 + 1% BSA) at a 1:1 dilution. Plates were allowed to incubate at room temperature for at least one hour. Plasma samples were thawed and diluted in PBS+BSA buffer to dilutions of 1:50, 1:250 and 1:1000 (plasma to buffer). Standard curve dilutions were prepared as seven-step twofold serial dilutions with a range of 4.8ug/mL to 0.0375ug/mL by diluting purified fathead minnow vitellogenin in PBS-BSA buffer. Internal standards were also included at 1:50 and 1:100 dilutions of control fathead minnow vitellogenin. A maximum binding control, a true-blank control and a BSA-coated well control were also included on each plate. A primary antibody solution was then prepared by diluting a polyclonal anti-fathead minnow vitellogenin antiserum (courtesy of Gerald LeBlanc, NC State University) with PBS-BSA buffer to a concentration of 1:10,000 (species specific antibody to PBS-BSA buffer). The primary antibody was then added to each sample dilution, the standard curve dilutions, the maximum binding control and the BSA-coated well control at a 1:1 volume to volume ratio. All dilutions were allowed to incubate at room temperature for a minimum of one hour, and not exceeding one and a half hours.

Following washing of the plates with a wash buffer in an automated plate washer, sample dilutions, standard curve dilutions, internal standards and controls were added to plates and allowed to incubate at room temperature for a minimum of one hour, and not exceeding one and a half hours. Plates were again washed with a wash buffer in an automated plate washer, followed by coating of a secondary antibody solution. The secondary antibody solution was prepared by dilution a horseradish peroxidase labeled anti-rabbit IgG (Sigma, St. Louis, USA) in PBS-BSA buffer to a dilution of 1:10,000 (antibody to PBS-BSA buffer). Plates were again allowed to incubate at room temperature for at least one hour, and not exceeding one and a half hours. Following the incubation period, plates were washed with a wash buffer in an automated

plate washer, and TMB substrate (Sigma, St. Louis, USA) was subsequently added to the plates and allowed to incubate for 15 to 20 minutes. Plates were then read at 620nm on a Multiskan EX (Thermo Electron) plate reader.

HISTOPATHOLOGY

Liver and gonads were exercised immediately following collection of blood. They were weighed to the nearest 0.001g, placed into pre-labeled histocassettes and placed in 10% buffered solution until the tissues could be histologically analyzed (Appendix A). Slides were analyzed for the sex of the fish, graded for the developmental stage of the gonad, and graded for the severity of liver vacuolization. Gonads were graded on a scale from 0 (immature) to 5 (completely spawned out). Livers were graded on a scale of 0 (no vacuoles) to 4 (vacuoles dominate). Hepatosomatic indicies (liver weight/whole body weight) and gonadosomatic indicies (gonad weight/whole body weight) were subsequently calculated.

SECONDARY SEX CHARACTERISTICS

Prior to the removal of the tail, male fathead minnow were evaluated for secondary sex characteristics modified after Smith (1978).The prominence of nuptial tubercles was scored on a scale of 0 (absent) to 3 (prominent). The dorsal pad was scored on a scale of 0 (absent) to 3 (prominent). Banding coloration intensity was scored on a scale of 0 (absent) to 3 (intense). For statistical analysis, the scores of the three secondary sex characteristics were summed and compared between treatments.

PREDATOR AVOIDANCE BEHAVIOR

At the completion of the exposure, ten larval fathead minnow were randomly sampled from each exposure tank and placed into separate 9.5-L pails (per exposure tank) that contained control water. Larvae were randomly taken from the pails and subjected to a predator avoidance behavior test (Appendix B). Each pail was completely analyzed before fish from another pail were subjected to the predator avoidance behavior test. Behavior tests were performed on one replicate from each treatment before moving on to the next set of replicates. Upon completion of the predator avoidance behavior test, larva were euthanized with MS222.

STATISTICAL ANALYSIS

All data sets were analyzed for assumption of normality using the Kolmogorov-Smirnov test for normality (PASW Statistics 18, IBM Corporation, Somers, NY). One-way ANOVA followed by Bonferroni post-hoc test was used to analyze data that met standards of normality. Kruskal-Wallis analysis followed by Dunn's post-hoc test was used to analyze data that did not meet standards of normality. A two-sample t-test was used for comparison of E2 levels between adult and larval exposure tanks. A significance level of 95% ($p < 0.05$) was used for all tests.

RESULTS

WATER QUALITY

Estradiol analysis of water indicated that 17β-estradiol (E2) levels were at concentrations relative to expected concentrations (Figure 10), allowing for control, low, and high concentration levels. The lowest variability was observed in the larval control treatments, however the control treatments demonstrated the highest variance between larval and adult tanks ($p = 0.0003$, two sample t-test). Control treatments had a median of 2.93 ppt, with an interquartile range of < 4.00. The low concentration treatments (10 ng/L) had a median value of 20.45 ppt, with an interquartile range of 24.15. High concentrations showed the largest variability and had median value of 35.73 ppt, and an interquartile range of 58.30. It is interesting to note that a large increase in E2 levels in all high concentration tanks coincided with an observed phytoplankton population crash. Water characteristics remained stable throughout the experiment. Mean (±SD) water characteristics during the exposures were: temperature = 22.0 ± 0.89°C; pH = 9.27 ± 0.31; and dissolved oxygen = 9.35 ± 0.69 mg/L.

