Novel aspects of bighead carp sperm storage and larval/juvenile rearing to address control of invasive Asian carp in the USA

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

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2020

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Abstract

Bighead carp (*Hypophthalmichthys nobilis*) is native to Asia and was introduced to the United States as a species of interest for aquaculture in the 1970s but escaped from captivity soon after. This species is currently widely spread throughout the Mississippi River Basin and may already be present in the Great Lakes, which could impact this important sport fishery. Current control efforts have focused on preventing further spread through constructing barriers and physical removal of adults from rivers. Alternative methods for biocontrol of invasive fish species are becoming increasingly attractive and show promise as another tool that can be used to control or eliminate invasive populations. These methods rely on genetic technology designed and implemented in aquaculture, such as sex reversal, polyploidy and mass offspring production for stocking. A series of experiments were conducted to investigate novel aspects of sperm storage to perform *in vitro* fertilization and intensive larval/juvenile rearing of bighead for application in biocontrol stocking programs.

The first experiment addressed the short term storage of sperm on ice and cryopreservation. There is a lack of available information on the short term storage of silver carp (*Hypophthalmichthys molitrix*) and bighead carp sperm. The quality of fresh silver carp sperm stored without dilution in a sperm motility inhibiting (extender) solution was found to decrease rapidly after 24 hours becoming agglutinated or forming clumps by the third day. When sperm was stored in an extender solution, 45 % motility was maintained after 3 days. The trials involving cryopreservation investigated the applicability of utilizing original or modified protocols previously described for common carp and silver carp. The trials conducted with modified protocols were not successful, as

post-thaw motility was sporadic or absent. However, the trials with original protocols using koi carp sperm identified post-thaw motility of at least 15 %. The protocol described by Bernath et al. (2016) had significantly higher post-thaw motility (75 %) of koi carp sperm than the other three protocols examined. A third trial with original protocols using silver carp sperm was also conducted but data is not yet available. Fertility trials were also conducted during the storage of sperm which utilized zebrafish *Danio rerio* ova as a surrogate. For the first time, silver carp sperm was found to successfully initiate the development of zebrafish ova with surviving hybrid embryos/larvae identified up through 48 hours post fertilization.

The second experiment attempted to optimize larval rearing of bighead carp by investigating a novel static method. I explored several environmental conditions, such as utilizing 24h light, salinity (feeding), algal turbidity, and continuous food availability as compared to a traditional water recirculating system. Various levels of salinity (2, 3, and 5 ppt) were examined in the novel rearing environment. A 2x2x3 full factorial ANOVA design was implemented to compare the main effects of larval rearing system, feeding regime, and stocking density (12.5, 25, and 50 fish L^{-1}) and the interaction effects between the main effects on survival, biomass, length, weight, proportion of large individuals, and specific growth rate. A desirability function was then constructed that would maximize survival (%), biomass (g L^{-1}) and proportion of large individuals to optimize larval rearing conditions. These data can be used in further experiments addressing hormonal sex reversal. Elevated salinity, 3 and 5 ppt, was found to increase survival (51.3 ± 3.5 % and 64.3 ± 10.1 %, respectively) and proportion of large individuals (0.76 ± 0.08 and 0.82 ± 0.02, respectively) as compared to 2ppt (33 ± 3 % and 0.63 ± 0.03). The desirability function identified the static rearing system, with high

stocking density (50 fish L^{-1}), initially fed *Artemia* nauplii as having the maximum desirability (0.733) for the selected variables. In addition, this methodology could be applied to any larval rearing program to select for conditions favorable to specific desired outcomes.

The third set of experiments consisted of two weaning trials of bighead carp, i.e. transitioning from live food to artificial diets. The first trial examined if larval rearing conditions (stocking density and live feeding regime) influenced the success of weaning bighead carp to a commercial diet (OTO). The second trial investigated how two formulated weaning diets, which included spirulina (SPN) or soybean meal (SBM), compared to the commercial diet. Aspects of growth, survival, feed conversion ratio, mineral concentrations of fish, and the rate of deformities recorded, were assessed. For trial 1, no significant effects of initial larval rearing conditions were identified between any of the parameters measured. However, the rate of deformities observed in fish at its conclusion was high (37 %). In the second trial, significant differences were observed in the growth, survival, mineral concentrations of fish, and the rate of deformities. Results for the OTO diet were similar between trials 1 and 2 and it had the best overall performance measured in the trials. Growth rates were similar between the two formulated diets but survival of SBM fed fish was significantly lower than OTO and SPN. There was an increased rate of deformities recorded for the formulated diets compared to control. This was most likely due to mineral imbalances which were recorded. The presumed cause of these severity of deformation was from an excess of zinc, which has previously been linked to vertebral deformities in another cyprinid species, the common minnow (*Phoxinus phoxinus)*.

Acknowledgments

I would like to thank my parents for their tremendous encouragement throughout this whole process. Without their support the completion of this thesis would not have been possible. I would also like to thank my advisor Dr. Konrad Dabrowski, who supported and encouraged me to pursue a path that was originally unexplored. Lastly, I would like to thank Mackenzie Miller, John Grayson, and everyone else who I have worked with at the Aquaculture Lab at OSU over the past 5 years who took the time to help me in planning and completing of all of the experiments presented and many more.

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Introduction

The spread of aquatic invasive species throughout the world is a consequence of the world's improved economic connectivity, or Globalization (Meyerson & Mooney, 2007). Occasionally, translocated species significantly impact aquatic ecosystems and communities after invasion, such as the Round Goby *Neogobius melanostomas* (Krakowiak & Pennuto, 2008) and Zebra Mussel *Dreissena polymorpha* (Nicholls & Hopkins, 1993) in the Great Lakes. Asian carps are believed to have a high potential for invasion and due to their capacity to grow to large sizes (40kg) and potential to influence the base of aquatic food webs are a species of concern. This group of invasive species has been a focus of U.S. fisheries managers over the past 15 years. Control efforts have been implemented across the Mississippi River Basin (MRB) to curtail the spread of Asian carp species (Tsehaye et al., 2013; Parker et al., 2015; Widloe et al., 2017). However, the only current feasible method to collapse US Asian carp populations is through overharvesting, which requires extensive governmental organization and commercial support to implement (Tsehaye et al., 2013).

Alternative methods have been proposed for potential control of aquatic invasive species, such as production and release of YY males (Gutierrez & Teem, 2006), triploids,

and Trojan Y chromosome (Thresher et al., 2014), which all have the potential to decrease population fecundity and may eventually lead to a collapse of invasive populations. We are proposing to go beyond what has been previously proposed through the production of an all-male tetraploid line of bighead carp *Hypopthalmichthys nobilis* that could potentially be released for biological control. A series of experiments were conducted to improve methods for the production and intensive culture of bighead carp which could be utilized to rear larvae and juveniles produced through polyploidy. While investigations important to chromosomal set manipulation techniques have been previously researched in grass carp *Ctenopharyngodon idella* and black carp *Mylopharyngodon piceus* (Shelton & Rothbard, 1993) there is a lack of understanding about the phytophagous Asian carps, silver carp *Hypopthalmichthys molitrix* and bighead carp. The storage of sperm, on ice and cryopreserved, was investigated, since limited investigations have been conducted with varying degrees of success (Sin, 1974; Withler, 1982; Lahnsteiner et al., 2000; Dzuba & Kopeika, 2002; Alvarez et al., 2003). A series of experiments optimizing larval/juvenile rearing with live feeds and the weaning (transitioning from live to formulated diets) of bighead carp were conducted. Analysis of growth, survival, production of fish suitable for hormonal sex reversal, and the impacts of formulated diets on the rate of observed deformities were assessed. This series of experiments will help create the foundation for a breeding and rearing program to develop a mono-sex tetraploid bighead carp release program that has the potential to collapse currently established invasive populations.

Background information

The term Asian carps refers to a group of four species and includes the filter-feeding bighead carp and silver carp, collectively referred to as bigheaded carps, as well as black carp and grass carp. The bigheaded carps have been of concern as they have expanded their range towards the Great Lakes (Buck et al., 2010). One focus of research has been assessing the suitability of Lake Erie and Lake Michigan to invasion and what impacts may occur (Cooke, 2016; Zhang et al., 2016; Alsip et al., 2019).

Bighead carp is a warmwater cyprinid species that is native primarily to southern and central Asia (Li & Fang, 1990). This species was brought to the U.S. in the 1970s to investigate its usefulness for controlling algae in effluent ponds, but they escaped from captivity in aquaculture research facilities in Arkansas around 1980 (Freeze & Henderson, 1982). Bighead carp are currently established throughout many MRB rivers, including the Illinois and Ohio rivers (Schofield et al., 2005). If the expansion of bigheaded carps continues its spread northward to the Great Lakes region it may impact a multi-billion dollar fishery (Southwick Associates, 2007).

Bighead carp are highly efficient filter feeders that grow very quickly, with fish attaining sizes of 18–23 kg in 4–5 years (Henderson, 1978) and can reach a maximum size of 40kg (Jennings, 1988). The maximum age of bighead carp is unknown, but fish aged 8–10 years old have been found in the USA (Long & Nealis, 2011). Bighead carp are believed to feed primarily on zooplankton (Burke et al., 1986), but they are opportunistic feeders that can utilize a wide variety of food items depending on what is

prevalent in the environment, including cyanobacteria (Henderson, 1976), detritus, and phytoplankton (Opuszyński, 1981).

Biological impacts and current control efforts

The effects of direct competition between bighead carp and native filter feeders are largely unknown. However, there are concerns that it will alter plankton assemblages, decreasing available food for native species. Sass et al. (2014) agrees with these concerns as they found that since the establishment of bigheaded carps in the Illinois River zooplankton communities have been altered with unknown consequences to food webs and native species. Irons et al. (2007) have found that the condition of gizzard shad *Dorosoma cepedianum* and bigmouth buffalo *Ictiobus cyprinellus*, native filter feeders, have decreased by 7% and 5%, respectively, since bigheaded carps became established within the La Grange Reach of the Illinois River. In a controlled experiment for limited resources, Schrank et al. (2003) found that age-0 bighead carp were able to outcompete age-0 American paddlefish *Polyodon spathula*, potentially because of their ability to switch prey items depending on availability. However, a study conducted by Sampson et al. (2009) in backwater lakes on the Illinois and Mississippi rivers found that diet overlap of bighead carp was greatest with gizzard shad *Dorosoma cepedianum*, with only mild overlap occurring between bigmouth buffalo, and that American paddlefish diets had almost no overlap. The discrepancy between these two studies on the diet overlap of American paddlefish and bighead carp is likely attributed to the differences in study

design, with a controlled limited resource experiment conducted by Schrank et al. (2007) forcing diet overlap between the age-0 fish, while Sampson et al. (2009) primarily looked at natural conditions between adults of both species. Decreased condition and growth of native species observed in these experiments provide evidence that bighead carp may be competitively superior and have diet overlap with native filter feeders. Bighead carp's high growth rate, large size, unknown life span, and ability to use a wide range of food sources has made it an invasive species of concern.

In 2010, the U.S. Fish and Wildlife Service listed bighead carp as an injurious species (FCC, 2011). The focus of fisheries managers throughout the MRB has been to remove Asian carps where possible (Tsehaye et al., 2013) and to prevent the establishment of new populations, especially within the Great lakes. A series of electric barriers were constructed in the Chicago Canal, a man-made waterway between the MRB and the Great Lakes Basin, in the late 2000s to prevent the spread of aquatic invasive species, including Asian carps (Jerde et al., 2011). However, the effectiveness of established countermeasures is not guaranteed. Two studies investigating the effectiveness of the electric barriers found weaknesses that could be exploited and allow fish passage. Parker et al., (2015) observed that small fish were able to pass further through the electric barriers before incapacitation occurred. Sparks et al., (2010) also found that disruptions to the electric field caused by metal barges allowed larger individuals to pass through these barriers. These studies prompted a re-evaluation of the electric field generated by these barriers, eventually increasing their operating settings.

Several different models have been constructed to assess the suitability of the Great Lakes and to predict what potential impacts bighead carp may have on the ecosystem (Cooke, 2016). These models were constructed to investigate the potential for invasion through the length and temperature suitability of Lake Erie tributaries for spawning, embryonic and larval development (George & Chapman, 2013), bioenergetics and food availability (Anderson et al., 2015; Cooke & Hill, 2010), multivariate methods (Cuddington et al., 2014), and ecological niche modeling (Herborg et al., 2007). The constructed models have also investigated potential ecological impacts in Lake Erie (Zhang et al., 2016) and Lake Michigan (Alsip et al., 2019). However, these models are often reliant on expert judgment (Zhang et al. 2016), as well as uncertain and non-species-specific data to make conclusions (Cooke, 2016). In general, all these models found that Lake Erie and Lake Michigan have at least some suitable habitat for bighead carp to reproduce and grow. While these models are imperfect, they demonstrate the need for more basic information on bigheaded carps and provide a basis for concern if these species should successfully invade and reproduce in the Great Lakes.

Sperm physiology and motility

The control of reproduction is an essential part of aquaculture production and the quality of both male and female gametes are both limiting factors (Bobe & Labbe, 2010). The factors which influence sperm quality include nutrition (Asturiano et al., 2001), stress (Campbell et al., 1992), time during the reproductive season (Dreanno et al.,

1999a) and sperm handling (Bobe & Labbe, 2008). The plasma membrane of fish spermatozoa tightly overlays the nucleus, with only a thin cytoplasmic layer between (Bobe & Labbe, 2010). The plasma membrane is essential both in motility activation and successful fertilization in fish, since spermatozoa of fish do not have an acrosome (Bobe & Labbe, 2010). Fish spermatozoa motility activation (initiation of flagellar beating) occurs when ionic changes are perceived by the plasma membrane when released into water. This process as well as the ion channels and progestin receptors involved have been previously described by Tubbs & Thomas (2008) in seatrout.

At the biophysical level, membrane lipids determine the plasma membrane fluidity, with both proteins and lipids influencing the sperms permeability to both ions and water, as aquaporins are believed to be absent (Labbe & Maisse, 2001). There are generally high levels of docosahexaenoic acid (DHA) found among lipids (>10%) leading to high unsaturated to saturated ratios of up to 1.3 (Bobe & Labbe, 2010).

Nuclear proteins of spermatozoa tend to vary by species. In previous investigations of nuclear protein composition the cyprinids common carp *Cyprinus carpio*, grass carp *Ctenopharyngodon idella* (Saperas et al., 1993a) and goldfish *Carassius auratus* (Munoz-Guerra et al., 1982) were found to only have histones, while other species only have protamines (Saperas et al., 1993b), or a combination of both such as in rainbow trout (Avramova et al., 1983). The differences in nuclear protein types are believed to impact the amount of chromatin condensation, with higher condensations leading to increased DNA resistance to chemical or mechanical damage (Bobe & Labbe, 2010).

The specific mechanisms of initiation of sperm motility are variable between species (Bobe & Labbe, 2008). In most species the activation of mature spermatozoa motility occurs when the osmolality differences between seminal plasma and water are perceived by the plasma membrane. An investigation into the molecular events that link ionic changes and sperm motility was conducted by Inaba (2008). This author found that dilution of external K^+ induces intracellular efflux of K^+ and increases Ca^+ which triggers a hyperpolarization of the plasma membrane and leads to an increase of adenylate cyclase activation as well as cAMP in *Salmonidae* species, but not for *Cyprinidae* (Inaba, 2008). While the events leading to the activation of spermatozoa motility are broadly understood, the specific processes are still under investigation (Bobe & Labbe, 2010). An interesting example is that testicular sperm of *Salmonidae* species cannot be activated (Morisawa & Morisawa, 1986). It must first go through a modification, involving an increase in the pH of the sperm (Morisawa & Morisawa, 1988) which triggers the production of intracellular cAMP while passing through the sperm ducts (Morisawa et al., 1993a). While modification processes of sperm are scantily investigated for other species, it is generally believed that the storage of testicular spermatozoa in a buffer at or above pH 8 increases the sperms ability to respond to signals which initiate motility (Bobe & Labbe, 2010).

For sperm energetics, energy metabolism when stored in the male gonads provides for the basal energy of sperm and during the high energy demand upon motility activation (Ingermann, 2008). After activation, species with different reproductive strategies (internal versus external fertilization) have varying ability to metabolize

extracellular substrate for energy (Terner, 1962; Gardiner, 1978; Bobe & Labbe, 2010). A reduced capacity to use extracellular glucose has been found in species that utilize external fertilization (Terner, 1962). This is likely due to poor membrane permeability (Gardiner, 1978) since both *Salmonidae* and *Cyprinidae* species have been found to have the enzymatic capacity for intracellular glycolysis (Lahnsteiner et al., 1992; Lahnsteiner & Patzner, 1998). Instead these species have a more efficient tricarboxylic acid incorporation (Mounib, 1967). ATP stores accumulated in spermatozoa before activation are the primary source of energy to maintain motility (Ingermann, 2008). After spermatozoa motility activation a rapid decrease of ATP occurs (Christen et al., 1987; Perchec et al., 1995, Dreanno et al., 1999a, 1999b, 2000).

Sperm quality assessment

Fertilization ability is the best indicator of sperm quality, but some assessment of quality before fertilization can be made. The motility of sperm is a major parameter used to measure sperm quality. It enables the assessment of not only the sperm plasma membrane (mediation of ionic activation signal and permeability to prevent loss of intracellular structures), but also the mitochondria (energy stores), and axoneme structure (efficiency of sperm movement) (Bobe & Labbe, 2010). Measurement of sperm motility is a good way to measure the impacts of collection procedures, dilution media, and sperm storage conditions (Bobe & Labbe, 2010). The easiest method for analysis of sperm motility is by estimating the percentage of motile sperm through direct observation with a

phase-contrast or dark-field microscope at 100x to 200x magnification after activation (Cosson, 2008; Bobe & Labbe, 2010). Other methods for sperm motility exist and were reviewed by Cosson (2008), such as the use of computer-assisted sperm analysis (CASA), but these methods require specialized equipment and software. Direct observation is generally considered reliable for analysis of sperm motility duration and general sperm motility capacity, but some common issues can occur (Cosson, 2008; Bobe & Labbe, 2010). The density of sperm on the slide can lead to over- or under-estimation and the sperm can stick to the glass slide and result in lower measurements (Cosson, 2008). While assessment of sperm quality can be useful, it is difficult to correlate quality assessments with fertilization capacity, as only one good spermatozoa from a large population is required (Bobe & Labbe, 2010). General fertilization protocols can call for application of up to 5 million spermatozoa per egg to achieve successful fertilization. Therefore, the improvement of even a low number of spermatozoa's fertilization ability could be sufficient and these improvements may not be detectable through analysis of sperm motility (Bobe & Labbe, 2010). It is generally accepted that while correlating sperm quality and fertilization is hard, it is also considered irrelevant and that a general improvement of sperm function, measured through motility, should increase the number of sperm which meet the requirements for successful fertilization (Lahnsteiner et al., 1998; Zilli et al., 2004). Overall, the measurement of *in vitro* parameters for sperm quality assessment is not a substitution for sperm fertilization ability but can be considered as a partial descriptor (Bobe & Labbe, 2010).

Fertilization success is the earliest estimator of sperm and egg quality as the ability to fertilize or be fertilized is the essence of gamete quality. The fertilization rate is easy to record in some species, including Cyprinids, which have transparent eggs. However, it can be challenging in other species that have opaque eggs, such as largemouth bass and rainbow trout (Bobe & Labbe, 2010). Fertilization success does not necessarily represent successful development as Avery & Brown (2005) have found that abnormal cleavages can result in early embryonic mortality. To account for this, the characterization of important developmental stages (eyed, hatching, and yolk sac resorption) can be used to determine mortalities between treatments (Bobe & Labbe, 2010) and may allow the identification of issues, such as the effect of sperm cryopreservation (Kopeika et al., 2003). Assessing larval malformation can also be used to investigate potential issues with sperm quality after cryopreservation, as was described by Horvath & Urbanyi (2000) for African catfish *Clarias gariepinus*. However, the presence of larval malformation is not always found when using cryopreserved sperm (Labbe et al., 2001). The combination of high levels of unsaturated lipids and chromatin compaction are likely important factors that enable fish spermatozoa to resist DNA damage and retain functionality after cryopreservation.

Sperm storage and handling is generally conducted between 0-4°C to reduce cell metabolism for all species of fish, irrespective of rearing temperature (Bobe & Labbe, 2008). This is due to spermatozoa being resistant to injury during chilling (Labbe et al., 1995). The dilution of sperm in an extender is not necessarily required during short term storage, but the addition of antibiotics has been found to always increase the duration of

sperm quality (Bobe & Labbe, 2010). If a dilution medium (sperm extender) is used, it needs to be kept in an appropriate solution which satisfies both the required pH and osmolality to inhibit motility initiation (Bobe & Labbe, 2010). These sperm extenders are generally developed for species with short sperm motility duration based on the seminal plasma composition of the species they are designed for, to maximize inhibition of motility activation (Bobe & Labbe, 2010).

Cryopreservation of sperm

Cryopreservation of sperm is another tool that is available in aquaculture which has several potential applications: genetic improvement programs, broodstock management, preservation of mutant or transgenic lines, and to help with species having reproductive problems like asynchronization of gamete production between genders (Asturiano et al. 2017). Cryopreservation can also be used as a tool for the conservation of threatened species to maintain genetic material from wild fish populations (Van Der Walt et al., 1993). Cryopreservation of fish gametes have largely focused on spermatozoa, because of its ease of collection for most species and its resistance to chilling (Labbe et al., 1995; Asturiano et al., 2017). The use of androgenesis, or the production of fish with only paternally inherited DNA, in combination with cryopreserved spermatozoa offers an interesting method for preservation of at least the male germplasm. While sperm cryopreservation has been studied extensively in several species (Asturiano et al., 2017), the wide diversity of fish species with varying

morphology and biology (Mattei, 1991) can present problems with the standardization of protocols. The lack of standardization both for the procedures involved in cryopreservation and the metrics to report outcomes (Rosenthal et al., 2010) are significant barriers involved in this field of research (Asturiano et al., 2017).

Cryopreservation involves many different variables. The basic concepts of sperm cryopreservation involve an extender, cryoprotectant concentration, and dilution ratios (Lahnsteiner et al., 2000; Asturiano et al., 2017). The working conditions for protocols can also be varied, such as sperm concentration determination, equilibration time, handling of sperm, freezing methods, thawing methods, osmolality measurements, and many more (Lahnsteiner et al., 2000; Asturiano et al., 2017). The main factors which influence the chance of a cell surviving cryopreservation include the temperature at which slow cooling is terminated, the concentration and toxicity of cryoprotectants, the permeability of the cell membrane, and the size of the cells (Kopeika & Kopeika, 2008). All these variables present a challenge when developing sperm cryopreservation protocols and each must be accounted for. The ability to alter any of these variables leads to significant challenges when trying to standardize cryopreservation protocols (Asturiano et al., 2017). Many of these protocols utilize specialized equipment or materials that make it difficult to replicate results and has led to the rejection of cryopreservation by the aquaculture industry (Asturiano et al., 2017).

Numerous protocols have been created which enable the successful long-term storage (years) of sperm in cyprinids (Babiak et al., 1997; Lahnsteiner et al., 2000; Li et al., 2013; Bernáth et al., 2016). Cryopreservation of bighead carp sperm has been

attempted previously in a limited number of investigations (Sin, 1974; Withler, 1982) and <1% sperm motility was found post thawing. Silver carp sperm cryopreservation has had more success with reports of >50 % motile sperm reported by Lahnsteiner et al. (2000), but significantly lower sperm velocity was found. Alvarez et al. (2003) was more successful, with reports of >75 % sperm motility post thawing and hatching rates using cryopreserved sperm ranging between 43-51%, which were not significantly different from control fertilizations. These authors also found that there was no impact of storage duration (up to 1-year post freezing) on motility or the sperms fertilization ability (Alvarez et al., 2003). Chen et al. (1992) investigated the effects of different concentrations of DMSO and freezing methods for the cryopreservation of silver carp sperm. These authors reported that the addition of the internal cryoprotectant DMSO at 10% resulted in the highest sperm motility duration without compromising sperm motility when activated with 0.75% NaCl, while activation with water compromised both sperm motility and duration (Chen et al., 1992). Sperm fertilization ability was high for both freezing methods examined, ampoule or plastic tube method, with 76.8 ± 9.7% (mean ± SD; n=8) and 94.8 ± 2.3% (n=16), respectively. Dzuba & Kopeika (2002) also found up to 30% of spermatozoa were motile after cryopreservation of silver carp sperm but swelling and cell disruption were common. This swelling is likely a main issue that must be overcome to successfully develop a cryopreservation protocol for this species. Each of these previously developed protocols utilized very different sperm extenders, but all of them found that ~10% DMSO and the use of semi-saline solution as an activator yielded the best results sperm motility and duration. However, many of these previously developed protocols utilize methods which are not replicable with currently available

materials (Chen et al., 1992; Alvarez et al., 2003), are unavailable in English (Chen et al., 1992; Dzuba & Kopeika, 2002), or require a very large dilution (Lahnsteiner et al., 2000) which makes it impractical to freeze large volumes of sperm.

Common carp sperm cryopreservation has been widely studied and many different protocols have been created (Babiak et al., 1997; Lahnsteiner et al., 2000; Li et al., 2013; Bernáth et al., 2016). We plan to modify and replicate these different common carp sperm cryopreservation protocols as well as to alter previously described silver carp protocols for available materials to identify the best one for silver and bighead carp. Impacts of extenders, internal cryoprotectants, equilibration duration, dilution ratios, freezing and thawing methods will be investigated to determine impacts on both pre-freeze and post-thaw motility of sperm. The resulting fertility of cryopreserved sperm will be assessed utilizing zebrafish eggs as a surrogate (Delomas & Dabrowski, 2016).

Rearing of Asian carps for aquaculture

Asian carps are an extremely important group of species for world aquaculture which accounted for 15 million metric tons, or about 30% of all production in 2016 (FAO, 2018). Grass carp (1st: 11%; 6,068,000 metric tons) is the most produced fish in the world, closely followed by silver carp (2nd: 10%; 5,301,000) and bighead carp (5th: 7%; 3,527,000). The filter-feeding bigheaded carps account for the majority of the 8.8 million metric tons of unfed fish produced in 2016 (FAO, 2018). Growth of these species is induced through the fertilization of ponds to promote the growth of their primary food

sources, zooplankton (Afzal et al., 2008; Cremer and Smitherman, 1980). The bigheaded carps are generally reared in extensive or pond polyculture, since there is limited diet overlap with other important cultured species past the larval stages (Cremer and Smitherman, 1980; Opuszyński, 1981; Pretto, 1976). While these species are often cultured without the addition of food, due to their importance several studies on larval and juvenile rearing have been conducted over the past 50 years.

Marciak & Bogdan (1979) attempted to determine the food requirements of grass carp, silver carp, and bighead carp. They conducted an intensive rearing experiment with each species by feeding them zooplankton from a sewage puddle initially dominated by rotifers and compared the results to previous research conducted in manured ponds (Tamas, 1978; Opuszyński, 1978). Fish were stocked to aquaria at a rate of 23 fish L^{-1} and natural food was fed ad libitum. After 10 days of the experiment two additional feeding treatments were created, one was fed a mix of both natural and a commercial diet with the other being switched to only a commercial diet for the remainder of the experiment (Marciak & Bogdan, 1979). They determined that rotifers became insufficient as a sole food source between 22–39 days of feeding as growth was much lower during this period than both previous and later periods. In the later periods the natural feed became dominated by *Daphnia* and subsequently performed as well as the mixed and artificial diets (Marciak & Bogdan, 1979). By 20 days of the experiment all groups had grown to sizes between 80–100mg and 18–22.4 mm. By the end of the 90-day experiment Silver carp (0.61–0.67 g) preformed similarly on each of the feeding regimes, while bighead carp (0.36–0.61 g) had higher growth on the natural feed. Grass carp

(0.741-1.553g) achieved the highest growth of all three species for all feeding regimes but the mixed diet was 2-fold higher than the artificial and natural diets alone. Compared with previous research conducted on pond culture (Opuszyński, 1969; Wolny, 1969) of these species during the same period they achieved much lower growth (grass carp = 6.9–53.0 g; silver carp = 2.5–37.0 g; bighead carp = 20–52 g).

Dabrowski (1984) examined the influence of fish initial weight when weaning from live to dry diets on survival and growth in four cyprinids. Tanks were stocked with 235–333 larvae L^{-1} of each common carp, grass carp, silver carp, or bighead carp. Fish were switched from zooplankton (*Brachionus calyciflorus*, *Synchaeta* sp., *Bosmina longirostris* and copepodites) to a dry diet to determine the adaptation weight or weight at which switching to dry feeds did not compromise growth or survival of a species. Two to three different initial dry diets were examined for each species since previous investigations by Bryant and Matty (1981) found that the composition of the initial dry diet also influenced this adaption weight. The formulated diets consisted of yeast (*Candida tropicalis*) from one of two sources, freeze-dried pork liver or krill, ascorbic acid, vitamin and mineral mixtures, as well as fish and soya oil. He found in a 14-day trial that common carp fed only zooplankton performed (weight = 46.3 mg; survival = 89.4 %) the best but all three adaptation weights achieved acceptable survival (68.4–88.3 %) with the largest adaptation weight (18.26 mg) achieving higher weights (35.4–36.2 mg) compared to lower (4.27 mg and 7.25mg) adaptation weights tested. The use of dry diets as initial feeds resulted in lower growth for both diets examined but decreased survival was only found for the diet that incorporated krill. The 15-day experiment with

grass carp and silver carp larvae found that switching to dry diets at 4.3 mg and 6.8 mg, respectively, resulted in significantly higher weights for all three diets in grass carp and two of three diets for silver carp. All three diets as initial feeds for grass carp performed poorly overall, but the diet with pork liver was comparable to the zooplankton control group. Results for silver carp initially fed dry diets followed a similar pattern, except the diet with pork liver achieved higher weights than the zooplankton control and was similar to the final weights of the fish weaned to dry diets. The final 27-day trial with bighead carp experienced issues with size heterogeneity. Most fish were 5.6 mg at the end of 15 days of feeding on zooplankton, but 18 individuals were identified that were much larger (42.2 mg). The initially fed zooplankton and dry diets were discontinued after 15 days and all remaining fish were switched to dry diets. At the end of the experiment survival was fairly low (23.4–38.5 %) but fish achieved mean weights of 78.7–109.5 mg. Bighead carp growth has previously been found to be highly variable during larval and early juvenile development (Dabrowski, 1984; Carlos, 1988; Fermin & Recometa, 1988; Rottmann et al., 1991; Garcia et al., 1999) with Lieder and Helms (1981) reporting that approximately 30% of fish grew more slowly. Overall, the experiment confirmed that the initial diet utilized for weaning is important and can be species-specific, but that if it is appropriate it can perform just as well or better than live feeds.

Rottmann et al. (1991) investigated the use of three different live feeds and two dry diets for the intensive culture of grass carp and bighead carp larvae for 21 days. The experiment utilized static tanks which had self-designed sponge filters for aeration and filtration. Grass carp were stocked at a rate of 13 fish L^{-1} and bighead carp were stocked

to 28 and 57 fish L^{-1}. The live feeds examined included *Artemia* nauplii, freshwater rotifers (*Brachionus rubens*), and nematodes (*Panagrellus* sp.) while the dry diets utilized were Ewos Larvstart and Fry Feed Kyowa A. For grass carp, weights (presented in a rather odd manner, dry weight of 50 individuals) and survival (63–95 %) were not significantly different between all diets but the lengths of fish were significantly different throughout each week of the experiment. At the end of the experiment, the rotifer fed fish had the highest length (18.4 ± 0.3 mm) followed by *Artemia* nauplii (16.0 ± 0.3 mm) with the other treatments being significantly lower. In the bighead trials, survival was 82–99% and 59–97% at 28 and 57 fish L^{-1}, respectively, with fish fed rotifers achieving significantly larger lengths than other diets. Interestingly, while *Artemia* nauplii did not achieve the best growth, it resulted in higher survival compared to rotifer fed fish. The nematode fed bighead carp performed similar to those fed *Artemia* nauplii, but the culture of these are more difficult than *Artemia* nauplii. While the dry diets utilized were found to be adequate in supporting growth and survival throughout the experiment, they were inferior compared with all live feeds at 57 fish L^{-1}.

Santiago and Reyes (1991) investigated the optimum dietary protein level for growth of bighead carp in a static rearing system. Seven isocaloric diets were formulated with between 20 and 50% dietary protein content and were fed to fish (mean initial weight = 3.8 ± 0.2 mg, total length 9.8 ± 0.1 mm) stocked at 5 fish L^{-1} for seven weeks. It is unclear if these fish were initially fed a live diet before the experiment began but based on the initial sizes of the fish, they did receive some feeding before the experiment began. These authors found that weights of fish began to differ significantly after 3 weeks of

feeding, with weight increasing from 20 to 30% dietary protein content but decreased as protein levels exceeded 30%. Survival between diets were not significantly different and were about 50% at the end of the experiment. The feed conversion ratio (FCR) and protein efficiency ratio of fish was highest in 25, 30, and 35 % protein diets. Increased dietary protein levels resulted in significantly higher moisture in the body composition of fish. Bighead carp juveniles reached an average weight of 254 mg on the diet with 30 % dietary protein after 7 weeks.

Garcia et al. (1999) investigated how low levels of salinity impacted the growth and survival of bighead carp at different stages of development (11–35 days post hatch [dph]). Fish were stocked to two tanks at 80 fish L^{-1} and were reared at 24–26 °C. These authors investigated the salinity tolerance of bighead carp to 0, 2, 4, 8, and 16 ppt water at three sizes for 96hr. The experiment found that the mean survival time of 11dph bighead fry kept at 4 ppt or higher (<3.3 hr) was significantly lower than both 0 and 2 ppt (95–96 hr). As fish grew to larger sizes, they appeared to become more tolerant to salinity, as 18 and 35 dph fish only saw decreases in survival at 6 and 8 ppt respectively. These authors also compared the growth of 18 dpf fish (mean weight = 19.5 mg) reared in 0, 2, 4, and 6 ppt water for 4 weeks. They found that fish reared in lower salinities (0 and 2 ppt) achieved significantly higher weights starting at 3 weeks compared to higher salinities (4 and 6 ppt).

Opuszyński has extensively researched the Asian carps, including research on thermal tolerances of larval and juveniles (Lirski & Opuszyński, 1988), weaning to formulated diets (Opuszyński et al., 1989), and their usefulness in pond culture

(Opuszyński, 1981). In Opuszyński et al. (1989) tanks were stocked at 200 fish L^{-1} and they investigated several different feeding regimes for 14 days to determine if grass carp, silver carp, and bighead carp could be solely reared on a formulated diet, or when weaning from live diets would not impact survival and growth of these species. These authors determined that bighead carp fed with live feeds till 6 or 9 days of the experiment before switching to a formulated diet resulted in survival similar to fish fed zooplankton for the entire period. However, the average weight of fish was only comparable with the live feed control (29 mg) when they were switched on day 10 to Ewos Larvstart C-10 (29 mg) but not a trout starter diet (15 mg). Sole feeding of four formulated diets were found to result in low survival (< 15 %) and very low growth (3–9 mg) for bighead carp, but grass carp performed better with weights for some diets (2–20 mg) being comparable to restricted live (12 mg) and mixed feeding (16 mg) and having similar survival. Silver carp performed poorly in the experiment due to a shift towards larger zooplankton in the live feeds that were provided to the fish which resulted in low survival and made comparisons difficult. The experiment identified a negative correlation for mortality and growth of silver carp (slope = –2.25) and bighead carp (slope = –3.88) between days 5–7 and 8–10 of the experiment for each species respectively. The variability of individual fish weight was found to be 2-fold higher (45–183%) for bighead carp compared to silver carp (23–86%) and grass carp (27–101%). Overall, the experiment found that the use of live diets had superior growth and survival compared to dry diets.

Opuszyński (1981) examined the usefulness of both silver carp and bighead carp duo-culture in common carp ponds. He determined that silver carp were a better

additional fish to be reared in these ponds. Neither of the two species were found to consume the feed that was given to the common carp, with detritus being the primary food source for these species. However, bighead carp were found to have a higher proportion of larger invertebrates (Cladocera and Copepoda species) in their diet which did overlap with common carp prey items, while silver carp primarily consumed smaller zooplankton (Rotifera). Overall production with the addition of either silver carp or bighead carp to ponds resulted in increased production (kg ha^{-1}) of 3–23% but silver carp did not impact the production of common carp while bighead carp decreased its production by ~20%.

Cremer & Smitherman (1980) conducted a study on the cage and pond culture of bighead (13.2 g) and silver carp (21.7 g) fingerlings with and without supplementary feeding for 159 days. The intestinal contents of silver carp were found to be similar between both the ponds and cages and were dominated by Chlorophyceae (81.9–84.1 %), but bighead carp were significantly different. Bighead carp diets were dominated by detritus (69.3 %) and zooplankton (23.6 %) in ponds but a switch to Chlorophyceae (66.8 %) with a lesser degree of detritus (25.3 %) and zooplankton (5.3 %) was found in cages. Bighead carp was found to filter much larger particle sizes, up to 3000 µm with most between 50–100 µm while silver carp only filter particles <100 µm with the majority being 17–50 µm. Bighead carp were found to consume artificial feeds supplied to both the ponds and cages, resulting in 57% increased growth compared to un-supplemented groups. This was confirmed by Afzal et al. (2008) that found bighead carp growth was 27% higher in monoculture ponds supplemented with a 22% crude protein diet. On the

other hand, silver carp were not found to consume artificial feeds that were supplied, and growth was similar between groups in each of the ponds and cages (Cremer & Smitherman, 1980). Significant differences in the weight of each species between pond and cage culture methods were found with silver carp and bighead carp reared in ponds being 62.5–80 % and 188–200% larger than comparable groups in cages.

Production of monosex populations

The production of monosex populations has been widely studied because of its application for increased yields in aquaculture (Dunham & Devlin, 1999; Pandian & Sheela, 1995). While it is possible to manipulate the genetics of fish to produce monosex populations (Mair et al., 1997), this review will focus specifically on the hormonal induction of sex reversal. Sex differentiation in teleosts is diverse and easily altered, enabling exogenous hormone control (Pandian & Sheela, 1995). Sex reversal is achieved through the use of androgen and estrogen steroids during the period of gonadal differentiation (Shelton, 1986) and currently, protocols exist for production of monosex populations of over 40 species (Pandian & Sheela, 1995), including silver carp (Mirza & Shelton, 1988), common carp (Gomelsky, 2003), and grass carp (Shelton, 1986). While steroid-induced sex-reversal can alter the phenotypic sex of fish, it is unable to alter its genotype (Shelton, 1986).

Mirza and Shelton (1988) separated silver carp gynogens of 65–135 mm initial size, ranging from 68 to 319 days post fertilization (dpf), into 10mm size categories and

either implanted fish or directly injected 17α-methyltestosterone to each experimental treatment for a duration of 95–324 days. Direct injection was not successful in sex reversing fish of any size group. However, the implantation treatment was successful in sex reversing silver carp to varying degrees (0–91 %). Older fish of the same size category were found to have a better response than younger fish to sex reversal, potentially due to damage caused during an important developmental period which occurs earlier in life (Mirza & Shelton, 1988). The most successful treatment these authors identified was for fish of 90–100 mm in length implanted with a silastic implant containing 17α-methyltestosterone at 319 days post fertilization. The results of sex reversal trials observed by Mirza & Shelton (1988) are similar to what was seen by Boney et al., (1984) in grass carp. Hormone filled implants were successful in inducing phenotypic sex reversal in 86% of larger (95-105mm) gynogenetic fish treated at 10 weeks and 96% of smaller (80-90mm) fish treated at 16 weeks until they reached at least 180mm and for at least 7.5 months (Boney et al., 1984). Like what was seen in Mirza and Shelton (1988), Boney et al. (1984) found that implantation of smaller fish (80-90mm) treated at 10 weeks had reduced sex reversal of only 26%. These previous investigations illustrate the importance of both the size and age of fish when hormone administration occurs as well as its duration of exposure.

Specific protocols have not been created for the sex reversal of bighead carp, but they would likely be comparable to those for grass carp and silver carp. Histological examination by Fatima et al. (2017) observed sex differentiation occurring in bighead carp and silver carp at 28 dph, while in grass carp it was identified at 45 dph when reared

at 28 °C. Indicating that current protocols developed for silver carp would likely apply to bighead carp.

Dietary supplementation of hormone treatment is the easiest and most preferred method of administering steroids to fish (Hunter & Donaldson, 1983). This strategy is not without its difficulties, as size heterogeneity can lead to discrepancy of feed uptake and result in different rates of hormonal intake (Pandian & Sheela, 1995). Therefore, fish need to be of similar sizes at the initiation of hormonal treatment. This is especially important for Cyprinid fishes, as in general a high intensity of treatment is required to achieve 100 % monosex populations (Pandian & Sheela, 1995). No previous investigations utilizing dietary supplementation of hormone treatment to sex reverse bighead carp or silver carp have been conducted. An experiment was conducted to examine if this method is feasible to produce monosex populations of bighead carp (initial size = 40mm) over 127 days once they reached >80mm in total length. The fish will be further grown out till they achieve sizes (>180mm) which enable histological examination of sex (Mirza & Shelton, 1988).

Control of aquatic invasive species

The control of invasive species is important because once established in a new ecosystem they have the potential to cause significant harm (Krakowiak & Pennuto, 2008; Nicholls & Hopkins, 1993). Early during an invasion, the elimination of aquatic invasive species can be accomplished with physical removal, biocides (such as rotenone),

barriers, or environmental modification (Meronek et al., 1996). However, once a population has become established and widespread the best method for removal becomes biological control (Thresher et al., 2014). The most readily available option to fisheries managers is the stocking of triploids (Thresher et al., 2014), because triploids are almost always sterile from the production of aneuploid gametes which result in nonviable offspring (Gomelsky et al., 2015). While it is the most readily available option for biological control, the development of new technologies has allowed for other options with greater effectiveness. The production and release of sterile male Sea lamprey *Petromyzon marinus* in the Great Lakes has also been conducted. Klassen et al. (2004) concluded that the release of sterile males at a ratio of 1:1 sterile to fertile males would be able to reduce a population to 3% of initial size within 4 generations. However, due to other control efforts, such as lampricides and barriers, being used simultaneously the effectiveness of this method has not been examined.

