PROPAGATION PRACTICES AND GENETIC RESOURCES IN LAKE STURGEON REHABILITATION

by

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A Thesis

submitted in partial fulfillment of the requirements of the degree

MASTER OF SCIENCE

IN

NATURAL RESOURCES (FISHERIES)

College of Natural Resources

UNIVERSITY OF WISCONSIN

Stevens Point, Wisconsin

March 27, 2009

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ABSTRACT

Over the past two centuries, lake sturgeon *Acipenser fulvescens* populations have suffered massive declines, throughout North America. Lake sturgeon once numbered 11 million in the Lake Michigan basin alone, but contemporary estimates place their current count at less than 10% of this historic abundance. This population decline has been attributed to the combined effects of water quality degradation, habitat fragmentation and alteration in the form of dam construction, and overexploitation. In response to these declines, the Wisconsin Department of Natural Resources (WDNR) began collecting lake sturgeon eggs in 1978 at the Fox and Wolf Rivers (Winnebago and Outagamie counties, Wisconsin) of the Lake Winnebago system for artificial rearing at the Wild Rose State Fish Hatchery (Waushara County, Wisconsin). Such efforts continue today, with on-site manual fertilization by hatchery personnel typically combining the eggs of one female with the mixed-milt from two to five males, after which all sturgeon progeny are reared together in communal hatchery ponds. The WDNR and Michigan Department of Natural Resources (MDNR) began rearing lake sturgeon at stream-side rearing facilities (SRFs) in 2006 and 2007, respectively. These facilities are located on the banks of four target streams, allowing the young sturgeon to imprint and one day potentially reestablish a spawning run in the stream. The spawning and rearing techniques used at the SRFs differ greatly from WDNR traditional hatchery methods, with the female's eggs split into five roughly equal lots which are fertilized by a single male per lot, after which female-based families are reared separately. The SRF spawning and rearing methods are designed to maximize genetic diversity and effective number of breeders (N_b) by minimizing the reproductive variance of broodstock within each cross in the facility. To determine the most genetically appropriate method for lake sturgeon artificial rearing, comparisons were made between the diversity produced at Wild Rose and the SRFs. The objectives of this study were to identify the presence and extent of sperm competition in mixed-milt crosses, characterize the genetic diversity present in each broodstock population, and evaluate the relative reproductive contributions of male broodstock to each cross at the facilities. Significant paternal bias was observed in two of three experimental mixed-milt crosses. This indicates that sperm competition was present in mixed-milt crosses of lake sturgeon and has the potential to significantly bias the genetic character of the resulting progeny towards the most successful males. Broodstock populations at Wild Rose and the SRFs were found to be genetically representative of their respective source populations, even during years characterized by difficult adult capture or facility problems that resulted in relatively small numbers of total broodstock. Significant paternal bias was detected in the final stock at the SRFs, by larval stage at Wild Rose, and in natural larval mortalities in the SRFs. This indicates that survival at the larval stage in the SRFs may be responsible for biased parental contribution at later stages. These data suggest that significant genetic gains can be made by furthering attempts to equalize reproductive contributions of male broodstock. This could be accomplished by hatching egg lots in separate containers and combining into female-based families for rearing only after hatch. Together with allowing progeny to imprint, these data suggest that stream-side rearing holds great promise for the re-establishment of genetically representative lake sturgeon spawning runs in the target streams.

ACKNOWLEDGEMENTS

I would first like to acknowledge the Great Lakes Fishery Trust and the Wisconsin Department of Natural Resources for providing the funding support for this project. I would like to give special thanks to my graduate advisor, Dr. Brian Sloss, for his support and guidance, along with his tireless pursuit of good science. I would like to thank Dr. Michael Bozek for providing input and innovative ideas throughout the course of this research. I would also like to acknowledge Dr. Christopher Hartleb for the time and effort he contributed to this research and thesis. I would like to extend special thanks to Brad Eggold, Ron Bruch, Steve Hogler, Mike Donofrio, and Steve Fajfer, from the WDNR and Ed Baker from the MDNR for providing samples, assistance, and insight throughout the entirety of this project. I would also like to thank Tom Burzynski (WDNR), Robert Elliott (USFWS), and J. Marty Holtgren (LRBOI). In addition, I would like to acknowledge Marc White and the Riveredge Nature Center staff, Steve Surendonk, and Becky Papke, along with all other technicians and volunteers working in support of lake sturgeon rearing efforts. I would like to thank Ryan Franckowiak for his patient instruction and guidance in the laboratory. I would like to acknowledge Andrea Musch for her ever-present support of graduate students within the research unit. I would also like to thank my fellow graduate students, particularly Jeremy Hammen, Benjamin Cross, Tim Kennedy, Brandon Spude, Sara Schmidt, and Meaghan Proctor for sample collection and other assistance throughout this research. I would like to acknowledge the staff of the UWSP library, particularly Sara Weisensel and the interlibrary loan staff for their support of student research. I would also like to extend special thanks to my parents, Pam and John Roffler, for their support over the years and for doing so much to get me to this point. I would also like to thank Bob and Gerlinde Tschurwald for providing room, board, and moral support every other weekend during the course of my studies. Lastly, I would like to express my deepest gratitude to my fiancée, Jennifer Tschurwald, for her unwavering patience, understanding, and good humor throughout the years.

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INTRODUCTION

Over the past two centuries, lake sturgeon *Acipenser fulvescens* populations have declined resulting in the species being listed as endangered, threatened, or of special concern throughout its entire range (Elliott et al. 2004). Lake Michigan once had as many as 11 million individuals (Hay-Chmielewski and Whelan 1997) comprising at least 33 suspected spawning populations (Zollweg et al. 2003). A majority of these populations are now extirpated or remain as small remnant populations. Lake Michigan's current lake sturgeon population estimates are <10% of the historic abundance (Gunderman and Elliot 2004). This decline has been attributed to a combination of degraded water quality associated with the development of the Lake Michigan basin, habitat fragmentation, especially dam construction, and overexploitation (Harkness and Dymond 1961; Rochard et al. 1990).

In degraded ecosystems, the use of proactive conservation is often necessary when continued population declines would compromise the recovery of a species through natural or artificial means (Anders 1997 and 1998). The use of artificial rearing for supplementing or re-establishing populations of an at-risk species (such as lake sturgeon) is known as conservation aquaculture (Anders 1997). When reintroducing an imperiled species through artificial propagation, a balance must be achieved between re-establishment of self-sustaining populations and the protection of any remnant populations occurring within the watershed. The former concern is largely a function of choosing an appropriate source population for propagation and using appropriate spawning and rearing strategies (Williams et al. 1988; Miller and Kapuscinski 2003). For lake sturgeon conservation aquaculture, a further concern is the negative genetic impacts of straying artificially-reared sturgeon on the numerous remnant lake sturgeon populations in the Lake Michigan basin.

The genetic character of a rearing facility's final product is influenced by selection based on the choice of broodstock, spawning/crossing strategies, and rearing conditions of the hatchery program. Given the difficulty of simulating a fish's natural environment, artificial rearing will differ tremendously from wild conditions. Domestication to conditions within a hatchery is a primary means by which the genetic character of hatchery fish can change (Reisenbichler et al. 2003). Domestication is a shift of genetic character in captively-reared populations brought about by any combination of selection (both intentional and unintentional) for a desired trait (e.g., growth rate, adult body size, etc.), nonrandom sampling of the broodstock, selection/adaptation to hatchery conditions (e.g., resistance to higher density, different prey items, etc.), and/or relaxation of selection pressures allowing maladapted individuals to survive and reproduce (Campton 1995; Waples 1999).

Concerns over straying and domestication of cultured lake sturgeon, coupled with a perceived risk of disrupting the genetic integrity of remnant, native lake sturgeon populations, have resulted in the stream-side rearing concept. Stream-side rearing consists of manually spawning lake sturgeon from a native broodstock based on strict crossing strategies, transferring the fertilized gametes to a stream-side rearing facility (SRF; a trailer outfitted as a miniature hatchery/rearing system) on the banks of the stream the fish will be stocked into, and rearing the fish to fall fingerlings for stocking. The SRF concept addresses the aforementioned concerns and uncertainties in regard to imprinting and straying by using water from the stream that will be the release site of the young sturgeon. Since the fish are reared from fertilization to release in the same facility, if imprinting occurs, it should only be to the unique chemical cues of the water within the SRF. In theory, this approach should maximize the probability of imprinting propagated fish to their release stream and the probability of successfully establishing a recruiting lake sturgeon population in the stream while minimizing the risk of straying to remnant native populations. These concerns over the lake sturgeon captive rearing process have brought about a shift from Wisconsin's traditional hatchery rearing methods to stream-side rearing. Traditional hatchery rearing utilizes mixed-milt crosses where the eggs of one female are fertilized with the milt of two to five males, after which the progeny of all families are combined for rearing. Stream-side rearing separates a female's eggs into five roughly equal lots. Each lot is then fertilized by a single male per lot (i.e., five males per female). After fertilization, progeny are combined into single female families for rearing.

This project aimed to assess the genetic impact of the different rearing practices being used in the SRFs compared to traditional hatchery practices employed at facilities such as the Wild Rose State Fish Hatchery (Wild Rose, WI). In particular, this project addresses concerns associated with mixed-milt spawning, equalization of family sizes, rearing of families separately, and differential survival/performance of families in SRF conditions versus traditional hatchery conditions (Figure 1.1). The three objectives of this study were:

1. To determine the extent and significance of sperm competition in mixed-milt crosses of lake sturgeon.
2. To determine if broodstock sampled from the Wolf River (Outagamie and Shawano counties, Wisconsin), Menominee River (Menominee County,

Michigan), and Sturgeon River (Houghton County, Michigan) were genetically representative of their respective source populations.

3. To determine the extent of reproductive disparity among male broodstock at both types of facilities (traditional hatchery and SRF) and associated reduction in genetic diversity.

Literature Review

Lake sturgeon life history. – The lake sturgeon is a freshwater species of prehistoric lineage endemic to the Great Lakes, Hudson Bay, and Mississippi River drainages (Houston 1987; Peterson et al. 2007). These sturgeon are dominant benthivores utilizing barbels located beneath their snout to detect prey such as mayfly nymphs (Emphemeroptera), fingernail clams (Sphaeriidae), and crayfish (Cambaridae; Baker 1980; Holey and Trudeau 2005). Lake sturgeon have long, scaleless bodies protected by rows of bony scutes that diminish with age and size (Priegel and Wirth 1971). The lake sturgeon is the longest-lived of all freshwater fish, with males typically reaching 55 years of age and females living up to 100 years or more (Roussow 1957; Baker 1980).

Lake sturgeon spawning behavior is unique among Great Lakes fish species in its combination of delayed maturation and intermittent spawning. Lake sturgeon reach sexual maturity at an advanced age, with males typically maturing at 14-16 years and females at 24-26 years (Priegel and Wirth 1971). Once sexually mature, lake sturgeon generally spawn intermittently, with males spawning every 1-4 years and females spawning every 3-7 years (Roussow 1957). This intermittent spawning, coupled with their long life expectancy, results in the potential for an individual sturgeon to reproduce several times over its lifetime.

Although lake sturgeon typically show wide migration within the Great Lakes, they exhibit philopatry by homing to their natal streams to spawn (Gunderman and Elliott 2004). This behavior, known as potamodromy, or migration strictly within freshwater systems, is unique to lake sturgeon among the North American *Acipenser* (Auer 1996b;

Bemis and Kynard 1997). The homing behavior is facilitated by the perceived ability of young sturgeon to imprint to the unique organic chemical cues of their birth waters. This process may occur as early as 10 days after hatching (Faber 1994). In a study of the spawning behavior of lake sturgeon within the Lake Winnebago system of eastern Wisconsin, Lyons and Kempinger (1992) found only 3 of 203 tagged sturgeon did not return to the same stream in which they were originally marked. Sturgeon within this system have been documented to travel over 200 km upstream from Lake Winnebago to spawning sites in the Wolf River (Kempinger 1988).

Lake sturgeon spawning relies on water temperature and proper habitat availability. In the Wolf River of the Lake Winnebago system, female sturgeon will move onto spawning sites when water temperatures reach 8.8^{o}C, with peak spawning typically occurring at 11.5-16.0^{o}C (Bruch and Binkowski 2002). Optimal spawning sites feature fresh, silt-free substrate of large rock, limestone, or granite (Auer 1996a; Bruch and Binkowski 2002). Spawning events typically last 2-4 s, during which the males strike the female's abdomen with their tails and caudal peduncles (Bruch and Binkowski 2002). While female sturgeon carry approximately 2,200 eggs/kg of female weight, individual spawning bouts typically involve ~1,500 eggs or less, allowing a female to continue spawning for several hours (Harkness and Dymond 1961; Bruch and Binkowski 2002).

Post-hatch and juvenile sturgeon rely on their stream of origin and proximate tributaries for nursery habitat. Kempinger (1988) studied the early life history of lake sturgeon over a four year period in the Lake Winnebago system and found that eggs typically hatched 8-14 d after fertilization. Larval drift downstream occurred primarily at night, with peak downstream movement at 9-10 d post-hatch. Young lake sturgeon utilize the Wolf River and connecting tributaries as nursery grounds, eventually moving into Lake Winnebago and/or the connecting chain of lakes for further growth as juveniles and adults (Bruch and Binkowski 2002).

Population declines. – Over the past two centuries, lake sturgeon populations have suffered massive declines resulting in the species being listed as endangered, threatened, or of special concern throughout its entire range (Elliott et al. 2004). Lake Michigan, in particular, once featured as many as 11 million individuals (Hay-Chmielewski and Whelan 1997) comprising at least 33 suspected spawning populations (Figure 1.2; Zollweg et al. 2003). A majority of these populations have been extirpated or remain as small remnant populations. Lake Michigan's current lake sturgeon population estimates are less than 10% of the historic abundance (Gunderman and Elliot 2004). This decline has been attributed to a combination of degraded water quality associated with the development of the Lake Michigan basin, habitat fragmentation, especially dam construction, and overexploitation (Harkness and Dymond 1961; Rochard et al. 1990).

