Quantifying Male Reproductive Performance of Blue Catfish (*Ictalurus furcatus*) to Improve Hatchery Production of Hybrid Catfish

by

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Abstract

Catfish farming constitutes about 60% of total U.S. aquaculture production, where the channel catfish, *Ictalurus punctatus* female × blue catfish, *I. furcatus* male hybrid accounts for >50% of the harvest. Current hatchery technology to produce hybrids requires the sacrifice of males for in vitro fertilization, and sperm are often of low quality/quantity, negatively affecting fertility and hatch rates. Thus, the overall objective of this thesis was to understand blue catfish male reproduction as it relates to hybrid production. Specific objectives were to: (i) compare sperm swimming kinematics and health metrics before and after cryopreservation; (ii) determine minimum quantity of frozen-thawed sperm required to maximize hatching success; (iii) decipher how early offspring development is affected when eggs were sired with fresh or frozen-thawed sperm; and iv) determine if there is a relationship between reproductive performance traits and sperm kinematic traits utilizing both fresh or cryopreserved sperm. Sperm kinematic traits and health metrics decreased after cryopreservation, adding greater than 5.0×10^4 sperm cryo or fresh?per egg had no significant effect on hatching success, and there was no decline in offspring performance when sired with cryopreserved sperm. Additionally, reproductive performance traits correlated with fresh and cryopreserved sperm kinematic traits. In conclusion, blue catfish male reproductive performance for more practical hybrid production was further quantified.

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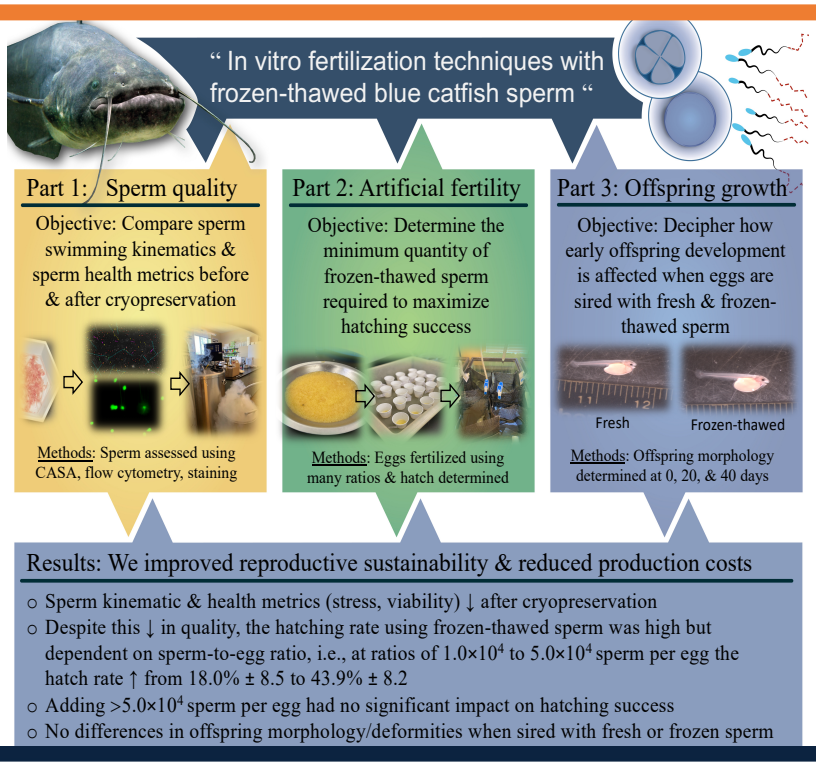
List of Abbreviations

US	United States of America	VCL	Curvilinear velocity
RAS	Recirculating aquaculture system	DNA	Deoxyribonucleic acid
USD	United States dollar	DPH	Days post-hatch
T_L	Total length	N_L	Notochord length
Y_S	Yolk sac area	E_D	Eye diameter
J_L	Jaw length	M_H	Myotome height
B_A	Body area	HBSS	Hank's balanced salt solution
IVF	In vitro fertilization	DH_2O	Deionized water
CASA	Computer Assisted Sperm Analysis	ROS	Reactive oxygen species
T	Testosterone	11-KT	11-ketotestosterone
RPM	Revolutions per minute	PCA	Principal Component Analysis

Chapter 1

In vitro fertilization with frozen-thawed blue catfish (*Ictalurus furcatus*) sperm and implications for gene banking



1.1 Abstract

Catfish farming constitutes roughly 60% of total U.S. finfish aquaculture production, for which the channel catfish female (*Ictalurus punctatus*) × blue catfish male (*I. furcatus*) hybrid accounts for greater than 50% of the harvest. Current hatchery technology to produce hybrids is labor intensive and requires the sacrifice of males. This is expensive and time-consuming, as blue catfish males require 4 to 7 years to mature. Sperm, even from mature fish, are often of low quality and quantity and do not necessarily give high fertility and hatch. Therefore, paternal gametes should be used sparingly, using the minimum quantity of sperm for fertilization, while maximizing offspring production. This is particularly important when using cryopreserved cells, as each males' sperm is in limited supply. To optimize the use of available sperm cells, the objectives of this study were to (i) compare sperm swimming kinematics and sperm health metrics before and after cryopreservation; (ii) determine the minimum quantity of frozen-thawed sperm required to maximize hatching success; and (iii) decipher how early offspring development is affected when eggs are sired with fresh and frozen-thawed sperm. Sperm kinematics were evaluated by computer-assisted sperm analysis, while health metrics (i.e., viability, oxidative stress, DNA fragmentation) were assessed using fluorescent imaging and flow cytometry. Over 2 spawning seasons, eggs from 18 females were collected and fertilized using cryopreserved sperm from 36 males at 10 sperm-to-egg ratios (ranging from 1.0×10^4 to 9.0×10^5 sperm per egg). Embryos sired with fresh and frozen-thawed sperm were then incubated under common environmental conditions and growth performance was documented over 40 days post-hatch. Generally, sperm kinematic traits [curvilinear velocity (VCL), motility, progressive VCL, and progressive motility] and health metrics (cell membrane viability, oxidative stress, and

DNA fragmentation) declined after cryopreservation. Despite this decrease in sperm quality, hatching success using cryopreserved sperm was high, but dependent on the sperm-to-egg ratio. For instance, at ratios of 1.0×10^4 to 5.0×10^4 sperm per egg, hatch increased from 18.0% ± 8.5 to 43.9% ± 8.2. Adding > 5.0×10^4 sperm per egg had no significant effect on hatching success. Furthermore, there were generally no differences in morphology or deformity rates in offspring sired with cryopreserved sperm, compared to a fresh control. These data improve understanding of frozen-thawed sperm quality for blue catfish to improve reproductive sustainability and reduce production costs. Cryopreservation may be a viable option for catfish farmers to improve reproductive efficiency and reduce costs associated with housing blue catfish males.

1.2 Introduction

In 2020, world aquaculture production reached a high of 122.6 million tonnes in live weight with a total farmgate sale value of $281.5 billion USD (FAO, 2022). Because the aquaculture sector is rapidly growing and is relatively new compared to the livestock sector, it offers tremendous opportunities for technological innovation to meet protein demands (Waite et al., 2014; Fry et al., 2016). Currently, the U.S. ranks 18th in global aquaculture production, with nearly 300 million kg of freshwater species harvested in 2019 (NMFS, 2022), with catfish making up most of the yearly harvests (Torrans and Ott, 2018; Engle et al., 2022).

Catfish farming in Alabama, Texas, Arkansas, and Mississippi accounts for nearly 60% of total U.S. freshwater aquaculture production, in which greater than 50% of the harvest is composed of the female channel catfish (*Ictalurus punctatu*s) × male blue catfish (*I. furcatus*) hybrid (Torrans and Ott, 2018; Hegde et al., 2021; Engle et al., 2022; NMFS, 2022). This is mainly because hybrid offspring produced by this cross are superior for pond culture as they exhibit higher growth rates and feed conversion (Dunham et al., 2008; Brown et al., 2011), disease resistance (Arias et al., 2012), tolerance to low dissolved oxygen (Dunham et al., 1983), higher meat production (Bosworth, 2012), and ease of harvest (Dunham and Masser, 2012), making them highly valuable for aquaculture.

Commonly achieved production rates for hybrids reach 13,000 kg/ha, almost double those observed for channel catfish (Brown et al., 2011; Bott et al., 2015). Remarkably, some farmers can achieve production rates approaching 20,000 kg/ha by utilizing hybrid catfish (Kumar et al., 2018). Despite high production rates, demands for hybrid fingerlings have continued to rise, resulting in annual shortages (Hu et al., 2014; Fantini-Hoag et al., 2022).

Although the hybrid industry has experienced sustained growth, there are still challenges associated with reproduction between parent species. Foremost, blue catfish males reach maturity after 4 to 7 years (Graham, 1999), and their sperm cannot be readily extracted by stripping, a common non-lethal practice for other fishes. Consequently, sperm collection for blue catfish is a lethal procedure, requiring the removal and maceration of the testes (Bart and Dunham, 1990). Additionally, since milt can only be acquired once per male there is a substantial investment in sperm. In contrast, female channel catfish reach sexual maturity earlier (after 3 years), and their eggs can be readily hand-stripped for in vitro fertilization (Dunham et al., 1999). These paternal complications suggest that the male contribution is a major bottleneck for the hybrid catfish industry. Recent evidence reveals that a male's contribution to progeny is important in determining offspring performance during the "critical" early life history stages (reviewed in Butts and Litvak, 2007a; Butts and Litvak, 2007b; Pitcher and Neff, 2007; Houde et al., 2015; Siddique et al., 2017; Myers et al., 2020a). Thus, good quality sperm is necessary for offspring performance (Bobe and Labbé, 2010). One beneficial way farmers can ensure "good" sperm is available year after year is to house high-quality cells in germplasm/sperm repositories.

To date, sperm cryopreservation protocols have been developed for commercial-scale applications to alleviate problems relating to male readiness for spawning (Bart et al., 1998). This technique will provide year-round access to sperm whenever females are in peak spawning condition (Hu et al., 2011, 2014). While sperm cryopreservation technology has not been readily adopted by the catfish industry, the USDA-ARS Germplasm Program currently houses blue catfish male sperm (>360 individuals; >9,000 units) frozen in perpetuity (USDA-ARS, 2022; Wang et al., 2022). The development and use of cryogenic technologies has garnered a great deal of interest and activity within the catfish industry. Unfortunately, a high degree of variability in

sperm kinematics between fresh and cryopreserved cells has been reported among males (Linhart et al., 2005; Hu et al., 2011, 2014; Wang et al., 2022). Thus, our limited understanding of the mechanisms involved in sperm form and function, especially as it pertains to frozen-thawed cells, is hindering industry progress. It is therefore of utmost importance that we improve our understanding of the physiological processes associated with sperm quality and quantity and paternal effects for blue catfish so that we can better predict fertility and industry-relevant offspring performance in a hatchery environment.

In vitro fertilization is a technique commonly implemented by hatcheries for fish that do not spawn readily in captivity. Sperm density, or how much sperm is used to fertilize a batch of eggs, can greatly impact fertility and hatch success. The optimal sperm-to-egg ratio varies depending on the species and its associated reproductive physiology, specific mating strategies, and gamete features (reviewed by Butts et al., 2012). Hybrid catfish aquaculture depends on the success of artificial fertilization to produce the annual cohort of fry, which is limited by a lack of natural hybridization between channel catfish and blue catfish. Due to the nature in which blue catfish sperm is collected, paternal resources should be utilized efficiently by using the minimum quantity of sperm from each male while still maximizing fertilization. This is particularly the case when using cryopreserved sperm, as each males' cells are in limited supply, and variability between initial and post-thaw activity of the sperm may impact fertility (Saksena et al., 1961; Makeeva and Emel'yanova, 1993; Small and Bates, 2001; Hu et al., 2011). Thus, further optimization of sperm to egg ratios is important to efficiently produce the valuable hybrid catfish (Myers et al., 2020b).

It is also especially important to determine the potential effects of using cryopreserved gametes on the generation of progeny. The cryopreservation process can result in cellular

damage (Wang et al., 2022), which can impair larval growth and survival (Nusbaumer et al., 2019; Zadmajid et al., 2019). Thus, we need to establish baseline data on how frozen-thawed sperm may impair development through their early life history. Assessing morphometrics in resultant offspring is one important way to examine this, as morphometrics are tied directly to survival and growth. For instance, total length, myotome height, and body area are typically correlated with fish swimming abilities, where increased size often translates into greater prey encounter and attack rates (Osse et al., 1997; Shepherd et al., 2000; Utne-Palm and Stiansen 2002; Ferreira do Nascimento et al., 2015). Yolk-sac area plays a vital role in growth performance during the earliest life stages (Butts and Litvak, 2007a; Sørensen et al., 2016), while eye diameter has been used as a proxy for visual acuity (Job and Bellwood, 1996; Tesser and Portella, 2011; Ferreira do Nascimento et al., 2015). Gape size, defined as the maximum size of prey that fish can consume, is usually quantified by jaw length. In turn, fry/fingerlings capable of feeding on larger food items have increased energy intake and thus can achieve higher growth rates (Rideout et al., 2005; Rahnama et al., 2015). Tracking deformities may also be used as an efficient tool to evaluate the developmental potential of progeny derived from frozen-thawed sperm (Zadmajid et al., 2019). Together, these performance traits provide an understanding of the effects of cryopreserved sperm, as well as the viability of resulting offspring.

In this study, our objectives were to (i) compare sperm swimming kinematics and health metrics before and after cryopreservation; (ii) determine the minimum quantity of frozen-thawed sperm required to maximize hatching success; and (iii) decipher how early offspring development is affected when eggs are sired with fresh and frozen-thawed sperm. Combined, these results will allow us to identify the impacts of cryopreservation on sperm cells and offspring created from cryopreserved milt, allowing for enhancement of current hybrid catfish

production techniques. Furthermore, this will provide farmers with solutions on how to advance hatchery efficiency and profitability, especially regarding reproductive techniques and technologies using frozen-thawed gametes.

1.3 Materials and Methods

All experiments were conducted at the Auburn University E.W. Shell Fisheries Center in Auburn, Alabama, USA (32.6524° N, 85.4860° W). Animal husbandry and experimental protocols were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC # 2019-3710). Water temperature for broodstock ponds during the sampling periods ranged from 19.9°C to 25.4°C in 2021 and 20.4°C to 27.3°C in 2022 with dissolved oxygen maintained at >7 mg/L for both seasons. All broodstock were fed a 32% protein diet (The Feed Lot, Montgomery, AL, USA) until the month leading up to spawning when protein in feed was increased to 36% (Fishbelt Feed Mill, Moorhead, MS, USA).

1.3.1. Female broodstock and spawning induction

Mature female channel catfish were collected from 0.1-acre earthen ponds. After collection, gravid females were held in soft mesh spawning bags (61.0 cm length × 91.4 cm height, 0.32 cm to 0.64 cm mesh, Lee Valley Tools, Ottawa, Ontario, Canada) suspended in fiberglass raceways (6.07 m × 0.91 m × 0.48 m) with a flow rate of ~80 L/min (Su et al., 2013; Chatakondi, 2014). Each female was administered two intraperitoneal injections of luteinizing hormone-releasing hormone analogue (LHRHa) totaling 100 μg/kg with a priming dose of 20 μg/kg (at 10:30 to

11:00 pm) and a resolving dose of 80 µg/kg 12 h later (10:30 to 11:00 am; Lambert, 1998; Su et al., 2013). Ovulation was checked every 4 h beginning 18 h after the resolving dose until eggs adhered to the bags.

1.3.2 Egg collection

Once ovulation was detected, females were anesthetized with 100 ppm MS-222 (tricaine methanesulphonate, Syndel, Ferndale, WA, USA) buffered with 100 ppm $NaHCO_3$ to minimize stress during handling and stripping. The urogenital pore was wiped dry, and eggs were released by applying gentle pressure on the abdomen toward the vent. Extra care was taken to avoid contamination from urine, blood, or feces. Eggs were stripped into a 30 cm diameter metal spawning pan lined with Crisco® All-Vegetable Shortening to prevent adherence to the pan. The total egg mass was weighed to the nearest ± 0.1 g (Ranger 3000 Compact Bench Scale, Ohaus, Parsippany, New Jersey, USA). Egg density was estimated by weighing and counting ~1 g of eggs with two replicate counts to obtain the total number of eggs/g. Eggs were aliquoted into 80 mL plastic cups lined with Crisco® at 250 eggs/cup.

1.3.3 Sperm collection and analyses

Mature blue catfish males were acquired from Jubilee Farms in Indianola, MS, USA (33.5002° N, 90.5815° W) and transported to the E.W. Shell Fisheries Center, where they were held in 0.1-acre ponds and fed to satiation (36% protein diet) every other day until the end of sampling. Males were euthanized following industry (and AU-IACUC) approved protocols and

testes were dissected from each fish using forceps and surgical scissors. Testes were separated from the peritoneum and blood vessels before being macerated through a fine mesh strainer (200 µm) into 50 mL centrifuge tubes. Sperm were then diluted to 1×10^9 cells/mL (Hu et al., 2014) with Hank's Balanced Salt Solution (HBSS; reverse osmosis deionized water with 8 g/L NaCl, 0.4 g/L KCl, 0.16 g/L $CaCl_2\times2H_2O$, 0.2 g/L $MgSO_4\times7H_2O$, 0.12 g/L $Na_2HPO_4\times7H_2O$, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, 1 g/L glucose; Tiersch, 1997). Sperm density was determined using a Neubauer hemocytometer following Myers et al. (2020b). Sperm samples were then stored at 4 °C until assessment and cryopreservation (*see Sections 2.3.1 to 2.3.4 and 2.4*).

