Molecular Regulation of Sperm Function, Environmental Interactions, and Diagnostic Biomarkers: An Integrated Omics Approach to Understanding Sperm Performance in Important Aquaculture Species

by

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Abstract

Reproduction is a critical component of both the economic viability and sustainability of aquaculture species. Technologies that optimize broodstock management and improve access to high-quality gametes can enhance productivity while reducing environmental impact. Emerging molecular tools provide powerful approaches for assessing sexual maturation and improving offspring production in aquaculture systems. The primary objective of this thesis was to utilize transcriptomic approaches to evaluate the quality of male gametes for major U.S. aquaculture species. The first study assessed the molecular regulation of mRNAs by miRNAs in oysters with sperm of differing qualities and cryotolerance. Our findings revealed differentially expressed RNAs and key mRNA×miRNA interactions that have roles in the regulation of oyster sperm performance. The second study investigated the role of ovarian fluid during blue catfish sperm activation to identify molecular mechanisms underlying changes in kinematic activity. Results demonstrated that the ovarian fluid microenvironment induces molecular changes in membrane properties, ion channel activity, signaling pathways, binding proteins, and energy metabolism prior to motility activation. These molecular changes promote hyperactivation of sperm cell motility. The third study evaluated circulating miRNAs as non-lethal biomarkers of sperm quality in blue catfish. Three differentially expressed miRNAs were identified between males with low-quality and high-quality sperm, highlighting their potential as diagnostic indicators. Together, these findings demonstrate the applications of reproductive molecular tools to improve industry efficiency, and sustainability in aquaculture production.

Artificial Intelligence (AI) Use Disclosure Statement

In the preparation of this thesis no Artificial Intelligence (AI) tools were used.

Digital Accessibility Disclosure Statement

In the preparation of this thesis, the following digital accessibility tools were used to ensure this document complies with federal requirements: Microsoft Word Accessibility Checker. The author acknowledges full responsibility for the intellectual content of this work and has made a good faith effort to comply with digital accessibility requirements in publishing, wherein the nature of the content does not significantly change in order to do so. Furthermore, all content has been reviewed and revised to meet these requirements prior to final publication

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List of Abbreviations

CASA- Computer Assisted Sperm Analysis
GO- Gene Ontology
BP- Biological Processes
MF- Molecular Function
CC- Cellular Component VCL- Velocity
mRNA- Messenger RNA
miRNA- MicroRNA
piRNA- Piwi-interacting RNA
tsRNA- tRNA-derived smallRNA
sRNA- smallRNA
rsRNA- rRNA-derived smallRNA
MRI- Motility Recovery Index
AUSL- Auburn University Shellfish Laboratory
ARPL- Aquatic Reproductive Physiology Laboratory
DI- deionized
ASW- Artificial Sea Water
SD- Standard Deviation
SEM- Standard Error
PE- Paired-end
DEG- Differentially Expressed Gene
PCA- Principal Component Analysis
NES- Normalized Expression Score
ATP- Adenosine Triphosphate
OF- Ovarian Fluid
CFC- cryptic female choice
GMMC- gamete mediated mate choice
IACUC- Institutional Animal Care and Use Committee
DO- dissolved oxygen
KEGG- Kyoto Encyclopedia of Genes and Genomes
VC- Variance component
GSI- Gonadosomatic Index
c-miRNA- Circulating microRNA
REML- Restricted Maximum Likelihood
MAPK- Mitogen-activated protein kinase
IgSF CAM- Immunoglobulin superfamily cell adhesion molecule
FA- Focal Adhesion
MHC- Major Histocompatibility complex
GRP- Gamete Recognition Proteins
NIFA- National Institute of Food and Agriculture
USDA- United States Department of Agriculture
ARS- Agriculture Research Station
Pro_{Mot} Progressive Motility
Pro_{VCL}- Progressive Curvilinear Velocity
CPM- Count Per Million
VEGF- Vascular endothelial growth factor

Chapter 1

Linking gene expression to sperm quality and cryotolerance in eastern oyster (*Crassostrea virginica*)

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Keywords: CASA; Cryopreservation; miRNA; mRNA; Aquaculture

1.1 Abstract

Access to high-quality gametes remains a constraint in eastern oyster (*Crassostrea virginica*) aquaculture. Germplasm cryopreservation offers a solution as it ensures access to gametes. However, for oysters there is high degree of individual variability in sperm quality and resistance to cryoinjury. Bioinformatic assessment is an emerging tool that can be used to understand and optimize gamete quality. Coding and non-coding RNAs provide an understanding of molecular mechanisms and genetic regulation involved in sperm development and function. Our objectives were to investigate how miRNA and mRNA profiles relate to fresh and frozen-thawed sperm quality, based on percent motility and swimming velocity. We hypothesized that non-coding miRNAs regulate mRNA transcription related to oyster sperm function. Sperm from 20 oysters were evaluated using Computer Assisted Sperm Analysis software before and after cryo-storage. Males were ranked for quality and cryotolerance based on fresh and recovered post-thaw kinematics. An integrated analysis of miRNA and mRNA transcription was conducted for six oysters with low-quality vs. high-quality fresh sperm (n = 12) and six oysters with low-cryotolerant vs. high-cryotolerant sperm (n = 12). Differential expression and enrichment analysis for both groups highlighted cilium, actin binding, chromosome segregation, the microtubule cytoskeleton, ATP activity, and DNA integrity, all important for cell structure and function. Regulatory interactions between miRNA and mRNA were also assessed, and 140 potential interactions identified. Our findings reveal RNAs that have critical roles in the regulation of oyster sperm performance, of which a functional understanding can provide the groundwork for precision breeding and more sustainable, profitable aquaculture.

1.2 Introduction

Global aquaculture surpassed capture fisheries as the main producer of aquatic animals for the first time in 2022, increasing 4.4% from 2020 for a total of 223.2 Mt (FAO, 2024). Molluscs comprised 17.7 Mt of production, 37.4% of which was from oysters (FAO, 2024). The eastern oyster (*Crassostrea virginica*) is a commercially important species that also plays a critical role as a keystone species with a natural distribution along the Atlantic coast of North America. In 2023, the eastern oyster represented the highest mollusc sales in the United States at a production value of $151 million USD (USDA, 2022). As the demand for aquaculture is growing, technological advancements must be made to improve efficiency and sustainability, and to support long-term industry growth. The production of many farmed aquatic species, including eastern oysters, is dependent on the accessibility of gametes from farm-raised broodstock or wild-caught populations. However, gamete quality remains one of the biggest bottlenecks for aquaculture production (Migaud et al., 2013). Eastern oysters are a high value species that can reproduce multiple times a season and mature quickly, making them good model species for research on gamete and reproduction optimization.

A solution that ensures continuous accessibility of gametes is germplasm cryopreservation. Gamete cryopreservation is a method of preserving genetic material indefinitely by freezing the cells to low temperatures (-196°C) with the use of liquid nitrogen to stop biological activity, DNA degradation, and cell apoptosis (Kumari and Maurye, 2021). The use of frozen-thawed germplasm to enhance hatchery production is expanding, as it provides hatcheries with gametes on demand, eliminating the need for male and female synchronization (Cabrita et al., 2010; Suquet et al., 2000; Kumari and Maurye 2021). Frozen germplasm also assists with selective breeding and maintaining

genetic diversity of genetically selected lines (Cabrita et al., 2010; Suquet et al., 2000; Kumari and Maurye, 2021). The successful freezing of spermatozoa has been reported in over 200 fish and shellfish species (Diwan et al., 2020). Despite optimized procedures for many species in which cells are viable post-thaw, there is large variation in the quality of spermatozoa due to individual variation and cryoinjury (Cabrita et al., 2010; Kumari and Maurye, 2021; Diwan et al., 2020). Furthermore, not all sperm samples withstand freezing to the same degree, meaning that some samples show high tolerance to the cryopreservation process and have high reproductive value post-thaw, while other sperm may have good viability when fresh but no tolerance to cryopreservation (Suo et al., 2024).

High-quality sperm cells are those that can fertilize and produce viable offspring (Bobe and Labbé, 2010; Valdebenito et al., 2015). In addition to direct measurements of fertilization, the quality of sperm has been assessed using Computer Assisted Sperm Analysis (CASA) software. CASA can be utilized to measure important cell characteristics for successful fertilization including curvilinear velocity (VCL) and percent of motile sperm (Nichols et al., 2021; Gallego et al., 2018; Bobe and Labbé, 2010; Rurangwa et al., 2004). Eastern oyster sperm cells exhibit helical swimming patterns, tracked as circular movement in 2D, that allow for more efficient movement in complex environments, and at higher velocities may allow for better chemotactic sensing during external fertilization (Nichols et al., 2021; Guasto et al., 2020; Mann & Luckenbach, 2013). Sperm motility is a measurement that integrates the quality of numerous cellular components that are all responsible for the activation and longevity of movement. This includes parameters such as fatty acid composition (Wood et al., 2025), plasma membrane integrity and mitochondrial membrane potential (Paniagua-Chávez et al., 2006; Boulais et al., 2017), energy metabolism and respiration activity (Geffen and Frayer, 1993; Lahnsteiner et al., 1998), and DNA fragmentation (Paniagua-

Chávez et al., 2006). While measurements of these parameters can be used to assess sperm quality, recent research has shown that emerging genomic tools such as high-throughput sequencing for genetic markers and bioinformatic analyses can offer more precise and efficient assessments of cell states (Hess et al., 2024; Long, 2020). Investigating molecular biomarkers associated with male reproduction, sperm quality, and sperm cryotolerance in aquaculture is needed to optimize gamete quality for use in hatcheries, gene-banking, and genetic selection.

Molecular biomarkers can be a valuable resource as predictors of sperm cell quality and fertilization potential (Hess et al., 2024; Zhang et al., 2025; Yalçın et al., 2025). While sperm cells are transcriptionally inert, they do carry a multitude of residual messenger RNAs (mRNAs) and non-coding RNA molecules resulting from spermatogenesis, as well as some RNA transmitted by extracellular vesicles, and a small amount from de novo transcription in mature cells (Santiago et al., 2021). These RNAs are transported to the oocyte during fertilization and have roles in embryonic development and epigenetic inheritance (Betlach and Erikson 1976; Jodar et al., 2013). Additionally, RNAs that remain within the sperm cells after spermatogenesis can provide a historical make-up of the cell and therefore, can be biomarkers of quality (Jodar et al., 2013).

Non-coding RNAs are highly prevalent in sperm and include microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), tRNA-derived small RNAs (tsRNAs), and rRNA-derived small RNAs (rsRNAs), all of which are small RNAs (sRNAs) that have important regulatory roles (Hua et al., 2019; Yue et al., 2022). In the reproduction of molluscs, coding and non-coding biomarkers identified in sperm and testes were found to impact sexual maturity, gamete quality, and gonadal development (Yalçın et al., 2025). Differentially expressed sRNAs have also been linked to sperm quality and embryo performance in molluscs due to differential regulatory

activity in gene expression. Recent research on the Pacific oyster (*Crassostrea gigas*) showed that the interaction between miRNAs and mRNAs influences the sexual development, gonad differentiation, and gametogenesis of the organism (Yue et al., 2022). The regulation of oyster gene expression is caused by miRNA interactions with mRNAs; these often result in the silencing or post-transcriptional regulation of gene expression (O'Brien et al., 2018). Nucleotides on the 5′end of the miRNA bind to the 3′end of complimentary untranslated regions of mRNA, resulting in the disruption of translation or degradation of the mRNA. This often results in the downregulation of those bonded mRNAs but can cause upregulation in specific mRNAs or upregulation of mRNAs through competing factors (O'Brien et al., 2018). Due to the prevalence of non-coding RNAs in sperm, epigenetic regulation of spermatogenesis may be of importance to quality and non-coding biomarkers may prove to be powerful indicators of sperm performance.

To achieve a goal of increasing the production of eastern oysters to meet growing aquaculture demands and generate lines with qualities desired by the industry (i.e., fast growth, environmental, and disease resilience), the focus is now on technological advancements including cryopreservation and breeding programs of domesticated oyster stocks (Yang et al., 2021). However, recent studies have found high variation in quality of eastern oyster gametes pre- and post-cryopreservation that may impede hatchery success (Yang et al., 2021; Yang et al., 2012). At present, the lack of understanding of molecular mechanisms, gene expression, and genetic regulation involved in eastern oyster sperm function prevents further progress in this area.

The identification of biomarkers for sperm cell quality and cryotolerance potential, based on kinematic performance measured by cell motility and VCL, would have major applications in the industry to develop gene banks and increase production outputs. Here, we investigated

miRNA and mRNA profiles of low- and high-quality eastern oyster sperm to further understand the relationship between gene expression and sperm performance. Additionally, we identified genes that indicate a low or high sperm cryotolerance using a Motility Recovery Index (MRI). As sperm kinematics are integral to male reproductive success, this study compares differential expression and pathway analyses between males with low vs. high sperm kinematics, and of males with sperm that had low vs. high motility recovery after the cryopreservation process. Our goal was to link coding and non-coding RNA biomarkers with motility-based sperm quality and cryotolerance in eastern oysters to support long-term industry growth.

1.2. Materials and Methods

1.2.1 Ethics statement

The study was conducted at Auburn University E.W. Shell Fisheries Center in Auburn, Alabama, U.S. (32.6524° N, 85.4860° W).

1.2.2 Oyster culture

Diploid oysters used in this study came from the Auburn University Shellfish Laboratory (AUSL) at Dauphin Island, Alabama (30°14’51.9” N, 88°4’46.9” W). Animals were transported at 14°C in a cooler with damp paper towels on top of ice packs to the Auburn University Aquatic Reproductive Physiology Laboratory (ARPL) in Auburn, Alabama on 25 June 2024, and 31 July 2024. The oysters were collected from AUSL broodstock line spawned in 2022. Sixty oysters were

held at ARPL in an Aquaneering Aquatic Habitat (Aquaneering., CA, U.S.) recirculating aquaculture system (AHAB) in 2.8 L tanks, or 6 L for wide individuals. The AHAB system contained a 110 L sump tank, 110 L biofilter with diffused air, UV light sterilizer (Item number MUVSS05, Aquaneering., CA, U.S.), and a heat-pump (Delta Star DSHP-4, Aqua Logic Inc., CA, U.S.). Each day oysters were fed LPB^{TM} Frozen Shellfish Diet to maintain a background density between 100,000 and 150,000 cells mL^{-1} (Reed Mariculture Inc., CA, U.S.). Approximately 20-30% of the system water was replaced daily with seawater prepared from Crystal Sea Marine mix (Marine Enterprises International, LLC., MD, U.S.). Seawater was mixed using tap water filtered through a reverse osmosis deionization system (Barracuda Glacial GPD RO/DI, AquaFX., FL, U.S.) to reach a salinity of 20 ppt. Water temperature was maintained at 18 ± 1°C and photoperiod was 12 h light/12h dark. Oysters were held in this system until used for experimentation.

1.2.3 Sperm collection and quality analyses

1.2.3.1 Sex determination and sperm collection

A subset of oysters was removed from tanks at multiple sampling dates between July and August 2024. A digital caliper (Item number 12777–830, VWR., PA, U.S.) and scale (TS200–02; Ohaus corporation., NJ, U.S.) were used to measure shell height (from hinge to opposite edge), shell length (across widest part), shell width (from left shell to right shell), and weight. Each oyster was released at the hinge using a shucking knife, with the left shell on the table, and the adductor muscle was severed to remove the right shell. The oyster tissues and shell were thoroughly rinsed with deionized water (DI) to remove any salt and dried with Kimwipes®. Genotypic sex was

determined by viewing the presence of eggs or sperm in ~1 µL sample of gonad tissue with a Zeiss Axiolab 5 microscope (Carl Zeiss Microscopy, LLC., NY, U.S.) equipped with 20× negative phase contrast objective (SAF "A-Plan" 20×/0.45 Ph-n 1). There were twenty-six mature male oysters identified by the presence of developed sperm cells, and as oysters are alternate hermaphrodites, the absence of eggs. Sperm were collected from male oysters by making shallow slices with a sterile scalpel into the gonad tissues, pipetting the sperm into 1.5 mL microcentrifuge tubes, and held at 4°C on an EcoTherm Chilling/Heating Dry Bath (Torrey Pines Scientific., CA, U.S.). If >1.5 mL of sperm was collected from a single oyster, all tubes were pooled into one 15 mL centrifuge tube and stored at 4°C. The gonad from both sides of each oyster were sampled, and as much sperm extracted as possible, while avoiding any digestive tissues.

1.2.3.2 Cell density determination and dilution

Cell density was determined for each male using a Neubauer hemocytometer. Sperm were diluted 1:500 in 650 mOsm kg^{-1} Ca-free HBSS, prepared by adjusting the standard 1 L HBSS recipe to 450 mL without the addition of $CaCl_2$ (0.137 M NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose) (Hanks, 1975; Paniagua-Chavez et al., 1998). The pH of HBSS was reduced to 6.0 using HCL to stop sperm motility (Nichols et al., 2021). Six males were discarded prior to cryopreservation due to low sperm density as they would not have produced enough straw replicates; the remaining twenty males were used for subsequent analyses. Diluted sperm samples were vortexed to homogenize for ~9 s, and 10 µL were pipetted onto a hemacytometer and allowed to settle. When sperm were settled onto the 5 × 5 grid, 5 squares were counted: top left and right, bottom left and right, and the center.

At least two replicates were counted for each male to determine the average cell density of the diluted sperm. The average count was multiplied by 5 to determine the number of cells on the entire grid, this was multiplied by 500 (the dilution factor), and then by 10,000 to determine the number of cells mL^{-1}. The amount of diluent needed to dilute the sperm to 1×10^9 cells mL^{-1} was then calculated to standardize the cryopreservation process. Sperm were held at 4°C until being diluted just prior to cryopreservation.

Once diluted to 1×10^9 cells mL^{-1}, two replicate 150 µL samples were taken from each male for transcriptomics. These samples were diluted into 350 µL of DNA/RNA Shield (Zymo Research., CA, U.S.) in 1.5 mL microcentrifuge tubes and held at 4°C for 24 h, then transferred to -80°C for storage until extraction.

1.2.3.3 Computer assisted sperm analysis

Fresh sperm motility was estimated within 1 h of dilution in 650 mOsm kg^{-1} Ca-free HBSS using CASA software (CEROS II, Hamilton Thorne Biosciences., MA, U.S.). Sperm activation solution was prepared by adjusting artificial sea water (ASW) (516 mM NaCl, 10.4 mM KCl, 11 mM $CaCl_2$, 34 mM $MgCl_2$, and 22 mM $MgSO_4$) (Nichols et al., 2021; Boulais et al., 2018) salinity from 40 to 20 ppt using ultrapure water (Advantage A10 Water Purification System, Milli-Q®., Merck KGaA, Germany). The activation solution was buffered to 8.8 pH with 20 mM Tris, and 0.4% Pluronic F-127 (Sigma Aldrich., MO, U.S.) was added to prevent the sperm from sticking to the glass slides during CASA. Diluted sperm samples and activation solution were held at 4°C on an EcoTherm Chilling/Heat Dry Bath (Torrey Pines Scientific., CA, U.S) while estimating sperm motility. To activate sperm, 1 µL of

diluted cells were pipetted into a 1.5 mL microcentrifuge tube with 10 to 20 µL of activation solution and mixed thoroughly with a pipette. Next, 5 µL of the activated sperm were immediately pipetted onto a 20 µm deep 2×-CEL chamber (Hamilton Thorne Biosciences., MA, U.S.), under the Zeiss Axiolab 5 microscope. Motility was recorded at 20× magnification using a negative phase contrast objective (SAF "A-Plan" 20×/0.45 Ph-n 1) at 30, 60, and 120 s post-activation. Each male had three technical replicate activations. Sperm curvilinear VCL (μm^{-1}) and total motility (%) were assessed. All CASA videos were manually checked for quality and sperm tracks were removed if the software classified non-motile sperm as motile, sperm were incorrectly tracked when multiple tracks crossed, and/or if sperm were drifting. Upon analysis of sperm kinematic it was found that there was no difference across time points, and all future analyses were conducted with the 30 s data.

1.2.4 Sperm cryopreservation

Once sperm were diluted into extender solutions at 1×10^9 cells mL^{-1}, they were mixed with 10% DMSO as a cryoprotectant and pipetted into 0.5 mL French straws (Minitube U.S., WI, U.S.) before being sealed with glass beads. Each straw held 200 µL of diluted sperm to increase straw replicates. For each male, 4 to 12 straws were filled. Straws were placed into metal racks and transferred to a control rate freezer (Planer Kryo 560–16, Cryo Associates Inc., MD, U.S.), where they were held at 4°C for 5 min, and then frozen at -20°C min^{-1} from 4 to -80°C. Straws were held at -80°C for 5 min and then plunged into a vapor-pressure liquid nitrogen storage system (MVE 815P-190F-GB, Cryo Associates Inc., MD, U.S.).

Straws were held in liquid nitrogen for 63 days before thawing. To thaw, straws were submerged in a 40°C water bath for 8 s, dried off with Kimwipes®, and emptied into 1.5 mL microcentrifuge tubes on a EcoTherm Chilling/Heat Dry Bath set at 4°C (Torrey Pines Scientific., CA, U.S). Motility of thawed sperm was then estimated following the same procedure outlined above for fresh sperm.

1.2.5 Transcriptomic Analyses

1.2.5.1 Sample Selection

Comparing males with high-quality vs. low-quality sperm.

Oysters were selected for RNA sequencing to compare low-quality vs. high-quality sperm based on the average motility (%) and average curvilinear VCL (μm^{-1}) from the three technical replicates recorded from CASA at 30 s post-activation (Fig. 1). Z-scores were calculated [$z = (x - \mu)/\sigma$] for both parameters to measure how far each male was from the population mean. This score was then used to determine males with the lowest and highest sperm quality. The z-scores of twenty oysters for both parameters were added together and the six individuals with the lowest combined z-score were considered low quality and the six with the highest were considered high quality.