Water pollution can have negative impacts on fish spawning and nursery areas and their trophic potential (Rochard et al. 1990). For example, pollution can result in disruptions in the life cycle of many aquatic insects leading to shortages of insect larvae and subsequent disruptions of the food web at higher trophic levels (Rochard et al. 1990). Lake sturgeon spawning and nursery habitat within the Great Lakes has been degraded as a result of sedimentation and pollution (Holey et al. 2000). Pollution can have more direct impacts on species through bioaccumulation and physiological disruptions. Longlived fish species such as lake sturgeon are particularly vulnerable to bioaccumulation of heavy metals and other pollutants (Auer 2003). Contaminants of concern in the Great Lakes region include mercury, DDT, and PCBs. The buildup of such contaminants possibly affects survival and reproductive success of lake sturgeon (Auer 2003). Water quality throughout the Great Lakes region has seen significant improvement since the passage of the Clean Water Act and the implementation of Federal Energy Regulatory Commission licenses (WDNR 2006).

Dams obstruct lake sturgeon spawning runs by preventing access to upriver spawning grounds and significantly altering the river's character through flow modifications (Auer 1996b; Auer 2003). Historic spawning runs of lake sturgeon have been shortened or completely blocked, often associated with extirpation of the local population (Auer 1996b). Remnant populations in fragmented streams find the substrate immediately below a dam site artificially stabilized causing a downstream shift of preferred sturgeon spawning habitat, where more natural river flows allow for regular turnover and freshening of the substrate for spawning (Auer 1996a). Reduced spawning site availability can decrease a population's overall spawning success. Kempinger (1988) found that the Shawano Dam on the Wolf River of the Lake Winnebago system featured sturgeon egg clumps as thick as 15 cm. Egg masses of such thickness may have lower hatch rates and increased susceptibility to disease and predation (Auer 1996b). Due to fluctuating flows at the dam during spawning periods, many of the eggs were dislodged or exposed to air and desiccated (Kempinger 1988). Auer (1996a) noted a positive response in lake sturgeon spawning activity by use of more natural near run-of-the-river flows at the Prickett hydroelectric facility on the Sturgeon River (Houghton and Baraga counties, Michigan).

Lake sturgeon populations suffered tremendous declines as a result of Great Lakes commercial fishing operations, even prior to being a target species. Before 1860, the lake sturgeon was perceived as a nuisance species by commercial fishermen (Baker 1980). Net damage, as a result of the juvenile sturgeon scutes and/or the adult weight, frustrated fisherman to the point that they often piled sturgeon carcasses and burned them (Scott and Crossman 1973; Baker 1980). After 1860, the lake sturgeon became a commercial target because of demand for its meat and roe for caviar (Peterson et al. 2007). Commercial fishing for lake sturgeon in Lake Michigan brought in a total of 1.74 million kg of fish in 1879, the first year such records were kept in the United States (Baldwin et al. 1979). Catch steadily declined until 1929, when the Lake Michigan lake sturgeon commercial fishery was closed for all states (Michigan re-opened their sturgeon fishery from 1950-1970; Baldwin et al. 1979; Baker 1980). Lake sturgeon numbers have yet to rebound, in part due to their delayed sexual maturity, high natural mortality of juveniles, and sensitivity to fishing gear (Houston 1987).

Lake sturgeon recovery has likely been hampered by the various introductions of non-native species to the Great Lakes. The result of species introductions include increased interspecific competition and direct predation of non-natives on native species (Auer 2003). An example of direct predation is the sea lamprey (*Petromyzon marinus*) which has been shown to regularly parasitize lake sturgeon, wounding and even killing some large sturgeon (Scott and Crossman 1973; Baker 1980; Auer 2003). Potential competitors for food resources include the round goby *Neogobius melanostomus* and the common ruffe *Gymnocephalus cernuus*.

In degraded ecosystems, the use of proactive conservation is often necessary when continued population declines would compromise the recovery of a species through natural or artificial means (Anders 1997; Anders 1998). The use of artificial rearing for supplementing or re-establishing populations of an at-risk species (such as lake sturgeon) is known as conservation aquaculture (Anders 1997). When reintroducing an imperiled species through artificial propagation, a balance must be achieved between re-establishment of self-sustaining populations and the protection of any remnant populations occurring within the watershed. The former concern is largely a function of choosing an appropriate source population for propagation and using appropriate spawning/crossing strategies and appropriate rearing strategies (Williams et al. 1988; Miller and Kapuscinski 2003). For lake sturgeon conservation aquaculture, a primary concern is the straying rate of artificially reared fish, which should be minimized to protect the numerous remnant populations in the Lake Michigan basin.

Propagation and domestication.—Artificial rearing of fish has become an increasingly controversial practice. Artificial rearing conditions often differ from natural conditions in several key areas, including lack of predation, rearing density, food type and availability, lack of turbidity, and substrate type (Einum and Fleming 2001; Belk et al. 2008). These differences impose an altered selection regime on fish reared within the facility, that can lead to domestication of the facility's population (Harada et al. 1998; Einum and Fleming 2001). Domestication is a shift in genetic character allowing maladapted individuals to survive and reproduce that can be brought about not only by selection/adaptation to hatchery conditions (i.e., resistance to higher density, different prey items, etc.) and/or relaxation of selection pressures, but by any combination of selection (both intentional and unintentional) for a desired trait (i.e., growth rate, adult body size, etc.) or nonrandom sampling of the broodstock (Campton 1995; Harada et al. 1998; Waples 1999). Domestication to conditions within a hatchery is the primary means by which the genetic character of a hatchery population can change and can often be detected through morphological, behavioral, or genetic comparisons (Reisenbichler et al. 2003). While many strictly supplementation programs have been successful at accomplishing their objectives, the rearing of self-sustaining populations for re-establishment would benefit from a lack of artificial selection/domestication (Vincent 1960).

The difference of hatchery populations from wild populations is a direct result of selection and rearing conditions within the facility (Busack and Currens 1995). The alteration of the rearing environment often meets the goal of high survival, but also brings about relaxation of several selective pressures that operate on wild populations (Busack and Currens 1995; Frankham 2007). This can result in selection for genetic variants dissimilar to those found in the wild or disadvantageous in the wild (Harada et al. 1998; Frankham 2007). The performance and survival of artificially-reared fish should be of great concern to hatchery operations, especially those attempting to supplement or reestablish populations.

Morphological variation. – Hatchery-reared fish are often found to differ morphologically from their wild counterparts. Swain et al. (1991) evaluated hatchery and wild populations of coho salmon *Oncorhynchus kisutch* across several morphological metrics. Hatchery-reared coho salmon in the study had significantly smaller head dimensions, smaller median fins, and thinner bodies, all of which were found to be environmentally-induced differences. This morphological difference in hatchery-reared fish was attributed to reduced demand for burst swimming compared to wild conditions (Fleming and Gross 1989; Swain et al. 1991). Artificial rearing has also contributed to similar morphological variations in endangered June sucker *Chasmistes liorus* (Belk et al. 2008). Hatchery-reared suckers exhibited differing morphology in regard to lateral body, ventral mouth, and lateral head views. Compared to lake-reared suckers, the hatchery also produced significantly more variation in body shape within and among families. Selective mortality of lake-reared fish was likely not a factor in the difference, as survival in lake-reared fish was high. Rather, rearing environment was considered the driving influence, especially the relatively benign hatchery conditions, which may have allowed for more unconstrained and varied development (Belk et al. 2008).

Rearing conditions and associated morphological distinctions can also have an effect on the progeny of hatchery-reared fish. Jonsson et al. (1996) found that wild-reared Atlantic salmon *Salmo salar* produced significantly larger eggs than hatchery-reared fish of a common gene pool. Given that both groups of fish were released for seaward migration as juveniles, the distinction is likely due to juvenile rearing disparities, such as faster growth, lower mortality, and higher density in the hatchery. Wild-reared salmon are thought to produce eggs of a certain size based on their feeding opportunities as juveniles, allowing for larger offspring during years of high competition (Jonsson et al. 1996). These morphological distinctions indicate domestication to hatchery conditions and the potential for the production of hatchery populations maladapted to survive in the wild.

Behavioral variation. – Artificial rearing not only has the potential to alter morphology but also key behaviors of fish within the facility. Anti-predator behavior is one of the most frequently studied behaviors in artificial rearing and has consistently been shown to be reduced by artificial rearing and domestication. Alvarez and Nicieza (2003) found hatchery-reared juvenile brown trout *Salmo trutta* to be insensitive to the risk of predation. Wild fish increased their use of refuges in response to predation risk, while hatchery-reared fish showed no response to predator proximity. Johnsson et al. (1996) also studied hatchery-reared juvenile brown trout and found a reduction in anti-predator behavior across three separate position measurements. Such difference in anti-predator behavior is not unique to salmonid species. Malavasi et al. (2004) found that both hatchery-reared and wild juvenile European sea bass *Dicentrarchus labrax* exhibited a significant response to predator exposure. Wild juveniles aggregated more quickly and showed greater mean shoal cohesiveness and mean shoal distance than hatchery juveniles (Malavasi et al. 2004).

Given the feeding regimes and high densities at most hatcheries, aggressive behavior is often beneficial for survival in a rearing facility. Aggression has been assessed for several species of hatchery-reared and wild fish. Sundstrom et al. (2004) compared juvenile hatchery and wild brown trout for levels of aggression towards food items and a novel object. Hatchery brown trout were found to be significantly more aggressive than wild origin fish. Swain and Riddell (1990) conducted mirror image stimulation tests for aggression in coho salmon. Artificially-reared coho salmon showed significantly higher levels of aggression than their wild counterparts. Increased levels of aggression can increase susceptibility to predation, lowering the survival likelihood of artificially-reared fish in the wild (Swain and Riddell 1990). Relative levels of aggression can also affect interactions between hatchery and resident fish. Metcalfe et al. (2003) assessed dominance in juvenile Atlantic salmon through territorial contests between hatchery-reared and wild fish. Hatchery fish were dominant over wild-origin fish reared in a common hatchery environment. However, wild-reared fish were significantly dominant over both groups, with difference in body size having no effect on the contests. This indicated that, despite increased levels of aggression common to hatchery-reared fish, they have a decreased ability to compete with wild fish in territorial contests (Metcalfe et al. 2003). The domestication of hatchery-reared fish and associated behavioral shifts can negatively impact not only their own likelihood of survival in the wild, but also any interactions with established resident populations (Brannon 1993).

Genetic variation. – The effects of both intentional and inadvertent selection pressures within an artificial rearing facility can often be detected through genetic analysis. Crozier (1998) used allozymes to detect a genetic difference of hatchery-reared Atlantic salmon from their wild gene pool. The difference was likely a direct result of earlier maturity in the hatchery population due to intentional selection for growth performance (Crozier 1998). Glover et al. (2004) studied the effect of feeding regime on differential family survival of hatchery brown trout. Families were fed either a typical hatchery food ration or a 75% lower food ration in an attempt to simulate the higher mortality of a natural environment. Microsatellite DNA profiling was necessary for family assignment of each individual due to communal rearing based on ration. While all families showed significantly lower mortality with an increase in food rations, the distribution of family mortality differed significantly based on feeding regime (e.g., one family showed the lowest mortality in the high feeding treatment but showed greater mortality than several other families in the low feeding treatment). This indicated a mechanism for inadvertent domestication selection simply through feeding regime or food availability (Glover et al. 2004).

The sampling of broodstock and the genetic variability therein is the starting point from which diversity is lost through the rearing process. Porta et al. (2007) compared four broodstocks of Senegalese sole *Solea senegalensis* with each stock being completely comprised of either wild or first generation hatchery-reared adults. The study showed a drastic reduction in genetic variation in one hatchery generation, indicated by a drop in allelic richness (A_r) and effective population size (N_e). Verspoor (1988) studied N_e in relation to artificial rearing and found a positive correlation to genetic variability within first-generation hatchery populations of Atlantic salmon. Positively correlated with decreased N_e, hatchery populations showed a 26% reduction in mean heterozygosity and a 12% reduction in alleles per locus (Verspoor 1988).

Post-release. – Concerns for the survival and/or spawning performance of hatchery-reared fish post-release has led to several studies attempting to simulate natural conditions and assess the survival and reproductive success of hatchery fish. A seminal study in this area compared the performance of wild and hatchery-reared steelhead trout *Oncorhynchus mykiss* (anadromous rainbow trout) in natural and hatchery environments (Reisenbichler and McIntyre 1977). While hatchery offspring showed significantly higher growth rates and survival in the hatchery pond, steelhead of wild ancestry showed significantly higher survival in natural streams. Fleming et al. (1996) attempted to compare farmed and wild Atlantic salmon spawning performance through construction of experimental spawning arenas. Artificially-reared females had only 20-40% of the spawning success of wild females, including fewer constructed nests, greater weight of unspawned eggs, and greater egg mortality. Farmed males achieved only 1-3% of the reproductive success of wild males, were less aggressive, courted less, and participated in fewer spawnings (Fleming et al. 1996). These comparison studies, while conducted within somewhat controlled environments, demonstrate a clear difference between artificially-reared fish and wild fish.

Accurately monitoring the performance and survival of hatchery fish in wild conditions requires long-term assessment, from release as juveniles to spawning as adults. Chilcote et al. (1986) compared the relative performance of hatchery and wild summer-run steelhead trout in a natural stream. During a four-year period, juvenile hatchery trout bearing a genetic tag were supplementally stocked into an existing steelhead population in the tributaries of the Kalama River, Washington. Upon returning as spawning adults, hatchery summer-run steelhead outnumbered their wild counterparts at least 4.5 to 1 in four successive summer spawns. Hatchery spawners also contributed approximately 62% of summer-run smolt offspring in the four years. However, the offspring of hatchery spawners showed disproportionately higher mortality over the four years, with a relative survival of only 28% that of wild spawner offspring (Chilcote et al. 1986). Skaala et al. (1996) performed a similar study on brown trout in Norway, stocking first-generation hatchery adults into two wild stocks. The genetically tagged hatchery fish spawned with indigenous fish, allowing for the assessment of reproductive contribution and the survival of hybrid and wild trout. Contribution of the hatchery spawners was estimated at 19.2% and 16.3% in the two wild stocks. Estimated survival rates of age-0 trout indicated approximately three times greater survival of wild trout (Skaala et al. 1996).

