

AFLP MARKERS DEMONSTRATE MALE HETEROGAMETY IN A
FATHEAD MINNOW (*Pimephales promelas*) POPULATION

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of DANIEL JOSEPH MISTAK find it satisfactory and recommend that it be accepted.

Chair

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Abstract

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The fathead minnow (*Pimephales promelas*) is an interesting study subject in environmental toxicology. To further develop its usefulness as a research aid, a genetic marker that can easily and quickly determine the sex of research subjects is necessary. Genetic analysis by amplified fragment length polymorphism (AFLP) of groups of fathead minnows from a research population demonstrated male heterogamety (an XX/XY method of sex determination). AFLP studies done on the individuals in the population also demonstrated male heterogamety. The male-specific bands were then removed from the AFLP gel, amplified and cloned into *E. coli*. Sequence analysis allowed for the creation of PCR primers that demonstrate specificity for male markers. This can be utilized by researchers to find the genetic sex of the fish in toxicological experiments where sex might have been affected by chemical treatments or other studies where phenotypic sex is not yet developed.

When tested with fish from separate populations, we found that the sex marker did not reach species wide. This is not surprising based on what others have found in different fish species, but we have laid the groundwork for later studies that can expand the usefulness of this marker.

This study demonstrated that information taken from AFLP can be utilized to create primers that can be utilized in genetic sex identification. Once a PCR primer can be made that works in the species at large, anyone interested in utilizing this species as a model organism for sex reversal would have useful tools. In order to provide a framework for later researchers, outbred fathead minnows have been sexed by histological methods and DNA has been extracted. These provide the necessary tools for researchers to test species wide markers.

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CHAPTER ONE

INTRODUCTION

It is the very nature of science to take empirical judgments and expand what information can be taken from that information into newer empirical judgments. The problem caused by this is that our empirical judgments often speed past our rational judgments and may often overlook very important questions: such as the consequences of our discoveries' effects on everyday life. A special problem is that many technological advancements, such as pharmaceuticals, are utilized before the scope of their impact is understood. It is becoming clear that some of these pharmaceuticals may not be broken down by sewage treatment and are found in the environment at large. These chemicals have demonstrated an effect on the life of phytoplankton (Harrass, Kinding, & and Taub, 1985), many plants (Batchelder, 1981, Batchelder, 1982), crustaceans, copepods and many other organisms (Halling-Sorensen, et al., 1998).

The question of how a certain chemical may be affecting the environment is one that needs to be analyzed before the technology should be utilized wholesale. Unfortunately, many of our technologies are already ubiquitous; the need for a method to demonstrate an environmental impact and current load is imperative. There are model organisms such as the plant *Thlaspi caerulescens* that can help demonstrate the environmental load of heavy metals (Assucao, Schat, & Aarts, 2003). Even if this method can be utilized to show how much of a chemical is currently in the environment, a way of demonstrating the dangers of the chemicals is needed.

Endocrine disruptors are a family of chemicals that is currently of interest to toxicologists. Endocrine disruptors are chemicals that closely resemble endogenous regulatory chemicals of the endocrine system. Researchers believe that these chemicals may interfere with normal cellular processes (Crisp, et al., 1998). There are many types of chemicals currently referred to as endocrine disruptors; these include: bisphenol A, polybrominated diphenyl ethers, phthalates, polychlorinated biphenyls (PCBs), vinclozlin, zearalenone and several pesticides. Because these chemicals closely resemble endogenous regulatory hormones, they may be dangerous to organisms exposed to them at specific stages of development. An example is the chemical bisphenol A. Industrially, it is used to create hard polycarbonate plastics and epoxy resins. This same chemical coincidentally binds with estrogen receptors, allowing them to induce transcription factors and cause undesirable results (Anstead, Carlson, & Katzenellenbogen, 1997). Researchers have already linked bisphenol A with neurodegenerative diseases (Ooe, Taira, Iguchi-Arigo, & Arigo, 2005), chromosomal non-disjunction (Hunt, Koehler, Susiarjo, Odges, Llagan, & Voigt, 2003), obesity (Sakurai, et al., 2004) and diabetes (Alonso-Magdalena, Morimoto, Ripoll, Fuentes, & Nadal, 2005) in lab tests on mice. Researchers have also found that other endocrine disrupting compounds may cause an epigenetic effect, i.e. one that is heritable in a way that is independent from genetic heredity (Anway, Cupp, Uzumcu, & Skinner, 2005). The chemicals that induce epigenetic inheritance can turn on or turn off different genes by means of DNA methylation. These methylation profiles are passed on the DNA without actually changing the genetic code itself. Another endocrine disruptor 17 α -ethinylestradiol, the main active ingredient in many types of birth control

pills, poses similar problems. Excess amounts of this chemical are excreted and not removed from sewage facilities (Ternes, Stumpf, Mueller, Haberer, Wilken, & Servos, 1999). This could pose a problem to those who depend on municipal water sources for water. One of the most interesting results of these experiments has been the noticeable phenotypic switch of fish that are exposed to high concentrations of these chemicals. A study on the fathead minnow (*Pimephales promelas*) demonstrated that exposure to 17 α -ethinylestradiol at levels greater than 4 ng/L over 305 days led to a sex ratio skewed from the control 50:50 to a ratio of 84:5 females to males with 11% of the fish demonstrating ovatestes, an intermediate sex (Länge, et al., 2000). Another study demonstrated a decrease in egg fertilization and demasculinization of males (Parrot & Blunt, 2004).