Other potential forms of biological control include the release of YY males (*Myy*) (Gutierrez & Teem, 2006) as well as the release of YY females (*Fyy)*, called the Trojan Y chromosome hypothesis (Thresher et al., 2014). Advances in aquaculture production technologies have led to the production of *Myy*, *Fxy* and *Fyy* to produce monosex populations in sexually dimorphic species resulting in greater production, such as in Nile Tilapia *Oreochromis niloticus* (Mair et al., 1997). This is accomplished through the feminization of *Mxy* using estrogen-like hormones. When spawning these *Fxy* with *Mxy*, about 25% of the resulting offspring are *Myy* (Gutierrez & Teem, 2006). Stocking *Myy* to waters with nuisance species allows them to reproduce in wild populations and all

resulting offspring will be male (Kennedy et al. 2018). This will skew the sex ratio towards more males and decrease population fecundity. Through the continued introduction of *Myy* to a population the number of females will continue to decrease eventually leading to the collapse of that population (Gutierrez & Teem, 2006). Once *Myy* are produced they can further be sex reserved into *Fyy* (Thresher et al., 2014). When these *Fyy* are released, through natural reproduction, all offspring will be males. An added benefit of this system over the release of *Myy* is that *Fyy* will naturally produce *Myy* offspring that further skew sex ratios (Thresher et al., 2014).

While the previously described methods for biocontrol are theoretically possible, their practical application is still in its infancy. Through translating the technologies developed for aquaculture, the implementation of these alternative methods for biological control are feasible, as was recently accomplished with the production and release of *Myy* to control non-native brook trout in Idaho, USA (Schill et al., 2016, 2017; Kennedy et al., 2018). While these systems for biocontrol shows promise, there are also problems with their application, such as for fish that have a ZW or polygenic sex determination system (Thresher et al., 2014).

Conclusion

While the specific impacts that Asian carp have on ecosystems they have invaded throughout the US are largely unknown, previous investigations and modeling have found that bighead carp are competitively superior to native filter feeders (Irons et al.,

2007; Schrank et al., 2003) and that they significantly alter plankton communities (Sass et al., 2014). Due to their potential to impact a 7-billion-dollar annual fishery in the Great Lakes (Southwick Associates, 2007), a concerted effort by fisheries managers has been made to control established populations through harvest and to prevent their spread outside of the MRB with barriers (Sparks et al., 2010). However, current control measures are unlikely to accomplish the goal of a complete population crash (Tsehaye et al., 2013). The development of a biological control program involving the production and release of all-male tetraploid bighead carp has the potential to accomplish this goal. Releasing all-male F_3 tetraploid bighead carp would produce 100% triploids when they mate with established diploid populations (Pandian et al., 1999). By only releasing male tetraploids it eliminates the possibility of tetraploid fish mating with each other, which could produce viable offspring. Triploid offspring resulting from mating stocked tetraploids and natural diploids could then reproduce with diploid populations, but due to aneuploid gamete production these offspring are not viable (Gomelsky et al., 2015). The impact of this process is likely similar to what has been hypothesized for the release of sterile male Sea Lamprey, with the potential to lead to crash a population within 5 generations (Klassen et al., 2004).

The production of triploid and tetraploid bighead Carp larvae has been accomplished (Aldridge et al., 1990). However, these experiments only used pressure shocks to induce polyploidy and the efficacy of heat and cold shocks have yet to be assessed. Aldridge et al. (1990) also was primarily focused on the shocking protocols for induction of triploidy and tetraploidy of bighead carp and no reports on survival past 48-

hr were made or could be found in the literature. While there is a lack of previous research for the production of triploid and tetraploid bighead larvae, meiotic and mitotic gynogens have been produced and grown to adulthood (Ye et al., 2008; Zhu et al., 2013; Sun et al., 2015; Liu et al., 2018). Protocols developed for meiotic or mitotic gynogenesis may be applicable to produce triploids and tetraploids, respectively. Liu et al. (2018) confirmed that bighead carp have an XY sex-determination system, the development of a Trojan Y, *Myy* (Gutierrez & Teem, 2006), or all-male tetraploid release program is feasible. The goal of the following experiments was specifically to improve the technology involved in the storage of bigheaded carp sperm and intensive larval and juvenile rearing of bighead carp which could be applied by a biological control program for Asian carps.

References

Afzal, M., Rab, A., Akhtar, N., Ahmed, I., Khan, M.F., and Qayyum, M. (2008). Growth performance of bighead carp *Aristichthys nobilis* (Richardson) in monoculture system with and without supplementary feeding. Pakistan Veterinary Journal *28*, 57–62.

Aldridge, F.J., Marston, R.Q., and Shireman, J.V. (1990). Induced triploids and tetraploids in bighead carp, *Hypophthalmichthys nobilis*, verified by multi-embryo cytofluorometric analysis. Aquaculture *87*, 121–131.

Alsip, P.J., Zhang, H., Rowe, M.D., Mason, D.M., Rutherford, E.S., Riseng, C.M., and Su, Z. (2019). Lake Michigan's suitability for bigheaded carp: The importance of diet flexibility and subsurface habitat. Freshwater Biology *00*, 1–19.

Alvarez, B., Fuentes, R., Pimentel, R., Abad, Z., Cabrera, E., Pimentel, E., and Arenal, A. (2003). High fry production rates using post-thaw silver carp (*Hypophthalmichthys molitrix*) spermatozoa under farming conditions. Aquaculture *220*, 195–201.

Anderson, K.R., Chapman, D.C., Wynne, T.T., Masagounder, K., and Paukert, C.P. (2015). Suitability of Lake Erie for bigheaded carps based on bioenergetic models and remote sensing. Journal of Great Lakes Research *41*, 358–366.

Asturiano, J.F., Sorbera, L.A., Carillo, M., Zanuy, S., Ramos, J., Navarro, J.C., and Bromage, N. (2001). Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture *194*, 173–190.

Asturiano, J.F., Cabrita, E., and Horvath, A. (2017). Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. General and Comparative Endocrinology *245*, 69–76.

Avery, T.S., and Brown, J.A. (2005). Investigating the relationship among abnormal patterns of cell cleavage, egg mortality and early larval condition in *Limanda ferruginea*. Journal of Fish Biology *67*, 890–896.

Avramova, Z., Uschewa, A., Stephanova, E., and Tsanev, R. (1983). Trout sperm chromatin. I. Biochemical and immunological study of the protein composition. European Journal of Cell Biology *31*, 137–142.

Babiak, I., Glogowski, J., Brzuska, E., and Adamek, J. (1997). Cryopreservation of sperm of common carp, *Cyprinus carpio* L. Aquaculture Research *28*, 567–571.

Bernáth, G., Żarski, D., Kása, E., Staszny, Á., Várkonyi, L., Kollár, T., Hegyi, Á., Bokor, Z., Urbanyi, B., and Horváth, Á. (2016). Improvement of Common Carp (*Cyprinus carpio*) sperm cryopreservation using a programable freezer. General and Comparative Endocrinology *237*, 78–88.

Bobe, J., and Labbe, C. (2008). Chilled storage of sperm and eggs. In: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in reproductive aquaculture: Marine and freshwater species Taylor and Francis, London (UK).

Bobe, J., and Labbé, C. (2010). Egg and sperm quality in fish. General and Comparative Endocrinology *165*, 535–548.

Boney, S.E., Shelton, W.L., Yang, S.-L., and Wilken, L.O. (1984). Sex reversal and breeding of grass carp. Transactions of the American Fisheries Society *113*, 348–353.

Bryant, P.L., and Matty, A.J. (1981). Adaptation of carp (*Cyprinus carpio*) larvae to artificial diets: 1. Optimum feeding rate and adaptation age for a commercial diet. Aquaculture *23*, 275–286.

Buck, E.H., Upton, H.F., Stern, C.V., and Nicols, J.E. (2010). Asian carp and the Great Lakes region. Congressional Research Report *12*, pp. 28.

Burke, J.S., Bayne, D.R., and Rea, H. (1986). Impact of silver and bighead carps on plankton communities of channel catfish ponds. Aquaculture *55*, 59–68.

Campbell, P.M., Pottinger, T.G., and Sumpter, J.P. (1992). Stress reduces the quality of gametes produced by rainbow trout. Biological Reproduction *47*, 1140–1150.

Carlos, M.H. (1988). Growth and survival of bighead carp (*Aristichthys nobilis*) fry fed at different intake levels and feeding frequencies. Aquaculture *68*, 267–276.

Chen, S.L., Liu, X.T., Lu, D.C., Zhang, L.Z., Fu, C.J., and Fang, J.P. (1992). Cryopreservation of spermatozoa of silver carp, common carp, blunt snout bream

and grass carp. Acta Zoologica Sinica *38*, 413–424. (in Chinese with English summary)

Christen, R., Gatti, J.-L., and Billard, R. (1987). Trout sperm motility. The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. European Journal of Biochemistry *166*, 667–671.

Cooke, S.L. (2016). Anticipating the spread and ecological effects of invasive bigheaded carps (*Hypophthalmichthys* spp.) in North America: a review of modeling and other predictive studies. Biological Invasions *18*, 315–344.

Cooke, S.L., and Hill, W.R. (2010). Can filter-feeding Asian carp invade the Laurentian Great Lakes? A bioenergetic modeling exercise: Bioenergetics of invasive Asian carp. Freshwater Biology *55*, 2138–2152.

Cosson, J.J. (2008). Methods to analyze the movements of fish spermatozoa and their flagella. In: Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology, Alpha Science International Ltd., Oxford, U.K., pp. 63–102.

Cremer, M.C., and Smitherman, R.O. (1980). Food habits and growth of silver and bighead carp in cages and ponds. Aquaculture *20*, 57–64.

Cuddington, K., Currie, W.J.S., and Koops, M.A. (2014). Could an Asian carp population establish in the Great Lakes from a small introduction? Biological Invasions *16*, 903–917.

Dreanno, C., Cosson, J., Suquet, M., Cibert, C., Fauvel, C., Dorange, G., and Billard, R. (1999a). Effects of osmolality, morphology perturbations and intracellular nucleotide content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. Journal of Reproductive Fertility *116*, 113–125.

Dreanno, C., Cosson, J., Suquet, M., Seguin, F., Dorange, G., and Billard, R. (1999b). Nucleotide content, oxidative phosphorylation, morphology, and fertilizing capacity of turbot (*Psetta maxima*) spermatozoa during the motility period. Molecular Reproductive Development. *53*, 230–243.

Dreanno, C., Seguin, F., Cosson, J., Suquet, M., and Billard, R. (2000). 1H-NMR and (31)PNMR analysis of energy metabolism of quiescent and motile turbot (*Psetta maxima*) spermatozoa. Journal of Experimental Zoology *286*, 513–522.

Dunham, R., and Devlin, R. (1999). Comparison of traditional breeding and transgenesis in farmed fish with implications for growth enhancement and fitness. Murray, J.D., Anderson, G.B., Oberbauer, A.M. and McGloughlin, M.M. (eds.) University of California Davis, pp. 209–229.

Dzuba, B.B., and Kopeika, E.F. (2002). Relationship between the changes in cellular volume of fish spermatozoa and their cryoresistance. Cryo Letters *23*, 353–360.

Fatima, S., Shoukat, A., Qamar, B., Mahmood, F., and Rafique, A. (2017). Histological study of sex differentiation in bighead carp (*Hypophthalmichthys nobilis*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and catla (*Catla catla*). Turkish Journal of Fisheries and Aquatic Sciences *17*, 1313–1316.

FAO (Food and Agriculture Organization of the United Nations) (2018). The state of world fisheries and aquaculture 2018- Meeting the sustainable development goals. Rome, *CC BY-NC-SA 3.0 IGO*, pp. 1–210.

FCC, (Federal Communications Commission) (2011). Rules and regulations. Federal Register *76*, 15857–15858.

Fermin, A.C., and Recometa, R.D. (1988). Larval rearing of bighead carp, *Aristichthys nobilis* Richardson, using different types of feed and their combinations. Aquaculture Research *19*, 283–290.

Freeze, M., and Henderson, S. (1982). Distribution and status of the bighead carp and silver carp in Arkansas. North American Journal of Fisheries Management *2*, 197–200.

Garcia, L.M.B, Garcia, C.M.H., Pineda, A.F.S., Gammad, E.A., Canta, J., Simon, S.P.D., Hilomen-Garcia, G.V., Gonzal, A.C., and Santiago, C.B. (1999). Survival and growth of bighead carp fry exposed to low salinities. Aquaculture International *7*, 241–250.

Gardiner, D.M. (1978). Utilisation of extracellular glucose by spermatozoa of two viviparous fishes. Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology *59*, 165–168.

George, A.E., and Chapman, D.C. (2013). Aspects of embryonic and larval development in bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. PLoS ONE *8*, e73829.

Gomelsky, B. (2003). Chromosome set manipulation and sex control in common carp: a review. Aquatic Living Resources *16*, 408–415.

Gomelsky, B., Schneider, K.J., Anil1, A., and Delomas, T.A. (2015). Gonad development in triploid ornamental koi carp and results of crossing triploid females with diploid males. North American Journal of Aquaculture *77*, 96–101.

Gutierrez, J.B., and Teem, J.L. (2006). A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y chromosome to cause extinction of an introduced exotic species. Journal of Theoretical Biology *241*, 333–341.

Henderson, S. (1976). Observations on the bighead and silver carp and their possible application in pond fish culture. Arkansas Game and Fish Commission, Little Rock.

Henderson, S. (1978). An evaluation of the filter feeding fishes, silver and bighead carp, for water quality improvement. In Smitherman R.O., W.L. Shelton, and J.H. Grover, (Eds.) *Culture of exotic fishes symposium proceedings.*, American Fisheries Society, Auburn, Alabama, 121–136.

Herborg, L.-M., Mandrak, N.E., Cudmore, B.C., and MacIsaac, H.J. (2007). Comparative distribution and invasion risk of snakehead (*Channidae*) and Asian carp (*Cyprinidae*) species in North America. Canadian Journal of Fisheries and Aquatic Sciences *64*, 1723–1735.

Horvath, A., and Urbanyi, B. (2000). The effect of cryoprotectants on the motility and fertilizing capacity of cryopreserved African catfish *Clarias gariepinus* (Burchell 1822) sperm. Aquaculture Research *31*, 317–324.

Hunter, G.A., and Donaldson, E.M. (1983). Hormonal sex control and its application to fish culture. In: Hoar, W.S., Randall, D.J., and Donaldson, E.M. (Eds.), Fish Physiology, Vol. 9B, Academic Press, New York, USA, pp. 223-301.

Inaba, K., Morisawa, S., and Morisawa, M. (1998). Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. Journal of Cell Science. *111*, 1105–1115.

Ingermann, R.L. (2008). Energy metabolism and respiration in fish spermatozoa. In: Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.), Fish spermatology. Alpha Science International Ltd, Oxford, UK, pp. 241–266.

Irons, K.S., Sass, G.G., McClelland, M.A., and Stafford, J.D. (2007). Reduced condition factor of two native fish species coincident with invasion of non-native Asian carps in the Illinois River, U.S.A. Is this evidence for competition and reduced fitness? Journal of Fish Biology *71*, 258–273.

Jennings, D.P. (1988). Bighead carp (*Hypophthalmichthys nobilis*): a biological synopsis. U.S. Fish and Wildlife Service, Washington, DC. Fish and Wildlife Service Biological Report *88*, 1–47.

Jerde, C.L., Mahon, A.R., Chadderton, W.L., and Lodge, D.M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. Conservation Letters *4*, 150–157.

Kennedy, P.A., Meyer, K.A., Campbell, M.R., Vu, N.V., and Schill, D.J. (2018). Survival and reproductive success of hatchery YY male brook trout stocked in Idaho streams. Transactions of the American Fisheries Society *147*, 419–430.

Klassen, W., Adams, J.V., and Twohey, M.B. (2004). Modeling the suppression of sea lamprey populations by the release of sterile males or sterile females. Journal of Great Lakes Research *30*, 463–473.

Kopeika, J., Kopeika, E., Zhang, T., Rawson, D.M., and Holt, W.V. (2003). Detrimental effects of cryopreservation of loach (*Misgurnus fossilis*) sperm on subsequent embryo development are reversed by incubating fertilised eggs in caffeine. Cryobiology *46*, 43–52.

Kopeika, E., and Kopeika, J. (2008). Variability of sperm quality after cryopreservation in fish. In: Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology, Alpha Science International Ltd., Oxford, U.K., pp. 347–396.

Krakowiak, P.J., and Pennuto, C.M. (2008). Fish and macroinvertebrate communities in tributary streams of eastern Lake Erie with and without round gobies (*Neogobius melanostomus*, Pallas 1814). Journal of Great Lakes Research *34*, 675–689.

Labbe, C., Maisse, G., Muller, K., Zachowski, A., Kaushik, S., and Loir, M. (1995). Thermal acclimation and dietary lipids alter the composition, but not fluidity, of trout sperm plasma membrane. Lipids *30*, 23–33.

Labbe, C., Martoriati, A., Devaux, A., and Maisse, G. (2001). Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Molecular Reproductive Development *60*, 397–404.

Labbe, C., and Maisse, G. (2001). Characteristics and freezing tolerance of brown trout spermatozoa according to rearing water salinity. Aquaculture *201*, 287–299.

Lahnsteiner, F., Patzner, R., and Weismann, T. (1992). Monosaccharides as energy resources during motility of spermatozoa in *Leuciscus cephalus* (*Cyprinidae*, Teleostei). Fish Physiology and Biochemistry *10*, 283–289.

Lahnsteiner, F., and Patzner, R.A. (1998). Sperm motility of the marine teleosts *Boops boops*, *Diplodus sargus*, *Mullus barbatus* and *Trachurus mediterraneus*. Journal of Fish Biology *52*, 726–742.

Lahnsteiner, F., Berger, B., Horvath, A., Urbanyi, B., and Weismann, T. (2000). Cryopreservation of spermatozoa in Cyprinid fishes. Theriogenology *54*, 1477–1498.

Li, S., and Fang, F. (1990). On the geographical distribution of the four kinds of pond-cultured carps in China. Acta Zoologica Sinica *36*, 244–255.

Li, P., Hulak, M., Li, Z. H., Sulc, M., Psenicka, M., Rodina, M., Gela, D., and Linhart, O. (2013). Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces protein phosphorylation in tyrosine and threonine residues. Theriogenology *80*, 84–89.

Lieder, U., and Helms, C. (1981). Erfahrungen beim Vorstrecken von Brut pflanzenfressender Cypriniden. Binnenfisch *28*, 3–8 (in German).

Lirski, A., and Opuszynski, K. (1988). Lower lethal temperatures for carp *Cyprinus carpio* L. and the phytophagous fishes *Ctenopharyngodon idella* Val. *Hypophthalmichthys-molitrix* Val. *Aristichthys-nobilis* Rich. in the first period of life. Roczniki Nauk Rolniczych *101*,11–30. (in Polish with English summary)

Liu, H., Pang, M., Yu, X., Zhou, Y., Tong, J., and Fu, B. (2018). Sex-specific markers developed by next-generation sequencing confirmed an XX/XY sex determination system in bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*). DNA Research *25*, 257-264.

Long, J.M., and Nealis, A. (2011). Age estimation of a large bighead carp from Grand Lake, Oklahoma. In Proceedings of the Oklahoma Academy of Science, pp. 15–18.

Mair, G.C., Abucay, J.S., Abella, T.A., Beardmore, J.A., and Skibinski, D.O.F. (1997). Genetic manipulation of sex ratio for the large-scale production of all-male tilapia *Oreochromis niloticus*. Canadian Journal of Fisheries and Aquatic Sciences *54*, 396–404.

Marciak, Z., and Bogdan, E. (1979). Food requirements of juvenile stages of grass carp *Ctenopharyngodon Idella* Val., silver carp *Hypophthalmichthys molitrix* Val., and bullhead carp *Aristichthys nobilis* Rich. In Stycznska-Jurewicz, E., Backiel, T., Jaspers, E., and Persoone, G., (Eds.). Cultivation of fish fry and its live food, Prinses Elisabethlaan 69, Belgium, European Mariculture Society, special publication *No. 4*, 140–148.

Mattei, X. (1991). Spermatozoon ultrastructure and its systematic implications in fishes. Canadian Journal of Zoology *69*, 3038–3055.

Meronek, T.G., Bouchard, P.M., Buckner, E.R., Burri, T.M., Demmerly, K.K., Hatleli, D.C., Klumb, R.A., Schmidt, S.H., and Coble, D.W. (1996). A Review of Fish Control Projects. North American Journal of Fisheries Management *16*, 63–74.

Meyerson, L.A., and Mooney, H.A. (2007). Invasive alien species in an era of globalization. Frontiers in Ecology and the Environment *5*, 199–208.

Mirza, J.A., and Shelton, W.L. (1988). Induction of gynogenesis and sex reversal in silver carp. Aquaculture *68*, 1–14.

Morisawa, S., and Morisawa, M. (1986). Acquisition of potential for sperm motility in rainbow trout and chum salmon. Journal of Experimental Biology *126*, 89–96.

Morisawa, S., and Morisawa, M. (1988). Induction of potential for sperm motility by bicarbonate and pH in rainbow trout and chum salmon. Journal of Experimental Biology *136*, 13–22.

Morisawa, S., Ishida, K., Okuno, M., and Morisawa, M. (1993a). Roles of pH and cyclic adenosine monophosphate in the acquisition of potential for sperm motility during migration from the sea to the river in chum salmon. Molecular Reproduction and Development *34*, 420–426.

Mounib, M.S. (1967). Metabolism of pyruvate, acetate and glyoxylate by fish sperm. Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology *20*, 987–992.

Munoz-Guerra, S., Azorin, F., Casas, T., Marcet, X., Maristany, M.A., Roca, J., and Subirana, J.A. (1982). Structural organization of sperm chromatin from the fish *Carassius auratus*. Experimental Cell Research. *137*, 47–53.

Nicholls, K.H., and Hopkins, G.J. (1993). Recent changes in Lake Erie (north shore) phytoplankton: cumulative impacts of phosphorus loading reductions and the zebra mussel introduction. Journal of Great Lakes Research *19*, 637–647.

Opuszyński, K. (1969). Production of plant feeding fish (*Ctenopharyngodon Idella* Val. And *Hypophthalmichthys molitrix* Val.) in carp ponds. Roczniki Nauk Rolniczych *91*, 221–307. (in Polish, English summary)

Opuszyński, K. (1978). Rearing of larvae and fry of silver carp *Hypophthalmichthys molitrix*. Proceedings of Conference on Aquaculture: "Cultivation of Fish Fry and its Live Food", Sept 23-28, 1977, Szymbark, Poland.

Opuszyński, K. (1981). Comparison of the usefulness of the silver carp and the bighead carp as additional fish in carp ponds. Aquaculture *25*, 223–233.

Opuszyński, K., Myszkowski, L., Okoniewska, G., Opuszyńska, W., Szlaminska, M., Wolnicki, J., and Woznieski, M. (1979). Rearing of common carp, grass carp, silver carp, and bighead carp larvae using zooplankton and/or different dry feeds. Polskie Archiwum Hydrobiologii *36*, 217–230.

Pandian, T., and Sheela, S.G. (1995). Hormonal induction of sex reversal in fish. Aquaculture *138*, 1–22.

Pandian, T., Venugopal, T., and Koteeswaran, R. (1999). Problems and prospects of hormone, chromosome and gene manipulations in fish. Current Science *76*, 369–386.

Parker, A.D., Glover, D.C., Finney, S.T., Rogers, P.B., Stewart, J.G., and Simmonds, R.L. (2015). Direct observations of fish incapacitation rates at a large electrical fish barrier in the Chicago Sanitary and Ship Canal. Journal of Great Lakes Research *41*, 396–404.

Perchec, G., Jeulin, C., Cosson, J., André, F., and Billard, R. (1995). Relationship between sperm ATP content and motility of carp spermatozoa. Journal of Cell Science *108*, 747–753.

Pretto, R. (1976). Polyculture systems with channel catfish as the principal species. Ph.D. Dissertation, Auburn University, Alabama, 190 pp.

Rosenthal, H., Asturiano, J.F., Linhart, O., and Horváth, Á. (2010). On the biology of fish gametes: summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. Journal of Applied Ichthyology *26*, 621–622.

Rottmann, R.W., Shireman, J.V., and Lincoln, E.P. (1991). Comparison of three live foods and two dry diets for intensive culture of grass carp and bighead carp larvae. Aquaculture *96*, 269–280.

Santiago, C.B., and Reyes, O.S. (1991). Optimum dietary protein level for growth of bighead carp (*Aristichthys nobilis*) fry in a static water system. Aquaculture *93*, 155–165.

Sampson, S.J., Chick, J.H., and Pegg, M.A. (2009). Diet overlap among two Asian carp and three native fishes in backwater lakes on the Illinois and Mississippi rivers. Biological Invasions *11*, 483–496.

Saperas, N., Lloris, D., and Chiva, M. (1993a). Sporadic appearance of histones, histone-like proteins, and protamines in sperm chromatin of bony fish. Journal of Experimental Zoology *265*, 575–586.

Saperas, N., Ribes, E., Buesa, C., Garcia Hegart, F., and Chiva, M. (1993b). Differences in chromatin condensation during spermiogenesis in two species of fish with distinct protamines. Journal of Experimental Zoology *265*, 185–194.

Sass, G.G., Hinz, C., Erickson, A.C., McClelland, N.N., McClelland, M.A., and Epifanio, J.M. (2014). Invasive bighead and silver carp effects on zooplankton communities in the Illinois River, Illinois, USA. Journal of Great Lakes Research *40*, 911–921.

Schill, D.J., Heindel, J.A., Campbell, M.R., Meyer, K.A., and Mamer, E.R. (2016). Production of a YY male Brook Trout broodstock for potential eradication of undesired Brook Trout populations. North American Journal of Aquaculture *78*, 72–83.

Schill, D.J., Meyer, K.A., and Hansen, M.J. (2017). Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. North American Journal of Fisheries Management *37*, 1054–1066.

Schofield, P.J., Williams, J.D., Nico, L.G., Fuller, P., and Thomas, M.R. (2005). Foreign non-indigenous carps and minnows (*Cyprinidae*) in the United States—A guide to their identification, distribution, and biology. Scientific Investigations Report *2005*, 5041.

Schrank, S.J., Guy, C.S., and Fairchild, J.F. (2003). Competitive interactions between age-0 bighead carp and paddlefish. Transactions of the American Fisheries Society *132*, 1222–1228.

Shelton, W.L. (1986). Broodstock development for monosex production of grass carp. Aquaculture *57*, 311–319.

Shelton, W., and Rothbard, S. (1993). Determination of the developmental duration (zeta(0)) for ploidy manipulation in carps. Israeli Journal of Aquaculture-Bamidgeh *45*, 73–81.

Sin, A.W. (1974). Preliminary results on cryogenic preservation of sperm of silver carp (*Hypophthalmichthys molitrix*) and bighead (*Aristichthys nobilis*). Hong Kong Fisheries Bulletin *4*, 33–36.

Southwick Associates (2007). Sportfishing in America: an economic engine and conservation powerhouse. American Sportfishing Association *Multistate Conservation Grant Program*.

Sparks, R.E., Barkley, T.L., Creque, S.M., Dettmers, J.M., and Stainbrook, K.M. (2010). Evaluation of an electric fish dispersal barrier in the Chicago Sanitary and Ship Canal. In: Chapman, D.C. and M.H. Hoff, (Eds.). Invasive Asian carps in North America, *American Fisheries Society, Symposium 74, Bethesda, Maryland,* 139–161.

Sun, Y., Yuan, Z., Tan, S., Fan, J., and Zhou, G. (2015). Induction of gynogenesis in red bighead carp (*Aristichthys nobilis* red var.). Journal of Fisheries of China *39*, 8–15. (in Chinese)

Tamas G. (1978). Rearing of common carp fry and mass cultivation of its food organisms in ponds. Proceedings of Conference on Aquaculture: "Cultivation of Fish Fry and its Live Food", Sept 23-28, 1977, Szymbark, Poland.

Terner, C. (1962). Oxidative and biosynthetic reactions in spermatozoa. In: Bishop D.W. (Ed.), Spermatozoan Motility, American Association for the Advancement of Science, Washington D.C., pp. 89–98.

Thresher, R.E., Hayes, K., Bax, N.J., Teem, J., Benfey, T.J., and Gould, F. (2014). Genetic control of invasive fish: technological options and its role in integrated pest management. Biological Invasions *16*, 1201–1216.

Tsehaye, I., Catalano, M., Sass, G., Glover, D., and Roth, B. (2013). Prospects for fishery-induced collapse of invasive Asian carp in the Illinois River. Fisheries *38*, 445–454.

Tubbs, C., and Thomas, P. (2008). Functional characteristics of membrane progestin receptor alpha (mPR[alpha]) subtypes: a review with new data showing mPR[alpha] expression in seatrout sperm and its association with sperm motility. Steroids *73*, 935–941.

Van Der Walt, L.D., Van Der Bank, F.H., and Steyn, G.J. (1993). The suitability of using cryopreservation of spermatozoa for the conservation of genetic diversity in African catfish (*Clarias gariepinus*). Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology *106*, 313–318.

Widloe, J., Widloe, T., Lederman, N., and Irons, K. (2017). Asian carp Removal Project in the Upper Illinois River. In 147th Annual Meeting of the American Fisheries Society. AFS, August 22.

Withler, F.C. (1982). Cryopreservation of spermatozoa of some freshwater fishes cultured in South and Southeast Asia. Aquaculture *26*, 395–398.

Wolny, P. (1969). Biological, technical and economical grounds for production of stocking material. II. Production of stocking material of three phytophagous fish species. Instruction of the Institute of Inland Fisheries *No. 37*.

Ye, Y., Wang, Z., and Wu, Q. (2008). Increasing the genetic uniformity of bighead carp [*Aristichthys nobilis* (Richardson)] by means of spontaneous diploidization of gynogenetically activated eggs: Genetic uniformity of bighead carp. Aquaculture Research *39*, 205–211.

Zhang, H., Rutherford, E.S., Mason, D.M., Breck, J.T., Wittmann, M.E., Cooke, R.M., Lodge, D.M., Rothlisberger, J.D., Zhu, X., and Johnson, T.B. (2016). Forecasting the impacts of silver and bighead carp on the Lake Erie food web. Transactions of the American Fisheries Society *145*, 136–162.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., and Vilella, S. (2004). Adenosine triphosphate concentration and beta-D-glucuronidase activity as indicators of sea bass semen quality. Biology of Reproduction *70*, 1679–1684.

Zhu, C., Sun, Y., Yu, X., and Tong, J. (2013). Centromere localization for bighead carp (*Aristichthys nobilis*) through half-tetrad analysis in diploid gynogenetic families. PLoS ONE *8*, 1-9.

Chapter 2: Short and long term storage of carp sperm

Introduction

The control of reproduction is an essential part of aquaculture production and the quality of both male and female gametes are both limiting factors (Bobe & Labbe, 2010). The factors which influence sperm quality include nutrition (Ciereszko & Dabrowski, 1995; Asturiano et al., 2001), stress (Campbell et al., 1992), time during the reproductive season (Dreanno et al., 1999), and sperm handling (Bobe & Labbe, 2008). Spermatozoa are in a quiescent state when they are in the testes of fish, but once they are released into fresh water, motility is initiated (Morisawa, 1985). Motility can range from 1-minute to 1-hour depending on the species (Billard, 1978), with common carp *Cyprinus carpio* and other sister species generally ranging from 80–110 seconds (Verma et al., 2009)

The specific mechanisms of initiation of sperm motility are variable between species (Bobe & Labbe, 2008). In most cases the activation of mature spermatozoa motility occurs when the osmolality differences between seminal plasma and water are perceived by the sperm plasma membrane. In investigations into the molecular events that link ionic changes and sperm motility in salmonids, Inaba (2008) found that dilution of external K^+ induces intracellular efflux of K^+ and Ca^+ which triggers a hyperpolarization of the plasma membrane and leads to an increase of adenylate cyclase

activation as well as cAMP. However, in *Cyprinidae* the mechanism of activation is based on a general shift of osmolality (Morisawa et al., 1993b). While the events leading to the activation of spermatozoa motility are broadly understood, the specific processes are still under investigation (Bobe & Labbe, 2010).

Sperm quality assessment

Fertilization ability is the best indicator of sperm quality, but some assessment of quality before fertilization can be made. The motility of sperm is a major parameter used to measure sperm quality. It enables the assessment of not only the sperm plasma membrane (mediation of ionic activation signal and permeability to prevent loss of intracellular structures), but also the mitochondria (energy stores), and axoneme structure (efficiency of sperm movement) *(*Bobe & Labbe, 2010). Assessment of sperm motility is an appropriate way to measure the impacts of collection procedures, dilution media, and sperm storage conditions (Cierezsko et al., 1999; Bobe & Labbe, 2010). The easiest method for analysis of sperm motility is by estimating the percentage of motile sperm through direct observation with a phase-contrast or dark-field microscope at 100x to 200x magnification within 1 minute after activation (Cosson, 2008; Bobe & Labbe, 2010).

While assessment of sperm quality can be useful, it can be inaccurate to correlate quality assessments with fertilization capacity, as only a single spermatozoon from a large pool (10^4 –10^6 per egg) is required (Bobe & Labbe, 2010). General fertilization protocols can call for application of up to 5 million spermatozoa per egg to achieve

successful fertilization. Therefore, the improvement of even a low number of spermatozoa's fertilization ability could be sufficient, even though it may not be detectable through the analysis of sperm motility (Bobe & Labbe, 2010). It is generally accepted that while correlating sperm quality and fertilization is hard, it is also considered irrelevant. A general improvement of sperm function, measured through motility, should increase the number of sperm which meet the requirements for successful fertilization (Lahnsteiner et al., 1998; Zilli et al., 2004).

Storage of sperm on ice

Sperm storage and handling is generally conducted between 0–4 °C to reduce cell metabolism for all species of fish, irrespective of optimum temperature (Rana et al. 1990; Bobe & Labbe, 2008). This is due to spermatozoa being resistant to injury during chilling (Labbe et al., 1995). The dilution of sperm in an extender is not necessarily required during short term storage, but the addition of antibiotics has been found in most cases to increase the duration of high sperm quality (Bobe & Labbe, 2010). If a dilution medium (sperm extender) is used, sperm needs to be stored in an immobilizing solution which satisfies both the required pH and osmolality to inhibit motility initiation *(*Bobe & Labbe, 2010). Sperm extenders are generally developed for species with short sperm motility duration based on the seminal plasma composition of the species they are designed for, to maximize inhibition of motility activation *(*Bobe & Labbe, 2010).

Previous investigations of short term storage of silver carp *Hypophthalmichthys molitrix* and bighead carp *H. nobilis* spermatozoa could not be found, but studies of common carp *Cyprinus carpio* and grass carp *Ctenopharyngodon idella* have been conducted (Hulata & Rothbard, 1978; Ravinder et al., 1997). Hulata & Rothbard (1978) examined the cold storage (0–5 °C) of common and grass carp sperm and found that there was no impact on the fertilization ability with and without the addition of a dilution medium up through 45 hours. Ravinder et al. (1997) examined several different sperm extenders applicability for the storage of common carp spermatozoa up to 24-hours. These authors found that if an appropriate sperm extender was used, it could improve the motility of spermatozoa over this period compared to unextended sperm (Ravinder et al., 1997).

Cryopreservation of sperm

Cryopreservation of sperm is a tool available to aquaculture which has several potential applications: genetic improvement programs, broodstock management, preservation of mutant or transgenic lines, and to help with species experiencing asynchronization of gamete maturation between genders (Asturiano et al. 2017). Cryopreservation can also be used as a tool for the conservation of threatened species to maintain genetic material in storage from wild fish populations (Van Der Walt et al., 1993). Cryopreservation of fish gametes have largely focused on spermatozoa, because of its ease of collection for most species and its resistance to chilling (Labbe et al., 1995;

Asturiano et al., 2017). While sperm cryopreservation has been studied extensively in several species (Asturiano et al., 2017), the wide diversity of fish species with varying morphology and biology (Mattei, 1991) can present problems with standardizing protocols. The lack of standardization for both the procedures involved in cryopreservation and the methods to report outcomes (Rosenthal et al., 2010) are significant barriers involved in this field of research (Asturiano et al., 2017).

Cryopreservation involves many different variables. The basic concepts of sperm cryopreservation involve choice of an extender, cryoprotectant concentration, and immobilizing solution dilution ratios (Lahnsteiner et al., 2000; Asturiano et al., 2017). The working conditions for protocols can also be varied, such as sperm concentration determination, equilibration time, handling of sperm, freezing methods, thawing methods, osmolality measurements, and many more (Lahnsteiner et al., 2000; Asturiano et al., 2017). The main factors which influence the cell surviving cryopreservation include the temperature at which slow cooling is terminated, the concentration and toxicity of cryoprotectants, the permeability of the cell membrane, and the size of the spermatozoa (Kopeika & Kopeika, 2008). All these variables present a challenge when developing sperm cryopreservation protocols and each must be accounted for. The ability to alter any of these variables leads to significant challenges when trying to standardize cryopreservation protocols (Asturiano et al., 2017).

Numerous protocols have been created which enable the successful long-term storage (years) of sperm in cyprinids (Babiak et al., 1997; Lahnsteiner et al., 2000; Li et al., 2013; Bernáth et al., 2016). Cryopreservation of bighead carp sperm has been

attempted previously in a limited number of investigations (Sin, 1974; Withler, 1982) and <1 % sperm motility was found post thawing. Silver carp sperm cryopreservation has had more success with reports of >50 % motile sperm reported by Lahnsteiner et al. (2000), but significantly lower sperm velocity was found. Alvarez et al. (2003) reported >75 % post thawing sperm motility and using cryopreserved sperm had hatching rates ranging between 43–51% which were not significantly different from control fertilizations. These authors also found that there was no impact of storage duration (up to 1-year post freezing) on motility or the sperms fertilization ability (Alvarez et al., 2003). Chen et al. (1992) investigated the effects of different concentrations of DMSO and freezing methods for the cryopreservation of silver carp sperm. These authors reported that the addition of the internal cryoprotectant DMSO at 10 % resulted in the longest sperm motility duration when activated with 0.75% NaCl. When activation was carried out with water both sperm motility and duration was compromised (Chen et al., 1992). Sperm fertilization ability was high for both freezing methods examined, ampoule or plastic tube method, with 76.8 ± 9.7 % and 94.8 ± 2.3 %, respectively (Chen et al., 1992). Dzuba & Kopeika (2002) also found up to 30% of spermatozoa were motile after cryopreservation of silver carp sperm but swelling and cell disruption were common. This swelling is likely a main issue that must be overcome to successfully develop a cryopreservation protocol for this species.

Each of these previously developed protocols utilized very different sperm extenders, but all of them found that ~10 % DMSO and the use of semi-saline solution as an activator yielded the best results for sperm motility and duration. Excellent motility in

DMSO cryoprotectants does not always correspond to high fertilization rates, and methanol proves to be better (Cierszko et al. 2006). However, many of these previously developed protocols utilize methods which are not replicable with currently available materials (Chen et al., 1992; Alvarez et al., 2003), are unavailable in English (Chen et al., 1992; Dzuba & Kopeika, 2002), or require a very large dilution (Lahnsteiner et al., 2000) which makes it impractical to freeze large volumes of sperm.

Objectives

There is a lack of basic information or replicable protocols for short and long term storage of silver and bighead carp spermatozoa. The objectives of these experiments were to identify how short term storage on ice with and without dilution in a sperm extender impacted sperm motility and fertilization ability. An investigation was also conducted to assess the applicability of common carp sperm cryopreservation protocols for silver carp sperm. Common carp sperm cryopreservation has been widely studied and many different protocols have been developed (Babiak et al., 1997; Lahnsteiner et al., 2000; Li et al., 2013; Bernáth et al., 2016). We planned to modify and replicate these cryopreservation protocols as well as to alter previously described silver carp protocols for available materials. Impacts of extenders, internal cryoprotectants, equilibration duration, dilution ratios, freezing and thawing methods will be investigated to determine impacts on both pre-freeze and post-thaw motility of sperm. The resulting fertilization ability of cryopreserved sperm will be assessed utilizing zebrafish ova as a surrogate (Delomas & Dabrowski, 2016).

Methods

Fish species and sources

Four different species are utilized throughout the experiments. Silver carp and bighead carp males were obtained from the Illinois River near Ottawa, Illinois by the Illinois Department of Natural Resources and fish (1.8-3.5 kg) were either transported with a fish hauler (2018) or sperm was immediately stripped at the dock and transported on ice (2019) back to Ohio State University (Columbus, OH). Koi carp *Cyprinus carpio* sperm utilized in the experiments was obtained from 1-2 year old broodstock ornamental Koi originally obtained from Reef Systems Coral Farm, Inc. (New Albany, OH). Koi carp sperm was included in the experiments because of its accessibility, large body of previous research, and it is also from the cyprinid family of fishes. Broodstock zebrafish *Danio rerio* used in the trials were originally from a AB/TL hybrid line (C. Beattie, Department of Neuroscience, The Ohio State University, Columbus, OH, USA) or fish from a local pet shop (GloFish brand).

Sperm motility

The motility of sperm was visually assessed using a compound microscope (Model BX41; Olympus, Center Valley, Pennsylvania) at 200 x magnification. The procedure to measure motility was the same throughout all sperm storage experiments. Briefly, 1 µL of sperm was spread on a coverslip and placed on a slide to examine if motility was present before activation. The sperm were activated with either 50 µL of 50mMol NaCl + 10 mMol Tris base buffered to pH 8.5 or DI water applied on a slide

before the coverslip was added. Motility was visually assessed every 30 s in the field up to 3 minutes post activation, over multiple fields ($n \geq 3$) and was recorded as a percentage.

Storage of sperm on ice

Trial 1 of short term storage of sperm stripped from live silver carp and macerated testes was conducted in 2018. The sperm used in the experiment was a mixture from two males. The macerated testes were a mixture collected from two fish that died due to the catching handling stress of manual stripping. Two replicates of 200 µL fresh sperm and three replicates of 200 µL macerated testes were transferred into 25 mL tissue culture flasks (BD Falcon, Bedford, MA) and one of three treatments were applied. In the first treatment, sperm/testes were stored without an extender added (unextended), while the other two treatments were diluted 1:4 in either Hank's Buffered Salt Solution (HBSS) or cyprinid Buffered Sperm Motility Inhibiting Solution (BSMIS) (Lahnsteiner et al., 2000). The tissue culture flasks allowed for the sperm to be evenly and thinly distributed over the bottom of the container. All containers were stored on ice (4.2 ± 1.2 °C) throughout the course of the experiment. Sperm motility was checked for each container at the onset of the experiment and during every subsequent 24-hours.