ANATOMICAL ENDPOINTS

Adult fathead minnows showed a dose-dependent decline in BCF with an increase in E2 concentrations (Figure 11). Males showed a significant difference ($p = 0.001$) between treatments, with control fathead minnows having a significant increase in BCF as compared to the 10 ng/L and 30 ng/L treatments. Females also showed a significant difference ($p = 0.001$) between treatments, with the 30 ng/L treatment showing a significant decrease in BCF as

compared to control and 10 ng/L treatments. Organosomatic indices (hepatosomatic index, HIS, Figure 12; gonadosomatic index, GSI, Figure 13) also did not differ between treatments in females. However, significant differences were observed between treatments in males for both organosomatic indices (HSI, $p < 0.001$; GSI, < 0.001). Differences in HSI and GSI were found between the control and 10 ng/L treatments, and the 10 ng/L and 30 ng/L treatments. The highest HSI was found in the 10 ng/L treatment in both males and females, although not at a significant level in females. The highest GSI was also found in the 10 ng/L treatment in both males and females, although again not at a significant level in females. Sum of secondary sex characteristics (dorsal pad, nuptial tubercles, banding coloration intensity) showed a significant dose-dependent decrease ($p < 0.001$), with differences between control and the 10 ng/L and 30 ng/L treatments (Figure 14).

HISTOLOGICAL ASSESSMENT

A difference in developmental stage of males was observed (Figure 15), with the 10 ng/L males being at a significantly further developmental stage than control ($p = 0.008$) and 30 ng/L males being at a stage between control and 10 ng/L males, although not at a significant level. No significant difference was observed in the developmental stage of females (Figure 15), though there is a slight dose-dependent decrease in developmental stage with an increase in E2. Females did have a dose-dependent increase in liver vacuolization with an increase in E2 (Figure 16), with the 30 ng/L treatment being significantly higher ($p = 0.002$) than control or 10 ng/L treatment females. Males also showed a dose-dependent increase in liver vacuolization with an increase in E2 (Figure 16), albeit not at a significant level.

PLASMA VITELLOGENIN CONCENTRATION

Males had a significantly higher plasma vitellogenin concentration ($p < 0.001$) in the control treatment as compared to the 10 ng/L or 30 ng/L treatments (Figure 17). Plasma vitellogenin concentrations were the lowest in the 10 ng/L treatment. Females showed the same plasma vitellogenin concentration pattern as the males (Figure 17), although not at a significant level ($p = 103$). Average plasma vitellogenin concentrations for the males were about 33% lower than those in the corresponding females.

PREDATOR AVOIDANCE BEHAVIOR

The ability of larval fathead minnows to respond to a stimulus simulating a predator showed no significant differences between treatments for all predator avoidance behavior endpoints (Figure 18). However, fish in the 10 ng/L treatment exhibited the best predator avoidance behavior response by having the shortest latency period, highest velocity and fastest total escape response. In contrast, control fish exhibited the worst predator avoidance behavior response by having the longest latency period, lowest velocity and slowest total escape response.

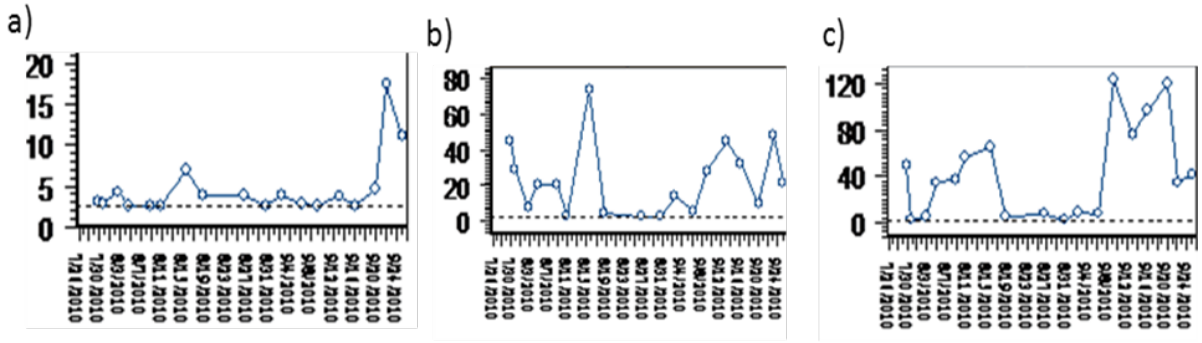

Figure 10. 17β-estradiol concentrations (ppt) in treatments: a) Control, b) Low, and c) High