Comparing males with high vs. low cryotolerance

To compare oysters having sperm with high tolerance to cryopreservation to those that did not, a motility recovery index (MRI) was calculated [MRI= $(Post\text{-}thaw\,Motility)/(Fresh\ Motility) \times 100$]. Oysters with a fresh percent motility < 20% were not included as there were insufficient cells recorded by CASA post-cryopreservation to use in analysis; therefore, eighteen males were analyzed. The top and bottom six oysters, based on MRI scores, were chosen for RNA sequencing and transcriptomic analysis, their average (± SD) MRIs were 35.73 ± 3.8 and 15.39 ± 5.8, respectively (Fig. 1).

1.2.5.2 Sperm kinematic statistical analysis

All statistical analyses were conducted using R (v.5.40) software. T-tests were utilized to evaluate significant differences for the motility (%) and VCL (μm/s) of low-quality vs. high-quality sperm, as well as the MRI between low-cryotolerant vs. high-cryotolerant sperm. Alpha was set at 0.05.

1.2.5.3 RNA extraction

Two technical replicates of fresh sperm from each male stored at -80°C in DNA/RNA Shield (Zymo Research, CA, U.S.) and of cryopreserved sperm were used for Total RNA extraction. For fresh sperm, samples were thawed at room temperature and pooled before extraction. For cryopreserved sperm, two straws were thawed at 40°C for 8 s in a water bath, pooled, then diluted in DNA/RNA Shield at a 2:1 shield to sperm ratio (Zymo Research, CA, U.S.). RNA was extracted from sperm samples following the protocol from the Quick RNA Miniprep

Plus kit for cells (Zymo Research, CA, U.S.). In brief, DNA/RNA Lysis buffer was added in a 1:1 ratio to the cell suspension and mixed. DNA was then removed from the suspension via Spin-Away Filter, and the RNA flow-through mixed 1:1 with 95% ethanol. The suspension was then filtered through a Zymo-Spin^{TM} IICR Column, treated with DNase I, and washed. The final elution volume for each sample was 50 µL to ensure a high final concentration of RNA. A Nanodrop One^{C} spectrophotometer (ThermoFisher Scientific., MA, U.S.) was used to measure concentration and purity (260/280) of RNA.

1.2.5.4 RNA Sequencing

Total RNA samples extracted from fresh and cryopreserved sperm were sent to Azenta Life Sciences (NJ, U.S.) for mRNA and sRNA library preparation and Illumina sequencing. Messenger (mRNA) sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep for Illumina with manufacturer's instructions (New England Biolabs, MA, U.S.). The polyA mRNA workflow was utilized to first isolate mRNA from the samples using poly(A) selection to remove non-polyadenylated RNA species, therefore, enriching for mRNA. Second, enriched mRNA samples were subjected to fragmentation at 94°C for 15 min, then priming, first and second strand cDNA synthesis, and end repair, before PCR enrichment and validation on the Agilent TapeStation (Agilent Technologies, CA, U.S.). Last, samples were sequenced on the Illumina NovaSeq X Plus platform using a 2 × 150 pb paired end (PE) configuration (Azenta Life Sciences, NJ, U.S.). Sample data were provided in demultiplexed raw FASTQ format at a minimum of 19 M PE reads.

Small RNA (sRNA) sequencing libraries were prepared using the NEB Small RNA library Prep Kit according to manufacturer's instructions (New England Biolabs, MA, U.S.) by first, purifying single-stranded sRNA molecules from total RNA samples by 3' adapter ligation, followed by primer hybridization and 5' adapter ligation. Then the sRNA, now enclosed by adapters, was converted to cDNA by first-strand cDNA synthesis and amplified with PCR. Samples were subjected to size selection and cleanup, then library validation on the Agilent TapeStation (Agilent Technologies, CA, U.S.), to ensure the integrity of the desired sRNAs before sequencing on the Illumina NovaSeq X Plus platform using a 2 × 150 pb PE configuration (Azenta Life Sciences, NJ, U.S.). Sample data were provided in demultiplexed raw FASTQ format at a minimum of 8 M PE reads.

1.2.5.5 Messenger RNA QC and alignment

Raw mRNA FASTQ sequencing files received from Azenta Life Sciences underwent an MD5 checksum to verify the integrity of the files, then quality control and preprocessing, where low-quality bases and adapter sequences were removed via Fastqc (v.0.10.1) and Trimmomatic (v.0.39) (Bolger et al., 2014). Reads were trimmed by removing the first 10 bp, or if they were of low quality, the first and last 30 bp were removed, and the average quality across every 6 bp was set to a phred score of 30. Remaining mRNA-seq reads shorter than 36 bp were then excluded from further analyses. The cleaned high-quality reads were then aligned to the *C-virginica* 3.0 genome obtained from NCBI with Hisat2 (v.2.2.0) (Refseq assembly number: GCF_002022765.2) (Kim et al., 2019). Read alignments were sorted using Samtools, and BAM files were used to generate a matrix of gene count data for each sample.

1.2.5.6 Small RNA QC, known miRNA alignment, and novel miRNA prediction

Raw sRNA FASTQ sequencing files were subject to the same integrity and quality control processes as the mRNA. Trimmomatic (v.0.39) parameters used were altered to reflect the length of sRNA reads (Bolger et al., 2014). Reads were trimmed if the first and last 3 bp were of low quality, the average quality across every 4 bp was set to a minimum phred score of 15, and then remaining mRNA-seq reads shorter than 18 bp were excluded from further analyses. The clean high-quality sRNA reads were mapped with Bowtie (Langmead et al., 2009) to the *C-virginica* 3.0 genome obtained from NCBI (Refseq assembly number: GCF_002022765.2). The mapped sRNA sequences were aligned against all known mature and precursor miRNA sequences in the miRBase20.0 using the quantifier.pl program from miRDeep2 (Friedländer et al., 2012) to obtain gene count expression and normalization of known miRNAs. Identification of possible novel miRNAs was performed using mirDeep2.pl software (Friedländer et al., 2012). As there are no known mature or precursor miRNA sequences from *C. virginica*, all known mollusc species in the miRBase20.0 database were concatenated to use as a species reference. To ensure that each unique sRNA read was only mapped to one annotation, sRNA reads were then filtered according to the following order: known miRNA>rRNA>tRNA>snRNA>snoRNA>exon>intron>novel miRNA.

1.2.5.7 Differential gene expression and functional annotation

Differential gene expression comparing gene count data between all sample group comparisons was evaluated using the DESeq2 (Love et al., 2014) package from R-Bioconductor 3.21. Un-normalized mRNA and miRNA gene count data and phenotype data were used to create DESeqDataSets (dds) as the program internally normalizes using the median of ratios method. Genes with <20 reads for mRNA and <50 reads for miRNA were removed from the dds to reduce noise, and then negative binomial generalized linear models were run. Statistically significant differentially expressed genes (DEGs) were characterized with an expression fold change ($|\log_2 FC| >$) 1, a basemean > 50, and a Benjamini and Hochberg (1995) adjusted $P < 0.05$. Significant DEGs were visualized using the ComplexHeatmap and ggplot packages in R (v.4.5.0) (Gu et al., 2016; Wickham, 2016).

Gene set enrichment analysis was then conducted for mRNA using the clusterProfiler (Yu, 2012) package from R-Bioconductor 3.21. The gseaGO function was utilized for enrichment annotation of the three major Gene Ontology (GO) categories [Molecular Function (MF), Biological Processes (BP), and Cellular Components (CC)]. Genes were ranked based on their Deseq2 “stat” value, and a minGSsize was set at 25 with a pvalueCutoff of 0.05.

1.2.5.8 mRNA miRNA integration analysis and network construction

Significant mRNA and miRNA DEGs were then analyzed with miRanda (v.3.3a) (Enright et al., 2003) using strict settings to identify possible miRNA-mRNA interactions. The interaction pairs were then further restricted by miranda scores to only include pairs with a Total Score >150 and a Total Energy > -10 (kcal/mol). Visualization of miRNA-mRNA interactions was conducted in Cytoscape (v.3.10.3) (Shannon et al., 2003).

1.2.6 qRT-PCR

Reverse transcription of mRNA samples was carried out using the Bio-Rad I-script cDNA synthesis kit according to instructions (Bio-Rad., Hercules, CA, U.S.). The synthesis reaction was held at 25°C for 5 min, incubated at 46°C for 20 min, and inactivated at 95°C for 1 min.

The qRT-PCR was then performed using Applied Biosystems Powerup Syber Green Master Mix (ThermoFisher Scientific., Waltham, MA, U.S.). Reactions were carried out in total reactions volumes of 10 µL, containing 4 µL of dilute cDNA, 0.5 µL of each primer, and 5 µL of master mix with the following cycle settings: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s, ending with 62°C for 5 s and 95°C for 50 s. Four mRNAs were analyzed for low- vs. high-quality sperm, and four were analyzed for low-cryotolerant vs. high-cryotolerant sperm (Table S1). *EF1*∝ and *ACTB* were used as housekeeping genes and all data were normalized to them during analysis using the $2^{-\Delta\Delta Ct}$ method.

1.3 Results

1.3.1 Morphometrics

Mean (± SD) oyster shell length, width, and height, were found to be 114.54 ± 12.06 mm, 82.75 ± 10.34 mm, and 43.38 ± 10.95 mm, respectively. Mean (± SD) oyster weight was 191.68

± 60.12 g. Initial sperm cell density ranged from 1.95×10^9 to 9.56×10^9 cells mL^{-1}, with a mean (± SD) density of $5.86 \times 10^9 \pm 1.96 \times 10^9$ cells mL^{-1}.

1.3.2 Sperm kinematics

To assess male reproductive potential and gamete quality we evaluated kinematic traits including percent motility and cell VCL using CASA software. Mean (± SD) sperm motility at 30 s across all twenty oysters was 33.33 ± 14.89% and mean (± SD) sperm VCL at 30 s across all oysters was 91.01 ± 25.28 μm^{-1}. For assessment of sperm with low-quality vs. high-quality, the average motility and VCL (± SD) of low-quality sperm were 15.68 ± 8.16% and 65.09 ± 20.88 μm^{-1}, respectively, and the average motility and VCL (± SD) of high-quality sperm were 50.21 ± 6.05% and 111.17 ± 11.98 μm^{-1} (Fig.1A, Fig.1B). The average combined z-score for the motility and VCL of low-quality males was 2.22 ± 0.86 vs. 1.76 ± 0.66 for high- quality males (Fig. 1C).

To evaluate sperm cell cryotolerance, pre- and post-thaw kinematics were compared using a recovery index. Low-cryotolerant sperm had an average MRI (± SD) of 15.36 ± 5.31% and high-cryotolerant sperm had an average MRI (± SD) of 35.76 ± 3.79% (Fig. 1D, E).

1.3.3 Identification of mRNAs

A total of 489,866,687 raw reads were obtained from seventeen mRNA libraries using high-throughput sequencing, with an average of 28,815,687 reads per sample (Table 1). After trimming reads with adapter, barcode, or primer contamination, and reads of low quality, there

were a total of 386,064,150 mRNA reads for downstream analysis, with an average of 22,709,656 reads (79.42%) per sample (Table 1). Clean reads were then aligned to the eastern oyster genome with an average mapping efficiency of 80.95% (Table 1).

1.3.4 Identification of miRNAs

A total of 272,381,734 raw reads were obtained from the small RNA (sRNA) libraries of the same seventeen males, with an average of 16,022,455 reads for each sample (Table 2). After trimming reads shorter than 18 bases, reads with low quality, and reads with adapter contamination, a total of 251,763,477 reads remained across all samples with an average of 14,809,616 (69.93 %) per sample (Table 2). Filtered sRNA reads were aligned with a resulting overall mapping efficiency of 69.79 % across samples (Table 2). In libraries comparing both quality and cryotolerance, sequences were predominantly 22 – 32 nucleotides in length (Fig. 2). Analysis of the distribution of different sRNAs within the samples showed that in total miRNAs accounted for 29.5% of the reads that were alignments across samples, however, many reads aligned to multiple miRNAs, meaning 5.7% of reads accounted for the total number of miRNA alignments (Fig. 3). This proportion indicates that reads mapping to multiple loci account for a high percentage the total number of alignments.

1.3.4.1 Differential expression and gene ontology for low-quality vs. high-quality sperm

For sperm of low-quality vs. high-quality, results of a Principal Component Analysis (PCA) showed that for mRNA, PC1 accounted for 57% of variance and PC2 accounted for 13%,

while for miRNA, PC1 accounted for 60% of variance and PC2 accounted for 16% (Fig.4A, Fig.4B). Comparison of the genes found in low- and high-quality sperm revealed that there were 33,324 mRNAs found in both groups, 1,082 mRNAs unique to low-quality sperm, and 1,843 mRNAs unique to high-quality sperm (Table S2, Fig.4C). For miRNAs there were 1,226 found in both groups, 267 miRNAs unique to low-quality sperm, and 244 unique to high-quality sperm (Table S2, Fig.4D). Differential expression analysis conducted with Deseq2 clustering revealed differentially expressed mRNAs and miRNAs (Padj < 0.05; $|\log_2 FC| > 1$) in low-quality sperm compared to high-quality sperm; there were a total of 65 significant differentially expressed mRNAs between sample groups and 106 miRNAs (Table 3). Volcano plots were used to further visualize expression patterns. For mRNA there were 48 up-regulated and 17 down-regulated genes, while for miRNA there were 72 up-regulated and 34 down-regulated genes (Fig. 4E, F). The twenty-five highest and lowest mRNA DEGs are visualized in a heatmap, where a z-score was used to show magnitude of differential expression between groups (Table S3, F4G).

Gene set enrichment analysis was then performed using clusterProfiler to further describe the role of gene expression in sperm quality performance. Overall, there were 626 significantly enriched GO terms ($P < 0.05$, SetSize > 25), 377 of which were categorized in BP, 109 in CC, and 140 in MF (Table S4). The top 8 GO terms from each category were collectively plotted based on the normalized expression score (NES) (Fig. 4H). Highly important GO-terms identified include *cilium*, *microtubule cytoskeleton*, *chromosome segregation*, *actin binding*, and *catalytic activity*.

1.3.4.2 Differential expression and gene ontology for low-cryotolerant vs. high-cryotolerant sperm

In the analysis of low-cryotolerant vs. high-cryotolerant sperm, a PCA showed that for mRNA, PC1 explained 15% of the variability between groups and PC2 explained 12%, while for miRNA, PC1 explained 61% of the variability and PC2 explained 33% (Fig.5A, B). In total, 35,969 of the mRNAs sequenced were found in both groups, while 1,330 were unique to low-cryotolerant sperm and 950 were unique to high-cryotolerant sperm (Table S2, Fig.5C). For miRNAs, 1,217 were found in both groups, while 177 were unique to low-cryotolerant sperm, and 290 were unique to high-cryotolerant sperm (Table S2, Fig.4D). In the differential expression analysis conducted with DeSeq2 (Padj < 0.05; $|\log_2 FC| > 1$), a total of 129 mRNA DEGs were identified, 30 of which were upregulated in the low-cryotolerant sperm, and 99 of which were downregulated (Table 3, Fig. 5E). A total of 139 miRNA DEGs were identified in low-cryotolerant sperm compared to high; 118 genes were up-regulated and 21 were down-regulated (Table 3, Fig. 5F). The twenty-five highest and lowest mRNA DEGs are visualized in a heatmap, where a z-score was used to show magnitude of differential expression between groups (Fig. 5G).

In the gene set enrichment analysis performed on low-cryotolerant vs. high-cryotolerant mRNA, there were 303 GO-terms identified ($P < 0.05$, SetSize > 25). Of these terms, 175 were in the BP category, 66 in CC, and 62 were MF (Table S5). The top 8 GO terms from each category were visualized based on their NES (Fig. 5H). GO-terms identified that may play crucial roles in sperm cryotolerance include many of the same that were important for quality such as *cilium*, *chromosome segregation*, and *microtubule cytoskeleton.* However, terms also included *condensed chromosome*, *spindle*, *mitotic cell cycles*, and *ATP dependent activity*, which may be crucial to maintain DNA integrity, repair damage, and restore ATP function.

1.3.5 Prediction of differentially expressed miRNA-mRNA interactions

Analysis of predicted target mRNA DEGS was conducted using miRanda (v.3.3a) with the sequences of differentially expressed miRNAs for both low-quality vs. high-quality sperm, and low-cryotolerant vs. high-cryotolerant sperm. Networks were constructed to show significant potential mRNA-miRNA interaction pairs (Total Energy > -10 & Total Score > 150) between miRNA families and differentially expressed mRNA. There were 19 interactions identified related to the quality of oyster sperm, where 6 miRNA families were predicted to interact with 12 different mRNAs (Fig. 6A). For cryotolerance, a total of 121 interactions were identified, where 12 miRNA families were found to interact with 45 mRNAs of both up- and down-regulation (Fig. 6B).

1.3.6 qRT-PCR validation results

To validate the RNA-seq data, two housekeeping genes and four DEGs were randomly selected from low- vs. high-quality (*LOC11126054*, *LOC111128695*, *LOC111123664*, and *LOC111111037*) and low- vs. high-cryotolerant (*LOC111123091*, *LOC111111994*, *LOC111131490*, and *LOC111111037*) differential expression results. Melt curve analysis for the genes obtained from qRT-PCR showed a single peak, indicating only one product was present. Relative expression the genes in low and high groups were consistent with the RNA-seq results, which confirmed that the transcriptomic data were reliable (Fig. S1).

1.4 Discussion

Genetic regulation and molecular mechanisms related to sperm cell quality and potential are integral to male eastern oyster reproductive success. It is therefore imperative to understand gene-level significance as the aquaculture industry turns to the development of gamete banks and selective breeding. The purpose of this study was to conduct an integrated analysis of miRNA and mRNA profiles related to eastern oyster sperm cell quality and cryotolerance as they relate to cell kinematics. This effort broadens our understanding of the regulatory role miRNAs play in gamete function.

Sperm, while often the smallest cells in an organism, are highly specialized for the sole purpose of fertilizing oocytes (Alberts et al., 2002). To fulfil this purpose, sperm are generally highly mobile and are structured to locate and fertilize, while also outcompeting other sperm cells (Alberts et al., 2002). There are three key features that sperm have across species, which allow for them to succeed: the head, which contains DNA and enzymes, the midpiece, where there are mitochondria to generate ATP, and the tail or flagellum, which propels the cell forward (Alberts et al., 2002; Alves et al., 2020). Cell motility and VCL are important for the ability of sperm to fertilize eggs. Specifically, motility is correlated to cell membrane integrity and mitochondrial membrane potential in eastern oysters, while VCL indicates both maturation of the cells and the ability for a cell to perform chemotaxis (Paniagua-Chávez et al., 2006; Boulais et al, 2017). When analyzing RNA regulation of sperm function, alterations in these key features and kinematics between samples are of importance.

In sperm, miRNAs have been linked to the regulation of functions and pathways including cell differentiation, proliferation, and apoptosis (Kotaja, 2014; Al-Gazi and Carroll,

2015). Recent studies have shown differential expression of miRNAs to be linked to low sperm counts (oligozoospermia), and poor sperm motility (asthenozoospermia) (Al-Gazi and Carroll, 2015). Studies in mice have even shown that miRNAs and related enzymes such as Dicer are crucial for the development of germ cells and essential for normal spermatogenesis (Hayashi et al., 2008). Many miRNAs are highly conserved between species in the animal kingdom, and some across the plant kingdom as well (Ha et al., 2008). Conserved miRNAs often have similar functions across lineages, such as miR-34a which has been found to be crucial for spermatogenesis and sperm motility in multiple species such as humans and zebrafish (*Danio rerio*) (Shi et al., 2024; Guo et al., 2017).

To understand the role of miRNAs and mRNAs in relation to sperm cell function of eastern oysters, we identified differentially expressed RNAs corresponding to motility and tolerance to freeze-thaw. The expression profiles between six male oysters with low-quality (low motility and VCL) sperm and six males with high-quality (high motility and VCL) revealed 106 novel and conserved miRNA and 65 mRNA DEGs. The comparison between six males with low-cryotolerance (low MRI) and six males with high-cryotolerance (high MRI) also identified 139 novel and conserved miRNA and 129 mRNA DEGs. In addition to genes that were differentially expressed, the mRNA and miRNA libraries for both quality and cryotolerance showed genes that were both unique and shared between the low and high groups. These sets of genes are crucial to pinpointing the specific regulatory interactions and pathways that are causing the disparity of sperm performance between males.

miRNAs

There were multiple processes identified by unique and differentially expressed miRNAs as important for sperm quality as described by kinematics. These processes include mitochondrial function and energy metabolism (miR-130, miR-217, miR-34, miR-277, miR-8, miR-27), ion channels and membrane potential (miR-135, miR-7, miR-9, miR-362, miR-449), cytoskeletal and flagellar regulation (miR-505, miR-34, miR-431, miR-199), and stress survival and apoptosis suppression (miR-210, miR-16, miR-15d, miR-24, miR-71, bantam). Previous research in other species have identified some of these miRNAs as biomarkers of sperm quality already. In crucian carp (*Carassius carassius*), miR-199 was found to be crucial for flagellar assembly as it negatively regulates *Tekt1* (Li et al., 2022). Human reproductive research has shown that miR-27a suppresses *CRISP2*, a protein that is important for modulating flagellar motility, the acrosome reaction, and gamete fusion (Zhou et al., 2023). Research on asthenozoospermia conducted on mice found that the upregulation of miR-24a-3p contributes to oxidative stress and energy metabolism disorder by disrupting *GSK3β* expression, however, when the miRNA was inhibited sperm motility increased (Chen et al., 2023). Overall, miRNAs have a regulatory role in necessary mechanisms for sperm motility and VCL, and sperm that exhibit high motility showed regulation of energy, DNA integrity, flagellar form and function, and regulation of ion channels and membrane potential.