Computer-Assisted Sperm Analysis (CASA)

CASA software (CEROS II, Hamilton Thorne Biosciences, Beverly MA, USA) was used to quantify sperm activity traits according to Myers et al. (2020b). In brief, 0.1 µL of sperm solution (diluted to 1×10^9 cells/mL) were pipetted in an 80 µm 2X-CEL chamber (Hamilton Thorne Biosciences, Beverly MA, USA) and activated with 12 µL DH_2O supplemented with 0.5% bovine serum albumin (126609, Merck Millipore, Burlington MA, USA) to prevent sperm from sticking to slides. Three technical replicate activations were completed per male, where sperm velocity (VCL; µm/s), percent motility, progressive VCL (µm/s), and progressive motility (%) were assessed at 10, 20, and 30 s post-activation. These kinematic traits were chosen as they typically correlate to fertility in both competitive and non-competitive spawning environments (Lahnsteiner et al., 1998; Gage et al., 2004; Linhart et al., 2005; Butts et al., 2011). Sperm samples and activation media were kept at 4ºC using an Echotherm™ Chilling/Heating Dry Bath (Torrey Pines Scientific, Carlsbad, CA, USA) for CASA analyses.

CASA videos were manually checked for quality control after experimentation following protocols from Butts et al. (2011). In brief, sperm tracks were removed from analysis if the software split the track of a single sperm, sperm were drifting, sperm swam out of the field of view before being adequately assessed, or if the software incorrectly combined crossing tracks of multiple sperm.

Sperm cell viability

Cell viability was assessed by flow cytometry using a Guava Muse Cell Analyzer® (EMD Millipore Corporation) following proprietary protocols in the Muse® Count and Viability kit (Luminex, Austin TX, USA). This kit utilizes two fluorescent dyes, of which one is a membrane-permeable dye that stains all cells with a nucleus and distinguishes cells from debris, while the second dye only stains dead or dying cells that have lost membrane integrity. First, sperm cells from each male were diluted in triplicate with HBSS ranging from 1×10^6 to 1×10^7 cells/mL. Next, 20 µL of cell suspension was mixed with 380 µL of Count and Viability Reagent in 1.7 mL microcentrifuge tubes. Tubes were then inverted and allowed to incubate for 5 min in the dark at room temperature (~21°C). After the 5 min incubation, count and viability were determined. The Cell Size Index sliders (gates) were adjusted to exclude cellular debris and include the cell populations (both living and dead cells). Another gate adjustment (viability discriminator) was used to separate the viable cells from the dead cells. These adjustments ensured no debris was counted as a cell and there was clear differentiation between the viable and dead cells.

Oxidative stress

Oxidative stress of sperm cells was assessed by flow cytometry following proprietary protocols in the Muse® Oxidative Stress Kit (Luminex, Austin, TX, USA) with three technical replicates per male. Sperm cells were diluted with 1X Assay Buffer to concentrations ranging from 1×10^6 to 1×10^7 cells/mL. Next, 10 µL of the sperm suspension was mixed with 190 µL Oxidative Stress Working Solution and incubated at 37ºC (Echotherm™ Chilling/Heating Dry Bath, Torrey Pines Scientific, Carlsbad, CA, USA) for 30 min. Two cell populations were determined: negative cells (ROS-) or live cells and cells exhibiting ROS activity (ROS+). A positive control, that completely oxidized cells, was used to differentiate between ROS- and ROS+ cell populations. This involved incubating the sperm at 37°C for 60 min in the presence of 2-methyl-1,4-naphthoquinone, 98% (Acros Organics, Geel, Belgium) at 2M.

DNA fragmentation

DNA fragmentation was assessed utilizing a HaloMax® DNA Fragmentation kit (Halotech DNA, Madrid, Spain). In brief, sperm samples were diluted between 1.5×10^7 and 2.0×10^7 cells/mL. Agarose gel was melted in a water bath at 95ºC and equilibrated to 37ºC for 5 min. Next, 50 µL of liquified agarose gel was combined with 25 µL of sperm. A 2 µL drop of cell suspension was then placed on a 24 mm × 24 mm glass coverslip with 5 technical replicate slides per male. Slides were then held at 4ºC for 5 min so the gel could solidify. Slides were then brought up to room temperature (~21ºC) before sperm processing and staining. Digital images were taken using a Zeiss Axio Imager A2 (Carl Zeiss Microscopy, LLC, White Plains, NY,

USA) with 40× Plan Achromat phase contrast objective, GFP longpass fluorescence filter, Axiocam 202 monochrome camera, and ZEN 2.5 Blue hardware license key. Digital images were then analyzed by counting fragmented cells, identified by their unclear margins and larger size, as a percentage of total cells. This was done by dividing the number of fragmented cells counted by the total cells counted, and then multiplying by one hundred.

1.3.4 Sperm cryopreservation

Sperm concentration was adjusted to 1×10^9 cells/mL with HBSS plus 10% methanol (Hu et al., 2011, 2014). Sperm suspensions were then pipetted into 0.5 cc straws, ~20 straws per male, (Minitube USA, Verona, WI, USA) before they were sealed with metal beads and positioned on metal racks (40 straws per rack). Straws were then placed in a control rate freezer (Planer Kryo 560-16, Cryo Associates Inc., Gaithersburg, MD, USA) for a ~30 min equilibration at 5ºC from the time methanol was added to the initiation of the freezing process. Straws were then frozen at a rate of -5ºC/min until they reached -80ºC. At the end of the freezing cycle, samples were immediately transferred into liquid nitrogen. Frozen samples were stored in a vapor-pressure liquid nitrogen storage system (MVE 815P-190F-GB, Cryo Associates Inc. Gaithersburg, MD, USA).

1.3.5 Embryo and fry husbandry conditions

Embryos and fry were housed in recirculating aquaculture systems (RAS). There were three RAS, each equipped with a UV filter (Emperor Smart DC2305, Pentair Aquatic Eco-Systems,

Apopka, Fl, USA), bead filtration system (Bubble Bead Filter XS10000, Aquaculture Systems Technology, Baton Rouge, LA, USA), 122 cm acrylic filter media cup (DVH Aquatics, Groningen, Netherlands), bag filter (Pall x-100, Pall Corporation, Port Washington, New York, USA), 0.5 hp pump (PerformancePro Cascade, Cascade Pump Company, Santa Fe Springs, CA, USA), 17 × 37.85 L (60 cm × 30 cm × 32 cm) polycarbonate aquaria, three 795 L circular blue tanks, two 190 L sump tanks, and a heat pump (AquaLogic Delta Star DSHP-9, Aqua Logic Inc, San Diego, CA, USA), or in-line heater (AquaLogic Titanium Evo Z31E, Aqua Logic Inc, San Diego, CA, USA). In addition, individual aquaria were equipped with diffused air to saturation, a water flow rate of ~7.5 L/min, maintained at 27.1 ± 0.5ºC, and 2 to 4 mesh baskets (18 cm × 16 cm × 14 cm) for incubating embryos.

All RAS were supplied with water of identical quality from a dechlorinated water tower supplied from Auburn City Water Works. Water quality was assessed using a spectrophotometer (D/R 2000 Direct Reading, Hach, Colorado, USA) and pH meter (pH30 meter, Oakton Instruments, Vernon Hills, IL, USA), where ammonia and nitrite levels were kept at 0 to 0.05 mg/L, hardness between 75 and 100 ppm, and dissolved oxygen between 7.75 and 8.05 mg/L. Oxygen levels and temperature were monitored daily with a YSI model 58 with 550A probe (YSI, Yellow Springs, OH, USA) to ensure they remained within recommended levels.

All unfertilized and dead embryos, identified by their white or opaque color and/or enlarged size, were removed daily using a plastic pipette or forceps to minimize fungal infections until hatch (Blaxter 1992; Small and Bates, 2001). Removal was done with minimal disturbance. Dead eggs that adhered to healthy embryos were left undisturbed to not cause unnecessary stress or damage to healthy embryos. For fry kept for experimentation after hatch, stocking densities were

kept at 100 fry/37.85 L tank. Fry were fed fry powder (Purina AquaMax Fry Powder, Purina Mills, St. Louis, Missouri, USA) three times daily to satiation.

1.3.6 Experiment 1: Comparison of fresh and cryopreserved sperm quality

Sperm samples from 43 blue catfish were collected from 6 to 13 May 2021 (average ± SD length and weight were 84.0 cm ± 4.5 and 4.4 kg ± 1.0, respectively) and from 40 males from 9 to 13 May 2022 (average ± SD length and weight were 72.0 cm ± 6.0 and 5.8 kg ± 1.3, respectively). For each male, an aliquot of sperm was cryopreserved, while another aliquot was used for the evaluation of fresh sperm quality. Sperm density, kinematics, and cell health metrics including viability, oxidative stress, and DNA fragmentation were then measured on both fresh and cryopreserved sperm (*see Sections 2.3.1 to 2.3.4 and 2.4*).

1.3.7 Experiment 2: Minimal sperm to egg ratio for fertilization using cryopreserved sperm

Eggs from 18 females (n = 6 in 2021; n = 12 in 2022) were crossed with the sperm from 2 males, on average, resulting in a total of 36 parental half-sibling families. Cryopreserved milt from 36 males (n = 24 in 2021; n = 12 in 2022) was thawed at 40ºC for 20 s before being transferred into 1.5 mL microcentrifuge tubes. Each year, sperm were diluted to obtain five sperm-to-egg ratios: 9.0×10^4, 1.0×10^5, 2.3×10^5, 4.5×10^5, and 9.0×10^5 sperm to egg in 2021, and 1.0×10^4, 2.5×10^4, 5.0×10^4, 7.5×10^4, and 1.0×10^5 sperm to egg in 2022. This enabled testing of a wide spectrum of ratios to see if adding more sperm inhibited hatch success (i.e., polyspermy)

and to find the lowest amount of sperm required for fertilization to conserve cells frozen in genetic repositories.

Fresh milt was also extracted from males (n = 15 in 2021; n = 16 in 2022) less than 12 h before fertilization occurred. All males were tested for sperm quality (*see Sections 2.3.1 to 2.3.4*) and stored at 4ºC. A fresh control ratio of 1.0×10^4 sperm per egg was used based on results from Myers et al. (2020b).

The final volume for fresh and cryopreserved sperm was standardized to 1.0 mL using HBSS with 3 replicates for each ratio. Mean aliquots of 250 ± 10 eggs were weighed according to each female's (n = 18) specific egg density. They were then transferred to 80 mL plastic cups lined with vegetable extract (Crisco® vegetable extract). Sperm solutions for each ratio were pipetted directly onto the eggs and allowed to sit for 2 min. Each cup was then activated with 5 mL of hatchery water mixed with Fuller's Earth (6 g/L; MP Biomedicals, Santa Ana, CA, USA) to reduce the clumping of eggs. Eggs were allowed to incubate with the sperm and Fuller's Earth solution for 3 to 5 min with continuous manual stirring. Stirring ensured sufficient gamete contact. Samples were then transferred into incubation aquaria. Hatch success was determined by calculating the total number of hatched larvae divided by the initial number of eggs in each basket (250 ± 10 eggs) multiplied by 100, to obtain a hatching percentage.

1.3.8 Experiment 3: Offspring performance from fresh and cryopreserved sperm

In 2022, fresh and cryopreserved milt from the same males (n = 9) were used for fertilization. Cryopreserved milt was thawed at 40ºC for 20 s before it was transferred into 1.5 mL microcentrifuge tubes. Males 1, 2, and 3 were used to fertilize Female 1; Males 4, 5, and 6

fertilized Female 2; and Males 7, 8, and 9 fertilized Female 3. For each female, an aliquot of fresh and cryopreserved sperm from each male was equally pooled so that all males had an equal chance of fertilizing an egg. The fresh and cryopreserved cells were kept separate to create two treatments, a fresh control and a frozen-thawed experimental treatment. All eggs were fertilized using a conservative sperm to egg ratio of 1×10^5 sperm per egg and there were six replicate fertilization events per treatment (fresh and cryopreserved) for each female (*see section 2.7 for fertilization techniques*).

Offspring morphology

At peak hatch, yolk-sac fry from the fresh and cryopreserved treatments were pooled, keeping each female and treatment separate. Yolk-sac fry from each treatment (n = 100) were gently pipetted into glass aquaria and reared for 40 days (*see section 2.5 for rearing conditions*). Digital images of 15 fish from the fresh and cryopreserved treatments were taken at 0-, 20-, and 40-days post-hatch (DPH) to track morphological development and deformities. Fish [3 females × 2 sperm treatments (fresh/cryopreserved) × 3 replicate tanks × 15 fish × 3 sampling periods = 810 fish] were euthanized prior to handling using 100 ppm MS-222 buffered with $NaHCO_3$. Digital images were taken using a Zeiss Discovery.V12 SteREO (Carl Zeiss Microscopy, LLC, White Plains, NY, USA) fitted with a Zeiss Plan S 1.0× FWD 81 mm objective, Axiocam 305 color camera, and ZEN 2.5 Blue hardware license key.

Measurements included total length (T_L; distance from the tip of the snout to the fork of tail), notochord length (N_L; distance from the tip of snout to end of notochord), yolk-sac area (Y_S), eye

diameter (E_D), jaw length (J_L), myotome height (M_H; body height immediately posterior to anus), and body area (B_A; excluding fin fold area and yolk sac) were acquired for each individual.

Determination of deformities

Deformities were identified and counted visually at 0, 20, and 40 DPH from the same fish used for morphology [3 females × 2 sperm treatments (fresh/cryopreserved) × 3 replicate tanks × 15 fish × 3 sampling periods = 810 fish)]. Deformities were determined according to Myers et al. (2020a). In brief, pericardial (edema), head (deformed head shape), eye (no eye/abnormal eye), yolk (abnormal lobe shape), tail (bent, curled, or missing), and spine (bent curvature) deformities were noted. Percent deformities were then calculated as the total deformed fish sampled divided by the total number of fish per aquaria, multiplied by 100 to obtain a percentage.

Offspring weight and survival

At 40 DPH, individual fish weights were taken using a 250 mL beaker (Fisher Scientific, Waltham, MA, USA) and scale (Scout® Balance Scale, Ohaus, Parsippany, New Jersey, USA). Fish were counted as they were weighed and the total amount of fish per tank was divided by the initial number of fish in the tank (n = 100) multiplied by 100 to obtain a survival percentage.

1.3.9 Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality using Shapiro-Wilk test, and homogeneity of variance using plots of residuals vs. predicted values. When necessary, data were $\log_{10}$ or arcsin square root transformed (for percentage data) to meet assumptions of normality and homoscedasticity. Post-hoc analyses were composed using Tukey's HSD multiple comparisons procedure. Treatment means were distinguished using the least-squared means method. Alpha was set at 0.05 for interactions and main effects. All values were expressed as means ± standard error.

Comparison of fresh and cryopreserved sperm quality

A series of repeated measures mixed-model ANOVAs were run to compare sperm kinematic traits between fresh and cryopreserved sperm treatments. These models contained the Time Post-Activation (10, 20, and 30 s post-activation; fixed factor) and Sperm Treatment (fresh or cryopreserved; fixed factor) main effects as well as the Time Post-Activation × Sperm Treatment interaction (fixed factor). The "subject" on the Repeated statement was Male ID (random factor). Each kinematic trait was analyzed separately for each spawning season utilizing these models. If a significant Time Post-Activation × Sperm Treatment interaction was identified, the models were broken-down to look at the effect of each sperm treatment at each time post-activation using paired t-tests. If no significant interaction was found, the Sperm Treatment and Time Post-Activation main effects were interpreted.

Using the 2021 data, a series of t-tests were run to compare viability, oxidative stress, and DNA fragmentation between treatments.

Minimum sperm to egg ratio for fertilization using cryopreserved sperm

Hatch success data were analyzed to assess hatch both across females (n = 18) and within females. This was done using several different approaches. First, hatch success data from all females were analyzed utilizing a mixed-model factorial ANOVA where Sperm-to-Egg Ratio (fixed factor), Female (random factor), Male nested within Female (random factor), Sperm-to-Egg Ratio × Female (random factor) and Sperm-to-Egg Ratio × Male nested within Female (random factor) were the model terms. This mixed-model factorial ANOVA model was run for each spawning season (in 2021 using "high" sperm-to-egg ratios from 9.0×10^4 to 9.0×10^5 sperm per egg and in 2022 using "low" ratios from 1.0×10^4 to 1.0×10^5). Post-hoc analysis was not performed on random factors. Variance components (VC) were generated instead, using the restricted maximum likelihood (REML) method, and expressed as a percentage. Variability among VCs >0 was also tested. Likelihood ratio statistics were constructed using *-2Res Log Likelihood* between the full model (as explained above) and a model with a given VC set to zero using the PARMS statement. The resulting chi-square statistics were one-tailed so these probabilities were halved to obtain a significance value for each VC (Littell et al., 1998, Messina and Fry, 2003; Fry, 2004). To compare fresh sperm (1.0×10^4 sperm per egg) and cryopreserved sperm (5.0×10^4 sperm per egg), a mixed-model ANOVA was used with Sperm Treatment as the fixed factor and Female as the random factor.

Secondly, hatch success data were analyzed for each individual female using a mixed-model ANOVA with the Sperm-to-Egg Ratio as the fixed factor and Male as the random effect.

Offspring performance from fresh and cryopreserved sperm

Offspring performance data were analyzed using a few different approaches to look at differences between treatments across females (n = 3 females) and within females. Firstly, morphological traits (T_L, N_L, Y_S, E_D, J_L, M_H, B_A) from eggs fertilized with fresh and frozen-thawed sperm were compared using a repeated measures mixed-model ANOVA. These models contained the Days Post-Hatch (0, 20, and 40 DPH; fixed factor), and Sperm Treatment (fresh and cryopreserved; fixed factor) main effects and the Days Post-Hatch × Sperm Treatment interaction (fixed factor). Female ID was the random factor (n = 18) with the rearing tank as the "subject" on the Repeated statement. If a non-significant interaction was detected, the Sperm Treatment and Days Post-Hatch main effects were interpreted. Yolk-sac area was analyzed separately using a mixed-model ANOVA since there was only one-time point for this measurement. Sperm Treatment was the fixed factor and Female ID was the random effect.