There is already substantial evidence of water-borne endocrine disruptors being very problematic to different species. For example, tributyltin (TBT) has already been indicted in the masculinizing of female neogastropod snails (Matthiessen & Gibbs, 1997). The obvious and immediate toxic effects are usually well noted, but there is the problem of long, slow, toxic effects that often go unnoticed. A spill of a toxic chemical, such as oil, often demands public attention, but there are chemicals that have the capability of leaching into the water almost unnoticed. The breakdown of polycarbonate plastics leads to the leaching of endocrine disrupting bisphenol A into water supplies (Hunt, Koehler, Susiarjo, Odges, Llagan, & Voigt, 2003). In the seventies, the breakdown of TNT demonstrated toxic effects on fathead minnow populations and two algal species (Smock, Stoneburner, & Clark, 1976). There is a need for a method to demonstrate endocrine-disrupting chemical effects in nature.

The problem seems clear: there needs to be an organism that can serve as a model for examining and understanding endocrine disruptions both in the lab and in the environment. Because of endocrine disruptor effects on the sex-determining pathways, an ideal candidate for a model species must have an understood method of sex determination as well as a genetic marker to effectively demonstrate the genetic sex. The zebra danio, *Danio rerio*, has a peculiar sex-determining mechanism that is not fully understood yet (Nanda, Scharf, Epplen, Feichtinger, & Schmid, 1992). Although the zebra danio has had much work invested into a genomic mapping program, it has not been widely utilized in toxicology studies. Other possible models have problems with life cycle length, unknown sex determination mechanism or reproductive limitations. The fathead minnow is a fish that could be useful for toxicology research. It has been used extensively on tests that study phthalates, heavy metals and many chemicals that are often found in water supply (Gustavson, Sonsthagen, Crunkilton, & Harkin, 1999). Scientists have already utilized them for histological examinations after exposure to various toxins. The fathead minnow is already an important tool for toxicology researchers, but their usefulness is stunted by a gap in knowledge about the genetic sex determination of the fish.

The fathead minnow (FHM) is already being used in environmental toxicology, including the area of endocrine disruption (Länge, et al., 2000). There are catalogues of assays for different toxicology screenings on FHM. These reports give detailed accounts of dosage amounts and timing, and ways to interpret these results (U.S. Environmental Protection Agency, 1986). But there is an important aspect of biology of this fish species that is not yet resolved. No mechanism for identifying the genetic sex

of individuals is available for the fathead minnow. Because of this, the usefulness and the reliability of research that makes assertions about apparent switches in sex are diminished. The fathead minnow's usefulness could be expanded from solely adult research to that which includes embryos, juveniles and also fish that may have undergone sex reversal. It will be much easier to gain information about possible developmental problems linked to endocrine disruption once a genetic sex-determining mechanism is identified for this species. This will improve the utility of this species as a model for toxicology research.

Fishes are, of course, highly divergent from mammals evolutionarily; however, that does not mean that they are not useful to understand mechanisms acting in mammalian systems. In fact, because many fish species demonstrate a genetic sex determination mechanism and a high sensitivity to endocrine disrupting chemicals they can actually demonstrate the effects these chemicals induce more fully. Once these are examined on the fish models, they can be translated to mammalian models. In most teleost fishes, a small region on the Y chromosome which is not present on the X takes on the role of a sex determining gene (Peichel, et al., 2004). The rest of the Y chromosome crosses over and recombines everywhere but in the small area where the sex locus is. Because an unrecombined gene may become the sex-determining region, it makes it difficult to locate candidate genes for sex determination. In fact, only one teleost fish has had its sex determining gene determined: a duplicated copy of *dmrt1* called *dmy* in the Japanese medaka (Nanda, et al., 2002). It is believed that *dmrt1* was translocated to the sex determining locus of the fish. Mutations changed the function of this gene and it became the sex determining gene for the fish. Furthermore, it has been

demonstrated that the gene, *dmy*, is not the universal sex-determining gene in fishes (Volff, Kondo, & Schartl, 2003). Because teleosts are beginning the process of evolving a sex determining gene, researchers can get a glimpse at the early life of a sex chromosome.

The FHM have been useful for researchers for practical reasons. Sexual dimorphism allows for phenotypic sexing. Cited literature takes for granted normal Mendelian 1:1 segregation (Länge, et al., 2000). This implies that current understanding is that there is a single locus responsible for sex determination in FHM and environmental conditions are not implicated in sex ratios. For example, although zebrafish are labeled with a ZW sex determination method, their sex is still highly dependent on environmental conditions (Devlin & Nagahama, 2002). There currently is no evidence that environmental conditions, such as temperature, other than environmental toxins, are utilized as signals for sex determination in the FHM. Because the fathead minnow is already useful for toxicology studies, it sets itself above the rest of model fish species as a research species in the field of endocrine disruption.