In addition to processes important for sperm motility including ion balance and membrane potential (miR-224), miRNAs that were differentially expressed or unique to low-cryotolerant vs. high-cryotolerant males revealed mechanisms crucial for motility retention after cryopreservation. miRNAs were identified that regulate processes related to oxidative stress and stress signaling (miR-190, let-7, bantam), membrane stabilization (miR-23), protein folding (miR-12), and apoptosis activation (miR-199, miR-17). Many of the functions of these miRNAs

have previously been explored in other species as well. In mice, the disruption of miR-17 was linked to the mTOR signaling pathway which led to poor spermatogenesis with depressed sperm production and apoptosis (Xie et al., 2016). An important miRNA regulator found in invertebrates, miR-bantam, was identified as well, this miRNA has been shown to prevent cell death and promote cell proliferation in *Drosophila* (Brennecke et al., 2003). The miR-let-7 family was also found to be expressed in cryotolerant sperm and is a well-known regulator of spermatogenesis (Tong et al., 2011). However, it also regulates cell proliferation and growth, and a low state of cell activity with low protein synthesis can lead to fewer misfolded proteins during the cryopreservation processes (Roush and Slack, 2007). In general, sperm that had poor motility retention post freezing tended to exhibit dysregulation of ion channels, destabilized membranes, activation of apoptosis, and stress signaling.

mRNAs

Gene set enrichment analysis of the expression of mRNAs also showed dozens of genes and pathways related to sperm cell motility and tolerance to freeze-thaw. Gene ontology terms related to *cilium assembly and organization, ciliary plasma, microtubule cytoskeleton and organizing center, actin binding and actin filament binding,* and *microtubule and tubulin binding* were all found to be upregulated in both high-quality and high-cryotolerant males. These pathways are related to sperm cell membrane stability, assembly, and flagellar beating (Mirvis et al., 2018; Dunleavy et al., 2019). Upregulation indicates that cells have a robust cytoskeleton structure, which may be allowing for good motility both pre- and post-thaw.

Additionally, the *nuclear division, chromosome segregation, mitotic cell cycle, chromosome segregation, spindle, condensed chromosome,* and *chromosomal region* gene ontology terms were also enriched in high-quality and high-cryotolerant males. These terms indicate an ability to preserve genomic integrity (García-Vielma et al., 2018 ; O'Connor., 2008).

Finally, the terms *protein tyrosine phosphatase activity, hydrolase activity acting on glycosyl bonds, catalytic activity acting on DNA,* and *ATP activity acting on DNA* showed enrichment. These are responses which indicate that cells are stressed, but that enzymatic regulation of cells is activated to repair the damage to restore ATP (Tomes et al., 2004; Kong et al., 2024; Li et al., 2014; Milanese et al., 2019).

miRNA × mRNA interaction analysis

The analysis of miRNA × mRNA interactions revealed many potential instances of gene regulation related to sperm function that further emphasized the importance of non-coding RNAs in gamete potential. One interaction with an SMC2-like gene found to be upregulated in low-quality males with upregulated families miR-200 and miR-1, has a role in the structure maintenance of chromosomes (Figure 6a., Harvey et al., 2002). The upregulation indicates that the sperm is responding to DNA damage or poor chromosome packaging, which may be a factor causing poor motility. miR-200 has previously been linked to motility regulation in zebrafish, where its deletion resulted in increased motility (Xiong et al., 2018).

A second notable interaction was identified between an ankryn-3-like gene that acts as a scaffolding protein and has a role in organizing ion channels, and three miRNA families: miR-26, miR-30, and miR-126 (Cunha et al., 2009). miR-26a has previously been associated with

sperm metabolism and oxidative phosphorylation, with research showing that it may regulate the glycometabolic pathway and influence cryopreservation success (Dorostghoal et al., 2020; Wang et al., 2020).

There was also an interaction identified between a CFAP-like gene and miR-142. This gene is a cilia and flagella associated protein that has function in the formation of flagella (Wu et al., 2023). Research into biomarkers of testicular cancer has previously found miR-142 to be a potential biomarker found in seminal plasma (Pelloni et al., 2017). While more research is needed to confidently link it to sperm function, miR-142 has also been found to play a key role in the paternal transgenerational inheritance (Zhang et al., 2024).

Applications in oyster aquaculture

Aquaculture production of animal species continues to grow to meet population demands for seafood products. As output increases, technological advancements must be made to sustain growth and to ensure economic and environmental sustainability. Gamete supply remains one of the biggest constraints to aquaculture (Migaud et al., 2013). Therefore, the ability to assess, select, or ensure the production of high-quality sperm that can be used for gene banking would aid in providing hatcheries and researchers with on-demand access to gametes. This would not only remove the accessibility constraint but would also allow for more selective breeding opportunities and out-of-season spawning from gene banks. Sperm RNAs have been researched as biomarkers of quality and fertility in model species since the early 2000's (Metha et al., 2024). Small RNAs have become a focus point due to their significant presence in sperm and their

ability to regulate multiple coding RNAs (Metha et al., 2024; Kocabaş and Kocabaş., 2023; Alves et al., 2020).

The expression of small RNAs, specifically miRNAs, has shown to be essential for the function of normal spermatogenesis and thus, miRNAs have the potential to be good biomarkers of male fertility (Bahmyari et al., 2024). In addition to fertility, studies have shown miRNAs to be important in the cryopreservation success of sperm cells and their ability to maintain viability and function (Predrosa et al., 2021). Looking to future applications of miRNA biomarkers for use in eastern oyster aquaculture, these markers can be useful in selecting males and for producing good males. First, miRNAs can be used to modulate expression of different genes involved in signaling pathways (Zheng et al., 2025). To do this, miRNA replacement molecules such as small double stranded RNAs, can be inserted to up-regulate the expression of specifically selected miRNAs (Klees et al., 2023). Second, miRNA antagonists or anti-miRNAs, such as single stranded anti-sense oligonucleotides that are complimentary to the mature form of a miRNA, can be used to inhibit or down-regulate a specific miRNAs expression (Klees et al., 2023). To apply the use of RNA biomarkers to eastern oyster aquaculture, future studies are needed in multiple populations to further understand and select specific species-wide genes to target. In addition to sperm kinematics, fertilization success and offspring survival should be studied to identify if the sperm transcriptome is indicative of the outcome. For use in gene banking, the effects of cryopreservation in RNA expression should also be researched, as miRNAs can protect against cryo-induced cellular damage as they can regulate pathways and target specific genes related to DNA damage response, apoptosis, and oxidative stress (Klees et al., 2023). The alteration of expression and any subsequent intergenerational effects of

cryopreservation or sperm quality should be understood to ensure successful application in industry.

1.5 Conclusion

The goal of this study was to investigate the role of miRNA- mRNA regulatory relationships in eastern oyster sperm kinematics and cryotolerance, with the objective of evaluating the potential use of RNAs as biomarkers. From the transcriptomic sequencing results, we found differentially expressed RNAs that regulate or are in gene sets with mechanisms impacting sperm kinematics, structure, membrane stability, and enzymatic response. Overall, sperm that had high kinematic indices also had enhanced mitochondrial efficiency, flagellar activation and ion channel signaling, and membrane fluidity. Sperm that was tolerant to cryopreservation showed suppressed apoptosis, tolerance to oxidative stress, membrane stability, and metabolism recovery. This shows that miRNA regulation of mRNAs plays a critical role in sperm function, and that RNAs therefore, have the potential to be used as biomarkers for sperm quality and cryotolerance. In practice, a functional understanding of eastern oyster sperm and RNA biomarkers can improve access to high-quality gametes for hatchery production and long-term industry growth.

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Resources

GitHub Repository: https://github.com/a-nowicki/eastern_oyster_miRNAxmRNA

NCBI BioProject: PRJNA1439917

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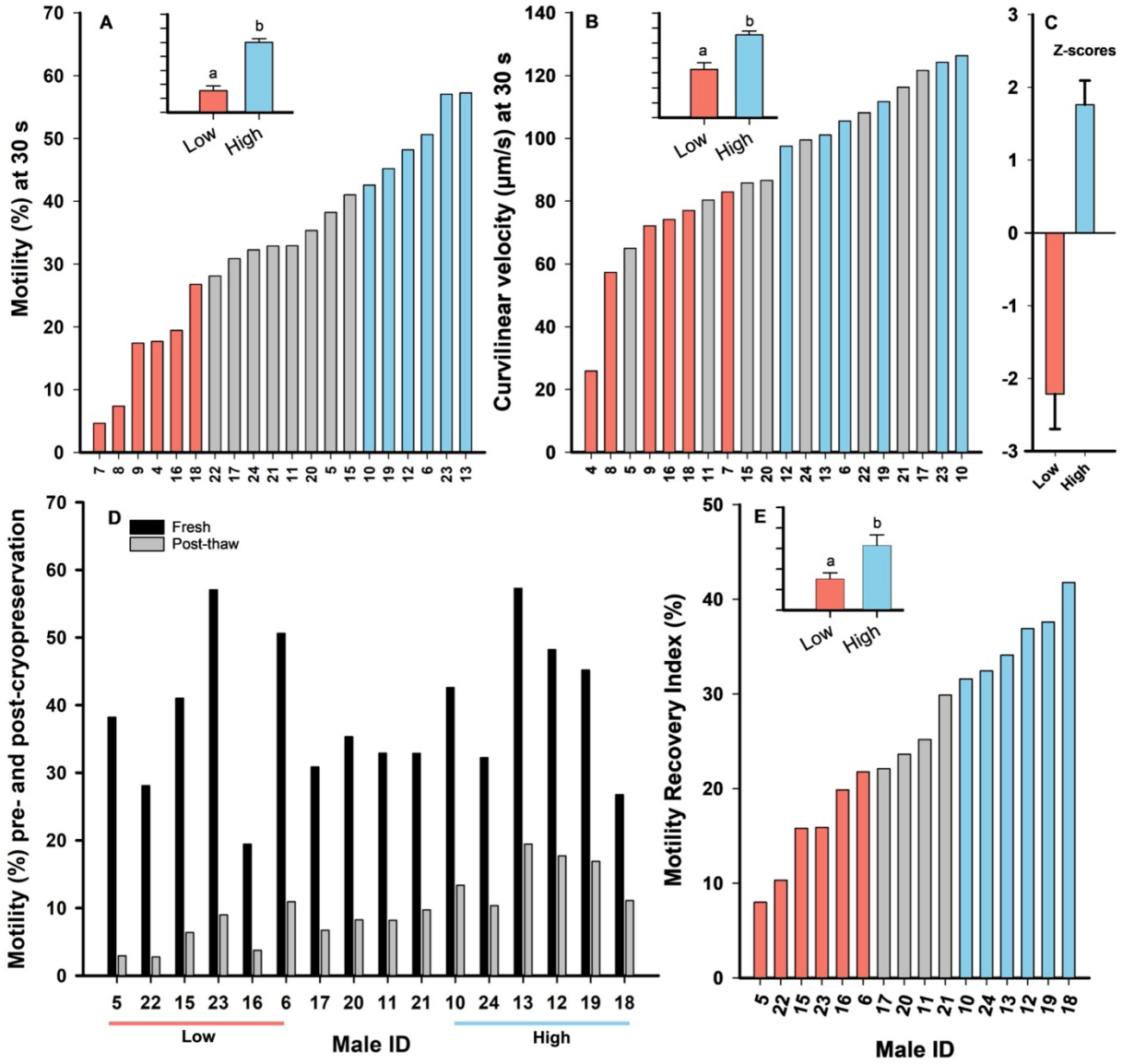


Figure 1.1. Selection of sperm samples for mRNA and sRNA sequencing for comparisons between low- vs. high-quality fresh eastern oyster (*Crassostrea virginica*) sperm, and low- vs. high-cryotolerant eastern oyster sperm. Males in red were selected as low samples, and males in blue were selected as high samples for both comparisons. Letters indicate that a t-test between the low and high groups was statistically significant (P <0.001). Error bars represent standard error. (A) Distribution of the percent of motile sperm for 20 male eastern oysters at 30 seconds post-activation in Artificial Sea Water (ASW). (B) Curvilinear VCL (μm^{-1}) of the sperm from 20 male eastern oysters at 30 seconds post-activation in ASW. (C) Results of a t-test between the

low- vs. high-quality males combined motility and VCL z-scores showed significant differences between groups (P <0.001). (D) Percent motility of 16 male eastern oysters' sperm at 30 seconds post-activation both pre- and post-cryopreservation, where the underlined male IDs correspond to low- and high-cryotolerant groups. (E) Distribution of the calculated Motility Recovery Index (MRI) for 16 males.

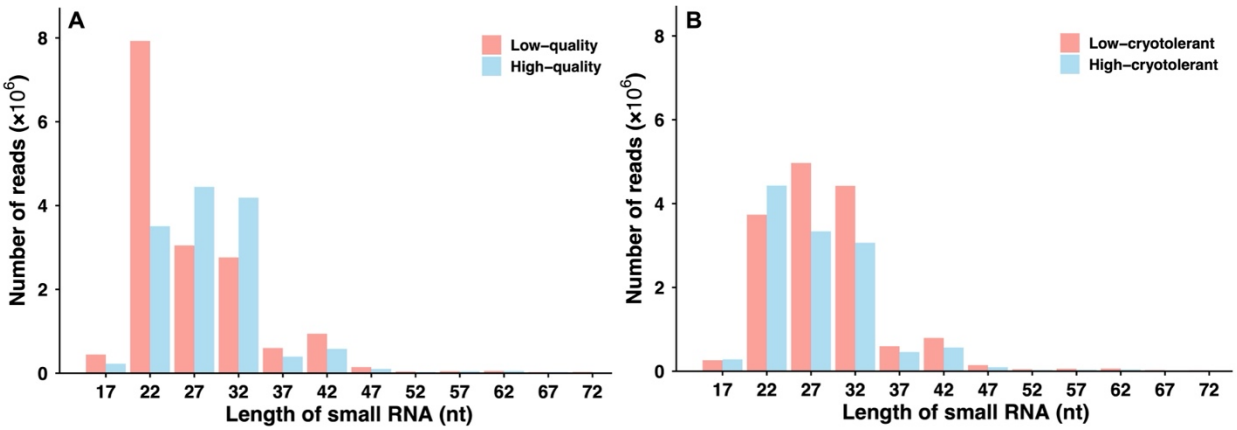


Figure 1.2. Length distribution of clean sRNAs in (A) samples used for low-quality vs high quality analysis, and (B) samples used in low-cryotolerant vs high-cryotolerant analysis. The y axis represents the number of reads identified in the clean libraries, and the x-axis represents the length of the small RNA reads in nucleotides.

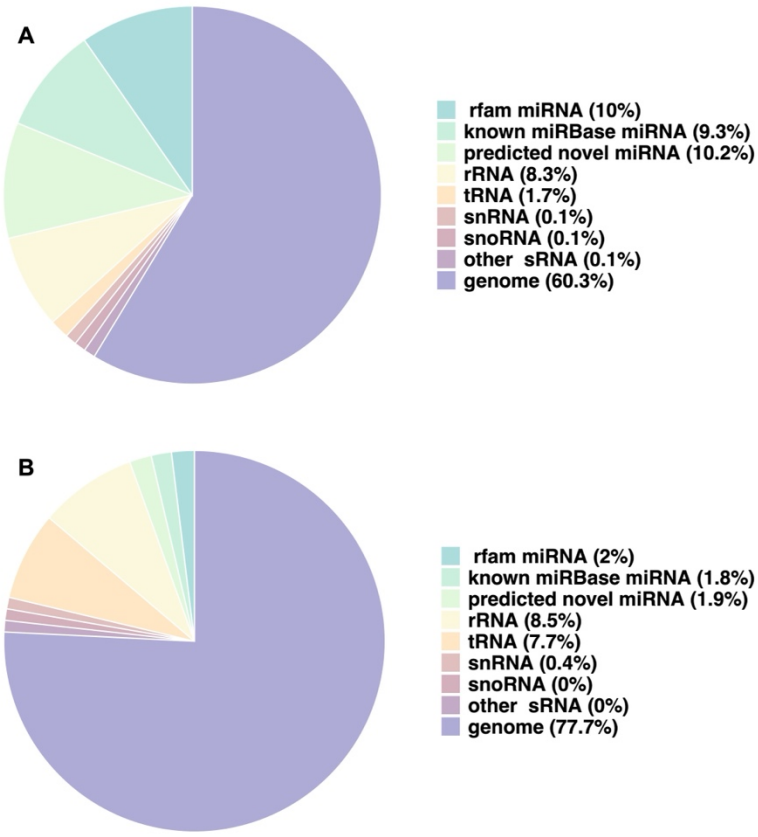
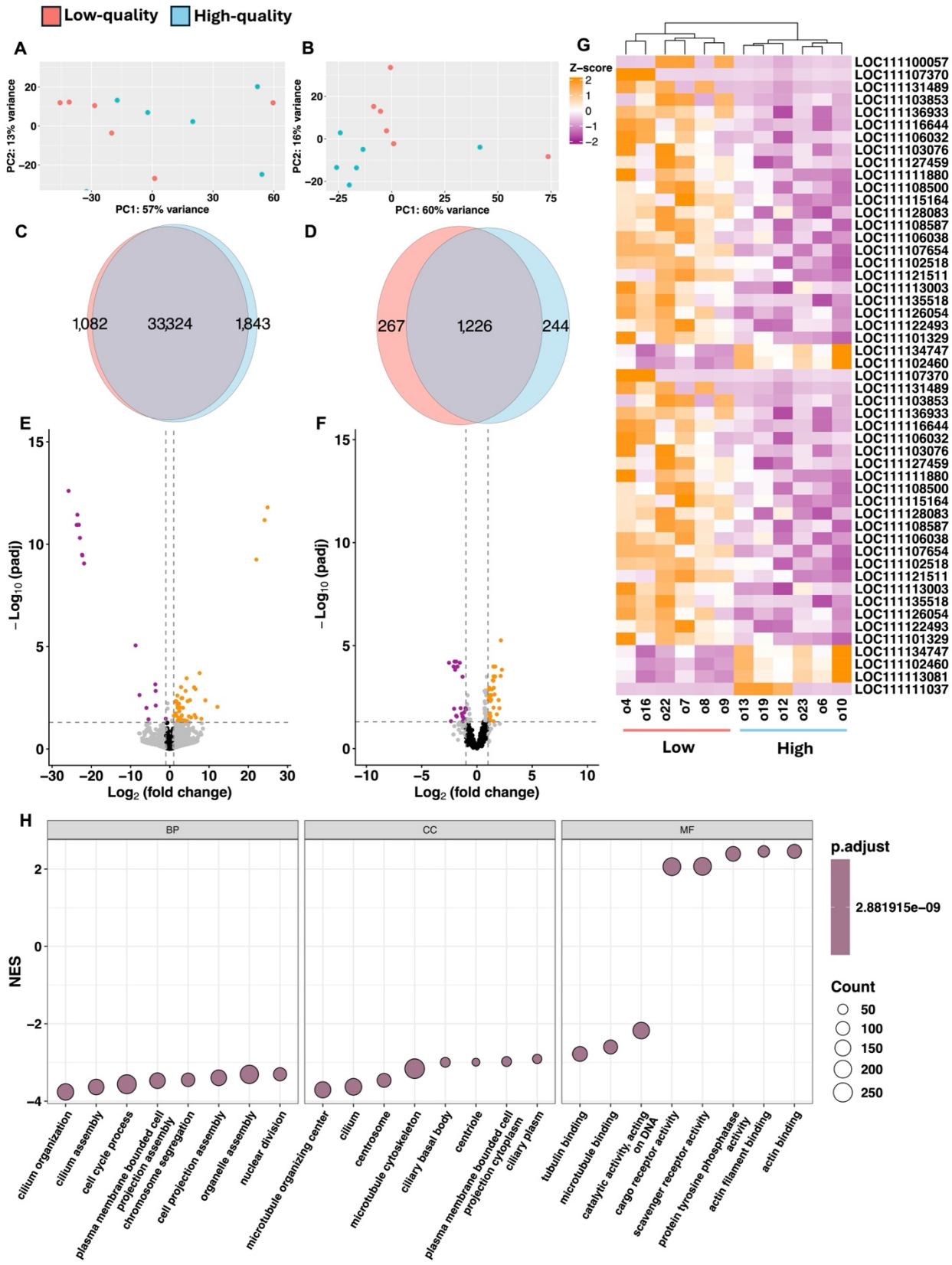


Figure 1.3. Distribution of sRNAs in clean sequencing files. (A) Indicates the distribution of sRNA annotations based on the proportions of reads that mapped to each type. (B) Indicates the distribution of total alignment annotations, including the contribution of repeat read alignments, by sRNA type.