Because eggs from silver carp were not available during extended storage, we attempted to correlate sperm motility with initiation of embryonic development of ova of cyprinid fish that can be obtained at short notice. A sperm fertilization ability check was

therefore conducted on remaining motile sperm 1- and 4-days post storage initiation utilizing zebrafish (another cyprinid species) ova as a surrogate, similar to methods described by Delomas & Dabrowski (2016). During these *in vitro* trials 20 µL of sperm from each container with motility was applied to ~150 zebrafish eggs and was activated with 500 µL of fresh hatchery water. Fertilization rates were recorded for each fertilization between 1.5–3 hours post activation. Eggs were incubated at 28 °C in net baskets and remaining embryos were counted every 24 hours following activation.

A second trial of storage of silver carp sperm on ice was conducted in September 2019. Sperm from eight different males was stripped and stored directly on ice in cell culture flasks. The sperm was transported back to the OSU Aquaculture Lab and the motility from each male was measured (8–9 hours post stripping). Two treatments were applied to the sperm (n=4), either storage without (unextended) or with a 1:4 dilution of sperm in HBSS. Motility for each replicate was evaluated 1- and 3-days post storage initiation.

Cryopreservation of sperm

Cryopreservation trials were conducted with koi carp (n= 2) and silver carp (n=3) sperm or bighead carp macerated testes (n=2). An initial equilibration trial was conducted to assess the impact of four different extenders with or without 10 % dimethyl sulfoxide (DMSO), an internal cryoprotectant, inclusion stored on ice (0–1 °C). The extenders and dilution ratios (sperm:extender) used in the trial were Alsever's solution (1:4) from

Alvarez et al. (2003), Grayling Solution pH 6.4 or 8.0 (1:4) from Bernáth et al. (2016), and a modified cyprinid BSMIS (1:6) from Lahnsteiner et al. (2000) with formulations in **Table 2.1**. The sperm utilized in this trial came from a single koi male. The Grayling pH 6.4 was tested because the extenders constituents were identical (but exact amounts were not given) to those listed by Chen et al. (1992). The osmolality (mOsm) of each extender with or without 10 % DMSO was measured using an Auto Osmometer (µOSMETTE, Model 504, Precision Systems Inc.). The motility of sperm was visually assessed 5-, 10-, 30-, 60-minutes and 1-day post equilibration. Results of sperm motility were used to determine equilibration times before cryopreservation. Osmolality of each mixture of sperm, extender, and internal cryoprotectant (DMSO) were measured before freezing.

Two different freezing methods were examined, either the pellet method or straw method. The pellet method followed the protocol outlined in Miller et al. (2018). In short, a block of dry ice (-79 °C) was drilled with holes (5-7 mm diameter) and approximately 100 µL of sperm/extender mixture was pipetted into the holes where they were allowed to freeze for 5 minutes into solid pellets. The pellets were then transferred to a metal colander in liquid nitrogen (LN_2). The pellets were collected from the LN_2 and placed dry inside capped cryovials which were again submerged in LN_2 for extended storage. The straw method was adapted from what was described by Lahnsteiner et al. (2000) for sperm cryopreservation of several cyprinid species. Briefly, 450 µL of the sperm and extender mixture was pipetted into 0.5 mL straws which were then capped with a silicate powder that sealed the straws when placed in water. The straws were placed 3 cm directly above LN_2 to freeze in the vapors for 10 minutes before being submerged in the LN_2 for

extended storage. Pellets from each group were thawed following Miller et al. (2018), where a single pellet was placed in a 20 mL glass vial which was then submerged in a 40 °C water bath for 20–30 s until the pellet was almost thawed. The vial was shaken to complete thawing and sperm motility was measured with multiple activation times up to 10-minutes post thawing.

A second trial of koi carp sperm cryopreservation was conducted, except rather than attempting to separately standardize the different equilibration, freezing, and thawing protocols they were directly followed. The straw method, previously described, was used in the second series of trials. Four different protocols were examined with a mixture of sperm from two koi males. The first protocol from Alvarez et al. (2003) involved diluting koi carp sperm 1:2 in Alsever's solution with 10 % DMSO. The mixture was equilibrated for 3 minutes before being placed 3 cm above LN_2 vapor for 25 minutes and was then submerged in LN_2. The frozen sperm straw was thawed in a 30 °C water bath for 13 seconds. The second protocol followed Bernáth et al. (2016) and involved diluting sperm 1:9 in Grayling solution pH 8 with 10 % methanol and equilibrated for 5 min before being placed in LN_2 vapor for 3 minutes prior to submersion. The frozen sperm was thawed in a 40 °C water bath for 13 seconds. The third protocol followed Lahnsteiner et al. (2000) where sperm was prediluted 1:6 in BSMIS before being additionally diluted 1:7 in BSMIS with 10 % DMSO + 0.5 % glycine. The straws were allowed to freeze in LN_2 vapor for 3 minutes before submersion in LN_2. The frozen sperm was thawed in a 25 °C water bath for 15 seconds. The last protocol followed Li et al. (2013) where sperm was diluted 1:1 in an extender developed

by the authors containing 11 % DMSO. The mixture equilibrated for 15 minutes before freezing in LN_2 vapor for 20 min before submersion. The frozen sperm was thawed in a 40°C water bath for 6 seconds. Sperm motility was assessed for each treatment at 1-, 3-, 6- and 9-minutes post thawing. The cryopreserved sperm fertility was assessed as was previously described with zebrafish ova being used as a surrogate. During each *in vitro* fertilization, the total amount of sperm applied to each batch of ova was controlled (8µL of cryopreserved sperm accounts for ~ 1.0×10^6 sperm per ova) for the different sperm densities from following the various cryopreservation protocols. Then 500 µL of water was used as an activator. An *in vitro* control with a zebrafish male was also included in the trial. Fertilization rate was calculated 1.5 hours post activation and surviving embryos were counted every subsequent 24 hours post fertilization.

The third cryopreservation trial replicated Trial 2 except that silver carp sperm was utilized instead of koi carp. The sperm from three silver carp males with >50% initial motility was combined for each of the protocols examined. An additional treatment consisting of bighead carp macerated testes (BH testes) that were cryopreserved using the protocol from Li et al. (2013) and another with silver carp sperm from a single male that followed the Lahnsteiner et al. (2000) protocol except the initial 1:6 dilution of sperm was with HBSS (HBSS & BSMIS). Motility analysis of the sperm is not included due to a problem with our equipment and the halt of research activities at the Ohio State University due to the coronavirus pandemic. However, a fertility trial was conducted with the cryopreserved sperm using zebrafish ova as a surrogate. In the trial, two fertilization with n= 168 ± 8 ova each were conducted for each cryopreserved sperm treatment.

During each *in vitro* fertilization the total amount of sperm applied to each batch of eggs was controlled (8µL of cryopreserved sperm for ~ 1.0 x 10^6 sperm per egg). Due to concerns that osmolality of the fertilization mixture would not reach a threshold for full sperm motility activation, a slight modification was made to the amount of activation water that was used in this experiment. The total solute for each extender/sperm mixture was calculated based on measurements from the previous cryopreservation trial. The activation water volume (500–3000 µL) for each fertilization was adjusted to reach an estimated value of approximately 90 mOsm, which is comparable to activator solutions used in other studies (Chen et al., 1992; Lahnsteiner et al., 2000). Control fertilizations were conducted *in vitro* with macerated testes from two zebrafish males at the beginning and end of the treatment fertilization to examine if ova quality decreased over time. An unfertilized batch of ova (no addition of sperm) activated with water was included.

Hybridization success (ploidy) was assessed by flow cytometry with surviving larvae at 52 hours post fertilization (hpf) following the protocol outlined by Delomas and Dabrowski (2016). Individual embryos were placed in 1.5 mL microcentrifuge tubes containing 800 µL staining solution (50 mg/L propidium iodide, 10 mg L^{-1} RNase A in Isoton II) with 1 µL of a 1:20 dilution of rainbow trout *Oncorhynchus mykiss* red blood cells (RBC) in Isoton II as an internal standard. Samples were refrigerated overnight and the following morning they were vortexed and aspirated through a 26 G needle with 1 mL syringe. Samples were filtered through 55 µm mesh and a 26 G needle. Twenty thousand gated events along a slope of 1 for forward scatter and side scatter were recorded per sample using an Attune™ NxT Acoustic Focusing Cytometer (Invitrogen,

Carlsbad, CA) to prevent the recording of "sticky cells". Relative nuclear DNA content was calculated as the ratio of mean fluorescence intensity between the sample peak and the trout RBC peak. This ratio was multiplied by the c-value for rainbow trout (Ohno et al., 1969) to obtain the sample c-value. Sample c-values were compared to the known c-values for zebrafish (1.68 pg) (Ciudad et al., 2002) and silver carp (1.02 pg) (Cui et al., 1991), with the expected hybridization to result in a c-value that was between each of these values (~1.35 pg).

Results

Storage of silver carp sperm on ice

Initial sperm motility was > 50 % for all treatments except for HBSS (sperm) which was 35 ± 25% (mean ± SE) due to low motility recorded in one of the replicates (Figure 2.1). Non-activated fresh stripped sperm motility was high (85 ± 5 %) indicating that some contamination occurred during the stripping process. Non-activated sperm of all other treatments were 0% except for BSMIS (sperm) which was 1 ± 0 %. Motility of activated sperm followed a pattern of gradually decreasing over time as sperm utilized stored energy reserves, but motility was present for at least 90 s after activation. Other than BSMIS (testes) there was low sperm motility (<10 %) measured after 1-day of storage with motility ceasing by 4-days. For the BSMIS (testes), sperm motility was 57 ± 9% and 17 ± 3% at 2- and 4-days, respectively, post storage.

For the first time, we identified that sperm of silver carp was able to initiate fertilization and development of zebrafish embryos (**Table 2.2)**. Two trials of fertility were conducted at 1- and 4-days post storage where we found that all treatments with motile sperm were able to fertilize zebrafish eggs. The mean fertilization rates during the trials were all <10 %, but surviving embryos were obtained 24 hpf for unextended (testes) and BSMIS (testes) treatments during the trial after 1 day of storage (**Figure 2.2**). Only one embryo remained at 48 hpf and did not survive to hatching.

Measurements of sperm motility over time for the second silver carp sperm storage on ice trial are presented in **Figure 2.3**. The motility of both groups of sperm, at 9 hours post storage, were similar. The mean motility recorded for the HBSS treatment was about 30 % and 40 % higher than the unextended treatment by day 1 and 3 of storage respectively. The sperm from males that was stored unextended started to agglutinate and formed clumps by the third day of storage at 4 ± 1°C.

Cryopreservation of sperm

The first cryopreservation trial attempted to adapt previously described cryopreservation protocols to a more uniform procedure. To test the effects of our modifications to these protocols an equilibration trial was conducted (**Figure 2.4**). In the trial both Grayling pH 8 or pH 6.4 and Alsever's solution were found to improve the motility of activated sperm throughout the first 90 minutes of storage compared to unextended sperm. The sperm in the modified BSMIS extender failed the activation test,

so the internal cryoprotectant trial for this extender was not conducted. Overall, the inclusion of the internal cryoprotectant (10 % DMSO) negatively impacted the recorded motility for each extender solution, but the effect was magnified for the Alsever's extender compared to both Grayling extenders. Equilibration times before cryopreservation were chosen using the results from this trial. The sperm + extender + internal cryoprotectant mixture was allowed to equilibrate for 25-, 5-, and 1-minute for the Grayling pH 6.4, Grayling pH 8.0, and Alsever's extenders, respectively, before each of the freezing methods were tested.

The results from the first cryopreservation trials are presented in **Table 2.3**. In general, the pellet method performed better than the straw method. However, the motility of cryopreserved sperm was erratic and was low or absent for all extenders, freezing, and thawing methods tested. Only the Grayling pH 8.0 extender had some consistency between freezing methods in the 40 °C for 13 s thawing method, but agglutination of cryopreserved sperm prevented a full analysis of the pellet method.

During the second trial of koi sperm cryopreservation the original protocols from each experiment were replicated as closely as possible. Measurements of sperm motility at the end of the equilibration period for each protocol are presented in **Figure 2.5**. Motility of sperm just prior to cryopreservation utilizing the Bernáth et al. (2016), Li et al. (2013), and Lahnsteiner et al. (2000) protocols were 10-20% less than unextended Koi sperm stored on ice with the Alvarez et al. (2003) protocol being 40% lower (**Figure 2.5**). The straws of cryopreserved sperm from each protocol were thawed and the sperm motility after being activated at 1-, 3-, 6-, and 9-min post-thawing are presented in

Figure 2.6. All cryopreserved sperm protocols had motile sperm after activation. The Bernáth et al. (2016) protocol performed the best with initial sperm motility being at least 70% during each of the activations. The Li et al. (2013) protocol had the 2nd highest sperm motility after activation but there was some variability measured as the time after thawing increased. The Lahnsteiner et al. (2000) and Alvarez et al. (2003) protocols had similar motility post thawing but were lower than the previous two protocols.

A fertility trial was conducted with cryopreserved koi carp sperm utilizing zebrafish eggs as a surrogate. This cross has been conducted previously and is described by Delomas & Dabrowski (2016). The fertilization rate and 24-hour survival for each of the cryopreservation protocols tested are presented in **Figure 2.7**. All cryopreserved sperm tested in the trial were found to fertilize zebrafish eggs. The Bernáth et al. (2016) Grayling and Lahnsteiner et al. (2000) BSMIS extenders performed best with 24-hour survival of embryos identified in each. No surviving embryos were identified for the Li et al. (2013) extender and Alvarez et al. (2003) Alsever's extender or at 48 hours after fertilization for any of the protocols.

The results for the third trial with cryopreserved silver carp fresh sperm and bighead carp testes are presented in **Figure 2.8**. Control fertilizations were comparable to previous *in vitro* fertilization attempts conducted using this protocol but a decrease of 9% in fertilization was recorded between the start and end controls. The percent fertilization was relatively high (30.5–43.7 %) for each of the cryopreservation protocols tested with means being about 20 % lower than fertilized controls in the experiment. However, the fertilization rate measured in the unfertilized control was exceptionally high (29.4 %).

The high fertilization rate in unfertilized ova indicates that their quality was compromised in the experiment. Only one surviving embryo was identified 24 hpf for the Bernáth et al. (2016) protocol. To confirm that the surviving larva from these trials was a true hybrid, and not a result of haploid embryonic development or spontaneous diploidization, flow cytometry was conducted (**Figure 2.9**). The surviving embryo for the Bernáth et al. (2016) protocol had a c-value = 1.31 pg compared to a c-value = 1.61 pg (n=2) for the control start group. Indicating that the surviving embryo was a true hybrid and that fertilization was successful in the Bernáth et al. (2016) protocol.

Discussion

The storage of sperm with and without dilution in an immobilizing solution had low motility after only 24 hours of storage in the first trial. This was likely due to contamination of the sperm with urine during the stripping process since non-activated motility of sperm was high (85 ± 5 %). Silver carp macerated testes diluted in BSMIS were found to have motile sperm throughout the entire 4-day storage trial, but initial motility decreased by 50% on day 3 and 4 of storage. The results from the second sperm storage on ice trial indicate that sperm stored without a diluent decreased motility rapidly by the third day. Other complementary observations of sperm motility by our lab concur with this finding, as bighead and silver carp unextended sperm agglutinated or had large sperm clumps by day 3 or 4 of storage depending on the volume. Compared to common carp, silver carp sperm stored on ice without an extender decreased viability more rapidly

(Ravinder et al. 1997). These results indicate that the sperm of silver carp should be diluted in a motility inhibiting solution if storage of over 24 hours is anticipated. The HBSS extender was found to effectively allow for storage of sperm up to 3 days but was not appropriate to store testes. Cyprinid BSMIS, in comparison allowed for storage of silver carp macerated testes for up to 4 days while maintaining sperm viability.

For the first time, we have shown that using zebrafish oocytes could be employed as a type of "fertility screen" for silver carp sperm. The fertilization rates from the first trial were quite low for this cross. But abnormal larvae were identified after 24 hours for two of the treatment groups from storage on ice and one after cryopreservation. Rather than relying on the rate of fertilization to assess sperm quality, it may be more appropriate to use the presence of abnormal embryos after gastrulation. Since both common carp sperm (Delomas & Dabrowski, 2016) and silver carp sperm have been identified as producing low-viability hybrids with zebrafish, the use of ova as a surrogate may be applicable to a wide range of cyprinid species. The availability of zebrafish ova throughout the year could allow for the expansion of examining cyprinid sperm fertilization ability after cryopreservation when female gametes are unavailable (out of season). This could potentially increase the research efficiency of creating and optimizing a sperm cryopreservation protocol that includes an estimate of the sperm fertilization ability, since zebrafish will be available year-round (Billard et al., 1995; Asturiano et al., 2017, Dabrowski & Miller, 2018). The inclusion of a control where no sperm will be applied appears to be particularly important with this screen. As was observed in the last fertilization ability trial, eggs can occasionally start development when no sperm is

applied. This phenomenon has been previously observed by Hubbs (1971) in experiments involving hybridization between percids. In his experiments he included unfertilized controls for each of his hybridization crosses and found that for most of the crosses some eggs did start development after activation but did not gastrulate (Hubbs, 1971). The exception was Yellow perch *Perca flavescens,* which were found to be able to gastrulate and did eventually hatch (Hubbs, 1971). He reasoned that this was a result of development without sperm addition, rather than the fish had an ovotestis, since these fish were characterized by delayed development and had very low survival, consistent with functional haploid development (Hubbs, 1971). Spontaneous diploidization is another possible explanation for the results obtained in the unfertilized control, as this phenomenon has been previously reported in zebrafish from our lab (Delomas & Dabrowski, 2016; Delomas & Dabrowski, 2017). However, this possibility is unlikely since no surviving embryos were identified at 24 hpf. The most likely case for the current experiment is that the eggs of zebrafish initiated development but did not gastrulate. To eliminate this issue, the use of flow cytometry on surviving embryos/larvae can confirm the presence of hybrids and eliminate the possibility of haploidy through evaluation of ploidy.

The modified cryopreservation protocols assessed were not very successful compared to their original protocol descriptions. Low motility was identified post thawing for these modified cryopreservation protocols and it was irregular with only the Grayling pH 8.0 extender consistently having motile sperm. Motility was found to be the highest for the Grayling pH 8.0 pellet method, but agglutination was an issue which

makes the application of this protocol unreliable. This issue was described by Bernáth et al. (2016) as being associated with glucose-based extenders, like the Grayling extender. However, these authors found that the sperm near the agglutinated area usually had the best motility (Bernáth et al., 2016). The pellet method does not utilize a large volume of liquid (~100 µL) for each pellet, so agglutination became an issue when utilizing this method. Chen et al. (1992) originally found that a slightly acidic extender solution (pH 6.4-6.6) resulted in the best motility after cryopreservation for bighead carp sperm but this result was not found to be transferable to koi carp.

The original protocols tested in trial 2 were all found to contain motile sperm. The Bernáth et al. (2016) protocol resulted in significantly higher post-thaw motility than the other protocols (ANOVA, $F= 16.4$, $df= 3,12$, $p= 0.0002$) while also having the highest measured fertilization ability and had surviving zebrafish embryos 24 hpf. Compared with the results of sperm motility (~40 ± 15 %) post thawing reported in Bernáth et al. (2016), the motility in trial 2 was much higher (72.5 ± 5 %). Mean motility for the Li et al. (2013) protocol in the trial was slightly less (36 ± 17 %) than what was found by the authors (47.2 %) and had the highest variability of the cryopreservation protocols tested. Both the Alvarez et al. (2003) and Lahnsteiner et al. (2000) protocols had similar results for sperm motility post-thaw and these results were much lower than reported by these studies. However, the Alvarez et al. (2003) protocol was designed for silver carp spermatozoa cryopreservation. The Lahnsteiner et al. (2000) protocol did show some potential as it had the highest percentage of surviving zebrafish embryos.

The results from the fertilization trial of the third cryopreservation trial with silver carp sperm is questionable for the initial fertilization rate since the unfertilized control had a fertilization rate of 29.2 %. However, there was one larva from the Bernáth et al. (2016) protocol which survived to 52 hours and was confirmed to be a hybrid with flow cytometry. This signifies that the cryopreservation of silver carp sperm with this protocol was successful, but due to complications of being unable to assess simultaneously motility and fertilization ability, the extent of this relation is unclear.

The current study confirmed the difficulties associated with the modification of existing cryopreservation protocols among different cyprinid species for easy application in aquaculture settings. The alterations that were made to these protocols resulted in unsatisfactory results and highlighted an issue with this field of study. Many of these protocols utilize specialized equipment or materials which make it difficult to replicate results and have led to the rejection of cryopreservation by the aquaculture industry (Asturiano et al., 2017). The cryopreservation protocol from Bernáth et al. (2016) was found to have the best results for both koi carp and silver carp in the present experiments. While the results are still somewhat inconclusive, this protocol shows promise as being appropriate for cryopreservation of silver carp sperm.

References

Alvarez, B., Fuentes, R., Pimentel, R., Abad, Z., Cabrera, E., Pimentel, E., and Arenal, A. (2003). High fry production rates using post-thaw silver carp (*Hypophthalmichthys molitrix*) spermatozoa under farming conditions. Aquaculture *220*, 195–201.

Asturiano, J.F., Sorbera, L.A., Carillo, M., Zanuy, S., Ramos, J., Navarro, J.C., and Bromage, N. (2001). Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture *194*, 173–190.

Asturiano, J.F., Cabrita, E., and Horvath, A. (2017). Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. General and Comparative Endocrinology *245*, 69–76.

Babiak, I., Glogowski, J., Brzuska, E., and Adamek, J. (1997). Cryopreservation of sperm of common carp, *Cyprinus carpio* L. Aquaculture Research *28*, 567–571.

Bernáth, G., Zarski, D., Kása, E., Staszny, A., Várkonyi, L, Kollár, T., Hegyi, A., Bokor, Z., Urbányi, B., and Horváth, A. (2016). Improvement of common carp (*Cyprinus carpio*) sperm cryopreservation using a programmable freezer. General and Comparative Endocrinology *237*, 78–88.

Bobe, J., and Labbe, C. (2008). Chilled storage of sperm and eggs. In: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in reproductive aquaculture: Marine and freshwater species Taylor and Francis, London (UK).

Bobe, J., and Labbé, C. (2010). Egg and sperm quality in fish. General and Comparative Endocrinology *165*, 535–548.

Billard, R. (1978). Changes in structure and fertilizing ability of marine and freshwater fish spermatozoa diluted in media of various salinities. Aquaculture *14*, 187–198.

Billard, R., Cosson, J., Perchec, G., and Linhart, O. (1995). Biology of sperm and artificial reproduction in carp. Aquaculture *129*, 95–112.

Campbell, P.M., Pottinger, T.G., and Sumpter, J.P. (1992). Stress reduces the quality of gametes produced by rainbow trout. Biological Reproduction *47*, 1140–1150.

Chen, S.L., Liu, X.T., Lu, D.C., Zhang, L.Z., Fu, C.J., and Fang, J.P. (1992). Cryopreservation of spermatozoa of silver carp, common carp, blunt snout bream and grass carp. Acta Zoologica Sinica *38*, 413–424. (in Chinese with English summary)

Ciereszko, A., and Dabrowski, K. (1995). Sperm quality and ascorbic acid concentration in rainbow trout semen are affected by dietary vitamin C: An across-season study. Biology of Reproduction *52*, 982–988.

Ciereszko, A., Dabrowski, K., Lin, F., Christ, S.A., and Toth, G.P. (1999). Effects of extenders and time of storage before freezing on motility and fertilization of cryopreserved muskellunge spermatozoa. Transactions of the American Fisheries Society *128*, 542–548.

Ciereszko, A., Dabrowski, K., Froschauer, J., and Wolfe, T.D. (2006). Cryopreservation of semen from lake sturgeon. Transactions of the American Fisheries Society *135*, 232–240.

Ciudad, J., Cid, E., Valesco, A., Lara, J.M., Aijan, J., and Orfao, A. (2002). Flow cytometry measurement of the DNA contents of G0/G1 diploid cells from three different teleost fish species. Cytometry *48*, 20–25.

Cosson, J.J. (2008). Methods to analyse the movements of fish spermatozoa and their flagella. In: Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology, Alpha Science International Ltd., Oxford, U.K., pp. 63–102.

Cui, J., Ren, X., and Yu, Q. (1991). Nuclear DNA content variation in fishes. Cytologia *56*, 425–429.

Dabrowski, K., and Miller, M. (2018). Contested paradigm in raising zebrafish (*Danio rerio*). Zebrafish *15*, 295–309.

Delomas, T.A., and Dabrowski, K. (2016). Zebrafish embryonic development is induced by carp sperm. Biology Letters *12*, 1–4.

Delomas, T.A., and Dabrowski, K. (2017). Heritability of spontaneous diploidization of maternal chromosomes in zebrafish *Danio rerio*. LARVI '17–Fish & Shellfish larviculture symposium, Hendry, C.I. (Ed). European Aquaculture Society, Special publication no. *XX*, Oostende, Belgium.

Dreanno, C., Cosson, J., Suquet, M., Cibert, C., Fauvel, C., Dorange, G., and Billard, R. (1999). Effects of osmolality, morphology perturbations and intracellular nucleotide content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. Journal of Reproductive Fertility *116*, 113–125.

Dzuba, B.B., and Kopeika, E.F. (2002). Relationship between the changes in cellular volume of fish spermatozoa and their cryoresistance. Cryo Letters *23*, 353–360.

Hubbs, C. (1971). Survival of intergroup percid hybrids. Japanese Journal of Ichthyology *18*, 65–75.

Hulata, G., and Rothbard, S. (1979). Cold storage of carp semen for short periods. Aquaculture *16*, 267–269.

Inaba, K., Morisawa, S., and Morisawa, M. (1998). Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. Journal of Cell Science. *111*, 1105–1115.

Kopeika, E., and Kopeika, J. (2008). Variability of sperm quality after cryopreservation in fish. In: Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology, Alpha Science International Ltd., Oxford, U.K., pp. 347–396.

Labbe, C., Maisse, G., Muller, K., Zachowski, A., Kaushik, S., and Loir, M. (1995). Thermal acclimation and dietary lipids alter the composition, but not fluidity, of trout sperm plasma membrane. Lipids *30*, 23–33.

Lahnsteiner, F., and Patzner, R.A. (1998). Sperm motility of the marine teleosts *Boops boops*, *Diplodus sargus*, *Mullus barbatus* and *Trachurus mediterraneus*. Journal of Fish Biology *52*, 726–742.

Lahnsteiner, F., Berger, B., Horvath, A., Urbanyi, B., and Weismann, T. (2000). Cryopreservation of spermatozoa in Cyprinid fishes. Theriogenology *54*, 1477–1498.

Li, P., Hulak, M., Li, Z. H., Sulc, M., Psenicka, M., Rodina, M., Gela, D., and Linhart, O. (2013). Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces protein phosphorylation in tyrosine and threonine residues. Theriogenology *80*, 84–89.

Morisawa, M. (1985). Initiation mechanisms of sperm motility at spawning in teleosts. Zoological Science *2*, 605–615.

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S., and Yasuda, K. (1993b). Effects of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. Journal of Experimental Biology *107*, 95–103.

Ohno, S., Muramoto, J., Klein, J., and Atkin, N.B. (1969). Diploid-tetraploid relationship in clupeoid salmonid fish. In: Darlington, C.D., and Lewis, K.R. (eds.). Chromosomes Today. Oliver & Boyd, Edinburgh, *Vol. 2*, 139–147.

Rana, K.J., Muiruri, R.M., McAndrew, B.J., and Gilmour, A. (1990). The influence of diluents, equilibration time and prefreezing storage time on the viability of *Oreochromis niloticus* (L.) spermatozoa. Aquaculture Research *21*, 25–30.

Ravinder, K., Nasaruddin, K, Majumdar, K.C., and Shivaji, S. (1997). Computerized analysis of motility, motility patterns and motility parameters of spermatozoa of carp following short-term storage of semen. Journal of Fish Biology *50*, 1309–1328.

Rosenthal, H., Asturiano, J.F., Linhart, O., and Horváth, Á. (2010). On the biology of fish gametes: summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. Journal of Applied Ichthyology *26*, 621–622.

Sin, A.W. (1974). Preliminary results on cryogenic preservation of sperm of silver carp (*Hypophthalmichthys molitrix*) and bighead (*Aristichthys nobilis*). Hong Kong Fisheries Bulletin *4*, 33–36.

Van Der Walt, L.D., Van Der Bank, F.H., and Steyn, G.J. (1993). The suitability of using cryopreservation of spermatozoa for the conservation of genetic diversity in African catfish (*Clarias gariepinus*). Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology *106*, 313–318.

Verma, D.K., Routray, P., Dash, C., Dasgupta, S., and Jena, J.K. (2009). Physical and biochemical characteristics of semen and ultrastructure of spermatozoa in six carp species. Turkish Journal of Fisheries and Aquatic Sciences *9*, 67–76.

Withler, F.C. (1982). Cryopreservation of spermatozoa of some freshwater fishes cultured in South and Southeast Asia. Aquaculture *26*, 395–398.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., and Vilella, S. (2004). Adenosine triphosphate concentration and beta-D-glucuronidase activity as indicators of sea bass semen quality. Biology of Reproduction *70*, 1679–1684.

Tables and figures

Table 2.1: Chemical formulas of the extenders that were tested in all storage on ice and cryopreservation trials. Trial 1 included an equilibration experiment before freezing with two different methods (pellet and straw) with the internal cryoprotectant of 10% DMSO. In Trial 2 the original cryopreservation protocols from four sources were replicated. The internal cryoprotectant for BSMIS and Alsever's was 10% DMSO and was 11% DMSO for the extender from Li et al. (2013) with 10% methanol being used with the Grayling extender.

Ingredient (mMol)	**Modified BSMIS**	**BSMIS**	**HBSS**	**Alsever's**	**Grayling**	**Li et al. (2013)**
NaCl	600	75	136.9	68.38	-	69.2
KCl	560	70	5.4	-	40	150.7
$CaCl_2$	16	2	1.7	-	-	2.2
$MgSO_4$	8	1	0.8	-	-	0.7
KH_2PO_4	-	-	0.4	-	-	-
Na_2HPO_4	-	-	0.3	-		-
Tris	160	20	-	-	30	-
Sodium Citrate	-	-	-	27.2	-	-
Dextrose	-	-	-	11.01	-	-
Glucose	-	-	5.6	-	200	-
$NaHCO_3$	-	-	4.2	-	-	2.7
pH	8	8	8	8	8.0/6.4	8
Osmolality (mOsm)						
Trial 1					pH 8.0/6.4	
Extender (Ext.)	1991	-	-	220	312/299	-
Ext. + IC	>6000	-	-	1603	3101/2287	-
Ext. + IC + Sperm	n/a	-	-	1735	1920/1866	-
Trial 2					pH 8.0	
Ext. + IC + Sperm	-	1661	-	2736	2016	5954

n/a: osmolality was not tested since cryopreservation was not attempted due to the inability to activate sperm during the equilibration experiment.
IC**:** Internal Cryoprotectant

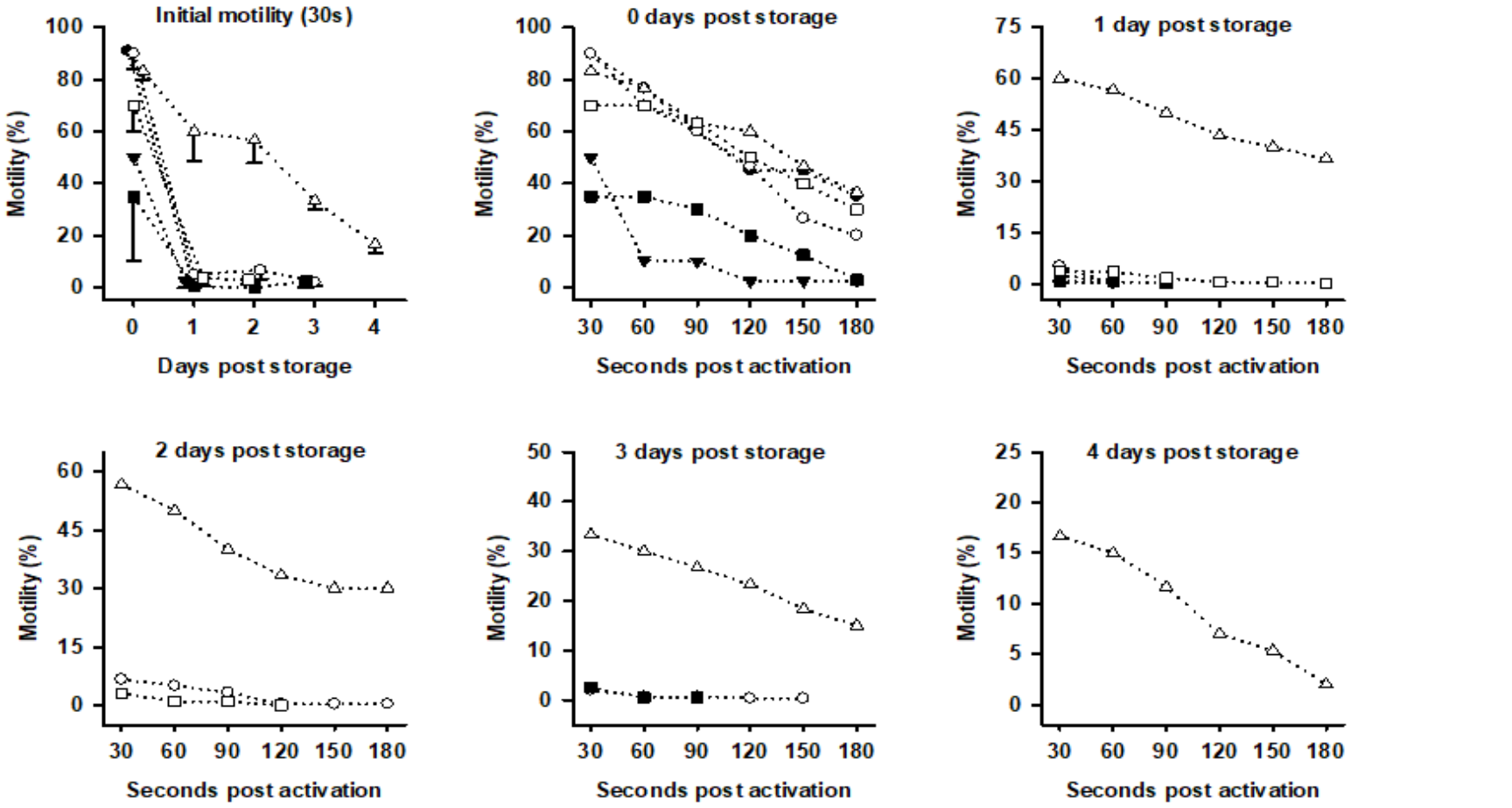

Figure 2.1 Graphs above display the initial sperm motility (30s after activation) of silver carp stripped sperm (stripped) or macerated testes (testes) throughout a storage on ice experiment with and without extenders. Daily measurements of motility (%) over the entire experiment for up to 180s post activation for each of the treatments are also shown. Treatments not shown on a graph were found to have no motility in any of the replicates.

Table 2.2: Results obtained during fertility trials with stored silver carp (SC) stripped sperm or from macerated testes stored on ice for 1 or 4 days used to fertilize zebrafish eggs. Data for the control group was obtained from zebrafish undergoing normal *in vivo* reproduction in breeding chambers. The unfertilized group consisted of eggs that were activated with water but were not fertilized with sperm. Values presented are mean ± standard deviation.

	1-Day post storage			4-Days post storage		
	Motility (%)	**Fertilization (%)**	**24-hr (%)**	**Motility (%)**	**Fertilization (%)**	**24-hr (%)**
Control	-	74.3 ± 13.2	52.9 ± 27.1	-	92.3	88.5
Unfertilized	-	-	-	-	2.6	0.0
SC sperm						
Fresh	0.5 ± 0.5	9.8 ± 4.7	0 ± 0	-	-	-
BSMIS	2.5 ± 2.5	8.9 ± 5.7	0 ± 0	-	-	-
HBSS	0.5 ± 0.5	6.3 ± 1.7	0 ± 0	-	-	-
SC Testes						
Fresh	5 ± 0	5.3 ± 0.9	0.3 ± 0.3	-	-	-
BSMIS	50 ± 10	2.8 ± 0.4	0.4 ± 0.4*	16.7 ± 3.3	9.8 ± 3.5	-
HBSS	5 ± 5	5.4 ± 1.2	0 ± 0	-	-	-

*1 surviving embryo at 48h

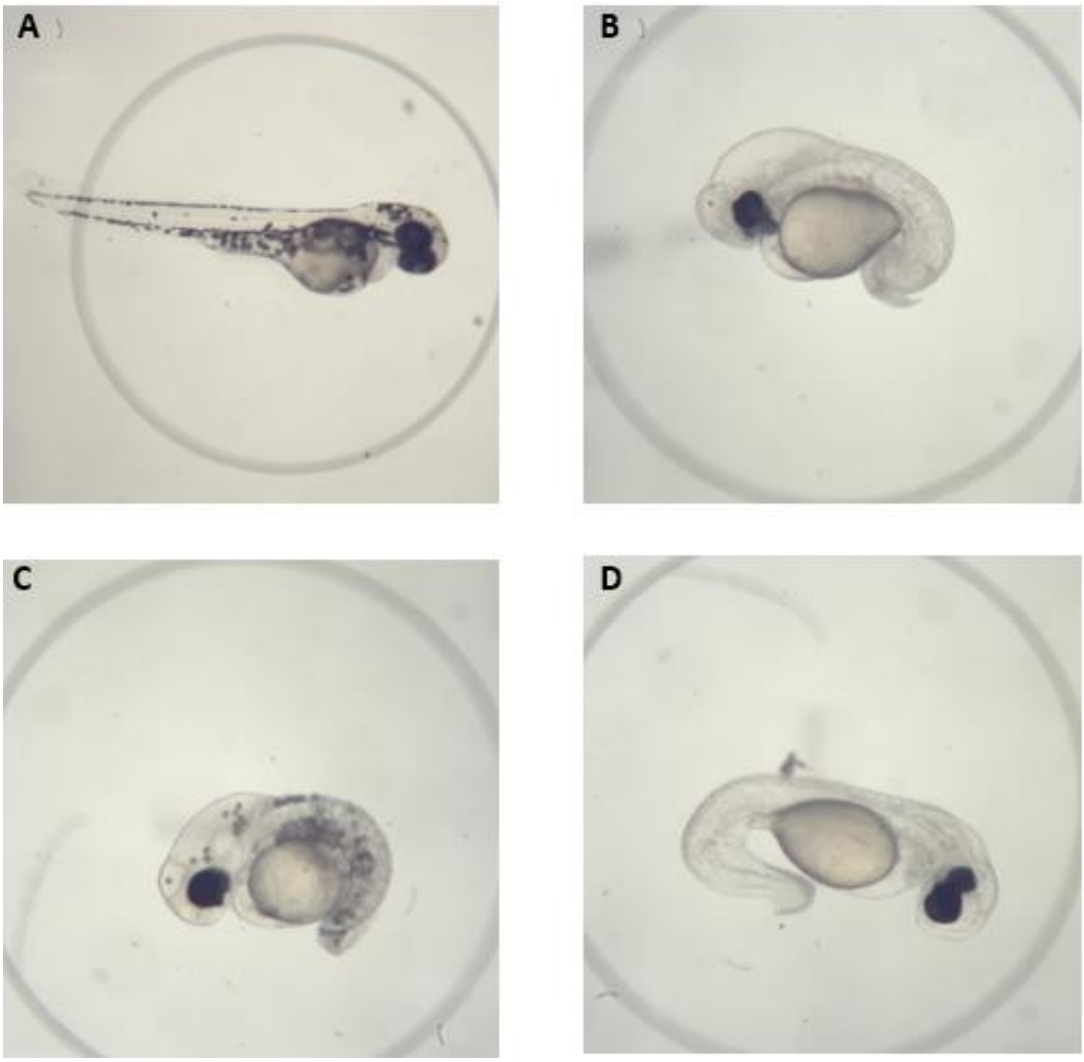

Figure 2.2: Images of embryos (24 hpf) taken during fertilization trials of stored silver carp sperm (1-day) used to fertilize zebrafish ova. A) zebrafish control B-D) zebrafish X silver carp hybrids. The circle in the image is 5mm in diameter.