Finding a sex-linked marker would be a great step in the direction of further understanding of sex determination and endocrine disruption. As previously stated, complete phenotypic sex reversal is not uncommon in many fishes, so a sex linked marker is the only truly reliable way of determining the original genetic sex of the animal. A sex marker is imperative to verify a sex reversal event. A second benefit of finding a sex marker is the contribution to the field of embryology. To test the genetic effects from different chemicals, it might be optimal to study effects on embryo development. Without the sex markers, it is necessary to wait until late in development

when they are larger for researchers to discern terminal sexual features. With defined sex markers, scientists could use a PCR test to find the genetic sex of the fish and sacrifice the embryos at any point of interest in development.

This thesis attempts to identify of a sex marker for the fathead minnow, as well as determine the method of sex determination in the species. Teleosts have different methods of sex determination between species (Devlin & Nagahama, 2002). In each species, it is necessary to determine which sex it heterogametic. Some fish demonstrate an XY (males are heterogametic) method of sex determination while others show a ZW (females are heterogametic) mode of sex determination. Complicating the matters more, some fish have a mix of both. Finding a distinctive sex-linked marker from males shows that the fish's sex is determined by an XY mechanism. Finding a marker in the females would show that it is a ZW mechanism.

Another example of why the fathead minnow would be an excellent model fish is that it can be utilized in environmental monitoring systems. Recently, the FHM has been utilized as an environmental watchdog. In Europe, introduced FHM were monitored downstream and upstream of beef cattle farms. Following animal development closely revealed that upstream of the farm, fish developed normally; however, downstream fish demonstrated drastic phenotypic abnormalities. Female fish were highly masculinized. The female fish demonstrated the typical male fat pad and head tubercles. This interesting find indicated that runoff beef hormones were causing the problems noted downstream (Durhan, et al., 2006). With the understanding of the fish's development, scientists can identify water quality issues in areas where there is no great visible threat yet. By using these fish as a veritable smoke alarm, researchers

will be able to better recognize the toxicological fires before they are out of hand.

Furthermore, the European Chemical Bureau, an organization for monitoring and testing environmental contaminants, utilizes the FHM extensively for its toxicology studies (European Chemicals Bureau, 2008).

To recapitulate, there is an ever-increasing realization that environmental toxins need investigation. These toxins come from the products and chemicals used every day and are found, often ubiquitously, in the air, soil and water. Of particular concern to researchers are the effects from water toxins. The fathead minnow has been an obvious and excellent choice thus far, but the lack of sex markers and information about its sex-determining system is a major gap in our current understanding of this organism. Identification of sex markers will facilitate this area of research and allow expansion into related new areas.

CHAPTER TWO

AFLP Markers Demonstrate Male Heterogamety in a Fathead Minnow (*Pimephales promelas*) Population

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Daniel Mistak contributed to the experimental design, collected and analyzed the data, and wrote this chapter.

Joseph Brunelli contributed to the experimental design and data analysis

Gary Thorgaard contributed to experimental design, the writing of this chapter and provided funding.

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Abstract- The fathead minnow (*Pimephales promelas*) is a widely used organism for aquatic toxicology research. A problem with using this organism reliably is that there is no strong evidence to demonstrate whether males or females exhibit heterogamety. We have used amplified fragment length polymorphisms (AFLPs) to find genetic markers that segregate consistently with male fish in a research population of fathead minnows. We were then able to use these results to construct a set of primers that amplified a male product in the fish, demonstrating male heterogamety in this species. Because the fathead minnow's utility as a model organism will be enhanced by an improved understanding of sex determination in this species and eventually isolating reliable sex-linked markers applicable to the species as a whole, this is an important advance.

Introduction

Identifying the sex of fish on a molecular basis and understanding sex determining mechanisms is important for research in sex determination, development and understanding of controlling factors. The fathead minnow is a fish that is currently being widely used for research in aquatic toxicology. Correctly assessing the genetic sex of a specimen is important for understanding what controlling factors may be associated with a change in the sex ratio of offspring from a test.

Teleost fishes have a diversity of sex determination (Devlin & Nagahama, 2002). The heterogametic sex varies among fish species (Bull, 1983). For example, Osteoglossiformes have demonstrated XY sex determination while Esociformes

demonstrate a ZW pattern of inheritance. Complicating matters further, Cypriniformes demonstrate XY, ZW and polygenic sex determining regulations. A commonly used model species, the zebrafish, has shortfalls because of its complex sex-determining pathway (Nanda, Scharl, Eppel, Feichtinger, & Schmid, 1992). Morphologically distinct sex chromosomes have only been described in a few research species (Hartley, 1987; Devlin & Nagahama, 2002). Furthermore, genetic sex linked markers are not nearly as readily available to researchers of fish as they are to researchers on mammals. There have been sex-linked markers identified in several *Oncorhynchus* (Pacific salmon) species (Devlin, McNeil, Solar, & Donaldson, 1994), Japanese medaka (Nanda, et al., 2002), stickleback (Griffiths, Orr, Adam, & Barber, 2000) and African catfish (Kovacs, Egedi, Bartfai, & Orban, 2001), among others, but only one sex determining gene from this group has been found: *dmy* in the medaka (Matsuda, Nagahama, Shinomiya, Sato, & Matsuda, 2002). Due to the difficulty of finding a sex marker, finding which sex exhibits heterogamety is an important initial step towards discovery of a sex marker.