Secondly, morphological traits were analyzed for each individual female using repeated measures mixed-model ANOVAs. Each ANOVA model contained the following fixed factors: Days Post-Hatch, Sperm Treatment, and the Days Post-Hatch × Sperm Treatment interaction. These models were then broken-down and interpreted at each DPH, as above.

Larval deformity data was analyzed to look at differences between treatments. Each fish was evaluated for pericardial, head, eye, yolk, tail, and spine deformities. There were few deformities observed within either treatment and no head deformities were observed at all. We summed the frequencies of each deformity (pericardial, eye, yolk, tail, and spine) across sampling days and all females to generate a 5×2 Chi-Square analysis to examine the effect of cryopreservation on

progeny deformity. Frequency of occurrence of deformities for the progeny from both fresh and cryopreserved sperm were compared using a Chi-square test (Zar, 2010).

Final wet weight and survival data were analyzed using mixed-model ANOVAs with Sperm Treatment as the fixed factor and female as the random effect. These two traits were analyzed separately for each female.

1.4 Results

1.4.1 Experiment 1: Comparison of fresh and cryopreserved sperm quality

Computer-assisted sperm analysis (CASA)

The Time Post-Activation × Sperm Treatment interaction was significant for VCL (2021: $F_{2,170} = 28.21$, $P < 0.0001$; 2022: $F_{2,186} = 108.72$, $P < 0.000$; Fig. 1.1AB) and progressive VCL (2021: $F_{2,170} = 84.61$, $P < 0.0001$; 2022: $F_{2,175} = 21.45$, $P < 0.0001$; Fig. 1.1CD) during the 2021 and 2022 spawning seasons. Therefore, the saturated models were decomposed to look at the effects of sperm treatment at each time post-activation. Besides VCL at 10 s post-activation in 2021 ($t_{34} = 1.70$, $P = 0.098$; Fig. 1.1A), the fresh sperm swam significantly faster than the cryopreserved sperm during both spawning seasons ($P \leq 0.022$; Fig. 1.1AB). Progressive VCL of the fresh sperm was also significantly higher at 20 s post-activation in 2021 ($t_{34} = 8.82$, $P < 0.0001$; Fig. 1.1C) and at 10, 20, and 30 s post-activation in 2022 ($t_{37} \geq 2.65$, $P \leq 0.012$; Fig. 1.1D).

The Time Post-Activation × Sperm Treatment interaction was not significant for sperm motility (2021: $F_{2,170} = 1.02$, $P = 0.363$; 2022: $F_{2,184} = 0.40$, $P = 0.673$; Fig. 1.2AD) nor for progressive motility (2021: $F_{2,170} = 1.44$, $P = 0.240$; 2022: $F_{2,183} = 0.57$, $P = 0.566$; Fig. 1.2GJ) during the 2021 and 2022 spawning seasons. As such, the Time Post-Activation and Sperm Treatment main effects were interpreted separately. During both spawning seasons, fresh sperm had significantly higher motility (2021: $F_{1,170} = 337.17$, $P < 0.0001$; 2022: $F_{2,184} = 748.76$, $P < 0.0001$; Fig. 1.2BE) and progressive motility (2021: $F_{1,170} = 399.67$, $P < 0.0001$; 2022: $F_{2,183} = 673.71$, $P < 0.0001$; Fig. 1.2HK) than the cryopreserved sperm. Motility ($F \geq 16.82$, $P < 0.0001$; Fig. 1.2CF) and progressive motility ($F \geq 13.63$, $P < 0.0001$; Fig. 1.2IL) also decreased over time in both years.

Sperm cell viability, oxidative stress, and DNA fragmentation

Mean (± SEM) cell viability significantly decreased from 80.7% ± 1.9 in fresh sperm to 29.3% ± 2.4 in cryopreserved sperm ($t_{21} = 19.14$, $P < 0.0001$; Fig. 1.3A). Furthermore, cryopreservation caused a significant increase in oxidative stress from 12.9% ± 0.97 in fresh sperm to 62.8% ± 2.14 in cryopreserved sperm ($t_{31} = 6.85$, $P < 0.0001$; Fig. 1.3B). Cryopreservation also increased the number of cells with fragmented DNA ($t_{16} = 22.25$, $P < 0.0001$; Fig. 1.4A-C).

1.4.2 Experiment 2: Minimal sperm-to-egg ratio for fertilization using cryopreserved sperm

Mean hatching success, using cryopreserved sperm at ratios of 1.0×10^4 to 1.0×10^5 sperm per egg in 2022 and 9.0×10^4 to 9.0×10^5 in 2021 ranged from 18.0% ± 8.55 to 54.6% ± 8.26 and from 49.4% ± 4.95 to 56.3% ± 4.93, respectively (Fig. 1.5AB). Sperm to egg ratio significantly influenced hatching success, where the total number of hatched yolk-sac fry increased from 1.0×10^4 to 2.5×10^4 sperm per egg and again at 5.0×10^4 sperm per egg ($F_{4,16.6} = 16.15$, $P < 0.0001$; Fig. 1.5A). Thereafter, adding greater than 5.0×10^4 sperm per egg had no significant effect on hatching success ($F_{4,264} = 1.84$, $P = 0.121$; Fig. 1.5AB).

The fresh control, at 1.0×10^4 sperm per egg, was then compared to the "ideal ratio" (5.0×10^4 of sperm per egg) using cryopreserved sperm. Results showed no significant difference in hatching success when eggs were sired with either fresh or frozen-thawed sperm at these ratios ($F_{1,48.1} = 2.33$, $P = 0.134$; Fig. 1.5C).

In 2021, using ratios ranging from 9.0×10^4 to 9.0×10^5 sperm per egg, the female VC was significant ($P < 0.0001$) for hatching success and explained over half of the model's variance (51.4%). The female VC was also highly significant in 2022 using ratios ranging 1.0×10^4 to 1.0×10^5 sperm per egg (VC = 59.7%, $P < 0.0001$). The female × sperm to egg ratio VC was also significant using the lower ratios (VC = 14.5%, $P < 0.0001$). All other VC were non-significant (all $P > 0.05$).

Sperm per egg ratio also showed variation in significance dependent on the female. For instance, adding greater than 5.0×10^4 sperm per egg had no effect on hatching success for Females 1-3 ($P < 0.0001$; Fig. 1.5D-F), while adding greater than 2.5×10^4 sperm per egg had no effect on hatching success for Females 4 and 5 ($P < 0.0001$; Fig. 1.5GH). Female 6 showed no

significance across ratios (Fig 1.5I). In addition, fertilizing eggs at higher sperm per egg ratios (from 9.0×10^4 to 9.0×10^5 sperm per egg in 2021) did not impact hatching success for Females 7-18 (Fig 1.5J-U).

1.4.3 Experiment 3: Offspring performance from fresh and cryopreserved sperm

Offspring morphology and deformities

Offspring morphology (Fig. 1.6A) was measured for all females combined to determine the effects of Days Post-Hatch and Sperm Treatment (Fig. 1.6). The Days Post-Hatch × Sperm Treatment interaction was not significant for T_L ($F_{2,36.1}$= 0.3, P = 0.705; Fig. 1.6B), N_L ($F_{2,34.4}$ = 0.45, P = 0.642; Fig. 1.6E), J_L ($F_{2,35.6}$ = 0.35, P = 0.704; Fig. 1.6H), E_D ($F_{2,32}$ = 0.65, P = 0.527; Fig. 1.6K), M_H ($F_{2,34.1}$ = 0.37, P = 0.692; Fig. 1.6N), and B_A ($F_{2,36.6}$ = 1.83, P = 0.175; Fig. 1.6Q). Sperm treatment ($F \geq 0.40$, $P \leq 0.528$) did not significantly impact the six morphological traits measured, where offspring had similar morphology when produced with either fresh or frozen-thawed sperm (Fig. 1.6C, F, I, L, O, R). However, days post-hatch did have a significant effect on these six morphological features (Fig. 1.6D, G, J, M, P, S) where morphological features increased in size from 0 to 20 days and again from 20 to 40 days. Y_S was also not impacted by sperm treatment ($F_{1,14}$ = 0.47, P = 0.503) at 0 DPH (fresh mean = 7.2 mm^2; cryopreserved mean = 7.6 mm^2).

Each female was then analyzed separately for all morphological traits (Fig. 1.7). Here, the Days Post-Hatch × Sperm Treatment interaction was significant for Female 1 E_D ($F_{2,9.38}$ = 5.33, P = 0.028. Fig. 1.7J), and Female 2 N_L ($F_{2,7.84}$ = 4.71, P = 0.045, Fig. 1.7E), M_H ($F_{2,7.67}$ = 9.69, P =

0.008, Fig. 1.7N), and B_A ($F_{2,8.34} = 8.04$, $P = 0.011$, Fig. 1.7Q). As such, these four models were broken-down and interpreted at each DPH. For Female 1, fish sired with frozen-thawed sperm had larger E_D at 20 DPH ($F_{2,7.84} = 4.71$, $P = 0.045$, Fig. 1.7J), with no differences detected at 0 and 40 DPH. For Female 2, fresh and frozen-thawed sperm had similar N_L across all sampling times ($F_{2,7.84} = 4.71$, $P \leq 0.059$, Fig. 1.7E), while eggs sired by frozen-thawed sperm had a smaller M_H ($F_{2,7.67} = 9.69$, $P = 0.008$, Fig. 1.7N) and B_A ($F_{2,8.34} = 8.04$, $P = 0.011$, Fig 1.7Q) than those sired by fresh sperm at 0 DPH; no differences were detected for these traits at 20 and 40 DPH. All other morphological traits had no significant Days Post-Hatch × Sperm Treatment interaction. Treatment was significant for Y_S ($P \leq 0.03$) where, in general, cryopreserved offspring had a higher Y_S (Female 1: fresh = 8.58 mm^2, cryopreserved = 10.62 mm^2; Female 2: fresh = 7.29 mm^2, cryopreserved = 6.21 mm^2; Female 3: fresh = 5.67 mm^2, cryopreserved = 5.89 mm^2).

There was no effect of cryopreservation on the frequency of occurrence of deformities (Chi-square df = 4, $P = 0.77$; Fig 1.6T-V).

Final offspring weight and survival

Fresh and cryopreserved treatments yielded no significant differences for total weight at 40 DPH ($F_{1,14} = 2.03$, $P = 0.176$, Fig. 1.8AB). Three females analyzed separately for total weight did not show significance when eggs were sired with fresh or frozen-thawed sperm: Female 1 ($F_{1,4} = 0.24$, $P = 0.651$, Fig. 1.8C), Female 2 ($F_{1,14} = 0.65$, $P = 0.464$, Fig. 1.8D), and Female 3 ($F_{1,14} = 1.99$, $P = 0.231$, Fig. 1.8E)

These results were also reflected in the survival data. Total survival across all three females was not significantly different between fresh and cryopreserved treatments ($F_{1,14} = 0.79$, $P = 0.390$, Fig. 1.9A). When females were analyzed separately, Female 1 ($F_{1,4} = 0.06$, $P = 0.815$, Fig. 1.9B), Female 2 ($F_{1,4} = 0.84$, $P = 0.411$, Fig. 1.9C), and Female 3 ($F_{1,4} = 0.91$, $P = 0.393$, Fig. 1.9D) survival across fresh and frozen-thawed treatments were not significant.

1.5 Discussion

Access to high quality gametes and offspring is one of the leading bottlenecks for hybrid catfish production in the U.S. (Bart and Dunham, 1996; Bobe and Labbé, 2010; Hu et al., 2014; Myers et al., 2020ab; Fantini-Hoag et al., 2022). In this study, several key findings to improve male gamete storage and hatchery production of hybrid catfish are reported: 1) post-thaw sperm quality showed a decrease in kinematic and health metrics; 2) adding $>5.0\times10^4$ frozen-thaw sperm per egg did not increase hatch success when all females were included, but ratio was also dependent on the female; and 3) frozen-thawed sperm generally had no impact on offspring morphology or occurrence of deformities within the first 40 DPH.

1.5.1 Experiment 1: Comparison of fresh and cryopreserved sperm quality

Over the years, cryopreservation protocols have been developed for aquatic organisms. Unfortunately, sperm damage resulting from the freezing-thawing process is often an unavoidable effect of these procedures. Sperm kinematic traits have been shown to predict the cells' ability to withstand the cryopreservation process (Butts et al., 2011; Duračka et al., 2023),

but more research is still needed to fill knowledge gaps and identify the main causes of poor sperm tolerance during cold storage. In this regard, both kinematic and health metrics are important for sperm form and function, where health metrics (i.e., DNA fragmentation, cell viability, and oxidative stress) are often related to kinematic parameters (Sanocka and Kurpisz, 2004; Cabrita et al., 2011). In turn, faster-swimming sperm are more likely to successfully fertilize an egg (Linhart et al., 2000; Gage et al., 2004, Butts et al., 2011). Thus, these sperm traits have a direct role in fertility and hatch success.

In our study, sperm kinematic and health metrics were both reduced following the freezing-thawing process. These results are consistent with previous findings for blue catfish (Hu et al., 2011, 2014), as well as other fish species (Butts et al., 2010; Cabrita et al., 2001, 2011; Gürler et al., 2016). For example, a study on blue catfish showed a decrease in post-thaw motility, but cryopreserved sperm could still serve as an alternative to fresh sperm (Hu et al., 2011). In rainbow trout (*Oncorhynchus mykiss*) a significant decrease in sperm motility and viability after cryopreservation was observed when compared to a fresh control (Cabrita et al., 2001). Together, these results (among others) show that cellular damage and decreases in post-thaw sperm quality are not species-specific.

A relationship between cell kinematics and health metrics in frozen-thawed sperm has also been studied. Typically, when kinematic metrics decrease, sperm health metrics have also shown a reduction in quality. In European sea bass (*Dicentrarchus labrax*), cryopreserved sperm decreased in viability, while DNA fragmentation increased (Cabrita et al., 2011). In accordance with these findings, there was a decrease in total motile and progressive sperm cells. This study also showed that sperm are exposed to oxidative stress during dilution in extender media, after exposure to cryoprotectants (in this case, dimethyl sulfoxide), and during the cooling process

(Cabrita et al., 2011). These results were also seen in rainbow trout (Cabrita et al., 2001, 2011; Gürler et al., 2016). In accordance with these studies, our recent findings (this study and Wang et al., 2022) collectively suggest that physiological and cellular processes are linked to sperm quality following the freezing process. Also, in our recent work (Wang et al., 2022) we investigated gene expression changes caused by cryopreservation using transcriptome profiles of fresh and cryopreserved sperm. Here, RNA-seq study identified 849 upregulated genes after cryopreservation, including members of all five complexes in the mitochondrial electron transport chain. This suggests a boost in oxidative phosphorylation activities during freezing and thawing which often leads to excessive production of reactive oxygen species (ROS) associated with cellular death. Finally, our functional enrichment analyses revealed changes in gene expression after cryopreservation to offset these negative effects of cryogenic storage. Further molecular work, combined with physiological and sperm kinematic assays, is urgently needed to provide novel insights into underlying damages caused by cryopreservation so that hatchery production can be optimized.

1.5.2 Experiment 2: Minimal sperm to egg ratio for fertilization using cryopreserved sperm

To accurately assess sperm quality and fertilization potential, assisted reproduction standards need to be established to control key variables like sperm density, as sperm number is highly correlated with quality (Gage et al., 2004). Hatching success will increase when more sperm are incorporated into an artificial spawning environment, but the benefit of adding more sperm will decrease as the total number of cells approaches the number required to fertilize all available eggs (Myers et al., 2020b). The optimal sperm to egg ratio is one of the most important

parameters when executing assisted reproduction. In commercial hatcheries, milt is often pooled from a large variety of males, using an excessive amount of sperm, and wasting limited paternal gametes (Myers et al., 2020b). Due to the limited supply of sperm and the expenses associated with obtaining it, utilizing the optimum sperm to egg ratio is especially important when utilizing cryopreserved cells.

As previously shown, cryopreserved sperm continuously underperforms when compared to fresh controls as shown not only in this study, but throughout the literature and across species. This shows that cryopreserved sperm undergoes cellular damage that may affect fertilization success (Wang et al., 2022). While frozen-thawed sperm is still able to fertilize eggs, it will take a higher sperm density to maximize hatch success from these cells. Previous studies have also looked at optimizing the number of cells used to maximize fertility (Bart and Dunham, 1996; Butts et al., 2009; Babiak et al., 2012; Butts et al., 2012; Siddique et al., 2015; Myers et al., 2020b; Yang et al., 2020), using fresh sperm and often in amounts exceeding what is required for successful fertilization and optimal hatch success. For example, Bart and Dunham (1996) tested ratios from 5.0×10^4 to 1.2×10^7 sperm per egg with success ranging between 17 to 87%, respectively. The best ratio for optimized fertilization was 1.25×10^5 sperm per egg when 450 eggs were fertilized (51.8% fertilization success; Bart and Dunham, 1996). Since this study, many new techniques and technologies have been developed to further optimize these protocols. These technological advances have shown a decrease in the amount of fresh sperm required for optimal hatch success. A study on blue catfish fertilization showed the optimal fresh sperm to egg ratio being 1.0×10^4 sperm per egg (~40%), utilizing much less sperm than what has been previously recommended for fertilization (Myers et al., 2020b). The addition of sperm after this ratio did not significantly change the hatching success of embryos. In the present study, this ratio

was used as the fresh control and when compared to our optimal cryopreserved ratio of 5.0×10^4, there was no significant difference. Together, these findings reiterate that throughout the cryopreservation process, there is cellular damage that is inflicted upon gametes, requiring more cryopreserved sperm to optimize fertilization. Thus, it is imperative to determine if cellular damage sustained by sperm affects offspring performance after hatch, as we discuss below.