A profound example of the post-release implications of domestication and intentional selection involves the coho salmon of the Lower Columbia River (LCR) and Oregon coastal streams. Because of a positive correlation between size and future survival and contribution, it was recommended that hatcheries maximize the growth of salmon prior to release (Hager and Noble 1976; Mahnken et al. 1982). This led hatchery personnel to intentionally select for early spawning individuals which allowed for longer growth periods within the hatchery (Rasch and Foster 1978). Returning hatchery salmon regularly spawned prior to native salmon in the LCR (Johnson et al. 1991). The early spawning of hatchery fish left their eggs susceptible to the scouring floods (also known as freshets) in late fall and early winter, which typically do not affect later-spawning wild fish (Nickelson et al. 1986; Chamberlain et al. 1991). Nickelson et al. (1986) studied a similar phenomenon in coho salmon populations in Oregon coastal streams. Included in the study were 15 streams stocked in late spring with juveniles and 15 unstocked streams, all 30 of which contained wild populations of coho salmon. Stocked streams showed a 41% increase in juvenile density in the summer following stocking. However, approximately 44% of wild juveniles in stocked streams were replaced by the larger hatchery juveniles. While there was no significant difference in resulting adult abundance between the stocked and unstocked streams, average time of spawning changed to approximately 2.5 weeks earlier in the stocked streams, due to a 48% drop in late spawners (i.e., wild fish) and a 70% increase in early spawners (i.e., hatchery fish).

These early spawners likely contributed little to overall juvenile recruitment as juvenile abundance was positively correlated only to density of late spawners. Nickelson et al. (1986) also cited freshets as a potential cause of poor contribution of early-spawning hatchery-reared fish.

The negative influences of domestication on performance are not limited to hatchery fish but can also influence native fish populations. In a conservation/rehabilitation effort, straying of artificially-reared fish is generally considered a detriment to establishing locally spawning populations and minimizing risks to neighboring, remnant populations. The local adaptations of wild populations can be disrupted or eliminated due to mixing of wild and artificially-reared fish accompanied by introgressive reproduction (Meffe 1986; Dittman and Quinn 1996; Grant 1997). This is of particular concern to programs attempting to supplement or reestablish populations of threatened or endangered species. Population re-establishment programs must therefore attempt to minimize domestication by preserving the genetic character of the wild broodstock population while maximizing the probability of progeny imprinting, potentially requiring a shift in traditional hatchery practices (Anders 1998).

Source. – The choice of broodstock for re-establishment of an imperiled fish species will vary depending on the environment, life history, or genetic lineage of prospective source populations (Williams et al. 1988; Miller and Kapuscinski 2003; Reisenbichler et al. 2003). While source populations for reintroduction efforts are typically selected based on high levels of genetic diversity, genetic similarity to the existing population, and environmental similarity, endangered species such as lake sturgeon offer few relatively large, stable populations to choose from (Allendorf and

Luikart 2007). The Wolf River lake sturgeon population is typically used by the Wisconsin Department of Natural Resources (WDNR) for artificial propagation of lake sturgeon because of its large population size (~22,000 spawning adult fish/year; Zollweg et al. 2003), while the Menominee River (a three population conglomerate containing >500 spawning adult fish/year; Zollweg et al. 2003) and Sturgeon River (~200 spawning adult fish/year; Zollweg et al. 2003) lake sturgeon populations are typically used by the Michigan Department of Natural Resources (MDNR) for lake sturgeon rearing (Ćzeskleba 1985; Hay-Chmielewski and Whelan 1997).

Even after the selection of an appropriate source population, genetic diversity can be lost if the broodstock sampled is not representative of the source population as a whole. The genetic diversity of the Wolf River, Menominee River, and Sturgeon River lake sturgeon populations have been quantified by DeHaan (2003), Welsh and McClain (2004), DeHaan et al. (2006), and Sloss and Kittel (2007), allowing for genetic comparison of broodfish to their source populations. In a study of lake sturgeon stocking in the Mississippi and Missouri Rivers, Drauch and Rhodes (2007) found that a single year's broodstock from the Wolf River was not genetically representative of the source population, likely due to the small number of parents used. Miller and Kapuscinski (2003) note the importance of obtaining a random sample of broodstock and sampling a sufficient number of broodstock, suggesting a minimum of 30-50 spawners be collected to minimize the risk of losing genetic diversity.

Spawning strategy. – During the fish rearing process, strategies should be employed that minimize the loss of genetic diversity from broodstock to final product. Conservation aquaculture focuses primarily on the maintenance of genetic diversity to increase the viability of the fish released and the likelihood of successful rehabilitation (Brannon 1993; Anders 1998; Reisenbichler et al. 2003). Populations with high levels of genetic diversity are better prepared to respond to environmental changes and develop adaptations to the local conditions (Frankham et al. 2002). Furthermore, loss of genetic diversity throughout the rearing process can increase the relatedness of a population and the likelihood of future inbreeding events (Allendorf and Luikart 2007). Therefore, maintaining the genetic diversity of the broodstock throughout the rearing process should be a top priority guiding the strategies employed in spawning broodfish and rearing the progeny.

The genetic diversity of the broodstock is the starting level from which loss must be minimized to produce a more viable and representative final product, especially when dealing with an imperiled species. Miller and Kapuscinski (2003) identified several methods for avoiding loss of diversity through domestication in the aquaculture process including the choice of proper mating scheme. Their suggestions included maintaining a one-to-one sex ratio and attempting to equalize or standardize the contribution of individual families (Miller and Kapuscinski 2003; Allendorf and Luikart 2007). Equalization of reproductive contribution faces two significant hurdles, sperm competition and differences in viability of offspring.

In a controlled environment with *in vitro* fertilization, male reproductive success has shown significant variance attributable to sperm competition potentially based on testis size, sperm length, sperm concentration, and a myriad of other factors (Iwamatsu et al. 1991; Stockley et al. 1997; Simmons and Kotiaho 2002; Oppliger et al. 2003). The effects of sperm competition when mixed-milt mating schemes are employed have been well documented for a vast array of species (Iwamatsu et al. 1991; Stockley et al. 1997; Simmons and Kotiaho 2002; Oppliger et al. 2003). When studying the spawning behavior of lake sturgeon in the Wolf River, Bruch and Binkowski (2002) noted the high likelihood of sperm competition due to the apparent inverse relationship between sperm concentration and time spent spawning. Sperm competition can lead to significant shifts in the genetic makeup of the next generation because of the dominance, hindrance, or complete absence of reproductive contributions from certain members of the broodstock (Campton 2004). However, even when a male has an advantage in sperm concentration, the competition of a pooled cross can be a counteracting force. Various salmonid species have been tested for relative reproductive contributions in pooled crosses, most of which showed no correlation between individual sperm concentration and the number of progeny sired. Relative reproductive contributions in these pooled crosses ranged from 0.5-88% (Gharrett and Shirley 1985; Withler 1988; Withler and Beacham 1994; Gile and Ferguson 1995; Hoysak et al. 2004). These results indicate a large degree of unpredictability when using a mixed-milt mating scheme. Among the typical recommendations for hatchery improvement is the use of a one-to-one mating system (Williams et al. 1988; Miller and Kapuscinski 2003; Campton 2004). This method preserves the genetic variation of the broodstock by removing the uncertainties of sperm competition. Nevertheless, the strategy of strict one-to-one crosses has risks associated with wasting the contributions of a single female if the lone male used to fertilize her eggs is sterile and requires a large increase in hatchery space and personnel effort.

If sperm competition is controlled, reproductive contribution of parent pairs to the next generation is based solely on viability, the survival of offspring to reproductive age

(Allendorf and Luikart 2007). Differences in viability can result in significant reproductive disparities, leading to shifts in genetic diversity and, potentially, domestication. Shifts in genetic diversity associated with differential viability result in a change in the effective population size (N_e) as a result of distinct, family-based advantage and resulting variance in reproductive success. The N_e is a measure roughly analogous to the number of breeders in a population. It is commonly used as an indicator of genetic variability of a population and to measure the relative risk of genetic drift (Frankham et al. 2002). The genetic concerns commonly associated with small population size (genetic drift, inbreeding, etc.) are actually results of low N_e. In fact, large populations with low N_e are just as likely to experience such deleterious conditions (Frankham et al. 2002). A single-year equivalent of N_e known as the effective number of breeders (N_b), approximates the genetic number of broodstock contributing to a year's progeny and is often used to assess hatchery protocols for the maintenance of genetic diversity (Campton 2004). Maximizing the N_b of the final product is the principle means by which a conservation aquaculture program can maintain the genetic characteristics of the broodstock and increase the overall genetic diversity in the resulting progeny that will form the foundation of the rehabilitated population.

One of the main influences on N_b in artificial rearing is the sex ratio of the broodstock, calculated as follows:

$$N_b = \frac{4N_f N_m}{N_f + N_m}$$

where N_f is the number of female broodstock spawned and N_m is the number of male broodstock spawned. Based on this equation, N_b is maximized with an equal sex ratio and declines with increasing skew toward either sex (Frankham et al. 2002). An equal, or one-to-one (1♀:1♂), mating ratio is repeatedly suggested for improvement of conservation aquaculture programs (Williams et al. 1988; Miller and Kapuscinski 2003; Campton 2004). The WDNR has traditionally spawned 1♀:5♂ (mixed-milt) for lake sturgeon propagation, primarily due to the high prevalence of males during broodstock capture. This strategy is also more consistent with the natural spawning behavior of lake sturgeon wherein several males compete for spawning with a single female and success for multiple males has been assumed (Bruch and Binkowski 2002).

The N_b of an artificially-reared population with unequal sex ratio is calculated as follows:

$$N_b = \frac{4N_{bf}\,N_{bm}}{N_{bf} + N_{bm}}$$

where N_{bf} is the effective number of female breeders and N_{bm} is the effective number of male breeders, with N_b maximized by equalization of family sizes within the facility (Hedrick 2005). When assessing a mating system most heavily influenced by variance in male reproductive success (i.e., mixed-milt crosses), effective number of female breeders is equal to the number of female broodstock. Effective number of male breeders is calculated as follows:

$$N_{bm} = \frac{N_m \bar{k}_m - 1}{\bar{k}_m - 1 + \left(\frac{V_k}{\bar{k}_m}\right)}$$

where V_k is the variance in progeny sired and $\bar{k}_m$ is the mean number of progeny sired by the males (Hedrick 2005). Variance in progeny sired is calculated as follows:

$$V_k = \sum_i x_i \left(k_i - \bar{k}_m\right)^2$$

where x_i is the proportion of progeny produced by male i and k_i is the number of progeny sired by male i (Hedrick 2005). Family size equalization is possible by use of one-to-one crosses and strict control of family size throughout the rearing process.

High family size variance can have negative consequences as shown by depressed N_e (relative to census size) in wild and hatchery-reared populations of various fish species (Turner et al. 2002; Araki et al. 2007). For example, Araki et al. (2007) found that high variance in family size was the primary cause of reduced effective size to census size (N_e/N_c) ratio in three generations of steelhead trout in Oregon. Despite the potential benefits of equalizing family sizes, various problems arise in the practical application of this principle. For example, if one family has particularly low survival (e.g., 20%), all other families would have to be limited to this lower percent. Theoretically speaking, if managing solely to maximize N_e, such an approach would be appropriate. Practically speaking, hatchery and management personnel are hesitant to cull a large proportion of healthy fish in these circumstances; especially if the fish are endangered, threatened, or of special concern such as the lake sturgeon. Generally, a goal of random survival probability will result in an N_e/N_c ratio that is unaffected by variance in family size (i.e., $N_e = N_c$). This is often an implicit goal of hatchery propagation plans.

Straying. – Various sturgeon species have shown imprinting to the chemical cues of their natal streams and subsequent philopatry. The most extensively studied sturgeon species in regard to imprinting and homing is the gulf sturgeon *Acipenser oxyrinchus desotoi*. Gulf sturgeon from various Gulf of Mexico tributaries regularly intermingle in the Atlantic Ocean during non-spawning times, but return to their natal streams for spawning (Wooley and Crateau 1985, USFWS 1995; Stabile et al. 1996; Heise et al.

2004). Stabile et al. (1996) found significant genetic differences between gulf sturgeon stocks of close geographic proximity and concluded strict home-site fidelity among the various subpopulations was the cause. Studies of lake sturgeon propagation in Russia indicated lake sturgeon imprint to their home stream about ten days after hatching (Faber 1994). Various agencies have raised concerns over the lack of knowledge regarding imprinting mechanisms and timing for several sturgeon species including the lake sturgeon (Zollweg et al. 2003). Moreover, with the apparent ability of young sturgeon to imprint to their birth waters, simulation of natural environments in artificial rearing is a priority (Miller and Kapuscinski 2003).

The counterpart to homing is a phenomenon known as straying, which is when a fish does not return to its natal stream and instead spawns elsewhere (Quinn 1993). Straying is of significant genetic concern to conservation aquaculture programs and presumably occurs when fish fail, or are not allowed, to properly imprint to their home stream (Miller and Kapuscinski 2003). In natural populations, straying can help facilitate the spread of a species to new or restored habitat, or can occur in response to environmental disruptions (Whitman et al. 1982, Leider 1989, Milner and Bailey 1989). In fact, gene flow (i.e., straying) is a key concept in the maintenance of healthy stocks and populations across a landscape (Allendorf and Luikart 2007). Straying rates vary greatly, even within the same species. For example, chinook salmon (*Oncorhynchus tshawytscha*) stocked from Columbia River hatcheries exhibited straying rates of 0-28.6% across six years (Quinn et al. 1991).

In a conservation/rehabilitation effort, straying is generally considered a detriment to establishing local spawning populations and minimizing risks to neighboring, remnant populations. The principle effect of philopatry is the creation of reproductively distinct populations that feature local adaptations specific to their particular environment (Meffe 1986; Dittman and Quinn 1996). These local adaptations can be disrupted or eliminated due to straying accompanied by introgressive reproduction (Grant 1997). When strays mix their genetic material with a population other than their own (i.e., gene flow) they can lower the overall fitness of the local population, a phenomenon known as outbreeding depression (Slatkin 1987, Stabile et al. 1996). The remnant populations of lake sturgeon in the Great Lakes show reproductive isolation brought about by spawning site fidelity, resulting in significant interpopulation variance (DeHaan et al. 2006). Outbreeding depression is most frequent and detrimental in crosses of such reproductively isolated and locally-adapted populations, such as crosses of remnant populations of lake sturgeon in Lake Michigan with fish of hatchery origin (Frankham et al. 2002). The straying of domesticated fish presents a scenario by which conservation aquaculture efforts may actually harm the overall resource through outbreeding depression within remnant, native populations.

