Identification of Molecular Biomarkers for Male Reproductive Success in Blue Catfish (*Ictalurus furcatus)*

by

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Abstract

The overall objective of this project was to understand the underlying physiological and molecular processes that are associated with sperm production and function. The primary objective was to identify RNA biomarkers in the blue catfish transcriptome associated with sperm quality that may ultimately lead to increased production efficiency for hybrid catfish. In the second chapter, mature blue catfish males (n = 5) were evaluated resulting in 174 known mature miRNAs detected in both testes and sperm. All known miRNAs from sperm were detected in testes, and 5 novel miRNAs were unique to sperm, therefore showing low tissue specificity. sRNAs with differential expression were assessed, revealing that testes and sperm exhibit different expression patterns. Sperm expressed more piwi-interacting RNA (piRNA, 33bp), whereas testes expressed more miRNA (22bp). KEGG enrichment for testes and testicular sperm showed that sRNAs were primarily involved in regulating genes involved with metabolism and endocytosis. In the third chapter, 43 males were assessed for sperm velocity, a determinate of fertilization success. From these 43 males, testis tissue from 4 individuals with low velocity and 4 with high velocity were sequenced for sRNAs and mRNAs. No differentially expressed sRNAs were detected between the two performance groups, suggesting regulatory roles of sRNAs are not directly related to sperm quality in *I. furcatus*. However, we identified two differentially expressed mRNA transcripts between low and high velocity groups, *aqp3a* and *ly97.3*, both upregulated in the high velocity group compared to the low velocity group. Utilizing these biomarkers in the future will include laparoscopic scopes and minimally invasive surgery to screen testis tissue for *ly97.3* and *aqp3a* expression levels in live blue catfish to predict their reproductive quality non-lethally.

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List of Abbreviations

US	United States of America	RIN	RNA integrity number
USD	US dollars	PCA	Principal component analysis
RAS	Recirculating aquaculture system	KEGG	Kyoto Encyclopedia of Genes and Genomes
IVF	*In-vitro* fertilization		
RNA	Ribonucleic acid	GO	Gene Ontology
DNA	Deoxyribonucleic acid	TPM	Transcripts per million
mRNA	Messenger RNA	FDR	False discovery rate
ncRNA	Non-coding RNA	T	Testosterone
miRNA	MicroRNA	11-KT	11-ketotestoserone
sRNA	Short RNA	CASA	Computer assisted sperm analysis
piRNA	Piwi-interacting RNA		
snRNA	Small nuclear RNA	SMI	Spermatogenic maturity index
snoRNA	Small nucleolar RNA	CV	Coefficient of variation
HBSS	Hank's Balanced Salt Solution	bp	Base pairs
RPM	Revolutions per minute	ATP	Adenosine triphosphate

Chapter 1

Introduction

1.1 Aquaculture

Aquaculture is the cultivation of aquatic organisms including finfish, mollusks, or aquatic plants. Global demand for aquatic products is rising, placing a higher demand on farming (Gephart et al., 2020). As of 2018, global aquaculture production is valued at 250 billion USD and supplies 20.5 kg of product for human consumption per capita (FAO, 2020). Over half of the fish that is consumed in the world is supplied by aquaculture production, and an additional 22 million tons of cultivated organisms are produced for non-food uses such as fish oil and fishmeal (FAO, 2020).

Compared to terrestrial food production, aquaculture is a new rapidly developing sector, and this opens up opportunities to create sustainability while still providing food products (Waite et al., 2014; Thomas et al., 2021). Though the United States (US) contribution to global production is minor (ranked 17th globally), there is opportunity to grow the industry due to continued innovations in recirculating systems (RAS), aquaponics, and other aquaculture technologies (National Marine Fisheries Service, 2021). Application of these new technologies such as assisted reproduction, improvement of feeds and feeding technologies, enhancement of production systems, disease management, and genetically improved strains have been shown to trigger development in shrimp, salmon, and tilapia industries (Kumar and Engle, 2016). Continued application of these techniques and technologies into other species, such as catfish would be especially advantageous as catfish make up the majority of US food fish sales (USDA NASS, 2022).