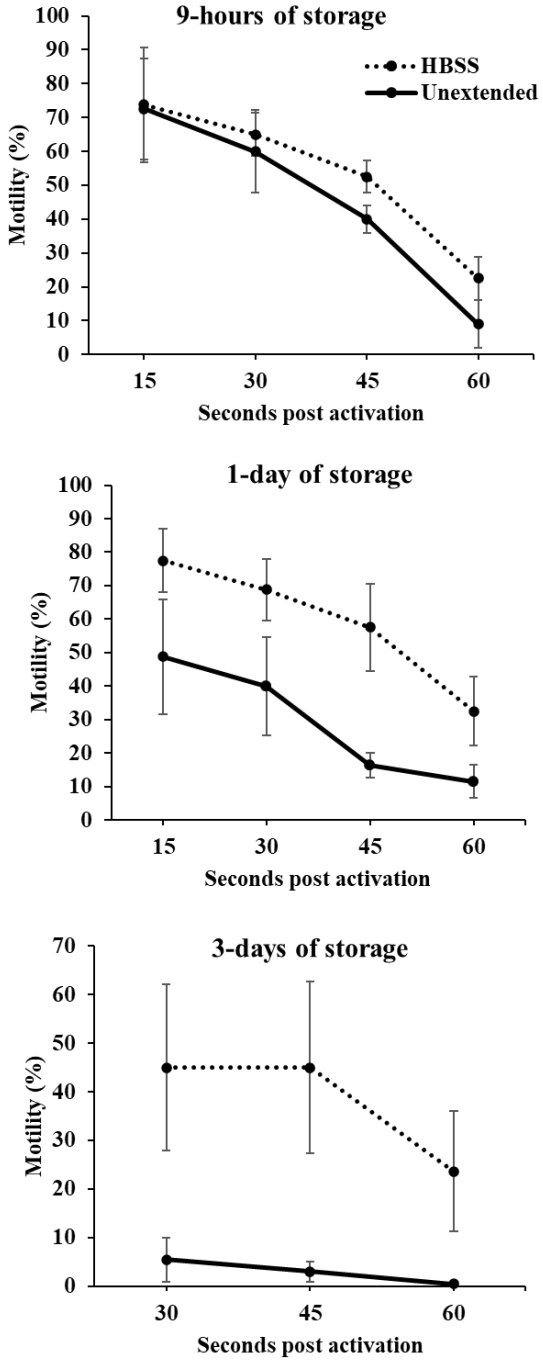

Figure 2.3 Graphs above display sperm motility after activation over time of silver carp sperm unextended or in a 1:4 dilution with HBSS throughout Trial 2 of storage on ice. Each treatment consisted of four replicates from different females and values presented are mean ± standard error. The graph for 9-hours post storage shows the initial motility of both treatments before the HBSS was added to the sperm

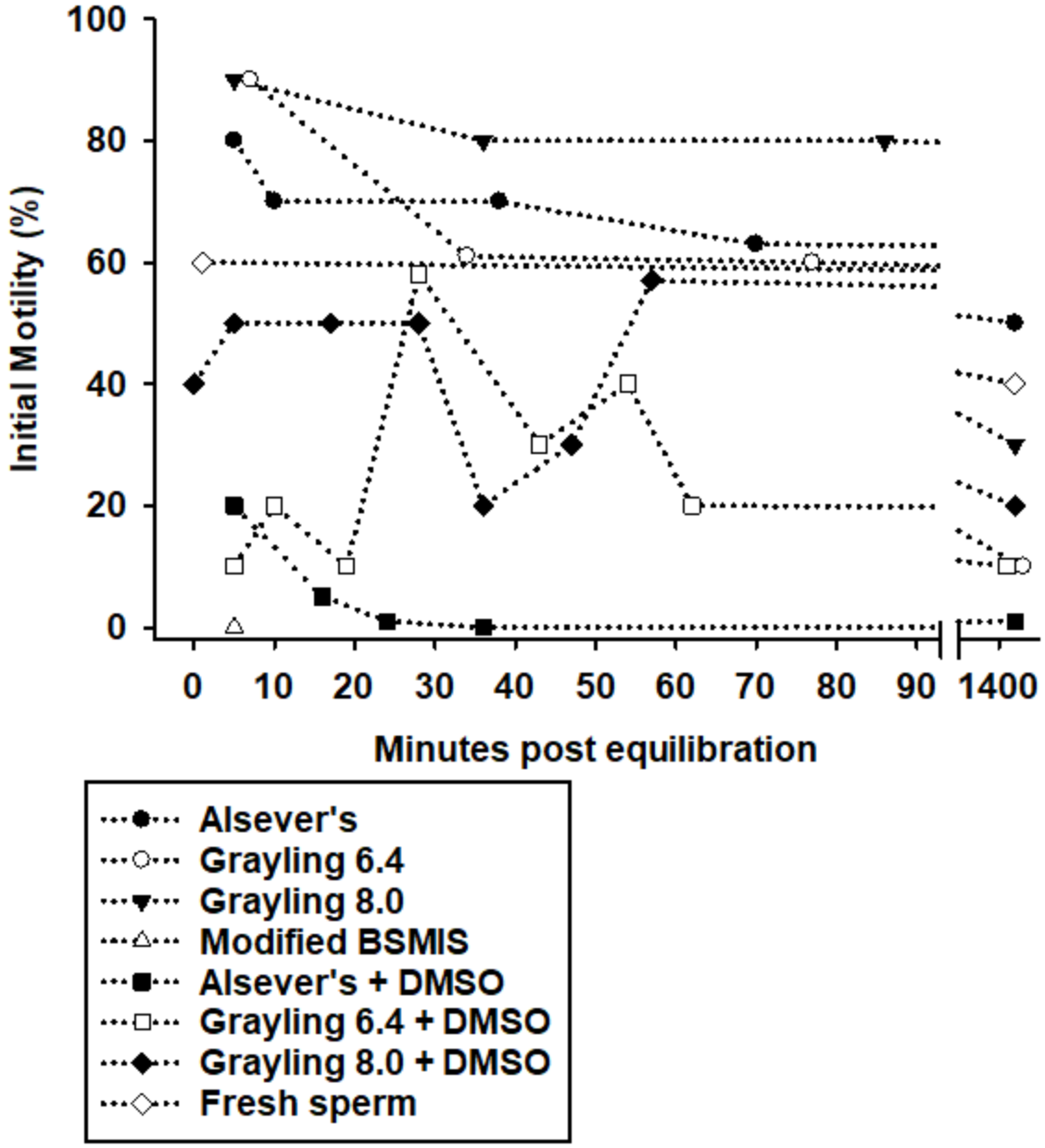

Figure 2.4: Initial sperm motility (30s after activation) over time after mixing sperm with either an extender or extender + internal cryoprotectant. Motility was recorded at regular intervals throughout the first 90 minutes and then again after 1-day (1400 minutes post equilibration).

Table 2.3: The table shows the initial sperm motility recorded (within the first 30 seconds of activation) for each extender solution tested during Trial 1 of koi carp sperm cryopreservation trials. Two different thawing and freezing methods are presented.

Thawing method	**25°C for 25 s**		**40°C for 13s**	
Freezing method	**Pellet**	**Straw**	**Pellet**	**Straw**
Alsever's (mpt)	(%)	(%)	(%)	(%)
1	15	0	0	n/a
4	0	0	10	< 1
7	0	1	1	0
10	n/a	1	0	0
Grayling 6.4 (mpt)				
1	5	0	5	3
4	0	0	< 1	0
7	0	0	0	0
10		0	0	0
Grayling 8.0 (mpt)				
1	5	2	Agg.	4
4	n/a	1	20	2
7	1	0	Agg.	0
10	n/a	0	3	n/a

Agg. - Agglutinated and unable to measure sperm motility
n/a - artifact from slide preparation made recording motility impossible
mpt – minutes post thawing

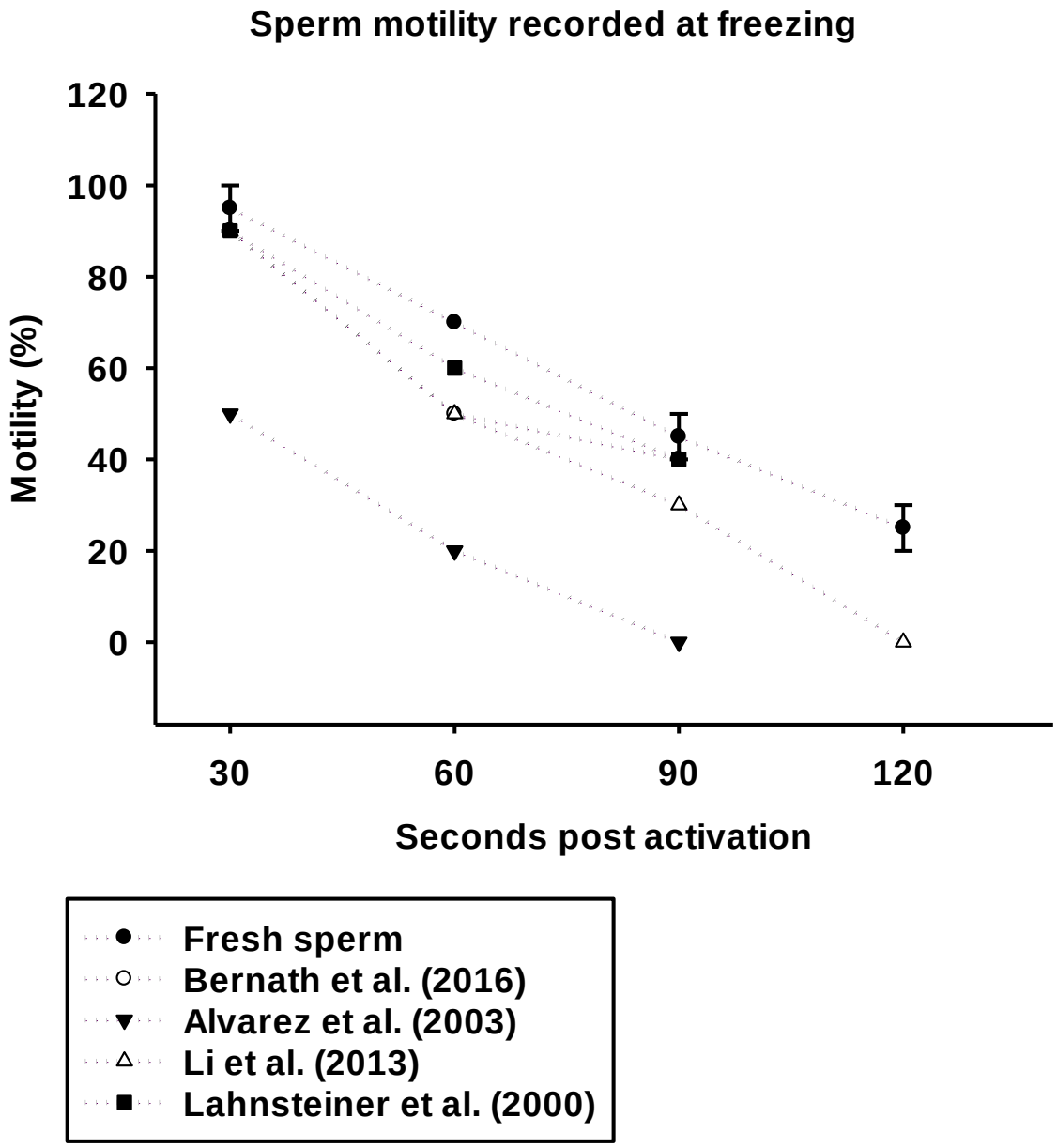

Figure 2.5: Koi carp **s**perm motility over time taken at the end of the equilibration time (time of freezing initiation) of the sperm + extender + internal cryoprotectant for each protocol examined. Fresh sperm is from two measurements of the un-extended sperm that was used in each of the cryopreservation protocols (mean ± standard deviation).

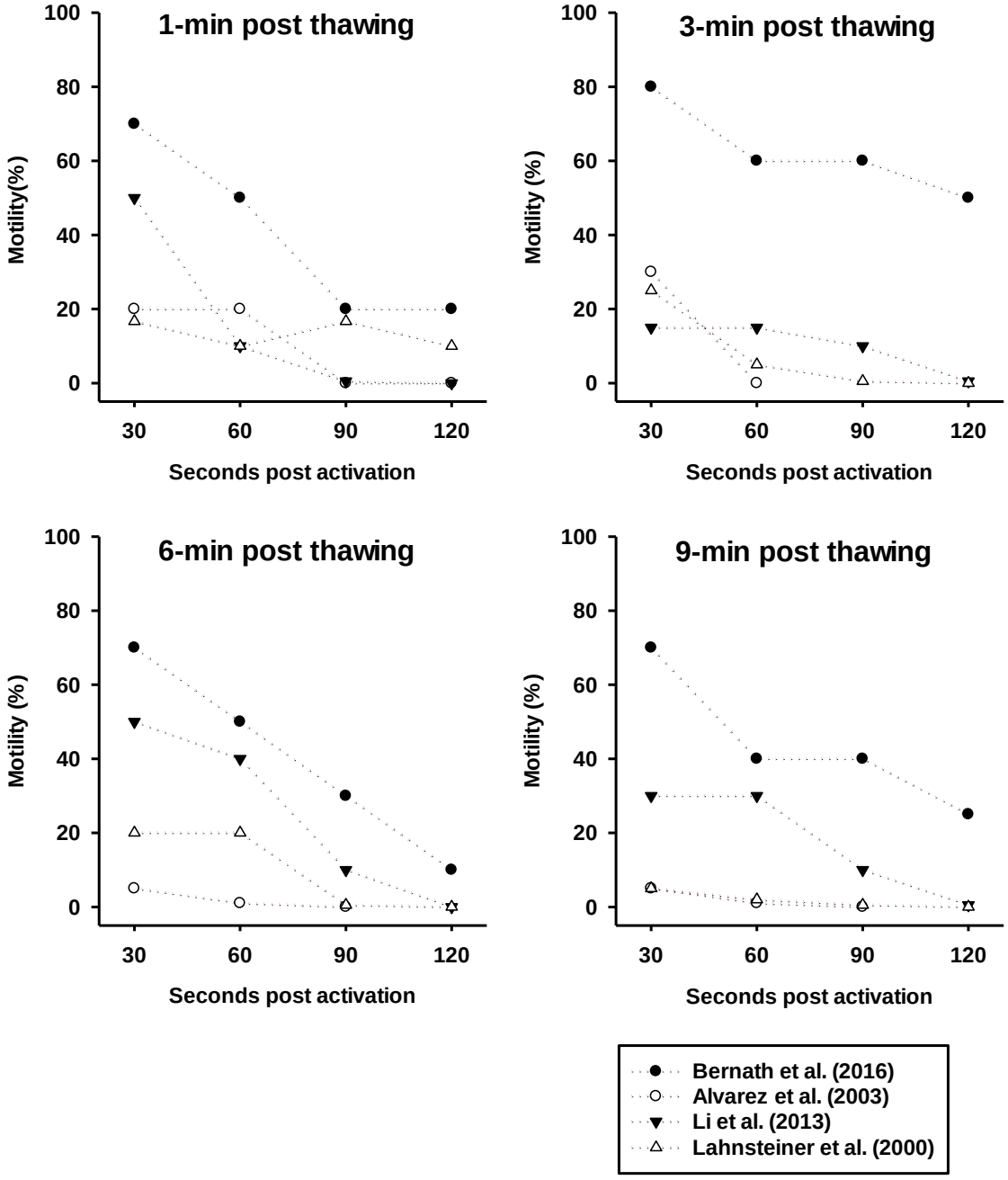

Figure 2.6: Koi carp **s**perm motility post-activation over time taken at 1-, 3-, 6-, and 9-min post thawing of cryopreserved sperm.

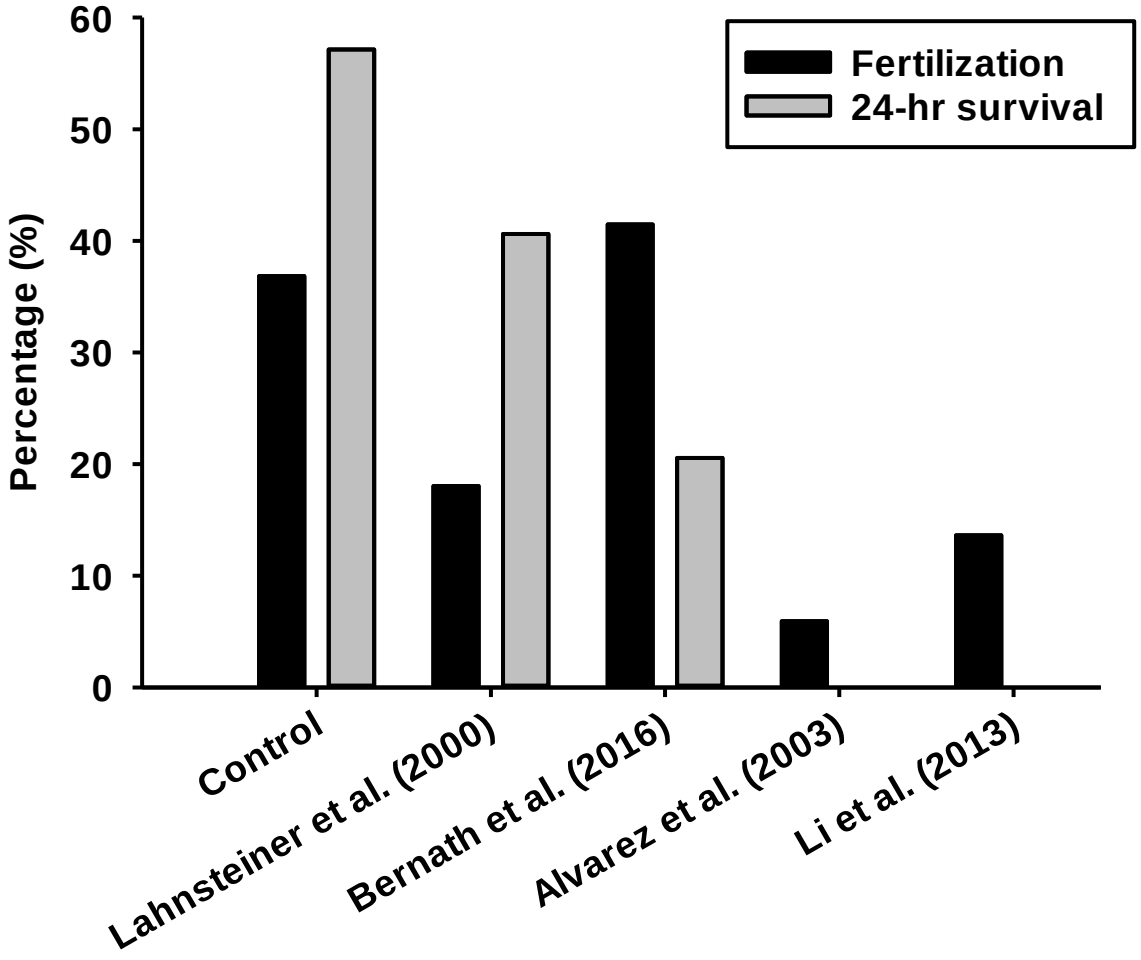

Figure 2.7 Percent fertilization and 24-hour survival of zebrafish ova fertilized *in vitro* with zebrafish macerated testes (control) and koi carp cryopreserved sperm or for each of the cryopreservation protocols examined. The control presented was an *in vitro* fertilization using macerated zebrafish testes.

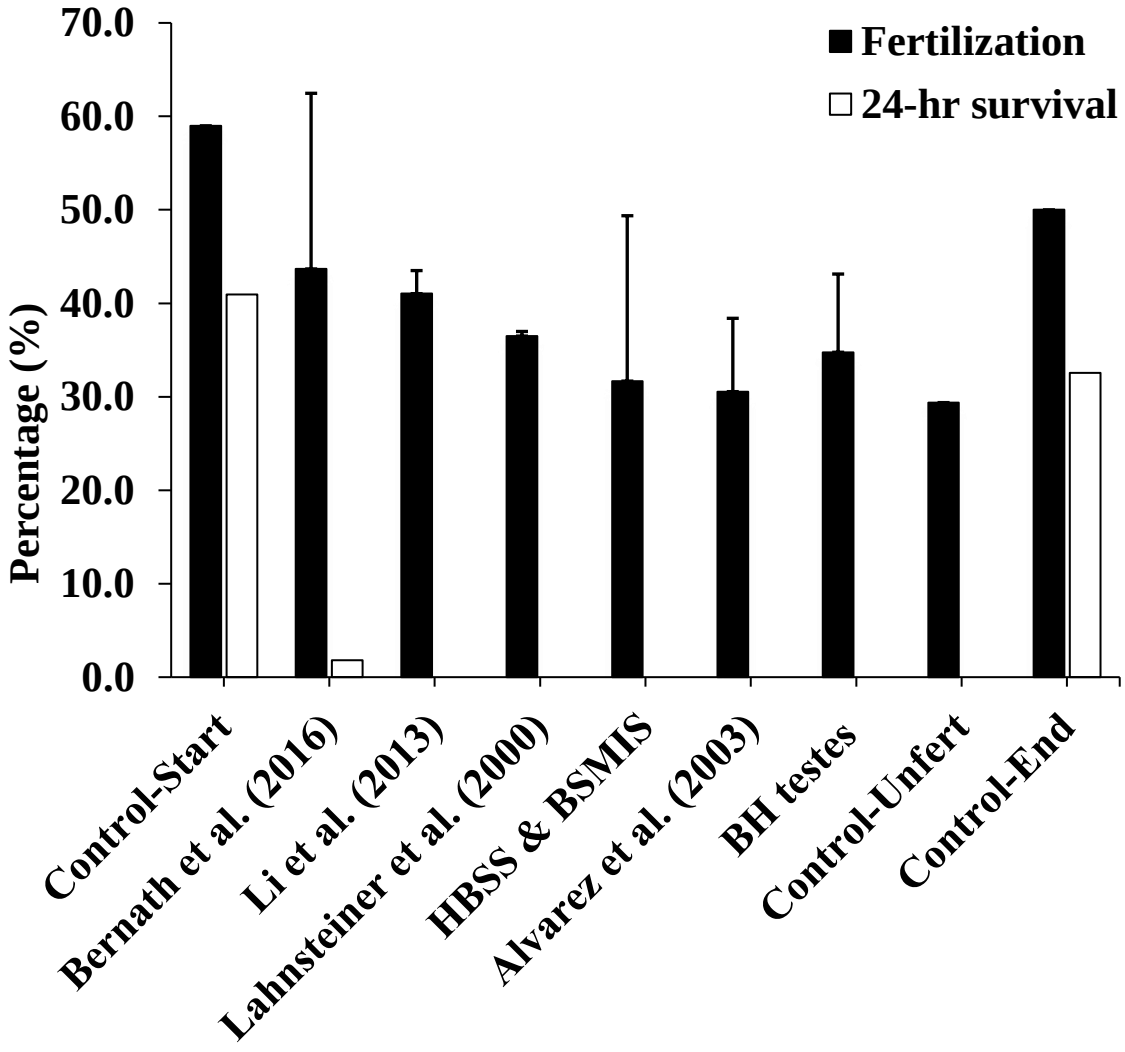

Figure 2.8 Percent fertilization and 24-hour survival of zebrafish ova fertilized *in vitro* with cryopreserved silver carp sperm or bighead carp (BH) carp testes {cryopreserved with protocol from Li et al. (2013)} for each of the cryopreservation protocols examined in Trial 3. The controls presented are from *in vitro* fertilization using macerated zebrafish testes at the start and end of the treatment fertilizations. An unfertilized control with no addition of sperm during fertilization is included.

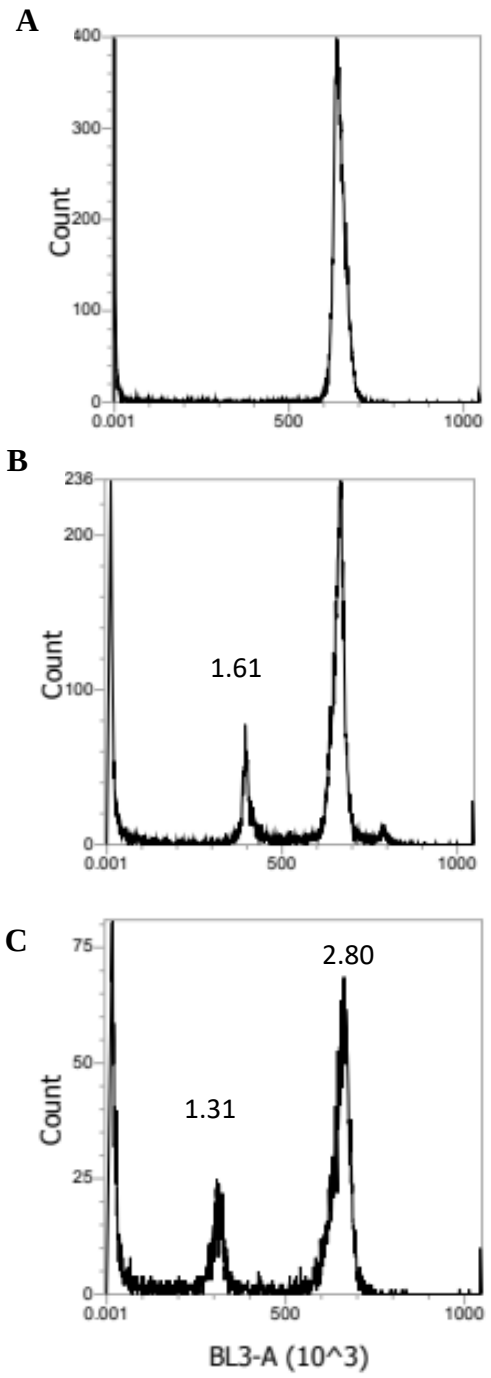

Figure 2.9 Results from flow cytometry for trial 3 of cryopreservation are shown above. A) Internal standard (rainbow trout red blood cells), B) embryo from Control-Start with internal standard, C) embryo from Grayling treatment with internal standard. The c-value for the sample is presented above each peak.

Chapter 3: Comparison and optimization of a novel larval rearing method for bighead carp *Hypophthalmichthys nobilis*

Introduction

Bighead carp *Hypophthalmichthys nobilis*, from the family *Cyprinidae*, is native to Asia (Li & Fang, 1990) and was introduced to the United States as a species of interest for aquaculture in the 1970s (Kolar et al., 2007) but escaped from captivity soon after (Freeze & Henderson, 1982). Bighead carp is currently widely spread throughout the Mississippi River Basin (Schofield et al., 2005) and is potentially threatening to invade the Great Lakes, which could impact a multi-billion dollar annual sport fishery (Southwick Associates, 2007).

The control of invasive species is important because once established in a new ecosystem they have the potential to cause significant harm (Krakowiak & Pennuto, 2008; Nicholls & Hopkins, 1993). Early during an invasion, the elimination of aquatic invasive species can be accomplished using physical removal, biocides (such as rotenone), barriers, or environmental modification (Meronek et al., 1996; Rayner & Creese, 2006). Fisheries managers are currently focused on the removal of bighead carp populations, such as in the upper Illinois River (Tsehaye et al., 2013), and to prevent the establishment of new populations. Electric barriers were constructed in the Chicago Canal to prevent the spread of migratory invasive fish species between the Mississippi

River and Great Lakes basins, including Asian carps (Jerde et al., 2011). But the effectiveness of current countermeasures is not guaranteed (Parker et al., 2015; Sparks et al., 2010).

Once a population has become established and widespread the best method for removal becomes biological control (Thresher et al., 2014). The most readily available option to fisheries managers is the stocking of sterile males, such as with Sea lamprey in the Great Lakes (Twohey et al., 2003), or triploids (Thresher et al., 2014) because triploids are almost always sterile due to the production of aneuploid sperm gametes resulting in nonviable offspring (Benfey, 1999; Gomelsky et al., 2015). However, there are other proposed alternative methods for biological control, such as the production and release of *Myy* (supermale or male with two Y chromosomes) or *Fyy* (female with two Y chromosomes), called the Trojan Y chromosome strategy (Gutierrez and Teem, 2006; Thresher et al., 2014), which both also have the potential to reduce population fecundity (Thresher et al., 2014) and may eventually lead to the total collapse of invasive populations if consistently stocked (Gutierrez & Teem, 2006; Thresher et al., 2014).

An important aspect of these alternative methods of biological control is that they are reliant on the ability to successfully produce hormonally sex-reversed fish. Advances in aquaculture production technologies have enabled the production of *Myy* and *Fyy* to be utilized in the production of monosex populations for better growth in sexually dimorphic species, such as in Nile Tilapia *Oreochromis niloticus* (Mair et al., 1997). Through translating the technologies developed for aquaculture, the implementation of these alternative methods for biological control are feasible, as was recently accomplished with

the production and release of *Myy* to control non-native brook trout in Idaho, USA (Schill et al., 2016, 2017; Kennedy et al., 2018).

Dietary supplementation of hormone treatment is the easiest and most preferred method of administering steroids to fish (Hunter and Donaldson, 1983). This strategy is not without its difficulties, as size heterogeneity can lead to discrepancy of feed uptake and result in different rates of the hormone intake (Pandian & Sheela, 1995). Therefore, fish need to be of similar sizes at the initiation of hormonal treatment. This is especially important for Cyprinid fishes, as in general a high intensity of treatment is required to achieve 100% monosex populations (Pandian & Sheela, 1995).

Size heterogeneity of cohorts in aquaculture is common and occurs throughout the entire production process (Araneda et al., 2013). The onset of size heterogeneity varies depending on the initial distribution as well as culture conditions, like stocking density and temperature (Araneda et al., 2013). The main factor which influences size heterogeneity is size-dependent, with the size that an individual is giving it a competitive advantage or disadvantage compared to others (Peacor et al., 2007). Bighead carp growth has previously been found to be highly variable during larval and early juvenile development (Dabrowski, 1984; Carlos, 1988; Fermin & Recometa, 1988; Rottmann et al., 1991; Garcia et al., 1999). Lieder and Helms (1981) reporting that approximately 30% of bighead carp progenies grew more slowly than the rest resulting in a bimodal size distribution and this trend appears to continue throughout adulthood for fish in the Mississippi River (Schrank and Guy, 2002). Minimizing size heterogeneity during early juvenile rearing is important as bighead carp stunted throughout this period were found to

have a decreased capacity to grow to larger sizes (Santiago et al., 2004). The production of fish that can grow to large sizes quickly is imperative for bighead carp culture as they are required to reach >2kg to complete sexual maturation (Kolar et al., 2005).

Feeds for larval stages of bighead carp are very diverse and include live organisms such as rotifers (fresh or marine), brine shrimp nauplii, nematodes (Rottmann et al., 1991) as well as commercial and formulated dry diets (Dabrowski, 1984; Opuszyński et al., 1989; Rottmann et al., 1991). Dabrowski (1984) and Marciak & Bogdan (1979) found that bighead carp larvae in natural environments initially consume rotifers followed by cladocerans and copepods. There are claims in the literature that some of the formulated artificial feeds, when used as the sole diet, can support bighead carp growth (Lieder and Helms, 1981; Carlos, 1988). However, most frequently live diets, such as initially supplying rotifers till the larvae reach 9mm and then switching to brine shrimp (*Artemia* sp.) nauplii, are suggested as being the best initial feeds for rearing of bighead carp (Van der Wind, 1979).

Larval rearing is a critical period for the successful aquaculture production of fishes (Dabrowski & Culver, 1991; Mahfuj et al., 2012), particularly in respect to cyprinids (Green & Smitherman, 1984; von Oertzen, 1985). A novel larval rearing approach concerning the rearing environment (light regime, salinity, turbidity), fish density, and live food concentration for zebrafish *Danio rerio*, another cyprinid fish, was recently outlined by our lab (Dabrowski and Miller, 2018) and this method was assessed for its suitability for bighead carp.

Objectives

The experiment was conducted to examine various larval bighead carp rearing methods. The objectives of the experiment were to compare a recirculating aquaculture system (RAS) and novel static rearing method with varying densities and two different feeding regimes to produce bighead carp juveniles suitable for dietary supplemented hormonal sex reversal. The experiment was designed to determine optimal conditions for larval rearing throughout an intensive culture period of 20 days. We specifically optimized for maximum survival and biomass (g/L) while reducing individual size heterogeneity. The potential effects of rearing at elevated salinities were also examined in the static rearing environment.

Materials and methods

Larval source, stocking, and general methods

Bighead carp larvae (8 days post fertilization; 1.14mg, 7.3 ± 0.5 mm) from two females were obtained from Osage Catfisheries (Osage Beach, MO) and combined before stocking. Fish were individually counted and randomly stocked, in triplicate, into containers utilized in each of the rearing systems described below at three different densities of 12.5, 25, and 50 larvae per liter which were low (LD), medium (MD), and high density (HD), respectively. Each density was offered two different feeding regimes, either 5 days of rotifers (*Brachionus plicatilis*) with a switch to *Artemia* nauplii on day 6 or 20 days of *Artemia* nauplii. The live feeds were offered ad libitum throughout daylight

hours, which was 4-6 x day^{-1} in the static system and 3-5 x day^{-1} in the RAS system, to maintain constant food availability. To increase the survival of marine live feeds utilized in the experiment, all tanks were maintained at 2 ppt salinity. However, larvae were also stocked, in triplicate, at 3 ppt and 5 ppt at MD under static system methods and were initially fed rotifers. The water temperature in the experiment was gradually raised to and maintained at 28°C, with individual aeration being provided to each tank. Water quality, including temperature (°C), salinity (ppt, parts per thousand), pH, oxygen (mg L^{-1}, %), and ammonia concentrations (mg $^{L-1}$) was measured daily throughout the experiment with a YSI handheld (YSI Multiprobe, Yellow Springs, OH).

Static rearing system

Static system rearing methods were similar to those outlined in Dabrowski and Miller (2018). Briefly, 8L poly-round containers, in a heated water bath, were initially filled with 4L of water and the volume of water was increased to 6L on day 6 of the experiment, effectively decreasing density by 1/3. Algae Nanno 3600 ® (Reed Mariculture Inc.) was added to the tanks to maintain turbidity between 10-20 NTU and a 24hr:0hr (Light:Dark) light regime was applied throughout the experiment. A 50% water change occurred on every third day, to ensure adequate water quality.

Recirculating aquaculture system (RAS) rearing system

The RAS system utilized in the experiment was equipped with mechanical and biofiltration as well as UV sterilization. The tanks in the system were filled with 10L of water and had 250-micron outlet screens. The system followed a natural light regime 15.5hr:8.5hr (Light:Dark) as it was operated in a greenhouse. Water replacement functionality of the system was not utilized throughout the experiment to maintain constant salinity levels. However, water was partially replaced by flushing water from the system every three days and was replenished with 2ppt water from an attached 600-L water reservoir.

Statistical analysis and sampling

A 2x2x3 full factorial ANOVA design was implemented (**Table 3.1**), in triplicate, to compare the main effects of larval rearing systems, feeding regime, and stocking density and the interaction between the main effects on survival (%), biomass (g/L), total length (mm) and weight (mg) at 10d and 20d of feeding as well as the proportion of large fish, specific growth rate (%/day) and weighted mean weight (mg) at 20d of feeding. Ten fish, fasted for at least 12h, from each tank were anesthetized with 50 mg/L of MS-222 before total length (mm) was taken with digital calipers and weight (mg) was measured with a top-loading balance. Fish were allowed to recover following measurements and were returned to their tanks. On day 20, the fish were additionally separated in small and large size categories and counted to calculate the proportion of large fish and survival (%) each tank. The biomass of each tank was calculated from the proportion of large and

small fish and the mean weight of each of the categories sampled. The calculated biomass was then divided by the total water volume of each tank to compare across different tank sizes. Specific growth rate (SGR; % day^{-1}) was calculated for the entire experiment as follows, $SGR = \frac{(\ln W_f - \ln W_i)}{days} * 100$, where W_i is the initial weight and W_f is the final weight measured over the course of the experiment. A one-way ANOVA was used to examine the effects between different salinity levels assessed in the experiment. Data was Box Cox transformed using the suggested optimal lambda and the data were tested for normality using Shapiro-Wilk's test (P>0.05 was considered normal). A Brown-Forsythe test was conducted to examine if the equal variance assumption was met (P >0.05 was considered to have equal variance). If transformed data was not normal, then an aligned rank transformation nonparametric factorial analysis using ANOVA procedures was conducted following Wobbrock et al. (2011). For analytical purposes in the experiments all main effects were considered as categorical variables. If a significant interaction was identified, then a post hoc least squared means Tukey's HSD was conducted for two and three effect interactions and a connecting letters report was generated to visualize differences between groups. Two effect interactions are presented when a three-way interaction was not significant. A desirability function was then constructed using responses measured to maximize survival (%), biomass (g L^{-1}) and the proportion of large fish (Jun et al., 2012).

Results

Water quality

Water quality data collected throughout the experiment is presented in **Figure 3.1**. The temperature of both systems was increased from ~24°C to 28°C over the first three days of the experiment and the mean water temperature was not significantly different between both systems. Salinity levels fluctuated slightly throughout the experiment, but mean salinity was maintained within ±0.5ppt of the desired level. Also, as to be expected, the RAS system had higher and less variable mean DO (% and mg L^{-1}) and lower and less variable pH and ammonia levels than the static rearing environment.

Model significance

The significance and goodness of the model (R^2) for the 2x2x3 factorial ANOVA for each of the parameters recorded is presented in **Table 3.2**. All models examined in the experiment were found to be significant ($P < 0.05$). In general, the predictors utilized in the models were acceptable, with growth and survival related models having the best fit. Only the proportion of large fish (Adj. $R^2 = 0.42$) was found to have an adjusted coefficient of determination < 0.58.

10-day samples

The effect of density (D), rearing system (S), and initial feed (F) on length, weight, survival, and biomass at 10d are shown in **Table 3.3**. The interaction of DxSxF was significant for all responses measured. Survival was similar between densities of *Artemia* fed fish in the static rearing environment but decreased with increasing density for rotifer fed fish in both rearing environments as well as *Artemia* fed fish in the RAS. This resulted in the biomass of static rearing system *Artemia* fed fish being higher overall and increasing with higher density while all other treatments had similar biomass across densities. Length and weight both followed similar trends, with static *Artemia* fed fish achieving the highest values at LD and then decreasing as density increased. Rotifer fed fish in the static system had higher length and weight than comparable fish in the RAS and were similar across densities likely due to reduced survival in the MD and HD groups. *Artemia* and rotifer fed fish in the RAS also had similar weight and length across densities.

20-day samples

The effect of density (D), rearing system (S), and initial feed (F) on survival, biomass, the proportion of large fish, and weighted mean weight (mg), as well as length, weight, and SGR for both small and large categorized fish at 20d, are shown in **Table 3.4**. The majority of mortalities in the experiment occurred throughout the first 10d with only minor decreases measured in survival between the 10d and 20d samples (**Figure**

3.2). A significant three-way interaction was present for survival measured at 20d with general trends between groups being consistent for both sample dates. The weighted mean weight and weight of large fish also had significant three-way interactions, but a clear trend across treatments was not apparent since these values were influenced by the survival, stocking density, and the proportion of large fish in each treatment. However, the data from both measurements did follow a similar pattern. The SGR of large fish additionally had a significant three-way interaction, with the static rearing system having higher values for each density and initial feed compared to the RAS. An exception to this trend occurred for the HD *Artemia* fed fish which were not significantly different between rearing systems.

Significant two-way interactions found for initial stocking density (D) and rearing system (S) are presented in **Figure 3.3**. Biomass was generally higher in the static rearing systems compared to the RAS. The length of large category fish in the static rearing system at LD was significantly higher than the RAS and fish reared in both systems at HD. The mean weight, length, and SGR of small category fish was highest in the static rearing system at LD with generally lower values in the RAS compared to the static system.

Significant two-way interactions found for initial feed (F) and rearing system (S) are presented in **Figure 3.4**. Biomass was highest in fish initially fed *Artemia* nauplii reared in the static system with rotifer fed fish being similar between each rearing system and fish initially fed *Artemia* nauplii in the RAS were lowest. The length and weight of small category fish and the length of large category fish initially fed rotifers in the static

rearing system were significantly higher than other groups. The proportion of large fish was highest in the fish initially fed *Artemia* nauplii in the static rearing system than the other groups.

Significant two-way interactions found for initial feed (F) and stocking density (D) are presented in **Figure 3.5**. The mean weight of small category fish initially fed rotifers and both groups at LD were higher than fish initially fed *Artemia* nauplii at MD and HD. The proportion of large fish was generally higher for both feeding regimes at HD and fish fed *Artemia* nauplii at MD and LD.

Salinity

The results obtained from the salinity trial are presented in **Table 3.5**. Survival in the experiment was significantly higher at increased levels of salinity and most of the mortality occurred during the first 10d of the experiment. There were no differences in mean weight or length found between the treatments at 10d, but significantly lower biomass was calculated in the 2 ppt treatment, due to the decreased survival. The biomass at 20d was not significantly different, but the weighted mean weight of the treatments was significantly lower in the 3 and 5 ppt treatments. The proportion of large fish recorded at the end of the experiment was highest in the 5 ppt treatment (0.82 ± 0.02) with the 2 ppt treatment (0.63 ± 0.03) being significantly lower. The mean length, weight, and SGR of both small and large category fish from the 2 ppt treatment were significantly higher than the 3 and 5ppt treatments.

Optimization

To optimize the larval rearing conditions, the models of biomass, survival, and proportion of large fish were simultaneously assessed following the methodology described by Montgomery (2005), which identified the static rearing environment, with high-density stocking (50 fish L^{-1}), initially fed *Artemia* nauplii as being optimal. The predictions for biomass, survival and proportion of large fish were 2.38 g L^{-1} (95% CI, 2.14–2.62), 70.3 % (95% CI, 60.1–80.6), and 0.768 (95% CI, 0.696–0.841) respectively, with the highest desirability function value of 0.733.

Discussion

Both live feeds provided in the experiment were readily accepted by bighead carp larvae in the experiment. Previous recommendations by Van der Wind (1979) to provide rotifers till the fish reach 9mm in length, did not seem to have an impact on the survival or growth of fish only fed *Artemia* nauplii. Instead, the biomass and survival of *Artemia* fed groups was generally higher than fish fed rotifers, but this was particularly apparent in the static rearing system.

The survival in the current experiment was lower overall compared to results presented by Rottman et al. (1991) utilizing *Artemia* and freshwater rotifers (*B. rubens*) in static conditions at densities 28 and 57 fish L^{-1}, similar to the current MD and HD. These authors reported a minimum survival of 80.4 ± 2.1% (mean ± SE) after 21d for rotifer fed fish at 57 fish L^{-1} but had the highest survival at 99.3 ± 1.8% in rotifer fed fish

at 28 fish L^{-1} (Rottman et al., 1991). The highest survival in the current experiment after 20 days was found in the static rearing system *Artemia* fed fish at HD (70.3 ± 5.9%; mean ± SD) and was lowest in RAS *Artemia* fed HD fish (13.3 ± 1.9%). Differences in stocking methods may be partially responsible for the decreased overall survival reported between these experiments. However, other bighead carp larval rearing experiments have also reported larger ranges of survival for zooplankton fed fish of 17-67% over 14 days (Dabrowski, 1984; Opuszyński et al., 1989).

Survival in the current experiment was highly variable between treatments and primarily occurred throughout the first week of feeding. This may potentially have been a consequence of utilizing a marine rotifer in the experiment. The salinity of tanks was maintained at about 2 ppt in the main experiment and the rotifers were observed to have short survival times (< 60 min) in this environment compared to *Artemia* nauplii. The results obtained from the salinity investigation support this idea, since survival significantly increased in the two elevated salinity treatments. This is contrary to what was previously reported by Garcia et al. (1999), who found that 11 dpf bighead carp held at a salinity of 4 ppt only had a mean survival time of 3.3 hours. These authors also noted that decreased growth was experienced in bighead carp juveniles (18 dpf) reared at salinities above 2 ppt (Garcia et al., 1999). The fish reared at elevated salinities did have decreased growth compared to the 2 ppt treatment, but this was likely impacted by the increased survival for these treatments since final biomass was not found to be significantly different. The proportion of large fish also significantly increased with salinity and leads to the question of if the increased availability of prey items may be

directly related to decreasing size heterogeneity which is prevalent in the culture of this species.

Size heterogeneity in the culture of cyprinid larvae has been previously described (Wohlfarth and Moav, 1972; Dabrowski, 1984; Opuszyński et al., 1989). Wohlfarth and Moav (1972) documented this effect in common carp *Cyprinus carpio*, with larger individuals gaining more weight than smaller ones and the deviation from the overall mean weight spreading out in an open-fan shape with culture duration. Opuszyński et al. (1989) found that variability in individual weight of fish was 2-fold higher (45–183%) for bighead carp compared to silver carp (23–86%) and grass carp (27–101%). Indicating that bighead carp experiences high size heterogeneity, even compared to closely related species. A bimodal weight distribution of common carp and silver carp occurred in larval feeding trials conducted by Dabrowski (1984) potentially due to supplying unsuitable food, either size of organisms and formulated diets offered or insufficient nutrients. In his experiment with bighead carp, he identified that an insufficient supply of food was provided between 8 and 14 days of feeding (Dabrowski, 1984). This resulted in decreased growth for most of the fish, but 18 individuals were identified which were significantly larger and believed to possess sizes that this species could achieve with an adequate supply of feed (Dabrowski, 1984).