Stream-side rearing. – The perceived threats of disrupting the genetic integrity of remnant, native lake sturgeon populations through the straying of cultured fish has resulted in the stream-side rearing concept. The stream-side rearing concept consists of manually spawning lake sturgeon from a broodstock based on strict crossing strategies, transferring the fertilized gametes to an SRF (a trailer outfitted as a miniature hatchery/rearing system) on the banks of the stream the fish will be stocked into, and rearing the fish to fall fingerlings for stock out. The SRF concept addresses the aforementioned concerns and uncertainties about imprinting and straying by using water from the stream that will be the release site of the young sturgeon. Since the fish are reared from fertilization to release in the same facility, imprinting will likely occur to the unique chemical cues of the water within the SRF. In theory, this approach should maximize the probability of imprinting propagated fish to their release stream and the probability of successfully establishing a recruiting lake sturgeon population in the stream while minimizing the risk of straying to remnant native populations.

The artificial rearing methods of SRFs differ from those traditionally used by the WDNR for lake sturgeon rearing at the Wild Rose State Fish Hatchery (hereafter referred to as Wild Rose). These differences are based on N_e concerns and focus on the sex ratio used and control of family size variance. The SRF crossing design features a 1♀:5♂ strategy with the female's eggs being separated into five equal lots prior to fertilization. Each lot is then fertilized individually with the milt of a single male and males are used for a single egg lot only. Family sizes are roughly equalized for each of the four females and reared to stock out. The Wild Rose sturgeon are crossed using a 1♀:5♂ strategy with all the eggs of one female being fertilized with mixed-milt from the five males and family size not equalized. The SRFs should predictably result in higher overall maintenance of genetic diversity due to the differences in crossing and rearing strategies.

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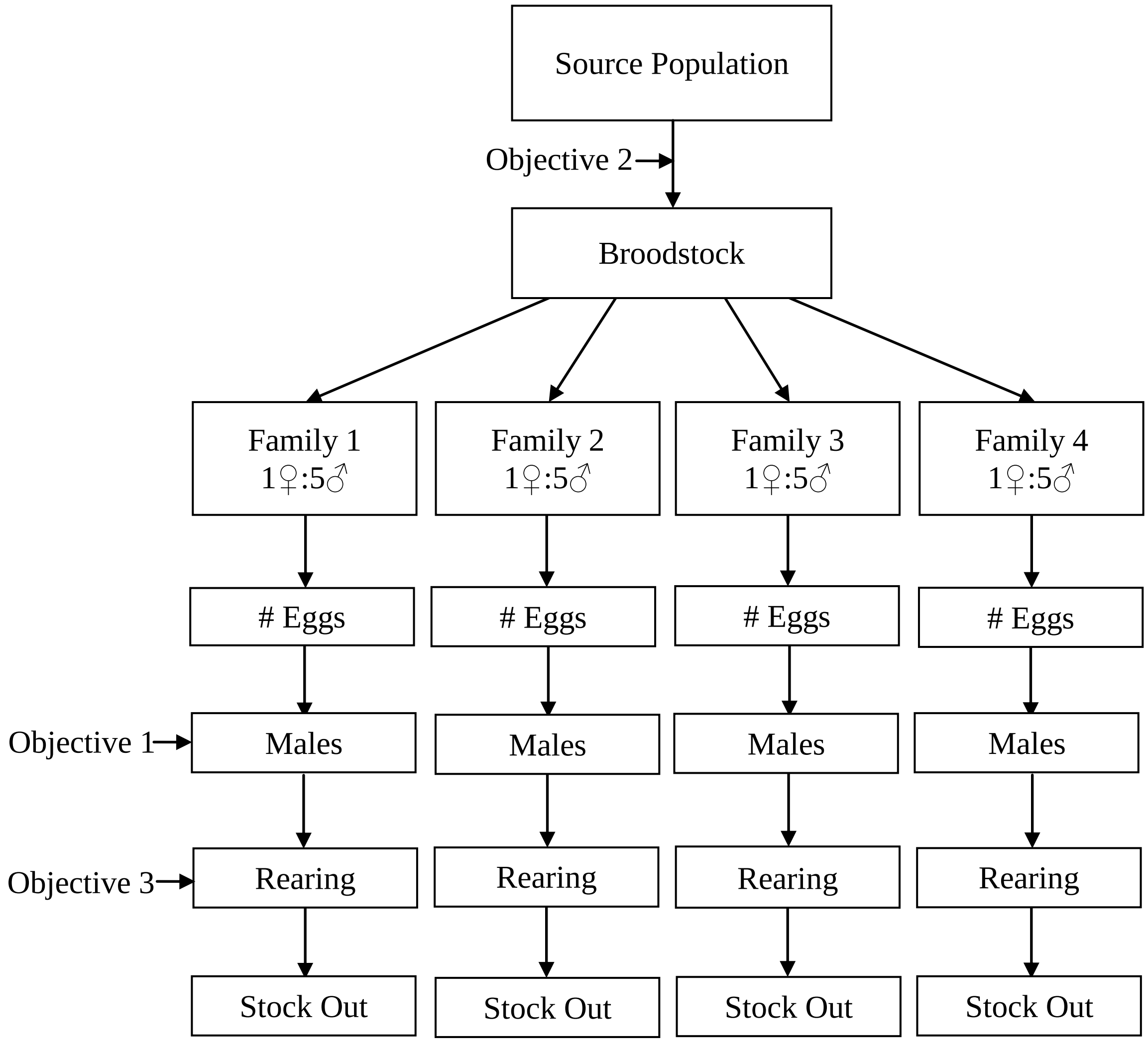

Figure 1.1. Schematic of the artificial rearing process for lake sturgeon, indicating several points at which selection and loss of genetic variability can occur, along with associated research objectives. Broodstock sampled from the source population can be selected for size, ease of capture, spawning time/location, gear bias, etc., potentially resulting in a genetically non-representative sample of the source (Objective 2). Family-based selection within the facility can occur based on sex ratio of the broodstock, number of eggs fertilized, and the spawning/hatching success of male broodstock. The latter is likely based largely on sperm competition when utilizing mixed-milt crosses to fertilize a female's eggs (Objective 1). Post-hatch selection can occur due to several variables including hatch time, size at hatch, water temperature, variety and availability of prey items, rearing density, and human/mechanical error. Such selection can result in significant reproductive disparity among male broodstock within each cross at a facility (Objective 3).

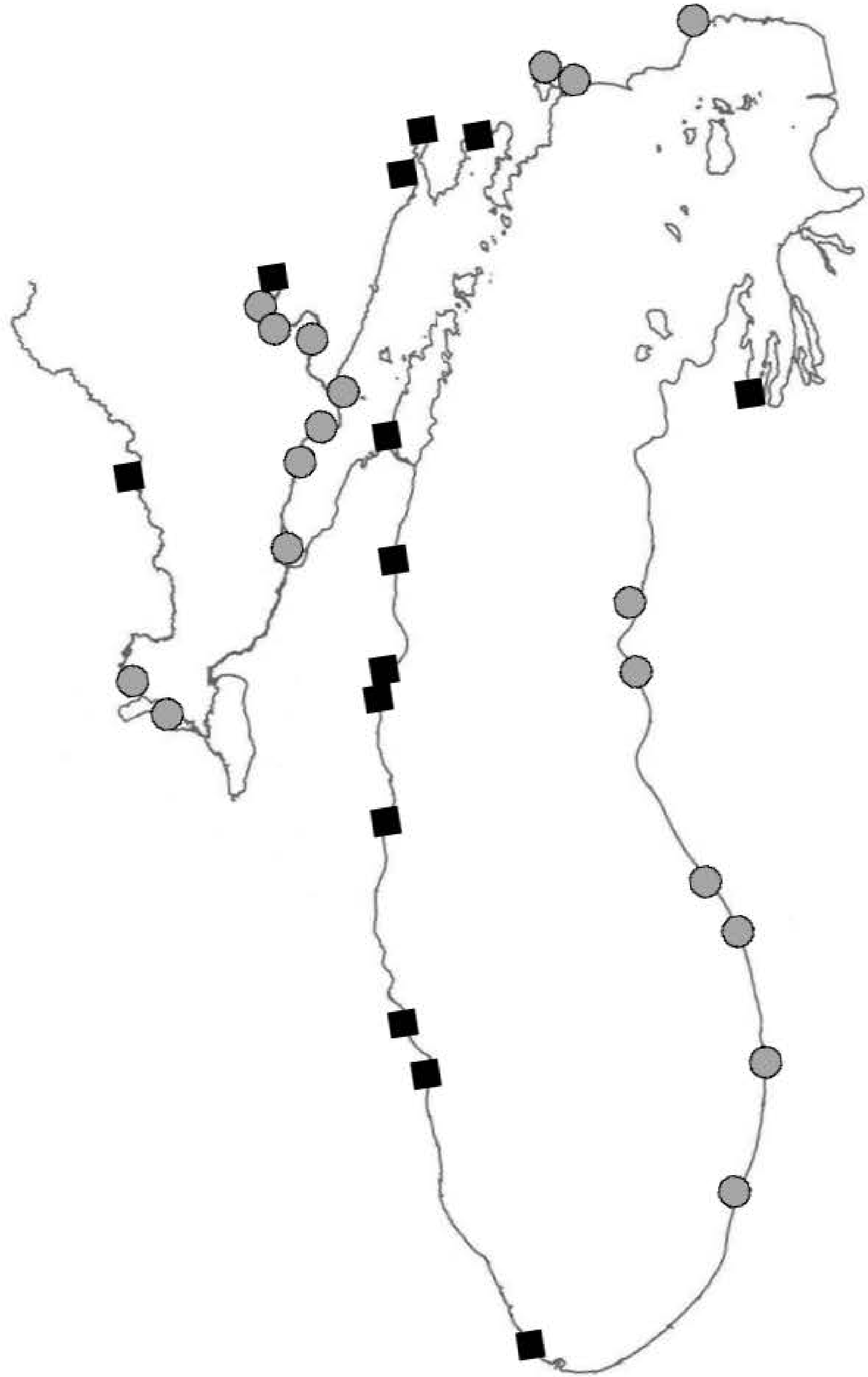

Figure 1.2. A map of Lake Michigan indicating the spawning sites of many of the basin's historic lake sturgeon populations (■ - extirpated populations, ● - remnant populations). Lake Michigan once featured at least 33 spawning populations of lake sturgeon (adapted from Zollweg et al. 2003).

Chapter II:

Sperm Competition in Mixed-milt Crosses of Lake Sturgeon

Abstract – Populations of lake sturgeon *Acipenser fulvescens* in the Lake Michigan basin have suffered massive declines over the past two centuries. In response to these declines, the Wisconsin Department of Natural Resources (WDNR) began collecting lake sturgeon eggs in 1978 at the Fox and Wolf Rivers (Winnebago and Outagamie counties, Wisconsin) of the Lake Winnebago system for artificial rearing at the Wild Rose State Fish Hatchery (Waushara County, Wisconsin). Such efforts continue today, with on-site manual fertilization by hatchery personnel typically combining the eggs of one female with the mixed-milt from two to five males. The use of a mixed-milt mating system introduces the risk of sperm competition between male broodstock. Significant reproductive bias due to sperm competition has the potential to decrease the genetic variability of the resulting progeny population, thereby increasing the likelihood of future inbreeding and, possibly, lowering viability and likelihood of survival and reproduction in the wild. The objective of this study was to determine if sperm competition occurs in one-to-five female-to-male mixed-milt crosses of lake sturgeon. To determine the appropriateness of mixed-milt crosses for artificial rearing of lake sturgeon, the progeny of three separate mixed-milt crosses were assessed for relative reproductive contribution of male broodstock by the use of six microsatellite loci and parentage analysis. The resulting paternal assignments showed significant bias in two of the three crosses. This indicated that sperm competition is present in mixed-milt crosses of lake sturgeon and has the potential to significantly bias the genetic variability of the resulting progeny toward the most successful males. Given that performance in a sperm competition scenario has not been correlated with viability of offspring or any other fitness-related

characteristic, the exclusion of certain paternal contributions as a consequence of mixed-milt mating systems represents a distinct risk to the genetic diversity and viability of lake sturgeon reared by this method.

INTRODUCTION

In degraded aquatic ecosystems, the use of proactive conservation in the form of reintroduction and/or supplementation is often necessary when continued population declines would compromise the recovery of a species through natural or artificial means (Anders 1997; Anders 1998). These programs typically aim to minimize the loss of genetic diversity from broodstock to final product, thereby maximizing viability of the fish released and the likelihood of successful rehabilitation (Brannon 1993; Anders 1998; Reisenbichler et al. 2003). A key recommendation for improving and maintaining genetic diversity is the use of a one-to-one mating system, intending to equalize the reproductive contribution from each member of the broodstock (Williams et al. 1988; Miller and Kapuscinski 2003; Campton 2004). Many hatcheries employ mixed-milt instead of one-to-one crosses, introducing the possibility of sperm competition, which is the artificial selection of phenotypic or life history traits based on the fertilization success of certain males (Campton 2004). The effects of sperm competition in mixed-milt crosses has been well documented in several species and can lead to significant shifts in the genetic makeup of the next generation because of the dominance, hindrance, or complete absence of reproductive contributions from certain members of the broodstock (Iwamatsu et al. 1991; Stockley et al. 1997; Simmons and Kotiaho 2002; Oppliger et al. 2003; Campton 2004).

Currently, the lake sturgeon *Acipenser fulvescens* is the focus of population re-establishment efforts in Wisconsin and Michigan. Lake sturgeon populations throughout the Great Lakes have suffered massive declines resulting in the species being listed as endangered, threatened, or of special concern throughout its entire range (Elliott et al.

2004). Lake Michigan, in particular, once featured as many as 11 million individuals; current population estimates are less than 10% of this historic abundance (Hay-Chmielewski and Whelan 1997; Gunderman and Elliot 2004). This decline has been attributed to the combination of degraded water quality associated with the development of the Lake Michigan basin, habitat fragmentation, especially dam construction, and overexploitation (Harkness and Dymond 1961; Rochard et al. 1990).