The fathead minnow is of particular interest because it is utilized in many toxicology research protocols. It has been used in environmental estrogen studies and widely used as a test organism for environmental toxins by the European Chemicals Bureau (European Chemicals Bureau, 2008). Although a 1:1 sex ratio appears common in this species (Länge, et al., 2000) there has been no published work that has demonstrated the method of sex determination in the fathead minnow. We have discovered an AFLP fragment linked to male sex in a research population. After

sequence analysis, a primer combination was created that allowed determination of the fish's sex through PCR analysis in the research population.

Materials and Methods

Fish and Sampling

Fathead minnows were obtained from Mid-Continent Ecology Division (Duluth, MN). Fish were euthanized using clove oil. Caudal fin samples were placed in 95% ethanol and stored at 4 degrees Celsius until DNA could be extracted. Phenotypic sex was determined by gross morphological analysis of the gonads.

AFLP Analysis

The overall strategy for identification of sex-linked AFLP markers followed similar studies in our laboratory (Brunnelli & Thorgaard, 2004; Felip, Young, Wheeler, Thorgaard, Neira, & Diaz, , 2005; Felip, et al., 2004) DNA was extracted from caudal fin clips. Tissues were digested by proteinase K digestion (Chen, et al., 2007). DNA was then precipitated by ammonium acetate/isopropyl precipitation. The resulting DNA pellet was washed twice in phenol, once in phenol chloroform and is finally reprecipitated by ammonium acetate/isopropyl precipitation. Extracted DNA was pooled based on sex in order to help eliminate non-sex-linked polymorphisms. Pools were made using 100 ng/ml of genomic DNA from 25 males and 25 females ($n=4-5$ fish/pool). Pooled samples were analyzed using *Bam*HI and *Mse*I restriction enzymes. AFLP-PCR conditions were already described in previous lab publications (Felip, Young, Wheeler, Thorgaard, Neira, & Diaz, 2005). Samples were separated by electrophoresis on denaturing 6% polyacrylamide gels and visualized by fluorescence imaging on a

Typhoon Imaging System (Molecular Dynamics). AFLP bands were manually analyzed to find differences between male and female pools. To determine the reliability of pooled families, primer combinations showing a possible sex marker were analyzed using the same AFLP primers to analyze individual members of the pools.

Cloning of Male-Specific Markers

Bands demonstrating a difference between males and females were extracted from the gel and placed in 50 ul of TE. DNA was reamplified, run on a 2% agarose gel and extracted by following GENECLAN® protocol. The fragment was ligated into PGEM®-T Easy vector and cloned into *E. coli*. Positive colonies were selected and directly amplified using T7 and SP6 primers. Products were extracted following GENECLAN® protocol and sequenced using T7 primers. Sequencing was then performed at the Molecular Biology Core at Washington State University.

Sequence Analysis

Primers were made using Primer3 online primer creating software (Rozen & Skaletsky). Primers used were: 5'-CATCCTATATGTCCTGCCTTAGC-3' for forward and reverse primers were 5'-CGATCTGCACCCTGTCATC-3'.

Analysis of Sex Marker

Primers were run in a 20 ul PCR reaction using Promega Taq polymerase. PCR reaction was 30 cycles with a 2 minute 94° predwell and a 2 minute 72° postdwell. Cycle temperatures were 94° melting temperature for 50 seconds, 62° annealing temperature for 50 seconds and a 72° elongation temperature for one minute. Analysis

was done on the family of fish from which the AFLP band was characterized. Banding was noted in the lanes containing male fish.

Results

Screening of male-specific bands

AFLP analysis of 4 pools of 4-5 individuals/pool over 169 primer combinations revealed an average of 35 bands per primer combination. About 5 bands were polymorphic (showed different banding patterns between pools) on average for each primer combination. From all of the polymorphisms, 4 male specific polymorphisms were discovered (Fig. 1). These were then analyzed on the individual basis and only two repeatable sex markers were found (Fig. 2). The other two candidate markers were shown to be artifacts of the pooling process. Although AFLP analysis of individuals showed 8 males that typed as females and one female that typed as male, these results were consistent among the two sex markers found. This could be explained by the difficulty of distinguishing undeveloped male testes from ovaries. The fact that these banding patterns were consistent among both of the repeatable markers suggests that the markers are viable, and in future studies, histological data should be utilized to remove errors in missexing.

Development of a PCR-based Genetic Sex Identification Method

Bands from the AFLP product were cloned into PGEM-T-Easy cloning kit and sequence was recovered (Fig. 3). This sequence was used to create a pair of primers that would demonstrate the sex of the fish. When the primers were used on all fifty of the test individuals, the males produced a product while the females did not (Fig. 4). The

missexed fish demonstrated the same banding patterns as previously discussed. This marker may not be able to be utilized outside of this research population because it can be difficult to find markers that work outside of specific research families or strains (Iturra, Medrano, Bagley, Lam, Vergara, & Marin, 1998; Felip, Young, Wheeler, Thorgaard, Neira, & Diaz, 2005) and because and because the sex-specific region of the Y chromosome appears to be relatively small (less than 1 Mb) in medaka (Matsuda, Nagahama, Shinomiya, Sato, & Matsuda, 2002) and in stickleback (Peichel, et al., 2004).