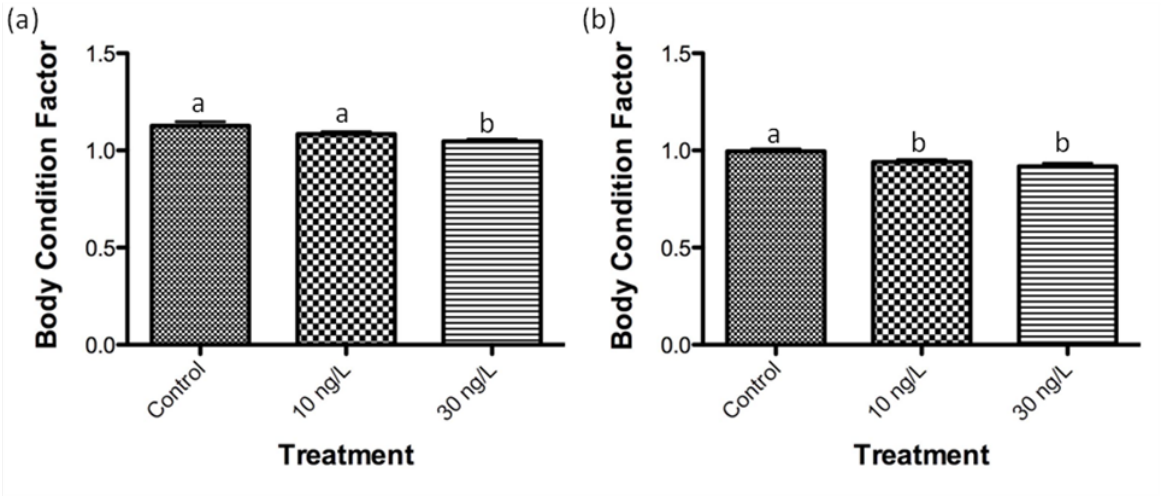

Figure 11. Body condition factors adult fathead minnow across three different 17β-estradiol treatments. Mean ± standard error. Letters indicate statistically significant differences between treatments. (a) Males (Kruskal-Wallis with Dunn's post-hoc test, $p < 0.05$); (b) Females (One-Way ANOVA with Bonferroni post-hoc test, $p < 0.05$)

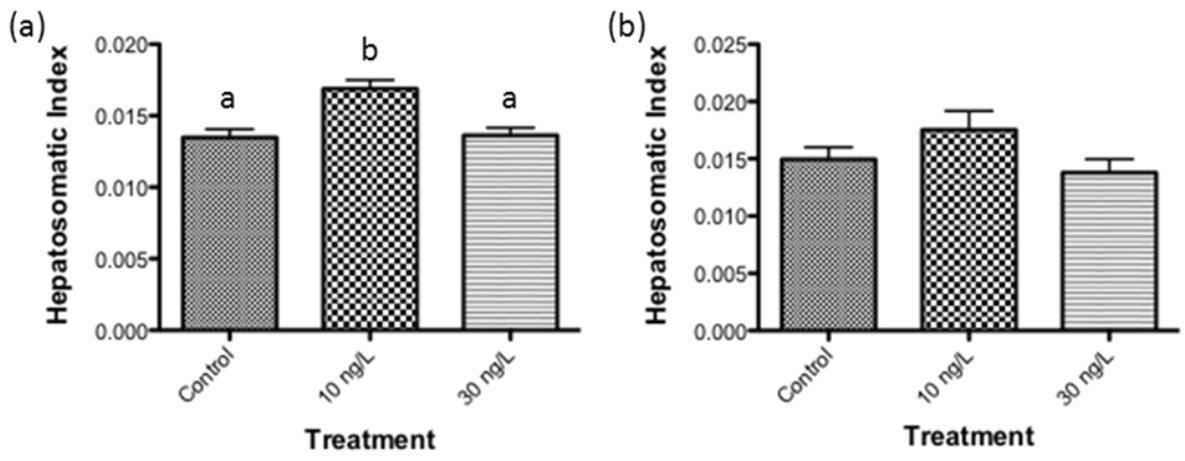

Figure 12. Hepatosomatic index of adult fathead minnow across three different 17β-estradiol treatments. Mean ± standard error. Letter represent statistically significant difference between treatments (One-way ANOVA with Bonferroni post-hoc test, $p < 0.05$). (a) Males; (b) Females

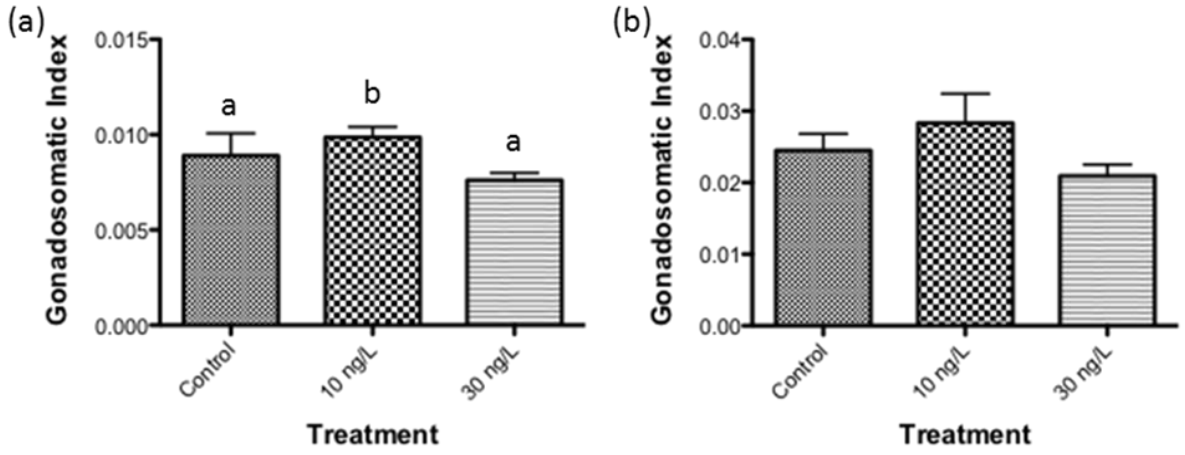

Figure 13. Gonadosomatic index of adult fathead minnow across three different 17β-estradiol treatments. Mean ± standard error. Letters represent statistically significant difference between treatments (Kruskal-Wallis with Dunn's post-hoc test, $p < 0.05$). (a) Males; (b) Females