The current experiment also identified two distinct size classes of fish. A majority of fish in each treatment were classified as large, but fish fed *Artemia* in the static system or at lower densities resulted in larger proportions of these individuals. This further supports the idea that early food availability affects the resulting size heterogeneity of

bighead carp progenies. Since increased food availability from longer live feed survival times and decreased intraspecific competition positively impacted the proportion of large fish in these treatments. Minimizing the size heterogeneity of progenies reared for dietary hormonal sex reversal is important because the amount of feed consumed directly relates to the rate of hormonal intake (Pandian & Sheela, 1995). This is particularly important for Cyprinid fishes as they generally require a high intensity of treatment over long periods to achieve 100 % monosex populations (Pandian & Sheela, 1995).

While many previous experiments with bighead carp have focused on the extensive rearing of bighead carp in ponds, they tend to focus on weight for growth assessment (Opuszyński, 1969, 1981; Cremer and Smitherman, 1980). Experiments conducted by Nagy et al. (1980) found that the growth of common carp in laboratory conditions is often highly correlated with growth in ponds. Fish in the experiment reached a mean weight of 17.8–58.5 mg after 10 days of feeding and were largely higher in the static system and at lower stocking densities. While it is hard to directly compare weights of fish between experiments due to differences in stocking density and sampling periods, some general conclusions can be made. Compared to Opuzynski et al. (1989), most of the fish in the current experiment achieved higher weights by 10 days of feeding contrasted to ad libitum feeding of bighead carp by 14 days (29 mg). The potential growth (42 ±30 mg) of bighead carp identified by Dabrowski (1984) after 15 days of feeding also falls within the range of weights recorded over the first period of the experiment. The weighted mean weight calculated in the experiment is comparable to the mean weight of a sample from a population. The range of the weighted mean weight in

the experiment was quite variable (102–424 mg). Other laboratory experiments with bighead carp are difficult to compare due to issues with food availability (Dabrowski, 1984), non-precise representation of weights over time (Marciak & Bogdan, 1979), or different methods for assessing weight (Rottman et al. 1991). However, the length of fish can allow for a more direct comparison of growth in these cases.

Marciak & Bogdan (1979) found that the length of bighead carp fed a natural diet reached 9.8 and 19.2 mm by 11 and 22 days of feeding. Rottman et al. (1991) found that fish reared under static conditions stocked at densities similar to MD and HD in the current experiment achieved mean lengths of 16.0–19.8 mm and 12.5–14.0 mm, respectively, after 21 days of feeding analogous diets. The reported lengths in these experiments were more like measurements of the small category fish after 20 days of feeding in the current experiment (MD = 16.1–19.3 mm, HD = 14.4–19.7 mm). The large category of fish, which was most fish reared, was much higher than previously reported values (MD = 29.7–37.4 mm, HD = 26.0–37.6 mm) over this period.

Conclusions

Bighead carp reared in the static rearing environment were found to perform similar to or in some cases better than the methods utilized in the RAS, especially at higher stocking densities. Most previous investigations of intensive cyprinid larval rearing have used systems where the water flows through tanks (Dabrowski, 1984; Szaminska & Drzybal, 1986; Opuszyński et al., 1989) or recirculates through filters

(Applebaum & Uland, 1979; Charlon and Bergot, 1984). But these system designs require frequent feeding to replenish live feeds which are washed out of the tanks over time (Rottman et al., 1991). Using static tanks prevents any potential feed from being unavailable due to being flushed from the culture environment before being consumed, increasing the consistency of food availability. While Rottman et al. (1991) did design a static rearing system capable of producing grass and bighead carp juveniles, this study is the first to directly compare the use of both methods. It provides direct evidence that a static rearing environment can outperform traditional systems by increasing survival and decreasing size heterogeneity when bighead carp larvae are initially fed *Artemia* nauplii.

The desirability function utilized in the current experiment identified the static rearing environment, with high density stocking (50 fish L^{-1}), initially fed *Artemia* nauplii as being optimal to rear bighead carp for dietary supplemented hormonal sex reversal. Using this type of statistical analysis can be an important and flexible tool to select rearing conditions that match the objectives of a breeding program. Its current usage in aquaculture research is extremely limited (Jun et al., 2012), but it has the capability of being widely applied in the optimization of rearing conditions and feeding of fishes.

References

Applebaum, S. and Uland, B. (1979). Intensive rearing of grass carp larvae *Ctenopharyngodon idella* (Valenciennes 1844) under controlled conditions. Aquaculture *17,* 175–179.

Araneda, M.E., Hernández, J.M., Gasca-Leyva, E., and Vela, M.A. (2013). Growth modeling including size heterogeneity: Application to the intensive culture of white shrimp (*P. vannamei*) in Freshwater. Aquacultural Engineering *56,* 1–12.

Benfey, T.J. (1999). The physiology and behavior of triploid fishes. Reviews in Fisheries Science *7*, 39–67.

Carlos, M.H. (1988). Growth and survival of bighead carp (*Aristichthys nobilis*) fry fed at different intake levels and feeding frequencies. Aquaculture *68*, 267–276.

Charlon, N., and Bergot, P. (1984). Rearing system for feeding fish larvae on dry diets. Trial with carp (*Cyprinus carpio* L.). Aquaculture *41*, 1–9.

Dabrowski, K. (1984). Influence of initial weight during the change from live to compound feed on the survival and growth of four cyprinids. Aquaculture *40*, 27–40.

Dabrowski, K., and Culver, D.A. (1991). The physiology of larval fish. Aquaculture Magazine *17*, 49–61.

Dabrowski, K., and Miller, M. (2018). Contested paradigm in raising zebrafish (*Danio rerio*). Zebrafish *15*, 295–309.

Fermin, A.C., and Recometa, R.D. (1988). Larval rearing of bighead carp, *Aristichthys nobilis* Richardson, using different types of feed and their combinations. Aquaculture Research *19*, 283–290.

Freeze, M., & Henderson, S. (1982). Distribution and status of the bighead carp and silver carp in Arkansas. North American Journal of Fisheries Management *2*, 197–200.

Garcia, L.M.B, Garcia, C.M.H., Pineda, A.F.S., Gammad, E.A., Canta, J., Simon, S.P.D., Hilomen-Garcia, G.V., Gonzal, A.C., and Santiago, C.B. (1999). Survival and growth of bighead carp fry exposed to low salinities. Aquaculture International *7*, 241–250.

Gomelsky, B., Schneider, K.J., Anil1, A., and Delomas, T.A. (2015). Gonad development in triploid ornamental koi carp and results of crossing triploid females with diploid males. North American Journal of Aquaculture *77*, 96–101.

Green, B.W., and Smitherman, R.O. (1984). Relative growth, survival and harvestability of bighead carp, silver carp, and their reciprocal hybrids. Aquaculture *37*, 87–95.

Gutierrez, J. B., and Teem, J. L. (2006). A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y Chromosome to cause extinction of an introduced exotic species. Journal of Theoretical Biology *241*, 333–341.

Hunter, G.A., and Donaldson, E.M. (1983). Hormonal sex control and its application to fish culture. In: Hoar, W.S., Randall, D.J., and Donaldson, E.M. (Eds.), Fish Physiology, Vol. 9B, Academic Press, New York, USA, pp. 223-301.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., and Lodge, D.M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. Conservation Letters *4*,150–157.

Jun, Q., Pao, X. Haizhen, W., Ruiwei, L., and Hui, W. (2012). Combined effect of temperature, salinity and density on the growth and feed utilization of Nile tilapia juveniles (*Oreochromis niloticus*). Aquaculture Research *43*, 1344–1356.

Kennedy, P.A., Meyer, K.A., Campbell, M.R., Vu, N.V., and Schill, D.J. (2018). Survival and reproductive success of hatchery YY male brook trout stocked in Idaho streams. Transactions of the American Fisheries Society *147*, 419–430.

Kolar C.S., Chapman, D.C., Courtenay, J.W.R., Housel, C.M., Williams, J.D., and Jennings, D.P. (2005). Asian carps of the Genus *Hypophthalmichthys* (Pisces, Cyprinidae) — A biological synopsis and environmental risk assessment. U.S. Fish and Wildlife Service *report 94400-3-0128*, pp. 175.

Kolar C.S., Chapman, D.C., Courtenay, J.W.R., Housel, C.M., Williams, J.D., and Jennings, D.P. (2007). Bigheaded carps: a biological synopsis and environmental risk assessment. American Fisheries Society, Bethesda, Special Publication *33*.

Krakowiak, P.J., and Pennuto, C.M. (2008). Fish and macroinvertebrate communities in tributary streams of eastern Lake Erie with and without round gobies (*Neogobius melanostomus*, Pallas 1814). Journal of Great Lakes Research *34*, 675–689.

Li, S., and Fang, F. (1990). On the geographical distribution of the four kinds of pond-cultured carps in China. Acta Zoologica Sinica *36*, 244–255.

Lieder, U., and Helms, C. (1981). Erfahrungen beim Vorstrecken von Brut pflanzenfressender Cypriniden. Binnenfisch *28*, 3–8 (in German).

Mahfuj, M.S., Hossain, M.A., and Sarower, M.G. (2012). Effects of different feeds on larval development and survival of ornamental koi carp, *Cyprinus carpio* (Linnaeus, 1748) larvae in laboratory condition. J. Bangladesh Agril. Univ. *10*, 179–183.

Mair, G.C., Abucay, J.S., Abella, T.A., Beardmore, J.A., and Skibinski, D.O.F. (1997). Genetic manipulation of sex ratio for the large-scale production of all-male tilapia *Oreochromis niloticus*. Canadian Journal of Fisheries and Aquatic Sciences *54*, 396–404.

Marciak, Z., and Bogdan, E. (1979). Food requirements of juvenile stages of grass carp *Ctenopharyngodon Idella* Val., silver carp *Hypophthalmichthys molitrix* Val., and bullhead carp *Aristichthys nobilis* Rich. In Stycznska-Jurewicz, E., Backiel, T., Jaspers, E., and Persoone, G., (Eds.). Cultivation of fish fry and its live food, Prinses Elisabethlaan 69, Belgium, European Mariculture Society, special publication *No. 4*, 140–148.

Meronek, T. G., Bouchard, P. M., Buckner, E. R., Burri, T. M., Demmerly, K. K., Hatleli, D. C., Klumb, R. A., Schmidt, S. H., and Coble, D. W. (1996). A review of fish control projects. North American Journal of Fisheries Management *16*, 63–74.

Montgomery, D.C. (2005). Design and analysis of experiments. John Wiley & Sons, New York, USA, 6^{th} *edn.*, pp. 405-444.

Nicholls, K.H., and Hopkins, G.J. (1993). Recent changes in Lake Erie (north shore) phytoplankton: cumulative impacts of phosphorus loading reductions and the Zebra mussel introduction. Journal of Great Lakes Research *sparks*, 637–647.

Opuszyński, K., Myszkowski, L., Okoniewska, G., Opuszyński, W., Szlaminska, M., Wolnicki, J., and Wozniewski, M. (1989). Rearing of common carp, grass carp, silver carp, and bighead carp larvae using zooplankton and/or different dry feeds. Polskie Archiwum Hydrobiologii *36*, 217–230.

Pandian, T.J., and Sheela, S.G. (1995). Hormonal induction of sex reversal in fish. Aquaculture *138*, 1–22.

Parker, A.D., Glover, D.C., Finney, S.T., Rogers, P.B., Stewart, J.G., and Simmonds, R.L. (2015). Direct observations of fish incapacitation rates at a large electrical fish

barrier in the Chicago Sanitary and Ship Canal. Journal of Great Lakes Research *41*, 396–404.

Peacor, S.D., Bence, J.A., and Pfister, C.A. (2007). The effect of size-dependent growth and environmental factors on animal size variability. Theoretical Population Biology *71*, 80–94.

Rayner, T.S., and Creese, R.G. (2006). A review of rotenone use for the control of non-indigenous fish in Australian fresh waters, and an attempted eradication of the noxious fish, *Phalloceros caudimaculatus*. New Zealand Journal of Marine and Freshwater Research *40*, 477–486.

Rottmann, R.W., Shireman, J.V., and Lincoln, E.P. (1991). Comparison of three live foods and two dry diets for intensive culture of grass carp and bighead carp larvae. Aquaculture *96*, 269–280.

Santiago, C.B., Gonzal, A.C., Aralar, E.V., and Arcilla, R.P. (2004). Effect of stunting of juvenile bighead carp *Aristichthys nobilis* (Richardson) on compensatory growth and reproduction. Aquaculture Research *35*, 836–841.

Schill, D.J., Heindel, J.A., Campbell, M.R., Meyer, K.A., and Mamer, E.R. (2016). Production of a YY male Brook Trout broodstock for potential eradication of undesired Brook Trout populations. North American Journal of Aquaculture *78*, 72–83.

Schill, D.J., Meyer, K.A., and Hansen, M.J. (2017). Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. North American Journal of Fisheries Management *37*, 1054–1066.

Schofield, P.J., Williams, J.D., Nico, L.G., Fuller, P., and Thomas, M.R. (2005). Foreign non-indigenous carps and minnows (*Cyprinidae*) in the United States—A guide to their identification, distribution, and biology. Scientific Investigations Report 2005-5041, pp. 103.

Schrank, S.J., and Guy, C.S. (2002). Age, growth, and gonadal characteristics of adult bighead carp, *Hypophthalmichthys nobilis*, in the Lower Missouri River. Environmental Biology of Fishes *64*, 443–450.

Southwick Associates. (2007). Sportfishing in America: An Economic Engine and Conservation Powerhouse. American Sportfishing Association, Multistate Conservation Grant Program.

Sparks, R.E., Barkley, T.L, Creque, S.M., Dettmers, J.M., and Stainbrook, K.M. (2010). Evaluation of an electric fish dispersal barrier in the Chicago Sanitary and Ship Canal. In: Chapman, D.C. and Hoff, M.H., (Eds.). Invasive Asian carps in North America, American Fisheries Society, Bethesda, Maryland, Symposium *74*,139–161.

Szlaminska, M., and Przybyl, A. (1986). Feeding of carp (*Cyprinus carpio* L.) larvae with artificial dry food, living zooplankton and mixed food. Aquaculture *54*, 77–82.

Thresher, R.E., Hayes, K., Bax, N.J., Teem, J., Benfey, T.J., and Gould, F. (2014). Genetic Control of Invasive Fish: Technological options and its role in integrated pest management. Biological Invasions *16*, 1201–1216.

Tsehaye, I., Catalano, M., Sass, G., Glover, D., and Roth, B. (2013). Prospects for fishery-induced collapse of invasive Asian carp in the Illinois River. Fisheries *38*, 445–454.

Twohey, M.B., Heinrich, J.W., Seelye, J.G., Fredricks, K.T., Bergstedt, R. A., Kaye, C. A., Scholefield, R. J., McDonald, R. B., and Christie, G. C. (2003). The sterile-male-release technique in Great Lakes sea lamprey management. Journal of Great Lakes Research *29*, 410–423.

Wobbrock, J.O., Findlater, L., Gergle, D., and Higgins, J.J. (2011). The Aligned Rank Transform for nonparametric factorial analyses using only ANOVA procedures. In: Proceedings of the 2011 Annual Conference on Human Factors in Computing Systems – CHI '11, ACM Press, Vancouver, BC, pp. 143.

Wohlfarth, G.W. and Moav, R. (1972). The regression of weight gain on initial weight in carp. I. Methods and results. Aquaculture *1*, 7–28.

Van der Wind, J.J. (1979). Techniques of rearing phytophagous fishes. FAO Fisheries Report *44*, 227–232.

von Oertzen, J.A. (1985). Resistance and capacity adaptation of juvenile silver carp, *Hypophthalmichthys molitrix* (Val.), to temperature and salinity. Aquaculture *44*, 321–332.

Tables and figures

Table 3.1 The structure of experimental design in the larval experiments, showing the design matrix coded in standard order and the factor levels in original units.

	Coded standard order			Factor level in original units		
Run	**System**	**Feed Regime**	**Density**	**System**	**Feed Regime**	**Density (fish L^{-1})**
1	+	+	+	RAS	Artemia	50
2	+	+	0	RAS	Artemia	25
3	+	+	-	RAS	Artemia	12.5
4	+	-	+	RAS	Rotifer	50
5	+	-	0	RAS	Rotifer	25
6	+	-	-	RAS	Rotifer	12.5
7	-	+	+	Static	Artemia	50
8	-	+	0	Static	Artemia	25
9	-	+	-	Static	Artemia	12.5
10	-	-	+	Static	Rotifer	50
11	-	-	0	Static	Rotifer	25
12	-	-	-	Static	Rotifer	12.5

All runs were replicated (n=3).

Table 3.2 Significance and goodness of fit for each model utilizing the 2x2x3 factorial ANOVA.

	10 days		
	P-value	**R^2**	**Adjusted R^2**
Length	0.0005	0.71	0.58
Weight	<0.0001	0.82	0.74
Survival	<0.0001	0.92	0.89
Biomass	<0.0001	0.91	0.86
	20 days		
Large fish ($\hat{p}$ =)	0.0079	0.61	0.42
Survival	<0.0001	0.91	0.86
Biomass	<0.0001	0.82	0.74
Weighted mean weight	0.0004	0.71	0.58
Large			
Length	0.0002	0.72	0.59
Weight	<0.0001	0.74	0.62
Specific growth rate	<0.0001	0.89	0.84
Small			
Length	<0.0001	0.87	0.80
Weight	<0.0001	0.87	0.82
Specific growth rate	<0.0001	0.85	0.78

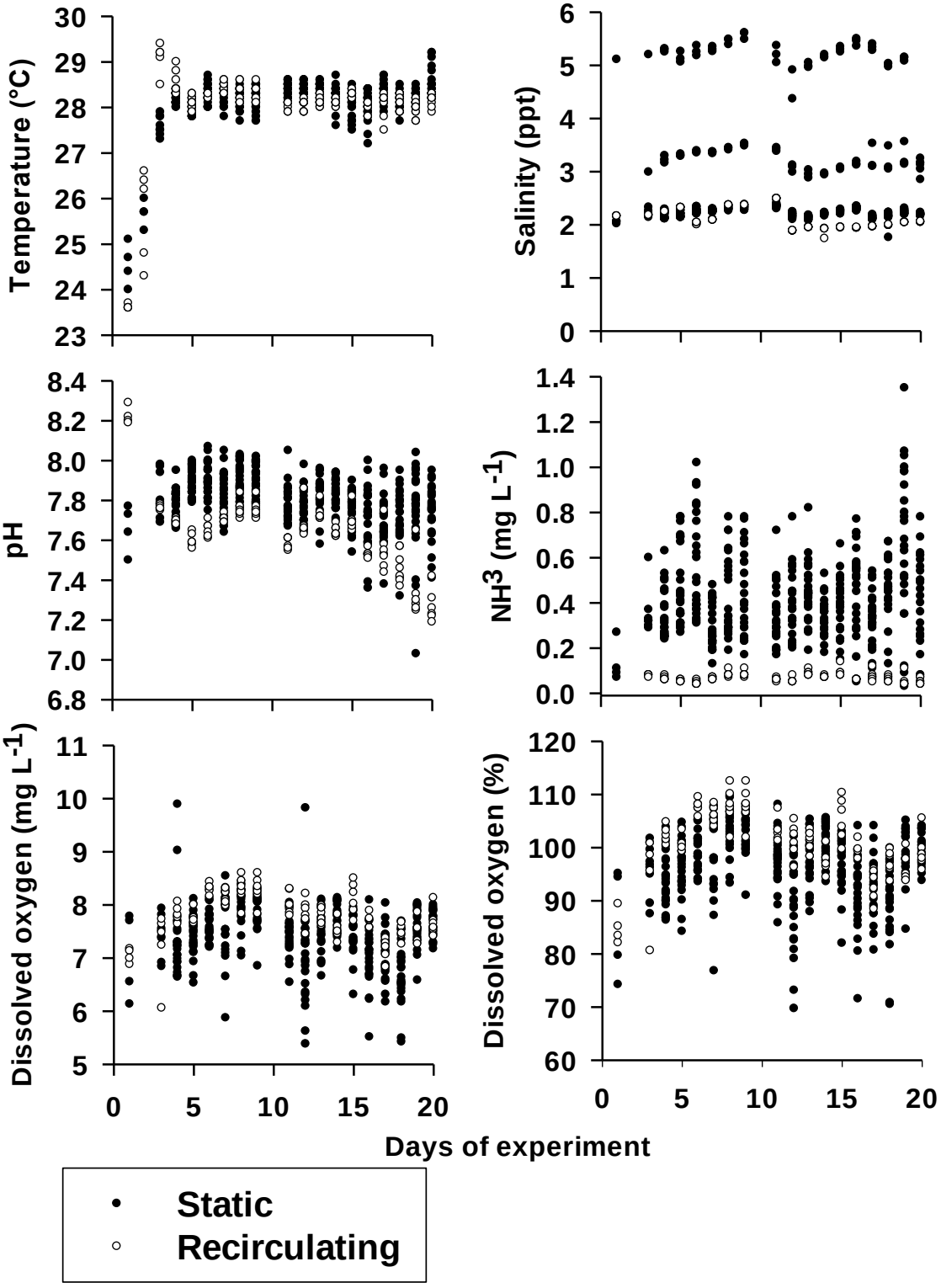

Figure 3.1 Water quality data for static and recirculating systems throughout the 20d experiment.

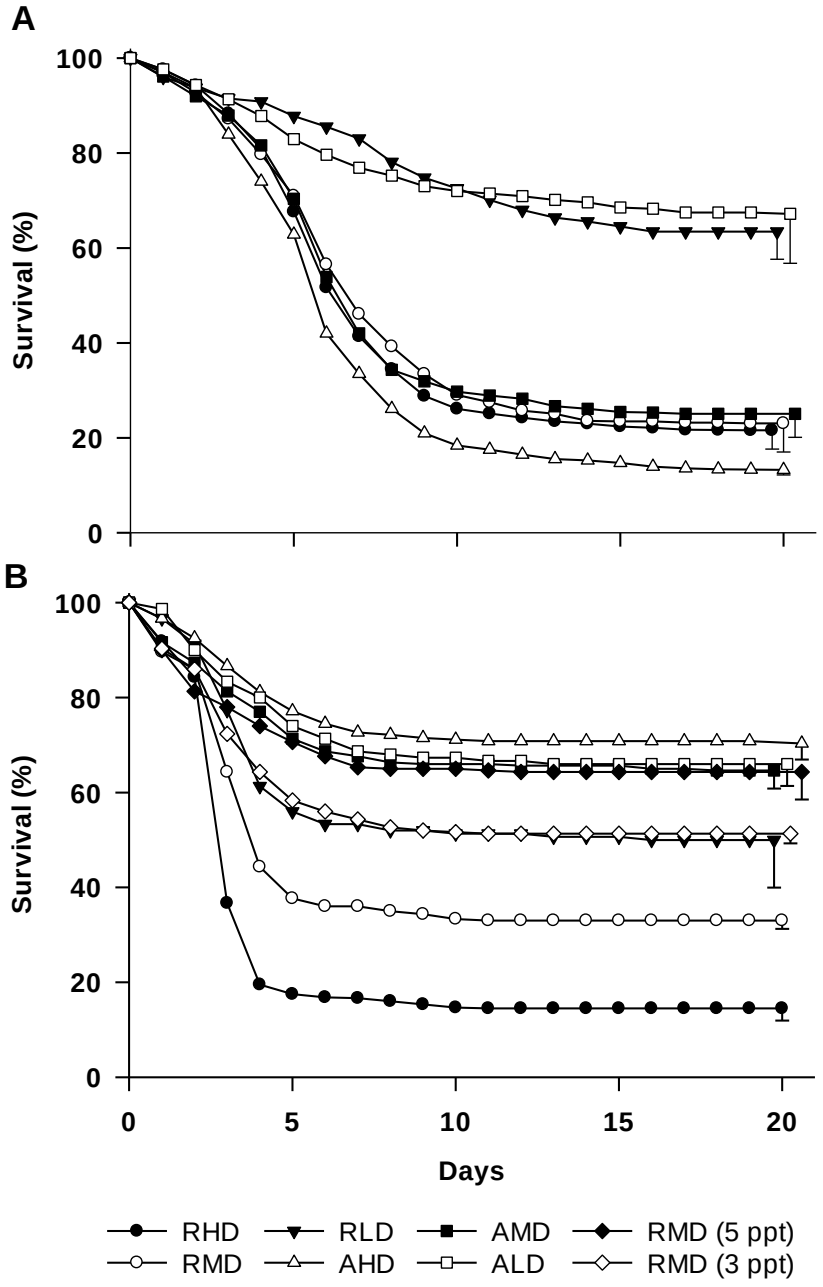

Figure 3.2 Mean survival recorded throughout the larval rearing optimization experiment and salinity investigation. A) survival in the RAS system, B) survival in the static rearing system. Standard error bars are given for the final measurements. (Rotifers at high density, RHD; rotifers at medium density, RMD; rotifers at low density, RLD; *Artemia* nauplii at high density, AHD; *Artemia* nauplii at medium density, AMD; *Artemia* nauplii at low density, ALD)

Table 3.3 Effects of initial stocking density, rearing system, and feed regime on growth and survival after 10d of the experiment. Post hoc least squared means Tukey's HSD was used to distinguish differences among groups when a significant three-way interaction of main effects was detected (P<0.05) by generating a connecting letters report. (*Artemia* nauplii, ART; rotifers, ROT)

Density (D)	**Low density (12.5 fish L^{-1})**				**Medium density (25 fish L^{-1})**				**High density (50 fish L^{-1})**				**Significance[a]**			**Interaction**			
System (S)	**Static**		**RAS**		**Static**		**RAS**		**Static**		**RAS**								
Feed (F)	**Art**	**Rot**	**Art**	**Rot**	**Art**	**Rot**	**Art**	**Rot**	**Art**	**Rot**	**Art**	**Rot**	**D**	**S**	**F**	**DxS**	**DxF**	**SxF**	**DxSxF**
Length (mm)	19.9 ± 0.7^{a}	18.3 ± 1.1^{ab}	17.2 ± 0.8^{abc}	17.1 ± 1.4^{abc}	17.8 ± 0.3^{ab}	18.4 ± 0.6^{ab}	17.6 ± 0.7^{ab}	16.5 ± 1.9^{bc}	16.6 ± 0.9^{bc}	17.9 ± 0.4^{ab}	16.6 ± 0.8^{bc}	14.6 ± 0.7^{c}	*	*	NS	NS	NS	NS	*
Weight (mg)	58.5 ± 6.0^{a}	50.7 ± 9.8^{abcd}	32.9 ± 3.5^{bcde}	31.9 ± 7.7^{bcde}	40.2 ± 1.4^{bcd}	48.4 ± 5.7^{ab}	34.5 ± 5.6^{bcde}	32.3 ± 13.2^{bcde}	30.2 ± 3.9^{cde}	45.8 ± 1.9^{abc}	27.2 ± 4.5^{de}	17.8 ± 2.9^{e}	*	*	NS	NS	NS	*	*
Survival (%)¥	66.0 ± 8.7^{a}	52.7 ± 18.1^{a}	71.5 ± 13.5^{a}	72.3 ± 6.1^{a}	66.3 ± 7.2^{a}	35.3 ± 4.5^{b}	29.7 ± 7.4^{bc}	28.5 ± 14.2^{bc}	71.7 ± 5.5^{a}	15.0 ± 4.8^{c}	18.4 ± 2.4^{bc}	26.1 ± 5.0^{bc}	*	*	*	*	NS	*	*
Biomass(gL^{-1})¥	0.32 ± 0.01^{bc}	0.22 ± 0.06^{c}	0.30 ± 0.07^{bc}	0.29 ± 0.08^{bc}	0.44 ± 0.05^{b}	0.29 ± 0.06^{bc}	0.25 ± 0.06^{c}	0.20 ± 0.02^{c}	0.72 ± 0.04^{a}	0.23 ± 0.08^{c}	0.25 ± 0.01^{c}	0.23 ± 0.05^{c}	*	*	*	*	*	*	*

¥ Nonparametric factorial analysis using aligned rank transform and ANOVA procedures (Wobbrock *et al.*, 2011)

[a] NS: not significant; *: $P < 0.05$

Table 3.4 Effects of initial stocking density, rearing system, and feed regime on growth and survival after 20d of the experiment. Fish were categorized as large or small within each treatment and the proportion of large fish and weighted mean weight were calculated. Post hoc least squared means Tukey's HSD was used to distinguish differences among groups when a significant three-way interaction of main effects was detected (P<0.05) by generating a connecting letters report. (*Artemia* nauplii, ART; rotifers, ROT)

Density (D)	**Low density (12.5 fish L^{-1})**				**Medium density (25 fish L^{-1})**				**High density (50 fish L^{-1})**				**Significance[a]**			**Interaction**			
System (S)	**Static**		**RAS**		**Static**		**RAS**		**Static**		**RAS**								
Feed (F)	**Art**	**Rot**	**Art**	**Rot**	**Art**	**Rot**	**Art**	**Rot**	**Art**	**Rot**	**Art**	**Rot**	**D**	**S**	**F**	**DxS**	**DxF**	**SxF**	**DxSxF**
Survival (%)[¥]	64.0±8.7^{a}	50.0±17.4^{ab}	67.2±18.0^{a}	63.5±10.1^{a}	64.7±6.7^{a}	33.0±3.0^{bc}	25.1±8.5^{cd}	23.1±10.4^{cd}	70.3±5.9^{a}	14.5±4.4^{cd}	13.3±1.9^{d}	21.6±6.8^{cd}	*	*	*	*	NS	*	*
Biomass (g L^{-1})	1.78±0.05	1.63±0.14	1.40±0.26	1.50±0.07	1.82±0.10	1.55±0.20	1.18±0.27	1.27±0.03	2.38±0.23	1.63±0.25	1.18±0.37	1.41±0.15	NS	*	NS	*	NS	*	NS
Large fish ($\hat{p}$ =)	0.82±0.05	0.62±0.08	0.76±0.02	0.73±0.08	0.82±0.04	0.63±0.03	0.67±0.08	0.68±0.04	0.77±0.03	0.74±0.09	0.67±0.06	0.74±0.06	NS	NS	*	NS	*	*	NS
Weighted mean weight (mg)	337±35^{a}	424±144^{ab}	173±40^{abc}	192±25^{abc}	169±9^{abc}	282±27^{bc}	214±116^{abc}	253±115^{abc}	102±5^{c}	348±51^{a}	185±74^{abc}	143±50^{bc}	*	*	*	NS	NS	*	*
Large																			
Length (mm)	36.5±0.9	41.7±4.7	31.2±2.5	32.3±2.7	29.7±0.8	37.4±2.0	33.6±5.5	35.1±4.6	26.0±0.6	37.6±2.4	31.9±4.0	29.1±3.9	*	*	*	*	NS	*	NS
Weight (mg)	394±24^{ab}	643±251^{a}	219±57^{abc}	258±57^{abc}	200±10^{abc}	419±34^{ab}	301±162^{abc}	350±146^{ab}	128±10^{c}	455±99^{a}	258±101^{abc}	185±67^{bc}	*	*	*	*	NS	*	*
SGR (% day^{-1})	26.4±0.3^{ab}	28.6±1.9^{a}	23.4±1.3^{d}	24.2±1.1^{cd}	23.0±0.3c^{b}	26.7±0.4^{ab}	24.6±2.5^{cd}	25.5±2.1^{cd}	20.8±0.4^{cd}	27.1±1.1^{ab}	24.0±2.3^{cd}	22.4±2.0^{cd}	NS	*	*	*	NS	*	*
Small																			
Length (mm)	22.4±0.9	23.0±1.2	16.3±0.7	16.5±0.6	16.1±0.5	19.3±1.3	16.7±0.4	17.5±2.8	14.4±0.2	19.7±0.7	14.8±2.6	15.7±2.0	*	*	*	*	NS	*	NS
Weight (mg)	79±9	84±11	28±5	27±5	25±2	49±12	28±6	36±15	16±3	62±12	24±12	31±11	*	*	*	*	*	*	NS
SGR (% day^{-1})	18.4±0.5	18.7±0.6	13.2±0.9	13.0±0.9	12.6±0.5	15.9±1.3	13.2±1.0	14.1±2.4	10.4±1.1	17.1±1.0	11.7±3.2	13.4±1.8	*	*	*	*	*	NS	NS

[¥] Nonparametric factorial analysis using aligned rank transform and ANOVA procedures (Wobbrock et al., 2011)

[a] NS: not significant; *: $P < 0.05$.

Table 3.5 Results obtained from the salinity trial conducted. The treatments were reared in the static system, at medium density (25 fish L^{-1}) and were initially fed rotifers (with a switch to *Artemia* nauplii on day 6 of feeding). Fish were categorized as being either small or large for samples taken at 20 days. Significance of one-way ANOVA is presented, and a connecting letters report was generated by Tukey's HSD post hoc tests to distinguish differences between treatments.

Salinity	2 ppt	3 ppt	5 ppt	ANOVA (p =)
		10 days		
Survival (%)	35.3 ± 4.5^{b}	54.3 ± 0.3^{a}	65.3 ± 10.1^{a}	0.0017
Biomass (g L^{-1})	0.29 ± 0.06^{b}	0.44 ± 0.02^{a}	0.49 ± 0.08^{a}	0.0036
Length (mm)	18.4 ± 0.6	18.2 ± 0.7	18.2 ± 0.4	0.9323
Weight (mg)	48.4 ± 5.7	48.6 ± 3.6	45.0 ± 1.7	0.9408
		20 days		
Survival (%)	33.0 ± 3.0^{b}	51.3 ± 3.5^{a}	64.3 ± 10.1^{a}	0.0006
Biomass (g L^{-1})	1.55 ± 0.20	1.50 ± 0.12	1.95 ± 0.26	0.0749
Large fish ($\hat{p}$ =)	0.63 ± 0.03^{b}	0.76 ± 0.08^{ab}	0.82 ± 0.02^{a}	
Weighted mean weight (mg)	282 ± 27^{a}	177 ± 26^{b}	182 ± 6^{b}	0.0020
Large				
Length (mm)	37.4 ± 2.0^{a}	30.9 ± 1.1^{b}	30.8 ± 0.2^{b}	0.0013
Weight (mg)	418.6 ± 33.6^{a}	226.3 ± 23.5^{b}	218.0 ± 2.2^{b}	<0.0001
SGR (% day^{-1})	26.7 ± 0.4^{a}	23.6 ± 0.5^{b}	23.5 ± 0.1^{b}	<0.0001
Small				
Length (mm)	19.3 ± 1.3^{a}	15.5 ± 0.5^{b}	15.1 ± 1.5^{b}	0.0084
Weight (mg)	49.2 ± 12.4^{a}	22.5 ± 5.0^{b}	21.0 ± 11.6^{b}	0.0253
SGR (% day^{-1})	15.9 ± 1.3^{a}	12.0 ± 1.1^{ab}	11.3 ± 2.7^{b}	0.0435

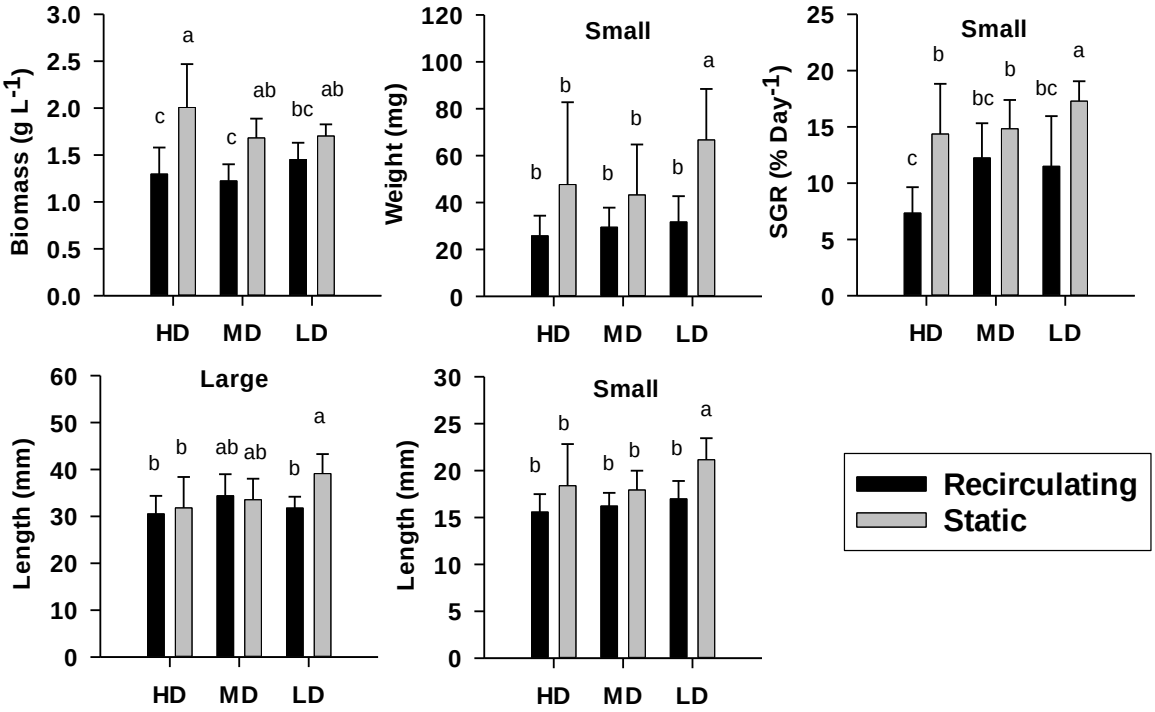

Figure 3.3 Significant interaction effects of stocking density and rearing system at 20 days on biomass, large category fish length, as well as small category fish length, SGR, and weight. A connecting letters report was generated by post hoc least squared means Tukey's HSD to visualize differences among groups (P<0.05).

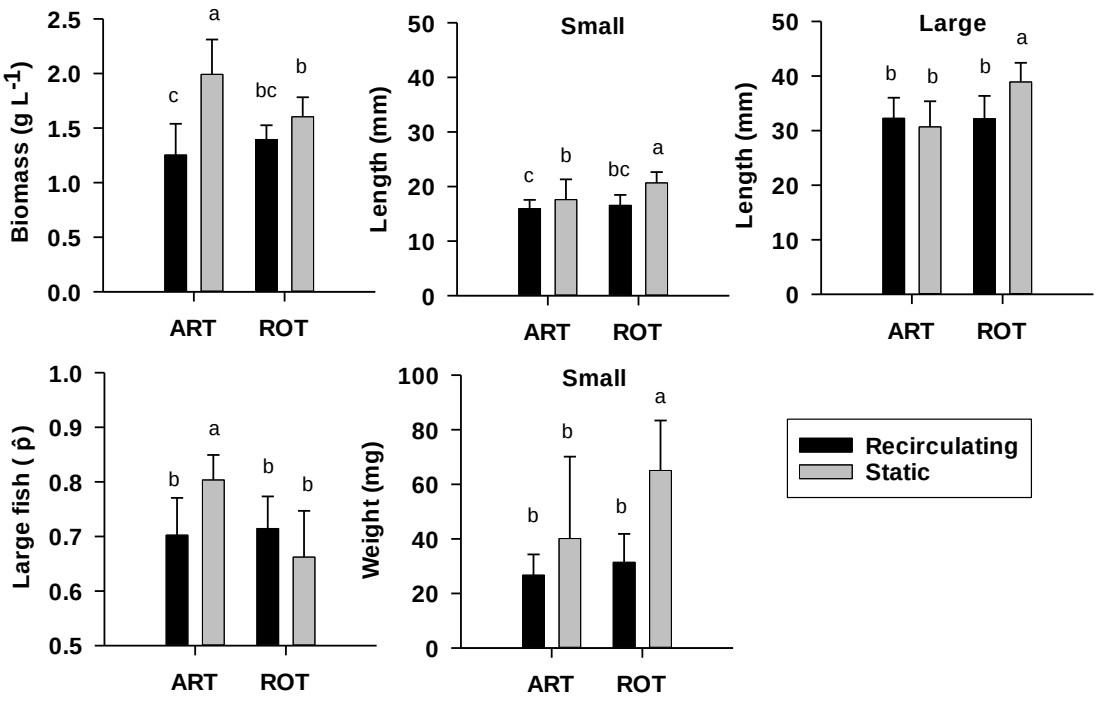

Figure 3.4 Significant interaction effects of rearing system and initial feed (*Artemia* nauplii, ART; rotifers, ROT) at 20 days on biomass, the proportion of large fish, large category fish length, and small category fish length and weight.

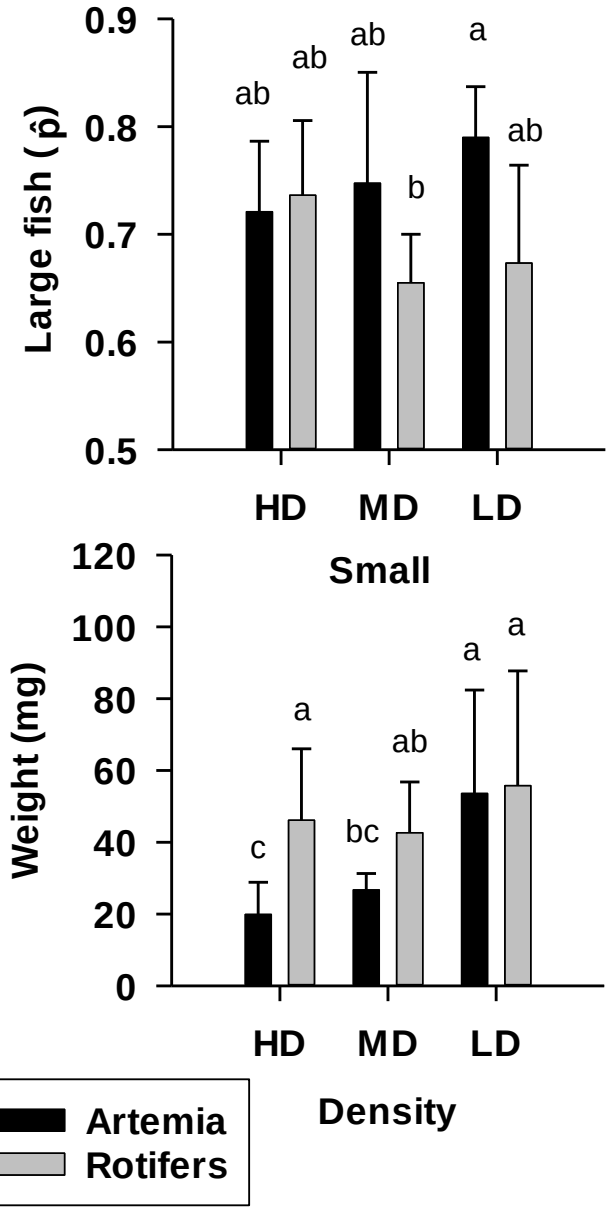

Figure 3.5 Significant interaction effect at 20 days of initial feed and stocking density (High density, HD; medium density, MD; low density, LD) on the proportion of large fish and small fish size weight. A connecting letters report was generated by post hoc least squared means Tukey's HSD to visualize differences among groups (P<0.05).