Discussion

AFLP analysis has demonstrated its suitability for finding sex-linked markers in many different organisms including fishes (Ama, Douek, Rabinowitz, & Rinkevich, 2007; Felip, et al., 2004; Chen, et al., 2007). We have found a genetic marker in a research population of fathead minnows. This marker could be useful for demonstrating the genetic sex of the fish in embryo studies or in studies where the fish may have experienced a sex reversal. Finding two sex markers, one of which was successfully converted into a PCR test, supports the interpretation that this species shows an XX/XY method of sex determination, with males being the heterogametic sex. Although individual AFLP analysis showed 8 males that typed as females and 1 female that typed as male, these results were consistent for both of the sex markers found. This could be explained by the difficulty of distinguishing undeveloped male testes from ovaries. These results indicated that histological data rather than gross anatomy data was needed for accurate sexing.

Discovery of sex markers is very important for understanding the genetic sex of a species of fish, especially in light of the lack of universal sex-determining mechanisms in fishes. While the markers we have identified are only able to distinguish sex within the Duluth population of fathead minnow, they still represent an important step for researchers using those fish. Studies in aquatic toxicology and endocrine disruption should now be advanced due to these results.

Conclusion

With the discovery of a sex-marker specific to the Duluth research population, we have taken the initial steps to finding a species-wide sex-marker and have implicated a male heterogametic sex-determining mechanism in the fathead minnow. No genetic sex-markers had previously been found in the fathead minnow. This research has laid the foundation for work with this research population or for identifying a sex-linked marker in the species as a whole.

CHAPTER THREE

ADDITIONAL RESEARCH

This research is an excellent beginning point or reference for later researchers to continue fruitfully in studies of sex marker identification and sex determination in the fathead minnow.

The original experimental design planned for checking the validity of an apparent sex marker on other fish families at large. Recapitulating chapter two, we used fish from the EPA lab in Duluth, Minnesota for AFLP analysis. Although no parentage history was available, these fish were apparently closely related to each other. In order to attempt to see if a sex marker found in this population would be useful for the fish species at large, fish were acquired from Carolina Biological Supply (Burlington, NC). In order to avoid ambiguity in future studies over the sex of the specimens, tissue samples were fixed with 10% formalin and prepared as hematoxylin and eosin stain. Caudal fin samples were taken from these fish. These slides allow researchers to histologically verify the sex of the fish (Figure 5). DNA was extracted from twenty-eight of the fifty caudal fin samples. This DNA was used with primers described in chapter two to demonstrate that the primer combinations and markers found were specific to the Duluth strain rather than species-specific. DNA samples are clean and ready for any type of genetic analysis that may follow my current research.

Another research path that was explored was using Clontech's "GenomeWalker™ Universal Kit." The provided protocol was used to construct

GenomeWalker libraries for male and female Duluth fathead minnows. The GenomeWalker allows researchers to walk out from a known section of DNA to isolate flanking sequences. This library likely will be very helpful in finding more sequence from a known region. GenomeWalker gave an additional 300 bp of sequence on the family sex marker, but the results were not helpful in finding species-wide specificity. Amplified DNA from the GenomeWalker was cloned into chemically competent cells as described by protocols for Invitrogen's TOPO® TA cloning.

CHAPTER FOUR

FUTURE DIRECTIONS

The research presented lays groundwork for future important research. It is not clear that a single model organism will serve all the needs for aquatic toxicology.

Nevertheless, with more research, the fathead minnow could be a much more important fish for toxicology research. The following avenues of research could solidify its role.

Development of a universal sex marker

The sex marker found in the current study is one that is only viable within the Duluth research population. To expand the markers usefulness, it needs to be effective species-wide. We have already found a method for finding a sex marker within this family. Once a sex marker is found, the sequence data from that should be used to construct primers, like we did previously. Once PCR primers are found to be successful, they should be tested on outbred individuals. We used fish from Carolina Biological supply to be our test subjects. Alternatively, new markers could be isolated using additional AFLP primer combinations tested in pools prepared from sexed individuals from both the Duluth and Carolina populations. The development of a species wide sex marker will allow researchers using the fish within the species to determine the sex of the fish in question.

Development and utilization of fathead minnow clonal lines

Creation of gynogenetic and androgenetic clones would also be useful for future studies. Creating clones of fish is a technique that has been utilized in the Thorgaard lab at Washington State University for many years (Young, Wheeler, Coryell, Keim, & Thorgaard, 1998). The process creates fish that are not only clones of each other, but are also completely homozygous. Even YY males survive. Genetic clones of fish are an excellent tool for many fish studies. Applications of androgenetic male and gynogenetic female clones are far-reaching. If viable YY clones are made, they could be mated to normal females to produce all-male offspring. Furthermore, these all-male offspring would be genetically identical if the cross were to homozygous females. This would allow for the testing of endocrine disruptors that may affect the sex of the fish. The genetic identity of these fish provides a reliable and convincing argument that a sex reversal in test fish is related solely to endocrine disrupting chemicals. Similarly, sex reversed females could be crossed to female fish in order to create all female offspring that could be used in masculinization studies.