1.5.3 Experiment 3: Offspring performance from fresh and cryopreserved sperm

At present, knowledge of the impact of cryopreservation is far from complete for blue catfish. As such, the industry needs a 'proof of concept' for the applicability of cryopreserved sperm for their hatchery management programs. Hence, it is important to decipher how offspring development is affected when eggs are fertilized with fresh and cryopreserved sperm so that farmers have confirmation that cryopreserved sperm can be used for fertility to synchronize gamete availability, conserve sperm, reduce male broodstock numbers, reduce risk of disease, and conserve genetic variability, thus strengthening sustainability. In this study, it was found that there were limited effects of cryopreservation on offspring development. These results have been shown in various other cases where fry morphology has not been affected using cryopreserved sperm (Chereguini et al., 2002; Sarder et al., 2013; Akter et al., 2016; Imsland et al. 2022). For instance, in a study on Pabda catfish (*Ompok pabda*),)), growth between cryopreserved and fresh treatments over a six-week period showed no significant differences between treatments (Sarker et al., 2013). While most morphological traits in our study reflect these same results, a difference in yolk-sac size was noticed when eggs were sired with frozen-thawed cells. The reason behind this phenomenon is unknown and should be investigated further. Overall, these results are very

promising. Nonetheless, more improvement still needs to be attained for increasing fertility and hatching rates before a commercial scale protocol can be offered to hatchery operators for commercial hybrid catfish production. It is also important to note that in previous studies, and this one alike, the time spent following the offspring only ranged a few months. Thus, further studies should look at hybrid catfish offspring sired from frozen-thawed sperm throughout the various grow-out phases to full maturity. As part of these future studies, disease resistance and poor water quality tolerance should also be examined. These future studies will allow farmers to see how these offspring perform long-term and whether any detrimental health defects develop that are not seen in the control group (fresh sperm) of catfish (sired from fresh sperm).

Lastly, growth and survival can be affected by the utilization of cryopreserved sperm (Nusbaumer et al., 2019), but these results are not reflected in our experiments. This study shows that there were not any significant differences overall or within females between growth and survival, similar to a study done on rainbow trout. Labbe et al. (2001) showed no differences were found between fresh and cryopreserved sperm treatments as it related to offspring survival.

1.6 Conclusions

Overall, our results revealed that, in blue catfish, sperm cryopreservation affects a variety of biological processes including sperm kinematics, sperm cell viability, ROS production, and sperm cell DNA fragmentation. These data are critical for the development and understanding of future gene banks. For example, understanding gamete interactions with cryogenics will allow us to equip both conservation and aquaculture sectors with the proper technologies to successfully fertilize eggs with cryopreserved gametes. This proof of concept also shows that sperm can be

frozen and reanimated without sacrificing the integrity and robustness of resulting offspring. The data presented in this study could aid in reducing hybrid catfish production costs associated with male broodstock holding and improve reproductive sustainability in catfish aquaculture. Through our innovative approaches to artificial reproduction, cryopreservation may be a viable option for hybrid catfish farmers to improve reproductive efficiency and lower economic costs of running a hatchery in the future.

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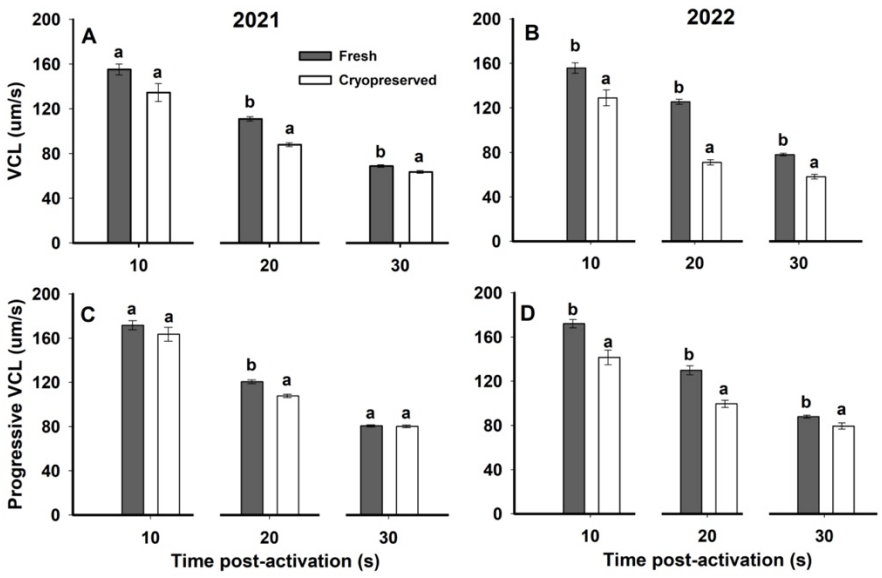


Fig. 1.1. Sperm from 83 blue catfish (*Ictalurus furcatus*) were cryopreserved with methanol at 1 × 10^9 sperm/mL. Straws were thawed and post-thaw curvilinear velocity (VCL; AB) and progressive VCL (CD) measured at 10, 20, and 30 s post activation during the 2021 and 2022 spawning seasons. Mean values ± standard errors are represented. Letters indicate a significant difference between treatments at each time post-activation based on results from T-tests.

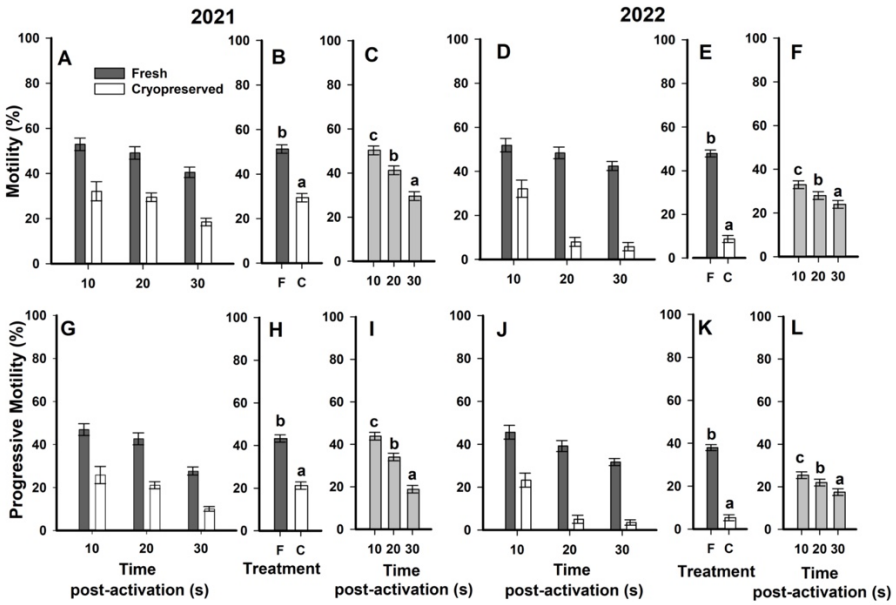


Fig. 1.2. Sperm from 83 blue catfish (*Ictalurus furcatus*) were cryopreserved with methanol at 1 × 10^9 sperm/mL. Straws were thawed and post-thaw motility (A-F) and progressive motility (G-L) of blue catfish sperm measured at 10, 20, and 30 s post activation during the 2021 and 2022 spawning seasons. Main effects were analyzed separately (Sperm Treatment: B, E, H, K; Time Post-Activation: C, F, I, L) and together (A, D, G, J) in the combined model. Average values ± standard errors are represented.

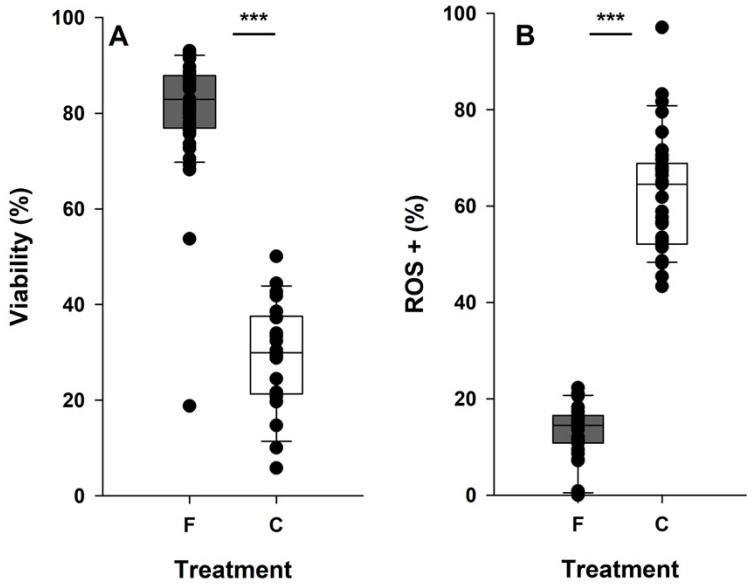


Fig. 1.3. Viability (A) and oxidative stress (B) of blue catfish (*Ictalurus furcatus*) sperm measured when fresh ("F") and after cryopreservation ("C"). Box heights indicate interquartile range, horizontal lines within indicate median and mean, and whiskers show the minimum and maximum values within the data range. Individual values are represented as dots.

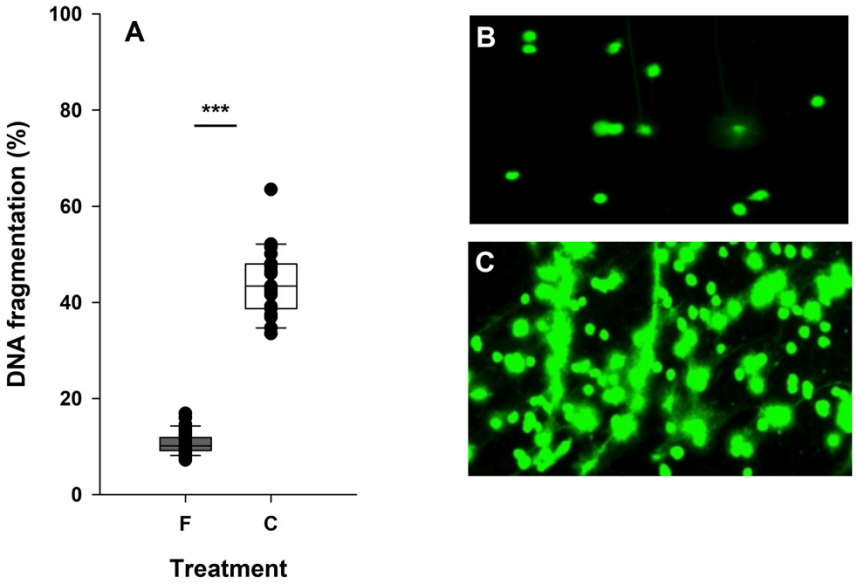


Fig. 1.4. DNA fragmentation of fresh ("F") and cryopreserved ("C") sperm in blue catfish (*Ictalurus furcatus*). Box heights indicate interquartile range, horizontal lines within indicate median and mean, and whiskers show the minimum and maximum values within the data range. Individual values are represented as dots (A). Photos of fresh (B) cells and frozen-thawed cells (C) using fluorescence microscopy. Fragmented cells are identified by unclear margins and larger size.

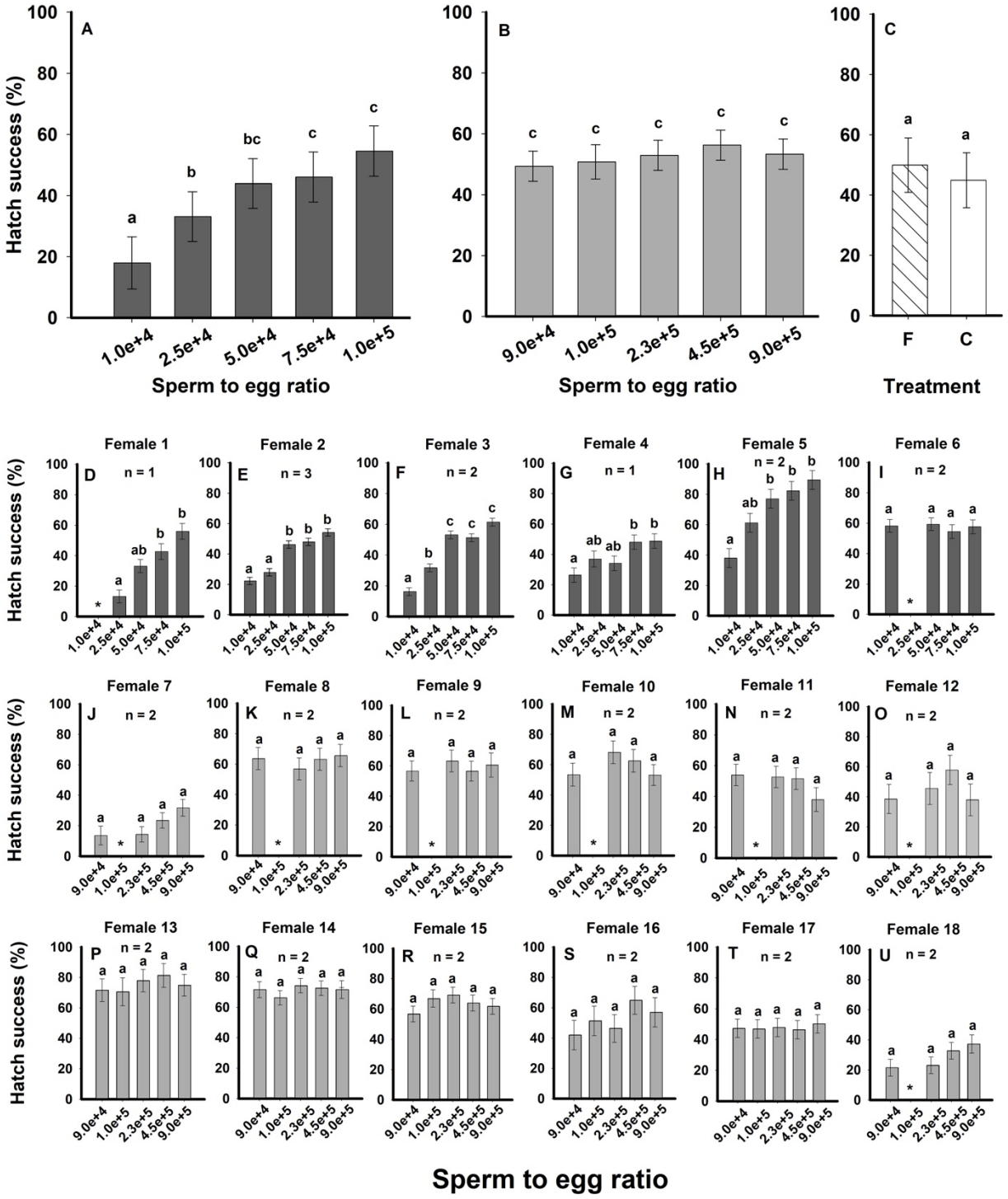


Fig. 1.5. Mean hatch success of hybrid catfish (*Ictalurus punctatus* female × *I. furcatus* male) embryos using cryopreserved sperm to egg ratios ranging from 1.0×10^4 to 1.0×10^5 (2022, A) and 9.0×10^4 to 9.0×10^5 (2021, B) sperm per egg. Results are shown for all females in each spawning

season. Hatch success was also compared between the fresh control ("F", 1.0×10^4 sperm per egg, C) and the newly determined ideal ratio for cryopreserved sperm ("C", 5.0×10^4 sperm per egg, C), and by individual females (1-18, D-U) where "n" represents the number of males used for fertilization. Bar color represents spawning season (dark grey = 2022, light grey = 2021). Sperm to egg ratios with different letters are significantly different based on ANOVA models ($P < 0.05$). Bars represent least square means for hatch success ± standard error.