The collection of lake sturgeon eggs for artificial rearing in Wisconsin was first attempted in spring of 1978 at the Fox and Wolf Rivers (Winnebago and Outagamie counties, Wisconsin) of the Lake Winnebago system (Folz et al. 1983). Since that time, sturgeon eggs have regularly been collected and manually fertilized by hatchery personnel for artificial rearing at Wild Rose State Fish Hatchery (Waushara County, Wisconsin; Ćzeskleba et al. 1985). The mating system employed during fertilization typically combines the eggs of one female and the mixed-milt of two to five males (Folz et al. 1983; Ćzeskleba et al. 1985). Pooling milt of several males prior to fertilization introduces the possibility of sperm competition, owing not only to potential differences in reproductive characteristics among the males (e.g., testis size, sperm length and/or motility, etc.), but also to varying levels of maturity, time spent spawning, and sperm "quality" or concentration (Iwamatsu et al. 1991; Stockley et al. 1997; Bruch and Binkowski 2002; Simmons and Kotiaho 2002; Oppliger et al. 2003).

Losses of genetic diversity, such as those associated with sperm competition and resulting high variance in male reproductive contribution, are considered detrimental to the success of conservation rearing programs (Withler 1988; Miller and Kapuscinski 2003; Campton 2004; Allendorf and Luikhart 2007). Minimizing the loss of genetic diversity of released fish maximizes the likelihood of their future survival and reproduction in wild conditions (Frankham et al. 2002; Allendorf and Luikhart 2007). The founding of a population produced by a relatively small number of spawners can reduce evolutionary potential and likelihood of future persistence (Frankham et al. 2002). To determine the appropriateness of mixed-milt mating systems for artificial rearing of lake sturgeon, the significance of sperm competition in such crosses must be assessed. The objective of this study was to determine if sperm competition occurs in one-to-five female-to-male mixed-milt crosses of lake sturgeon.

METHODS

Study Design and Sample Collection

To determine the extent of sperm competition in lake sturgeon crosses, three mixed-milt crosses of Wolf River lake sturgeon were performed at the Great Lakes Water Institute (University of Wisconsin-Milwaukee), specifically for this objective. Male broodstock spawned for this study ranged from 100-183 cm in length, whereas female broodstock ranged from 140-200 cm in length. The progeny of each cross were euthanized at larval stage and stored separately from the progeny of other crosses. Detailed records were kept of which broodstock were crossed to produce each group of progeny.

Genetic samples for sperm competition assessment were larval fish (n = 82-83/cross) and fin-clips from the adults that spawned them. Each sample was assigned an individual archive number and placed into a labeled tube with 95% ethanol. These samples were submitted to the Molecular Conservation Genetics Laboratory (MCGL) at the University of Wisconsin-Stevens Point (UWSP) for genetic analysis.

Each cross consisted of five males, simultaneously tested in one-to-one crosses to confirm fertility, and one female, with the total progeny of each cross stored separately. Given a known mother, each individual offspring was assigned to its father through genetic parentage assignment that allowed for the comparison of the relative reproductive contributions of each male.

DNA Extraction

Genomic DNA was extracted from each tissue sample using the Promega Wizard® Genomic DNA purification kit (Promega Corp., Madison, WI) with an in-house modification for a 96-well format. DNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and checked for quality (presence of high molecular weight fragments) via electrophoresis on a 1% agarose gel. DNA concentrations were normalized to a standard concentration (20 ng/μl) for subsequent genotyping.

Microsatellite Genotyping

Six microsatellite loci previously identified for use in lake sturgeon studies were used in this study (May et al. 1997; McQuown et al. 2002; Welsh et al. 2003; Table 2.1). Samples were amplified by polymerase chain reaction (PCR) with one primer for each locus end-labeled with a commercially-available fluorescent dye (Table 2.1). Genotyping was performed on an ABI Prism™ 377XL automated DNA sequencer (Applied Biosystems, Inc. Foster City, CA). A known size standard (GeneFlo™ 625 DNA Ladder, Chimerx Corp., Milwaukee, WI) was included in each sample to allow for accurate sizing of alleles. Allele sizes were estimated by GeneScan® software (Applied Biosystems Inc.), allele calls were manually confirmed, and the resulting data was direct-count, multi-locus genotypes.

Data Analysis

To determine if sperm competition was occurring as a result of mixed-milt crosses, the known mother, the potential fathers, and individual progeny from each of the three female crosses were genotyped and paternity analysis performed. Parental assignment for lake sturgeon progeny was conducted using PROBMAX v1.3 (Danzmann 1997). This program uses multilocus genotype data of all possible parents along with that of the progeny and the mating history of the adults to determine the most likely parents for each individual progeny. Each male's reproductive contribution was compared to the expected value of 20% of the total progeny using chi-square analysis.

Differential reproductive contribution and associated loss of genetic diversity can result in a change in the effective population size (N_e). The N_e is a measure roughly analogous to the number of breeders in a population. It is commonly used as a predictor of genetic variability in a population and to measure the relative risk of genetic drift (Frankham et al. 2002). Populations with low N_e are at greater risk of genetic drift, inbreeding, etc. (Frankham et al. 2002). A single-year equivalent of N_e, known as the effective number of breeders (N_b), is often used to determine the level of genetic diversity retained through a hatchery process (Campton 2004). Maximizing the N_b of the final product is the principle means by which a conservation aquaculture program can maintain the genetic characteristics of the broodstock and increase the overall genetic diversity in the resulting progeny that will form the foundation of the rehabilitated population.

The N_b of an artificially-reared population with unequal sex ratio is calculated as follows:

$$N_b = \frac{4N_{bf}\,N_{bm}}{N_{bf} + N_{bm}}$$

where N_{bf} is the effective number of female breeders and N_{bm} is the effective number of male breeders (Hedrick 2005). The N_{bf} is often set equal to the number of female broodstock when dealing with mixed-milt crosses, and N_{bm} is calculated as follows:

$$N_{bm} = \frac{N_m\bar{k}_m - 1}{\bar{k}_m - 1 + \left(\frac{V_k}{\bar{k}_m}\right)}$$

where V_k is the variance in progeny sired and $\bar{k}_m$ is the mean number of progeny sired by the males (Hedrick 2005). Variance in progeny sired (V_k) is calculated as follows:

$$V_k = \sum_i x_i \left(k_i - \bar{k}_m\right)^2$$

where x_i is the proportion of progeny produced by a male *i* and k_i is the number of progeny sired by male *i*. (Hedrick 2005).

To quantify the genetic implications of mixed-milt spawning, we compared observed N_b for the three experimental crosses versus expected N_b with equal contribution from male broodstock. We then extrapolated the total of the resulting N_b estimates to determine N_e output over 20 years, which is approximately equal to a single lake sturgeon generation and a recommended time frame for lake sturgeon conservation rearing programs (Zollweg et al. 2003).

Progeny assignment for Cross A was hampered by an inheritance issue in Male 2 at *Spl120*. Fifteen Cross A progeny featured alleles at other loci unique to Male 2, yet could not be automatically assigned to Male 2 due to incompatibility at *Spl120*. This inheritance issue was confirmed through several replicate genotyping runs, along with the genotyping of additional progeny from Cross A, some of which showed the same issue. These steps were taken to rule out genotyping error and the inheritance issue is a unique occurrence in this cross. Due to the presence of unique Male 2 alleles at other loci, however, these progeny were assigned to Male 2 and are included in the results and analysis of this chapter. To my knowledge, such an inheritance issue has not been reported in any other lake sturgeon studies at the MCGL or other laboratories.

RESULTS

Three experimental crosses with one female and five males per cross were sampled and genetically tested (18 total fish). A total of 247 larval progeny were genotyped, of which 199 could be assigned to a unique father. Numbers of progeny assigned were 55, 71, and 73 (Crosses A, B, and C, respectively). A total of 34 alleles were observed across the six loci and the numbers of alleles per locus ranged from four (*AfuG63*) to nine (*Afu68b*).

Paternal Assignment

The variation present in the six microsatellite loci selected proved to be sufficient to assign 86.6% of progeny genotyped (214 of 247; Table 2.2). All males were fertile in the one-to-one fertilization tests. Cross A had 85.4% of progeny assigned (70 of 82), Cross B had 86.6% assignment (71 of 82), and Cross C had 88.0% assignment (73 of 83). Over the three crosses, individual fathers contributed between 4.2% and 52.1% of assigned progeny within their cross. Cross A showed ranges of 4.3% to 40.0% contribution, Cross B showed ranges of 4.2% to 52.1% contribution, and Cross C showed ranges of 11.0% to 27.4% contribution.

Chi-square analysis indicated significant bias in male reproductive contribution in two of the three crosses surveyed (Table 2.2). Cross A showed significant reproductive disparity ($\chi^2 = 54.64$, df = 4, $p < 0.001$), as did Cross B ($\chi^2 = 53.86$, df = 4, $p < 0.001$). Cross C had more balanced contribution, resulting in a lack of significant reproductive disparity ($\chi^2 = 5.97$, df = 4, $p > 0.200$).

Effective Number of Breeders

Effective breeder estimates ranged from 2.71 (Cross B) to 3.32 (Cross C; Table 2.2). All three crosses showed a decrease in N_b versus expected N_b based on random male reproductive variance. Proportional reduction in N_b as a result of male reproductive variance ranged from 0.3% (Cross C) to 18.6% (Cross B). Total N_b extrapolated over a lake sturgeon generation (20 years) equaled an N_e of 175.81 versus an expected N_e of 199.48. This indicates that the output of a 20-year conservation rearing program with a similar level of male reproductive variance present in our experimental crosses would show an 11.9% proportional reduction in N_e versus expectations of random male contribution.

DISCUSSION

The objective of this study was to determine if sperm competition occurs in one-to-five female-to-male mixed-milt crosses of lake sturgeon. Sperm competition was present in mixed-milt crosses of lake sturgeon and has the potential to significantly bias reproductive contributions of male broodstock, thereby reducing N_b and the overall genetic output of the mating crosses. The significant reproductive bias observed in this study was consistent with several other mixed-milt studies in a variety of fish species (Gharrett and Shirley 1985; Withler 1988; Gile and Ferguson 1995; Hoysak et al. 2004).

Given the confirmed fertility of the male broodstock used in this study, the observed reproductive disparity could be a result of several methodological and/or biological variables. Previous studies have cited inconsistencies in the storage, pooling, and application of milt as possible contributing factors for reproductive disparity (Gharrett and Shirley 1985; Withler 1988; Withler and Beacham 1994). Mixed-milt crosses of lake sturgeon in Wisconsin have typically mixed the milt of two to five males for storage in glass vials on ice (Folz et al. 1983; Ćzeskleba et al. 1985). Milt from the males is usually collected first, after which a female is located, captured, and stripped of eggs, a process which requires milt storage ranging from 15 min to > 2 h (Ćzeskleba et al. 1985). Hatchery personnel attempt to not utilize milt exposed to water, but if such exposure was undetected, issues of sperm motility and sperm age could affect milt within the vials (Stoss 1983; Ćzeskleba et al. 1985; Hoysak et al. 2004). Biological disparities cited in other sperm competition studies include the relative maturity of male broodstock and the proximity to spawning females (Defraipont and Sorensen 1993; Withler and Beacham 1994; Liley and Kroon 1995; Zheng et al. 1997). Given the lake sturgeon reproductive strategy of several spawning opportunities over a relatively long lifespan (for males, every 1-4 years over ~55 years; Roussow 1957), there exists the potential for a continuum of relative maturity among males at a spawning site. Also, Bruch and Binkowski (2002) noted an inverse relationship between sperm concentration and time spent spawning. Randomly capturing male broodstock of equal advantage in the variables listed above is unlikely. Nevertheless, all males used in this study were shown to be viable in the one-to-one spawnings. Therefore, concerns over male fertility do not solely account for the observed variance.

Given the conservation aquaculture goal of maintaining genetic diversity, mating systems should not be utilized that result in the exclusion or reduction of reproductive contribution from any member of the broodstock (Anders 1998). In an artificial rearing system, choice of breeding scheme is the initial and most effective control over reproductive variance and loss of genetic diversity (Allendorf and Luikart 2007). Choosing the proper mating scheme to minimize reproductive variance increases the likelihood of equal family contribution, with selection being driven by rearing conditions and hatchery practices, which are much more easily adjusted than the variables of sperm competition.

The loss of genetic diversity through genetically detrimental hatchery methods during lake sturgeon conservation rearing has been suggested as a key impediment to successful rehabilitation of populations within the Great Lakes (Holey et al. 2004). Equalization of reproductive contribution to maintain genetic diversity is repeatedly identified as a high priority in conservation aquaculture operations (Withler 1988; Miller and Kapuscinski 2003; Campton 2004; Allendorf and Luikart 2007). Sperm competition resulting in high variance in male reproductive contribution is a primary means by which genetic variability can be lost in hatchery programs using mixed-milt crosses. The potential 20-year reduction in N_e shown by this study indicates the likelihood of reduced genetic output from facilities with similar variance in male reproductive success. Given that these fish were sampled immediately post-hatch, facilities with similar reproductive variance may actually suffer further reductions in N_b by the end of the rearing process due to further artificial selection prior to stocking.

The high reproductive variance found in mixed-milt crosses of lake sturgeon and the potential for the loss of genetic variability indicated mixed-milt crosses are genetically inappropriate for lake sturgeon conservation aquaculture and population rehabilitation purposes. The success of supplemental or rehabilitation breeding programs depends on the ability to maximize the genetic diversity of released fish, thereby allowing environmental conditions in the wild to dictate the most beneficial genes and gene complexes (Allendorf and Luikart 2007). Decreases in genetic diversity are of particular concern for programs attempting to reestablish spawning populations of an imperiled species such as lake sturgeon. The founding of a population by a relatively small number (i.e., genetic effective number) of individuals can result in a loss of allelic diversity, thereby reducing evolutionary potential and likelihood of persistence (Frankham et al. 2002).

Future Research

The results of this study indicate the need for further research into the mechanisms and variables of sperm competition in mixed-milt crosses of lake sturgeon. While this project was sufficient to determine the presence and extent of sperm competition in such crosses, further information should be gathered by expansion of the study design. Incorporating replicates of experimental crosses would allow for insight into the heterogeneity of male broodstock performance in sperm competition. Previous studies of sperm competition in fish have identified significant heterogeneity in individual male contribution in replicated sperm competition trials against the same males (Gharrett and Shirley 1985; Withler 1988).