A benefit of creating clones is a researcher does not need to concern himself with sex markers for the whole fathead population anymore. Duluth fish could be utilized after several cycles of inbreeding. Inbreeding will increase homozygosity and this might mitigate the problems associated with inbreeding depression while trying to create homozygous fish. Egg and sperm could be taken from fish selected from these families. Formation of androgenetic fish versus formation of gynogenetic fish is quite different. FHM have not been used for the production of clones; however, the work has already been done on fish from the family Cyprinidae (Corley-Smith, Lim, & Brandhorst, 1996). For androgenetic fish, eggs are collected from a female fish. The eggs are then

irradiated either by UV-light or gamma radiation in order to destroy maternal DNA. Sperm is added, activation occurs and the first mitotic division is blocked by heat shock. This makes embryos that are 100% identical at all loci. To perform gynogenesis, similar methods are used. Sperm are irradiated to destroy their nuclear DNA. The irradiated sperm are used to initiate mitosis in the egg. The first mitotic cell division is blocked, resulting in homozygous embryos. Gametes from each of the homozygous doubled haploids are utilized to make a second generation that will allow the offspring to be not only homozygous, but also identical to their siblings. This technique has been used previously in the Thorgaard lab to create clonal lines of rainbow trout, *Oncorhynchus mykiss* (Young, Wheeler, Coryell, Keim, & Thorgaard, 1998). AFLP analysis is then utilized on the fish to test for homozygosity among offspring.

Research has demonstrated that the creation of clones is not as difficult as many would think. It has been demonstrated that creating clonal lines does not follow the traditional ideas of dominance hypothesis of inbreeding depression (Patton, Kane, Wheeler, & Thorgaard, 2007). This is a seemingly worthwhile endeavor because of the experimental freedom that comes from the production of clones.

In order to test the usefulness of the FHM in endocrine disruption tests, sensitivity to endocrine disruption chemicals is key issue. Once a clone is created, its sensitivity to endocrine disruption can be tested. Ideally, this could be done using the FHM clones. If clones can be made, a cross between male and female fish will be made, which would result in all-male offspring. In Länge's study, the researchers found that exposure to >4ng/L of EE2 led to a sex ratio of 84:5 female to male fish with 11% of the fish demonstrating an intermediate sex named ovatestes. Fish in this experiment

were exposed for 305 days post fertilization (Länge, et al., 2000). It is probable that we would not need to expose the fish for this long a time. FHM have a sexual life cycle of four months. Since these fish are gonochoristic fish (fish that remain one sex for their life) they only need to be exposed during sexual differentiation. Fish that have not been exposed could be used as controls. Once the fish have reached sexual maturity, examination of the gonads would reveal the phenotypic sex. Following this, genetic analysis using the Duluth sex marker could be demonstrated by PCR analysis to validate the findings.

It should be noted that this study could be done without clones. Now that there is a sex marker that is associated with male fish, it is possible to do a mating of Duluth research fish, expose them to a chemical of interest, and analyze the results based on the genetic marker found.

The clones are a very useful tool. Besides the possibilities mentioned, there is also the possibility of creating a linkage map. As clonal lines are established, different sensitivity levels to toxins may become evident. Using techniques similar to QTL analysis of rainbow trout crosses (Zimmerman, Evenhuis, Thorgaard, & Ristow, 2004), toxin trials and genetic analysis can be utilized to try and find candidate genes of sensitivity. This could have far reaching implications to toxicology, epidemiology, immunology and other areas of research. With the foundation provided by the Duluth sex marker, the further research may prove to be very important for diverse areas of study.

Figure Legends

Figure 1: AFLP analysis on pooled male and female fathead minnows demonstrates a male specific sex marker. The first three pools of each respective sex contain four fish while the fourth pool contains five fish. Male pools demonstrate a differential banding pattern from female pools for the BamHI GAC-MseI gaa primer combination. Four other sex markers were found, but only this one was discovered to be viable for later studies.

Figure 2: AFLP analysis on individual fathead minnows from the pools supports a male-specific sex-linked marker. Males 3, 5, 7, 8, 10, 12, 18, 21 and female 16 consistently sexed incorrectly in all individual AFLP analysis and PCR tests.