Low-quality
High-quality
A
PC2: 13% variance
PC1: 57% variance
B
PC2: 16% variance
PC1: 60% variance
C
1,082
33,324
1,843
D
267
1,226
244
E
−Log10 (padj)
Log2 (fold change)
F
−Log10 (padj)
Log2 (fold change)
G
Z-score
LOC111100057
LOC111107370
LOC111131489
LOC111103853
LOC111136933
LOC111116644
LOC111106032
LOC111103076
LOC111127459
LOC111111880
LOC111108500
LOC111115164
LOC111128083
LOC111108587
LOC111106038
LOC111107654
LOC111102518
LOC111121511
LOC111113003
LOC111135518
LOC111126054
LOC111122493
LOC111101329
LOC111134747
LOC111102460
LOC111107370
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LOC111103853
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LOC111111037
o4 o16 o22 o7 o8 o9 o13 o19 o12 o23 o6 o10
Low
High
H
BP
CC
MF
NES
cilium organization
cilium assembly
cell cycle process
plasma membrane bounded cell projection assembly
chromosome segregation
cell projection assembly
organelle assembly
nuclear division
microtubule organizing center
cilium
centrosome
microtubule cytoskeleton
ciliary basal body
centriole
plasma membrane bounded cell projection cytoplasm
ciliary plasm
tubulin binding
microtubule binding
catalytic activity, acting on DNA
cargo receptor activity
scavenger receptor activity
protein tyrosine phosphatase activity
actin filament binding
actin binding
p.adjust
2.881915e−09
Count
50
100
150
200
250

Figure 1.4. (A) Principal Component Analysis (PCA) of low-quality (red) vs. high-quality (blue) eastern oyster (*Crassostrea virginica*) sperm mRNA. (B) PCA of low-quality (red) vs. high-quality (blue) eastern oyster sperm miRNA. (C) Venn diagram plot showing mRNAs unique to low-quality sperm in red, mRNAs unique to high-quality sperm in blue, and overlapping mRNAs in purple. (D) Venn diagram plot showing miRNAs unique to low-quality sperm in red, miRNAs unique to high-quality sperm in blue, and overlapping miRNAs in purple. (E) Volcano plot showing Differentially Expressed Genes (DEGs) from mRNAs between low- and high-quality sperm, where significantly down-regulated genes are shown in purple and significantly up-regulated genes are shown in orange ($|log2FC| > 1$ & $Padj < 0.05$). (F) Volcano plot showing miRNA DEGs between low- and high-quality sperm, where significantly down-regulated genes are shown in purple and significantly up-regulated genes are shown in orange ($|log2FC| > 1$ & $Padj < 0.05$). For both volcano plots the vertical dashed lines represent a $|log2FC| = 1$ and the horizontal dashed line represents $Padj < 0.05$. (G) Heatmap of the top 25 down- and up-regulated mRNAs expressed as a z-score, the intensity of the purple and orange is correlated with the levels of down- and up- regulation respectively. The low-quality samples are clustered on the left, and the right cluster is the high-quality samples. (H) Bubble-plot illustrates top 8 gene ontology (GO) terms for BP, CC, and MF, from mRNA gene set enrichment analysis for eastern oyster sperm quality. The y-axis represents the normalized expression score, and the x-axis represents the description of the GO term. The color of the bubble represents their significance (*P*adj), and the size represents the number of genes identified in that term. Complete lists of DEGs and GO terms are provided in supplemental material (Table S3, S4).

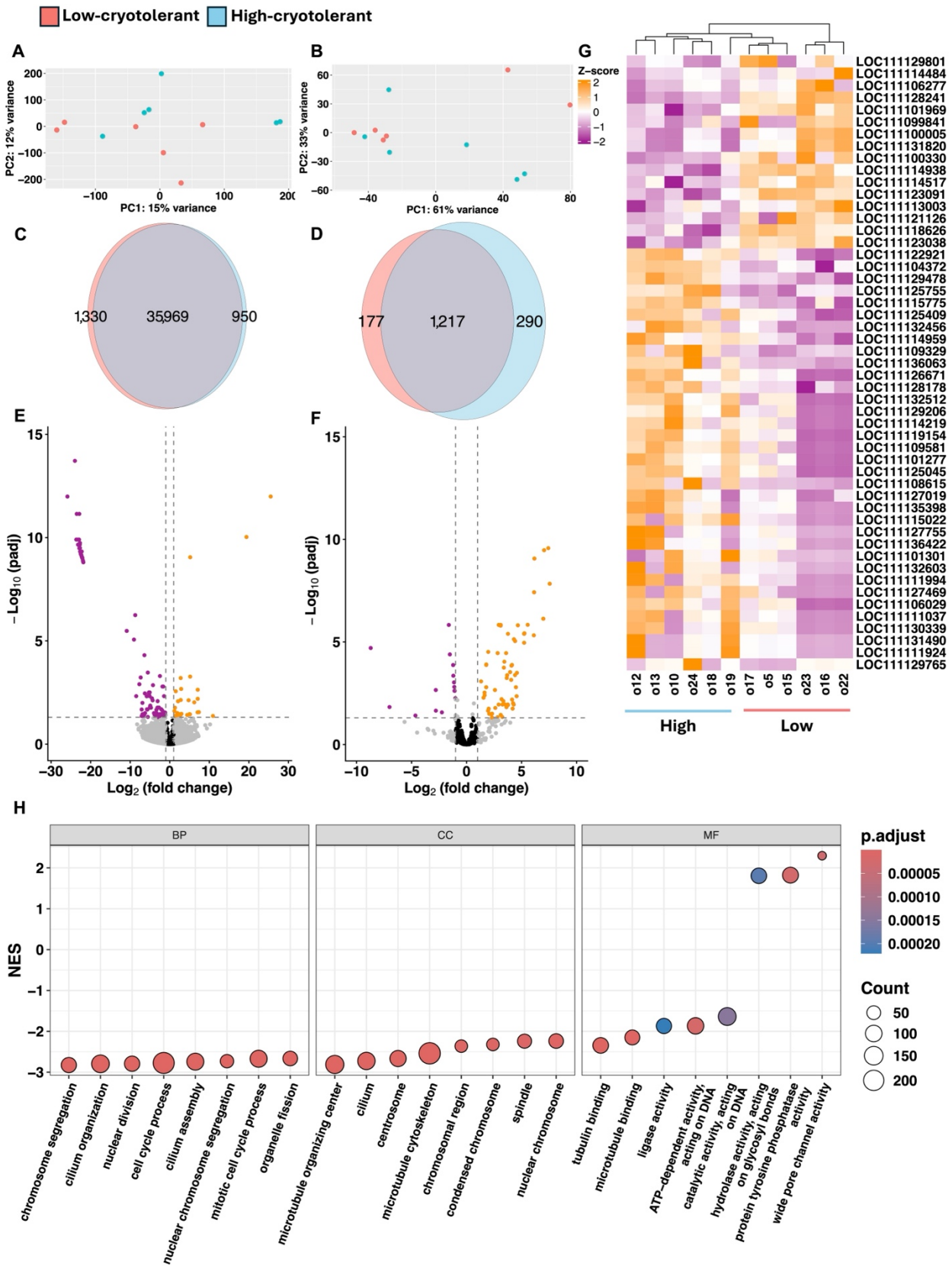
Low-cryotolerant
High-cryotolerant
A
PC2: 12% variance
PC1: 15% variance
B
PC2: 33% variance
PC1: 61% variance
C
1,330
35,969
950
D
177
1,217
290
E
−Log10 (padj)
Log2 (fold change)
F
−Log10 (padj)
Log2 (fold change)
G
Z-score
LOC111129801
LOC111114484
LOC111106277
LOC111128241
LOC111101969
LOC111099841
LOC111100005
LOC111131820
LOC111100330
LOC111114938
LOC111114517
LOC111123091
LOC111113003
LOC111121126
LOC111118626
LOC111123038
LOC111122921
LOC111104372
LOC111129478
LOC111125755
LOC111115775
LOC111125409
LOC111132456
LOC111114959
LOC111109329
LOC111136063
LOC111126671
LOC111128178
LOC111132512
LOC111129206
LOC111114219
LOC111119154
LOC111109581
LOC111101277
LOC111125045
LOC111108615
LOC111127019
LOC111135398
LOC111115022
LOC111127755
LOC111136422
LOC111101301
LOC111132603
LOC111111994
LOC111127469
LOC111106029
LOC111111037
LOC111130339
LOC111131490
LOC111111924
LOC111129765
o12 o13 o10 o24 o18 o19 o17 o5 o15 o23 o16 o22
High
Low
H
BP
CC
MF
NES
chromosome segregation
cilium organization
nuclear division
cell cycle process
cilium assembly
nuclear chromosome segregation
mitotic cell cycle process
organelle fission
microtubule organizing center
cilium
centrosome
microtubule cytoskeleton
chromosomal region
condensed chromosome
spindle
nuclear chromosome
tubulin binding
microtubule binding
ligase activity
ATP-dependent activity, acting on DNA
catalytic activity, acting on DNA
hydrolase activity, acting on glycosyl bonds
protein tyrosine phosphatase activity
wide pore channel activity
p.adjust
0.00005
0.00010
0.00015
0.00020
Count
50
100
150
200

Figure 1.5. (A) Principal Component Analysis (PCA) of low-cryotolerant (red) vs. high-cryotolerant (blue) eastern oyster (*Crassostrea virginica*) sperm mRNA. (B) PCA of low-cryotolerant (red) vs. high-cryotolerant (blue) eastern oyster sperm miRNA. (C) Venn diagram plot showing mRNAs unique to low-cryotolerant sperm in red, mRNAs unique to high-cryotolerant sperm in blue, and overlapping mRNAs in purple. (D) Venn diagram plot showing miRNAs unique to low-cryotolerant sperm in red, miRNAs unique to high-cryotolerant sperm in blue, and overlapping miRNAs in purple. (E) Volcano plot showing Differentially Expressed Genes (DEGs) from mRNAs between low- and high-cryotolerant sperm, where significantly down-regulated genes are shown in purple and significantly up-regulated genes are shown in orange ($|log2FC| > 1$ & $Padj < 0.05$). (F) Volcano plot showing miRNA DEGs between low- and high-cryotolerant sperm, where significantly down-regulated genes are shown in purple and significantly up-regulated genes are shown in orange ($|log2FC| > 1$ & $Padj < 0.05$). For both volcano plots the vertical dashed lines represent a $|log2FC| = 1$ and the horizontal dashed line represents $Padj < 0.05$. (G) Heatmap of the top 25 down- and up- regulated mRNAs expressed as a z-score, the intensity of the purple and orange is correlated with the levels of down- and up-regulation respectively. The low-cryotolerant samples are clustered on the left, and the right cluster is the high-cryotolerant samples. (H) Bubble-plot illustrates top 8 gene ontology (GO) terms for BP, CC, and MF, from mRNA gene set enrichment analysis for eastern oyster sperm cryotolerance. The y-axis represents the normalized expression score, and the x-axis represents the description of the GO term. The color of the bubble represents their significance (*P*adj), and the size represents the number of genes identified in that term. Complete lists of DEGs and GO terms are provided in supplemental material (Table S3, S5).

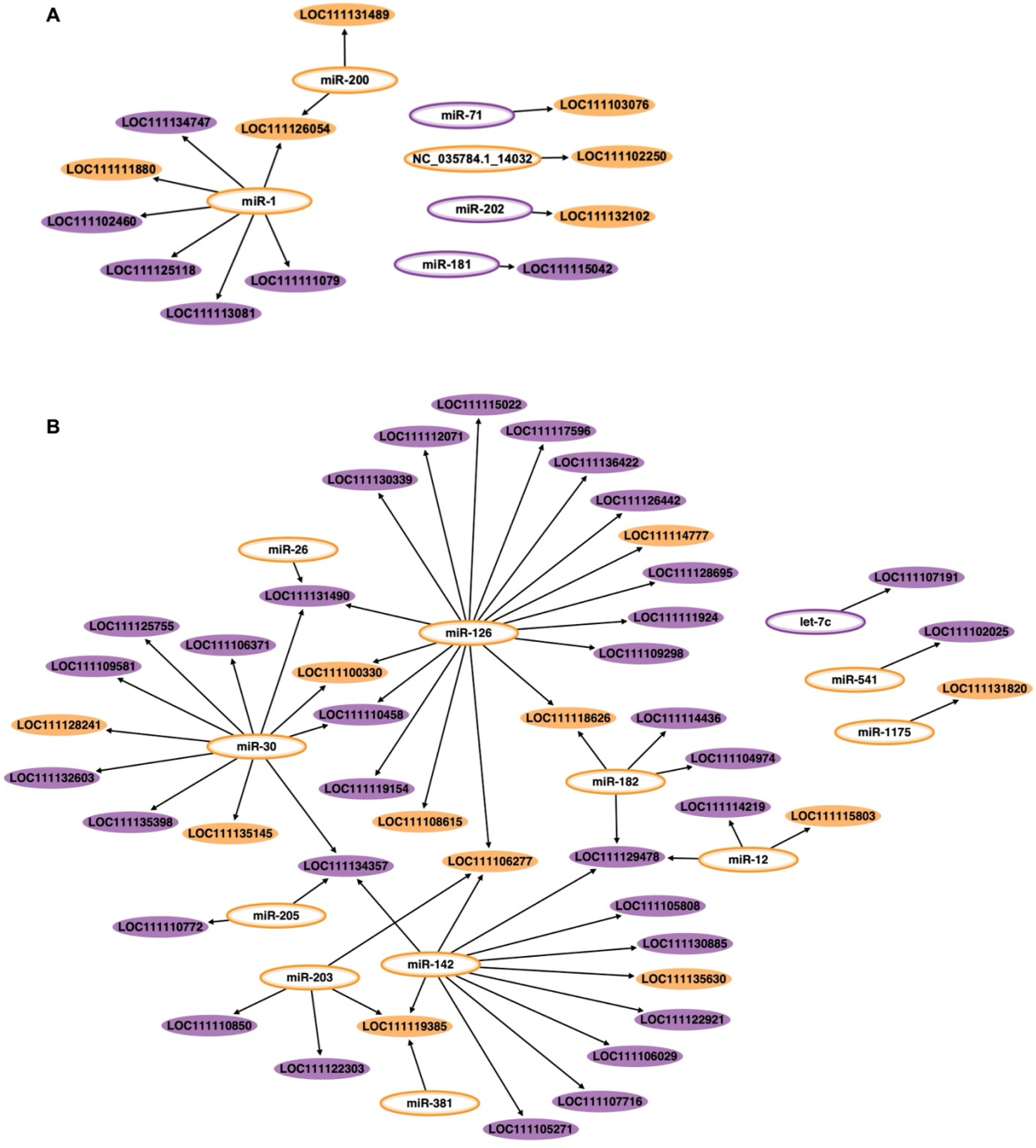

Figure 1.6: Interaction network analysis between (A) low-quality and high-quality eastern oyster (*Crassostrea virginica*) sperm mRNAs and miRNAs, and (B) low-cryotolerant and high-cryotolerant eastern oyster sperm mRNAs and miRNAs, as predicted using miRanda v3.3a. Filled orange ellipses represent up-regulated mRNAs, filled purple ellipses represent down-

regulated mRNAs, orange outline ellipses represent up-regulated miRNA families, and purple outlined ellipses represent down-regulated miRNA families.

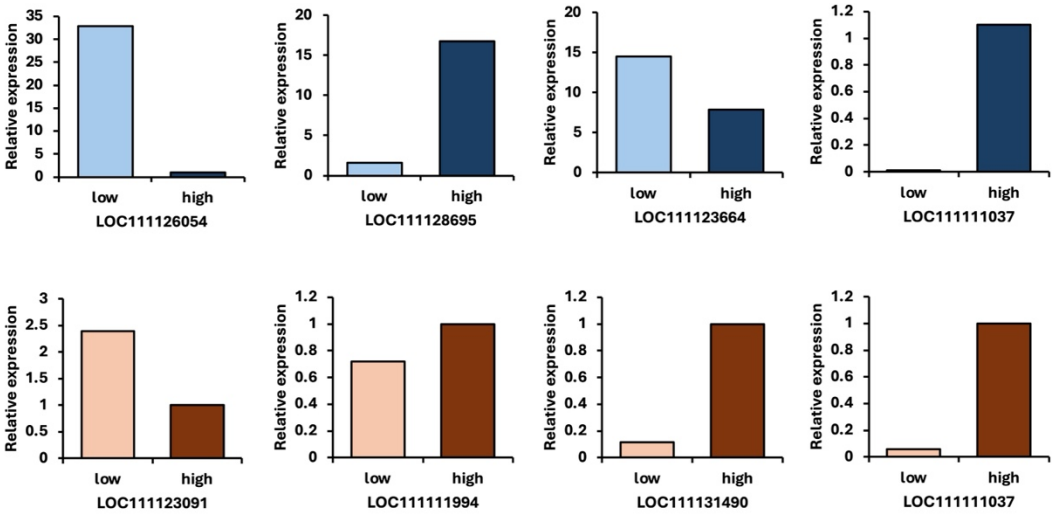

Figure S1.1: Validation of RNA sequencing results with qRT-PCR. Four genes (*LOC11126054*, *LOC111128695*, *LOC111123664*, and *LOC111111037*) were validated for low- vs high-quality males, and four genes (*LOC111123091, LOC111111994*, *LOC111131490*, and *LOC111111037*) were validated for low- vs high-cryotolerant males. Two housekeeping genes (*EF1α*, *ACTB*) were used for normalization in all samples. All data was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Sequencing, quality control, and mapping metrics for extracted eastern oyster (*Crassostrea virginica*) sperm mRNA.

mRNA

Sample ID	Quality	Cryotolerance	Total reads	Clean reads	% Clean reads	% Mapped reads
o10	High	High	21,934,197	16,923,373	77.16	84.16
o12	High	High	133,749,985	105,242,522	78.69	82.73
o13	High	High	20,181,843	15,761,803	78.1	82.78
o15	-	Low	28,848,738	24,035,227	83.31	82.49
o16	Low	Low	20,627,595	15,732,034	76.27	78.39
o17	-	Low	19,614,998	15,418,882	78.61	82.62
o18	-	High	27,657,517	24,289,872	87.82	77.17
o19	High	High	24,464,689	18,338,526	74.96	81.5
o22	Low	Low	20,397,752	14,793,333	72.52	80.24
o23	High	Low	20,019,044	14,356,935	71.72	81.98
o24	-	High	19,182,118	15,279,864	79.66	73.8
o4	Low	-	20,297,774	15,564,348	76.68	81.66
o5	-	Low	28,282,885	25,218,827	89.17	79.92
o6	High	-	19,167,087	14,379,934	75.02	79.39
o7	Low	-	20,179,593	15,715,347	77.88	80.9
o8	Low	-	24,449,444	19,033,723	77.85	82.04
o9	Low	-	20,811,428	15,979,600	76.78	81.98
Average			28,815,687	22,709,656	79.42	80.95
Total Reads			489,866,687	386,064,150		

Table 2. Sequencing, quality control, and mapping metrics for extracted eastern oyster (*Crassostrea virginica*) sperm sRNA.

sRNA						
Sample ID	**Quality**	**Cryotolerant**	**Total reads**	**Clean reads**	**% Clean reads**	**%Mapped reads**
o10	High	High	14,422,647	13,841,801	95.97	56.47
o12	High	High	15,109,690	14,513,103	96.05	60.33
o13	High	High	15,896,380	15,303,789	96.27	63.66
o15	-	Low	9,458,573	8,798,367	93.02	78.57
o16	Low	Low	12,270,022	11,393,792	92.86	62.66
o17	-	Low	27,368,030	22,465,373	82.09	61.12
o18	-	High	10,124,111	9,040,411	89.30	63.89
o19	High	High	15,162,662	14,273,397	94.14	70.56
o22	Low	Low	14,057,644	13,284,748	94.50	87.01
o23	High	Low	12,603,327	11,776,565	93.44	66.25
o24	-	High	26,734,154	24,602,359	92.03	55.80
o4	Low	-	26,780,714	25,000,907	93.35	88.97
o5	-	Low	8,110,149	7,208,482	88.88	81.43
o6	High	-	13,304,515	12,705,457	95.50	54.16
o7	Low	-	14,401,728	13,431,997	93.27	78.29
o8	Low	-	15,561,040	14,604,763	93.85	76.75
o9	Low	-	21,016,348	19,518,166	92.87	80.59
Average			16,022,455	14,809,616	69.93	69.79
Total reads			272,381,734	251,763,477		

Table 3. Number of differentially expressed mRNAs, known miRNAs, and novel miRNAs, in low-quality vs. high-quality eastern oyster (*Crassostrea virginica*) sperm, and in low-cryotolerant vs. high-cryotolerant eastern oyster sperm. Differential expression was assessed using Deseq2, and significance was evaluated as |log2FC| > 1 and *P*adj < 0.05.

-	-	Up-regulated genes	Down-regulated genes	Total DEGs by type
mRNA	Low-quality vs. High-quality	48	17	65
	Low-cryotolerance vs. High-cryotolerance	30	99	129
known miRNA	Low-quality vs. High-quality	55	29	84
	Low-cryotolerance vs. High-cryotolerance	114	14	128
novel miRNA	Low-quality vs. High-quality	17	5	22
	Low-cryotolerance vs. High-cryotolerance	4	7	11

	Total DEGs
Low-quality vs. High-quality	171
Low-cryotolerance vs. High-cryotolerance	268

Table S1. Sequences for primers used in qPCR validation. EF-1α and ACTB were used as housekeeping genes for normalization.

Gene		Sequence
EF1-α	Forward	ACAGGCTGACTGTGCTGTGT
	Reverse	CGTTGAAACGGCTCTCACTGT
ACTB	Forward	AGAGTGTTTGACCCAGAGCCA
	Reverse	CGTCATGGAGCCCTCTACGG
LOC111126054	Forward	TGGCTAGCACCGTTGAGTCAT
	Reverse	ACTCCCATCTACTGCTGCCTT
LOC111111037	Forward	AGGGCTTTAAATACAAGGCAGGGA
	Reverse	CAGCTGTTAGCACCACACCCA
LOC111128695	Forward	CTCAACATCCCTTTGCTTCATGCT
	Reverse	CTGCAGTCTTCTTTGTATCCCTTCT
LOC111123664	Forward	ACCTGTGCATCCGAATAATCAAAC
	Reverse	CACATGGATAAGAACAGCAGGGA
LOC111131490	Forward	ACAGTAGCTCCTGGACCGAA
	Reverse	CACCTATGGATTCTGCCACCCA
LOC111123091	Forward	CGCCAAAGTCTCAGTCTCGC
	Reverse	ACGGCTTCGTAAAGTCCGGT
LOC111111994	Forward	TGTTCGTCTGTTCCGTTCCA
	Reverse	CGTTGGCTCTCTTGACTATCCTCAT

Chapter 2

Ovarian fluid induces molecular remodeling of sperm to enhance performance in a teleost fish

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Keywords: Hyperactivation; Gamete biology; CFC; Molecular; RNA

2.1 Abstract

For most externally fertilizing teleost fish, sperm remain immotile until release into the external environment, where physicochemical cues trigger motility. In many species, ovarian fluid present at spawning further enhances sperm swimming performance, enabling cells to swim longer and at higher velocities than in water alone, including blue catfish (Ictalurus furcatus). This study aimed to identify molecular pathways associated with sperm-environment interactions between: (i) sperm combined with 100% ovarian fluid (inactive); (ii) sperm activated with 100% DH2O (control); and (iii) sperm activated with 25% ovarian fluid and 75% DH2O (hypermotile). We hypothesized that the physical and biochemical properties of ovarian fluid instigate regulation of key signaling pathways and ion channels during sperm activation that are critical for motility and fertilization potential. Our findings suggest that the micro-environment provided by ovarian fluid stimulates molecular changes of cell membrane properties, ion channels, signaling pathways, binding proteins, and energy metabolism even prior to motility activation. These molecular changes allow for the induced hyperactivation of sperm cell motility and velocity, as well as increased longevity. Results also indicate that there is a significant female effect on gene expression of sperm cells exposed to ovarian fluid, providing evidence of gamete compatibility that may impact fertility. This study provides the first insights into ovarian fluid-mediated molecular mechanisms that enhance sperm activation in a teleost fish.