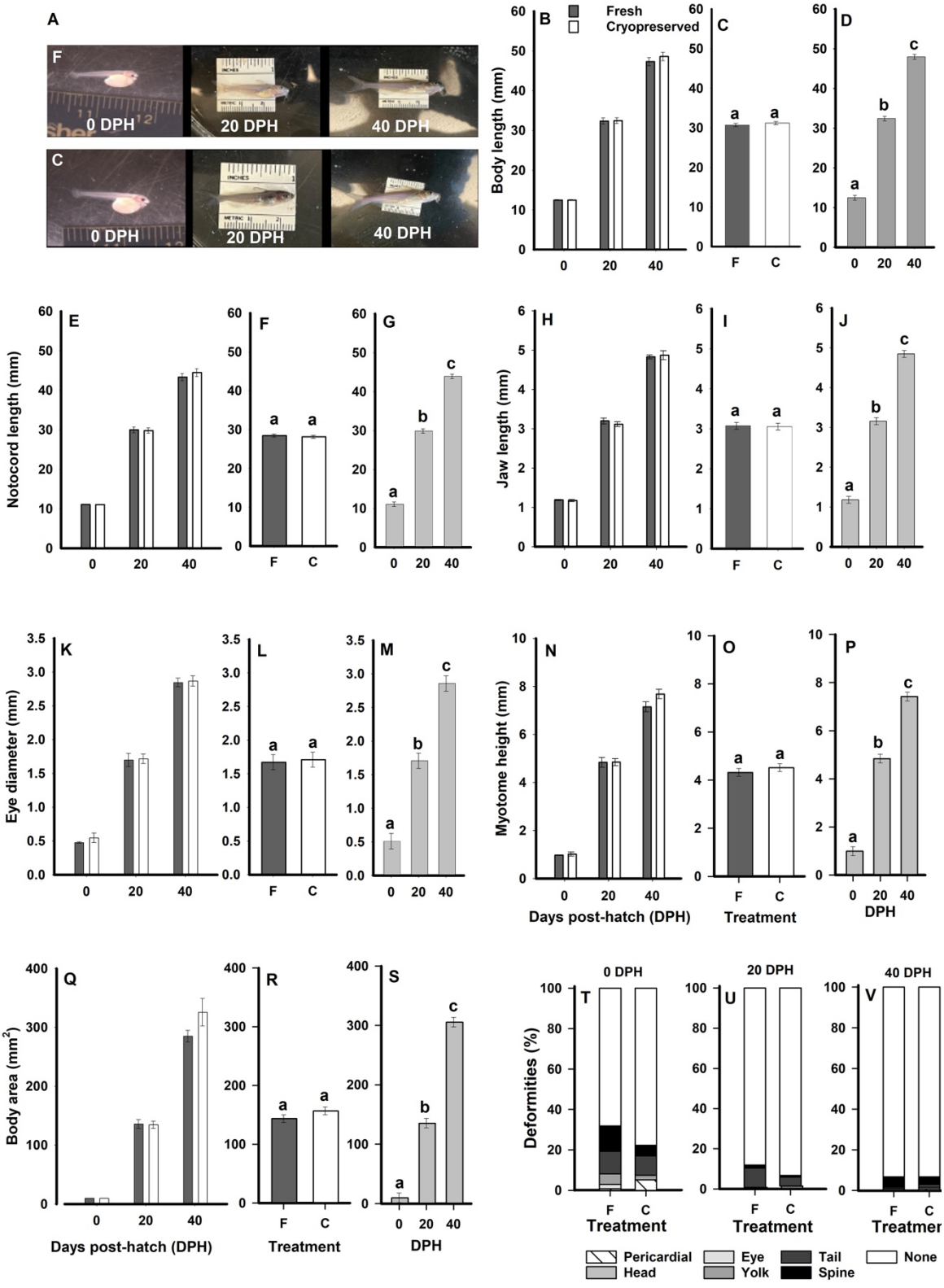

A
F
0 DPH
20 DPH
40 DPH
C
0 DPH
20 DPH
40 DPH
B
Fresh
Cryopreserved
Body length (mm)
C
D
E
Notocord length (mm)
F
G
H
Jaw length (mm)
I
J
K
Eye diameter (mm)
L
M
N
Myotome height (mm)
Days post-hatch (DPH)
O
Treatment
P
DPH
Q
Body area (mm²)
Days post-hatch (DPH)
R
Treatment
S
DPH
0 DPH
20 DPH
40 DPH
T
U
V
Deformities (%)
Treatment
Pericardial
Head
Eye
Yolk
Tail
Spine
None

Fig. 1.6. Morphological traits of hybrid catfish (*Ictalurus punctatus* female × *I. furcatus* male) fry at three early stages (A-S). Bars indicate least-square means ± standard error at each time point and treatment. Letters above bars indicate significant differences based on ANOVA models. Bars sharing the same letter do not differ in significance. Occurrence of deformities for each class (%, T-V) are shown for each time post-hatch.

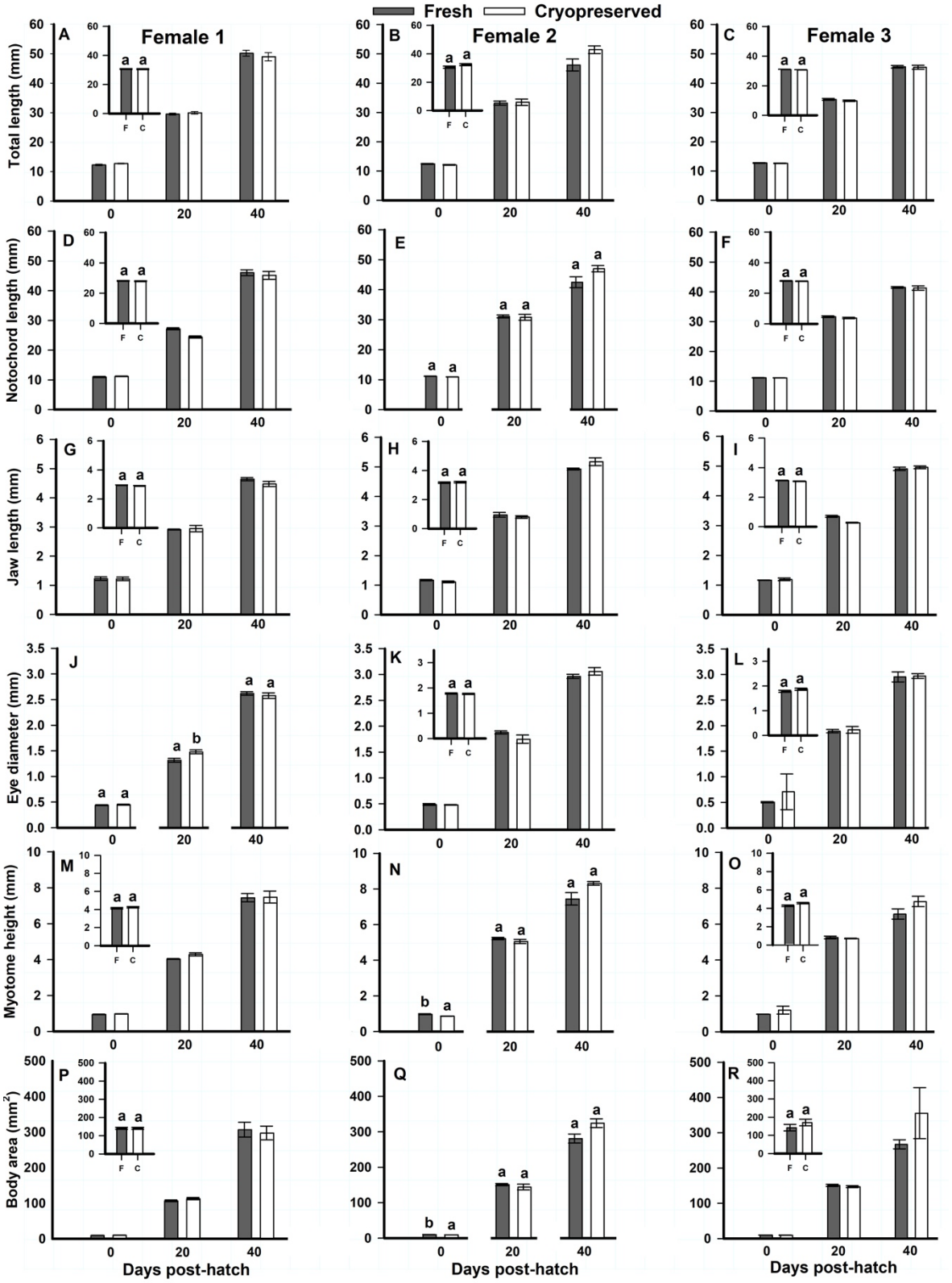


Fig. 1.7. Morphology traits of hybrid catfish (*Ictalurus punctatus* female × *I. furcatus* male) fry from individual females at three times post hatch. Main effects were analyzed separately (Sperm

Treatment: A-D, F-I, K-M, O,P,R; Days Post-Hatch: E, J, N, Q). Average values ± standard errors are represented. Letters indicate a significant difference between treatments at each time point.

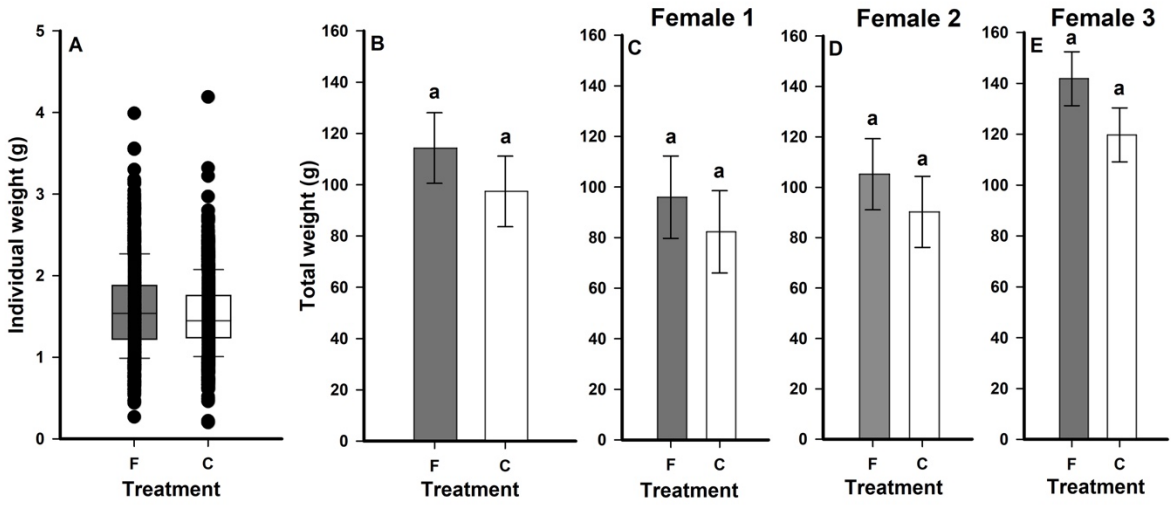


Fig. 1.8. Weights of hybrid fry (*Ictalurus punctatus* female × *I. furcatus* male) at 40 days post-hatch. Individual fish weights across treatments are expressed as dots on the box plot (A). Box heights indicate interquartile range, horizontal lines within indicate median and mean, and whiskers show the minimum and maximum values within the data range. Each bar is the least square means ± standard error of each treatment both across all females (B) and within each female (C-E). There was no significance across females or treatments.

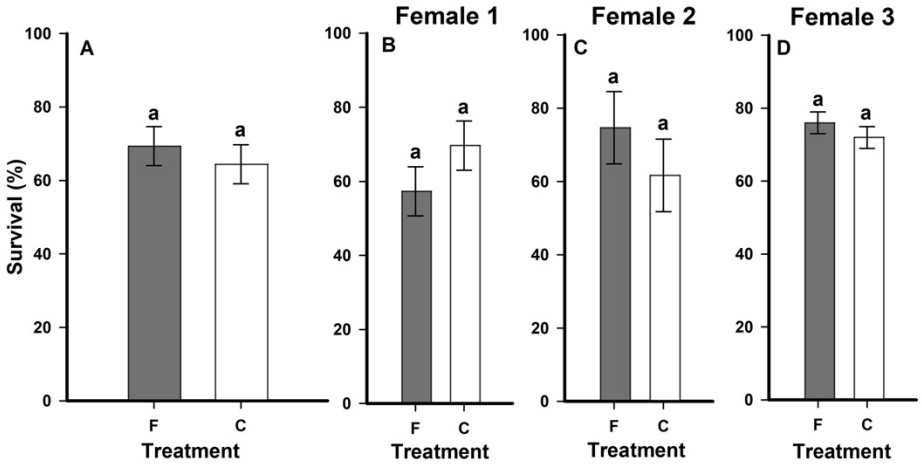
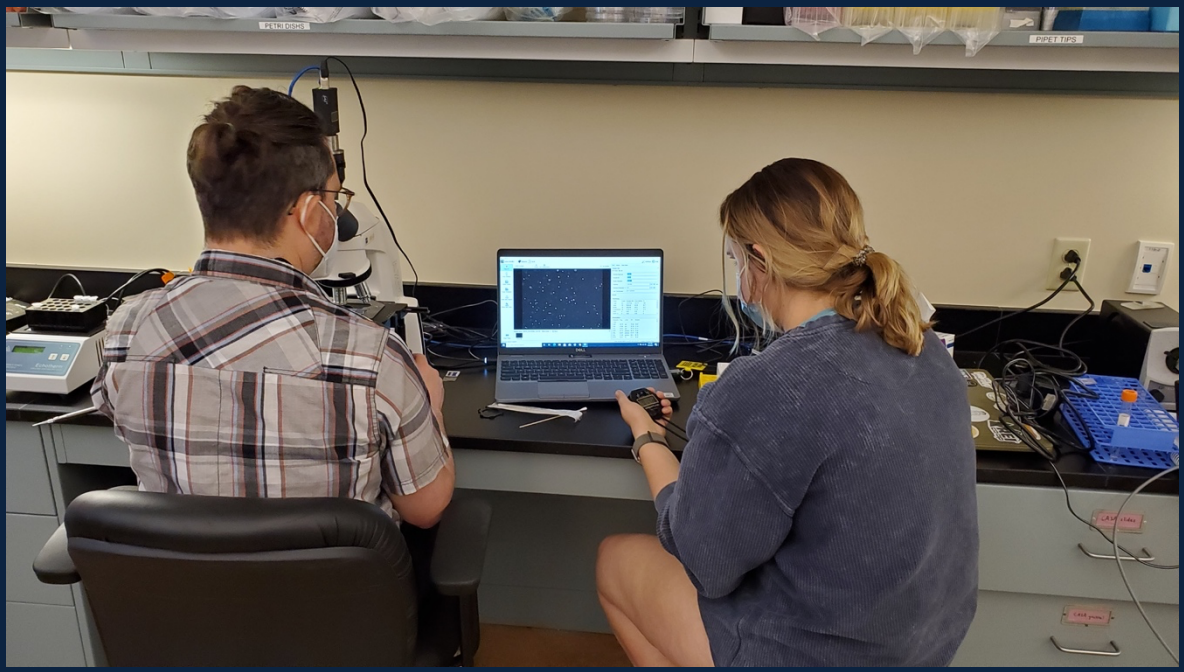


Fig. 1.9. Survival of hybrid fry (*Ictalurus punctatus* female × *I. furcatus* male) at 40 days post-hatch. Each bar is the least square means ± standard error of each treatment both across all females (A) and within each female (B-D). There was no significance across females or treatments.

Chapter 2

Exploring how male body size attributes and androgens impact fresh and frozen-thawed sperm kinematics in blue catfish (*Ictalurus furcatus*)



2.1. Abstract

Catfish farming accounts for about 60% of total United States finfish aquaculture production. Of this, the channel catfish female (*Ictalurus punctatus*) × blue catfish male (*I. furcatus)* hybrid makes up greater than 50% of the total harvest. Recent evidence has shown that the male contribution to offspring is important during the early life history stages. This emphasizes the need for good quality male gametes as highly variable sperm can contribute to a considerable amount of variation in hatch success and offspring performance. The objective of this study was to determine correlations between male (n = 83) reproductive performance traits (body weight, body morphology, genital morphology, androgens, and testes weight) and sperm kinematics [average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), and percent motility] utilizing both fresh and frozen thawed sperm over two spawning seasons. In general, our results revealed that blue catfish male reproductive performance traits correlated to fresh and post-thaw sperm kinematic traits when using individual correlations. Significance was also detected among these traits using canonical correlation. Specifically, males that had a smaller genital size and higher androgen concentrations had a higher initial swimming velocity (10 s) and were swimming slower towards the end of the motility duration (30 s). Together, these data advance our understanding of assisted reproduction to improve reproductive efficiency for hatcheries and enhance living and cryopreserved gamete banks for genetic breeding and production.

2.2. Introduction

Wild-caught fish stocks are declining and as the world population grows, the demand for high-quality protein is increasing. Aquaculture is playing a pivotal role in global food security, providing sources of protein outside of traditional land-based agriculture (Fry et al. 2016). The United States ranks 18th in global aquaculture production with nearly 300 million kg of freshwater species harvested in 2019 (NMFS, 2022). Most of this harvest is made up of catfish (Engle et al., 2022), where farming occurs in Alabama, Texas, Arkansas, and Mississippi and accounts for greater than 60% of total United States freshwater aquaculture production (Johnson, 2021). Overall, greater than 50% of catfish yield is composed of the female channel catfish, *Ictalurus punctatu*s, × male blue catfish, *I. furcatus*, hybrid (Torrans and Ott, 2018; Hegde et al., 2021; Engle et al., 2022; NMFS, 2022). Their popularity is primarily due to their ability to thrive in pond culture and in-pond raceway systems, making them highly valuable in the aquaculture industry (Dunham et al., 1983; Dunham et al., 2008; Brown et al., 2011; Bosworth, 2012; Dunham and Masser, 2012, Fantini-Hoag et al., 2022). For example, some farmers can achieve production rates as high as 20,000 kg/ha when growing and harvesting hybrid catfish in ponds (Kumar et al., 2018). Despite the growing hybrid industry, a major bottleneck is getting high quality blue catfish gametes.

Blue catfish males reach maturity after 4 to 7 years (Graham, 1999), and their sperm cannot be readily extracted by hand-stripping, which can be accomplished in carps and salmonids. Consequently, sperm collection is a lethal procedure, requiring the removal and maceration of the testes (Bart and Dunham, 1990). Furthermore, housing and processing blue catfish males requires a substantial economic investment. Recent evidence also shows that a male's

contribution to offspring is important in determining their performance during early life history stages (Butts and Litvak, 2007ab; Pitcher and Neff, 2007; Houde et al., 2015; Siddique et al., 2017; Myers et al., 2020b). This emphasizes the need for good quality gametes as highly variable sperm, both from wild-caught and cultivated broodstock, can contribute to a considerable amount of variation in hatch success and early offspring performance, as observed using in vitro fertilization (IVF) experiments (Linhart et al., 2000; Gage et al., 2004, Butts et al., 2010, 2011). This variation is due to various inherent factors and/or broodstock rearing conditions (Bobe and Labbé, 2010) and can cause major economic losses, especially when milt from selectively bred, high-quality individuals must be discarded, or poor performing males are used for fertilization. Some farmers even report a considerable decline in well-developed testes as the spawning season ends, which is another inconvenience for late season spawning and hybrid production (Bosworth et al., 2018). Thus, developing innovative strategies to forecast sperm quality could have major ramifications for more practical and efficient hybrid catfish hatchery production.

In teleost fishes, both age and size (Van Eenennaam and Doroshov, 1998; Bosworth et al., 2018) have been shown to affect maturation and development of testes along with sperm quality (Casselman and Montgomerie 2004; Rogers et al., 2006; Azlina et al., 2011; Lesyna and Barnes, 2016). Furthermore, genital papillae can be a common indicator of sexual maturity (Har, 2000; Kruger et al., 2013), where papillae length and width have been positively correlated with maturation of the testes (Bond and Forsgren, 2022). Gonadal maturity has been linked to sperm production (Chatakondi and Davis, 2012; Kazemi et al., 2019) and significant correlations between body size attributes and quantitative milt characteristics have been reported (Babiak et al. 2006; Butts et al., 2010).