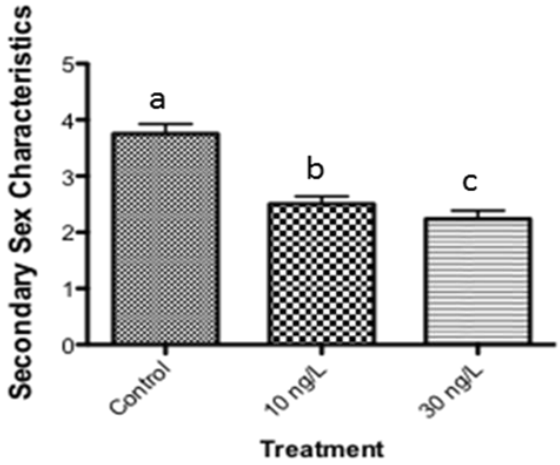

Figure 14: Secondary sex characteristics of adult male fathead minnow to cross three different 17β-estradiol treatments. Mean ± standard error. Letters represent statistically significant difference between treatments (Kruskal-Wallis with Dunn's post-hoc test, $p < 0.05$)

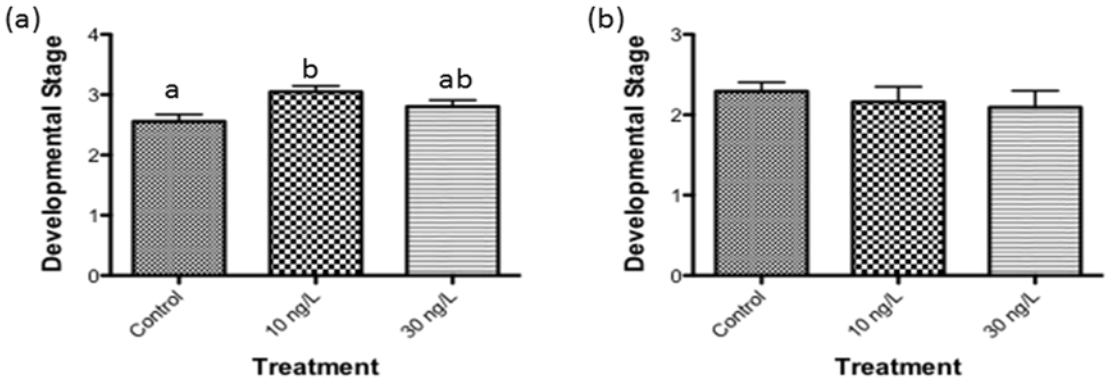

Figure 15. Developmental stages of adult fathead minnow across three different 17β-estradiol treatments. Mean ± standard error. Letters represent statistically significant difference between treatments (One-way ANOVA with Bonferroni post-hoc test, $p < 0.05$). (a) Males; (b) Females

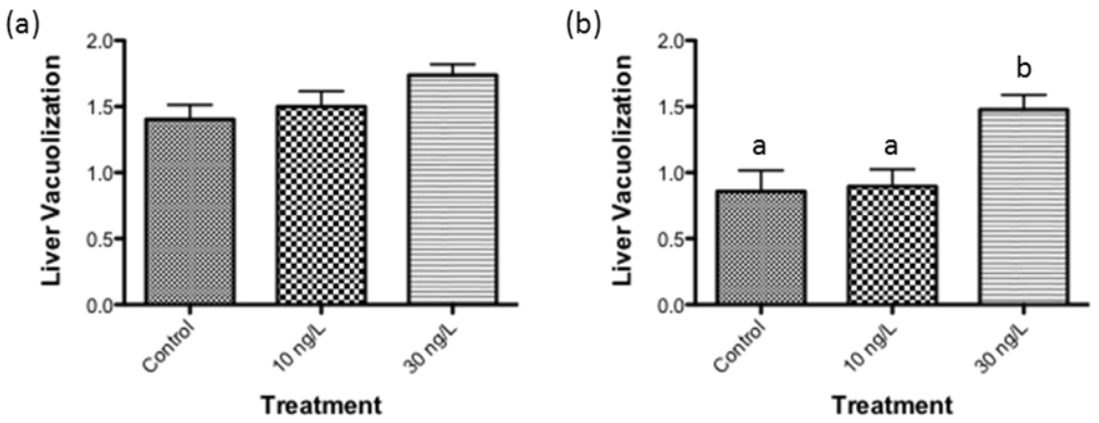

Figure 16. Live vacuolization of adult fathead minnow across three different 17β-estradiol treatments. Mean ± standard error. Letters represent statistically significant difference between treatments (Kruskal-Wallis with Dunn's post-hoc test, $p < 0.05$). (a) Males; (b) Females