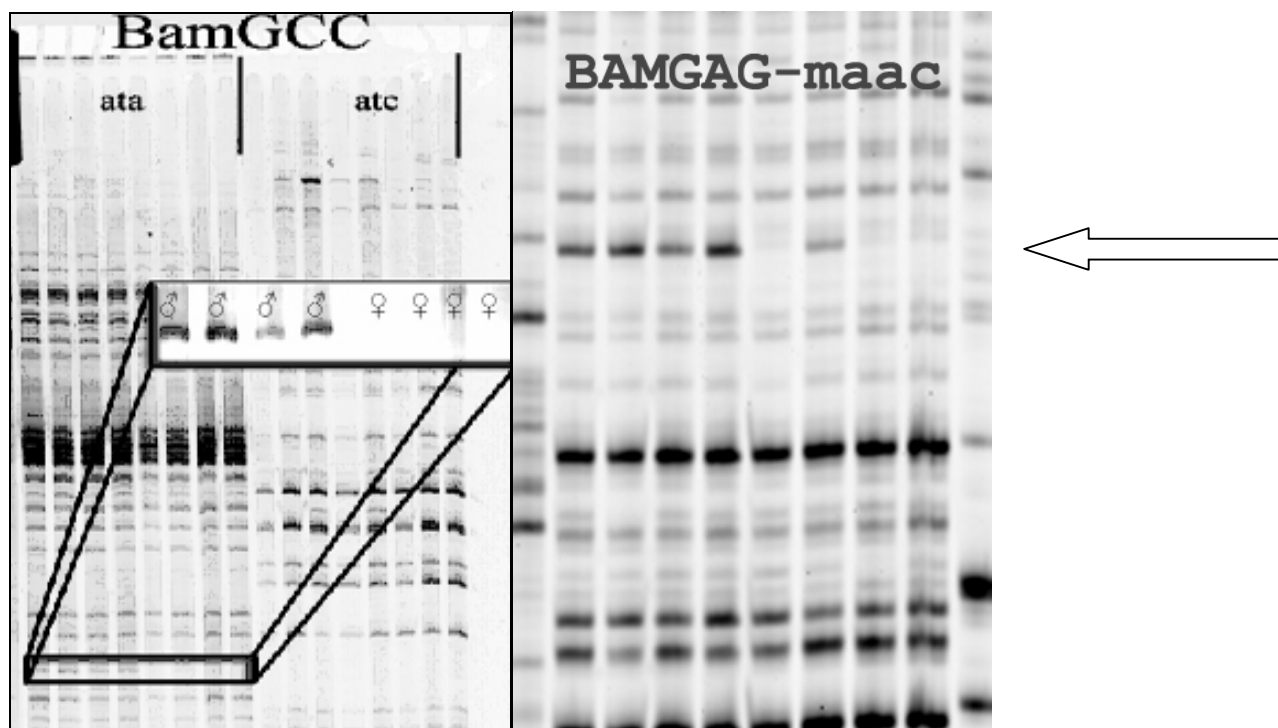
Figure 3: Sequence data from sex-linked fathead minnow AFLP markers were used to create primers are bold on the sequence. This information was collected from bands on the BamHI GAC- MseI GAA primer combination.

Figure 4: PCR analysis from primers created were tested in the original fathead minnow individuals. This figure demonstrates 3 males and 3 females that accurately sexed.

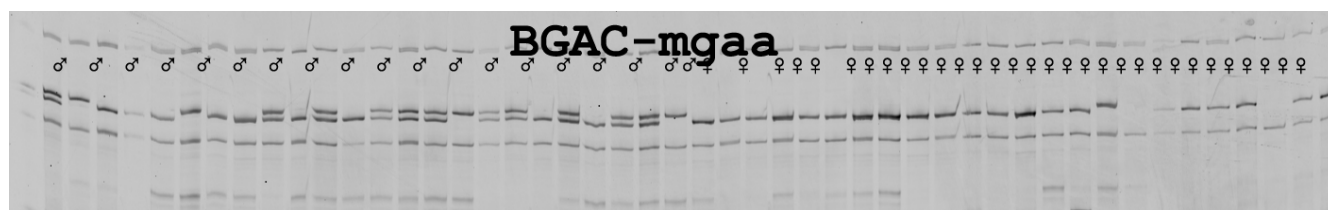
Figure 5a and b: Example of histology slides from fathead minnows created for future studies. Gonads were removed from fish and fixed in 10% formalin. A single H&E slide was made. Figure a shows the female with arrows pointing to characteristic oocytes.

Figure 5b demonstrates the male with arrow pointing at testes.

[FIG 1]



[FIG 2]



[FIG 3]