While body morphology, external genital size, and gonad maturity can be good indicators of sperm performance, they are not the only potential signs of male quality. Blood sex hormones also correlate to sperm quality (Tessaro et al., 2019), where androgens, such as testosterone (T) and 11-ketotestosterone (11-KT), participate in several processes initiating reproductive maturity in male fishes (Kazemi et al., 2019; Tessaro et al., 2019). These hormones start the onset of maturation and cell differentiation, multiplication of spermatogonia, regulation of spermatogenesis, formation of spermatocytes, and spermiation (Billard et al., 1982; Amiri et al., 1996, 1999; Plant and Marshall, 2001; Schultz et al., 2010). Androgens are also related to gonad development (Butts et al., 2012) and sperm production can be predicted based on stage of gonadal development and blood concentration (Chatakondi and Davis, 2012). While age and temperature impact androgen levels, the relationships between these sex hormones remain high at spermiation (Alavi et al., 2012) and decrease at the conclusion of spawning (Amiri et al., 1996; Barannikova et al. 2004, 2008). As a result, blood hormone analysis can play a key role in choosing a male in peak spawning condition and selecting for high quality gametes.

Understanding how body size attributes and androgens impact sperm kinematics is especially important when utilizing cryopreservation techniques, as selectively bred lines often need to be frozen in perpetuity. Protocols for freezing blue catfish sperm have been developed for commercial-scale applications (Bart et al., 1998, Hu et al., 2011, 2014), but this cryopreservation technology has not been readily adopted by the catfish industry.

Despite cryopreservation not yet being utilized commercially, the USDA-ARS Germplasm Program currently houses blue catfish sperm (USDA-ARS, 2022). Unfortunately, there is a high-degree of variability in frozen-thawed sperm kinematics when compared to that of fresh cells (Linhart et al., 2005; Hu et al., 2011, 2014; Wang et al., 2022, Montague et al., Unpublished

Data). Therefore, it is imperative that factors that impact sperm quality be elucidated for this economically important catfish species.

The objective of this study was to determine correlations between body weight, body size morphology, genital size, blood T and 11-KT concentrations, and testes weight (hereafter, collectively termed "reproductive performance traits") and sperm kinematics [average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), and percent motility] comparing both fresh and frozen-thawed sperm. Together, these data will advance our understanding of assisted reproduction to improve reproductive efficiency for hatcheries and enhance living and cryopreserved sperm banks for genetic breeding and production.

2.3. Materials and methods

Animal husbandry and experimental protocols were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). In 2021 and 2022, mature blue catfish males were obtained from Jubilee Farms in Indianola, MS, USA (33.5002° N, 90.5815° W) and transported to the Auburn University E.W. Shell Fisheries Center (32.6524° N, 85.4860° W) in Auburn, Alabama, USA, where all experimental procedures were conducted. Water temperature for broodstock ponds during the sampling periods ranged from 19.9°C to 25.4°C in 2021 and 20.4°C to 27.3°C in 2022 with dissolved oxygen maintained at >7 mg/L for both seasons. All broodstock were housed in 0.04-ha earthen ponds (~150 pounds of fish per 0.04 ha) and fed a 36% protein diet (Fishbelt Feed Mill, Moorhead, MS, USA) three times per week until satiation.

2.3.1. Catfish morphology

Males (n = 83) were euthanized following industry-approved protocols. Individual male blood samples (two replicates, each ≥ 5 mL of blood) were taken for androgen assays (*see Section 2.3.2*). Following blood collection, body weight (kg), length (cm), head circumference (cm), hump circumference (cm), and head width (cm) measurements were recorded. External genitalia were also digitally imaged for length (cm), width (cm), and area (cm^2) quantification using ImageJ analyses software (Schneider et al., 2012).

2.3.2. Androgen assays

Blood was collected from each fish prior to dissection and stored at 4°C in sterile, non-heparinized tubes, allowing the blood and serum to separate for <24 h. Serum samples were collected off the top of the sample and spun down at 4°C for 10 min at 4,000 rpm to ensure no red blood cells were present. Two replicate samples were collected from each male (>1 mL). Blood serum samples were stored at -20°C until further hormonal analysis could be completed using T (582701, Cayman Chemical Company, Ann Arbor MI, USA) and 11-KT (582751, Cayman Chemical Company, Ann Arbor MI, USA) ELISA kits following manufacturer protocols. For each hormone, serum was assayed in triplicate across two T and two 11-KT assay plates yielding an intra-assay variation of 4.3% in 2021 and 10.6% in 2022 for T and 25.8% in 2021 and 13.9% in 2022 for 11-KT. Inter-assay variation was 10.7% in 2021 and 18.9% in 2022 for T and 19.9% in 2021 and 14.5% in 2022 for 11-KT. Relevant cross reactivity for T was 19-nortestoserone (140%), 5α-dihydrotestosterone (27.4%), 5β-dihydrotestosterone (18.9%),

methyltestosterone (4.7%), androstenedione (3.7%), 11- KT (2.2%), 5-androstenediol (0.51%), epi-testosterone (0.2%), progesterone (0.14%), testosterone enanthate (0.11%), androsterone (0.05%), androsterone sulfate (0.04%), testosterone sulfate (0.03%), DHEA sulfate (0.02%), estradiol (<0.01%), and testosterone glucuronide (<0.01%). Relevant cross-reactivity for the 11-KT assay included adrenosterone (2.9%), 4-androsten-11β,17β-diol-3-one (0.01%), 5α-androstan-17β-ol-3-one (<0.01%), 5α-androsten-3β,17β-diol (<0.01%), and T (<0.01%). Absorbance plate readings were taken on a Cytation 3 Plate Reader where sensitivity (80% B/B_0) was 6 pg/mL in the range of 3.9 to 500 pg/mL for T and 1.3 pg/mL in the range of 0.78 to 100 pg/mL for 11-KT using 405 nm absorption for both androgens.

2.3.3. Sperm collection

Males were euthanized following industry-approved protocols. Sperm samples from 43 blue catfish were collected from 6 to 13 May 2021 and from 40 males from 9 to 13 May 2022 (Table 2.1). Testes were dissected from each fish using forceps and surgical scissors and stored in Hank's Balanced Salt Solution (HBSS; reverse osmosis deionized water with 8 g/L NaCl, 0.4 g/L KCl, 0.16 g/L $CaCl_2 \times 2H_2O$, 0.2 g/L $MgSO_4 \times 7H_2O$, 0.12 g/L $Na_2HPO_4 \times 7H_2O$, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, 1 g/L glucose; Tiersch, 1997) until processing (<30 min). Testes were separated from the peritoneum and blood vessels before they were weighed and macerated through a fine mesh strainer (200 μm) into 50 mL tubes.

2.3.4. Sperm density

Small aliquots of milt (< 5 µL) were diluted with HBSS at dilutions ranging from 1:50 to 1:200, depending on individual male density as observed under a Zeiss Axio Imager A2 (Carl Zeiss Microscopy, LLC, White Plains, NY, USA) at 20× magnification. Cells were then counted using a Neubauer Hemocytometer following protocols from Myers et al. (2020c). In brief, cells were counted at 20× magnification where five squares (1 mm^2) were quantified. The mean of counts for each sperm dilution were calculated, and two counting reps were averaged. This value was then used to calculate the total cells/mL. HBSS was then added to the milt based on the density of the sample and standardized to 1×10^9 cells/mL. Sperm samples were assessed for swimming kinematics (*see Section 2.3.5*) and stored at 4 °C until cryopreservation (<5 h; see *Section 2.3.6*).

2.3.5. Computer Assisted Sperm Analysis

Computer Assisted Sperm Analysis (CASA) software (CEROS II, Hamilton Thorne Biosciences, Beverly MA, USA) was used to quantify sperm kinematic traits according to Myers et al. (2020a). In brief, sperm were pipetted in an 80 µm 2X-CEL chamber slide (Hamilton Thorne Biosciences, Beverly MA, USA) and activated with DH_2O supplemented with 0.5% bovine serum albumin (126609, Merck Millipore, Burlington MA, USA) to prevent cells from sticking to slides. Sperm samples and activation media were kept at 4ºC using an Echotherm™ Chilling/Heating Dry Bath (Torrey Pines Scientific, Carlsbad, CA, USA) for CASA analyses.

Three technical replicate activations were completed per male, where VAP, VCL, VSL, and percent motility were assessed at 10, 20, and 30 s post-activation to stimulate the beginning, middle, and end of the sperm activation period (Myers et al., 2020a). Furthermore, these four kinematic traits were chosen as they typically correlate to a male's fertilization ability (Lahnsteiner et al., 1998; Gage et al., 2004; Linhart et al., 2005; Butts et al., 2011).

Following experimentation, CASA videos were manually checked for quality utilizing protocols from Butts et al. (2011). In brief, sperm tracks were removed from analysis if the software split the track of a single sperm, sperm were drifting, sperm swam out of the field of view before being adequately assessed, or if the software incorrectly combined crossing tracks of multiple sperm. Files were then exported for statistical analyses.

2.3.6. Sperm cryopreservation

Sperm concentration was adjusted to 1×10^9 cells/mL based on sperm cell counts (*see Section 2.3.4*) with HBSS plus 10% methanol as a cryoprotectant (Hu et al., 2011, 2014). Sperm suspensions were then pipetted into 0.5 cc straws, with ~20 straws per male (Minitube USA, Verona, WI, USA), before they were sealed with metal beads and positioned on chilled metal racks (40 straws per rack). These racks were then placed in a controlled rate freezer (Planer Kryo 560-16, Cryo Associates Inc., Gaithersburg, MD, USA) for equilibration at 5ºC. This equilibration time started when the methanol was added to the sperm solutions and lasted ~30 min. Straws were then frozen at a rate of -5ºC/min until they reached -80ºC when they were immediately plunged into liquid nitrogen. Frozen samples were stored in a vapor-pressure liquid

nitrogen storage system (MVE 815P-190F-GB, Cryo Associates Inc. Gaithersburg, MD, USA) until post-thaw analysis could take place.

2.3.7. Statistical analyses

Data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA) and R statistical software (v. 4.3.0; R Core Team 2023). A series of t-tests were run to compare reproductive performance traits and sperm kinematics between the two spawning seasons.

Next, a two-tier approach was employed to explore how reproductive performance traits impact fresh and frozen-thawed sperm kinematics. Firstly, correlations (Pearson correlation; SAS PROC CORR) within and between the reproductive performance and sperm kinematics traits were described at 10, 20, and 30 s post-activation. Secondly, multivariate statistics considered each male as an integrated unit to delineate patterns between reproductive performance and sperm kinematic traits. Specifically, canonical correlation (*CANCOR* package, R) was performed. Because many of the traits were correlated (*see Section 2.4*), principal component analysis (PCA) was used as a solution to multicollinearity and to simplify the complexity of datasets, where principal components (PCs) were constructed for the reproductive performance as well as the fresh and frozen-thawed sperm kinematic traits. Separate PCs were generated for both spawning seasons. Loadings for PCs were cut off at 0.6 (Guadagnoli and Velicer 1988). Seven males had missing hormone data (n = 4 in 2021, n = 3 in 2022), which were predicted and imputed using the *imputePCA* function in the R package *missMDA*. Nine other males had inadequate sperm volumes for cryopreservation, thus were omitted from the analysis. Canonical

variates had a cut-off level of 0.3 (Tabachnick and Fidell, 2019) and Wilks' Lambda F-approximation was used to determine significance (Nimon et al., 2010; Tabachnick and Fidell, 2019). Alpha was set at 0.05.

2.4. Results

Descriptive statistics for the reproductive performance traits over both spawning seasons are shown in Table 2.1. Significant changes in body weight, head circumference, hump circumference, and head width were found, where males were generally larger in 2022. Genital pore length, width, and area measurements were not statistically significant between years, despite having higher mean values in 2022. A similar trend was observed for 11-KT and T, where no statistical significance was detected, but T concentration increased in 2022. Males collected in 2022 also had significantly bigger testes with higher sperm density. Both T and 11-KT had a high-degree of variation (CVs ranged from 52.07 to 90.09% for T and 59.52 to 123.90% for 11-KT) when compared to the other reproductive performance traits.

Descriptive statistics for the kinematic traits are shown in Tables 2.2 and 2.3 for the fresh and cryopreserved sperm, respectively. In 2021, VCL and VSL both significantly increased for the fresh sperm at 10 s, while in 2022, VAP, VCL, and VSL significantly increased at 20 and 30 s (Table 2.2). Sperm motility at 30 s also increased in 2022. Following freezing, VAP, VCL, VSL, and motility all increased at 10, 20, and 30 s in 2021. Overall, variation in sperm kinematic traits increased in 2022 (CVs ranged from 8.69 to 53.54% vs. 9.35 to 194.67% in 2021).

2.4.1. Correlations within/between the reproductive and sperm kinematic traits

Correlations within reproductive performance and sperm kinematic traits

Bubble plots show correlations for the reproductive performance (Fig. 2.1) and sperm kinematic traits (Fig 2.2). In 2021 and 2022, fish body attributes, such as weight, length, head circumference, hump circumference, and head width were typically positively correlated. In general, genital traits (i.e., genital length, width, and area) were highly correlated. Androgens, T and 11-KT, were not correlated in 2021 ($r = 0.26$, $P = 0.13$), but positively correlated in 2022 ($r = 0.88$, $P < 0.01$). In general, velocity (VAP, VCL, VAP) and motility traits were correlated for both the fresh and cryopreserved sperm during both spawning seasons.

Correlations between the reproductive performance and fresh sperm kinematic traits

Significant correlations were detected between reproductive performance traits and fresh sperm kinematics in 2021 (Table 2.4). In brief, genital length was positively related to VAP, VCL, and VSL at 10 s as well as motility at 10, 20, and 30 s. Genital area had a positive correlation with motility at 30 s and 11-KT had positive correlations with VAP, VCL, and VSL at 30 s. Lastly, testes weight had a negative correlation with VAP, VCL, and VSL at 20 s.

Significant correlations were also detected in 2022 (Table 2.4), where weight, length, head circumference, hump circumference, and head width were all negatively correlated with VAP at 20 s. Hump circumference was negatively correlated with VCL and VSL at 10. Genital length,

width, and area were also negatively correlated with VAP at 30 s, VCL at 10 s, VSL at 10 s. These same traits were positively correlated with VCL, VSL, and motility at 30 s. Testes weight was negatively correlated with VAP at 10 s and sperm density was positively correlated to VCL at 10 s.

Correlations between the reproductive performance and cryopreserved sperm kinematic traits

Mostly negative correlations were detected between the reproductive performance traits and cryopreserved sperm kinematics in 2021 (Table 2.4). In brief, weight and hump circumference were negatively correlated with VAP at 10 and 20 s as well as VCL at 10 s and VSL at 20 s. Hump circumference was also negatively correlated with VSL at 30 s and motility at 10 s. Similarly, head circumference was negatively correlated with VAP at 20 and 30 s along with VCL at 10 s and VSL at 20 and 30 s. Head width was negatively correlated with VAP, VCL, VSL, and motility at 10 s, and VAP and VSL at 20 s. Lastly, genital width was negatively correlated with VAP and VSL at 30 s.

Significant correlations were again detected between reproductive performance traits and cryopreserved sperm kinematics in 2022 (Table 2.4). To highlight, fish weight and hump circumference were negatively correlated with VAP and VSL at 30 s. Similarly, length, head circumference, and head width were negatively correlated with VAP, VCL, and VSL at 30 s. Additionally, head width was negatively correlated with VCL at 10 s. Testes weight was positively correlated with VAP at 30 s and VCL at 30 s, and sperm density was positively correlated with VAP, VCL, and VSL at 10 s.

2.4.2. Multivariate statistical approaches

Fresh sperm

For the fresh sperm in 2021, three PCs described the reproductive performance axis for the canonical correlation (Table 2.5). PC1 (0.35 variance explained) was composed of body attributes, such as fish weight, length, head circumference, hump circumference, and head width, while PC2 (0.21 variance explained) included all the genital traits (i.e., genital length, width, and area). PC3 (0.13 variance explained) included the androgens (T, 11-KT) and sperm density. For the sperm kinematic axis, PC1 (0.43 variance explained) included VAP at 10 s, VCL at 10 s, VSL at 10 s, and motility at 10, 20, and 30 s. PC2 (0.42 variance explained) was composed of VAP, VCL, and VSL at 20 and 30 s (Table 2.6). Canonical correlation revealed a non-significant Wilks' Lambda F-approximation ($F_{6, 176} = 1.37$, $P > 0.24$), where no significant canonical variates were detected.

Similarly in 2022, three PCs described the reproductive performance axis for the canonical correlation (Table 2.5). PC1 (0.38 variance explained) included the body attributes, fish weight, length, head circumference, hump circumference, and head width, while PC2 (0.27 variance explained) included all the genital traits, and PC3 (0.16 variance explained) included the androgens (Table 2.5). For the sperm kinematic axis (Table 2.6), PC1 (0.30 variance explained) was composed of VCL and VSL at 10 and 30 s. Meanwhile, PC2 (0.25 variance explained) was composed of VAP at 10 s and motility at 10, 20 and 30 s, while PC3 (0.26 variance explained) was composed of VAP at 20 and 30 s and VCL and VSL at 20 s.

Canonical correlation revealed a significant Wilks' Lambda F-approximation, where canonical variate 1 was marginally significant ($F_{9, 82.9} = 2.00$, $P = 0.049$; Fig. 2.3A). F-statistics were not statistically significant for canonical variates 2 and 3 ($P > 0.35$). The correlation was 0.55 (Fig. 2.3C), where the first canonical variate accounted for 33.3% of the variance in morphology and 33.3% for sperm kinematics. Redundancy analysis was also performed to determine how much variation was explained for the independent and dependent variables for the significant canonical pair. The canonical variate extracted 9.9% of the variance for both axes.