2.2 Introduction

In most externally fertilizing fish, sperm remain immotile within the testis and seminal plasma. Upon release into the external aquatic environment, sperm motility is triggered by physicochemical cues (Pérez, 2020). Depending on the species, changes in ion concentrations, osmotic pressure, pH, and temperature can contribute to regulation of sperm activation and motility (Alavi & Cosson, 2005, 2006). In many fish species, the presence of ovarian fluid (OF) in the external environment or activation medium has been shown to induce sperm hyperactivation, resulting in significant increases in the number of mobile cells, swimming velocity, and overall longevity (Diogo et al., 2010; Myers et al., 2020a; Wojtczak et al., 2007; Zadmajid et al., 2019). The interval between sperm activation and fertilization represents a critical window for externally fertilizing fish, as sperm must rapidly locate the egg and enter the micropyle prior to sperm-egg binding. OF-mediated activation is therefore of great interest, as enhanced sperm performance may substantially increase fertility outcomes (Gallego & Asturiano, 2019; Wood et al., 2025).

Sperm-egg chemotaxis is an important chemical signaling mechanism in which chemoattractants direct sperm toward the site of fertilization (Kholodnyy et al., 2020). Chemotaxis is influenced by the composition of OF, a complex mix of organic and inorganic constituents, which exhibits interspecies and intraspecies variation (Hatef et al., 2009; Myers et al., 2020b; Nynca et al., 2015). Consequently, females within a species produce distinct microenvironments in which chemotactic signaling and fertilization occur (Beirão et al., 2015; Rosengrave et al., 2008, 2009). In many species, female×male interactions arising from intraspecific sperm selection have been described as cryptic female choice (CFC) (Firman et al.,

2017; Rosengrave et al., 2016). Studies investigating CFC in fish have shown that OF biochemically and physiologically mediates sperm performance (Johnson et al., 2020). Additionally, a genetic component to female×male interactions has been identified, where sperm-OF interactions bias sperm activation and fertilization toward unrelated (Gasparini & Pilastro, 2011) or related fish (Butts et al., 2012). Therefore, CFC derived from OF composition is a critical determinant of fertility and evolution outcomes in fish species, as it enables reproductive control at the gametic level (Devigili et al., 2018; Firman et al., 2017). Such mechanisms may facilitate increased fertility and improved offspring genetic quality in native environments or under hatchery-based in vitro fertilization conditions.

Despite extensive research on functional interactions and physiological changes associated with sperm hyperactivation in OF across many fish species (Beirão et al., 2014, 2019; Dietrich et al., 2007; Rosengrave et al., 2024), the molecular mechanisms underlying this phenomenon remain poorly understood. To the best of our knowledge, transcriptional changes in sperm cells themselves, prior to entering the micropyle in external fertilizers, have not been investigated, leaving the unresolved question of what molecular and functional changes sperm undergo during this critical window.

Although sperm have conventionally been considered transcriptionally dormant, increasing evidence suggests that RNAs retained from spermatogenesis maintain functional and regulatory potential (Jodar et al., 2013). These RNAs can influence sperm motility, stress-responses, and fertilization potential (Cabrita et al., 2014; Jodar et al., 2013). In externally fertilizing fish, where sperm undergo rapid activation under highly variable environmental conditions, such molecular regulation may be particularly important for controlling ion flux, chemotaxis, energy metabolism, and cell structure.

In this study we investigated molecular mechanisms that sperm undergo upon activation in the presence or absence of OF. Our objectives were to identify differential expression in the sperm transcriptome that contributes to the increased motility and velocity seen during hyperactivation. We also aimed to identify molecular responses, including signaling pathways and regulatory mechanisms, that underpin sperm performance when cells are activated in 25% OF vs. deionized water (dH2O). We hypothesized that the physical, biochemical, and organic properties of OF trigger the regulation of key signaling pathways and ion channels during sperm activation, thereby influencing sperm swimming.

This work was conducted using channel catfish (Ictalurus punctatus) OF and cryopreserved blue catfish (I. furcatus) sperm as a model system. Catfish are the most produced food fish in the U.S., with a large portion comprised of the channel♀×blue ♂ hybrid (2023 Census of Aquaculture). Production of hybrids requires in vitro fertilization, as channel and blue catfish are reproductively isolated. As aquaculture expands, improving fertilization outcomes during artificial spawning is a key goal. Previous research on blue catfish sperm activation in OF demonstrated that sperm velocity, motility, and longevity were enhanced compared to activation in a control media (distilled H2O without OF), and that female×male interactions played a significant role (Myers et al., 2020a). Understanding gametic interactions that enhance activation of sperm can not only improve aquaculture but can also progress our knowledge of the mechanisms underlying fertilization in fish.

2.3 Materials and methods

2.3.1 Ethics statement

All experimental animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) (#2023-5229).

2.3.2 Blue catfish and channel catfish sampling

The catfish used in this study were obtained from Heartland Catfish Company (Itta Bena, MS) and transported to the E.W. Shell Fisheries Center in Auburn, Alabama, U.S. (32.6524° N, 85.4860° W) on 21 May 2025. Fish were held in two 0.02–0.04 ha earthen ponds equipped with aerators. Sampling occurred on 26–29 May 2025. Pond temperatures and dissolved oxygen (DO) (mean±SD) in the ponds were 26.6±0.88 °C and 8.06±0.55 (mg/L) respectively. The catfish were collected from ponds via seine net. Blue catfish were euthanized by a lethal blow to the head following IACUC guidelines. Channel females were strip spawned to collect OF.

2.3.3 Sperm collection, dilution, and cryopreservation

Blue catfish sperm were sampled, the cell concentration evaluated and diluted, and sperm cryopreserved following (Liyanage et al., 2025). In brief, the testes were removed via surgical incision, and sperm separated from tissue then suspended in HBSS. Sperm cell suspensions were stored at 4°C until density was assessed in duplicate with a hemocytometer, at which point sperm from each male were diluted in HBSS at 1×10^9 cells/mL for cryopreservation. Cells were frozen at -5°C/min from 4 to -80°C and held at -80°C for 5 min. Frozen straws were stored in liquid nitrogen (Fig. 1).

2.3.4 Ovarian fluid collection

Spawning of female catfish was induced using a two-dose LHRHa (Syndel U.S., DC. U.S.) injection protocol. The first injection, a priming dose of 20 µg/kg, followed 12 h later by the second injection, a resolving dose of 100 µg/kg. Fish were checked for ovulation every 4 h, and females were hand-stripped into a mesh lined container. The mesh was pre-sprayed with vegetable oil to prevent the eggs from sticking, and the OF was drained from the eggs for ~10–15 min while rotating the eggs. The OF was transferred into 15 mL tubes and centrifuged at 4°C and 4,000 rpm for 10 min to allow for any blood and cells to settle. The supernatant was transferred to a new 15 mL tube and pH of the OF was recorded for each female. Remaining OF was held at 4°C until use (Fig. 1).

2.3.5 Sperm activation

2.3.5.1 Thawing cryopreserved sperm

To thaw, 2 straws from each male were submerged in a 40°C water bath for 20 s, wiped dry and contents emptied into 1.5 mL tubes. Thawed sperm were held on an EcoTherm Chilling/Heat Dry Bath at 4°C (Torrey Pines Scientific., CA, U.S.) (Fig. 1).

2.3.5.2 Sperm activation treatments

Three treatment groups were produced to evaluate sperm performance when activated in OF vs. DH_2O: 1) Sperm combined with OF (inactive treatment); 2) sperm activated with DH_2O (control treatment); and 3) sperm activated with 25% OF and 75% DH_2O (hypermotile treatment) (Fig. 1).

2.3.5.3 Computer assisted sperm analysis

Progressive sperm motility and velocity was evaluated using CASA (CEROS II software, Hamilton Thorne Biosciences, MA, U.S.); Progressive cells had a STR (%)>45 and VAP (μm/s)>45. Three replicate sperm activations were conducted for each treatment for 6♂ crossed with up to 6♀ pending adequate volume of OF. For the inactive and hypermotile treatments this resulted in 30 pairings. For the control treatments CASA was evaluated each time new straws were thawed. Sperm performance was recorded with the following procedure: 0.3 μL of thawed sperm was pipetted onto an 80 μm 2X-CEL chamber, 12 μL of the treatment media was then pipetted into the chamber to activate. Progressive motility (%), and progressive curvilinear velocity (VCL; μm/s) were assessed at 30 s post-activation. Videos were manually checked for quality prior to statistical analysis.

2.3.5.4 Transcriptomic sampling

For each of the three treatments (inactive, control, and hypermotile) two replicate samples were produced for transcriptomics using a 3♂ × 3♀ factorial combination with half of the males and females analyzed for CASA (Fig. 1). Inactive samples consisted of 500 μL of OF in which 50

μL of thawed sperm was added, 30 s after combining the samples were lysed with 500 μL of DNA/RNA Shield (Zymo Research., CA, U.S.). For control samples, 50 μL of thawed sperm for each male was combined with 2,000 μL of DH_2O (same sperm to media ratio as CASA) and samples were lysed with 2,000 μL of DNA/RNA Shield at 30 s. The hypermotile treatment was sampled following the same method as the control, except 500 μL of OF and 1,500 μL of DH_2O were combined with 50 μL of thawed sperm. Lysed samples were held at -80°C until total RNA extraction was completed following the Zymo Quick RNA Miniprep Plus kit protocol for cells. Total RNA samples were sent to Azenta Life Sciences for mRNA library preparation and Illumina sequencing in a 2×150 bp paired-end (PE) configuration.

2.3.6 Transcriptomic analysis

MD5 checksum was performed for transferred files to verify their integrity prior to analysis. Raw data quality was analyzed with *FastQC* (v.0.10.1), followed by trimming of adapter sequences and low-quality bases, as well as filtering of short reads with *Trimmomatic* (v. 0.39). Clean reads were subsequently aligned to the Billie_1.0 reference assembly (NCBI RefSeq assembly GCF_023375685.1) with *HiSat2* (v.2.2.0). Remaining unmapped reads were aligned with the Coco_2.0 reference assembly (NCBI RefSeq assembly GCF_001660625.3) utilizing the same parameters.

Differential gene expression analysis was carried out in R (v.4.5.0), where all comparisons were evaluated with *DESeq2* (Love et al., 2014). Statistically significant differentially expressed genes (DEGs) were characterized with an expression fold change ($|\log_2 FC| >$) 2, and a Benjamini and Hochberg P adj < 0.05. Visualization of differential expression utilized the *ComplexHeatmap*

and *ggplot* packages in R (Gu, 2022). Female and male variance effects were evaluated with the *variancePartition* package (Hoffman & Schadt, 2016).

Overrepresentation analyses of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were carried out for significant DEGs using the *clusterProfiler* package (Yu et al., 2012). The *enrichGO* function was utilized for annotation of the three major Gene Ontology (GO) categories [Molecular Function (MF), Biological Processes (BP), and Cellular Components (CC)]. The *enrichKEGG* function was utilized to identify overrepresented KEGG terms in the significant DEG data. Significantly enriched terms were characterized by a P adj < 0.05.

2.3.7 RT-qPCR

The Bio-Rad I-script cDNA synthesis kit was used to reverse transcribe extracted mRNA samples according to the provided protocol (Bio-Rad., CA, U.S.). The synthesis reaction was held at 25°C for 5 min, incubated at 46°C for 20 min, and inactivated at 95°C for 1 min.

Applied Biosystems Powerup Syber Green Master Mix was then used to perform RT-qPCR. Reactions were carried out in total reaction volumes of 10 µL, containing 4 µL of dilute cDNA, 0.5 µL of each primer, and 5 µL of master mix with the following cycle settings: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s, ending with 62°C for 5 s, and 95°C for 50 s. Four genes were used for validation: LOC128599035, *cxcl18b*, *sparc*, and LOC128599148. *Ef*1-alpha and *actb* were used as internal controls and all data was analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.3.8 Sperm kinematics

Sperm kinematics data were analyzed using SAS statistical analysis software (v.9.4; SAS Institute, Inc., 2016; NC, U.S.). Alpha was set at 0.05. Significant differences in progressive motility and progressive velocity between treatment groups was analyzed using one-way ANOVA followed by Dunnet's post-hoc test (PROC MIXED). Female×male interaction significance in progressive motility and progressive velocity were evaluated with random effects models (PROC MIXED), and variance components (VCs) were constructed using the restricted maximum likelihood (REML) method.

2.4 Results

2.4.1 Female traits and sperm kinematics

Female catfish weight ranged from 1.8–2.8 kg, and total egg mass stripped ranged from 194 g–400 g, with the volume of OF collected going from 2.5–12 mL. The mean (±SD) pH of OF was 7.93±0.32.

Sperm kinematic assessment at 30 s post-activation identified significant differences between hypermotile and control performance. Analysis of progressive motility (%) found that males A, E, S, and V had a higher proportion of cells moving forward when activated with 25% OF than in dH_2O ($P < 0.05$, Fig. 2A). VC analysis of progressive motility further identified that female explains 13.57% of the variation, and male explains 40.08% of the variation ($P < 0.05$, Fig. 2B). Analysis of sperm exhibiting progressive velocity (μm/s) found that all males had a

higher proportion of rapidly moving cells when activated with 25% OF than in dH_2O ($P < 0.05$, Fig. 2C). VC analysis of progressive velocity further identified that male explains 41.91% of the variation, and the interaction of female×male explains 16.27% of the variation ($P < 0.05$, Fig. 2D).

2.4.2 RNA sequencing statistics

Sperm from three males were sampled from all three treatments, (inactive, control, hypermotile) and used to prepare twenty-one sequencing libraries (see Supplemental Table S1.). A total of 605,329,613 raw PE reads were generated, with an average of 28,825,220 per sample. After trimming, an average of 26,382,901 (91.54%) clean reads were remaining in each sample. The clean reads were then mapped to the Billie_1.0 genome. Remaining unmapped reads were subsequently mapped to the Coco_2.0 genome. Total alignment rates ranged from 60.75%–96.04% with an average of 77.77%.

2.4.3 Treatment effect

Reads mapped to the Billie_1.0 genome were further investigated to understand sperm response. Differential expression analysis conducted with DESeq2 in R comparing inactive, control, and hypermotile sperm revealed significant changes between treatments. A Principal Component Analysis (PCA) explained 88% of total variance in gene expression, where PC1 accounted for 73% and PC2 15% (Fig. 3A). Samples clustered according to treatment, where hypermotile and inactive samples were more similar to each other than to the control. Further

comparison of gene expression between samples found that there were genes unique to treatments and shared between treatments (Fig. 3B). Hypermotile sperm had 333 unique genes, inactive sperm had 605, and control sperm 343. There was a total of 21,377 genes shared between all treatments. Hypermotile and inactive sperm were the most similar with 985 additional shared genes between them, and hypermotile and control sperm were the least similar with 288 additional shared genes.

2.4.4 Differential expression

There was a total of 2,443 significant DEGs identified between hypermotile and control sperm (P adj < 0.05; $|\log_2 FC|>2$), where 2,415 were down-regulated in control and up-regulated in hypermotile, and 28 were up-regulated in control and down-regulated in hypermotile (Fig. 4A). There was a total of 101 significant DEGs identified between hypermotile and inactive sperm (P adj < 0.05; $|\log_2 FC|>2$), where 45 were down-regulated in inactive and up-regulated in hypermotile, and 56 were up-regulated in inactive and down-regulated in hypermotile (Fig. 4B). There was a total of 4,156 significant DEGs identified between control and inactive sperm (P adj < 0.05; $|\log_2 FC|>2$), where 461 were down-regulated in inactive and up-regulated in control, and 3,695 were up-regulated in inactive and down-regulated in control (Fig. 4C).

2.4.5 Gene Ontology and KEGG

Overrepresentation analysis of DEGs revealed 197 significantly enriched GO terms in hypermotile sperm compared to control sperm (P adj < 0.05). Of the significant terms, 136 were from BP, 38 CC, and 29 MF. Subsequent enrichment mapping identified several highly connected clusters centered on actin cytoskeleton formation and organization, cell migration and

signaling, environment interaction, and activity regulation (Fig. 5A). KEGG analysis of the same genes found 18 enriched terms (P adj < 0.05). Enrichment mapping for KEGG analysis of DEGs further identified core clusters of importance. Like in the GO map, the actin cytoskeleton was highly prevalent and interconnected. However, emphasis was further placed on focal adhesion and signaling complexes, pathways related to calcium and kinase mediated signaling, signaling control, and membrane organization (Fig. 5B). These results support the idea that hyperactivation of sperm cells requires a rapid but coordinated structural remodeling of actin in the flagella, mechanosensitive regulation, signaling, and cell-environment interaction.

For DEGs that were enriched in the control sperm compared to in the hypermotile, GO analysis found 3 significantly enriched CC terms (P adj < 0.05). The terms *external side of plasma membrane*, *side of membrane*, and *cell surface*, indicate that cells have proteins exposed outside the cell membrane. As surface proteins usually have roles as receptors, ion channel regulators, and in membrane structure, their enrichment potentially reflects that cells are in a state of being ready to interact with a reproductive environment. There were no significant KEGG terms for genes upregulated in control sperm when compared to hypermotile sperm.

As previously mentioned, the hypermotile sperm and inactive sperm were the most similar of the treatments. This was also reflected in GO and KEGG analysis, where there was only one significant GO term enriched in the inactive sperm, *canonical NF-kappaB signal transduction* (IKBA) (P adj < 0.05). In humans, IKBA signaling has been found to orchestrate sperm motility through modulation of fatty acid β-oxidation, and in its phosphorylated form it suppresses motility (Y. Li et al., 2023). These results indicate that IKBA is restricting motility of sperm prior to osmotic shock.

Finally, when comparing control sperm to inactive sperm, one BP GO term was found to be enriched in the control treatment, the *ubiquitin-dependent protein catabolic process via the N-end rule pathway* (*P* adj<0.05). However, there were 190 significant GO terms enriched in the inactive sperm, 104 BP, 49 CC, and 37 MF (*P* adj<0.05). Enrichment mapping of these terms identified some of the same interconnected clusters that were identified in the hypermotile vs. control sperm, they are related to the actin cytoskeleton, cell surface signaling, and activity regulation. There were also 24 significantly enriched KEGG pathways identified (*P* adj<0.05). Analysis of enrichment map clusters revealed very similar results to the hypermotile vs. control KEGG results, with nodes representing signaling pathways, actin, and focal adhesion. These results indicate that the presence of OF causes a response in sperm cells, where environment interaction mechanisms, flagella structure, the cell membrane, and signaling, are all primed even when there is no motility activation.

2.4.6 Female effect

The effect of female on sperm gene expression was evaluated by comparing DESeq2 models with a likelihood ratio test to identify if terms removed in the 'reduced' model explained a significant amount of variance in the data. There were 2,632 genes identified where expression varied by female beyond what could be explained by male variation (*P* adj < 0.05). PCA analysis was performed to visualize the distribution pattern among samples, where PC1 (73%) and PC2 (12%) accounted for 83% of total variation among gene expression (Fig. 6A). Clustering of samples indicates that differences in gene expression between females represent a large portion of variance between samples.

Subsequent variance partitioning analysis revealed that for the average gene, female effect explains ~ 5% of the variance (Fig. 6B). However, there are genes where female effect explains >90% of variance. Variance analysis also found that male effect explains ~1% of variance (Fig. 6B). A heatmap was used to visualize the top and bottom 20 genes that vary by female effect (P adj < 0.05; $|\log_2 FC|>2$) (Fig. 6C).

GO and KEGG analyses were performed to identify the function of genes whose expression varies significantly among females (P adj < 0.05). There were 91 GO terms found, 40 BP, 36 CC, and 15 MF. Enrichment mapping showed highly interconnected nodes related to cilium assembly, organization, and movement, as well as the actin and microtubule cytoskeletons, response to oxygen levels, and cell motility (Fig. 7A). Additionally, 23 KEGG pathways were identified. Enrichment clusters identified included signaling pathways, cytoskeleton regulation, focal adhesion, and apoptosis (Fig. 7B).

2.4.7 RT-qPCR

For RNA-seq validation, two housekeeping genes (*ef*1-alpha and *actb*) and four significant DEGs were randomly selected (LOC128599035, *cxcl18b*, *sparc*, LOC128599148). Melt curve analysis for the genes obtained from qRT-PCR showed a single peak, indicating only one product was present. The $\log_2 FC$ of the four genes was consistent with the RNA-seq differential expression results, which confirmed that the transcriptomic data were reliable (see Supplementary Figure S1).

2.5 Discussion

The function, performance, and compatibility of gametes can have crucial influence on the reproductive success of individuals and of species (Kosman & Levitan, 2014). In external fertilizers, sperm competition and CFC are known to be driven by physiological processes that facilitate fertilization at cellular and molecular levels, a form of selection known as gamete mediated mate choice (GMMC) (Kekäläinen & Evans, 2018). Understanding the biochemical and molecular interactions that dictate and bias fertilization may have important benefits for spawning teleost fish that can be applied to interpreting ecological impacts as well as increasing production in the aquaculture sector. This study was the first of its kind to analyze the transcriptomic changes during *in vitro* activation of teleost fish sperm with and without OF to identify genes, pathways, and their biological roles associated with sperm activity. Our results both confirmed the extended duration of motility and increased velocity of blue catfish sperm when activated with OF (Myers et al., 2020a), and also identified when and what molecular changes are occurring during activation. The comparison between hypermotile, inactive, and control sperm revealed DEGs that enrich for reproductive processes associated with cell activation, swimming performance, and environment interaction.