Specific expansions to the overall study design would allow for the assessment of female-based disparity in male reproductive contribution. Female-based disparity could be assessed by replicating milt mixtures and using them to fertilize individual egg lots of various females. Hatching each lot separately and assigning parentage of larval fish will allow for comparisons of relative male contribution to each female's lot of eggs. Comparing individual male contributions to the replicates proposed in the previous paragraph will allow for detection of significant variation in male contribution based on female.

Several other crossing strategies and mating systems suggested for conservation rearing could be assessed in future studies. Among the suggestions for the utilization of all males captured during broodstock sampling is the mixing of milt from overlapping males (i.e., 1 and 2, 2 and 3, 1 and 3; Miller and Kapuscinski 2003). While typically suggested to address infertility concerns, this mating system is meant to decrease the concerns of sperm competition while also allowing for the utilization of a greater number of male broodstock. Overlapping crosses could be assessed for paternity bias and its effect on N_b to determine if such a mating system is genetically appropriate for lake sturgeon conservation rearing. The splitting of all broodstock gametes into equal lots so that each male can be spawned with each female, a practice known as factorial mating, has been suggested for species with a small number of broodfish and programs meant to maximize genetic diversity (Miller and Kapuscinski 2003). Larval fish hatched under a factorial mating system could be pooled based on mother and assessed for variance in male reproductive contribution (now simply a function of fertility and hatching success). Significant gains in N_b compared to other mating systems would indicate the potential genetic gains of employing such a system, provided the likely marked increase in rearing space and handling time is available and cost-effective.

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Table 2.1. PCR conditions for two co-load reactions used in this study, including loci, allele size range, number of alleles per locus (*A*), locus-specific primers and labels, 10x PCR buffer, dNTPs, $MgCl_2$, and *Taq* polymerase concentrations. Afu68b is from May et al. (1997) and McQuown et al. (2002), Spl120 is from McQuown et al. (2002), and all other loci are from Welsh et al. (2003).

Co-Load	Locus	Range	*A*	Label	Primer (μM)	10x Buffer	dNTPs (mM)	$MgCl_2$ (mM)	*Taq* (units)
A	AfuG112[1]	246-266	5	NED™	0.30	1x	0.80	1.50	0.50
	AfuG63[1]	127-143	4	NED™	0.20	1x	0.80	1.50	0.50
	Afu68b[1]	162-194	9	6FAM™	0.20	1x	0.80	1.50	0.50
B	Spl120[2]	254-286	5	NED™	0.20	1x	0.80	1.50	0.50
	AfuG122[1]	161-179	6	6FAM™	0.20	1x	0.80	1.50	0.50
	AfuG9[1]	125-152	7	6FAM™	0.20	1x	0.80	1.50	0.50

[1] 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 52°C for 45 s, and 72°C for 2 min, ending with a final elongation of 72°C for 5 min.

[2] 94°C for 1 min, followed by 20 cycles of 92°C for 30 s, and 70°C for 40 s (decrease 0.5°C per cycle), followed by 20 cycles of 92°C for 30 s, and 60°C for 40 s (increase 1 s per cycle).

Table 2.2. Male reproductive contributions to three mixed-milt crosses of lake sturgeon, Male is the ID of each male used in a mixed-milt cross with an individual female, N is the number of progeny sired by a given male, Prop. is the proportion of each male's contribution to a cross, df is the chi-square test degrees of freedom, χ^2 is the chi-square test statistic, p is the chi-square estimated p-value compared to equal contribution, V_k is the variance in male reproductive contribution for each cross, $\bar{k}_m$ is the mean number of progeny produced by each male, N_b is the estimate of effective number of breeders, N_b exp. is the expected estimate of effective number of breeders based on random contribution, % is the percent reduction in N_b given the bias in male reproductive contribution, N_{e20} is the total N_b (all crosses) extrapolated over 20 years of rearing, N_{e20} exp. is N_b exp. over 20 years, and $\%_{20}$ is % over 20 years.

Female	Male	N	Prop.	df	χ^2	p	V_k	$\bar{k}_m$	N_b	N_b exp.	%	N_{e20}	N_{e20} exp.	$\%_{20}$
A	1	28	0.400	4	60.09	<0.001	159.46	14.0	2.96	3.32	10.8	179.80	199.48	9.9
	2	27	0.386											
	3	7	0.100											
	4	5	0.071											
	5	3	0.043											
B	6	37	0.521	4	53.86	<0.001	287.47	14.2	2.71	3.33	18.6			
	7	17	0.239											
	8	3	0.042											
	9	9	0.127											
	10	5	0.070											
C	11	18	0.247	4	5.97	>0.200	16.14	14.6	3.32	3.33	0.3			
	12	13	0.178											
	13	8	0.110											
	14	20	0.274											
	15	14	0.192											

Chapter III:

Genetic Assessment of Traditional Hatchery and Stream-Side Rearing Techniques for the Re-establishment of Lake Sturgeon Populations

Abstract – Over the past two centuries, lake sturgeon *Acipenser fulvescens* populations have suffered massive declines. In response to these declines, the Wisconsin Department of Natural Resources (WDNR) artificially rears lake sturgeon at the Wild Rose State Fish Hatchery (Waushara County, Wisconsin) for restoration and supplemental stocking. Methods employed in the program include on-site manual fertilization by hatchery personnel, typically combining the eggs of one female with the mixed-milt from two to five males, and rearing of all sturgeon progeny together in communal hatchery ponds. Concerns exist regarding the effectiveness of the program to establish lake sturgeon spawning runs and the rate of straying these fish exhibit, thereby threatening native, remnant populations in Lake Michigan. The WDNR and Michigan Department of Natural Resources (MDNR) began rearing lake sturgeon at stream-side rearing facilities (SRFs) in 2006 and 2007, respectively. These facilities are located on the banks of four target streams, allowing the young sturgeon to potentially imprint and reestablish a spawning run in the stream. The spawning and rearing techniques used at the SRFs differ greatly from Wisconsin traditional hatchery methods, with the female's eggs split into five roughly equal lots which are fertilized by a single male per lot, after which female-based families are reared separately from one another. The SRF spawning and rearing methods are designed to maximize genetic diversity and effective number of breeders (N_b) by minimizing the reproductive variance of broodstock within each cross in the facility. The objectives of this study were to compare the genetic diversity found within each broodstock group to that of their source population and to determine the extent of

reproductive disparity among male broodstock at each rearing facility, along with associated reductions in genetic diversity. Ten microsatellite loci were used to characterize the genetic diversity present in each broodstock group and evaluate the relative reproductive contributions of male broodstock to each cross at the facilities. All broodstock used were found to be genetically representative of their respective source populations, even during years characterized by difficult adult capture or facility problems that resulted in relatively small numbers of total broodstock. Significant paternal bias was also detected in final progeny of the SRFs, in the larval stage at Wild Rose, and in natural larval mortalities in the SRFs. This indicates that survival at the larval stage in the SRFs may be responsible for biased parentage at later stages. These data suggest significant genetic gains can be made by furthering attempts to equalize reproductive contributions of male broodstock. This could be accomplished by hatching egg lots in separate containers and combining into female-based families for rearing only after hatch. Together with allowing progeny to imprint, these data suggest that stream-side rearing holds great promise for the re-establishment of genetically representative lake sturgeon spawning runs in the target streams.

INTRODUCTION

The lake sturgeon is a freshwater benthic species endemic to the Great Lakes, Hudson Bay, and Mississippi River drainages (Houston 1987; Peterson et al. 2007). Over the past two centuries, lake sturgeon populations have suffered massive declines resulting in the species being listed as endangered, threatened, or of special concern throughout its entire range (Elliott et al. 2004). Lake Michigan, in particular, once featured as many as 11 million individuals, with current population estimates less than 10% of the historic abundance (Hay-Chmielewski and Whelan 1997; Gunderman and Elliot 2004). This decline has been attributed to the combination of degraded water quality associated with the development of the Lake Michigan basin, habitat fragmentation, especially dam construction, and overexploitation (Harkness and Dymond 1961; Rochard et al. 1990).

In response to lake sturgeon population declines, the Wisconsin Department of Natural Resources (WDNR) and Michigan Department of Natural Resources (MDNR) artificially rear sturgeon at several facilities for stocking in Lake Michigan. These include traditional hatcheries such as the Wild Rose State Fish Hatchery (Waushara County, Wisconsin) and several recently constructed stream-side rearing facilities (SRFs). Stream-side rearing was developed to allow the young sturgeon the potential to imprint to their birth waters a reestablish spawning runs in the target streams. Wisconsin traditional hatchery methods include a mating system typically combining the eggs of one female and the mixed-milt of two to five males, after which all progeny are reared communally (Folz et al. 1983; Ćzeskleba et al. 1985). Stream-side rearing methods divide a female's eggs into roughly equal lots, which are fertilized by a single male per lot, after which female families are reared separately.

To assess the genetic consequences of the various spawning and rearing strategies for artificial rearing of lake sturgeon (i.e., traditional hatchery vs. SRF methods), the maintenance of genetic diversity at several points of the rearing process must be assessed. The first objective of this study was to determine whether broodstock groups sampled for gamete collection were genetically representative of their respective source populations. The second objective was to quantify the significance and effect of reproductive disparity among male broodstock at all rearing facilities.

METHODS

Study Design and Sample Collection

To determine the genetic effect of current lake sturgeon artificial rearing methods, tissue samples for genetic analysiswere collected from both types of facilities (traditional hatchery and SRF). Sampling varied by year and facility, but overall project sampling involved three points in the lake sturgeon artificial rearing process (broodstock, larval fish, and final progeny; Table 3.1). Broodstock tissue samples consisted of fin-clips taken from all spawning adults during broodstock capture. Larval fish tissue samples consisted of either a subsample of progeny (Wild Rose 2006) or natural production mortalities, with all sampling occurring less than three weeks post-hatch. Final stock tissue samples consisted of fin-clips from a subsample of final stock progeny or fin-clips from all final stock progeny during years of relatively low production (Table 3.1).

Study streams assessed in this project were on the western shore of Lake Michigan in Wisconsin and the Upper Peninsula of Michigan and the southern shore of Lake Superior in the Upper Peninsula of Michigan (Figure 3.1). Broodstock sampled from the Wolf River (Outagamie County, Wisconsin) provided gametes for the Wild Rose State Fish Hatchery (Waushara County, Wisconsin) and the SRFs located on the Manitowoc River (Manitowoc County, Wisconsin) and the Milwaukee River (Ozaukee County, Wisconsin). Broodstock sampled from the Menominee River (Menominee County, Michigan) provided gametes for the SRFs located on the Cedar River (Menominee County, Michigan) and the Whitefish River (Delta County, Michigan). Broodstock sampled from the Sturgeon River (Houghton County, Michigan) provided gametes for the SRF located on the Ontonagon River (Ontonagon County, Michigan).

DNA Extraction

Genomic DNA was extracted from each tissue sample using the Promega Wizard® Genomic DNA purification kit (Promega Corp., Madison, WI) with an in-house modification for a 96-well format. DNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and checked for quality (presence of high molecular weight fragments) via electrophoresis on a 1% agarose gel. DNA concentrations were normalized to a standard concentration (20 ng/μl) for subsequent genotyping.

Microsatellite Genotyping

Ten microsatellite loci previously identified for use in lake sturgeon studies were used in this study (May et al. 1997; McQuown et al. 2002; Welsh et al. 2003; Table 3.1). Samples were amplified by polymerase chain reaction (PCR) with one primer for each locus end-labeled with a commercially-available fluorescent dye (Table 3.2). Genotyping was performed on an ABI Prism™ 377XL automated DNA sequencer (Applied Biosystems, Inc. Foster City, CA). A known size standard (GeneFlo™ 625 DNA Ladder, Chimerx Corp., Milwaukee, WI) was included in each sample to allow for sizing of alleles. Allele sizes were determined by GeneScan® software (Applied Biosystems Inc.), allele calls were manually confirmed, and the resulting data were direct-count, multi-locus genotypes.

Data Analysis

Broodstock Diversity.—To determine whether broodstock sampled from the Wolf River, Menominee River, and Sturgeon River were genetically representative of their respective populations, genetic diversity of each broodstock group was compared to that of the individual source populations (DeHaan 2003; Welsh and McClain 2004; DeHaan et al. 2006; Sloss and Kittel 2007). Allele count data from each broodstock group was tested for independence from source population allele counts by use of a G-test performed in Genepop v3.4 (Raymond and Rousset 1995). Resulting p-values for each locus were combined using Fisher's method, providing an overall p-value for each broodstock group (Fisher 1925).

Progeny Diversity.—Paternal assignment for lake sturgeon progeny in this study was performed in PROBMAX v1.3 (Danzmann 1997). This program uses multilocus genotype data of all possible parents along with that of the progeny and the mating history of the adults to determine the most likely parents for each individual progeny. Each male's reproductive contribution to a spawning cross was compared to the expectation of equal contribution using chi-square analysis. Resulting p-values for each cross were combined using Fisher's method, providing an overall p-value for each facility (Fisher 1925).

Shifts in genetic diversity associated with differential reproductive contribution can result in a change in the effective population size (N_e). The genetic concerns commonly associated with small population size (genetic drift, inbreeding, etc.) are actually results of low N_e. In fact, large populations with low N_e are just as likely to experience such deleterious conditions (Frankham et al. 2002). A single-year equivalent of N_e, known as the effective number of breeders (N_b), approximates the genetic number of broodstock contributing to a year's progeny and is often used to assess hatchery protocols for the maintenance of genetic diversity (Campton 2004). The N_b of an artificially-reared population is calculated as follows:

$$N_b = \frac{4N_{bf} N_{bm}}{N_{bf} + N_{bm}}$$

where N_{bf} is the effective number of female breeders and N_{bm} is the effective number of male breeders (Hedrick 2005). When assessing a mating system most heavily influenced by variance in male reproductive success (i.e., mixed-milt crosses), effective number of female breeders is equal to the number of female broodstock. The N_{bm} is calculated as follows:

$$N_{bm} = \frac{N_m \bar{k}_m - 1}{\bar{k}_m - 1 + \left(\frac{V_k}{\bar{k}_m}\right)}$$

where V_k is the variance in progeny sired and $\bar{k}_m$ is the mean number of progeny sired by the males (Hedrick 2005). Variance in progeny sired is calculated as follows:

$$V_k = \sum_i x_i \left(k_i - \bar{k}_m\right)^2$$

where x_i is the proportion of progeny produced by a male *i* and k_i is the number of progeny sired by male *i*. (Hedrick 2005). To quantify the genetic implications of mixed-milt spawning, we calculated observed N_b for each spawning cross, and then combined N_b estimates by facility to compare observed N_b for each facility versus expected N_b with random contribution from male broodstock. We also calculated the percent reduction in N_b to quantify the effect of male reproductive variance on estimates of N_b.