To interpret canonical variate 1, males that had smaller genital size (canonical loading = -0.918) and higher androgen concentrations (canonical loading = 0.313) also had higher initial VCL and VSL at 10 s and slower VCL and VSL at 30 s (canonical loading = 0.787; Fig. 2.3BC). As well, males that had smaller genital size and higher androgen concentrations had lower sperm velocities (VAP, VCL, VCL) at 20 s and VAP at 30 s post-activation (canonical loading = -0.56; Fig 2.3BC).

Cryopreserved sperm

For the cryopreserved sperm in 2021, body attributes (PC1; 0.38 variance explained), genital traits (PC2; 0.27 variance explained), and androgens in conjunction with sperm density (PC3; 0.16 variance explained) described the reproductive performance axis for the canonical correlation (Table 2.5). For the sperm kinematic axis (Table 2.6), PC1 (0.41 variance explained) was composed of VAP, VCL, and VSL at 10 and 20 s, while PC2 was composed of VAP, VCL, and VSL at 30 s (0.28 variance explained). Canonical correlation revealed a Wilks' Lambda F-approximation, where no canonical variates were significant ($F_{6, 60} = 1.64$, $P > 0.15$).

Similarly in 2022, three PCs described the reproductive performance axis for the canonical correlation, where PC1 (0.36 variance explained) included the body attributes, fish weight, length, head circumference, hump circumference, and head width, PC2 (0.21 variance explained) included all the genital traits, and PC3 (0.14 variance explained) included the androgens (Table 2.5). For the sperm kinematic axis (Table 2.6), PC1 (0.33 variance explained) was composed of VAP and VCL at 10 and 30, and VSL at 30 s. Meanwhile, PC2 (0.26 variance explained) was composed of VAP, VCL, and VSL at 20 s. Lastly, PC3 (0.20 variance explained) was composed of motility at 10 and 20 s. Canonical correlation revealed a Wilks' Lambda F-approximation, where no canonical variates were significant ($F_{9, 80} = 1.02$, $P > 0.43$).

2.5. Discussion

Catfish hybrids are in high demand by the aquaculture industry, but the capacity to produce hybrid embryos can be a limiting factor due to a lack of natural hybridization between the two species and the necessity to sacrifice males for IVF (Bart and Dunham, 1996; Argue et al., 2003). Observations at our facilities and reports from commercial hatcheries indicate a high degree of male-to-male, seasonal, and age variations for testes size, and milt quality for this economically important species (Bobe and Labbé, 2010; Bosworth et al., 2018; Myers et al., 2020abc). Thus, a comprehensive understanding of male reproductive performance and mechanisms regulating sperm quantity are urgently needed. In this study, we showed that annual variations can have a profound effect on male blue catfish reproduction. In addition, we found significant correlations between reproductive performance traits and sperm kinematics at individual and multivariate levels.

Relationships between primary and secondary reproductive performance traits have been explored in other fishes (Locatello et al., 2008; Pitcher et al., 2009). For example, in coho salmon (*Onorhynchus kisutch*) body size and sperm velocity were not significantly related (Pitcher et al., 2009), while the authors linked male spawning coloration to sperm velocity. In Atlantic cod (*Gadus morhus*) body size attributes, spermatocrit, and seminal plasma were all significantly related to sperm activity variables across the annual reproductive season (Butts et al., 2010). In our study, we showed a series of significant correlations between reproductive performance traits and sperm kinematics. For example, both years showed significant relationships with genital morphology and fresh sperm kinematic traits, however, these correlations were inconsistent between years. These annual variations likely reflect differences in male breeder performance, such as family origin, social status, and husbandry conditions (i.e., annual temperature profiles), which have all been shown to directly impact sperm physiology (reviewed in Alavi et al., 2007).

In this study, we focused on both fresh and frozen-thawed sperm. Thus far, many studies have explored how fresh sperm quality predicts cryopreservation success (Butts et al., 2011; Carvalho et al., 2017; Dietrich et al., 2017; Ciereszko et al., 2020; Duračka et al., 2023) yet knowledge on other aspects which forecast male cryo-tolerance is limited. To overview some of the limited literature on this topic, Butts et al (2011) found significant positive relationships between frozen-thawed motility and velocity and fertility, while Dietrich et al. (2017) found that carp (*Cyprinus carpio*) sperm freezability was linked to different seminal plasma proteins that are involved in the maintenance of sperm plasma membrane integrity. Lastly, a review focused on the search for new freezability biomarkers using transcriptomics, proteomics, and bioinformatics to decipher mechanisms underlying cryotolerance in male gametes (reviewed in

Duračka et al., 2023). Very few studies have looked at how male reproductive performance traits impact post-thaw sperm kinematics, as reported here.

In our study, we found many novel correlations between reproductive performance traits and cryopreserved sperm kinematics, but like the fresh correlations, there was high variability between years. For instance, body weight, head circumference, hump circumference, head width, and genital width all had significant negative relationships with sperm kinematics in 2021. Contradictorily, in 2022, there was a lack of genital morphology correlations, but body weight, head circumference, hump circumference, and head width still had negative correlations. Furthermore, body length, testes weight, and sperm density all had significant correlations with sperm kinematics, where testes weight and sperm density were both positively correlated.

Another notable finding is the general lack of individual correlations between androgens and sperm kinematic traits. In 2021, 11-KT correlated with velocity parameters, but no correlation was detected for fresh sperm during 2022 or for the cryopreserved sperm during both years. This was unexpected based on the role of hormones in reproduction. For example, androgens typically remain high during spermiation (Alavi et al., 2012) but decrease at the conclusion of the spawning season, coinciding with decreases in sperm density and quality (Amiri et al., 1996; Babiak et al. 2006; Barannikova et al. 2004, 2008; Hachero-Cruzado et al., 2013; Cejko et al., 2018). These hormones have also been related to reproductive performance traits in other species. For example, in Chinook salmon (*Onocorhynchus tshawytscha*), T and 11-KT were both positively related to gonadal investments in sneaker males (Butts et al., 2012). It was also shown that 11-KT concentration was related to total mass and hump depth for hooknose males (Butts et al., 2012). While only a few correlations were detected between androgens and sperm traits at an

individual level (as mentioned above), these hormones together made up one of the PCs for our canonical correlation analyses, which considered males as an integrated unit.

No canonical correlations were detected across the fresh and cryopreserved treatments in 2021. One canonical correlation was detected in 2022, where fresh sperm was marginally significant indicating that as genital morphology gets smaller and hormone concentration increases, sperm display quicker initial velocity and decreased longevity. This could be related to a series of sperm morphological, physiological and health related metrics. For example, in other fish species, the size and shape of sperm has been associated with velocity and fertilization ability (Gage et al., 2004). Significant correlations between sperm concentration and quality have been reported (Babiak et al. 2006). Oxidative stress has been shown to cause sperm cell apoptosis, DNA strand breakages, and mitochondria impairment (Cabrita et al., 2014). Additionally, sub-optimal seminal plasma constituents (i.e., enzyme or protein concentration) have been shown to impair protective functions of seminal plasma, consequently decreasing sperm quality (Butts et al. 2011). At present, the picture is far from being complete for blue catfish. Despite significance in this canonical correlation, it is best to be cautious with the interpretation, especially when making statements about relevance of a relationship. This is because it is still a matter of debate on whether canonical correlation analysis is more closely equivalent to a descriptive expression of the data or if the analysis more closely resembles a predictive model (Wang et al., 2020).

2.6. Conclusions

Overall, our results revealed that blue catfish male reproductive performance traits are linked to fresh and post-thaw sperm kinematic traits when using individual correlations. Significance was also detected among these traits using a multivariate approach. Together, these data will help us to better assess male sperm quality as a basis for hatchery production. Results may also enable us to address problems associated with male-to-male variation, so in the future we can transform a "bad" sperm donor into a "good" sperm donor to maintain effective living/cryopreserved germplasm repositories for blue catfish breeding and hatchery production.

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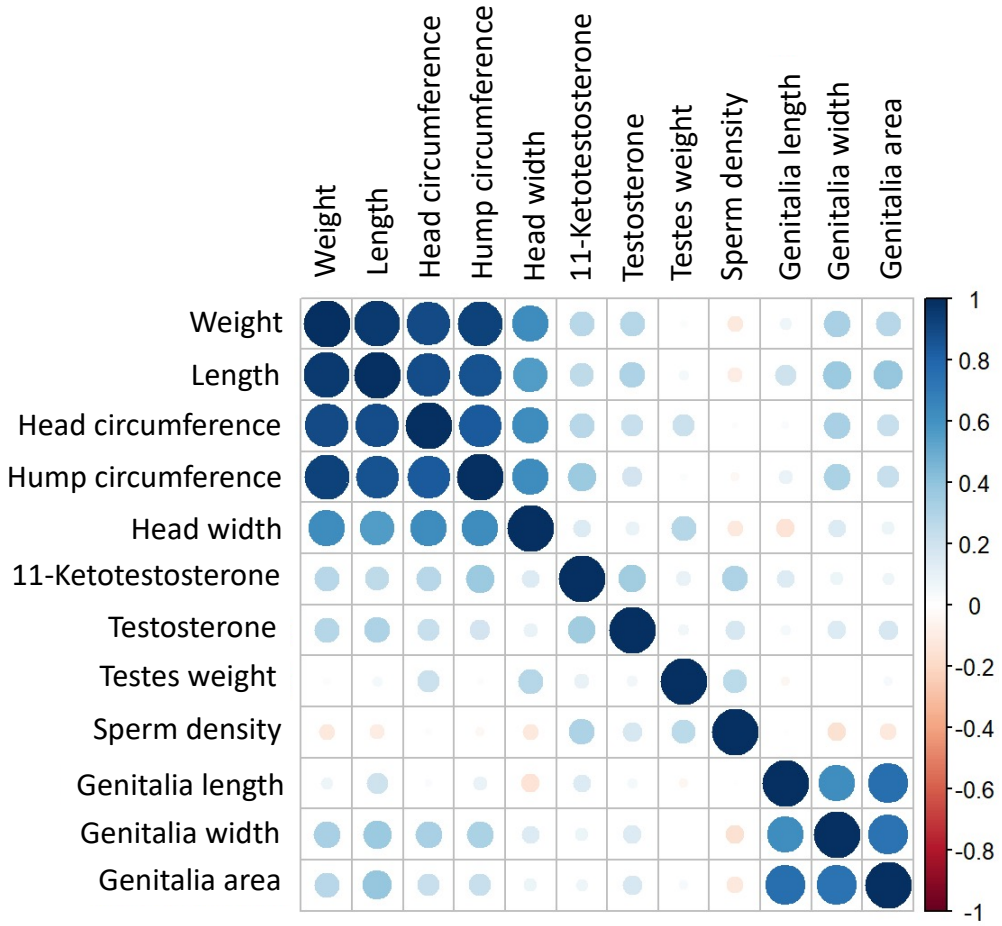

Figure 2.1. Bubble plot showing male reproductive performance traits from blue catfish (*Ictalurus furcatus*) males in 2021. Increased bubble size and shade indicate how positively (blue) or negatively (red) correlated each reproductive performance trait is to the next.

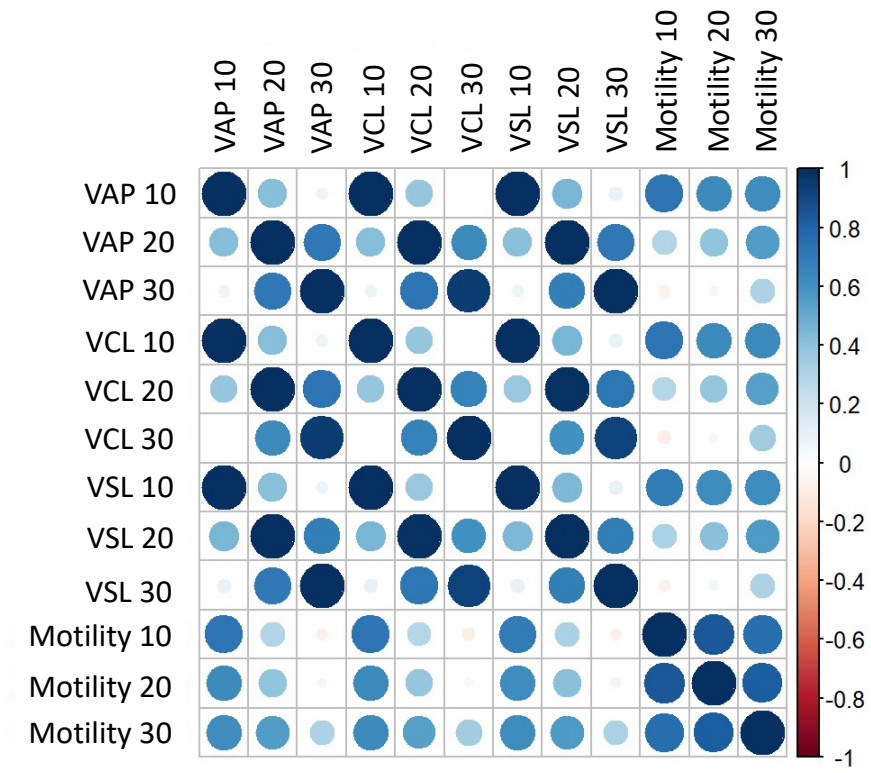

Figure 2.2. Bubble plot showing fresh sperm traits from blue catfish (*Ictalurus furcatus*) males in 2021. Increased bubble size and shade indicate how positively (blue) or negatively (red) correlated each sperm trait is to the next.

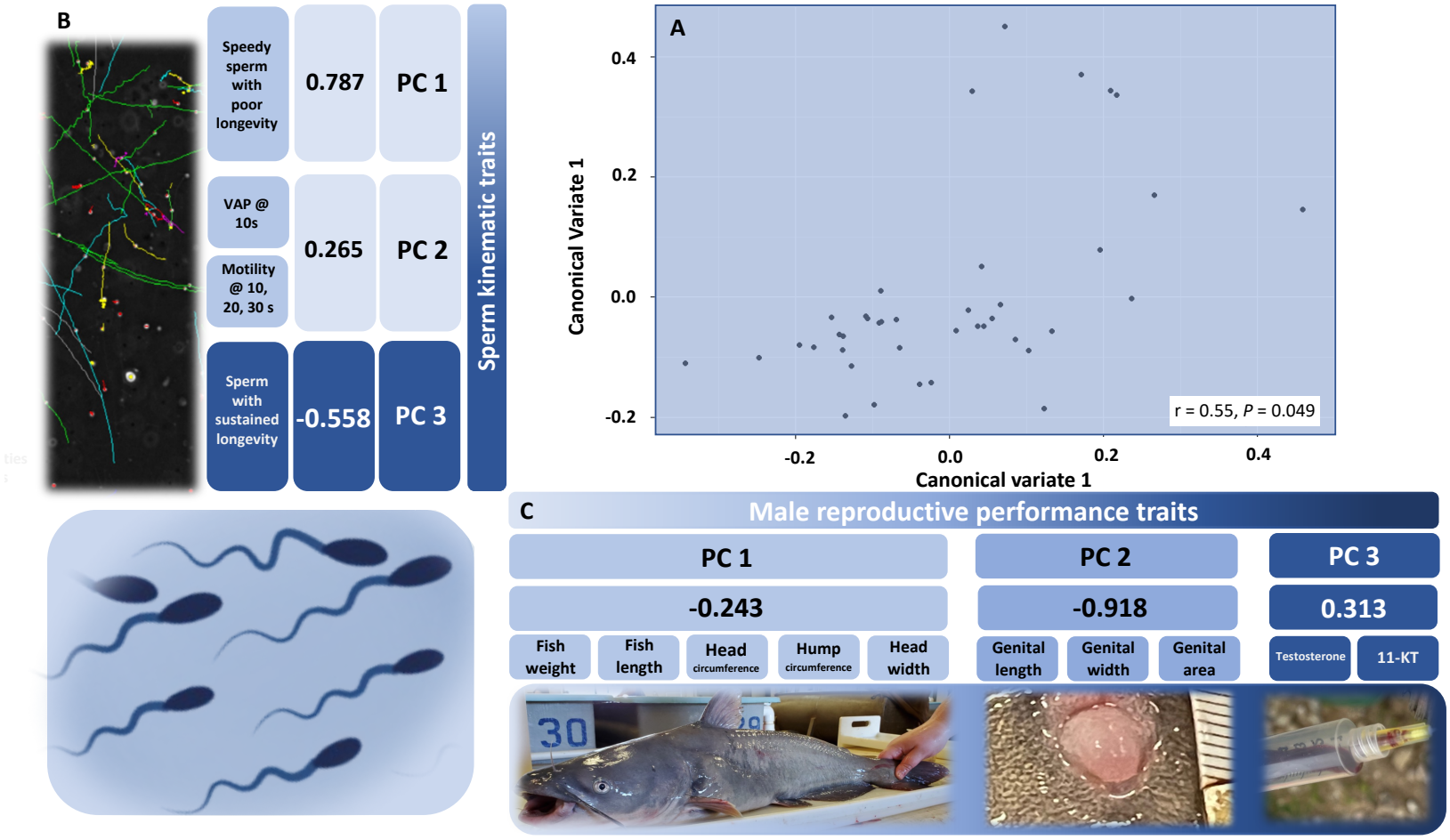

Figure 2.3. Canonical correlation analysis (A) was used to link blue catfish (*Ictalurus furcatus*) male reproductive performance traits (B) to fresh sperm kinematic traits (C). Data is shown for the 2022 spawning season. Wilks' Lambda F-approximation indicated only

one canonical variate was significant. Canonical loadings are reported for each principal component to make up the independent and dependent axes.

Table 2.1. Quantitative characteristics of blue catfish (*Ictalurus furcatus*) male (n = 43 in 2021; n = 40 in 2022) reproductive performance traits. The mean, median, standard deviation (SD), coefficient of variation (CV), minimum (Min), and maximum values (Max) are reported for each reproductive performance trait as well as results obtained from t-tests to compare 2021 vs. 2022.