Sperm activation is a process that happens in milliseconds as cells move from an immotile state to a motile one (Pérez, 2020). The importance of ion channels for sperm activity has been confirmed in many species including humans, mice, and fish (Alavi & Cosson, 2006; Pérez, 2020; Wang et al., 2021). The CatSper channel, a Ca^{2+} selective plasma membrane channel is highly studied for its role in sperm motility activation and regulation as it controls the influx of calcium to the flagella (Lissabet et al., 2020; Wang et al., 2021). This channel is organized along the principal piece of the flagellums actin cytoskeleton, where the structure and

organization of ion channels is critical for hypermotile cells (Gervasi et al., 2018). Our results showed that in hypermotile and inactive cells compared to control, the regulation, organization, and assembly of actin and actin-based processes were enriched. This indicates that OF may increase sperm activity in part by regulating actin remodeling, allowing for the improved function of CatSper channels. Additional enrichment was seen in OF treatments in the signaling pathways: *Mitogen-activated protein kinase* (MAPK), *Vascular Endothelial Growth Factor* (VEGF), and *Immunoglobulin superfamily cell adhesion molecule* (IgSF CAM). Each of these are conserved proteins that have previously been linked to spermatogenesis, sperm motility, and reproductive success through their role in facilitating signal transmission by phosphorylation cascades (Iyibozkurt et al., 2009; Kumar et al., 2024; Xiao et al., 2013).

Osmotic shock is also important for the sperm motility activation of many teleost fish (Bates et al., 1996; Pérez, 2020; Takei et al., 2012). The osmolality of seminal plasma suppresses sperm motility, and the osmotic change upon release into an external environment initiates flagellar movement. Sperm morphological changes have been found to coincide with this change in many fish species soon after release. Changes include plasma membrane swelling in low osmolality and membrane shrinking in high osmolality (Krasznai, 2003; Márián et al., 1993; Pérez, 2020). The rapid water movement and increased cell membrane permeability and fluidity from osmotic pressure can increase ion flux across membranes, altering sperm activity. Osmotic pressure fluctuation also initiates stretch-activated or mechanosensitive membrane proteins in fish species such as puffer fish (*Takifugu niphobles*) and carp (*Cyprinus carpio*), where it was found that the presence of Gadolinium, a stretch-activated ion channel blocker, almost fully stopped sperm motility (Krasznai et al., 2003). We found that sperm treated with OF was enriched for focal adhesions (FAs), which are large multi-protein complexes that act as

mechano-transducers. Meaning, FAs trigger intracellular signaling upon detecting mechanical strain (Kuo, 2013; Roa-Espitia et al., 2016). Through stretch activated signaling, FAs organize the actin cytoskeleton, drive cell migration, and contribute to acrosome integrity in guinea pig (*Cavia porcellus*) sperm (Roa-Espitia et al., 2016). Our results also found FAs to be highly interconnected with regulation of the actin cytoskeleton and signaling pathways, and they are, therefore, of interest for enhanced sperm motility.

Environment-sperm interactions, including gamete interactions, are also of importance for broadcast spawning species. Female derived signaling has been found in externally fertilizing species where there have been induced changes in sperm motility from interactions with OF and egg-derived chemical factors (Rosengrave et al., 2009). Environment interaction and sperm chemotaxis are driven by OF composition, which is a complex mix of proteins, glycoproteins, hormones, enzymes, and metabolites. We identified many significant cellular process and pathways important to protein binding, signaling, and directional movement enriched in OF treatments (see Supplemental Table S2). *GTP binding*, *GTPase activity*, *cell migration*, *integrin binding*, and *glycosaminoglycan binding* are a few molecular functions stimulated in hypermotile and inactive sperm that indicate cells are detecting external signals (proteins) and responding with directional navigation as previously found in mammalian studies (Mahé et al., 2023; Merc et al., 2021). Additional enrichment for protein kinase activity related pathways supports OF influenced environment interaction and chemotactic signaling (Ijiri et al., 2012; Teves et al., 2009). Overall, these results indicate that OF micro-environments guide sperm physiological responses through specific pathways and signaling cascades.

Further analysis of the role of the OF environment revealed that there are female induced effects on sperm, which causes female×male gamete interactions, and therefore, CFC. These

results confirmed previous findings in blue catfish (Myers et al., 2020a), and align with what has been seen in many other fish species. Genes that were identified as significantly different between females were associated with mechanisms involved in cell motility, response to reactive oxygen species, ion channels, signaling pathways, and chemotactic movement. Overall, this indicates that there are non-random sperm-female interactions, and perhaps even gamete-gamete interactions, that strongly influence the fertilizing ability of cells. Previous research in external fertilizers has found that on a cellular level sperm cells swim towards unfertilized genetically compatible eggs (Evans et al., 2012). In Chinook salmon (*Oncorhynchus tshawytscha*), the major histocompatibility complex (MHC) has been investigated for its role in the genetic compatibility of mates (Garner et al., 2010; Gessner et al., 2017). In many species, females select for males with a dissimilar MHC as it can improve offspring pathogen resistance (Garner et al., 2010). In salmon it has been found that individuals are more aggressive towards fish with similar MHC, however, MHC influence on fertilization success and sperm-egg interaction may be locust dependent in addition to the genetic distance (Gessner et al., 2017). Research into non-random mating in multiple marine invertebrates and zebrafish (*Danio rerio*) has also found that gamete compatibility is mediated by gamete recognition proteins (GRPs) (Gert et al., 2023; Kosman & Levitan, 2014). As we found membrane protein related processes to be significantly affected by female, GRPs and other chemoattractant components of OF may be of high importance in external fish fertilization.

It is clear from the results of kinematic and transcriptomic analyses that OF micro-environments initiate molecular changes in sperm cells that stimulate enhanced movement. Interestingly, the transition in gene expression was found to occur when sperm encounters OF regardless of if the sperm is motile or not. In many internally fertilizing species, sperm acquire

the ability to fertilize only after contact with the female reproductive tract through a process called capacitation (Ruffenach, 2009). Capacitation is where sperm cells undergo changes like membrane restructuring and organization, increased intracellular ion concentrations, protein modifications, and signaling pathway events. This allows for the acrosome reaction, and hyperactivated motility where vigorous tail movement is developed that helps sperm navigate the female tract and penetrate the egg (Jin & Yang, 2017). As previously mentioned, most teleost fish eggs have a micropyle that allows entry for sperm to penetrate the egg, and therefore, their sperm do not require an acrosome or acrosome reaction. As such, teleost fish sperm do not undergo the same capacitation as seen in other species. However, our results indicate that OF in teleost fish stimulates a similar reaction in externally released sperm cells. This is evident due to the activation of pathways with roles in membrane fluidity, transport, and ion channel regulation. Additionally, fish sperm with OF exhibit a hyperactivated state measured by increased mobility over those that are not exposed to OF. When combined with the prominent female effect that is seen across teleost species, it is evident that OF, CFC via GMMC, are crucial to reproductive outcomes. Looking towards the future, the utilization of sexual selection on the molecular scale could be an effective method of understanding and improving management and aquaculture of fish species.

In summary, these findings indicate that the OF micro-environment stimulates molecular changes in conjunction with the physiological and mechanical changes seen in sperm performance. As a result, OF can alter sperm fertilization potential and facilitate gamete compatibility.

Conflicts of interest

The authors declare no conflicts of interest

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Data availability

NCBI: BioProject PRJNA1423719

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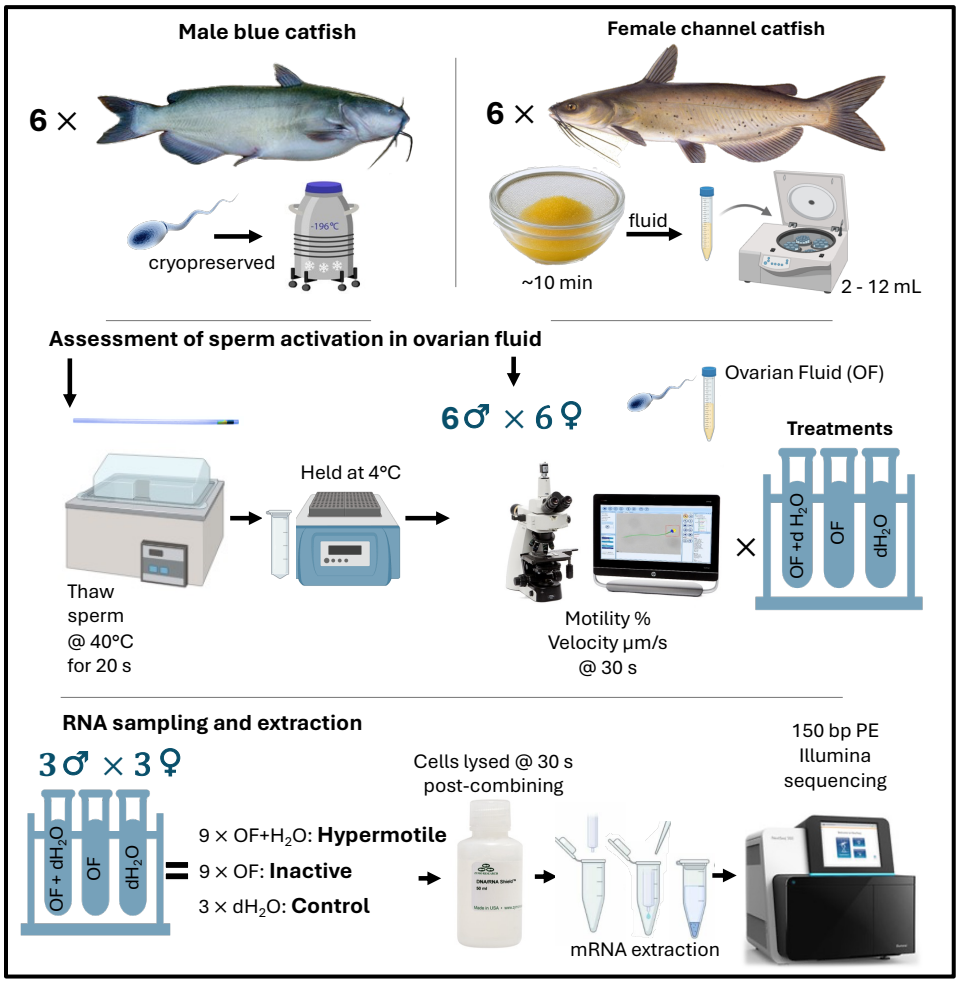

Figure 2.1. Experimental design for the sampling of blue catfish (*Ictalurus furcatus*) sperm and channel catfish (*Ictalurus punctatus*) OF, the kinematic assessment of sperm activation in a dH_2O control media and in a 25% OF media, and the RNA sampling and extraction of sperm samples for transcriptomic analysis.

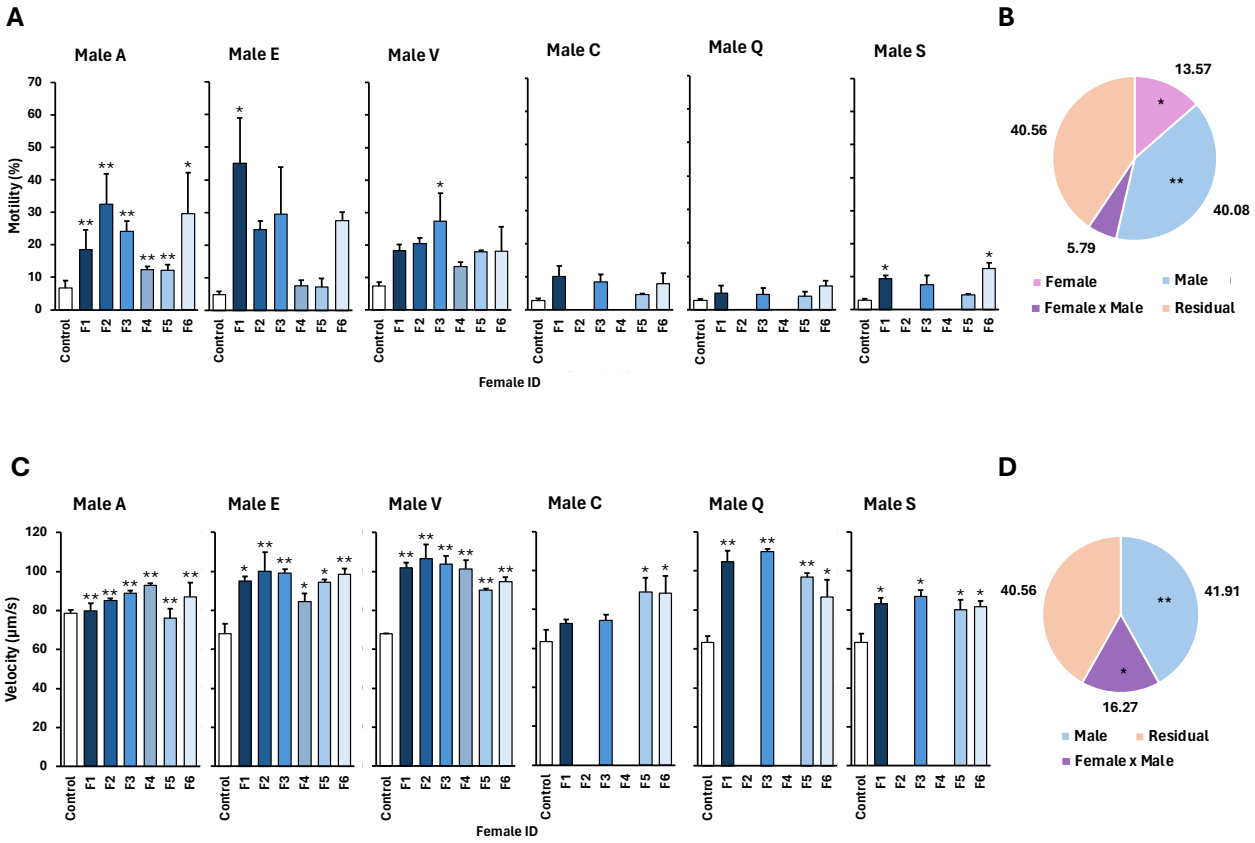

Figure 2.2. Sperm progressive motility and progressive velocity analysis between six male blue catfish (*Ictaurus furcatus*) and six female channel catfish (*Ictalurus punctatus*). A) Mean (±SEM) percent of progressive motile cells at 30 s post-activation in hypermotile (blue bars) and control treatments (white bars). The hypermotile sperm were significantly different from the control for Males A, E, V, and S ($P < 0.05$). B) Pie chart showing female, male, and female×male progressive motility variance components. C) Mean (±SEM) progressive velocity (µm/s) of cells at 30 s post-activation in hypermotile (blue bars) and control treatments (white bars). The hypermotile sperm were significantly different from the control for alle Males ($P < 0.05$).). B) Pie chart showing female, male, and female×male progressive velocity variance components. Significant values were denoted by ∗ = P < 0.05, ∗∗ = P < 0.001.

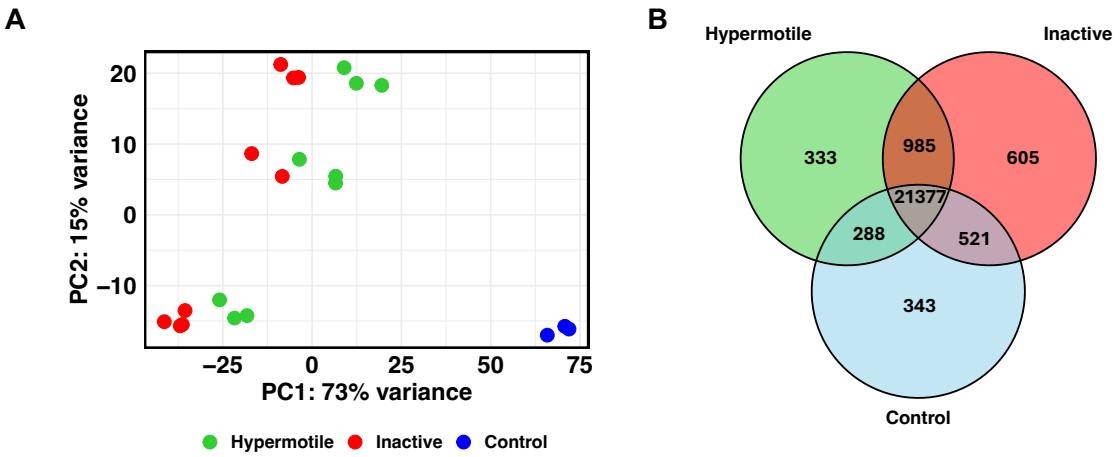

Figure 2.3. RNA-sequencing variance between three treatments: 1) hypermotile sperm activated in 25% OF in green, 2) inactive sperm in red, and 3) control sperm activated in dH_2O in blue. A) Principal component analysis (PCA) plot shows PC1 (73%) vs. PC2 (15%) explaining a total of 88% variance. Sample clusters indicate that gene expression varies by sperm treatment. B) Venn diagram showing the number of blue catfish genes expressed in each treatment that are either unique to a treatment, shared between two treatments, or shared between all treatments.

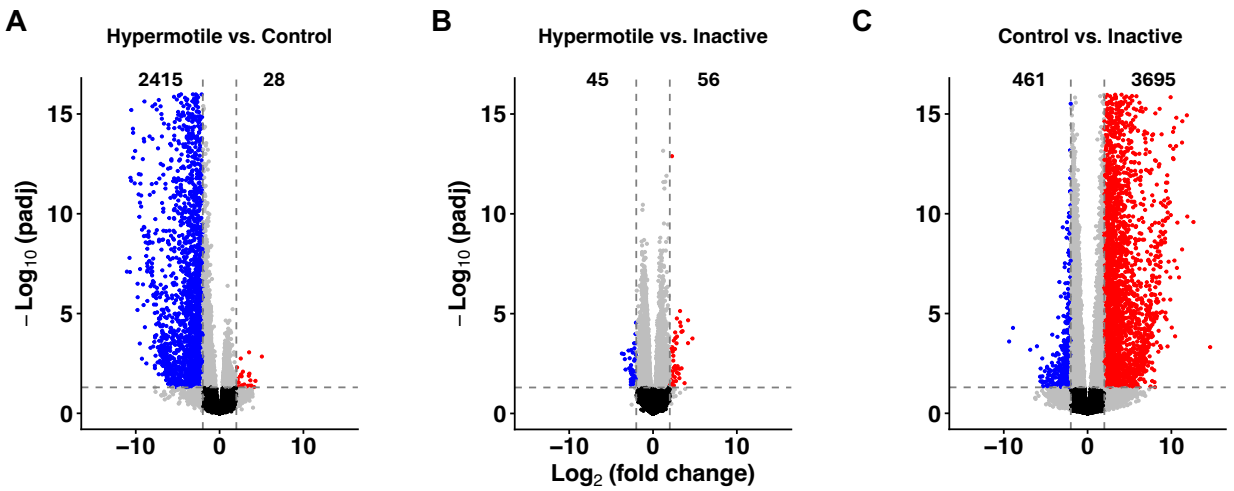

Figure 2.4. Differential gene expression visualized with volcano plots for three treatment comparisons: A) Hypermotile vs. control sperm activation, B) Hypermotile vs. inactive sperm, and C) Control activation vs. inactive sperm. The reference group for each comparison is shown on the left of each figure, and the number is the significant up-regulated DEGs in that treatment.

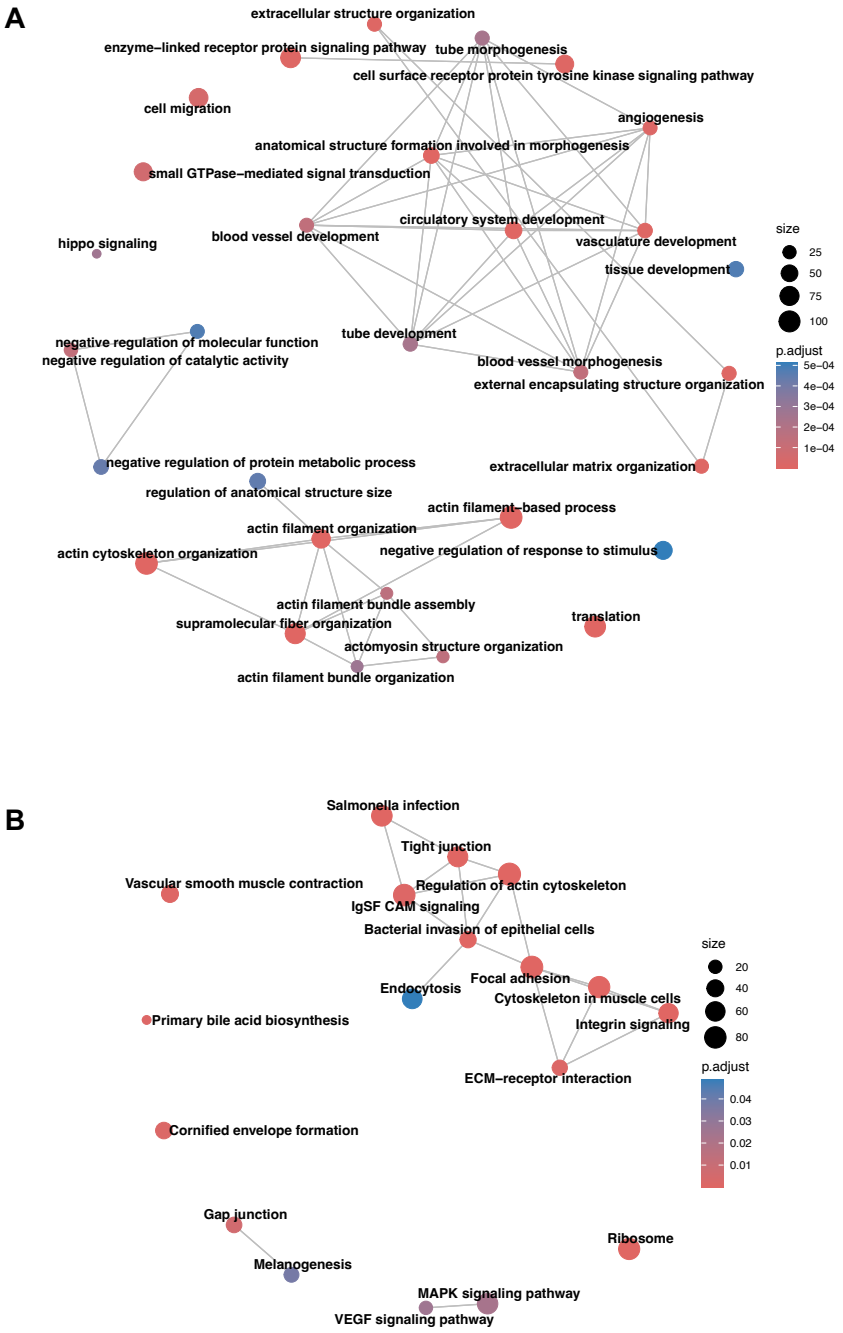

Figure 2.5. Enrichment analysis of significantly upregulated genes in hypermotile treatment compared to the control treatment. A) Enrichment map plot of overrepresented Gene Ontology terms for biological processes, molecular functions, and cellular components. B) Enrichment map of overrepresented KEGG pathways. The size of the nodes represents the number of genes

associated with each term, the color of the nodes represents the adjusted *P*-value, and the edges represent the gene overlap between terms.