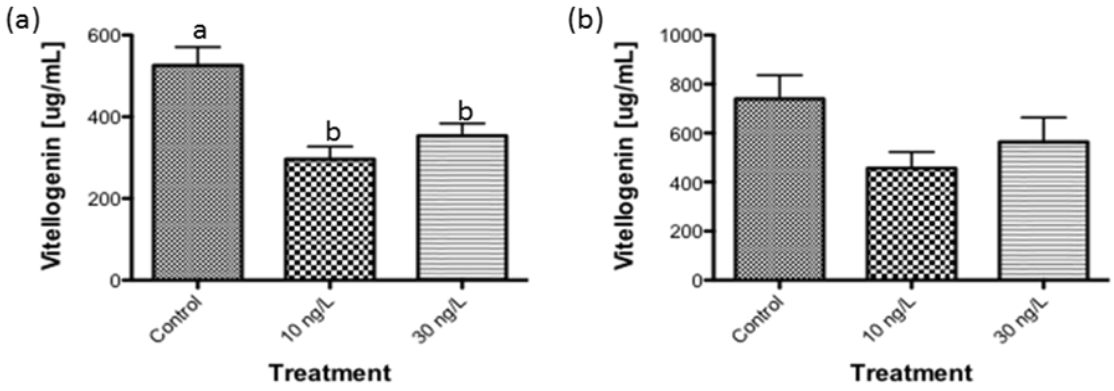

Figure 17. Plasma vitellogenin concentration of adult fathead minnow across three different 17β-estradiol treatments. Mean ± standard error. Letters represent statistically significant difference between treatments (Kruskal-Wallis with Dunn's post-hoc test, $p < 0.05$). (a) Males; (b) Females

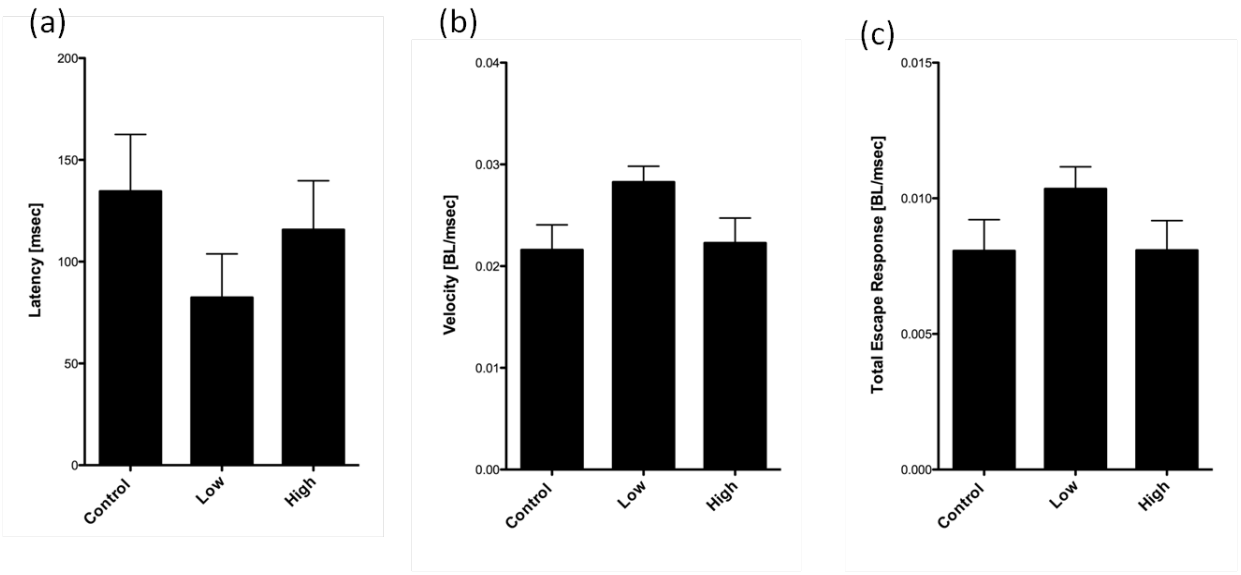

Figure 18. Predator avoidance behavior of larval fathead minnows across three 17β-estradiol treatments. Mean ± standard error. (a) Latency; (b) Velocity; (c) Total Escape Response.

DISCUSSION

A majority of toxicological exposure experiments are conducted as partial generation studies; studies which are carried out for duration of time that is less than the complete life cycle of the organism. These studies are beneficial in that they can show possible exposure effects in a shorter amount of time that a multi-generational study is able to. However, not all exposure effects may be observed during a partial generation study. Effects that only manifest after long-term accumulation in the body, or effects that are only present on the subsequent generation will not be measured during a partial generation study. Partial generation tests are also predominately performed on one life stage of the organism. Differences within an organism, such as metabolism

or size, at different life stages may affect the interaction a chemical has on the organism. With larvae being a smaller size than adults, a lower concentration of a chemical may cause more drastic effects on the larvae than the adult. The purpose of my experiment was to add to the limited knowledge as to the possible differences in exposure effects that 17β-estradiol (E2) may have on different life stages of the fathead minnow.