	2021						2022							
	Mean	**Median**	**SD**	**CV**	**Min**	**Max**	**Mean**	**Median**	**SD**	**CV**	**Min**	**Max**	**t-statistic**	***df***
Body morphology														
Weight (kg)	4.28	4.07	0.96	22.39	2.70	6.76	5.79	5.91	1.33	22.95	3.43	8.31	5.92***	70.5
Length (cm)	71.15	70.00	4.46	6.26	61.20	80.50	72.03	72.00	6.03	8.38	61.00	84.00	0.76	81.0
Head circumference (cm)	35.40	34.50	2.87	8.09	29.50	42.00	39.58	39.75	3.57	9.01	32.50	46.00	5.91***	81.0
Hump circumference (cm)	38.71	38.00	3.50	9.04	32.00	47.00	44.54	45.50	4.50	10.11	36.00	53.00	6.61***	81.0
Head width (cm)	12.34	12.00	1.21	9.83	10.50	15.50	14.69	14.50	2.01	13.67	10.00	18.50	6.38***	63.2
Urogenital pore														
genital length (cm)	0.77	0.76	0.14	17.50	0.49	1.08	0.78	0.79	0.17	21.17	0.55	1.33	0.28	81.0
genital width (cm)	0.56	0.55	0.10	17.31	0.36	0.78	0.58	0.55	0.18	32.10	0.31	1.19	0.49	81.0
genital area (cm)	0.43	0.42	0.11	24.89	0.21	0.68	0.48	0.48	0.18	36.49	0.22	1.07	1.73	63.2
Androgens														
11-ketotestosterone	0.06	0.06	0.04	59.52	0.004	0.12	0.06	0.04	0.07	123.90	0.003	0.30	0.17	52.2
Testosterone	0.22	0.22	0.11	52.07	0.003	0.48	0.31	0.23	0.28	90.09	0.0001	1.14	1.81	47.3
Testes weight (g)	10.68	10.58	3.51	32.82	3.26	19.55	12.34	12.52	3.93	31.86	5.31	22.07	2.03*	81.0
Sperm density (cells/mL)	3.24×10^{9}	3.24×10^{9}	1.12×10^{9}	34.51	6.87×10^{8}	5.65×10^{9}	4.71×10^{9}	4.38×10^{9}	2.00×10^{9}	42.43	8.90×10^{8}	1.01×10^{10}	4.51***	60.2

*$P < 0.05$, **$P < 0.01$, **$P < 0.001$

Table 2.2. Quantitative characteristics of blue catfish (*Ictalurus furcatus*) male (n = 43 in 2021; n = 40 in 2022) fresh sperm performance traits. The mean, median, standard deviation (SD), coefficient of variation (CV), minimum (Min), and maximum values (Max) are reported for each kinematic trait with results obtained from t-tests to compare 2021 vs. 2022.

Sperm	**2021**						**2022**							
kinematics	**Mean**	**Median**	**SD**	**CV**	**Min**	**Max**	**Mean**	**Median**	**SD**	**CV**	**Min**	**Max**	**t-statistic**	***df***
Velocity (µm/s)														
VAP $_{10\,s}$	169.29	168.28	18.12	10.70	128.84	214.45	168.50	169.80	53.11	37.10	120.70	204.20	0.19	80.5
VAP $_{20\,s}$	103.48	103.42	11.04	10.67	77.45	131.45	117.52	118.89	39.87	39.87	78.81	147.51	4.89***	81.0
VAP $_{30\,s}$	58.40	58.00	6.13	10.49	42.35	71.84	68.04	66.80	21.96	21.96	54.24	82.17	6.38***	75.4
VCL $_{10\,s}$	175.70	173.60	17.10	9.75	137.64	218.59	163.30	171.70	19.41	11.11	102.70	210.74	2.35*	62.4
VCL $_{20\,s}$	111.27	112.07	10.77	9.68	86.18	138.35	127.02	128.44	14.73	11.83	97.91	152.19	6.02***	75.3
VCL $_{30\,s}$	69.16	69.02	6.01	8.69	52.61	79.50	85.97	81.61	7.27	9.35	65.62	126.60	5.69***	45.9
VSL $_{10\,s}$	164.20	163.69	18.45	11.12	121.57	209.48	151.23	157.03	50.77	36.76	89.65	198.89	2.53*	68.2
VSL $_{20\,s}$	101.43	101.11	10.92	10.77	74.84	127.06	117.04	119.30	38.64	38.79	91.22	146.26	5.84***	81.0
VSL $_{30\,s}$	56.46	56.84	6.33	11.20	38.79	70.14	74.79	71.05	21.49	37.08	52.13	117.60	6.07***	46.5
Motility (%)														
Motility $_{10\,s}$	57.90	58.64	13.42	23.17	30.96	86.08	54.44	56.28	16.63	31.38	20.77	81.13	1.07	76.9
Motility $_{20\,s}$	52.06	50.72	15.25	29.30	22.20	86.13	51.34	52.59	17.25	36.67	16.47	83.90	0.21	79.3
Motility $_{30\,s}$	38.82	37.92	13.22	34.06	16.22	66.40	45.88	47.05	13.35	32.39	22.23	70.03	2.46*	80.0

VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity; $*P < 0.05$, $**P < 0.01$, $**P < 0.001$

Table 2.3. Quantitative characteristics of blue catfish (*Ictalurus furcatus*) male (n = 43 in 2021; n = 40 in 2022) cryopreserved sperm performance traits. The mean, median, standard deviation (SD), coefficient of variation (CV), minimum (Min), and maximum values (Max) are reported for each kinematic trait with results obtained from t-tests to compare 2021 vs. 2022.

Sperm	**2021**						**2022**							
kinematics	**Mean**	**Median**	**SD**	**CV**	**Min**	**Max**	**Mean**	**Median**	**SD**	**CV**	**Min**	**Max**	**t-statistic**	***df***
Velocity (µm/s)														
VAP $_{10\,s}$	120.90	120.40	14.32	11.85	71.00	145.80	78.59	76.93	14.54	18.50	56.75	110.09	12.5***	72.0
VAP $_{20\,s}$	74.47	74.57	11.06	14.85	42.33	96.94	54.32	54.77	14.62	26.91	24.21	114.64	6.62***	72.0
VAP $_{30\,s}$	49.96	50.58	6.24	12.49	38.73	64.06	42.67	41.14	9.40	22.33	28.11	69.97	4.23***	64.7
VCL $_{10\,s}$	131.82	131.56	13.94	10.58	83.79	155.31	90.05	87.98	14.34	15.93	69.07	123.82	12.67***	72.0
VCL $_{20\,s}$	87.97	87.13	9.98	11.35	60.73	110.81	70.79	69.91	14.50	20.38	42.18	117.61	5.99***	67.7
VCL $_{30\,s}$	63.61	64.40	6.80	10.69	51.13	75.58	58.26	54.57	12.35	21.45	37.62	85.50	2.62*	58.5
VSL $_{10\,s}$	116.72	115.66	14.11	12.09	68.06	139.25	74.49	73.63	14.83	19.91	51.07	106.15	12.51***	72.0
VSL $_{20\,s}$	71.75	70.88	11.01	15.34	40.40	94.40	50.32	49.38	15.49	30.78	19.66	114.04	6.91***	68.5
VSL $_{30\,s}$	47.29	47.81	6.49	13.71	33.43	61.91	39.68	37.09	9.35	23.89	22.05	66.10	4.36***	66.1
Motility (%)														
Motility $_{10\,s}$	40.25	39.75	14.52	36.07	4.38	73.78	13.10	10.27	12.00	91.16	3.50	70.00	8.80***	72.0
Motility $_{20\,s}$	29.50	27.75	11.22	38.03	9.33	54.70	8.70	4.90	13.14	150.98	1.17	67.73	7.28***	72.0
Motility $_{30\,s}$	18.53	17.10	9.92	53.54	2.55	44.16	6.38	3.03	11.73	194.67	0.75	67.70	4.93***	70.5

VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity; $*P < 0.05$, $**P < 0.01$, $**P < 0.001$

Table 2.4. Correlations between blue catfish (*Ictalurus furcatus*) male reproductive performance traits and fresh and cryopreserved sperm kinematic traits over two spawning seasons (2021, 2022).

Reproductive performance	Fresh kinematics	r
2021		
Genital length	VAP $_{10\,s}$	0.36*
Genital length	VCL $_{10\,s}$	0.36*
Genital length	VSL $_{10\,s}$	0.36*
Genital length	Motility $_{10\,s}$	0.38*
Genital length	Motility $_{20\,s}$	0.37*
Genital length	Motility $_{30\,s}$	0.51**
Genital area	Motility $_{30\,s}$	0.34*
11-Ketotestosterone	VAP $_{30\,s}$	0.38*
11-Ketotestosterone	VCL $_{30\,s}$	0.36*
11-Ketotestosterone	VSL $_{30\,s}$	0.37*
Testes weight	VAP $_{20\,s}$	-0.32*
Testes weight	VCL $_{20\,s}$	-0.347*
Testes weight	VSL $_{20\,s}$	-0.31*
2022		
Weight	VAP $_{20\,s}$	-0.42**
Length	VAP $_{20\,s}$	-0.34*
Head circumference	VAP $_{20\,s}$	-0.36*
Hump circumference	VAP $_{20\,s}$	-0.49***
Hump circumference	VCL $_{10\,s}$	-0.4**
Hump circumference	VSL $_{10\,s}$	-0.46 **
Head width	VAP $_{20\,s}$	-0.32*
Genital length	VAP $_{30\,s}$	-0.4**
Genital length	VCL $_{10\,s}$	-0.34*
Genital length	VCL $_{30\,s}$	0.36*
Genital length	VSL $_{10\,s}$	-0.4*
Genital length	VSL $_{30\,s}$	0.32*
Genital length	Motility $_{30\,s}$	0.33*
Genital width	VAP $_{30\,s}$	-0.35*

Reproductive performance	Cryopreserved kinematics	r
2021		
Weight	VAP $_{10\,s}$	-0.35*
Weight	VAP $_{20\,s}$	-0.36*
Weight	VCL $_{10\,s}$	-0.38*
Weight	VSL $_{20\,s}$	-0.35*
Head circumference	VAP $_{20\,s}$	-0.36*
Head circumference	VAP $_{30\,s}$	-0.37*
Head circumference	VCL $_{10\,s}$	-0.34*
Head circumference	VSL $_{20\,s}$	-0.36*
Head circumference	VSL $_{30\,s}$	-0.4*
Hump circumference	VAP $_{10\,s}$	-0.38*
Hump circumference	VAP $_{20\,s}$	-0.36*
Hump circumference	VCL $_{10\,s}$	-0.39*
Hump circumference	VSL $_{10\,s}$	-0.36*
Hump circumference	VSL $_{20\,s}$	-0.35*
Hump circumference	VSL $_{30\,s}$	-0.36*
Hump circumference	Motility $_{10\,s}$	-0.38*
Head width	VAP $_{10\,s}$	-0.53***
Head width	VAP $_{20\,s}$	-0.35*
Head width	VCL $_{10\,s}$	-0.53***
Head width	VSL $_{10\,s}$	-0.49**
Head width	VSL $_{20\,s}$	-0.335*
Head width	Motility $_{10\,s}$	-0.38*
Genital width	VAP $_{30\,s}$	-0.34*
Genital width	VSL $_{30\,s}$	-0.33*
2022		
Weight	VAP $_{30\,s}$	-0.32*
Weight	VSL $_{30\,s}$	-0.34*
Length	VAP $_{30\,s}$	-0.38*

Genital width	VCL $_{10\ s}$	-0.35*	Length	VCL $_{30\ s}$	-0.37*
Genital width	VCL $_{30\ s}$	0.34*	Length	VSL $_{30\ s}$	-0.39*
Genital width	VSL $_{10\ s}$	-0.41**	Head circumference	VAP $_{30\ s}$	-0.42**
Genital width	VSL $_{30\ s}$	0.33*	Head circumference	VCL $_{30\ s}$	-0.36*
Genital width	Motility $_{30\ s}$	0.32*	Head circumference	VSL $_{30\ s}$	-0.44**
Genital area	VAP $_{30\ s}$	-0.37*	Hump circumference	VAP $_{30\ s}$	-0.33*
Genital area	VCL $_{10\ s}$	-0.36*	Hump circumference	VSL $_{30\ s}$	-0.33*
Genital area	VCL $_{30\ s}$	0.36*	Head width	VAP $_{30\ s}$	-0.43**
Genital area	VSL $_{10\ s}$	-0.42**	Head width	VCL $_{10\ s}$	-0.36*
Genital area	VSL $_{30\ s}$	0.34*	Head width	VCL $_{30\ s}$	-0.43**
Genital area	Motility $_{30\ s}$	0.*34	Head width	VSL $_{30\ s}$	-0.46**
Testes weight	VAP $_{30\ s}$	-0.49***	Testes weight	VAP $_{30\ s}$	0.32*
Sperm density	VCL $_{10\ s}$	0.33*	Testes weight	VCL $_{30\ s}$	0.44**
			Sperm density	VAP $_{10\ s}$	0.41**
			Sperm density	VCL $_{10\ s}$	0.43*
			Sperm density	VSL $_{10\ s}$	0.41

*$P < 0.05$, **$P < 0.01$, **$P < 0.001$

VAP = Average path velocity

VCL = Curvilinear velocity

VSL = Straight line velocity

Table 2.5. Principal component analysis for male reproductive performance traits in blue catfish (*Ictalurus furcatus*). Eigenvalues, proportion of variance, and loadings for male reproductive performance traits over two spawning seasons (2021, 2022).

	2021						2022					
	Fresh			Cryopreserved			Fresh			Cryopreserved		
Male reproductive performance	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**
Eigenvalue	4.71	2.20	1.54	4.86	3.06	1.93	4.90	3.03	1.93	4.76	2.19	1.61
Proportion of variance	0.35	0.21	0.13	0.38	0.27	0.16	0.38	0.27	0.16	0.36	0.21	0.14
Loadings												
Weight	0.95	0.13	0.15	0.97	0.08	-0.06	0.97	0.09	-0.06	0.96	0.12	0.12
Length	0.90	0.25	0.17	0.96	0.06	0.01	0.96	0.07	0.01	0.91	0.26	0.12
Head circumference	0.92	0.10	0.13	0.97	0.04	-0.04	0.97	0.05	-0.04	0.94	0.17	0.13
Hump circumference	0.91	0.18	0.18	0.93	0.14	-0.07	0.93	0.15	-0.08	0.94	0.12	0.14
Head width	0.78	-0.08	-0.12	0.90	0.00	-0.02	0.90	0.00	-0.02	0.78	-0.17	-0.16
Genital length	-0.08	0.90	0.12	-0.04	-0.14	0.96	0.10	0.97	-0.01	0.18	0.08	0.79
Genital width	0.26	0.84	-0.03	-0.05	0.13	0.97	0.08	0.98	-0.02	0.24	0.05	0.56
Genital area	0.16	0.92	0.01	-0.04	0.58	0.06	0.13	0.97	-0.04	0.08	0.02	0.07
11-Ketotestosterone	0.21	0.04	0.77	-0.19	0.01	0.16	-0.04	-0.14	0.96	-0.18	-0.10	0.80
Testosterone	0.19	0.10	0.68	0.09	0.97	-0.01	-0.06	0.13	0.97	-0.10	0.91	0.10
Testes weight	0.11	0.01	0.03	0.08	0.98	-0.02	-0.02	0.59	0.05	0.29	0.81	-0.03
Sperm density	-0.20	-0.11	0.64	0.13	0.97	-0.04	-0.18	0.00	0.16	0.18	0.91	-0.02

Table 2.6. Principal component analysis for fresh and cryopreserved sperm kinematic traits in blue catfish (*Ictalurus furcatus*). Eigenvalues, proportion of variance, and loadings for male reproductive performance traits over two spawning seasons (2021, 2022).

	2021				2022					
Sperm	**Fresh**		**Cryopreserved**		**Fresh**			**Cryopreserved**		
kinematics	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**
Eigenvalue	6.49	3.67	8.47	1.73	4.19	2.99	2.49	4.34	3.51	1.58
Proportion of variance	0.43	0.42	0.41	0.28	0.30	0.25	0.26	0.33	0.26	0.20
Loadings										
VAP $_{10\ s}$	0.93	0.09	0.86	0.14	0.17	0.89	0.13	0.60	0.36	0.61
VAP $_{20\ s}$	0.40	0.85	0.85	0.39	0.23	0.07	0.90	-0.10	0.96	0.05
VAP $_{30\ s}$	-0.06	0.96	0.26	0.93	0.03	-0.27	0.66	0.95	-0.14	-0.14
VCL $_{10\ s}$	0.93	0.10	0.84	0.11	0.89	0.27	0.25	0.62	0.34	0.59
VCL $_{20\ s}$	0.36	0.87	0.84	0.42	-0.01	0.16	0.94	-0.09	0.91	0.07
VCL $_{30\ s}$	-0.10	0.92	0.18	0.91	-0.95	0.16	0.05	0.83	-0.23	0.02
VSL $_{10\ s}$	0.92	0.09	0.86	0.11	0.89	0.29	0.25	0.59	0.39	0.59
VSL $_{20\ s}$	0.43	0.83	0.84	0.40	0.03	0.17	0.92	-0.10	0.95	0.04
VSL $_{30\ s}$	-0.04	0.95	0.25	0.91	-0.94	0.23	0.07	0.94	-0.13	-0.17
Motility $_{10\ s}$	0.89	-0.04	0.37	0.20	0.19	0.83	0.09	-0.22	0.06	0.75
Motility $_{20\ s}$	0.83	0.11	0.38	0.33	-0.05	0.79	-0.01	-0.01	-0.06	0.71
Motility $_{30\ s}$	0.77	0.37	0.45	0.43	-0.22	0.71	-0.05	0.52	-0.06	0.40