RESULTS

Broodstock Diversity

A total of 92 lake sturgeon broodstock were sampled and used by the WDNR and the Michigan Department of Natural Resources (MDNR) over the three years of this study (Table 3.1). Numbers of broodstock per facility ranged from five (Cedar 2007 and Whitefish 2007) to 38 (Wild Rose 2006 and Milwaukee 2006). All broodstock groups were found to be statistically representative of their respective source populations, based on G-tests of allele counts at each locus, combined with Fisher's method (Table 3.3). Even relatively small broodstock groups of five and seven (Michigan 2007 and 2008) were statistically representative of source populations of the Menominee River and Sturgeon River, respectively. Production at the Milwaukee River SRF in 2007 suffered a loss of two families of progeny due to pump issues, resulting in a final broodstock group size of 12 (rather than the full complement of 24 broodstock), but still retained allele counts statistically representative of the Wolf River source population (Table 3.3).

Progeny Diversity

A total of 369 larval lake sturgeon progeny were genotyped over the three years of this study (Table 3.1) Numbers of larval progeny genotyped per facility ranged from 46 (Cedar 2007 and Whitefish 2007) to 133 (Milwaukee 2008). Of the larval progeny genotyped, paternity was successfully resolved for 333 (90.2%). Individual male contribution to crosses within rearing facilities ranged from 0.0% (Wild Rose 2006 and Milwaukee 2008) to 100% (Wild Rose 2006; Table 3.4). The Ontonagon River SRF (2008) was the only rearing facility not showing reproductive bias among male broodstock (Table 3.4). The Ontonagon facility larval progeny also maintained N_b at the greatest rate, losing only 1.2% of expected N_b based on random reproductive contribution. All other facilities showed biased paternity by larval stage, with percent reductions in N_b ranging from 14.5% to 34.0% (Table 3.4). Estimates of N_b at the larval stage ranged from 2.30 (Cedar 2007) to 13.55 (Wild Rose 2006), whereas expected N_b ranged from 3.18 (Cedar 2007 and Whitefish 2007) to 18.45 (Wild Rose 2006).

A total of 744 final stock fish were genotyped over the three years of this study (Table 3.1). Numbers of final stocked fish genotyped per facility/year ranged from 27 (Milwaukee 2006) to 266 (Milwaukee 2008). Of the stocked fish genotyped, paternity was successfully resolved for 617 (82.9%). Individual male contribution to crosses within rearing facilities ranged from 0.0% (Manitowoc 2007, Milwaukee 2007, Milwaukee 2008) to 100.0% (Milwaukee 2006; Table 3.5). The Milwaukee River SRF (2006) was the only facility to rear stocked progeny not showing reproductive bias among male broodstock (Table 3.5). The lack of bias was evident despite the large range of male reproductive contribution to crosses at the facility (0.0% to 100.0%), and is likely an artifact of relatively small sample size (19 progeny assigned paternity). All other facilities showed biased paternity by final stock stage. Percent reductions in N_b ranged from 1.1% to 41.4%, with the Milwaukee River SRF (2007) retaining N_b at the greatest rate (Table 3.5). The Milwaukee River SRF (2006) produced an estimated N_b slightly greater than expected, however the small sample size of this year could be influencing this estimate. Estimates of N_b at stock out for each tested facility ranged from 2.18 (Cedar 2007) to 11.66 (Manitowoc 2007).

The percentage of overall progeny successfully assigned paternity varied by state, with Wisconsin producing a total of 80.2% assignable progeny (536 of 668) and

Michigan producing a total of 93.0% assignable progeny (414 of 445). This is likely a function of relative diversity among the male broodstock used in production. Percent reduction in N_b by final stock stage was similar by state, with Wisconsin facilities losing 17.8% and Michigan facilities losing 18.7%. However, mean N_b per facility over the three years of this study favored Wisconsin, 5.98 to 2.66, likely a function of greater broodstock numbers used in Wisconsin facilities across each year of this study.

DISCUSSION

The loss of genetic diversity and the choice of genetically detrimental hatchery methods during lake sturgeon conservation rearing have been suggested as key impediments to successful rehabilitation of populations within the Great Lakes (Holey et al. 2000). Two primary means of avoiding a loss of genetic diversity in propagation programs are to sample adequate broodfish to represent the native diversity and to equalize reproductive contribution among broodfish.

Broodstock Diversity

The genetic diversity found in broodstock groups used in this study was consistently representative of the source populations based on allele count distributions. Broodstock groups as small as five were found to be statistically representative of their source populations based on comparisons of allele counts. This is in conflict with a previous study of lake sturgeon stocking in the Mississippi and Missouri Rivers, in which Drauch and Rhodes (2007) found that a single year's broodstock from the Wolf River was not genetically representative of the source population (based on allelic richness), likely due to the small number of broodstock used. This indicates that any inadvertent or intentional selection during broodstock sampling (e.g., gear bias, spawning location, size, ease of capture) has not significantly biased the genetic diversity of broodstock sampled from the larger source populations. However, while all broodstock groups had allele count distributions statistically representative of their respective source populations, each broodstock group lost at least 28% of the alleles previously documented in their source population (DeHaan 2003; Welsh and McClain 2004; DeHaan et al. 2006; Sloss and Kittel 2007). While hatchery crews cannot be expected to knowingly account for a greater number of alleles during broodstock sampling, such proportional reductions in observed versus available alleles indicates the importance of maximizing broodstock number. Such maximization can help lessen the impact of any intentional or inadvertent selection for or against individual fish.

The observed reduction in N_b based on number of broodstock indicates the importance of capturing and successfully spawning a full complement of broodstock, based on the space and capability of the rearing facility. For example, final stock progeny from the Ontonagon River SRF (production year 2008) had the tightest range of male reproductive contribution and the lowest percent reduction in N_b based on equal male contribution. However, due to a relatively small number of broodstock resulting in only one spawning cross, total N_b output was only 3.35 (Table 3.5). Had the Ontonagon facility spawned the full four female crosses, with the same number of progeny produced and the same variance in male reproductive contribution, total N_b output would have been 13.41, higher than any other rearing facility in this study.

Maximization of broodstock numbers for conservation rearing is a primary concern in the maintenance of genetic diversity (Frankham et al. 2002; Miller and Kapuscinski 2003). Drauch and Rhodes (2007) noted that the relatively high level of genetic diversity found in reintroduced populations of lake sturgeon in the Mississippi and Missouri Rivers is likely the result of a large number of broodstock used over several years of hatchery production. The SRFs in this study are designed for the rearing of four families of progeny produced by four separate 1♀:5♂ broodstock crosses. Adhering to the goal of 24 total broodstock allows for the opportunity to maximize the diversity of a facility's population. Maximizing N_e and allelic diversity throughout a conservation rearing program increases the likelihood of survival and reproduction in the wild and the re-establishment of populations within target streams.

Progeny Diversity

The high variance in male reproductive success detected at the larval stage during this project indicated that similar disparity at stock out is not simply an effect of long-term rearing conditions. While rearing conditions seemed to produce male-mediated success in some facilities (e.g., poor survival of Male A5 progeny beyond larval stage at Ontonagon 2008), conditions within the egg incubation containers may also cause or contribute to high male reproductive variance. Over the period of this study, egg lots at Wisconsin SRFs were typically recombined into female-based families 4-7 h post-fertilization (B. T. Eggold, WDNR, personal communication). While egg incubation containers are designed to circulate water and provide all eggs with sufficient oxygen, any male-mediated disparity (e.g., relative position of fertilized eggs) may contribute to high male reproductive variance. Combined with disparities in fertilization ability among male broodstock, inconsistencies during incubation and/or hatching could encourage male-mediated selection resulting in a predominance of progeny from the most successful males (e.g., high proportion of Male A4 progeny at larval stage and final stock at Whitefish 2007 and Cedar 2007).

Variance in male reproductive success is often a function of hatchery mating strategy. Mixed-milt spawning crosses have repeatedly been shown to suffer the effects of sperm competition and produce significantly biased paternity in fish progeny (Gharrett and Shirley 1985; Withler 1988; Withler and Beacham 1994; Hoysak et al. 2004). Progeny sampled immediately post-hatch from mixed-milt crosses of lake sturgeon have shown significantly biased paternity (see Chapter 2). Pooling milt of several males prior to fertilization introduces the possibility of sperm competition, owing not only to potential differences in reproductive characteristics among the males (e.g., testis size, sperm length and/or motility, etc.), but also to varying levels of maturity, time spent spawning, and sperm concentration (Iwamatsu et al. 1991; Stockley et al. 1997; Bruch and Binkowski 2002; Simmons and Kotiaho 2002; Oppliger et al. 2003). Further hampering the likelihood of equal contribution are any methodological inconsistencies on the part of hatchery personnel (e.g., milt storage time, exposure of milt to water, incomplete pooling of milt, surface area and depth of ova; Stoss 1983; Gharrett and Shirley 1985; Withler 1988; Withler and Beacham 1994; Hoysak et al. 2004).

High variance in reproductive success among adults has been cited as the main cause of reduced genetic diversity in studies of hatchery-reared and wild fish (Turner et al. 2002; Araki et al. 2007). Aside from sperm competition concerns, disparity at several other factors can bring about significant bias in reproductive contribution. The ability of a male to effectively fertilize his lot of eggs may be dependent on a variety of biological variables listed above. Capturing males of equal advantage in all reproductive variables is unlikely, thereby granting reproductive advantage to certain males of a cross, even absent sperm competition and prior to any selection during hatching or rearing within the facility. Male milt may also decrease in potency due to milt storage time or exposure to water, thereby promoting a differential in fertilization ability of certain vials of milt (Stoss 1983; Hoysak et al. 2004).

The primary recommendation of this study is to further reduce male reproductive variance during lake sturgeon conservation rearing. The significant bias in paternity often evident at larval stage indicates such reductions in male reproductive variance should focus on hatch success and/or larval survival. Given the limited rearing space in the SRFs, expanding the program to include more crosses is unlikely. The goal of maximizing genetic diversity in each of the four crosses is therefore paramount to the long-term success of the rearing program. One key improvement would be eliminating any male-mediated selection during incubation and hatching, perhaps by incubating each male's egg lot in a separate hatch container and combining only after the hatch into female-based rearing groups.

Allowing each male's lot of eggs to incubate and hatch separately would eliminate any potential for male-mediated selection within the incubation containers. Such an approach is not cost-prohibitive (~$100/hatching kit; Aquatic Eco-Systems, Inc., Apopka, FL; E. A. Baker, MDNR, personal communication), nor time/space consuming, as eggs would no longer have to be combined and all five containers can still feed into the same rearing tank. Larval progeny or production mortalities could then be assessed for significant paternal bias, which, if found, would likely be attributable to the fertilization ability of individual males, methodological inconsistencies on the part of hatchery personnel, or rearing conditions within the facility. If further equalization was desired, hatchery personnel could attempt to standardize the storage of male gametes during broodstock capture, perhaps by holding all five males until the female is captured and stripped of eggs. Extracting milt from the males immediately following egg collection would greatly decrease any differential in milt storage time and would be unlikely to slow the overall process of broodstock collection, as male lake sturgeon are readily available at most spawning sites.

Overall, this study suggested the SRFs have the potential to produce acceptable N_b but genetic gains could be achieved without the need to expand broodstock sampling. Reducing the variance in male reproductive success in larval and final stock fish would be a key improvement not only to the genetic diversity produced at the facilities, but also to the likelihood of the persistence of the fish in the wild. Together with allowing the progeny to imprint, these results indicate that stream-side rearing holds great promise for the re-establishment of genetically-representative sturgeon spawning runs through artificial propagation.

Future Research

The high male reproductive variance observed in this study and the wide range of potential contributing factors suggests research into sturgeon rearing strategies would likely yield improvements for maximizing N_b. The continued use of SRFs for lake sturgeon rearing over the next several years provides an opportunity to study conditions and methodologies unique to those observed during the course of this study. The SRFs are expected to set production goals based on a set quantity of progeny, as opposed to genetic diversity goals of the first three years. On its own, this shift will encourage the maximization of total progeny, regardless of any differences in family size. This shift in priority presents the opportunity to determine the genetic effects of hatchery practices based on production, not the maintenance of genetic diversity. Comparisons can be made not only to the relative diversity produced by any new methods or strategies, but also to total production and the cost-benefit of any gains in production numbers at the sake of genetic diversity.

At the completion of extensive renovations, the Wild Rose State Fish Hatchery is expected to resume lake sturgeon rearing in the coming years. If Wisconsin traditional hatchery spawning and rearing methods are reinstated, samples could be obtained from larval and final stock progeny to provide further insight to the data collected from Wild Rose in this study. Final stock samples would be especially beneficial, as they would allow for direct comparison to the genetic output from the SRFs of this study. If spawning and/or rearing methods are revised to more closely mimic those of the SRFs, progeny samples from Wild Rose will provide insight into facility-specific genetic output. The potential would then exist to experimentally rear lake sturgeon family replicates under different rearing conditions. Differences related to rearing densities, food availability, climate control, etc., could have direct impact on progeny survival and the level of genetic diversity at stock out.