Results from the organosomatic indices (hepatosomatic index, HSI; and gonadosomatic index, GSI) of the adult fathead minnows indicate the strongest response to estrogenic exposure can be seen in the 10 ng/L treatment, however not at a significant level in females. This supports previous studies that have seen an increase in response to estradiol exposure at lower levels than at high estradiol levels (Hyndman et al. 2010, Panter et al. 1998). The increase in GSI in females would be expected after exposure to estradiol because of the influence estradiol would have on the increase of egg production. Although developmental stage of females was not significantly different, and control females had the highest developmental stage, this is not entirely unexpected. Although an increase in estradiol may cause eggs to develop faster, it would also cause an increase in egg development at different stages. This is seen through the GSI, in which there was an increased production of eggs in the female. It is interesting that E2 exposure would cause increased GSI in males as well. This could be expected if intersex became apparent, which was not the case. The increase in estradiol actually caused an increase in developmental stage (increased sperm) in the males. In both GSI and developmental stage, the males did see a significant increase in both from control to the 10 ng/L treatment.

The increase in HSI is another biomarker that would be expected after E2 exposure. Increased estradiol in fish could cause an increase in vitellogenin production, thereby increasing the weight of the liver. Another possible cause for increased liver weight could be an increase in

liver vacuolization, which can be seen in fish after stressful conditions are encountered. Female fathead minnows had the highest HSI in the 10 ng/L treatment with control and 30 ng/L treatments having similar HSI. The 30 ng/L treatment had a significantly higher amount liver vacuolization than the control or 10 ng/L treatments, with the control and 10 ng/L treatments having similar levels of vacuolization. For vitellogenin concentrations in females, the control treatment had the highest concentration, although not to a significant level. The same relationships are observed in the male fathead minnow. Liver vacuolization is dose-dependent with increasing vacuolization associated with higher E2 levels. Vitellogenin concentrations for males are highest in the control treatment. The variation seen in vitellogenin concentrations, liver vacuolization and HSI do not follow an expected correlation pattern for males or females.

The sum scores of secondary sex characteristics show feminization of males in a dose dependent manner, with decreasing secondary sex characteristic scores as E2 levels increase. This supports previous findings by Shappell et al. (2010) that also saw a decrease in secondary sex characteristics in males at higher E2 levels. Having decreased secondary sex characteristics could have the possibility to lower the chances that a male will successfully find a female for mating, lowering his reproductive fitness. The male may be seen by females as an inadequate mate. Males, along with females, also showed a dose-dependent decrease in body condition with an increase in E2. This decrease in body condition together with decreased secondary sex characteristics could cause exposed males to have an even lower reproductive fitness.

Larval fathead minnow showed corresponding exposure effects after a predator avoidance behavior test. The 10 ng/L treatment demonstrated the largest change from the control measurements. The 30 ng/L treatment showed similar predator avoidance behavior as the control treatment. The 10 ng/L treatment larvae showed the fastest total escape response after

stimulation. In a corresponding study (conducted at the USGS Yankton Field Research Station), juvenile fathead minnows showed similar results in which the juveniles in the 10 ng/L treatment showed the fastest predator escape response. However, this may not be advantageous. If a predator escape response is only needed at a certain speed, anything faster than that would be a waste of energy expended. This increase in response may be caused by an increase in cortisol in the organism. Because of an increased production of cortisol, production of hormones would most likely be reduced. This reduced production of the other hormones may have detrimental effects on other aspects of the organism.

Overall, this experiment shows similar effects to estrogenic exposure between different life stages of the fathead minnow. Exposures to E2 appears to have a greater effect on adults, larva and juveniles at a concentration of 10 ng/L as compared to 30 ng/L. All three life stages also showed increased effects with exposure to lower levels of E2 as compared to the high E2 levels. Effects of observed in fish exposed to 30 ng/L more closely resembled those observed in the controls compared to those exposed to 10 ng/L. However, these deductions are made with some hesitation. Although these patterns were observed between treatments, the number of observations that were significant are limited. But the reasonable number of samples per treatment makes me reasonably confident in drawing these deductions.

Chapter V

CONCLUSIONS FOR CONTINUED RESEARCH IN CHEMICAL POLLUTION WITHIN THE AQUATIC ENVIRONMENT

Over the last half of a century, the general public has become increasingly concerned with the harmful effects that pollutants may have on our aquatic ecosystems. Originally, most of the concern was over how the chemicals may cause harm to the human population. Over the years, however, concern over the protection of aquatic life as well as human health has steadily increased as our understanding of the complex interactions that take place within the aquatic environment has improved. To protect humans and aquatic life, the United States government enacted the Clean Water Act in 1972. In subsequent years, the Clean Water Act has been amended numerous times to improve the effectiveness of the act. Other regulations have also been implemented that contribute to the protection of aquatic life, including the Endangered Species Act. The Endangered Species Act contributes to the protection of aquatic life by not only implementing restrictions directly on the species, but also on the habitat in which the species lives.