Chapter 4: Weaning of bighead carp and implications on the rate of observed deformities

Introduction

The use of live feeds as an initial diet for larval fish is currently essential for most cultured fish species (Dhont et al., 2013). The technology for the culture of these live feeds {primarily rotifers (freshwater and marine), *Artemia* nauplii (brine shrimp), and copepods} has improved drastically since they were initially used in hatchery production. However, their use in aquaculture is often both labor-intensive and costly, (Bakerville-Bridges & Kling, 2000; Callan et al., 2003; Dhont et al., 2013) making it important to transition to artificial diets as early as possible (Curnow et al., 2006). The successful replacement of live feeds with artificial diets tends to depend on the species, with most commercially grown freshwater fish having the capacity to utilize them as first feeds (Curnow et al., 2006; Sales, 2011; Vandecan et al., 2011). But a meta-analysis conducted by Sales (2011) across 27 cultured freshwater species found that fish initially fed an artificial diet were on average 2.5 times more likely to die. Fish only fed an artificial diet also experience slower growth than fish provided live feeds in their diet (Sales, 2011; Dhont et al., 2013). The transitioning of fish from live to dry diets, called weaning or feed training, is a critical stage of aquaculture production.

The goal of a larval weaning protocol is to gain the benefits of live feeds (increased larval survival and growth) but also allow the transition to a more reliable diet once fish have developed the ability to utilize artificial diets. A successful change to an artificial diet often requires a more developed digestive system that produces enzymes capable of metabolizing larger macromolecules (Jimenez-Martinez et al., 2012). Co-feeding is a common strategy used for feed training fish (Rosenlund et al., 1997, Dhont et al., 2013). During this period, both live feeds and artificial diets are simultaneously provided with a decreasing proportion of live feeds being offered over time (Rosenlund et al., 1997, Nhu et al., 2010; Dhont et al., 2013). This transition period can last anywhere from as short as 3 to over 18 days in duration and the artificial diet fed can significantly impact growth and/or survival depending on when weaning is initiated (Opuszyński et al. 1989; Rosenlund et al., 1997, Nhu et al., 2010,).

The culture of bighead carp *Hypophthalmichthys nobilis* often occurs without the use of additional food in ponds (FAO, 2018), but the use of artificial diets as first feeds have been investigated for intensive rearing of bighead carp. In general, these studies determined that live feeds performed superior to the artificial diets examined (Lieder and Helms, 1981; Dabrowski, 1984; Opuszyński et al., 1989; Rottmann et al., 1991; Carlos, 1988). It is suggested that bighead carp larvae be provided rotifers initially and switched to *Artemia* nauplii after they reach 9mm in length (Van der Wind, 1979). Since live foods are an important part of larval bighead carp rearing, several investigations have been made to determine an optimal weaning protocol (Opuszyński et al., 1989; Dabrowski, 1984; Marciek & Bogdan, 1979). Marciek & Bogdan (1979) found that transitioning to

either a mixed diet (natural and artificial feed) or artificial diet after 10 days of live feeds was successful but compromised growth slightly when compared to fish on a natural diet. Opuszyński et al. (1989) confirmed this finding, but also determined that weaning could begin as early as on day seven of feeding without negatively impacting survival and growth. However, Brant & Matty (1981) suggest that the initiation of weaning should consider the weight of fish, rather than age, to better understand when it is appropriate to switch to an artificial diet. While both studies identified when weaning could occur, they did not investigate whether these different methods affected the rate deformities.

Deformities involving the lateral line and spinal truncation have been recorded for bighead carp in both wild and aquaculture settings (Subba, 2004; Jawad & Kousha, 2011). But the cause of these observed deformities was not determined. Both larval diet and rearing conditions can impact the presence of deformities (spinal, opercular, jaw, etc.) as fish grow to larger sizes (Roo et al., 2005). The inbreeding of fish in a hatchery also decreases the genetic heterozygosity of progeny and often results in increased rates of deformities (Garcia-Celdran et al., 2015). The presence of deformities in progenies can significantly impact the success of a breeding program (Georgakopoulou et al., 2010). Fish with abnormalities have a reduced potential for normal physiological development, such as decreased growth and higher mortality (Andrades et al., 1996; Karahan et al., 2013). While the causes of deformities in bighead carp are unknown, research on deformities in the related cyprinid common carp *Cyprinus carpio* has identified several possible sources (Satoh et al., 1983; Backiel et al., 1984; Geurden et al., 1997).

Satoh et al. (1983) found that the deletion of trace minerals (Zn, Mn, Cu, Co, Mg) in the diet for common carp depressed growth and increased the incidence of dwarfism and cataracts by 40-70%. The deletion of only Mn from the diet performed worse than the deletion of all trace minerals from the diet (Satoh et al., 1983). Geurden et al. (1997) tested the effects of polar lipid supplementation on purified soybean meal based diets. They observed that un-supplemented diets had significantly higher rates of deformity (14–24 % higher) than most of the 2% polar lipid supplemented diets (Geurden et al., 1997). Both the live and artificial diets provided to fish are required to contain the appropriate lipids and minerals for normal growth during larval and early juvenile development. Diets that are insufficient to meet the nutritional requirements of fish can result in increased abnormalities which become observable as the fish grow (Roo et al., 2005). Stocking density also may influence the rate of deformities, but this is generally attributed to insufficient nutrition from increased competition (Garcia et al., 2013). While the diet of fish is an important aspect that impacts the rate of deformities, the environmental or rearing conditions a fish experiences can also influence it.

A study conducted by Backiel et al. (1984) explored the potential for thermal effluent canals as a location for the cage culture of common carp. These cages were placed across and down a section of the canal in flowing water and stocked with different sizes of fish (Backiel et al., 1984). The locations of the cages in the canal were found to significantly influence the presence of skeletal deformities (Backiel et al., 1984). This was linked to water velocity, with cages that experienced higher water velocities having increased observed deformities (Backiel et al., 1984). The initial stocking size of the fish

was also important, with smaller fish (5 g) having higher rates than larger fish (20 g). The overall rate of spinal deformities (lordosis and scoliosis) recorded in the experiment were 20, 15, and 64 percent for normal, slightly, and severely deformed fish respectively (Backiel et al., 1984). Temperature during embryonic (Sfakianakis et al., 2006) and early ontogeny has also been shown to influence the presence of haemal deformities, but the specific response pattern was unclear in Gilthead seabream *Sparus aurata* (Georgakopoulou et al., 2010).

Deformities often do not become apparent until fish grow to larger sizes. Early life stage diet and rearing conditions are important to optimize for production (growth and survival) but the influence of these conditions on the rate of deformities should also be accounted for. In the current experiments, fish that underwent a larval optimization study were weaned to a commercial diet to investigate if initial rearing conditions influenced the rate of observed deformities. A second experiment was conducted to assess if utilizing different weaning diets influenced the deformities recorded.

Objectives

The objectives of the current experiments were to investigate how weaning bighead carp juveniles to an artificial diet affects growth, dry feed acceptance, and the rate of observed deformities. The first trial examined whether initial rearing or environmental conditions (stocking density and feeding regime) had any observable effect during grow out. In the second trial, the initial rearing conditions were held constant but three different

diets were provided to the fish to examine if nutrition was a limiting factor to the successful production of these fish.

Methods

Fish source, stocking, and general methods

In trial 1 bighead carp larvae (8 days post fertilization; 2.3 mg) were obtained from Osage Catfisheries, Inc. (Osage Beach, MO) in July 2018. The trial was conducted immediately following the completion of a larval rearing optimization study which examined 2 different feeding regimes (rotifers for 5 days switched to 15 days of *Artemia* nauplii or 20 days of *Artemia* nauplii) and three initial stocking densities (12.5, 25, and 50 fish L^{-1}) in triplicate. The fish were reared in 10-L tanks with individual aeration and 250µm outlet screens in a recirculating aquaculture system equipped with mechanical- and bio-filtration as well as UV sterilization. Water temperature was maintained at 28 ± 1°C throughout the trial. After the larval rearing experiment 30 fish were restocked to their original treatment tanks and trial 1 was conducted to examine if initial larval rearing conditions influenced weaning to an artificial diet. Impacts of weaning and the resulting growth, feed acceptance, and the rate of observed deformities for the artificial diet were measured for 78 days. The fish were co-fed *Artemia* nauplii and a commercial artificial diet, Otohime (OTO; Reed Mariculture, CA) B1, during the first 5 days of the experiment with OTO being fed 3 times per day throughout the trial at a rate of 5% biomass day^{-1}.

Spinal deformities were recorded during the final samples similar to Backiel et al. (1984) with some additional abnormalities recorded (jaw, opercular, eye). Deformities were classified into two categories, slightly deformed and severely deformed. Slightly deformed (SLD) fish were those considered slightly abnormal, either exhibiting very marginal spinal deformities which required close inspection, cataracts or missing eyes, opercular malformation, or minor jaw deformities. Fish categorized as severely deformed (SVD) were very abnormal having multiple slight deformities, cataracts in both or completely missing eyes, easily recognized spinal deformities, either severe curvature (scoliosis) or dorsal uplift of the caudal peduncle (lordosis), or had severe jaw deformities (fused lower jaw).

Trial 2 was conducted the following year from July to October 2019. Larvae of bighead carp were similarly obtained from Osage Catfisheries, Inc. (Osage Beach, MO). The initial larval rearing period (20 days) was standardized for all fish (2 days rotifers switched to 18 days of *Artemia n*auplii, stocked at 14 fish L^{-1}) in the experiment. After the larval rearing period, all surviving fish were combined and were randomly distributed (n= 38 per tank) to three different randomly assigned artificial diet treatments in triplicate. The rearing system utilized in trial 2 was the same as was described for trial 1 with temperature maintained at 28 ± 1.5°C throughout the trial.

The trial was conducted to examine if the weaning diet used for bighead carp significantly influenced the rate of deformities observed as fish grow to larger sizes. The trial assessed the impact of each diet on growth, feed acceptance and the rate of observed

deformities for 63 days. The fish were co-fed *Artemia* nauplii and an artificial diet during the first 3 days of the experiment with each artificial diet being fed 3 times per day throughout the trial. Artificial feeds were offered at an initial rate of 10% biomass day^{-1} and this was gradually reduced to 4% biomass day^{-1} to prevent overfeeding from occurring. The pellet sizes provided for the formulated artificial diets were size-matched as close as possible to the commercial diet that was used in the experiment.

Two formulated artificial diets, a spirulina powder (SPN) or soybean meal (SBM) with squid meal based diets (**Table 4.3**), were examined in the experiment. Spirulina was chosen as a main diet ingredient because substitution of 32% of the protein in the diet with green algae (*Scenedesmus* meal) for grass carp *Ctenopharyngodon idella* diets were found to increase growth and decrease the rate of deformities compared to higher substitution amounts and a commercial feed (Meske et al., 1978). Soybean meal was selected as the second main diet ingredient because it is a common ingredient found in commercial carp diets. Squid meal was selected as a fish meal replacement for the diets because it is a diet attractant for other fish (Li et al., 2019). The third diet in the trial was the same commercial artificial diet, Otohime, which was fed in trial 1 (**Table 4.2**).

Statistical analysis

Data collected in both trials was box cox transformed using the suggested optimal lambda and was tested for normality using Shapiro-Wilks test (P >0.05 was considered normal). A Brown-Forsythe's test ($\alpha = 0.05$) was conducted to examine if the equal

variance assumption was met for parametric statistical procedures. Specific growth rate (SGR; % day^{-1}) was as follows, $SGR = \frac{(\ln W_f - \ln W_i)}{days} * 100$, where W_i was the initial weight and W_f was the final weight measured with days being the number of days since previous samples were taken. The feed conversion ratio was calculated as, $FCR = \frac{amount\ fed}{biomass\ gain}$ for each period of the experiments. All values presented in the experiment are mean ± standard deviation and results were considered significant at $\alpha = 0.05$.

In both trials, comparisons between treatments for growth and deformity related assessments were conducted using one-way ANOVA procedures. Tukey's HSD post hoc tests were used to distinguish differences among treatments when main effects were detected, and a connecting letter report was generated. Proximate body composition and mineral analysis was conducted using a Welch's t-test for Trial 1. Minerals were examined using Aligned Rank Transformation non-parametric 2x3 factorial ANOVA procedures with the main effects of diet and presence of deformity following Wobbrock et al. (2011). Significant main effects are presented when a significant interaction effect was not found. If a significant effect was identified then a post hoc least squared means Tukey's HSD or Student's t-test was conducted for interaction and main effects, respectively, and a connecting letters report was generated to visualize differences between groups

Sampling

For Trial 1, ten fish, fasted for at least 24 hours, from each tank were anesthetized with 100 mg L^{-1} of MS-222 (Tricane mesylate, Sigma Aldrich) before total length (mm) was taken with a ruler and weight (mg) was measured with a top-loading balance. Fish were allowed to recover following measurements and were returned to their tanks. In Trial 2, the biomass of each tank was measured, and the total number of fish were counted to determine the mean weight. After each trial a visual investigation for the presence of deformities was conducted.

Mineral analysis in Trial 1 was conducted on (n= 4) normal and deformed (slight and severe) fish from each replicate of larvae initially fed *Artemia* nauplii stocked at 25 fish L^{-1}. This was expanded in Trial 2 to include (n=5) normal and deformed fish from each tank in the experiment. The processing fish for mineral analysis was the same for each of the trials. Briefly, samples were snap-frozen in liquid nitrogen (-196 °C), freeze-dried for 48-hours to determine water content, and the whole body of individual fish was ground into a powder. Samples were sent to the Ohio State University (OSU) Ohio Agriculture Research and Development Center's (OARDC) Service Testing and Research (STAR) lab to measure ash, nitrogen, and the major suite of elements by inductively coupled plasma mass spectrometry (ICP) after ashing and acid dissolution with standard methods (AOAC, 2002).

Results

Trial 1

Samples taken at the beginning of the experiment did not significantly differ in either weight or length (**Table 4.1**). All treatments readily accepted the commercial diet that was provided, with mean FCR values ranging from 0.71–0.90 and 0.70–1.18 for the first and second periods (**Table 4.1**). The survival of fish in the trial was also high and no significant differences were identified between treatments for either period (**Table 4.1)**. The mean length and weight were also similar between treatments throughout the trial (**Table 4.1**). The SGR was comparable between treatments for the first period but was significantly different in the second period with fish fed rotifers in high density being higher than fish fed *Artemia* in medium density ($p= 0.0039$). Proportions of deformities of each category recorded at the end of the trial were not significantly different (**Table 4.1**). The overall rates of deformities recorded for the experiment were 0.63 ± 0.15, 0.26 ± 0.12, and 0.10 ± 0.07 for normal, slight, and severe, respectively.

The proximate analysis of the normal and deformed fish from the trial found that there were no significant differences in moisture, nitrogen, and ash (**Table 4.2**). All the minerals examined in the trial were similarly found to not be significantly different for normal and deformed fish (**Table 4.2**).

The initial larval rearing conditions did not impact the growth, survival, mineral concentrations or the proportions of deformities measured in trial 1. But the overall rate

of deformities was quite high. These findings led us to question if the diet used in Trial 1 was potentially responsible for the relatively high rates of deformities recorded.

Trial 2

Survival in the experiment was relatively high for both the OTO and SPN treatments but SBM was significantly lower by day 14 of the trial (ANOVA, F = 16.30, df =2, 8, p = 0.0038; **Figure 4.1)**. SBM mortality continued to increase until it leveled out after 28 days of feeding (**Figure 4.1**). After Trial 2 the survival of OTO and SPN were significantly higher than SBM (ANOVA, F = 20.52, df =2, 8, p = 0.0021; **Figure 4.1**).

The initial mean weight of fish in the experiment for the randomly assigned diets were not significantly different (ANOVA, F = 1.0305, df =2, 8, p = 0.4124; **Figure 4.1**). But the mean weight of fish fed OTO was significantly more than the two formulated diets by day 14 of the experiment and maintained this increased growth throughout the entire trial. The final mean weight of OTO fed fish was almost 3 fold higher than both formulated diets (ANOVA, F = 30.72, df =2, 8, p = 0.0007; **Figure 4.1**). This increased growth is illustrated by the significantly higher SGR recorded for the OTO treatment during the first two sample periods (**Figure 4.2**). In general, the SGR of all treatments decreased over time (**Figure 4.2)**. An opposite trend became apparent in FCR, with all diet treatments increasing throughout the experiment (**Figure 4.2)**. The OTO treatment had significantly lower FCR throughout most of the trial than both formulated diets (**Figure 4.2)**. FCR of SPN was also significantly lower than SBM during the first 28 days

of the experiment but they became similar throughout the latter portion of the trial (**Figure 4.2)**.

The proportions of deformities recorded at the end of Trial 2 are presented in **Figure 4.3**. The normal categories of fish were significantly different between treatments with normal fish making up a larger proportion of fish in the OTO group compared to the formulated diets. The slight deformity category was also significantly different from the SBM diet having a larger proportion than the OTO diet. The severe deformity category was not significantly different between the diet treatments.

The combined effects of diet and deformity for the non-parametric factorial analysis are presented in **Table 4.4**. There were no significant interaction effects between the main effects identified in the analysis, but significant main effects for both deformity and diet were found and respective p-values for the effects are given in **Table 4.4**. Significant main effects of diet on the mineral concentrations are presented in **Figure 4.4**. In general, concentrations of minerals in fish fed both formulated diets had significantly higher K, Na, Zn, Fe, and Mn. The only exception was for Mn, which was only significantly different between SPN and OTO diet treatments. Significant main effects of the presence of deformity on mineral concentrations are shown in **Figure 4.5**, with the normal category of fish found to have significantly higher Ca and Mn than deformed fish.

Discussion

The initial larval rearing conditions of stocking density and feeding regime examined in trial 1 were not found to significantly influence survival, growth, and feed acceptance/utilization of bighead carp juveniles. These results indicate that OTO was an appropriate weaning diet for bighead carp at the adaptation weights investigated. While not directly comparable, the growth that fish achieved in this trial was higher (1.68-2.54 g) at 63 days of feeding than was achieved after 90 days (0.36-0.61 g) by Marciek & Bogdan (1979).

The adaptation age (20 days of feeding) and weights of fish examined in the trial ranged between 185-350 mg and no negative impacts on fish survival or growth were recorded. Adaptation weight has been suggested as being a superior metric to age for examining when the onset of weaning be successful (Bryant and Matty, 1981). The adaptation age and weights of the current trial were much higher than were previously found appropriate by Opuszyński et al. (1989) of 7 days, Maciek & Bogdan (1979) of 10 days and 10-20 mg and Dabrowski (1983) of 15 days and 5.4 mg. The current experiment successfully weaned bighead carp to artificial diets, but it was quite conservative in its investigation of adaptation weight. The mean weights of fish after 10 days of feeding in the previous larval rearing optimization trial were already as high or higher (18-59 mg) than all previous attempts. These results suggest that the initial live feeding period could potentially be cut down by at least half, greatly decreasing labor and the cost of utilizing live feeds. However, these previous studies did not investigate how early weaning impacted the presence of deformities and additional investigation needs to be conducted

to assess if initiation of weaning earlier impacts their formation since a relatively high overall rate of deformities were recorded in trial 1 (37 %).

Throughout both trials most of the severe and slightly deformed fish in the experiments had spinal deformities, but operculum deformities were also common. Spinal deformities in cultured fish can significantly impact not only the costs associated with production, but also their survival and growth (Berillis, 2015). Some marine hatcheries regularly experience rates of deformities from 7-20% in juveniles and occasionally up to 100% of the cultured fish are deformed (Gerorgakopoulou et al., 2010). The causes of skeletal deformities in fish are not well understood but have been linked to genetic, environmental, and nutritional factors (Dabrowski, 1982; Fernandez et al. 2008) with onset possibly arising during embryonic or early ontogeny (Berillis, 2015).

The rate of deformities observed in fish fed the Otohime diet were similar between both trial 1 and 2. Suggesting that, even though this diet had the best performance in the experiments, it may responsible for the elevated rate of deformities recorded. In the current experiments, no significant differences in the mineral concentrations were identified between normal and deformed fish fed Otohime. Therefore, it is likely that the mineral composition of this diet was appropriate, and the causes of deformities observed in this treatment were from another source. Other aspects of the diet may have been nutritionally deficient for bighead carp and impacted the rate of observed deformities, such as polar lipid content (Geurden et al. 1997), vitamin C deficiency (Sato et al., 1982; Madsen & Dalsgaard, 1999), vitamin A excess (Hilton, 1983; Dedi et al., 1997; Fernandez et al. 2008), or vitamin K deficiency (Udagawa, 2001;

Roy and Lall, 2007) which have all been linked to the formation of skeletal abnormalities. However, this is unclear as there is similarly the potential that an environmental, or even a genetic, factor that was unaccounted for in the experiment, such as temperature (Sato et al. 1982), may have been responsible.

The SPN diet was found to have high survival and diet acceptance throughout the experiment was better than SBM, indicating that spirulina powder may be a good protein source and diet attractant for bighead carp. Meske et al. (1979) found that the inclusion of *Scenedesmus* meal (32% of diet) for formulated grass carp diets increased growth and decreased the presence of deformities. Grass carp growth further improved linearly from 0 to 90 % inclusion of *Scenedesmus* but decreased at 100% (Meske et al., 1979). This same experiment with common carp resulted in increased growth up to 40% before plateauing and then significantly decreasing above 60% with high mortality recorded for >70% inclusion (Meske et al., 1979). Responses to algal inclusion in carp diets appear to be species-specific, but some inclusion in the diet is beneficial. The natural diet of bighead carp is broad and often includes algae when detritus or zooplankton are unavailable (Cremer and Smitherman, 1980) which may make this species uniquely adapted to utilizing algal protein sources.

Survival was significantly lower for the SBM treatment than the other diets even though growth comparable to SPN. The SBM treatment also consistently had the highest FCR and diet acceptance was poor throughout the experiment. Soybean meal has been successfully used as an alternative protein source for several carp species (Meske et al., 1979; Kom et al., 1997). The protein efficiency ratio soybean meal in carp is above 90%

in common carp (Pongmaneerat & Watanabe, 1993) but it also has antinutritional properties that can lead to decreased growth (Viola et al., 1982). Partial inclusion of fish meal (Viola et al., 1982; Kom et al., 1997) and supplementation of soy-based diets with lysine (Viola et al. 1982) or polar lipids (Geurden et al., 1997) can help to alleviate some of these negative impacts. Kom et al. (1997) found that there were no negative impacts on common carp growth when soybean meal inclusion in the diet was as high as 75% of the protein source. However, Wang et al. (2015) found that, even within carp species, the natural diet of the species influenced the successful inclusion of soy protein. These authors found that the herbivorous grass carp more efficiently utilized soybean meal compared to carnivorous black carp *Mylophargyngodon piceus* and omnivorous gibel carp *Carassius auratus gibelio* because this species is adapted to utilization of plant-based proteins (Wang et al., 2015). Bighead carp, which could be considered a detritivore or planktivore (Cremer & Smitherman, 1980), performed poorly on the SBM diet which only had 35% inclusion of plant-based proteins. The surviving fish in this treatment also had the highest rates of deformities recorded in the experiment.

The increased rate of deformities measured in the SPN and SBM diet treatments were likely a result of inappropriate dietary nutrition in both diets. The diet formulations, other than the experimental ingredient tested, were the same and resulted in similar rates of deformities recorded for each. These diets were supplemented with a vitamin mix and Vitamin C so deficiency-related deformities from Vitamin C (Sato et al., 1982; Madsen & Dalsgaard, 1999) and Vitamin K (Udagawa, 2001; Roy and Lall, 2007) are unlikely.

The mineral contents of the diets are a more likely source of the observed increase in deformities.

Proximate analysis of the diets revealed that the mineral profiles of the formulated diets and OTO were different. Ca in all diets were similar, but significantly lower concentrations were observed in deformed fish. Low Ca concentration is often correlated with skeletal deformities, as it is important in bone development (Muramoto, 1981), but actual Ca deficiency in fish is rare (Berillis, 2015). However, phosphorus imbalances in diets can result in skeletal deformities with deficiencies resulting in bone malformation in several fish species (Ogino et al., 1979; Watanabe et al., 1980; Roy and Lall, 2003) and excesses negatively impact bone mineralization (Berillis, 2015). Proximate analysis of the formulated diets found lower P concentrations than OTO but analysis of mineral concentrations of fish were not significantly different. Muramoto (1981) found that the ratio of Ca/P in the vertebrae of fish was an indicator of the presence of deformities in common carp, with lower ratios found in deformed fish as a response to Cd exposure. The ratios of Ca/P for normal and deformed fish in the current experiment were not found to be different, but variation was found for the ratios between the diets**.** Both formulated diets (SBM & SPN) resulted in elevated concentrations of K, Na, Zn, Fe, and Mn in fish which may have influenced the increased rate of deformities. Deformities in fish have not previously been associated with imbalances of K, Na, or Fe but both Zn (Bengtsson, 1974; Messaoudi et al., 2009; Zhu et al., 2011; Kessabi et al., 2013) and Mn (Satoh et al., 1983) are linked to skeletal malformation. Satoh et al. (1983) found that Mn deficient diets for common carp resulted in low growth and increased

prevalence of cataracts and dwarfism. No information could be found on whether Mn excess causes deformities in fish, even though Mn supplementation in diets has been previously studied (Shim & Lim 1990; Lorentzen et al. 1996). Based on the data collected the elevated concentration of Zn identified in fish fed the formulated diets is the most likely source of the increased rate of deformities found. The baseline level of Zn for this species is uncertain, but previous reports of bighead carp meal and wild fish have found that the concentration is around 128-137 μg g^{-1} (Leung et al., 2015; Bowzer et al., 2015). This range is similar to the Zn concentration that was identified in the OTO treatment (146-148 μg g^{-1}) with the formulated diets (289-377 μg g^{-1}) being 2-fold higher. While Zn is an essential mineral, previous investigation of another cyprinid, the common minnow *Phoxinus phoxinus*, by Bengtsson (1979) found that exposure of this species to Zn resulted in skeletal deformities. Interestingly the Zn concentrations of the formulated diets were found to be much lower than in the OTO diet even though elevated levels of Zn were identified in fish fed the formulated diets. This is likely a result of the difference in bioavailability of Zn between the commercial and formulated diets. However, more investigation is need to specifically identify if this is the mechanism responsible for increased accumulation of Zn measured in the experiment.

Conclusions

The initial artificial diet fed to bighead carp significantly influenced the rate of deformities, survival, and growth of fish, but the initial larval rearing conditions of density and larval diet did not. The current experiment only examined some of the

environmental conditions which have previously been identified as sources of deformities. Further investigation needs to be conducted to assess if the temperature (Sato et al., 1982), tank shape/size, or water velocity (Backiel et al., 1984) in the tanks may have influenced the rate of deformities.

No significant interaction effect between deformity and diet were found for minerals during trial 2. The formulated diets examined led to an increased rate of deformities compared to the commercial diet Otohime, potentially due to excess Zn accumulation which has been linked to increased deformities in wild populations (Bengtsson, 1974; Messaoudi et al., 2009; Kessabi et al., 2013) and laboratory investigations (Zhu et al., 2011). While spirulina showed some promise as a diet ingredient for bighead carp, more investigation needs to be conducted to assess how various inclusion rates influence its growth and whether algal protein sources in the diet improve the rate of deformities, as was found for grass carp (Meske et al. 1979). A more in-depth study also needs to be conducted to identify a suitable weaning diet for this species which maximizes their growth potential while not increasing the rate of deformities. Both are required to design a successful rearing program for bighead carp.

References

Andrades, J.A., Becerra, J., and Fernández-Llébrez, P. (1996). Skeletal deformities in larval, juvenile and adult stages of cultured gilthead sea bream (*Sparus aurata* L.). Aquaculture *141*, 1–11.

AOAC International (2002). Official Methods of Analysis of AOAC International. AOAC International, Gaithersburg, Md.

Backiel, T., Kokurewicz, B., and Ogozalek, A. (1984). High incidence of skeletal anomalies in carp, *Cyprinus carpio*, reared in cages in flowing water. Aquaculture *43*, 369–380.

Bakerville-Bridges, B. and Kling, L.J. (2000). Early weaning of Atlantic cod (*Gadus morhua*) larvae onto a microparticulate diet. Aquaculture *189*, 109–117.

Bengtsson, B.E. (1974). Vertebral damage to minnows *Phoxinus phoxinus* exposed to zinc. Oikos *25*, 134–139.

Berillis, P. (2015). Factors that can lead to the development of skeletal deformities in fishes: A review. Journal of Fisheries Sciences *9*, 17–23.

Bowzer, J., Trushenski, J., Rawles, S., Gaylord, T.G., & Barrows, F.T. (2015). Apparent digestibility of Asian carp- and common carp-derived fish meals in the feeds for hybrid striped bass *Morone saxatilis* (F) x *M. chrysops* (M) and rainbow trout *Oncorhynchus mykiss*. Aquaculture Nutrition *21*, 43–53.

Bryant, P.L., and Matty, A.J. (1981). Adaptation of carp (*Cyprinus carpio*) larvae to artificial diets: 1. Optimum feeding rate and adaptation age for a commercial diet. Aquaculture *23*, 275–286.

Callan, C., Jordaan, A., and Kling, L.J. (2003). Reducing *Artemia* use in the culture of Atlantic cod (*Gadus morhua*). Aquaculture *219*, 585–595.

Carlos, M.H. (1988). Growth and survival of bighead carp (*Aristichthys nobilis*) fry fed at different intake levels and feeding frequencies. Aquaculture *68*, 267–276.

Cremer, M.C., and Smitherman, R.O. (1980). Food habits and growth of silver and bighead carp in cages and ponds. Aquaculture *20*, 57–64.

Curnow, J., King, J., Partridge, G., and Kolkovski, S. (2006). Effects of two commercial microdiets on growth and survival of barramundi (*Lates calcarifer* Bloch) larvae within various early weaning protocols. Aquaculture Nutrition *12*, 247–255.

Dabrowski, K. (1982). Further study on dry diet formulation for common carp. Rivista Italiana di Piscicoltura e Ittiopatologio *XVII*, 11–29.

Dabrowski, K. (1984). Influence of initial weight during the change from live to compound feed on the survival and growth of four cyprinids. Aquaculture *40*, 27–40.

Dedi, J., Takeuchi, T., Seikai, T., Watanabe, T., and Hosoya, K. (1997). Hypervitaminosis A during vertebral morphogenesis in larval Japanese flounder. Fisheries Science *63*, 466–473.

Dhont, J., Dierckens, K., Strottrup, J.G., Van Stappen, G., Wille, M., and Sorgeloos, P. (2013). Rotifers, artemia and copepods as live feeds for fish larvae in aquaculture. In: Advances in aquaculture hatchery technology, Allan (eds.), Woodhead Publishing, Cambridge, 157–202.

FAO (Food and Agriculture Organization of the United Nations) (2018). The state of world fisheries and aquaculture 2018- Meeting the sustainable development goals. Rome, *CC BY-NC-SA 3.0 IGO*, pp. 1–210.

Fernández, I., Hontoria, F., Ortiz-Delgado, J.B., Kotzamanis, Y., Estévez, A., Zambonino-Infante, J.L., and Gisbert, E. (2008). Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*). Aquaculture *283*, 102-115.

Garcia, F., Romera, D.M., Gozi, K.S., Onaka, E.M., Fonseca, F.S., Schalch, S.H.C., Candeira, P.G., Guerra, L.O.M., Carmo, F.J., Carneiro, D.J., Martins, M.I.E.G., and Portella, M.C. (2013). Stocking density of Nile tilapia in cages placed in a hydroelectric reservoir. Aquaculture *410-411*, 51–56.

Garcia-Celdran, M., Ramis, G., Machado, M., Estevez, A., Afonso, J.M., Maria-Dolores, E., Penalver, J., and Armero, E. (2015). Estimates of heritability and genetic correlations of growth and external skeletal deformities at different ages in a reared gilthead sea bream (*Sparus aurata* L.) population sourced from three broodstocks along the Spanish coasts. Aquaculture *445*, 33–41.

Georgakopoulou, E., Katharios, P., Divanach, P., and Koumoundouros, G. (2010). Effect of temperature on the development of skeletal deformities in gilthead seabream (*Sparus aurata* Linnaeus, 1758). Aquaculture *308*, 13–19.

Geurden, I., Charlon, N., Marion, D., and Bergot, P. (1997). Influence of purified soybean phospholipids on early development of common carp. Aquaculture International *5*, 127–149.

Hilton, J.W. (1983). Hypervitaminosis A in rainbow trout (*Salmo gairdneri*) toxicity signs and maximum tolerable level. The Journal of Nutrition *113*, 1737–1747.

Jawad, L.A., and Kousha, A. (2011). A case of vertebral coalescences and lateral line deformity in *Hypophthalmichthys nobilis* (Richardson, 1844) obtained from aquaculture activity in Iran. Boll. Mus. Reg. Sci. nat. Torino *28*, 29–36.

Jimenez-Martinez, L. D., Alvarez-Gonza´lez, C. A., Tovar-Ramı´rez, D., Gaxiola, G., Sanchez-Zamora, A., Moyano, F.J., Alarco´n, F.J., Ma´rquez-Couturier, G., Gisbert, E., Contreras-Sa´nchez, W.M., Perales-Garcı´a, N., Arias-Rodrı´guez, L., Indy, J.R., Pa´ramo-Delgadillo, S., and Palomino-Albarra´n, I.G. (2012). Digestive enzyme activities during early ontogeny in common snook (*Centropomus undecimalis*). Fish Physiology and Biochemistry *38*, 441–454.

Karahan, B., Chatain, B., Chavanne, H., Vergnet, A., Bardon, A., Haffray, P., Dupont-Nivet, M., and Vandeputte, M. (2013). Heritabilities and correlations of deformities and growth related traits in the European sea bass (*Dicentrarchus labrax*, L) in four different sites. Aquaculture Research *44*, 289–299

Kessabi, K., Annabi, A., Hassine, A.I.H., Bazin, I., Mnif, W., Said, K., and Messaoudi, I. (2013). Possible chemical causes of skeletal deformities in natural populations of *Aphanius fasciatus* collected from the Tunisian coast. Chemosphere *90*, 2683–2689.

Kom, M.K., Ozkok, E., and Han, I.K. (1997). Effect of soybean meal and full-fat soybean for fish meal protein replacement on the growth performance of carp fingerlings. Korean Journal of Animal Nutrition and Feedstuffs *21*, 391–398.

Leung, H.M., Leung, A.O.W., Wang. H.S., Ma, K.K., Liang, Y., Ho, K.C., Cheung, K.C., Tohidi, F., and Yung, K.K.L. (2014). Assessment of heavy metals/metalloid (As, Pb, Cd, Ni, Zn, Cr, Cu, Mn) concentrations in edible fish species tissue in the Pearl River Delta (PRD) China. Marine Pollution Bulletin *78*, 235–245.

Li, L., Fang, J.G., Liang, X.F., Alam, M.S., Liu, L.W., and Yuan, X.C. (2019). Effect of feeding stimulants on growth performance, feed intake and appetite regulation of mandarin fish, *Siniperca chuatsi*. Aquaculture Research *50*, 3684–3691.

Lieder, U., and Helms, C. (1981). Erfahrungen beim Vorstrecken von Brut pflanzenfressender Cypriniden. Binnenfisch *28*, 3–8 (in German).

Lorentzen, M., Maage, A., and Julshamn, K. (1996). Manganese supplementation of a practical, fish meal based diet for Atlantic salmon parr. Aquaculture Nutrition *2*, 121–125.

Madsen, L., and Dalsgaard, I. (1999). Vertebral column deformities in farmed rainbow trout (Oncorhynchus mykiss). Aquaculture *171*, 41-48.

Marciak, Z., and Bogdan, E. (1979). Food requirements of juvenile stages of grass carp *Ctenopharyngodon Idella* Val., silver carp *Hypophthalmichthys molitrix* Val., and bullhead carp *Aristichthys nobilis* Rich. In Stycznska-Jurewicz, E., Backiel, T., Jaspers, E., and Persoone, G., (Eds.). Cultivation of fish fry and its live food, Prinses Elisabethlaan 69, Belgium, European Mariculture Society, special publication *No. 4*, 140–148.

Meske, C., Pfeffer, E., Ahrensburg, and Gottingen (1978). Growth experiments with carp and grass carp. Arch. Hydrobiol. Beih. Ergebn. Limnol. *11*, 98–107.

Messaoudi, I., Deli, T., Kessabi, K., Barhoumi, S., Kerkeni, A., and Saïd, K. (2009). Association of spinal deformities with heavy metal bioaccumulation in natural populations of grass goby, *Zosterisessor ophiocephalus* Pallas, 1811 from the Gulf of Gabès (Tunisia). Environmental Monitoring and Assessment *156*, 551–560

Muramoto, S. (1981). Vertebral column damage and decrease of calcium concentration in fish exposed experimentally to cadmium. Environmental Pollution (Series A) *24*, 125–133.

Nhu, V.C., Dierckens, K., Nguyen H.T., Hoang, T.M.T., Le, T.L., Tran, M.T., Nys, C., and Sorgeloos, P. (2010). Effect of early co-feeding and different weaning diets on the performance of cobia (*Rachycentron canadum*) larvae and juveniles. Aquaculture *305*, 52–58.

Opuszyński, K., Myszkowski, L., Okoniewska, G., Opuszyński, W., Szlaminska, M., Wolnicki, J., and Wozniewski, M. (1989). Rearing of common carp, grass carp, silver carp, and bighead carp larvae using zooplankton and/or different dry feeds. Polskie Archiwum Hydrobiologii *36*, 217–230.

Pongmaneerat, J., and Watanabe, T. (1993). Nutritional evaluation of soybean meal for rainbow trout and carp. Nippon Suisan Gakkaishi *59*, 157–163.

Roo, F.J., Hernández-Cruz, C.M., Fernández-Palacios, H., and Izquierdo, M.S. (2005). Development of skeletal deformities in gilthead sea bream (*Sparus aurata*) reared under different larval culture and dietary conditions. In: Larvi '05 – Fish & Shellfish Larviculture symposium. Hendry, C.I., Van tappen, G., Wille, M., and Sorgeloos, P. (Eds.), European Aquaculture Society, Special Publication *No. 36*, Oostende, Belgium.

Rosenlund, G., Stoss, J., and Talbot, C. (1997). Co-feeding marine fish larvae with inert and live diets. Aquaculture *155*, 183–191.

Rottmann, R.W., Shireman, J.V., and Lincoln, E.P. (1991). Comparison of three live foods and two dry diets for intensive culture of grass carp and bighead carp larvae. Aquaculture *96*, 269–280.

Roy, P.K., and Lall, S.P. (2003). Dietary phosphorus requirement of juvenile haddock (*Melanogrammus aeglefinus* L.). Aquaculture *221,* 451–468.
Roy, P.K., and Lall, S.P. (2007). Vitamin K deficiency inhibits mineralization and enhances deformity in vertebrae of haddock (*Melanogrammus aeglefinus* L.). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology *148*, 174-183.
Sales, J. (2011). First feeding of freshwater fish larvae with live feeds versus compound diets: a meta-analysis. Aquaculture International *19,* 1217–1228.
Sato, M., Kondo, T., Yoshinaka, R., and Ikeda, S. (1982). Effect of dietary ascorbic acid levels on collagen formation in rainbow trout, Bulletin of the Japanese Society of Scientific Fisheries *48*, 553–556.
Sato, M., Kondo, T., Yoshinaka, R., and Ikeda, S. (1983). Effect of water temperature on the skeletal deformity in ascorbic acid-deficient rainbow trout. Bulletin of the Japanese Society of Scientific Fisheries *49*, 443-446.
Satoh, S., Yamamoto, H., Takeuchi, T., and Watanabe, T. (1983). Effects of growth and mineral composition of carp of deletion of trace elements or magnesium from fish meal diet. Bulletin of the Japanese Society of Scientific Fisheries *49*, 431–435.
Sfakianakis, D.G., Georgakopoulou, E., Papadakis, I.E., Divanach, P., Kentouri, M., Koumoundouros, G., (2006). Environmental determinants of haemal lordosis in European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquaculture *254*, 54–64.
Sfakianakis, D.G., Renieri, E., Kentouri, M., and Tsatakis, A.M. (2015). Effect of heavy metals on fish larvae deformities: A review. Environmental Research *137*, 246–255.
Shim, K.F., and Lim, C.P. (1990). The effect of dietary level of manganese on red tilapia. Singapore Journal of Primary Industries *18*, 24–33.
Subba, B.R. (2004). Anomalies in bighead carp *Aristichthys nobilis* and African catfish *Clarias gariepinus* in Biratnagar, Nepal. Our Nature *2*, 41–44.
Udagawa, M. (2001). The effect of dietary vitamin K (phylloquinone and menadione) levels on the vertebral formation in mummichog *Fundulus heteroclitus*. Fisheries Science *67*, 104-109.
Van der Wind, J.J. (1979). Techniques of rearing phytophagous fishes. FAO Fisheries Report *44*, 227–232.
Vandecan, M., Diallo, A., and Melard, C. (2011). Effect of feeding regimes on growth and survival of *Clarias gariepinus* larvae: replacement of *Artemia* by a commercial feed. Aquaculture Research *42*, 733–736.
Viola, S., Mokady, S., Rappaport, U., and Arieli, Y. (1981/1982). Partial and complete replacement of fishmeal by soybean meal in feeds for intensive culture of carp. Aquaculture *26,* 223–236.
Wang, C., Zhu, X., Han, D., Jin, J., Yang, Y., and Xie, S. (2015). Responses to fishmeal and soybean meal-based diets by three kinds of larval carps of different food habits. Aquaculture Nutrition *21*, 552–568.
Wobbrock, J.O., Findlater, L., Gergle, D., and Higgins, J.J. (2011). The Aligned Rank Transform for nonparametric factorial analyses using only ANOVA procedures.

In: Proceedings of the 2011 Annual Conference on Human factors in Computing Systems – CHI ’11, ACM Press, Vancouver, BC, pp. 143.