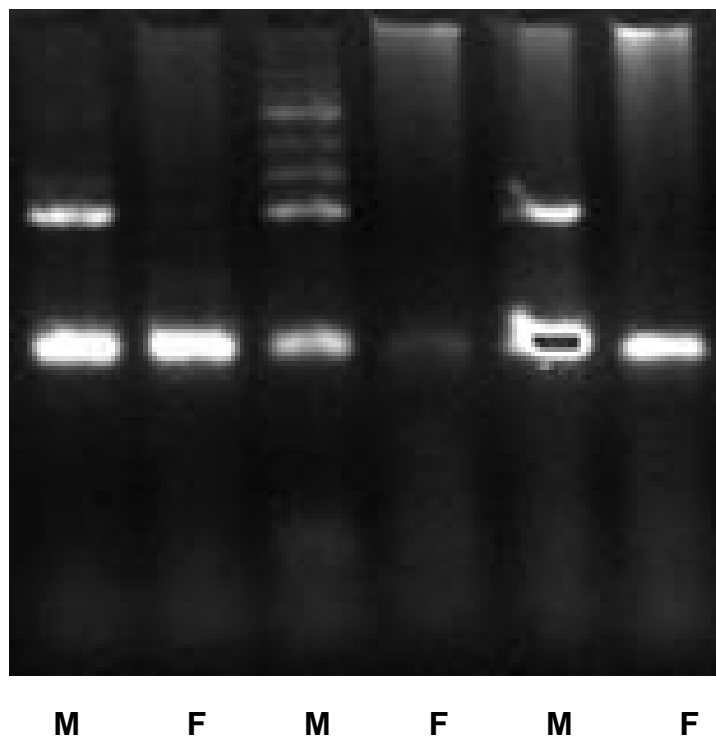
LOWER MOLECULAR WEIGHT BAND

GATGAGTCCTGAGTAAGAATGAAATGGCAATAATTTATTCACACCTTTACAATTGAA
TCCAACGTATCATCAGCGCTCGGATTCGCTTCGGATTCGCATGGTGCTGTCAAGTA
CAACTTGCAAGTCTATCTGTGAGGTAACTAAACGCTATTGGCTATTTGAAAAGGGA
GAGGAGCC**CATCCTATATGTCCTGCCTTAGC**TTTCTGTTTCAATGGAAATTACGTCA
ACGCATCCAAGGCACTTTGAGACACGGCAGGCAAAAACGGTAATTTTTTTACTGGT
CAGTTTTACGATTTTGGGAAATTTGTATTCAATAACATGTTGTCTTTC:::AGAAAA
AAAGTACATATTTAGATCTTTATTTTACTACACTTTGTCTGTTTGCCTGTGTATATTT
CGCCTAAATTCCTCAAAAGTTTGCATTGTAAATCATCCCGCTGCTCCGTGATCG**CG**
ATCTGCACCCTGTCATCGCACACACCGTTAGAAAAGCTCTCTCTTTCTCCTCTTCA
ATGCTGTCGGATCAGTNACGCAGTCA

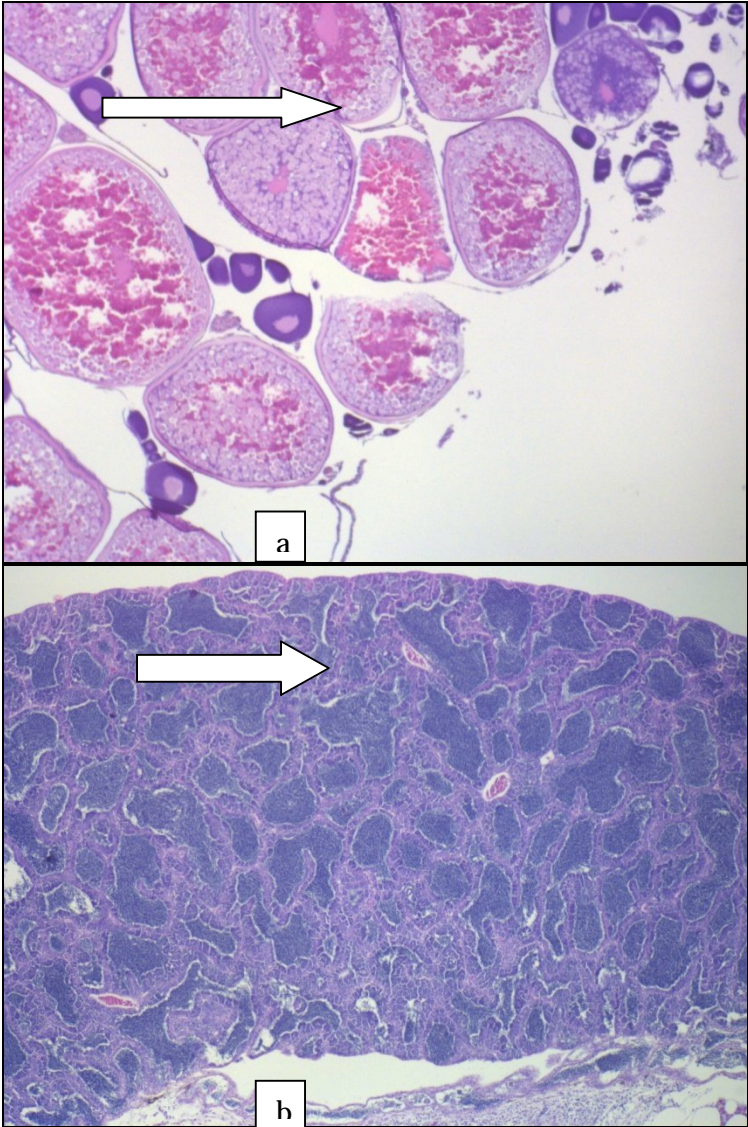
HIGHER MOLECULAR WEIGHT BAND

GCCCTTNNANGAGTAAGAATGAAATGGCAATAATTTATTCACAACCTTTACAATTGAA
TCCAACGTATCATCAGCGCTCGGATTCGCTTCGGATTCGCATGGTGCTGTCAAGTA
CAACTTGCAAGTCTATCTGTGTGGTAACTAAACGCTATTGGCTATTTGAAAAGGGA
GAGGAGCC**CATCCTATATGTCCTGCCTTAGC**TTTCTGTTTCAATGGAAATTACGTCA
ACGCATCCAAGGCACTTTGAGACACGGCAGGCAAAAACGGTAATTTTTTCACTGGT
CAGTTTTACGATTTTGGGAAATTTGTATTCAATAACATGTTGTCTTTCAGACTTGTGC
TGAAAAAAGTACATATTTAGATCTTTATTTTACTACACTTTGTCTGTTTGCCTGTGT
ATATTTGCCTAAATTCCTCAAAAGTTTGCATTGTAAATCATCCCGCTGCTCCGTGA
TCG**CGATCTGCACCCTGTCATCGCACACACCGTTNAGAAAAGCTCTCTCTTTCTCGT**
CTTCAATGCTGTCGGATCAGT:ACGCAGTCA

[FIG 4]



[FIG 5]



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