Unfortunately, because of the limitations of population sizes and the Endangered Species act itself, the protection of aquatic species from chemical pollution is accomplished through the use of a surrogate species. These surrogate species are commonly termed “model” species. Model species are the most commonly used species in toxicological studies. The reason that these species are chosen for toxicological studies is because of their short life cycle, their ease of availability and their ease of maintenance in the laboratory (Ankley and Villeneuve 2006). The model species that are most often used for studying effects of endocrine active compounds are the fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), bluegill sunfish (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*). A considerable benefit to using a model species in toxicology, especially with emerging contaminants, is the ease in relatability of exposure effects to a chemical in a species with known exposure effects to a wide array of chemicals. This allows for a faster implementation of regulations in light of new chemicals. What is not as well known is how do these model species actually represent other species where the effects are not known. Model species are frequently represented in multiple habitats throughout the nation. Endangered species, by definition, are not as widely distributed because of some constraint on the species. It could easily be assumed that a species which has no difficulty adapting to different habitats might not adequately represent a species with limited adaptive ability.

Existing guidelines for the testing of chemicals for the recommendation of government regulations include the use of a limited number of surrogate species tested of a short exposure period (Stephan et al. 1985). These limited number of surrogate species consist of members from nine different families, and are used to represent all species within the habitat. These nine species are tested, and their resulting effects are complied to create a recommendation based on a

calculated Criterion Maximum Concentration value that is meant to protect 95% of the species in that habitat. These tests are also commonly conducted over a short exposure period, representing only a portion of the complete life cycle of the organism. Knowledge as to the effects of accumulation over an extended period, or the effects over multiple generations is commonly not known. Too often, chemicals are not tested beyond these limited species over the limited exposure period.

The Rio Grande silvery minnow (*Hybognathus amarus*) is a species that is listed as an endangered species by the United States government (US Fish & Wildlife Service 2007) and is considered by some as one of the most endangered species in the country. It has seen an extensive decrease in habitat range during the past half of a century, currently residing in around 7% of its historical home range (US Fish & Wildlife Service 2007). The fathead minnow is the surrogate species that the government uses for the Rio Grande silvery minnow (Buhl 2002). While the Rio Grande silvery minnow has seen a decline in home range along the Rio Grande River, the fathead minnow has continued to be observed in all reaches of the river (Carter 1995). It is believed that a major cause for the reduction in home range for the Rio Grande silvery minnow is the introduction of multiple dams and diversions along the river (US Fish & Wildlife Service 2007). It is possible that the fathead minnow has not seen this decline because of its prevalence as a bait fish, allowing for a continual reintroduction into the river. Another possibility could be found in the reproductive methods employed by each species. Rio Grande silvery minnow are pelagic spawners where eggs are released, fertilized and carried downstream. Upon maturation, the Rio Grande silvery minnow historically would swim upstream to reside as adults. With the building of dams and diversions, the migration patterns of the Rio Grande silvery minnow have been obstructed. Fathead minnows, on the other hand, are nest spawners

where eggs remain in the same area where they are spawned. This would be beneficial for a species living in an area with multiple dams and diversions that would obstruct migration.

Through my experiments I attempted to address two issues seen in the field of aquatic toxicology. The first is the limited number of species that are tested against chemicals for exposure effects and the use of these exposure effects to represent all species in the environment, even when the relatability of these species to vulnerable species it is not clearly understood. The second issue I attempted to address is the limited knowledge of effect difference between different life stages of the same species.

I found that there was a difference in exposure effects between the Rio Grande silvery minnow and its surrogate species, the fathead minnow. The Rio Grande silvery minnow showed an increased sensitivity to 17β-estradiol as compared to the fathead minnow. While there was a difference in sensitivity between these two species, both species showed an even greater difference when compared to a third species, the bluegill sunfish, which was also exposed concurrently. The results indicate that phylogenetic relationships may assist in understanding possible exposure effects of species with unknown effect responses, these relationships should not be used to assume that exposure effects in one species will completely represent the effects in on another species.

I also found that, with respect to fathead minnow, life stages show similar exposure effects when exposed to 17β-estradiol. Adult, larval and juvenile fathead minnow showed the same increase in response to 10 ng/L 17β-estradiol as compared to a control treatment. A subsequent decrease in response was observed when exposed to 30 ng/L 17β-estradiol as compared to the 10 ng/L treatment. These results indicate that testing of chemicals on one life

stage of the fathead minnow could possibly be used to represent the effects that chemical may have on other life stages.

Although my study indicates that similar exposure effects are observed at different life stages of the fathead minnow, they do not attempt to answer the effects that may be seen after prolonged exposure throughout the entire life cycle, and subsequent exposure effects that are passed on to following generations. My involvement in the study on fathead minnow life stages was only during the first of three years. Conclusions drawn from the completion of the study will allow for a better understanding as to the effects of chemical accumulation in an organism and effects on subsequent generations.

The questions and conclusions drawn from my study may paint a bleak picture of the field of aquatic toxicology. This is not my intent nor is it what I think should be deduced from my project. What should be recognized is the opportunity available for future research, and the need for continued research. In order to have a complete understanding of the effects chemical pollution can have on the environment, we need to be willing to explore all possible effects. Complacency has never been a part of the scientific community, nor do I see it ever happening. There will always be the next generation of scientists eagerly waiting to try to understand the complexity of the environment and the part we, as humans, play in it.