Zhu, B., Wu, Z.F., Li, J., and Wang, G.X. (2011). Single and joint action toxicity of heavy metals on early developmental stages of Chinese rare minnow (*Gobiocypris rarus*). Ecotoxicology and Environmental Safety *74*, 2193–2202.

Tables and Figures

Table 4.1: Data obtained from Trial 1 of the experiment for each of the different initial stocking densities and feeding regimes (n= 10, 3 replicates). The specific growth rate (SGR) and feed conversion ratio (FCR) are presented for the period since previous samples were taken. Deformities were recorded after the experiment at 108 days post fertilization (dpf). Significance of one-way ANOVA ($P < 0.05$) is indicated by different letters generated by Tukey's HSD post hoc test to visualize differences between treatments. Values are mean ± standard deviation.

Initial rearing conditions	**Low density (12.5 fish L^{-1})**		**Medium density (25 fish L^{-1})**		**High density (50 fish L^{-1})**		**ANOVA (P =)**
	Artemia	**Rotifers**	**Artemia**	**Rotifers**	**Artemia**	**Rotifers**	
30 dpf (initial)							
Length (mm)	31 ± 2	32 ± 3	34 ± 6	35 ± 5	32 ± 4	29 ± 4	0.5745
Weight (mg)	219 ± 57	258 ± 57	301 ± 162	350 ± 146	258 ± 101	185 ± 67	0.5180
73 dpf							
Survival (%)	100 ± 0	100 ± 0	100 ± 0	97.5 ± 2.2	97.8 ± 3.8	100 ± 0	0.7978
Length (mm)	58 ± 5	58 ± 4	61 ± 7	62 ± 5	59 ± 5	56 ± 3	0.6735
Weight (g)	1.89 ± 0.5	1.95 ± 0.43	2.36 ± 1.09	2.54 ± 0.81	2.04 ± 0.53	1.68 ± 0.36	0.6447
SGR (% day^{-1})	5.0 ± 0.4	4.7 ± 1.0	4.8 ± 0.2	4.7 ± 0.3	4.9 ± 0.7	5.2 ± 0.4	0.8573
FCR	0.79 ± 0.13	0.90 ± 0.12	0.79 ± 0.06	0.87 ± 0.14	0.85 ± 0.23	0.71 ± 0.07	0.5435
108 dpf							
Survival (%)	94.4 ± 5.1	96.7 ± 3.3	86.7 ± 10.0	96.1 ± 4.2	91.1 ± 10.1	96.7 ± 3.3	0.6389
Length (mm)	74 ± 4	80 ± 4	76 ± 7	82 ± 7	73 ± 9	80 ± 4	0.4321
Weight (g)	4.38 ± 0.94	5.1 ± 0.77	4.65 ± 1.5	6.12 ± 1.85	4.37 ± 1.12	5.10 ± 0.77	0.5281
SGR (% day^{-1})	2.4 ± 0.2^{ab}	2.8 ± 0.3^{ab}	2.0 ± 0.4^{b}	2.5 ± 0.1^{ab}	2.2 ± 0.3^{b}	3.2 ± 0.4^{a}	0.0039
FCR	0.95 ± 0.13	0.71 ± 0.01	1.18 ± 0.23	0.77 ± 0.06	1.06 ± 0.34	0.7 ± 0.12	0.1241
Deformities ($\hat{p}$ =)							
Normal	0.71 ± 0.26	0.67 ± 0.07	0.58 ± 0.27	0.57 ± 0.06	0.64 ± 0.13	0.62 ± 0.03	0.8779
Slight	0.17 ± 0.11	0.24 ± 0.06	0.29 ± 0.22	0.32 ± 0.04	0.24 ± 0.13	0.32 ± 0.09	0.6789
Severe	0.12 ± 0.16	0.09 ± 0.02	0.13 ± 0.06	0.11 ± 0.03	0.12 ± 0.09	0.06 ± 0.07	0.8201

Table 4.2: Proximate body composition and mineral analysis for normal and deformed fish (n=4, 3 replicates) from the treatment initially fed *Artemia* nauplii and reared at medium density (25 fish $^{L-1}$) in Trial 1. The data was analyzed using Welch's t-test and the resulting p-value is presented. Values are mean ± standard deviation. WW %: percent of wet weight.

	Normal	**Deformed**	**Welch's t-test**
Weight (g)	5.67 ± 1.63	5.96 ± 1.87	0.8493
Moisture (% WW)	78.25 ± 1.03	77.64 ± 1.88	0.6531
Dry Matter (%)			
Nitrogen	8.13 ± 0.36	8.21 ± 0.29	0.7890
Ash	5.73 ± 0.46	5.50 ± 0.50	0.5904
Ca	2.74 ± 0.24	2.59 ± 0.25	0.5182
P	1.90 ± 0.14	1.80 ± 0.16	0.4408
K	0.97 ± 0.03	0.93 ± 0.06	0.3276
Na	0.52 ± 0.02	0.53 ± 0.04	0.7373
ug/g			
Mg	991 ± 62	944 ± 71	0.4331
Zn	168 ± 25	153 ± 18	0.4369
Fe	72.1 ± 4.4	74.8 ± 5.3	0.5417
Al	21.8 ± 7.1	22.4 ± 9.8	0.9270
Cu	6.5 ± 0.9	6.8 ± 0.8	0.7358
Mn	0.9 ± 0.1	0.9 ± 0.2	0.8707

Table 4.3: Formulations of the diets that were offered in Trial 2 of the experiment and proximate and mineral analysis of each. The proximate analysis provided by Reed Mariculture inc. (Campbell, CA) was used for Otohime diets fat content.

Ingredient (%)	Diet			
	Spirulina (SPN)	Soybean (SBM)	Otohime (OTO) B1	Otohime (OTO) B2
Squid meal	35	35	-	-
Spirulina powder	35	0	-	-
Soy protein concentrate	0	29	-	-
Squid oil	3.8	3.8	-	-
Soybean oil	0	1.7	-	-
Wheat meal	1	5.3	-	-
Dextrinized starch	9	9	-	-
CPSP	8	8	-	-
Mineral mix	4	4	-	-
Vitamin mix	4	4	-	-
Vitamin C	0.1	0.1	-	-
Choline chloride	0.1	0.1	-	-
Proximate analysis (%)				
Protein	58.9	58.1	62.1	62.7
Fat	8.04	8.06	> 8.0	> 8.0
Moisture	8.84	7.16	6.53	6.15
Ca	2.72	2.40	2.40	2.79
P	1.66	1.52	2.52	2.75
Na	1.23	0.48	1.05	1.18
K	1.57	1.63	1.21	1.28
Mg	0.25	0.26	0.33	0.44
µg/g				
Fe	942	737	549	539
Al	457	384	96	98
Mn	189	192	54	74
Cu	71	54	51	42
Zn	83	82	233	264

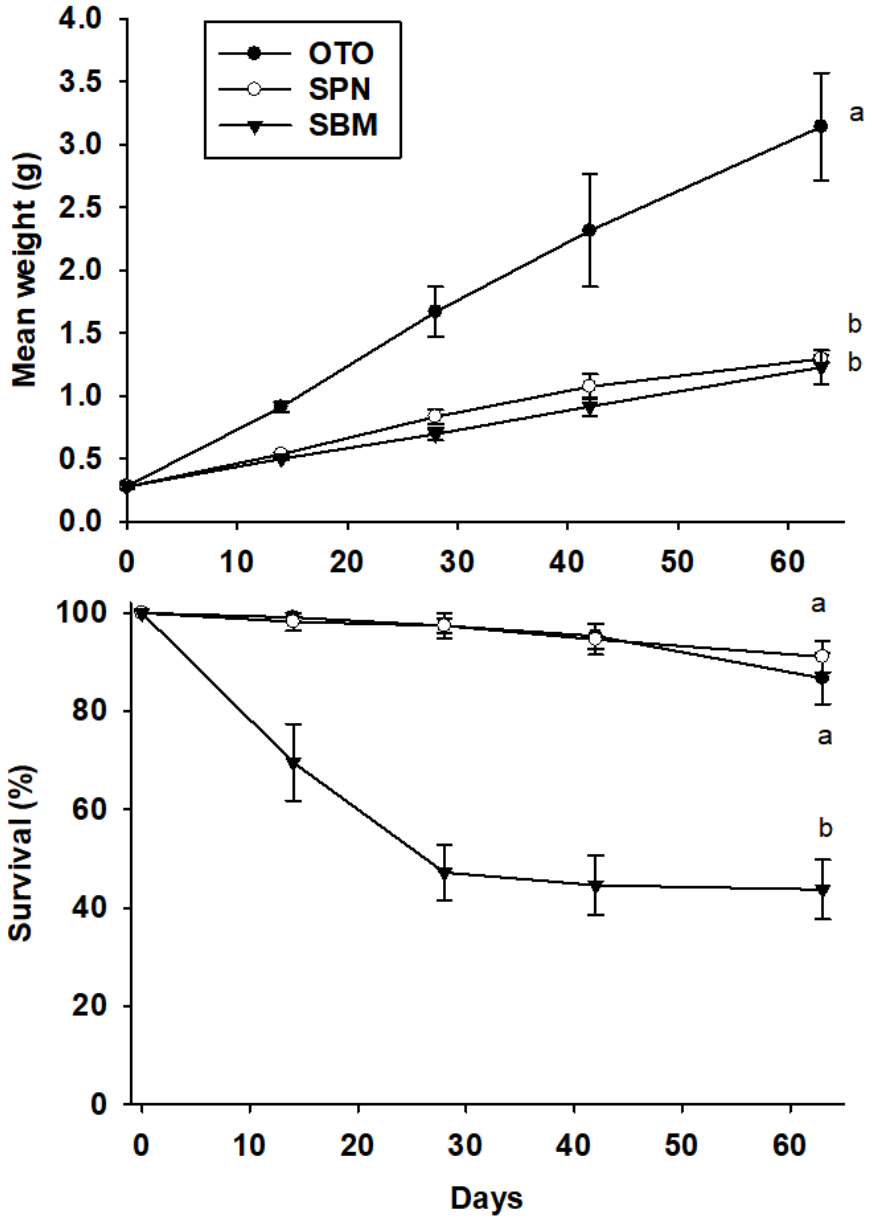

Figure 4.1: Mean weight and survival of bighead carp throughout Trial 2 are presented for the Otohime (OTO), Spirulina (SPN) and Soybean meal (SBM) diets that were examined from the start of weaning. Significance of one-way ANOVA ($P < 0.05$) for final samples is indicated by different letters generated by Tukey's HSD post hoc test. Values are mean ± standard deviation.

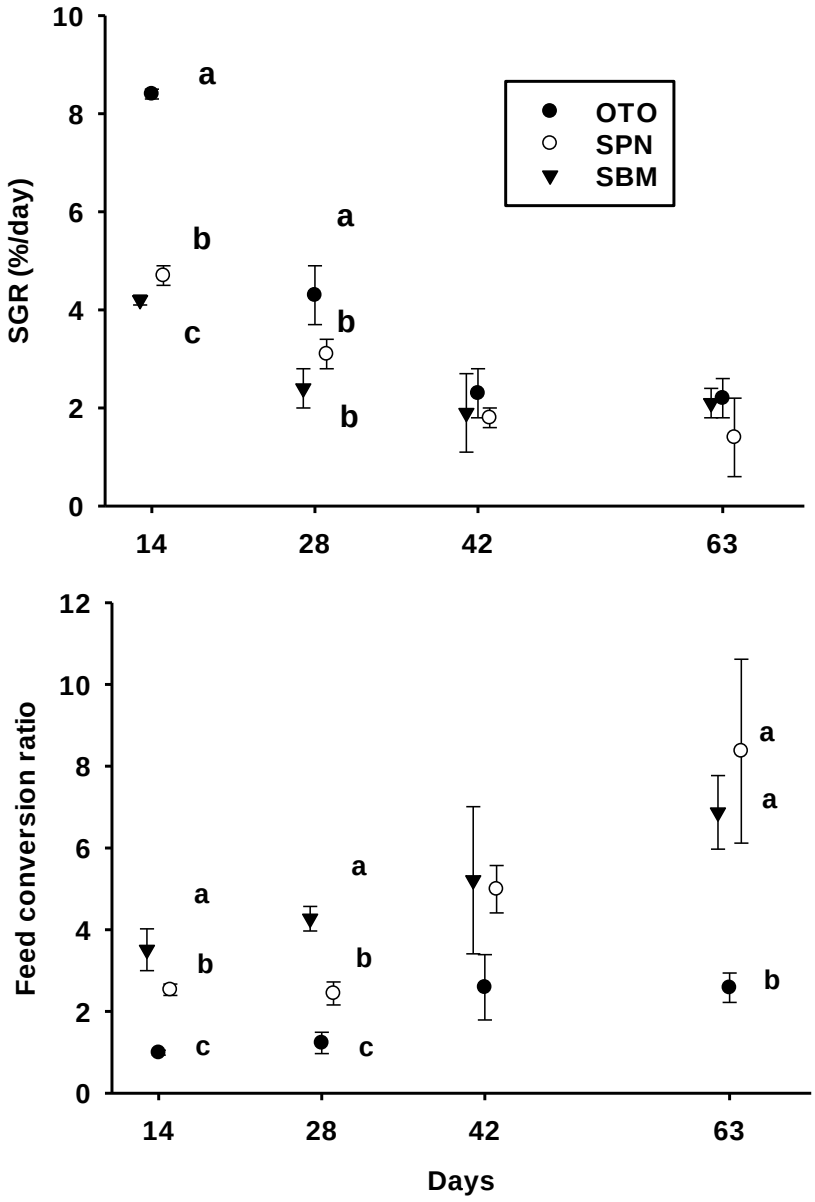

Figure 4.2: Specific growth rate (SGR) and feed conversion ratio of bighead carp throughout Trial 2 are presented for the Otohime (OTO), Spirulina (SPN) and Soybean meal (SBM) diets that were examined from the start of weaning. Significance of one-way ANOVA ($P < 0.05$) between treatments during each sample period is indicated by different letters generated by Tukey's HSD post hoc test. Values are mean ± standard deviation.

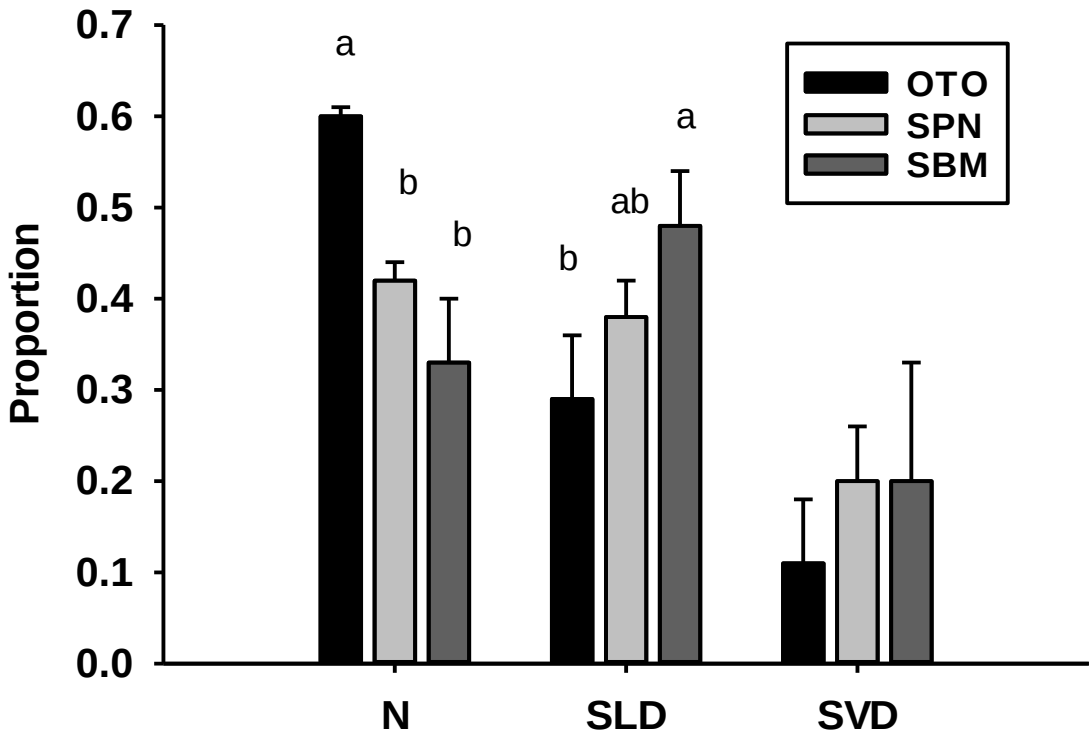

Figure 4.3: Proportions of normal (N), slight deformities (SLD) and severe deformities (SVD) bighead carp after Trial 2 are presented for the Otohime (OTO), Spirulina (SPN) and Soybean meal (SBM) diets. Significance of one-way ANOVA ($P < 0.05$) between treatments for each category is indicated by different letters generated by Tukey's HSD post hoc test. Values are mean ± standard deviation.

Table 4.4: Effects of deformity for fish fed Otohime (OTO), soybean meal (SBM), and spirulina (SPN) diets on mineral concentrations using Aligned Rank Sign non-parametric 2x3 full factorial analysis with ANOVA procedures. Differences between treatments for interaction effects are shown with a connecting letter report generated using the least significant means Tukey's HSD post hoc test. Values are mean ± standard deviation.

Deformity (D)	**Normal**			**Deformed**			**Significance (P =)**		**Interaction**
Diet (F)	**OTO**	**SPN**	**SBM**	**OTO**	**SPN**	**SBM**	**D**	**F**	**DxF**
Dry Matter (%)									
Ca	2.53 + 0.18	3.26 + 0.55	2.48 + 0.84	2.63 + 0.13	2.07 + 0.08	2.20 + 0.11	0.0403	0.4090	0.0579
P	1.70 + 0.09	2.20 + 0.39	1.96 + 0.76	1.77 + 0.04	1.55 + 0.09	1.70 + 0.08	0.1166	0.7930	0.2539
K	0.89 + 0.06	1.28 + 0.11	1.29 + 0.07	0.92 + 0.05	1.18 + 0.15	1.32 + 0.07	0.7319	<0.0001	0.4431
Na	0.31 + 0.02	0.62 + 0.08	0.66 + 0.01	0.33 + 0.02	0.63 + 0.13	0.73 + 0.07	0.3382	<0.0001	0.7423
µg/g									
Mg	967 + 37	1226 + 187	1150 + 299	978 + 37	965 + 73	1041 + 35	0.1146	0.2930	0.3228
Zn	148 + 19	377 + 51	366 + 94	146 + 14	289 + 19	365 + 27	0.1863	<0.0001	0.2241
Fe	69.9 + 15	98.5 + 7	92.8 + 5	72.8 + 6	89.6 + 10	98.4 + 13	0.9749	0.0021	0.4515
Cu	18.2 + 9.4	16.9 + 3.9	24.3 + 3.6	13.9 + 5.2	28.9 + 12.5	25.1 + 8.5	0.4514	0.1771	0.2263
Al	6.5 + 2.7	10.0 + 2.9	6.1 + 1.1	5.8 + 2.3	6.1 + 1.4	9.3 + 3.2	0.5248	0.4130	0.3069
Mn	1.3 + 0.3	3.8 + 1.3	2.4 + 0.2	1.2 + 0.3	2.1 + 0.3	2.0 + 0.3	0.0019	0.0210	0.0972

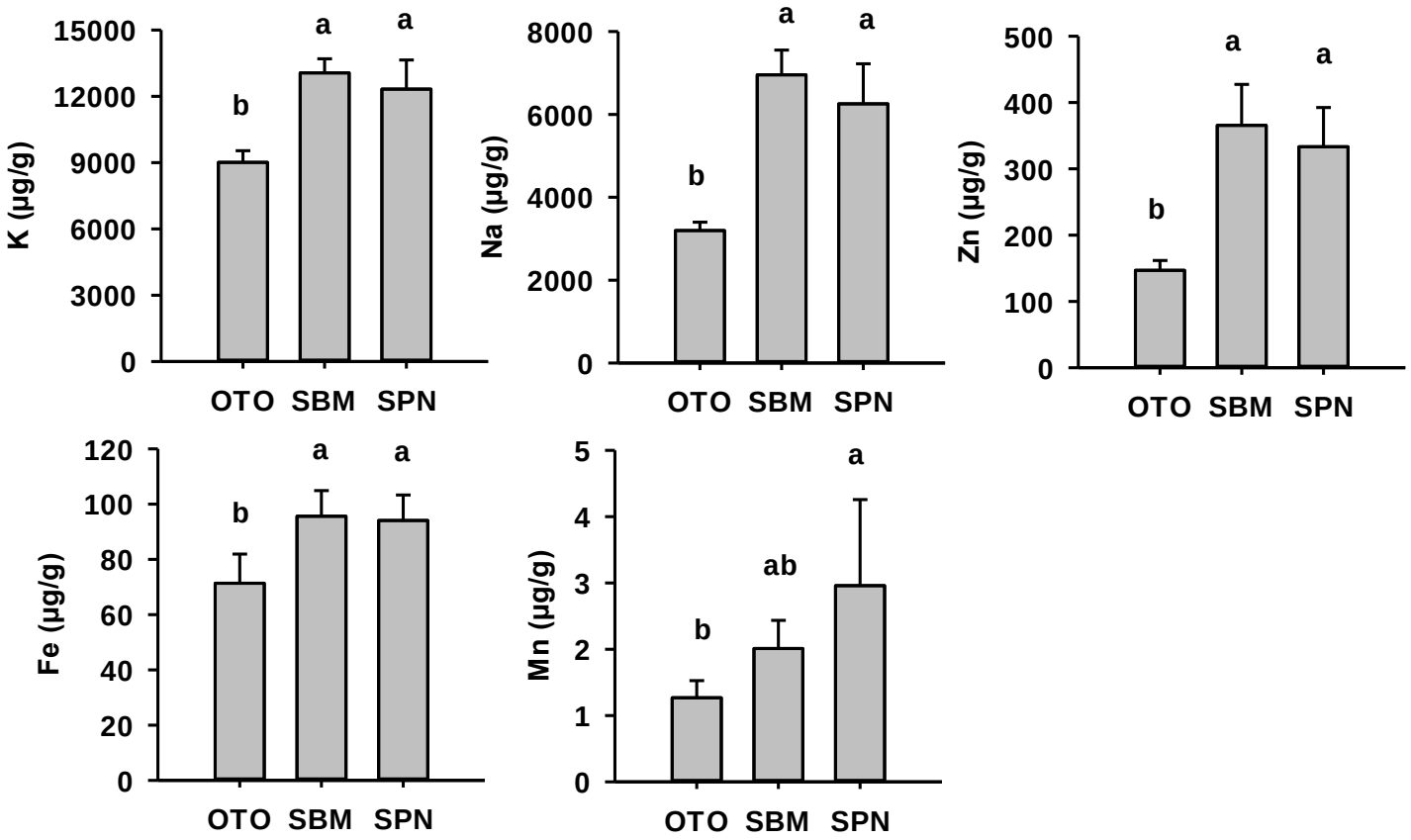

Figure 4.4: Significant main effect identified between mineral concentrations of fish fed Otohime (OTO), soybean meal (SBM), and spirulina (SPN) diets using Aligned Rank Sign non-parametric 2x3 full factorial analysis using ANOVA procedures. Differences between treatments were determined and a connecting letter report was generated using Tukey's HSD post hoc test. Values are mean ± standard deviation.

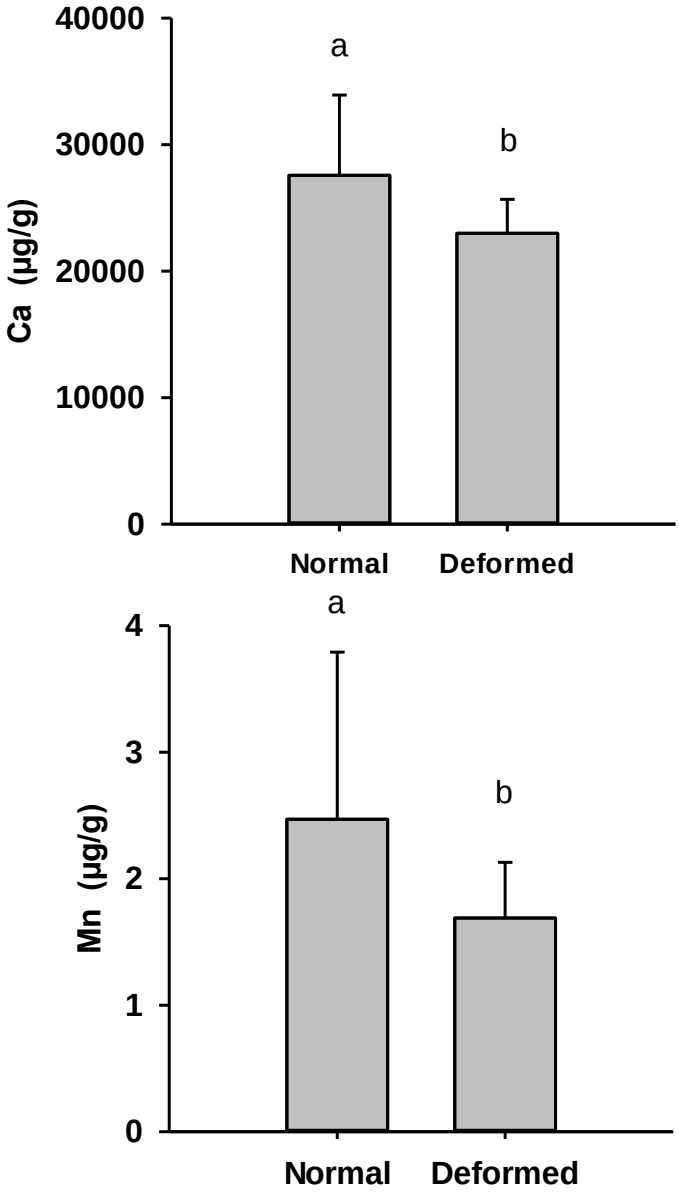

Figure 4.5: Significant main effects identified between mineral concentrations of normal and deformed category fish using Aligned Rank Sign non-parametric 2x3 full factorial analysis using ANOVA procedures. Differences between categories are were determined and a connecting letter report was generated using Tukey's HSD post hoc test. Values are mean ± standard deviation.

Chapter 5: Potential applications and future work

The development of breeding programs to produce fish for alternative methods of biocontrol of invasive species are currently in their preliminary stages. The theory for the practical application of the Trojan Y hypothesis (*Fyy*) and *Myy* release have been modeled by Gutierrez & Teem (2006), Thresher et al. (2014), and Schill et al. (2017) which show great promise. But at this time only one actual implementation of these methods has been accomplished for brook trout *Salvelinus fontinalis* (Kennedy et al., 2018). These authors have confirmed that stocked *Myy* brook trout can survive and successfully reproduced with wild stocks (Kennedy et al. 2018) but the overall impacts of this program on population control have not yet been reported. The release of sterile males was also accomplished for biocontrol of sea lamprey *Petromyzon marinus* and Klassen et al. (2004) predicted that a 1:1 release of sterile to fertile males could result in a 97% decrease in population after only 4 generations. However, due to the combined efforts of an integrated invasive control program involving the use of sterile males, lampricides, and habitat modification the impact of this biocontrol strategy could not be evaluated.

We recently proposed a novel method for biocontrol of Asian carp populations which involves the production and stocking of all-male populations of tetraploid bighead

carp. The potential impact of release with this novel biocontrol method has not yet been modeled, but in theory it has the potential to outperform previously implemented biocontrol methods involving sterile males and *Myy.* This is due to the compounding effect that would result from the offspring produced from wild diploid (2n) crossed with tetraploid (4n) being triploid (3n). These 3n offspring are physiologically sterile due to the production of aneuploid gametes (Gomelsky et al. 2015), essentially resulting in a two-tiered application of biocontrol. Triploid males of atlantic cod *Gadus morhua* (Feindel et al., 2010) and zebrafish *Danio rerio* (Fisher, Delomas & Dabrowski, unpublished) where both found to successfully reproduce and fertilize ova, but all surviving offspring died shortly after hatching. Future work needs to be conducted to assess what impacts might be expected at specific stocking levels utilizing this new biocontrol method. The series of investigations that were conducted in this thesis were designed to examine novel aspects involved in the implementation of this newly proposed form of biocontrol. However, the results from these experiments would also be applicable in implementing a *Fyy* or *Myy* breeding program for biocontrol.

The experiments with sperm storage were designed to alleviate issues that were experienced while conducting *in vitro* fertilization required for tetraploidy induction. Procurement of captive broodstock fish of bighead carp *Hypophthalmichthys nobilis* and silver carp *H. molitrix* to develop a breeding program is difficult, as only one bighead carp and none for silver carp were identified within the continental US. This is likely due to the listing of these species as injurious under the Lacey Act, which regulates both the transport and housing of these species in aquaculture facilities (FCC, 2011). While the

establishment of a new broodstock utilizing offspring from currently maintained bighead carp is feasible, this species reaches sexual maturity at >2 kg which can take from 3-8 years to occur (Kolar et al., 2005). Leaving the capture and spawning of wild fish to develop a breeding program as the quickest option. Unfortunately, we discovered that the stress involved in capturing these fish often led to asynchronous spawning in our laboratory environment. To alleviate issues identified with fresh storage of sperm, such as clumping and agglutination of sperm stored without dilution in a motility inhibiting solution (extender), we examined the effectiveness of common extenders. The dilution of silver carp sperm in Hank's Balanced Salt Solution 1:4 was effective in increasing the duration of acceptable motility by 2-3 days. This extended duration will considerably help to conduct *in vitro* fertilization with these species.

The experiments investigating sperm cryopreservation of Asian carp are not directly related to the creation of a breeding program for biocontrol but could be useful once it has been established. The cryopreservation of either neomale (*Myy*) or 4n Asian carp sperm would allow these stocks to preserved and potentially used as a genetic bank which could speed up the formation of new biocontrol programs throughout the country (Martinez-Paramo et al., 2017). When *Myy* sperm is used to fertilize XX female (*Fxx*) gametes the resulting progeny would be *Mxy*, effectively an all-male population. The all-male progeny could then undergo hormonal sex reversal with estrogen-like compounds to produce XY females (*Fxy*) (Pandian & Sheela, 1995). An advantage of using this method versus traditional applications of *Myy* production is that any females that are identified after sex reversal could be considered *Fxy*. This eliminates the step which requires

confirmation of genotype with sex-specific markers (Schill et al., 2016) since *Fxx* would not be produced by this cross. There is also the possibility of directly producing *Myy* through androgenesis (Pandian & Koteeswaran, 1998). Asian carp 4n sperm could also be used to establish a biocontrol program which is focused on stocking 3n. While 3n fish can be produced by applying a physical shock to embryos after fertilization to prevent the extrusion of the 2nd polar body, the production of 100 % 3n fish is not guaranteed and must undergo confirmation of ploidy before progenies should be stocked (Pandian & Koteeswaran, 1998). These fish also suffer from high rates of abnormalities due to the shocking process which produces them (Weber et al., 2014). Crossing a 2n female with a 4n male has significant advantages. The resulting progeny would all be 3n and could eliminate the need to examine large numbers of fish to confirm ploidy. Another advantage is that a physical shock applied to the embryos is not required and abnormalities or delays of development in offspring that can occur are minimized (Weber et al. 2014).

The results obtained from replicating original cryopreservation protocols in chapter 2 with koi carp sperm were successful, but the replication of these protocols with silver carp sperm is still ongoing. The results obtained from the fertility trial show that there is some promise for their application to Asian carp. This is due to the identification and confirmation of a zebrafish X silver carp hybrid larvae that survived to 52 hours post fertilization. Further investigation will be conducted which analyzes the post-thaw motility of the cryopreserved sperm. Another fertilization trial should also be conducted

with the remaining sperm to better assess the actual fertilization rates of the sperm, since the fertilization rate measured in the unfertilized control was abnormally high.

The experiments in chapter 3 were designed to optimize larval production of bighead carp for intensive rearing, but also to examine the effectiveness of the novel static rearing system designed by our lab. The static system utilized in the experiment was developed to improve the growth and survival of sensitive larvae which had undergone genetic manipulation techniques. The system was initially tested with zebrafish gynogens (Delomas & Dabrowski, 2016), or fish with only maternally inherited DNA, and found that this rearing system was well suited. We have also previously examined the effectiveness of this system with cichlids and percids to varying degrees of success. The results obtained for the experiments revealed that this system was well suited to rearing bighead carp larvae, as the fish in the novel static system, fed *Artemia* nauplii, at 50 fish L^{-1} were found to have the highest desirability (0.733) of treatments tested. As was explained earlier, fish that undergo genetic manipulation techniques, such as tetraploid or triploid induction, tend to suffer from abnormalities and lower growth (Weber et al., 2014). Utilizing the novel static rearing system described could help to increase the survival and growth of these fish. Potentially increasing the chances to successfully develop the first generation of bighead carp tetraploids. The specific impacts that this rearing system had on rearing triploid or tetraploid bighead carp were unable to be evaluated due to issues related to the production of these progenies by our lab. Future work needs to be conducted with triploid and tetraploid fish to determine if this novel static rearing system can improve the rearing of these progenies. Another benefit of

utilizing the static rearing system is that there is no chance for escapement of produced fish to natural waters and it is compliant with housing these injurious species under the Lacey Act, which requires double containment measures (FCC, 2011).

The experiment in chapter 4 is also relevant to the implementation of the proposed biocontrol method. Even under normal commercial aquaculture conditions deformities can compromise the effectiveness of a breeding program (Gerorgakopoulou et al., 2010). Fish produced using genetic manipulation techniques already suffer from increased rates of deformities. The experiment was designed to evaluate if currently establish methods for rearing bighead carp in our lab may further compound this issue and then examine if the newly developed larval rearing protocols from chapter 3 could also have influenced the presence of deformities. We found that the commercial diet that is regularly used by our lab consistently resulted in rates of deformity being high (37 %) and there was no observable effect of larval stocking density or initial live feeding regime. The artificial diets produced in the second trial were aimed at attempting to reduce the prevalence of deformities. We were unsuccessful in this attempt, but we did confirm that the diet could significantly impact the rate of deformities recorded. Further investigation is required to determine if the baseline rate of deformities for fish found was due to the use of the commercial diet (Satoh et al., 1983) or were caused by an environmental factor, such as temperature (Sfakianakis et al., 2006; Gerorgakopoulou et al., 2010) or water velocity (Backiel et al., 1984)

Other investigations by our lab are currently underway to develop protocols to produce monosex and tetraploid bighead carp progenies. Once these endeavors are

completed the experiments presented here will help to provide information that is relevant to the initiation of a breeding program for biocontrol of Asian carps.

References

Backiel, T., Kokurewicz, B., and Ogozalek, A. (1984). High incidence of skeletal anomalies in carp, *Cyprinus carpio*, reared in cages in flowing water. Aquaculture *43*, 369–380.

Delomas, T.A., and Dabrowski, K. (2016). Zebrafish embryonic development is induced by carp sperm. Biology Letters *12*, 1–4.

FCC, (Federal Communications Commission) (2011). Rules and regulations. Federal Register *76*, 15857–15858.

Feindel, N.J., Benfey, T.J., and Trippel, E.A. (2010). Competitive spawning success and fertility of triploid male Atlantic cod *Gadus morhua*. Aquaculture Environment Interactions *1*, 47–55.

Georgakopoulou, E., Katharios, P., Divanach, P., and Koumoundouros, G. (2010). Effect of temperature on the development of skeletal deformities in gilthead seabream (*Sparus aurata* Linnaeus, 1758). Aquaculture *308*, 13–19.

Gomelsky, B., Schneider, K.J., Anil1, A., and Delomas, T.A. (2015). Gonad development in triploid ornamental koi carp and results of crossing triploid females with diploid males. North American Journal of Aquaculture *77*, 96–101.

Gutierrez, J. B., and Teem, J. L. (2006). A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y Chromosome to cause extinction of an introduced exotic species. Journal of Theoretical Biology *241*, 333–341.

Kennedy, P.A., Meyer, K.A., Campbell, M.R., Vu, N.V., and Schill, D.J. (2018). Survival and reproductive success of hatchery YY male brook trout stocked in Idaho streams. Transactions of the American Fisheries Society *147*, 419–430.

Klassen, W., Adams, J.V., and Twohey, M.B. (2004). Modeling the suppression of sea lamprey populations by the release of sterile males or sterile females. Journal of Great Lakes Research *30*, 463–473.

Kolar C.S., Chapman, D.C., Courtenay, J.W.R., Housel, C.M., Williams, J.D., and Jennings, D.P. (2005). Asian carps of the Genus *Hypophthalmichthys* (Pisces, Cyprinidae) — A Biological synopsis and environmental risk assessment. U.S. Fish and Wildlife Service *report 94400-3-0128*, pp. 175.

Martinez-Paramo, S., Horvath, A., Labbe, C., Zhang, T., Robles, V., Herraez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R., and Cabrita, E. (2017). Cryobanking of aquatic species. Aquaculture *472*, 156–177.

Pandian, T.J., and Koteeswaran, R. (1998). Ploidy induction and sex control in fish. Hydrobiologia *384*, 167–243.

Schill, D.J., Heindel, J.A., Campbell, M.R., Meyer, K.A., and Mamer, E.R. (2016). Production of a YY male Brook Trout broodstock for potential eradication of undesired Brook Trout populations. North American Journal of Aquaculture *78*, 72–83.

Schill, D.J., Meyer, K.A., and Hansen, M.J. (2017). Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. North American Journal of Fisheries Management *37*, 1054–1066.

Sfakianakis, D.G., Georgakopoulou, E., Papadakis, I.E., Divanach, P., Kentouri, M., Koumoundouros, G., (2006). Environmental determinants of haemal lordosis in European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquaculture *254*, 54–64.

Thresher, R.E., Hayes, K., Bax, N.J., Teem, J., Benfey, T.J., and Gould, F. (2014). Genetic Control of Invasive Fish: Technological options and its role in integrated pest management. Biological Invasions *16*, 1201–1216.

Weber, G., Hostuttler, M.A., Cleveland, B.M., and Leeds, T.D. (2014). Growth performance comparison of intercross-triploid, induced triploid, and diploid rainbow trout. Aquaculture *433*, 85–93.

Combined References

Afzal, M., Rab, A., Akhtar, N., Ahmed, I., Khan, M.F., and Qayyum, M. (2008). Growth performance of bighead carp *Aristichthys nobilis* (Richardson) in monoculture system with and without supplementary feeding. Pakistan Veterinary Journal *28*, 57–62.

Aldridge, F.J., Marston, R.Q., and Shireman, J.V. (1990). Induced triploids and tetraploids in bighead carp, *Hypophthalmichthys nobilis*, verified by multi-embryo cytofluorometric analysis. Aquaculture *87*, 121–131.

Alsip, P.J., Zhang, H., Rowe, M.D., Mason, D.M., Rutherford, E.S., Riseng, C.M., and Su, Z. (2019). Lake Michigan's suitability for bigheaded carp: The importance of diet flexibility and subsurface habitat. Freshwater Biology *00*, 1–19.

Alvarez, B., Fuentes, R., Pimentel, R., Abad, Z., Cabrera, E., Pimentel, E., and Arenal, A. (2003). High fry production rates using post-thaw silver carp (*Hypophthalmichthys molitrix*) spermatozoa under farming conditions. Aquaculture *220*, 195–201.

Anderson, K.R., Chapman, D.C., Wynne, T.T., Masagounder, K., and Paukert, C.P. (2015). Suitability of Lake Erie for bigheaded carps based on bioenergetic models and remote sensing. Journal of Great Lakes Research *41*, 358–366.

Andrades, J.A., Becerra, J., and Fernández-Llébrez, P. (1996). Skeletal deformities in larval, juvenile and adult stages of cultured gilthead sea bream (*Sparus aurata* L.). Aquaculture *141*, 1–11.

AOAC International (2002). Official Methods of Analysis of AOAC International. AOAC International, Gaithersburg, Md.

Applebaum, S. and Uland, B. (1979). Intensive rearing of grass carp larvae *Ctenopharyngodon idella* (Valenciennes 1844) under controlled conditions. Aquaculture *17*, 175–179.

Araneda, M.E., Hernández, J.M., Gasca-Leyva, E., and Vela, M.A. (2013). Growth modeling including size heterogeneity: Application to the intensive culture of white shrimp (*P. vannamei*) in Freshwater. Aquacultural Engineering *56*, 1–12.

Asturiano, J.F., Cabrita, E., and Horvath, A. (2017). Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. General and Comparative Endocrinology *245*, 69–76.

Asturiano, J.F., Sorbera, L.A., Carillo, M., Zanuy, S., Ramos, J., Navarro, J.C., and Bromage, N. (2001). Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture *194*, 173–190.

Avery, T.S., and Brown, J.A. (2005). Investigating the relationship among abnormal patterns of cell cleavage, egg mortality and early larval condition in *Limanda ferruginea*. Journal of Fish Biology *67*, 890–896.

Avramova, Z., Uschewa, A., Stephanova, E., and Tsanev, R. (1983). Trout sperm chromatin. I. Biochemical and immunological study of the protein composition. European Journal of Cell Biology *31*, 137–142.

Babiak, I., Glogowski, J., Brzuska, E., and Adamek, J. (1997). Cryopreservation of sperm of common carp, *Cyprinus carpio* L. Aquaculture Research *28*, 567–571.

Backiel, T., Kokurewicz, B., and Ogozalek, A. (1984). High incidence of skeletal anomalies in carp, *Cyprinus carpio*, reared in cages in flowing water. Aquaculture *43*, 369–380.

Bakerville-Bridges, B. and Kling, L.J. (2000). Early weaning of Atlantic cod (*Gadus morhua*) larvae onto a microparticulate diet. Aquaculture *189*, 109–117.

Benfey, T.J. (1999). The physiology and behavior of triploid fishes. Reviews in Fisheries Science *7*, 39–67.

Bengtsson, B.E. (1974). Vertebral damage to minnows *Phoxinus phoxinus* exposed to zinc. Oikos *25*, 134–139.

Berillis, P. (2015). Factors that can lead to the development of skeletal deformities in fishes: A review. Journal of Fisheries Sciences *9*, 17–23.

Bernáth, G., Zarski, D., Kása, E., Staszny, A., Várkonyi, L, Kollár, T., Hegyi, A., Bokor, Z., Urbányi, B., and Horváth, A. (2016). Improvement of common carp (*Cyprinus carpio*) sperm cryopreservation using a programmable freezer. General and Comparative Endocrinology *237*, 78–88.

Billard, R. (1978). Changes in structure and fertilizing ability of marine and freshwater fish spermatozoa diluted in media of various salinities. Aquaculture *14*, 187–198.