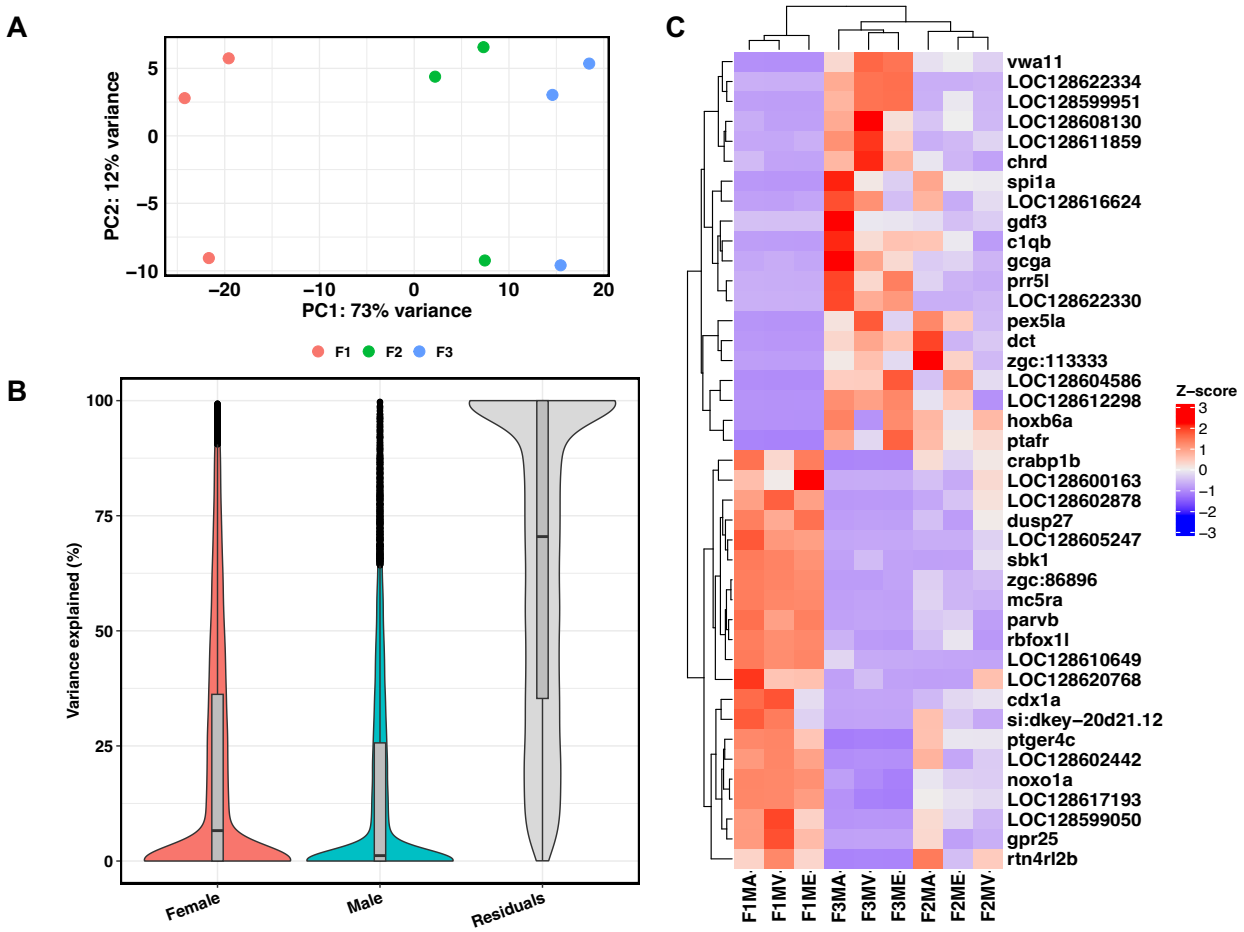

Figure 2.6. Analysis of the effect of female on hypermotile activation of sperm. A) component analysis (PCA) plot shows PC1 (73%) vs. PC2 (12%) explaining a total of 85% variance between all hypermotile samples. Female 1 activations are shown in red, female 2 in green, and female 3 in blue. Clusters indicate that gene expression varies between females and show similarity between males. B) Violin plot of (%) variance explained on y-axis, by Female in red, Male in blue , and Residuals in grey on x-axis. Horizonal black line shows median variance. Where the plots are wide, it indicates that many genes varied by the corresponding percent. C) Heatmap of the 20 highest and lowest genes by log2FC that showed a significant female effect (adjusted $P < 0.05$). Color represents the z-score, a measure of how many standard deviations the log2FC was from the mean, with red showing positive and blue negative.

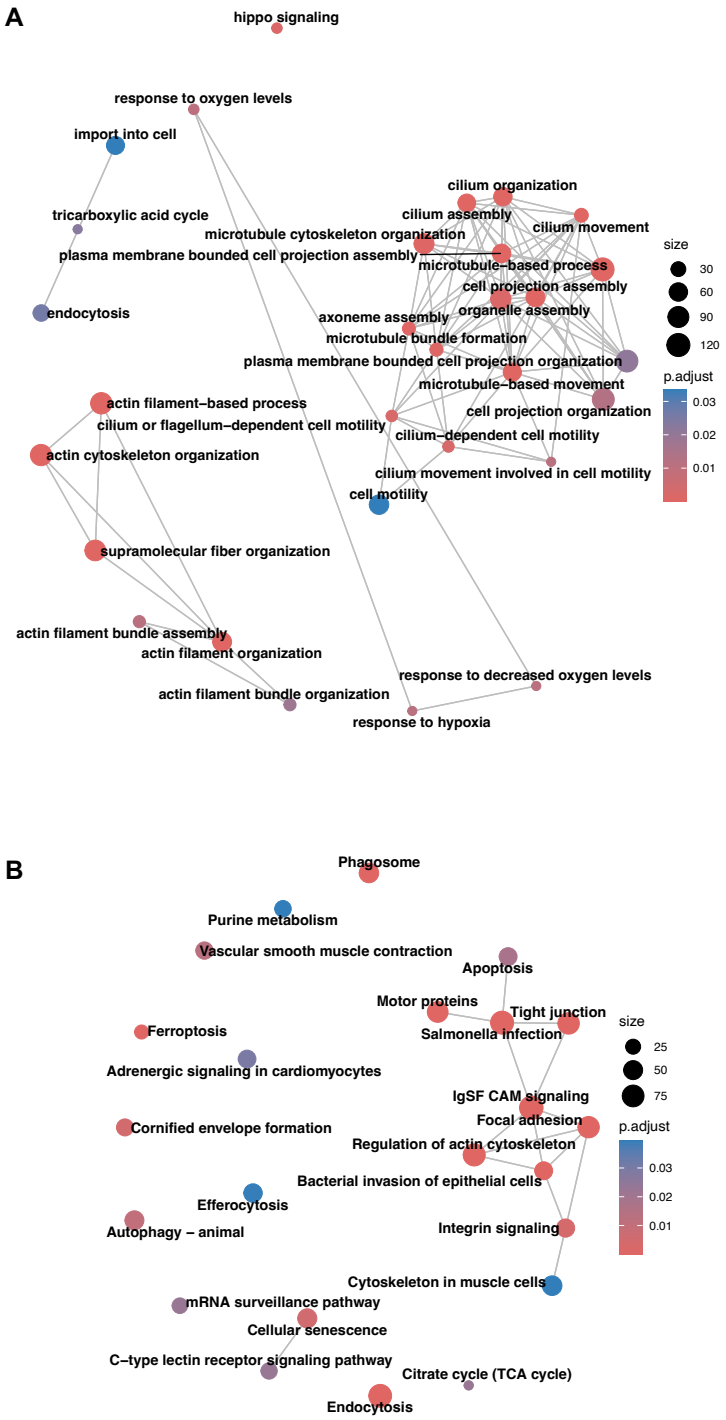

Figure 2.7. Enrichment analysis of genes that have a significant effect from female. A) Enrichment map plot of overrepresented Gene Ontology terms for biological processes, molecular functions, and cellular components. B) Enrichment map of overrepresented KEGG pathways. The size of the nodes represents the number of genes associated with each term, the

color of the nodes represents the adjusted *P*-value, and the edges represent the gene overlap between terms.

Supplemental:

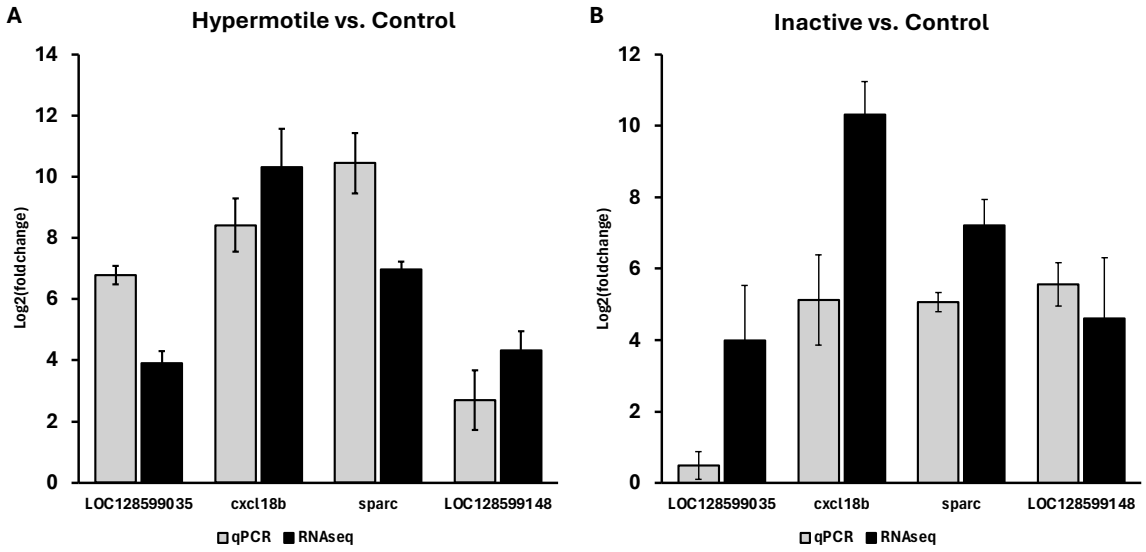

Figure S2.1. Log_2FC in the expression of differentially expressed genes identified by RNA-seq and validated by real-time qPCR between A) hypermotile sperm activated in OF and control sperm activated in dH_2O, and B) inactive sperm in OF and control sperm activated in dH_2O**.** *Ef*1-alpha and *actb* genes were used for RT-qPCR data normalization, and DEseq2 median of ratios was used to normalize RNA-seq read counts.

Table S1. RNA sequencing read quality control and mapping statistics.

Sample	Treatment	Raw reads	Clean reads	% Clean	% Mapped to Billie	% Mapped to Coco	% Total mapped
F1MA-H2O	hypermotile	26,090,532	24,358,755	93.36	33.79	35.49	60.75
F1ME-H2O	hypermotile	25,382,529	23,422,490	92.28	43.07	39.51	69.70
F1MV-H2O	hypermotile	28,978,213	26,428,204	91.20	41.33	38.26	67.71
F2MA-H2O	hypermotile	28,495,776	26,208,169	91.97	40.12	37.43	66.29
F2ME-H2O	hypermotile	26,885,698	24,481,173	91.06	46.35	34.33	68.56
F2MV-H2O	hypermotile	27,286,168	24,609,993	90.19	53.76	40.71	76.93
F3MA-H2O	hypermotile	29,183,226	26,644,712	91.30	42.51	56.98	81.62
F3ME-H2O	hypermotile	25,929,595	23,545,157	90.80	44.42	47.98	76.57
F3MV-H2O	hypermotile	28,772,287	26,307,901	91.43	37.14	45.36	69.93
MA	control	34,187,835	30,326,430	88.71	83.16	29.28	91.73
ME	control	24,129,449	21,311,019	88.32	83.28	30.98	92.56
MV	control	32,497,649	29,490,486	90.75	91.05	30.02	96.04
F1MA-OF	inactive	24,031,164	22,599,668	94.04	47.53	49.97	79.34
F1ME-OF	inactive	32,549,218	30,358,517	93.27	49.3	45.89	77.72
F1MV-OF	inactive	30,485,705	28,049,626	92.01	49.45	45.76	77.73
F2MA-OF	inactive	31,323,345	28,705,139	91.64	47.73	47.43	77.31
F2ME-OF	inactive	31,325,918	28,491,624	90.95	53.28	43.1	78.17
F2MV-OF	inactive	33,070,104	30,797,890	93.13	41.57	49.97	76.34
F3MA-OF	inactive	29,333,964	27,053,543	92.23	43.87	50.21	77.93
F3ME-OF	inactive	27,818,718	25,649,326	92.20	50.35	58.94	86.34
F3MV-OF	inactive	27,572,520	25,201,107	91.40	49.26	56.24	83.80
Average		28,825,220	26,382,901	91.54	51.06	44	77.77
Total		605,329,613	554,040,929				

Chapter 3

Circulating blood miRNAs as non-lethal biomarkers of sperm kinematic performance in blue catfish, *Ictalurus furcatus*

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Keywords: Aquaculture; Reproduction; Motility; Velocity; Gene Expression

3.1 Abstract

Catfish production is the leading domestic aquaculture industry in the U.S. for both economic value and product output. The hybrid catfish, a cross between channel females (*Ictalurus puntatus*) and blue males (*I. furcatus*), is produced by over 60% of farms due to its increased performance in growth, survival, and disease resistance over either parent species. However, the production of hybrid catfish comes at high economic and time cost as the blue males don't reach sexual maturity until 4-7 years of age. At which point the male must be sacrificed to harvest their sperm for *in vitro* fertilization with channel female eggs since the two species are reproductively isolated. Therefore, it is of the utmost importance to harvest males that are ready to reproduce with high-quality gametes.

As such, our objectives were to evaluate circulating miRNAs as non-lethal biomarkers of sperm quality in blue catfish. We collected blood serum and sperm from 42 blue males. Computer assisted sperm analysis was used to evaluate the quality of each male's sperm, and miRNA from low-quality (n=6) and high-quality (n=6) males were sequenced. Three differentially expressed miRNAs were identified between males with low-quality and high-quality sperm, highlighting their potential as diagnostic indicators. Together, these findings demonstrate the applications of reproductive molecular tools to improve industry efficiency, and sustainability in aquaculture production.

3.2 Introduction

At a value of $479 million, catfish are the highest-produced U.S. domestic aquaculture species in both economic value and total output (USDA, 2023). Hybrid catfish, produced by crossing channel catfish (*Ictalurus furcatus*) females ♀ with blue catfish (*I. furcatus*) males ♂, are used by ~60% of farms due to their ability to outperform either parent species in growth, disease resistance, and tolerance to changes in water quality (Venugopalan et al., 2021; Dunham and Masser, 2012). However, biological barriers prevent natural reproduction of these species. Production, therefore, requires farmers to euthanize mature blue catfish to manually remove their testes and extract sperm for the artificial feralization of channel catfish eggs (Dunham, 2023). This creates challenges for farmers, as males only reach sexual maturity after 4 years of age (Liyanage et al., 2025; Martin et al., 2025). Additionally, there is limited research on methods to evaluate sperm quality prior to euthanasia. As a result, the ability to collect high quality sperm comes at a high economic cost and time investment for the industry.

Previous research on paternal impacts in blue catfish has shown that larger males produce hybrid larvae that are significantly larger with higher survival rates at 40 days post-hatch than larvae sired by smaller males (Wood et al., 2025). Males with a higher gonadosomatic index (GSI) also produced smaller larvae with lower survival than males with low GSI. Finally, sperm kinematics including percent motility and progressive velocity, were positively correlated with hybrid hatch success (Wood et al., 2025). While reproductive indices, such as GSI and sperm kinematics provide valuable insight into male fertility, they require lethal sampling due to the necessity of harvesting testes. To improve the efficiency and success of the catfish aquaculture industry, there is a need for non-lethal methods to access to quality sperm. Molecular biomarkers

found in blood may offer a solution by enabling the predictive of male fertility without the need for euthanasia.

Spermatogenesis is a highly complex process that relies on many epigenetic, transcriptional, and environmental factors for gene regulation and expression (Shi et al., 2024). Mature micro-RNAs (miRNAs) are short (18-25 nucleotide) single-stranded, endogenous, non-coding RNAs that are epigenetically regulated and linked to the regulation of gene expression through their ability to bind to the 3′-unstranslated regions of protein coding genes (O'Brien et al., 2018). Each miRNA may have multiple target mRNA regions, and each mRNA may have multiple regions for different miRNA to bind; this often results in the translation disruption or degradation of the mRNA (O'Brien et al., 2018). Many studies have demonstrated that miRNAs are crucial factors for cell differentiation, proliferation and apoptosis, as well as the growth and metabolism of cells. Some studies have further shown that miRNAs play important roles in the stages of spermatogenesis (Shi et al., 2024). Given the goal of identifying non-lethal biomarkers of male fertility, circulating miRNAs (c-miRNAs) present in blood are of particular interest.

c-miRNAs have shown potential in human biomedical research and in several aquatic species, including fish, for predicting reproductive success (Cardona et al., 2021; Cito et al., 2020a). In female rainbow trout (*Oncorhynchus mykiss*) miR-202-5p has been established as a candidate biomarker of egg quality (Cardona et al., 2021). In humans, recent studies on male infertility suggest that miRNA expression levels in blood plasma are different in males with abnormal sperm quality and quantity. In patients with non-obstructive azoospermia, a condition where there is low to no sperm count in the ejaculate, it was found that miR-20a-5p in the blood plasma was significantly higher than in control patients (Cito et al., 2020a). There are, however, no studies that investigate c-miRNAs related to male fish gametes or spermatogenesis.

Because blood can be collected from blue catfish without the need for euthanasia, and c-miRNAs show potential as biomarkers of reproductive status, establishing predictive c-miRNAs for sperm quality represents a valuable opportunity to improve reproductive efficiency and hatchery outcomes. In this study, we aimed to identify c-miRNAs in blue catfish blood serum that may serve as biomarkers of male sperm quality, with focus on sperm exhibiting high kinematic indices. Our objective was to develop a non-lethal method for male selection prior to euthanasia, enabling farmers to identify individuals with high-quality sperm while reducing wasted time and gametic resources, therefore, improving economic outcomes. Here, we collected blood serum from 42 male blue catfish, measured sperm kinematics using Computer Assisted Sperm Analysis (CASA) and selected males that had low-quality (n = 6) and high-quality (n = 6) sperm based on total motility, progressive motility, and progressive velocity. Small RNA (sRNA) sequencing was then performed on these samples to identify and evaluate potential c-miRNAs as biomarkers of sperm quality.

3.3 Materials and Methods

3.3.1 Ethics Statement

All experimental animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC #PRN 2020–3710 and PRN 2023–5229).

3.3.2 Blue catfish sampling

Male blue catfish used in this study were obtained from Heartland Catfish Company (Itta Bena, MS) and transported to the E.W. Shell Fisheries Centers in Auburn, Alabama, U.S. (32.6524° N, 85.4860° W) on 21 May 2025. Fish were held in 0.02 to 0.04 ha earthen ponds equipped with aerators. Sampling occurred between 26 to 29 May 2025. Pond temperature (average ± SD) was 26.6 ± 0.88°C) and dissolved oxygen (DO) concentration (average ± SD was 8.06 ± 0.55 (mg/L) were recorded each morning. Forty-two males were collected from ponds via seine net and euthanized by a lethal blow to the head following AU-IACUC Guidelines and industry standards.

3.3.3 Serum collection

Upon euthanasia 8 to12 mL blood samples were immediately collected from the caudal vein behind the anal fin using 20 G sterile needles and transferred into a 15 mL non-heparinized centrifuge tubes without anticoagulant. Samples were placed in an ice filled Styrofoam cooler on paper towel and the immediately transferred to a 4°C Eppendorf Centrifuge 5702R (Eppendorf, Hamburg, Germany). Serum was separated from total blood samples by centrifuging at 4,400 RPM for 10 min while holding at 4°C. If separation was not complete after 10 min, the samples were recentrifuged for 10 min intervals up to 30 min. Serum was pipetted into three 1.5 mL microcentrifuge tubes and stored at –80°C until miRNA extraction.

3.3.4 Sperm collection and dilution

Sperm collection and cell density quantification followed (Liyanage et al., 2025; Martin et al., 2025; Wood et al., 2025).