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APPENDICES

APPENDIX A

HISTOPATHOLOGY

TISSUE PROCESSING

Histo-cassettes containing fish tissues were removed from 10% buffered formalin and placed into cassette baskets. Tap water was run over the cassette baskets for a few minutes to remove the 10% buffered formalin. Cassette baskets were then placed into a plastic container and a 50% ethanol solution was added to the container until the cassette baskets were submerged. A stir bar was placed into the containers, and the containers were then placed on a stir plate. The stir plate was subsequently turned to a low setting and the cassette baskets were allowed to sit in the 50% ethanol solution for a minimum of two hours, but no more than 24 hours. After the incubation period, cassette baskets were removed and allowed to drain for a minute to remove as much excess 50% ethanol solution as possible from the histo-cassettes. The 50% ethanol solution was removed from the container and the cassette baskets were placed back into the container. The submersion steps were repeated for a 75% ethanol solution, 95% ethanol solution, 100% ethanol I solution, 100% ethanol II solution, xylene I, and xylene II. Upon completion of

the final xylene bath, cassette baskets were placed into a warm, liquid paraffin bath for a minimum of 24 hours but no more than 72 hours.

TISSUE EMBEDDING

Cassette baskets were removed from the paraffin bath and placed into the cassette holding area of the Microm Ec 350-1 embedding machine (Thermo Scientific). For the juvenile Rio Grande silvery minnow from the first year study, the sections of fish were placed laterally into paraffin molds and covered in warm paraffin. For the juvenile fish from the model species study, a small piece (about 2mm) of the anterior region of the tail section was removed and placed horizontally into paraffin molds. The pieces were then covered in warm paraffin. For the multi-generation study, a small portion (about 1mm around) of the gonad and liver were removed and placed into paraffin molds. The pieces were then covered in warm paraffin. For all studies, after the pieces were covered with paraffin, the labeled piece of the histo-cassette was placed on top of the mold (for identification purposes) and excess pieces of tissue were placed on top of the histo-cassette. Warm paraffin was added to mold the histo-cassettes with the tissue block. Molds were then placed on a Tissue-Tek Cryo Console (Miles Scientific) and allowed to sit for a minimum of 15 minutes. Molds were then removed from the chilling plate and allowed to sit at room temperature for at least 24 hours before samples were removed from the mold.

SECTIONING AND STAINING

Histo-cassettes were placed in the cassette holder on the 2030 BIOCUT microtome (Leica Reichert-Jung). Sections were made at a thickness of 4 µm. Sectioned ribbons were placed onto the water surface of a warm water bath that had tissue section adhesive (Richard-Allan Scientific) mixed in with the water. Ribbons were then placed onto pre-labeled slides and set aside to allow drying of the ribbons to the slide. Once the slides were dry, they were subsequently placed into slide staining racks and placed into an Autostainer XL automatic stainer (Leica Reichert-Jung). The slides were stained using a common haematoxylin and eosin counter stain procedure modified after Gabe (1976). Slides were then removed from the rack upon completion of the counter staining procedure and, using cryoseal-60 mounting medium (Richard-Allan Scientific), micro cover glass (VWR Scientific) was placed over the samples on the slides. The slides were then allowed to dry for a minimum of 72 hours to ensure complete drying of the cryoseal.

APPENDIX B

C-START PREDATOR AVOIDANCE BEHAVIOR TEST

C-start predator avoidance behavior tests were conducted as modified after McGee et al. (2009) and Painter et al. (2009). Fish were placed into the testing arena and allowed to acclimate while swimming freely. The temperature of the arena water was measured using a Fluke 62 Mini IR Thermometer and recorded. Upon acclimation, the tester would wait until the fish swam into the center portion of the grid paper that was marked with a square. The center portion was drawn using grid paper and each side of the square at least 2.5 times longer than the fish with each corner remaining at least one body length away from the side of the test arena. Once the fish swam into the center portion, the tester would depress the video trigger and switch on the power strip simultaneously. If a C-start behavior was observed, the video would be edited to start at the point when the stimulus was initiated and end at 100 frames past the C-Start behavior. The videos were then saved as an .AVI file. If no C-start behavior was observed, it was recorded as no reaction for the 1st try and initiating of c-start behavior would be attempted again. If a C-start was observed, the video edited to appropriate length (as described above) and then saved as an .AVI file. The file would be labeled as being the second attempt. If no C-start behavior was observed, it was recorded as no reaction both tries. A short interval of video

around 100 frames long, starting at the initiation of the stimulus, was saved as an .AVI file and labeled as being a no reaction.

Upon returning to St. Cloud State University, AVI files were converted to MOV files for analysis using NIH Image. The length of the fish was calculated by labeling the anterior-most tip of the snout and the posterior-most tip of the tail, in addition to two points representing 1-mm on the grid. Latency period (time of induction of the behavior) was calculated by marking the point at which the stimulus was initiated and measuring the length of time until an evasive maneuver was initiated. Escape velocity was measured by recording the distance the fish swam during the first 40 msec post initiation of an evasive maneuver, and dividing the calculated body length by this distance. Total escape response was calculated by dividing the calculated body length by the latency period plus 40 sec (body length/ (latency + 40 msec)). Videos were not considered if: (1) latency response was less than 5 ms (false start), (2) the first movement of the fish after the stimulus was a non-escape turn or (3) the fish swam out of the field of view during the recording.