Billard, R., Cosson, J., Perchec, G., and Linhart, O. (1995). Biology of sperm and artificial reproduction in carp. Aquaculture *129*, 95–112.

Bobe, J., and Labbe, C. (2008). Chilled storage of sperm and eggs. In: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in reproductive aquaculture: Marine and freshwater species Taylor and Francis, London (UK).

Bobe, J., and Labbé, C. (2010). Egg and sperm quality in fish. General and Comparative Endocrinology *165*, 535–548.

Boney, S.E., Shelton, W.L., Yang, S.-L., and Wilken, L.O. (1984). Sex reversal and breeding of grass carp. Transactions of the American Fisheries Society *113*, 348–353.

Bowzer, J., Trushenski, J., Rawles, S., Gaylord, T.G., & Barrows, F.T. (2015). Apparent digestibility of Asian carp- and common carp-derived fish meals in the feeds for hybrid striped bass *Morone saxatilis* (F) x *M. chrysops* (M) and rainbow trout *Oncorhynchus mykiss*. Aquaculture Nutrition *21*, 43–53.

Bryant, P.L., and Matty, A.J. (1981). Adaptation of carp (*Cyprinus carpio*) larvae to artificial diets: 1. Optimum feeding rate and adaptation age for a commercial diet. Aquaculture *23*, 275–286.

Buck, E.H., Upton, H.F., Stern, C.V., and Nicols, J.E. (2010). Asian carp and the Great Lakes region. Congressional Research Report *12*, pp. 28.

Burke, J.S., Bayne, D.R., and Rea, H. (1986). Impact of silver and bighead carps on plankton communities of channel catfish ponds. Aquaculture *55*, 59–68.

Callan, C., Jordaan, A., and Kling, L.J. (2003). Reducing *Artemia* use in the culture of Atlantic cod (*Gadus morhua*). Aquaculture *219*, 585–595.

Campbell, P.M., Pottinger, T.G., and Sumpter, J.P. (1992). Stress reduces the quality of gametes produced by rainbow trout. Biological Reproduction *47*, 1140–1150.

Carlos, M.H. (1988). Growth and survival of bighead carp (*Aristichthys nobilis*) fry fed at different intake levels and feeding frequencies. Aquaculture *68*, 267–276.

Charlon, N, and Bergot, P. (1984). Rearing system for feeding fish larvae on dry diets. Trial with carp (*Cyprinus carpio* L.). Aquaculture *41*, 1–9.

Chen, S.L., Liu, X.T., Lu, D.C., Zhang, L.Z., Fu, C.J., and Fang, J.P. (1992). Cryopreservation of spermatozoa of silver carp, common carp, blunt snout bream and grass carp. Acta Zoologica Sinica *38*, 413–424. (in Chinese with English summary)

Christen, R., Gatti, J.L., and Billard, R. (1987). Trout sperm motility. The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. European Journal of Biochemistry *166*, 667–671.

Ciereszko, A., and Dabrowski, K. (1995). Sperm quality and ascorbic acid concentration in rainbow trout semen are affected by dietary vitamin C: An across-season study. Biology of Reproduction *52*, 982–988.

Ciereszko, A., Dabrowski, K., Lin, F., Christ, S.A., and Toth, G.P. (1999). Effects of extenders and time of storage before freezing on motility and fertilization of cryopreserved muskellunge spermatozoa. Transactions of the American Fisheries Society *128*, 542–548.

Ciereszko, A., Dabrowski, K., Froschauer, J., and Wolfe, T.D. (2006). Cryopreservation of semen from lake sturgeon. Transactions of the American Fisheries Society *135*, 232–240.

Ciudad, J., Cid, E., Valesco, A., Lara, J.M., Aijan, J., and Orfao, A. (2002). Flow cytometry measurement of the DNA contents of G0/G1 diploid cells from three different teleost fish species. Cytometry *48*, 20–25.

Cooke, S.L. (2016). Anticipating the spread and ecological effects of invasive bigheaded carps (*Hypophthalmichthys* spp.) in North America: a review of modeling and other predictive studies. Biological Invasions *18*, 315–344.

Cooke, S.L., and Hill, W.R. (2010). Can filter-feeding Asian carp invade the Laurentian Great Lakes? A bioenergetic modeling exercise: Bioenergetics of invasive Asian carp. Freshwater Biology *55*, 2138–2152.

Cosson, J.J. (2008). Methods to analyse the movements of fish spermatozoa and their flagella. In: Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology, Alpha Science International Ltd., Oxford, U.K., pp. 63–102.

Cremer, M.C., and Smitherman, R.O. (1980). Food habits and growth of silver and bighead carp in cages and ponds. Aquaculture *20*, 57–64.

Cuddington, K., Currie, W.J.S., and Koops, M.A. (2014). Could an Asian carp population establish in the Great Lakes from a small introduction? Biological Invasions *16*, 903–917.

Cui, J., Ren, X., and Yu, Q. (1991). Nuclear DNA content variation in fishes. Cytologia *56*, 425–429.

Curnow, J., King, J., Partridge, G., and Kolkovski, S. (2006). Effects of two commercial microdiets on growth and survival of barramundi (*Lates calcarifer* Bloch) larvae within various early weaning protocols. Aquaculture Nutrition *12*, 247–255.

Dabrowski, K. (1982). Further study on dry diet formulation for common carp. Rivista Italiana di Piscicoltura e Ittiopatologio *XVII*, 11–29.

Dabrowski, K. (1984). Influence of initial weight during the change from live to compound feed on the survival and growth of four cyprinids. Aquaculture *40*, 27–40.

Dabrowski, K., and Culver, D.A. (1991). The physiology of larval fish. Aquaculture Magazine *17*, 49–61.

Dabrowski, K., and Miller, M. (2018). Contested paradigm in raising zebrafish (*Danio rerio*). Zebrafish *15*, 295–309.

Dedi, J., Takeuchi, T., Seikai, T., Watanabe, T., and Hosoya, K. (1997). Hypervitaminosis A during vertebral morphogenesis in larval Japanese flounder. Fisheries Science *63*, 466–473.

Delomas, T.A., and Dabrowski, K. (2016). Zebrafish embryonic development is induced by carp sperm. Biology Letters *12*, 1–4.

Delomas, T.A., and Dabrowski, K. (2017). Heritability of spontaneous diploidization of maternal chromosomes in zebrafish *Danio rerio*. LARVI '17–Fish & Shellfish larviculture symposium, Hendry, C.I. (Ed). European Aquaculture Society, Special publication no. *XX*, Oostende, Belgium.

Dhont, J., Dierckens, K., Strottrup, J.G., Van Stappen, G., Wille, M., and Sorgeloos, P. (2013). Rotifers, artemia and copepods as live feeds for fish larvae in aquaculture. In: Advances in aquaculture hatchery technology, Allan (eds.), Woodhead Publishing, Cambridge, 157–202.

Dreanno, C., Cosson, J., Suquet, M., Cibert, C., Fauvel, C., Dorange, G., and Billard, R. (1999a). Effects of osmolality, morphology perturbations and intracellular nucleotide content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. Journal of Reproductive Fertility *116*, 113–125.

Dreanno, C., Cosson, J., Suquet, M., Seguin, F., Dorange, G., and Billard, R. (1999b). Nucleotide content, oxidative phosphorylation, morphology, and fertilizing capacity of turbot (*Psetta maxima*) spermatozoa during the motility period. Molecular Reproductive Development. *53*, 230–243.

Dreanno, C., Seguin, F., Cosson, J., Suquet, M., and Billard, R. (2000). 1H-NMR and (31)PNMR analysis of energy metabolism of quiescent and motile turbot (*Psetta maxima*) spermatozoa. Journal of Experimental Zoology *286*, 513–522.

Dunham, R., and Devlin, R. (1999). Comparison of traditional breeding and transgenesis in farmed fish with implications for growth enhancement and fitness. Murray, J.D., Anderson, G.B., Oberbauer, A.M. and McGloughlin, M.M. (eds.) University of California Davis, pp. 209–229.

Dzuba, B.B., and Kopeika, E.F. (2002). Relationship between the changes in cellular volume of fish spermatozoa and their cryoresistance. Cryo Letters *23*, 353–360.

FAO (Food and Agriculture Organization of the United Nations) (2018). The state of world fisheries and aquaculture 2018- Meeting the sustainable development goals. Rome, *CC BY-NC-SA 3.0 IGO*, pp. 1–210.

Fatima, S., Shoukat, A., Qamar, B., Mahmood, F., and Rafique, A. (2017). Histological study of sex differentiation in bighead carp (*Hypophthalmichthys nobilis*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and catla (*Catla catla*). Turkish Journal of Fisheries and Aquatic Sciences *17*, 1313–1316.

Feindel, N.J., Benfey, T.J., and Trippel, E.A. (2010). Competitive spawning success and fertility of triploid male Atlantic cod *Gadus morhua*. Aquaculture Environment Interactions *1*, 47–55.

Fernández, I., Hontoria, F., Ortiz-Delgado, J.B., Kotzamanis, Y., Estévez, A., Zambonino-Infante, J.L., and Gisbert, E. (2008). Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*). Aquaculture *283*, 102-115.

Fermin, A.C., and Recometa, R.D. (1988). Larval rearing of bighead carp, *Aristichthys nobilis* Richardson, using different types of feed and their combinations. Aquaculture Research *19*, 283–290.

Freeze, M., & Henderson, S. (1982). Distribution and status of the bighead carp and silver carp in Arkansas. North American Journal of Fisheries Management *2*, 197–200.

Garcia, F., Romera, D.M., Gozi, K.S., Onaka, E.M., Fonseca, F.S., Schalch, S.H.C., Candeira, P.G., Guerra, L.O.M., Carmo, F.J., Carneiro, D.J., Martins, M.I.E.G., and Portella, M.C. (2013). Stocking density of Nile tilapia in cages placed in a hydroelectric reservoir. Aquaculture *410-411*, 51–56.

Garcia-Celdran, M., Ramis, G., Machado, M., Estevez, A., Afonso, J.M., Maria-Dolores, E., Penalver, J., and Armero, E. (2015). Estimates of heritability and genetic correlations of growth and external skeletal deformities at different ages in a reared gilthead sea bream (*Sparus aurata* L.) population sourced from three broodstocks along the Spanish coasts. Aquaculture *445*, 33–41.

Gardiner, D.M. (1978). Utilisation of extracellular glucose by spermatozoa of two viviparous fishes. Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology *59*, 165–168.

Georgakopoulou, E., Katharios, P., Divanach, P., and Koumoundouros, G. (2010). Effect of temperature on the development of skeletal deformities in gilthead seabream (*Sparus aurata* Linnaeus, 1758). Aquaculture *308*, 13–19.

George, A.E., and Chapman, D.C. (2013). Aspects of embryonic and larval development in bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. PLoS ONE *8*, e73829.

Geurden, I., Charlon, N., Marion, D., and Bergot, P. (1997). Influence of purified soybean phospholipids on early development of common carp. Aquaculture International *5*, 127–149.

Gomelsky, B. (2003). Chromosome set manipulation and sex control in common carp: a review. Aquatic Living Resources *16*, 408–415.

Gomelsky, B., Schneider, K.J., Anil1, A., and Delomas, T.A. (2015). Gonad development in triploid ornamental koi carp and results of crossing triploid females with diploid males. North American Journal of Aquaculture *77*, 96–101.

Green, B.W., and Smitherman, R.O. (1984). Relative growth, survival and harvestability of bighead carp, silver carp, and their reciprocal hybrids. Aquaculture *37*, 87–95.

Gutierrez, J. B., and Teem, J. L. (2006). A model describing the effect of sex-reversed YY fish in an established wild population: The use of a Trojan Y Chromosome to

cause extinction of an introduced exotic species. Journal of Theoretical Biology *241*, 333–341.
Henderson, S. (1976). Observations on the bighead and silver carp and their possible application in pond fish culture. Arkansas Game and Fish Commission, Little Rock.
Henderson, S. (1978). An evaluation of the filter feeding fishes, silver and bighead carp, for water quality improvement. In Smitherman R.O., W.L. Shelton, and J.H. Grover, (Eds.) *Culture of exotic fishes symposium proceedings.*, American Fisheries Society, Auburn, Alabama, 121–136.
Herborg, L.-M., Mandrak, N.E., Cudmore, B.C., and MacIsaac, H.J. (2007). Comparative distribution and invasion risk of snakehead (*Channidae*) and Asian carp (*Cyprinidae*) species in North America. Canadian Journal of Fisheries and Aquatic Sciences *64*, 1723–1735.
Hilton, J.W. (1983). Hypervitaminosis A in rainbow trout (*Salmo gairdneri*) toxicity signs and maximum tolerable level. The Journal of Nutrition *113*, 1737–1747.
Horvath, A., and Urbanyi, B. (2000). The effect of cryoprotectants on the motility and fertilizing capacity of cryopreserved African catfish *Clarias gariepinus* (Burchell 1822) sperm. Aquaculture Research *31*, 317–324.
Hubbs, C. (1971). Survival of intergroup percid hybrids. Japanese Journal of Ichthyology *18*, 65–75.
Hulata, G., and Rothbard, S. (1979). Cold storage of carp semen for short periods. Aquaculture *16*, 267–269.
Hunter, G.A., and Donaldson, E.M. (1983). Hormonal sex control and its application to fish culture. In: Hoar, W.S., Randall, D.J., and Donaldson, E.M. (Eds.), Fish Physiology, Vol. 9B, Academic Press, New York, USA, pp. 223-301.
Inaba, K., Morisawa, S., and Morisawa, M. (1998). Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. Journal of Cell Science. *111*, 1105–1115.
Ingermann, R.L. (2008). Energy metabolism and respiration in fish spermatozoa. In: Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.), Fish spermatology. Alpha Science International Ltd, Oxford, UK, pp. 241–266.
Irons, K.S., Sass, G.G., McClelland, M.A., and Stafford, J.D. (2007). Reduced condition factor of two native fish species coincident with invasion of non-native Asian carps in the Illinois River, U.S.A. Is this evidence for competition and reduced fitness? Journal of Fish Biology *71*, 258–273.
Jawad, L.A., and Kousha, A. (2011). A case of vertebral coalescences and lateral line deformity in *Hypophthalmichthys nobilis* (Richardson, 1844) obtained from aquaculture activity in Iran. Boll. Mus. Reg. Sci. nat. Torino *28*, 29–36.
Jennings, D.P. (1988). Bighead carp (*Hypophthalmichthys nobilis*): a biological synopsis. U.S. Fish and Wildlife Service, Washington, DC. Fish and Wildlife Service Biological Report *88*, 1–47.
Jerde, C. L., Mahon, A. R., Chadderton, W. L., and Lodge, D.M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. Conservation Letters *4*,150–157.

Jimenez-Martinez, L. D., Alvarez-Gonza´lez, C. A., Tovar-Ramı´rez, D., Gaxiola, G., Sanchez-Zamora, A., Moyano, F.J., Alarco´n, F.J., Ma´rquez-Couturier, G., Gisbert, E., Contreras-Sa´nchez, W.M., Perales-Garcı´a, N., Arias-Rodrı´guez, L., Indy, J.R., Pa´ramo-Delgadillo, S., and Palomino-Albarra´n, I.G. (2012). Digestive enzyme activities during early ontogeny in common snook (*Centropomus undecimalis*). Fish Physiology and Biochemistry *38*, 441–454.

Jun, Q., Pao, X. Haizhen, W., Ruiwei, L., and Hui, W. (2012). Combined effect of temperature, salinity and density on the growth and feed utilization of Nile tilapia juveniles (*Oreochromis niloticus*). Aquaculture Research *43*, 1344–1356.

Karahan, B., Chatain, B., Chavanne, H., Vergnet, A., Bardon, A., Haffray, P., Dupont-Nivet, M., and Vandeputte, M. (2013). Heritabilities and correlations of deformities and growth related traits in the European sea bass (*Dicentrarchus labrax,* L) in four different sites. Aquaculture Research *44*, 289–299.

Kennedy, P.A., Meyer, K.A., Campbell, M.R., Vu, N.V., and Schill, D.J. (2018). Survival and reproductive success of hatchery YY male brook trout stocked in Idaho streams. Transactions of the American Fisheries Society *147*, 419–430.

Kessabi, K., Annabi, A., Hassine, A.I.H., Bazin, I., Mnif, W., Said, K., and Messaoudi, I. (2013). Possible chemical causes of skeletal deformities in natural populations of *Aphanius fasciatus* collected from the Tunisian coast. Chemosphere *90*, 2683–2689.

Klassen, W., Adams, J.V., and Twohey, M.B. (2004). Modeling the suppression of sea lamprey populations by the release of sterile males or sterile females. Journal of Great Lakes Research *30*, 463–473.

Kolar C.S., Chapman, D.C., Courtenay, J.W.R., Housel, C.M., Williams, J.D., and Jennings, D.P. (2005). Asian carps of the Genus *Hypophthalmichthys* (Pisces, Cyprinidae) — A Biological synopsis and environmental risk assessment. U.S. Fish and Wildlife Service *report 94400-3-0128*, pp. 175.

Kolar C.S., Chapman, D.C., Courtenay, J.W.R., Housel, C.M., Williams, J.D., and Jennings, D.P. (2007). Bigheaded carps: a biological synopsis and environmental risk assessment. American Fisheries Society, Bethesda, Special Publication *33*.

Kom, M.K., Ozkok, E., and Han, I.K. (1997). Effect of soybean meal and full-fat soybean for fish meal protein replacement on the growth performance of carp fingerlings. Korean Journal of Animal Nutrition and Feedstuffs *21,* 391–398.

Kopeika, E., and Kopeika, J. (2008). Variability of sperm quality after cryopreservation in fish. In: Alavi, S.M.H., Cosson, J.J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology, Alpha Science International Ltd., Oxford, U.K., pp. 347–396.

Kopeika, J., Kopeika, E., Zhang, T., Rawson, D.M., and Holt, W.V. (2003). Detrimental effects of cryopreservation of loach (*Misgurnus fossilis*) sperm on subsequent embryo development are reversed by incubating fertilised eggs in caffeine. Cryobiology *46*, 43–52.

Krakowiak, P.J., and Pennuto, C.M. (2008). Fish and macroinvertebrate communities in tributary streams of eastern Lake Erie with and without Round Gobies (*Neogobius melanostomus,* Pallas 1814). Journal of Great Lakes Research *34*, 675–689.

Labbe, C., and Maisse, G. (2001). Characteristics and freezing tolerance of brown trout spermatozoa according to rearing water salinity. Aquaculture *201*, 287–299.

Labbe, C., Maisse, G., Muller, K., Zachowski, A., Kaushik, S., and Loir, M. (1995). Thermal acclimation and dietary lipids alter the composition, but not fluidity, of trout sperm plasma membrane. Lipids *30*, 23–33.

Labbe, C., Martoriati, A., Devaux, A., and Maisse, G. (2001). Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Molecular Reproductive Development *60*, 397–404.

Lahnsteiner, F., and Patzner, R.A. (1998). Sperm motility of the marine teleosts *Boops boops*, *Diplodus sargus*, *Mullus barbatus* and *Trachurus mediterraneus*. Journal of Fish Biology *52*, 726–742.

Lahnsteiner, F., Berger, B., Horvath, A., Urbanyi, B., and Weismann, T. (2000). Cryopreservation of spermatozoa in Cyprinid fishes. Theriogenology *54*, 1477–1498.

Lahnsteiner, F., Patzner, R., and Weismann, T. (1992). Monosaccharides as energy resources during motility of spermatozoa in *Leuciscus cephalus* (*Cyprinidae*, Teleostei). Fish Physiology and Biochemistry *10*, 283–289.

Leung, H.M., Leung, A.O.W., Wang. H.S., Ma, K.K., Liang, Y., Ho, K.C., Cheung, K.C., Tohidi, F., and Yung, K.K.L. (2014). Assessment of heavy metals/metalloid (As, Pb, Cd, Ni, Zn, Cr, Cu, Mn) concentrations in edible fish species tissue in the Pearl River Delta (PRD) China. Marine Pollution Bulletin *78*, 235–245.

Li, P., Hulak, M., Li, Z. H., Sulc, M., Psenicka, M., Rodina, M., Gela, D., and Linhart, O. (2013). Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces protein phosphorylation in tyrosine and threonine residues. Theriogenology *80*, 84–89.

Li, L., Fang, J.G., Liang, X.F., Alam, M.S., Liu, L.W., and Yuan, X.C. (2019). Effect of feeding stimulants on growth performance, feed intake and appetite regulation of mandarin fish, *Siniperca chuatsi*. Aquaculture Research *50*, 3684–3691.

Li, S., and Fang, F. (1990). On the geographical distribution of the four kinds of pond-cultured carps in China. Acta Zoologica Sinica *36*, 244–255.

Lieder, U., and Helms, C. (1981). Erfahrungen beim Vorstrecken von Brut pflanzenfressender Cypriniden. Binnenfisch *28*, 3–8 (in German).

Lirski, A., and Opuszynski, K. (1988). Lower lethal temperatures for carp *Cyprinus carpio* L. and the phytophagous fishes *Ctenopharyngodon idella* Val. *Hypophthalmichthys-molitrix* Val. *Aristichthys-nobilis* Rich. in the first period of life. Roczniki Nauk Rolniczych *101*,11–30. (in Polish with English summary)

Liu, H., Pang, M., Yu, X., Zhou, Y., Tong, J., and Fu, B. (2018). Sex-specific markers developed by next-generation sequencing confirmed an XX/XY sex determination system in bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*). DNA Research *25*, 257-264.

Long, J.M., and Nealis, A. (2011). Age estimation of a large bighead carp from Grand Lake, Oklahoma. In Proceedings of the Oklahoma Academy of Science, pp. 15–18.

Lorentzen, M., Maage, A., and Julshamn, K. (1996). Manganese supplementation of a practical, fish meal based diet for Atlantic salmon parr. Aquaculture Nutrition *2*, 121–125.

Madsen, L., and Dalsgaard, I. (1999). Vertebral column deformities in farmed rainbow trout (Oncorhynchus mykiss). Aquaculture *171*, 41-48.

Mahfuj, M.S., Hossain, M.A., and Sarower, M.G. (2012). Effects of different feeds on larval development and survival of ornamental koi carp, *Cyprinus carpio* (Linnaeus, 1748) larvae in laboratory condition. J. Bangladesh Agril. Univ. *10*, 179–183.

Mair, G.C., Abucay, J.S., Abella, T.A., Beardmore, J.A., and Skibinski, D.O.F. (1997). Genetic manipulation of sex ratio for the large-scale production of all-male tilapia *Oreochromis niloticus*. Canadian Journal of Fisheries and Aquatic Sciences *54*, 396–404.

Marciak, Z., and Bogdan, E. (1979). Food requirements of juvenile stages of grass carp *Ctenopharyngodon Idella* Val., silver carp *Hypophthalmichthys molitrix* Val., and bullhead carp *Aristichthys nobilis* Rich. In Stycznska-Jurewicz, E., Backiel, T., Jaspers, E., and Persoone, G., (Eds.). Cultivation of fish fry and its live food, Prinses Elisabethlaan 69, Belgium, European Mariculture Society, special publication *No. 4*, 140–148.

Martinez-Paramo, S., Horvath, A., Labbe, C., Zhang, T., Robles, V., Herraez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R., and Cabrita, E. (2017). Cryobanking of aquatic species. Aquaculture *472*, 156–177.

Mattei, X. (1991). Spermatozoon ultrastructure and its systematic implications in fishes. Canadian Journal of Zoology *69*, 3038–3055.

Meronek, T. G., Bouchard, P. M., Buckner, E. R., Burri, T. M., Demmerly, K. K., Hatleli, D. C., Klumb, R. A., Schmidt, S. H., and Coble, D. W. (1996). A review of fish control projects. North American Journal of Fisheries Management *16*, 63–74.

Meske, C., Pfeffer, E., Ahrensburg, and Gottingen (1978). Growth experiments with carp and grass carp. Arch. Hydrobiol. Beih. Ergebn. Limnol. *11*, 98–107.

Messaoudi, I., Deli, T., Kessabi, K., Barhoumi, S., Kerkeni, A., and Saïd, K. (2009). Association of spinal deformities with heavy metal bioaccumulation in natural populations of grass goby, *Zosterisessor ophiocephalus* Pallas, 1811 from the Gulf of Gabès (Tunisia). Environmental Monitoring and Assessment *156*, 551–560

Meyerson, L.A., and Mooney, H.A. (2007). Invasive alien species in an era of globalization. Frontiers in Ecology and the Environment *5*, 199–208.

Mirza, J.A., and Shelton, W.L. (1988). Induction of gynogenesis and sex reversal in silver carp. Aquaculture *68*, 1–14.

Montgomery, D.C. (2005). Design and analysis of experiments. John Wiley & Sons, New York, USA, 6th *edn.*, pp. 405-444.

Morisawa, M. (1985). Initiation mechanisms of sperm motility at spawning in teleosts. Zoological Science *2*, 605–615.

Morisawa, S., and Morisawa, M. (1986). Acquisition of potential for sperm motility in rainbow trout and chum salmon. Journal of Experimental Biology *126*, 89–96.

Morisawa, S., and Morisawa, M. (1988). Induction of potential for sperm motility by bicarbonate and pH in rainbow trout and chum salmon. Journal of Experimental Biology *136*, 13–22.

Morisawa, S., Ishida, K., Okuno, M., and Morisawa, M. (1993a). Roles of pH and cyclic adenosine monophosphate in the acquisition of potential for sperm motility during migration from the sea to the river in chum salmon. Molecular Reproduction and Development *34*, 420–426.

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S., and Yasuda, K. (1993b). Effects of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. Journal of Experimental Biology *107*, 95–103.

Mounib, M.S. (1967). Metabolism of pyruvate, acetate and glyoxylate by fish sperm. Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology *20*, 987–992.

Munoz-Guerra, S., Azorin, F., Casas, T., Marcet, X., Maristany, M.A., Roca, J., and Subirana, J.A. (1982). Structural organization of sperm chromatin from the fish *Carassius auratus*. Experimental Cell Research. *137*, 47–53.

Muramoto, S. (1981). Vertebral column damage and decrease of calcium concentration in fish exposed experimentally to cadmium. Environmental Pollution (Series A) *24*, 125–133.

Nhu, V.C., Dierckens, K., Nguyen H.T., Hoang, T.M.T., Le, T.L., Tran, M.T., Nys, C., and Sorgeloos, P. (2010). Effect of early co-feeding and different weaning diets on the performance of cobia (*Rachycentron canadum*) larvae and juveniles. Aquaculture *305*, 52–58.

Nicholls, K.H., and Hopkins, G.J. (1993). Recent changes in Lake Erie (north shore) phytoplankton: cumulative impacts of phosphorus loading reductions and the Zebra mussel introduction. Journal of Great Lakes Research *sparks*, 637–647.

Ohno, S., Muramoto, J., Klein, J., and Atkin, N.B. (1969). Diploid-tetraploid relationship in clupeoid salmonid fish. In: Darlington, C.D., and Lewis, K.R. (eds.). Chromosomes Today. Oliver & Boyd, Edinburgh, *Vol. 2*, 139–147.

Opuszyński, K. (1969). Production of plant feeding fish (*Ctenopharyngodon Idella* Val. And *Hypophthalmichthys molitrix* Val.) in carp ponds. Roczniki Nauk Rolniczych *91*, 221–307. (in Polish, English summary)

Opuszyński, K. (1978). Rearing of larvae and fry of silver carp *Hypophthalmichthys molitrix*. Proceedings of Conference on Aquaculture: "Cultivation of Fish Fry and its Live Food", Sept 23-28, 1977, Szymbark, Poland.

Opuszyński, K. (1981). Comparison of the usefulness of the silver carp and the bighead carp as additional fish in carp ponds. Aquaculture *25*, 223–233.

Opuszyński, K., Myszkowski, L., Okoniewska, G., Opuszyńska, W., Szlaminska, M., Wolnicki, J., and Woznieski, M. (1979). Rearing of common carp, grass carp, silver carp, and bighead carp larvae using zooplankton and/or different dry feeds. Polskie Archiwum Hydrobiologii *36*, 217–230.

Pandian, T., and Sheela, S.G. (1995). Hormonal induction of sex reversal in fish. Aquaculture *138*, 1–22.

Pandian, T.J., and Koteeswaran, R. (1998). Ploidy induction and sex control in fish. Hydrobiologia *384*, 167–243.

Pandian, T., Venugopal, T., and Koteeswaran, R. (1999). Problems and prospects of hormone, chromosome and gene manipulations in fish. Current Science *76*, 369–386.

Parker, A.D., Glover, D.C., Finney, S.T., Rogers, P.B., Stewart, J.G., and Simmonds, R.L. (2015). Direct observations of fish incapacitation rates at a large electrical fish barrier in the Chicago Sanitary and Ship Canal. Journal of Great Lakes Research *41*, 396–404.

Peacor, S.D., Bence, J.A., and Pfister, C.A. (2007). The effect of size-dependent growth and environmental factors on animal size variability. Theoretical Population Biology *71*, 80–94.

Perchec, G., Jeulin, C., Cosson, J., André, F., and Billard, R. (1995). Relationship between sperm ATP content and motility of carp spermatozoa. Journal of Cell Science *108*, 747–753.

Pongmaneerat, J., and Watanabe, T. (1993). Nutritional evaluation of soybean meal for rainbow trout and carp. Nippon Suisan Gakkaishi *59*, 157–163.

Pretto, R. (1976). Polyculture systems with channel catfish as the principal species. Ph.D. Dissertation, Auburn University, Alabama, 190 pp.

Rana, K.J., Muiruri, R.M., McAndrew, B.J., and Gilmour, A. (1990). The influence of diluents, equilibration time and prefreezing storage time on the viability of *Oreochromis niloticus* (L.) spermatozoa. Aquaculture Research *21*, 25–30.

Ravinder, K., Nasaruddin, K, Majumdar, K.C., and Shivaji, S. (1997). Computerized analysis of motility, motility patterns and motility parameters of spermatozoa of carp following short-term storage of semen. Journal of Fish Biology *50*, 1309–1328.

Rayner, T.S., and Creese, R.G. (2006). A review of rotenone use for the control of non-indigenous fish in Australian fresh waters, and an attempted eradication of the noxious fish, *Phalloceros caudimaculatus*. New Zealand Journal of Marine and Freshwater Research *40*, 477–486.

Roo, F.J., Hernández-Cruz, C.M., Fernández-Palacios, H., and Izquierdo, M.S. (2005). Development of skeletal deformities in gilthead sea bream (*Sparus aurata*) reared under different larval culture and dietary conditions. In: Larvi '05 – Fish & Shellfish Larviculture symposium. Hendry, C.I., Van tappen, G., Wille, M., and Sorgeloos, P. (Eds.), European Aquaculture Society, Special Publication *No*. *36*, Oostende, Belgium.

Rosenlund, G., Stoss, J., and Talbot, C. (1997). Co-feeding marine fish larvae with inert and live diets. Aquaculture *155*, 183–191.

Rosenthal, H., Asturiano, J.F., Linhart, O., and Horváth, Á. (2010). On the biology of fish gametes: summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. Journal of Applied Ichthyology *26*, 621–622.

Rottmann, R.W., Shireman, J.V., and Lincoln, E.P. (1991). Comparison of three live foods and two dry diets for intensive culture of grass carp and bighead carp larvae. Aquaculture *96*, 269–280.

Roy, P.K., and Lall, S.P. (2003). Dietary phosphorus requirement of juvenile haddock (*Melanogrammus aeglefinus* L.). Aquaculture *221*, 451–468.

Roy, P.K., and Lall, S.P. (2007). Vitamin K deficiency inhibits mineralization and enhances deformity in vertebrae of haddock (*Melanogrammus aeglefinus* L.). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology *148*, 174-183.

Sales, J. (2011). First feeding of freshwater fish larvae with live feeds versus compound diets: a meta-analysis. Aquaculture International *19*, 1217–1228.

Sampson, S.J., Chick, J.H., and Pegg, M.A. (2009). Diet overlap among two Asian carp and three native fishes in backwater lakes on the Illinois and Mississippi rivers. Biological Invasions *11*, 483–496.

Santiago, C.B., and Reyes, O.S. (1991). Optimum dietary protein level for growth of bighead carp (*Aristichthys nobilis*) fry in a static water system. Aquaculture *93*, 155–165.

Santiago, C.B., Gonzal, A.C., Aralar, E.V., and Arcilla, R.P. (2004). Effect of stunting of juvenile bighead carp *Aristichthys nobilis* (Richardson) on compensatory growth and reproduction. Aquaculture Research *35*, 836–841.

Saperas, N., Lloris, D., and Chiva, M. (1993a). Sporadic appearance of histones, histone-likee proteins, and protamines in sperm chromatin of bony fish. Journal of Experimental Zoology *265*, 575–586.

Saperas, N., Ribes, E., Buesa, C., Garcia Hegart, F., and Chiva, M. (1993b). Differences in chromatin condensation during spermiogenesis in two species of fish with distinct protamines. Journal of Experimental Zoology *265*, 185–194.

Sass, G.G., Hinz, C., Erickson, A.C., McClelland, N.N., McClelland, M.A., and Epifanio, J.M. (2014). Invasive bighead and silver carp effects on zooplankton communities in the Illinois River, Illinois, USA. Journal of Great Lakes Research *40*, 911–921.

Sato, M., Kondo, T., Yoshinaka, R., and Ikeda, S. (1982). Effect of dietary ascorbic acid levels on collagen formation in rainbow trout, Bulletin of the Japanese Society of Scientific Fisheries *48*, 553–556.

Sato, M., Kondo, T., Yoshinaka, R., and Ikeda, S. (1983). Effect of water temperature on the skeletal deformity in ascorbic acid-deficient rainbow trout. Bulletin of the Japanese Society of Scientific Fisheries *49*, 443-446.

Satoh, S., Yamamoto, H., Takeuchi, T., and Watanabe, T. (1983). Effects of growth and mineral composition of carp of deletion of trace elements or magnesium from fish meal diet. Bulletin of the Japanese Society of Scientific Fisheries *49*, 431–435.

Schill, D.J., Heindel, J.A., Campbell, M.R., Meyer, K.A., and Mamer, E.R. (2016). Production of a YY male Brook Trout broodstock for potential eradication of undesired Brook Trout populations. North American Journal of Aquaculture *78*, 72–83.

Schill, D.J., Meyer, K.A., and Hansen, M.J. (2017). Simulated effects of YY-male stocking and manual suppression for eradicating nonnative brook trout populations. North American Journal of Fisheries Management *37*, 1054–1066.

Schofield, P.J., Williams, J.D., Nico, L.G., Fuller, P., and Thomas, M.R. (2005). Foreign non-indigenous carps and minnows (*Cyprinidae*) in the United States—A guide to their identification, distribution, and biology. Scientific Investigations Report 2005-5041, pp. 103.

Schrank, S.J., and Guy, C.S. (2002). Age, growth, and gonadal characteristics of adult bighead carp, *Hypophthalmichthys nobilis*, in the Lower Missouri River. Environmental Biology of Fishes *64*, 443–450.

Schrank, S.J., Guy, C.S., and Fairchild, J.F. (2003). Competitive interactions between age-0 bighead carp and paddlefish. Transactions of the American Fisheries Society *132*, 1222–1228.

Sfakianakis, D.G., Georgakopoulou, E., Papadakis, I.E., Divanach, P., Kentouri, M., Koumoundouros, G., (2006). Environmental determinants of haemal lordosis in European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquaculture *254*, 54–64.

Sfakianakis, D.G., Renieri, E., Kentouri, M., and Tsatakis, A.M. (2015). Effect of heavy metals on fish larvae deformities: A review. Environmental Research *137*, 246–255.

Shelton, W., and Rothbard, S. (1993). Determination of the developmental duration (zeta(0)) for ploidy manipulation in carps. Israeli Journal of Aquaculture-Bamidgeh *45*, 73–81.

Shelton, W.L. (1986). Broodstock development for monosex production of grass carp. Aquaculture *57*, 311–319.

Shim, K.F., and Lim, C.P. (1990). The effect of dietary level of manganese on red tilapia. Singapore Journal of Primary Industries *18*, 24–33.

Sin, A.W. (1974). Preliminary results on cryogenic preservation of sperm of silver carp (*Hypophthalmichthys molitrix*) and bighead (*Aristichthys nobilis*). Hong Kong Fisheries Bulletin *4*, 33–36.

Southwick Associates (2007). Sportfishing in America: an economic engine and conservation powerhouse. American Sportfishing Association *Multistate Conservation Grant Program*.

Sparks, R.E., Barkley, T.L, Creque, S.M., Dettmers, J.M., and Stainbrook, K.M. (2010). Evaluation of an electric fish dispersal barrier in the Chicago Sanitary and Ship Canal. In: Chapman, D.C. and Hoff, M.H., (Eds.). Invasive Asian carps in North America, American Fisheries Society, Bethesda, Maryland, Symposium *74*,139–161.

Subba, B.R. (2004). Anomalies in bighead carp *Aristichthys nobilis* and African catfish *Clarias gariepinus* in Biratnagar, Nepal. Our Nature *2*, 41–44.

Sun, Y., Yuan, Z., Tan, S., Fan, J., and Zhou, G. (2015). Induction of gynogenesis in red bighead carp (*Aristichthys nobilis* red var.). Journal of Fisheries of China *39*, 8–15. (in Chinese)

Szlaminska, M., and Przybyl, A. (1986). Feeding of carp (*Cyprinus carpio* L.) larvae with artificial dry food, living zooplankton and mixed food. Aquaculture *54*, 77–82.

Tamas G. (1978). Rearing of common carp fry and mass cultivation of its food organisms in ponds. Proceedings of Conference on Aquaculture: “Cultivation of Fish Fry and its Live Food”, Sept 23-28, 1977, Szymbark, Poland.

Terner, C. (1962). Oxidative and biosynthetic reactions in spermatozoa. In: Bishop D.W. (Ed.), Spermatozoan Motility, American Association for the Advancement of Science, Washington D.C., pp. 89–98.

Thresher, R.E., Hayes, K., Bax, N.J., Teem, J., Benfey, T.J., and Gould, F. (2014). Genetic Control of Invasive Fish: Technological options and its role in integrated pest management. Biological Invasions *16*, 1201–1216.

Tsehaye, I., Catalano, M., Sass, G., Glover, D., and Roth, B. (2013). Prospects for fishery-induced collapse of invasive Asian carp in the Illinois River. Fisheries *38*, 445–454.

Tubbs, C., and Thomas, P. (2008). Functional characteristics of membrane progestin receptor alpha (mPR[alpha]) subtypes: a review with new data showing mPR[alpha] expression in seatrout sperm and its association with sperm motility. Steroids *73*, 935–941.

Twohey, M.B., Heinrich, J.W., Seelye, J.G., Fredricks, K.T., Bergstedt, R. A., Kaye, C. A., Scholefield, R. J., McDonald, R. B., and Christie, G. C. (2003). The Sterile-Male-Release Technique in Great Lakes Sea Lamprey Management. Journal of Great Lakes Research *29*, 410–423.

Udagawa, M. (2001). The effect of dietary vitamin K (phylloquinone and menadione) levels on the vertebral formation in mummichog *Fundulus heteroclitus*. Fisheries Science *67*, 104-109.

Van Der Walt, L.D., Van Der Bank, F.H., and Steyn, G.J. (1993). The suitability of using cryopreservation of spermatozoa for the conservation of genetic diversity in African catfish (*Clarias gariepinus*). Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology *106*, 313–318.

Van der Wind, J.J. (1979). Techniques of rearing phytophagous fishes. FAO Fisheries Report *44*, 227–232.

Vandecan, M., Diallo, A., and Melard, C. (2011). Effect of feeding regimes on growth and survival of *Clarias gariepinus* larvae: replacement of *Artemia* by a commercial feed. Aquaculture Research *42*, 733–736.

Verma, D.K., Routray, P., Dash, C., Dasgupta, S., and Jena, J.K. (2009). Physical and biochemical characteristics of semen and ultrastructure of spermatozoa in six carp species. Turkish Journal of Fisheries and Aquatic Sciences *9*, 67–76.

Viola, S., Mokady, S., Rappaport, U., and Arieli, Y. (1981/1982). Partial and complete replacement of fishmeal by soybean meal in feeds for intensive culture of carp. Aquaculture *26*, 223–236.

Wang, C., Zhu, X., Han, D., Jin, J., Yang, Y., and Xie, S. (2015). Responses to fishmeal and soybean meal-based diets by three kinds of larval carps of different food habits. Aquaculture Nutrition *21*, 552–568.

Weber, G., Hostuttler, M.A., Cleveland, B.M., and Leeds, T.D. (2014). Growth performance comparison of intercross-triploid, induced triploid, and diploid rainbow trout. Aquaculture *433*, 85–93.

Widloe, J., Widloe, T., Lederman, N., and Irons, K. (2017). Asian carp removal project in the Upper Illinois River. In 147th Annual Meeting of the American Fisheries Society. AFS, August 22.

Withler, F.C. (1982). Cryopreservation of spermatozoa of some freshwater fishes cultured in South and Southeast Asia. Aquaculture *26*, 395–398.

Wohlfarth, G.W. and Moav, R. (1972). The regression of weight gain on initial weight in carp. I. Methods and results. Aquaculture *1*, 7–28.

Wolny, P. (1969). Biological, technical and economical grounds for production of stocking material. II. Production of stocking material of three phytophagous fish species. Instruction of the Institute of Inland Fisheries *No. 37*.

Ye, Y., Wang, Z., and Wu, Q. (2008). Increasing the genetic uniformity of bighead carp [*Aristichthys nobilis* (Richardson)] by means of spontaneous diploidization of gynogenetically activated eggs: Genetic uniformity of bighead carp. Aquaculture Research *39*, 205–211.

Zhang, H., Rutherford, E.S., Mason, D.M., Breck, J.T., Wittmann, M.E., Cooke, R.M., Lodge, D.M., Rothlisberger, J.D., Zhu, X., and Johnson, T.B. (2016). Forecasting the impacts of silver and bighead carp on the Lake Erie food web. Transactions of the American Fisheries Society *145*, 136–162.

Zhu, B., Wu, Z.F., Li, J., and Wang, G.X. (2011). Single and joint action toxicity of heavy metals on early developmental stages of Chinese rare minnow (*Gobiocypris rarus*). Ecotoxicology and Environmental Safety *74*, 2193–2202.

Zhu, C., Sun, Y., Yu, X., and Tong, J. (2013). Centromere localization for bighead carp (*Aristichthys nobilis*) through half-tetrad analysis in diploid gynogenetic families. PLoS ONE *8*, 1-9.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., and Vilella, S. (2004). Adenosine triphosphate concentration and beta-D-glucuronidase activity as indicators of sea bass semen quality. Biology of Reproduction *70*, 1679–1684.