3.3.5 Computer assisted sperm analysis (CASA)

3.3.5.1 Kinematic analysis

CASA (CEROS II software, Hamilton Thorne Biosciences, U.S.) was used to analyze sperm samples for kinematic traits including percent motility, curvilinear velocity (VCL; μm/s), percent progressive motility (Pro_{Mot}), and progressive curvilinear velocity (Pro_{VCL}; μm/s). Kinematic traits were picked following results from a previous study that correlated sperm percent motility and progressive velocity with hybrid catfish hatch successes (Wood et al., 2025).

In short, three technical replicate activations were carried out for each male using the following procedure: 1 μL of diluted sperm was activated with 12 μL of activation media (dH_2O supplemented with 5% bovine serum albumin) in an 80 μm 2X-CEL chamber (Hamilton Thorne Biosciences, U.S.). Replicates were recorded under a light microscope at 10-, 20-, and 30 s post-activation. Diluted sperm samples and activation media were held at 4°C in 1.5 mL microcentrifuge tubes on an Echotherm™ Chilling/Heating Dry Bath (Torrey Pines Scientific, CA, U.S.) for the duration of the analysis.

CASA videos were manually checked for quality control prior to statistical analysis. Sperm tracks were removed from videos in a few scenarios; if cells were seen drifting rather than swimming across the cameras field of vision, if the software mistakenly split a single sperm track

into multiple or combined tracks that crossed paths, and if the software mistakenly categorized still sperm as motile or vice versa.

3.3.5.2 Predictive kinematics

The low-quality and high-quality groups were also evaluated for predicted hatch success using a previously established linear regression model that establishes VCL as a kinematic parameter with predicative ability (Wood et al., 2025). The evaluated equation was $[y = 0.91x + 27.62]$ where x is VCL μm/s and y is the predicted hatch percent. While we are cognizant that it was built using data from only one female, it does show that curvilinear velocity has major ramifications for hatchery success.

3.3.6 Transcriptomic Analysis

3.3.6.1 Male selection

The average ± SD of kinematic traits for the three technical replicate activations were calculated at 10-, 20-, and 30 s. Trends between males with high motility and velocity were consistent across timepoints, and as the most cells were active at 10 s, this timepoint was used for further analysis. To find the males of low-quality and high-quality, a Z-score was calculated ($Z = (x - \mu)/\sigma$) for each kinematic trait: motility, VCL, Pro_{Mot} , and Pro_{VCL}. The four Z-scores were combined and ranked from lowest to highest. The six bottom and six top males were

selected for further analysis and miRNA extraction. A t-test was conducted, and it was found that the low-quality and high-quality groups were significantly different ($P < 0.05$).

3.3.6.2 miRNA extraction

For the twelve males chosen for miRNA extraction, frozen serum samples were thawed, and extraction was performed according to the Qiagen Serum/Plasma miRNeasy Advanced Kit (cat. no. 217204) (QIAGEN, Denmark). Samples were quality controlled using an Agilent High Sensitivity RNA Screentape on the Agilent 4200 Tapestation System (Agilent, CA, U.S.).

3.3.6.3 Small RNA sequencing

Serum miRNA samples for the low-quality (n = 6) and high-quality (n = 6) males were sent to Azenta Life Sciences (NJ, U.S.) for miRNA library preparation and Illumina sequencing. Sequencing libraries were prepared using the NEB Small RNA library Prep Kit, according to manufacturer's instructions (New England Biolabs). First, samples underwent 3' adapter ligation, followed by primer hybridization and 5' adapter ligation. Then the miRNA, now enclosed by adapters, were converted to cDNA by first-strand cDNA synthesis and amplified with PCR. Samples were subjected to size selection and cleanup, then library validation on the Agilent TapeStation, to ensure the integrity of the desired miRNAs prior to sequencing on the Illumina NovaSeq X Plus platform using a 2 × 150 bp PE configuration (Azenta Life Sciences).

3.3.6.4 Transcriptomic analysis

Raw FASTQ sequencing files received from Azenta Life Sciences underwent a MD5 checksum to verify the integrity of the files, then quality control and preprocessing where low-quality bases and adapter sequences were removed via Fastqc (V 0.10.1) and Trimmomatic. The clean reads were then aligned to the Billie1.0 reference genome (NCBI RefSeq Assembly: GCF_023375685.1) and mapped to known mature and precursor catfish miRNAs in the miRbase2.0 database using the quantifier.pl program from miRDeep2 (Friedländer et al., 2012). Sequences of previously reported catfish miRNAs and zebrafish (*Danio rerio*) miRNAs were used as a reference to detect novel miRNAs with miRDeep2.pl software (Friedländer et al., 2012). Predicted novel miRNAs were filtered for sequences with >50 reads and >90% estimated true positive probability, and remaining novels were annotated, where possible, with BLAST against all known teleost miRNAs in the miRBase2.0 database. Unique and shared known and novel miRNAs between low-quality and high-quality males were also identified. miRNAs were categorized as expressed in a male if they had > 1 count per million (CPM) to normalize reads to the library size ($CPM = \frac{\#\ of\ reads\ mapped\ to\ miRNA}{Total\ number\ of\ reads} * 1 \times 10^6$).

3.3.6.5 Differential expression analysis

Differential expression of miRNAs was conducted using the DESeq2 (Love et al., 2014) package from R-Bioconductor 3.21. Genes with fewer than 50 reads were not analyzed for differential expression to reduce noise. Statistically significant differentially expressed genes (DEGs) were characterized by an adjusted $P < 0.05$.

3.4 Results

3.4.1 Sperm kinematics

Analysis of CASA results from the forty-two males revealed an average motility of 51.18, 40.97, and 37.22% at 10-, 20- and 30 s post activation respectively. The average velocity of those males was 163.25-, 117.37-, and 78.19 μm/s at 10-, 20-, and 30 s post-activation. As the same trends were seen across the three timepoints, further analysis was conducted on six low-quality and six high-quality male CASA results from 10 s.

The average motility (± SD) of the low-quality group was 20.21 ± 6.28%, compared to 81.99 ± 3.38% for the high-quality males (P <0.05, Fig.1A). Similarly, the average progressive motility (± SD) was 15 .08 ± 5.61% for the low-quality males, whereas the high-quality males averaged 76.56 ± 3.56% (P <0.05, Fig.1B). The average velocity (± SD) of the low-quality group was 117.37 ± 24.96 μm/s, compared to 184.72 ± 6.64 μm/s for the high-quality males (P < 0.05, Fig. 1C). The same trend was seen in progressive velocity (± SD), where low-quality males averaged 136.14 ± 22.83 μm/s, and high-quality males averaged 193.03 ± 4.68 μm/s (P < 0.05, Fig. 1D). A Z-score was calculated for each parameter for every male and then added for one combined Z-score. The low-quality group had an average (±SD) score of -9.07 ± 1.68, and the high-quality group score averaged 6.49 ± 0.71 (P <0.05, Fig. 1EF).

The predicted hatch for low quality males ranged from 31.88 to 60.41 % with an average (± SD) of 45.65 ± 9.48 % (Table 1). For high-quality males the predicted hatch ranged from 67.77 to 75.09 % and averaged 71.24 ± 2.52 % (Table 1).

3.4.2 miRNA analysis

For the twelve males sequencing libraries, there were an average of 7,484,191 reads after trimming (Table 2). An average of 1,658,566 of the clean reads were mapped to known mature catfish miRNAs in miRBase2.0 for each male, and an average of 130,604 reads mapped to predicted mature novel miRNAs (Table 2). There were 102 total predicted novel miRNAs, and 47 were annotated using existing miRNA sequences with a BLAST alignment score >30 and percent identity >94%. Annotated miRNA families included let-7, miR-29, miR-30, miR-19 and miR-143. Many of these miRNAs have previously been linked to spermatogenesis, testis maturation, germ cell differentiation, and sperm motility

There were 246 known and novel miRNAs found to be shared between groups, 6 known and novel miRNAs unique to low-quality males, and 11 known and novel miRNAs unique to high-quality males (Table 3, Fig. 1G). Differential expression analysis of miRNAs between low- and high-quality males revealed three significant genes, *ipu-miR-19a*, *ipu-miR-126a*, and *ipu-miR-143* (*P*adj < 0.05, Fig. 1H). A heatmap was used to visualize the expression of each DEG for each male (Fig. 1I).

3.5 Discussion

Circulating miRNAs found in serum have shown high potential for use as diagnostic biomarkers (Cito et al., 2020b). Our results indicate that there is differential expression of 3 c-miRNAs between male blue catfish with low-quality sperm compared to males with high-quality sperm. These c-miRNAs constitute potential biomarkers for blue catfish gametes that can be

sampled in a non-lethal manner. If applied in aquaculture, the ability to evaluate male reproductive potential, without sacrificing the fish, can increase efficiency and decrease costs for hatcheries.

Previous studies on c-miRNAs, from the blood plasma or serum of freshwater and marine teleost fish, have identified differential expression related to temperature, environmental stress, immune response, and sex determination (Cao et al., 2023; Houdelet et al., 2023). In Atlantic cod (*Gadus* morhua *L*.), the expression of inflammation related *miR-21*, and stress related *miR-155*, were both elevated in fish reared at 9°C compared to those reared at 4°C (Magnadóttir et al., 2020). However, growth-related *miR-206* expression was higher in the 4°C rearing group. Analysis of serum exosomes in tongue sole (*Cynoglossus semilaevis*) found 26 miRNAs enriched that were related to innate immunity, *miR-133-3p* specifically was reported to increase the expression of pro-inflammatory cytokines TNF-α, IL-6, and IL-8 (Sun et al., 2022). In many fish species and studies, c-miRNAs have been identified in serum and plasma that can distinguish a male from a pseudo male or a female at the juvenile stage, where there are often no differences in appearance (Deng et al., 2024; Geffroy et al., 2025; Sun et al., 2017). There are, however, very limited studies that have investigated c-miRNAs in relation to gamete quality.

For female fish, blood plasma analyses conducted on rainbow trout (*Oncorhynchus mykiss*) have revealed that the miRNAome changes due to metabolic and reproductive state (Cardona et al., 2021). For reproductive success specifically, *miR-202-5p* was identified as a candidate biomarker to predict egg quality and ovulation (Cardona et al., 2021). For male fish however, c-miRNA expression in relation to sperm quality has not previously been investigated. Therefore, our findings of differentially expressed blood c-miRNAs between groups with contrasting sperm function serve as a novel method of broodstock evaluation.

We identified the overexpression of *miR-143*, *miR-126a*, and *miR-19a* in the blood of blue catfish males with low-quality sperm, when compared to males with high-quality sperm. Previous research in humans has identified that *miR-143* promotes cell senescence, limits Sertoli cell proliferation, and causes dysfunction of the blood-testis barrier (Xiao et al., 2024). Specifically, *miR-143-3p* regulates testicular function and fertility. When endogenous *miR-143-3p* is diminished or negatively regulated, the aging of testis is postponed and germ cells are seen in higher numbers (Liang et al., 2024). *miR-143-3p* has also been linked to the regulation of sperm motility and function in boar, where its suppression is shown to increase gamete quality (Ding et al., 2022). The link between sperm performance and *miR-143* marks it as a potential candidate biomarker in humans and boar. Our results show that it may also be a diagnostic marker for blue catfish, as we identified poor sperm performance in males with high expression of *miR-143*.

miR-126 is one of the most studied miRNAs in humans and across vertebrate taxa. Extensive research in humans has demonstrated the role of *miR-126* in vascular disorders and cancer, as well as highlighted its role in angiogenesis, immune response, inflammation, and tumor growth (Guo et al., 2025). The role of *miR-126* in spermatogenesis has also been previously demonstrated in porcine cells, where it has been shown to have a regulatory role targeting pathways related to Sertoli cell proliferation (Tang et al., 2021). In zebrafish, *miR-126a* has been identified as a regulator of vascular integrity and angiogenesis as it modulates the formation of lymphangioblasts and the signaling of chemokine chemoattractant activity (Chen et al., 2016). Vascular integrity and the vascular endothelial growth factor (VEGF) have been shown to increase sperm kinematics when present in seminal plasma (Iyibozkurt et al., 2009). The VEGF pathway and angiogenesis are also crucial to the formation of microvasculature to

support spermatogenesis. The upregulation we found in low-quality males for *miR-126a* suggests that it is inhibiting processes related to sperm development and function, which makes this a c-miRNA of interest for determining male quality.

The third differentially expressed miRNA, *miR-19a*, has previously been associated with oligoasthenozoospermia and male infertility in human fertility research (Abu-Halima et al., 2022). Oligoasthenozoospermia is a disorder characterized by low sperm count (oligospermia) and low sperm motility (asthenozoospermia). Upregulation of *miR-19a* and the downregulation of corresponding target genes that are needed for normal spermatogenesis are, therefore, subjects for potential biomarkers of male infertility (Abu-Halima et al., 2022). In zebrafish, *miR-19a* has also been found to respond to environmental stressors and function in immune response by regulating *socs3b* and the *IL-6/STAT3* signaling pathway (Zhao et al., 2022). While further studies are needed to understand the role of *miR-19a* in teleost spermatogenesis, it's links to immune-related gene expression regulation and human spermatogenesis show that it may have an important role in deciding sperm performance. Upregulation of *miR-19a* in response to immune stress may be linked to the stressed fish producing poor gametes. There may also be genes in fish species crucial for spermatogenesis that are regulated by *miR-19a*. As such, *miR-19a* is a potential candidate diagnostic biomarker of sperm performance in blue catfish.

We analyzed c-miRNAs linked to sperm motility and velocity in teleost fish for the first time. Our results confirmed that there is potential for non-lethal sampling for novel diagnostic biomarkers for sperm quality of blue catfish. While this study identified three c-miRNAs that have previously been studied in human fertility research, as well as been found in other fish species, their confidence could be increased by further studies. This initial study utilized a small

sample size of fish from the same genetic line. Further research on larger populations of multiple breeding lines can confirm the predictive ability of the c-miRNA biomarkers.

3.6 Conclusion

Circulating miRNAs *miR-143*, *miR-19a*, and *miR-126a* are promising non-invasive biomarkers of blue catfish sperm kinematics. The over expression of these miRNAs in the blood appears to be linked to decreased cell motility and velocity. To further validate this finding, larger studies that include more samples and multiple populations should be conducted to confirm our evaluations applicability across the species.

Conflicts of interest

The authors declare no conflicts of interest

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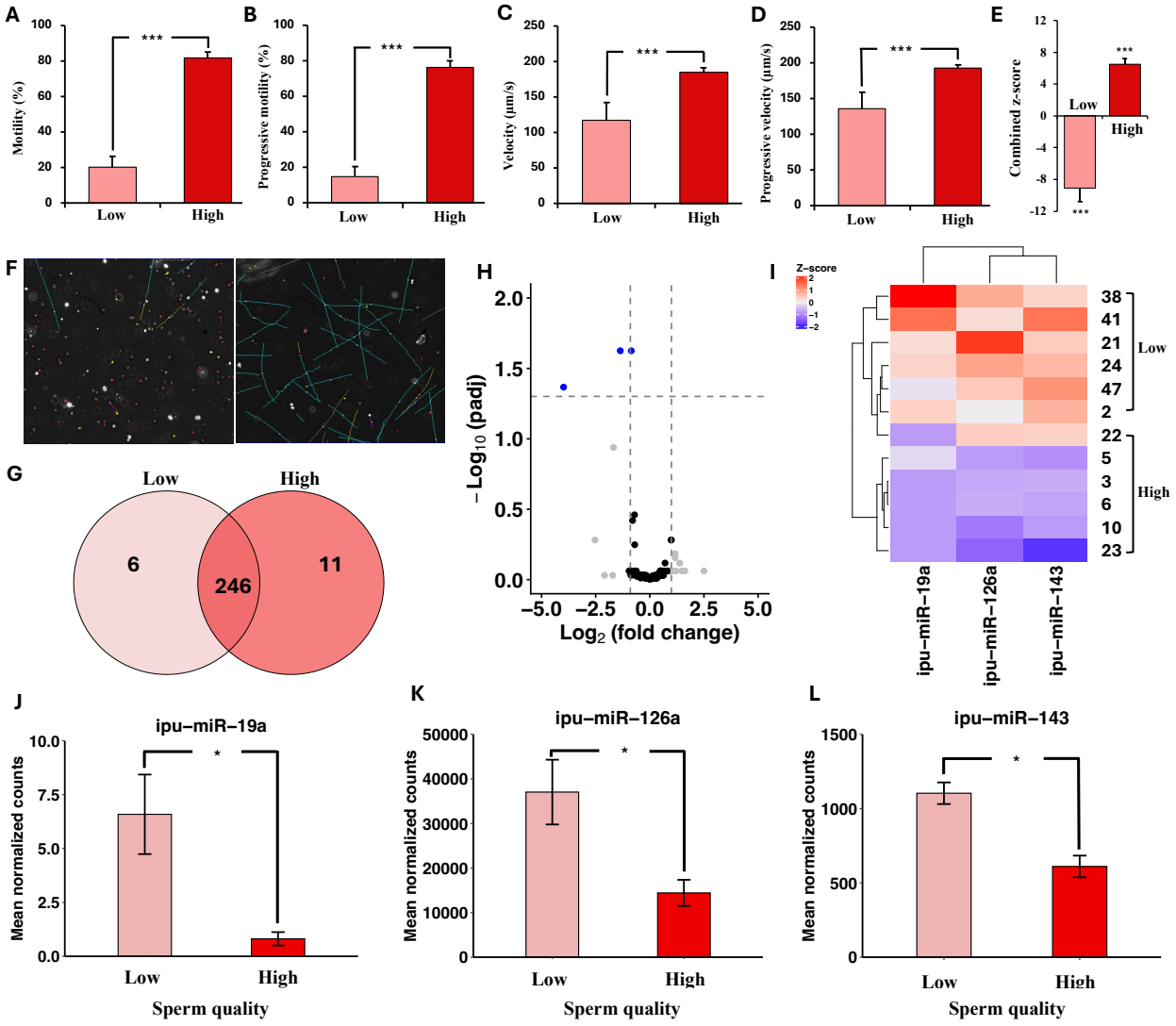

Figure 1. Sperm kinematics assessed at 10 s post-activation and corresponding blood serum c-miRNA expression analysis for six blue catfish (*Ictalurus furcatus*) with low-quality sperm and six with high-quality sperm. A) Percent of motile cells calculated as the mean percent of cells with an average path velocity (VAP) higher than 20 μm/s in low- and high-quality males. B) Percent of motile cells calculated as the mean percent of cells with a VAP higher than 45 μm/s in low- and high-quality males. C) Mean curvilinear velocity (VCL μm/s) of low- and high-quality males. D) Mean VCL of cells with a VAP higher than 45 μm/s for low- and high-quality males. E) Sum of the z-score ($Z = (x - \mu)/\sigma$) for motility, progressive motility, velocity, and

progressive velocity, of low- and high-quality males. F) Computer assisted sperm analysis recording for (left) a male with low-quality sperm 10 s post-activation, and (right) a male with high-quality sperm 10 s post-activation. Green lines show motile cells, blue lines, show progressive motile cells, red shows static cells. G) Venn-diagram of unique and shared known and novel miRNAs found in low- and high-quality males. H). Volcano plot visualizing differential miRNA expression between low- and high-quality males. Points in blue represent significant genes (Padj < 0.05). I) Heatmap visualizing the expression for each male of significantly differentially expressed genes (DEGs)(Padj < 0.05). J) Mean count of ipu-miR-19a reads normalized in DeSeq2 for low- and high-quality males. K) Mean count of ipu-miR-126a reads normalized in DeSeq2 for low- and high-quality males. L) Mean count of ipu-miR-143 reads normalized in DeSeq2 for low- and high-quality males. Error bars represent standard deviation, P <0.05 is *, P <0.001 is ***.

Table 1. Predicted hybrid catfish hatch success for low-quality and high-quality blue catfish (*Ictalurus furcatus*) males calculated using linear regression function established by (Wood et al., 2025).

Group	VCL (µm/s)	Predicted Hatch ~ VCL (µm/s)
Low	81.12	31.88
	114.24	44.46
	113.91	44.33
	130.82	50.76
	156.21	60.41
	107.89	42.05
Average	117.37	45.65
High	182.68	70.47
	194.83	75.09
	181.13	69.88
	188.88	72.82
	185.22	71.44
	175.58	67.77
Average	184.72	71.24
	Equation	y=0.38x+1.05

Table 2. Circulating serum miRNA sequencing and mapping statistics for six high-quality and six low-quality male blue catfish (*Ictalurus furcatus*).

High	Clean reads	Mapped to known	Mapped to novel	Low	Clean reads	Mapped to known	Mapped to novel
	8,377,990	1,270,883	99,316		7,865,657	1,826,093	101,283
	6,831,287	1,941,062	121,666		7,758,588	952,127	74,884
	6,891,270	1,673,809	90,503		7,145,599	2,120,198	122,339
	6,725,834	782,892	46,137		7,519,112	2,424,099	406,239
	6,716,258	1,938,025	153,349		7,912,029	1,815,871	138,119
	6,154,192	1,207,390	95,620		9,912,479	1,950,340	117,794
Total	41,696,831	8,814,061	606,591	**Total**	48,113,464	11,088,728	960,658
Average	6,949,472	1,469,010	101,099	**Average**	8,018,911	1,848,121	160,110

Table 3. Gene IDs of unique known and novel circulating serum miRNAs for blue catfish (*Ictalurus furcatus*) with low-quality and high-quality sperm kinematics. The number of males with greater than 1 count per million.

Unique gene ID	Group	# of males
NC_071275.1_26857	low-quality	1
miR-17a	low-quality	1
miR-190a	low-quality	1
miR-19a	low-quality	2
miR-7559	low-quality	1
miR-7563b	low-quality	1
NC_071261.1_9774	high-quality	1
NC_071266.1_16918	high-quality	4
NC_071267.1_17396	high-quality	1
miR-138	high-quality	3
miR-182	high-quality	1
miR-29c	high-quality	3
miR-728	high-quality	1
miR-7555	high-quality	4
miR-7563a	high-quality	1
miR-7566	high-quality	1
miR-7569	high-quality	1