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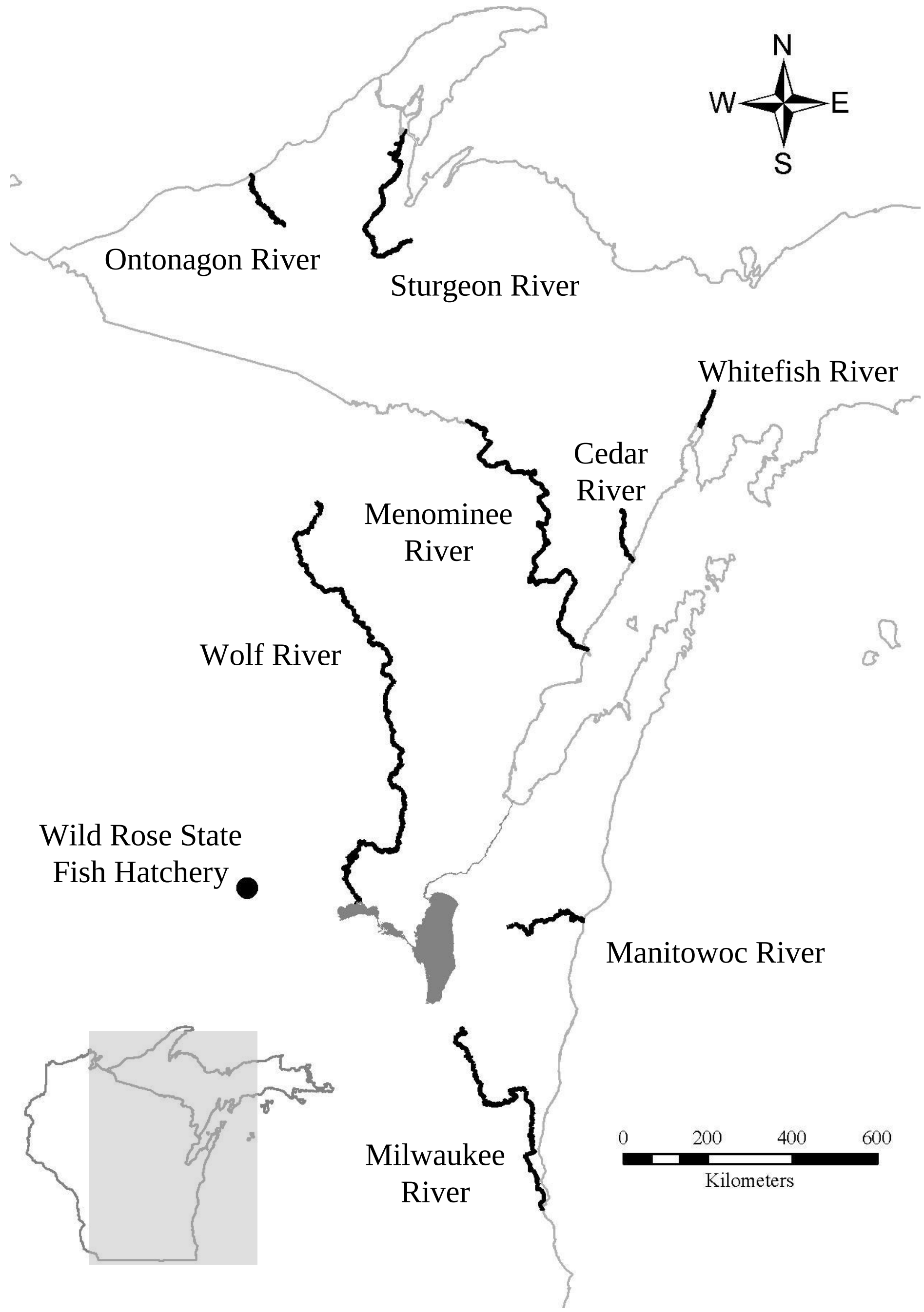

Figure 3.1. Study sites for the genetic assessment of lake sturgeon artificial rearing methods. The Wolf River contains the source population for the Wild Rose State Fish Hatchery and SRFs on the Manitowoc River and Milwaukee River. The Menominee River contains the source population for SRFs on the Cedar River and Whitefish River. The Sturgeon River contains the source population for the SRF on the Ontonagon River.

Table 3.1. Number of fish from which tissue samples for the genetic analysis of lake sturgeon artificial rearing occurred by facility and year. Broodstock samples consisted of fin-clips taken during broodstock capture at the Wolf River (WI) and Menominee River (MI), larval samples consisted of a subsample of progeny (2006 and 2007) or natural mortalities (2008), and final stock samples consisted of fin-clips from a subsample of the final stock or all final stock during years of relatively low production (Milwaukee 2006 and Manitowoc 2007).

	2006			2007			2008		
	Brood	Larval	Final	Brood	Larval	Final	Brood	Larval	Final
Wild Rose Hatchery	38	72							
Milwaukee River SRF	38		27	12		119	18	120	266
Manitowoc River SRF				24		64			
Cedar River SRF				5	46	92			
Whitefish River SRF				5	46	84			
Ontonagon River SRF							7	85	92

Table 3.2. PCR conditions for four co-load reactions used in this study, including loci, allele size range, number of alleles per locus, locus-specific primers and labels, 10x PCR buffer, dNTPs, $MgCl_2$, and *Taq* polymerase concentrations. Afu68b is from May et al. (1997) and McQuown et al. (2002), Spl120 is from McQuown et al. (2002), and all other loci are from Welsh et al. (2003).

Co-Load	Locus	Range	*A*	Label	Primer (µM)	10x Buffer	dNTPs (mM)	$MgCl_2$ (mM)	*Taq* (units)
A	AfuG112[1]	242-266	7	NED™	0.30	1x	0.80	1.50	0.50
	AfuG63[1]	127-147	5	NED™	0.20	1x	0.80	1.50	0.50
	Afu68b[1]	153-193	11	6FAM™	0.20	1x	0.80	1.50	0.50
B	Spl120[2]	254-290	6	NED™	0.20	1x	0.80	1.50	0.50
	AfuG9[1]	124-156	9	6FAM™	0.20	1x	0.80	1.50	0.50
	AfuG74[1]	218-226	2	6FAM™	0.20	1x	0.80	1.50	0.50
C	AfuG160[3]	131-147	3	6FAM™	0.20	1x	0.80	1.50	0.50
	AfuG56[2]	262-274	3	6FAM™	0.20	1x	0.80	1.50	0.50
	AfuG195[4]	161-165	2	HEX™	0.20	1x	0.80	1.50	0.50
D	AfuG204[3]	141-145	2	6FAM™	0.20	1x	0.80	1.50	0.50

[1] 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 52°C for 45 s, and 72°C for 2 min, ending with a final elongation of 72°C for 5 min.
[2] 94°C for 1 min, followed by 20 cycles of 92°C for 30 s, and 70°C for 40 s (decrease 0.5°C per cycle), followed by 20 cycles of 92°C for 30 s, and 60°C for 40 s (increase 1 s per cycle).
[3] 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 67°C for 45 s (decrease 0.5°C per cycle), and 72°C for 2 min, with a final elongation of 72°C for 5 min.
[4] 95°C for 1.5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 45 s, and 72°C for 2 min, with a final elongation of 72°C for 2 min.

Table 3.3. Genetic diversity of broodstock used during lake sturgeon artificial rearing efforts in Wisconsin and the Upper Peninsula of Michigan from 2006-2008, along with respective source populations (shown in bold) from which the broodstock were sampled (Welsh and McClain 2004; DeHaan et al. 2006). Location is the river of origin for the source populations or state in which broodstock were used, Year is the production year, N is the estimated number of spawning adults for the source populations (Zollweg et al. 2003) or the number of broodstock used at each rearing facility, ♀:♂ is the female-to-male spawning ratio, $\bar{A}$ is mean alleles per locus, H_o is observed heterozygosity, H_e is expected heterozygosity, df is the chi-square test degrees of freedom, χ^2 is the chi-square value from G-tests at each locus combined using Fisher's method for the comparison of broodstock to source, and p is the chi-square estimated p-value. Of special note is the 2007_M Wisconsin broodstock group, which is a subset of the 2007 Wisconsin broodstock and contains the only twelve adults whose progeny remained at stock out.

Location	Year	N	♀:♂	$\bar{A}$	H_o	H_e	df	χ^2	p
Wolf River		**~ 22,000**		**4.60**	**0.5300**	**0.5600**			
Wisconsin	2006	38	7:31	4.33	0.5311	0.5291	14	17.76	>0.20
	2007	24	4:20	4.00	0.5953	0.5482	16	17.61	>0.20
	2007_M	12	2:10	3.40	0.6083	0.5368	18	23.51	>0.10
	2008	18	3:15	4.11	0.5500	0.5022	18	28.77	>0.05
Menominee River		**~ 500**		**4.50**	**0.5600**	**0.5400**			
Michigan	2007	5	1:4	3.70	0.5167	0.5561	16	11.02	>0.80
Sturgeon River		**~ 200**		**4.67**	**0.5440**	**0.6130**			
Michigan	2008	7	1:6	3.30	0.6143	0.5495	12	18.81	>0.05

Table 3.4. Male reproductive contributions to larval lake sturgeon progeny, Facility is the rearing facility, Year is the production year, ♀:♂ is the female-to-male spawning ratio of contributing crosses, N is the number of larval progeny with full paternal assignment, Prop. is the range of individual male contribution to crosses in each facility, df is the chi-square test degrees of freedom, χ^2 is the chi-square test statistic, p is the chi-square estimated p-value compared to equal contribution, V_k is the total variance in male reproductive contribution for each facility, $\bar{k}_m$ is the mean number of progeny produced by each male, N_b is the total estimate of effective number of breeders, N_b exp. is the expected total estimate of effective number of breeders based on random contribution, and % is the percent reduction in N_b given the bias in male reproductive contribution. The Wild Rose progeny were a subsample of the larval production, while all other facilities' progeny are natural production mortalities from within three weeks post-hatch.

Facility	Year	♀:♂	N	Prop.	df	χ^2	p	V_k	$\bar{k}_m$	N_b	N_b exp.	%
Wild Rose	2006	6:27	59	0.0-100.0	10	23.89	<0.010	15.08	14.0	13.55	18.45	26.4
Cedar	2007	1:4	43	2.3-67.4	3	46.95	<0.001	230.41	10.8	2.30	3.18	27.7
Whitefish	2007	1:4	41	4.9-56.1	3	24.27	<0.001	97.17	10.3	2.72	3.18	14.5
Milwaukee	2008	3:15	111	0.0-69.1	6	112.23	<0.001	597.96	22.2	6.57	9.95	34.0
Ontonagon	2008	1:6	79	10.3-28.2	5	9.78	>0.050	28.19	13.2	3.38	3.42	1.2

Table 3.5. Male reproductive contributions to final stock lake sturgeon progeny, Facility is the rearing facility, Year is the production year, ♀:♂ is the female-to-male spawning ratio of contributing crosses, N is the number of progeny with full paternal assignment, Prop. is the range of individual male contribution to crosses in each facility, df is the chi-square test degrees of freedom, χ^2 is the chi-square test statistic, *p* is the chi-square estimated p-value compared to equal contribution, V_k is the total variance in male reproductive contribution for each facility, $\bar{k}_m$ is the mean number of progeny produced by each male, N_b is the total estimate of effective number of breeders, N_b exp. is the expected total estimate of effective number of breeders based on random contribution, and % is the percent reduction in N_b given the bias in male reproductive contribution.

Facility	Year	♀:♂	N	Prop.	df	χ^2	*p*	V_k	$\bar{k}_m$	N_b	N_b exp.	%
Milwaukee	2006	5:22	19	0.0-100.0	10	9.65	>0.050	4.10	3.7	5.81	5.80	-
Manitowoc	2007	4:20	49	0.0-60.0	8	20.89	<0.010	24.10	9.8	11.66	13.07	10.8
Milwaukee	2007	2:10	86	0.0-33.3	4	12.55	<0.020	24.67	17.2	6.56	6.63	1.1
Cedar	2007	1:4	91	1.1-70.3	3	109.57	<0.001	1219.92	22.8	2.18	3.19	31.7
Whitefish	2007	1:4	79	1.3-63.3	3	82.06	<0.001	604.44	19.8	2.45	3.19	23.2
Milwaukee	2008	3:15	212	0.0-97.6	6	43.43	<0.001	4850.23	42.4	5.85	9.98	41.4
Ontonagon	2008	1:6	81	5.0-28.8	5	19.37	<0.005	44.62	13.5	3.34	3.42	2.3

Appendix 3.1. Length (in cm) of all lake sturgeon broodstock successfully spawned for Wild Rose and the SRFs from 2006 – 2008.

Year	State	ID	Length
2006	Wisconsin	Female A	177.80
		Male A1	170.18
		Male A2	156.21
		Male A3	144.78
		Male A4	148.59
		Male A5	161.29
		Female B	165.10
		Male B1	130.81
		Male B2	144.78
		Male B3	161.29
		Male B4	113.03
		Female C	184.15
		Male C1	115.57
		Male C2	156.21
		Male C3	146.05
		Male C4	158.75
		Female D	176.53
		Male D1	154.94
		Male D2	118.11
		Male D3	114.30
		Male D4	151.13
		Male D5	129.54
		Female E	191.77
		Male E1	146.05
		Male E2	110.49

Appendix 3.1. Continued.

Year	State	ID	Length
		Male E3	120.65
		Male E4	151.13
		Male E5	179.07
		Female F	154.94
		Male F1	139.70
		Male F2	104.14
		Male F3	133.35
		Male F4	116.84
		Female G	177.80
		Male G1	149.86
		Male G2	157.48
		Male G3	142.24
		Male G4	129.54
2007	Wisconsin	Female A	166.37
		Male A1	152.40
		Male A2	116.84
		Male A3	149.86
		Male A4	130.81
		Male A5	142.24
		Female B	166.37
		Male B1	134.62
		Male B2	134.62
		Male B3	102.87
		Male B4	134.62
		Male B5	139.70

Appendix 3.1. Continued.

Year	State	ID	Length
		Female C	166.37
		Male C1	149.86
		Male C2	152.40
		Male C3	137.16
		Male C4	167.64
		Male C5	148.59
		Female D	177.80
		Male D1	171.45
		Male D2	154.94
		Male D3	143.51
		Male D4	167.64
		Male D5	148.59
2007	Michigan	Female A	137.00
		Male A1	120.00
		Male A2	125.00
		Male A3	112.00
		Male A4	117.00
2008	Wisconsin	Female A	181.86
		Male A1	154.94
		Male A2	132.59
		Male A3	170.43
		Male A4	162.56
		Female B	173.74
		Male B1	132.84
		Male B2	145.29

Appendix 3.1. Continued.

Year	State	ID	Length
		Male B3	153.16
		Male B4	133.10
		Male B5	152.40
		Female C	182.88
		Male C1	152.40
		Male C2	135.89
		Male C3	153.92
		Male C4	157.48
		Male C5	160.02
		Female D	169.16
		Male D1	151.38
		Male D2	154.94
		Male D3	169.83
		Male D4	160.53
		Male D5	152.91
2008	Michigan	Female A	159.50
		Male A1	141.00
		Male A2	151.00
		Male A3	153.00
		Male A4	136.00
		Male A5	133.50
		Male A6	145.00