1.2 Catfish Aquaculture

Catfish are extremely important for the US aquaculture sector, where farms in Mississippi, Arkansas, Alabama, and Texas account for 70% of total freshwater aquaculture production (Torrans and Ott, 2018). Within these states, the channel catfish (*Ictalurus puncatatus*) ♀ × blue catfish (*I. furcatus*) ♂ hybrid constitutes over half of the harvest (Torrans and Ott, 2018). Compared to channel catfish, these hybrid catfish have vastly increased production rates of up to 13,000 kg/ha (Brown et al., 2011; Bott et al., 2015), with some farmers even approaching commercial production rates of up to 20,000 kg/ha using specialized intensive pond-based production systems (Kumar et al., 2018). Additionally, when compared to channel catfish, they are superior for pond culture due to improved disease resistance (Dunham et al., 2008; Arias et al., 2012), low oxygen tolerance (Dunham et al., 1983), improved growth and feed conversion rates (Brown et al., 2011; Dunham et al., 2008), carcass yield (Bosworth et al., 2004; Bosworth, 2012), and harvestability (Dunham and Masser, 2012).

Fig 1- Specialized pond setup – 6 unit raceway (Kumar et al, 2018); Image Source: Travis Brown

The hybrid catfish industry has sustained growth due to the positive attributes of hybrids however, there are still challenges, especially considering issue related to reproduction. Channel × blue catfish hybrids are usually the result of *in vitro* fertilization (IVF), as the parent species

rarely hybridize naturally (Dunham et al., 1999). This adds to complexity of spawning when compared to channel catfish that spawn in ponds with less human intervention (Dunham and Masser, 2012). Additionally, there is a disconnect between males and females in time to maturity. Channel catfish females reach sexual maturity earlier at 3 years of age (Dunham et al., 1999) compared to blue catfish males that reach maturity after 4 to 7 years (Graham, 1999). Furthermore, the male's sperm cannot be expressed by stripping and as a result sperm collection is a lethal process that requires the dissection, removal, and maceration of the testes to release gametes (Bart and Dunham, 1990). These factors compound such that there is a high initial investment to acquire sperm, especially when compared to channel catfish females that can be readily hand stripped (Dunham et al., 1999).

All of these above paternal complications suggest that sperm production is a major bottleneck for the hybrid catfish industry. To address this issue, sperm cryopreservation protocols have been developed at a commercial scale, allowing sperm to be available when females are in peak spawning condition (Bart et al., 1998; Hu et al., 2011, 2014). Unfortunately, cryogenic technology has yet to be widely adopted by the industry. Additionally, as is common among many aquatic species there has been a high degree of variability reported in the quality of sperm between males that ultimately impacts offspring performance as well as efficiency of producing embryos (Bobe and Labbé, 2010). Our current limited understanding of underlying mechanisms involved in sperm function is the main reason for lack of progress. Therefore, it is important to focus our efforts in this area and increase understanding of physiological and molecular processes that are associated with male gamete production and function.

1.3 RNA Biomarkers

RNA transcripts can be described as snapshots as they capture what is occurring in cells at a specific period in time, and therefore can be reliable, efficient biomarkers (Qian et al., 2014; Herkenhoff et al., 2018). The complete set of RNA transcripts, otherwise known as a transcriptome, is made up of two categories of RNA, messenger RNA (mRNA) and non-coding RNA or ncRNA (Okazaki et al., 2002; Lindberg and Lundeberg, 2010). The distinction between the two is that mRNA are translated into proteins, whereas ncRNAs are not (Hubé and Francastel, 2018). However, this does not mean ncRNAs are unimportant; for instance, ncRNAs are involved in post-translational regulation of genes associated with spermatogenesis and oogenesis (Robles et al., 2019). One subset of ncRNA are microRNAs (miRNAs), they are short ncRNAs typically around 21 base pairs, and play key roles in regulation of gene expression networks (Bizuayehu and Babiak, 2014; O'Brien et al., 2018). Studies of ncRNA in fish show tremendous potential to be used as biomarkers as they currently are used in mammals (Bizuayehu and Babiak, 2014). As for mRNAs, though it is known that sperm are transcriptionally inactive, studies in mammals have shown that residual mRNAs from spermatogenesis have roles in early embryonic development and ultimately impact fertility (Ostermeier et al., 2002; Lalancette et al., 2008; García-Herrero et al., 2011; Johnson et al., 2011). mRNAs have also been successfully investigated for use as biomarkers for reproductive quality in aquaculture species (Sullivan et al., 2015; Myers et al., 2020).

Because variation in sperm quality leads to high variability in reproductive success and latent effects in larvae of fish aquaculture species (Bobe and Labbé, 2010), investigating molecular biomarkers associated with sperm quality are urgently needed to optimize efficiency in

aquaculture (Herráez et al., 2017). As hybrid catfish are economically important for aquaculture in the US, it is important to continue to investigate best practices for their production.

1.4 Objectives

The overall goal of this research was to further understand the underlying physiological and molecular processes that are associated with sperm production and function. The primary objective was to identify RNA biomarkers in the blue catfish transcriptome that may ultimately lead to increased production efficiency for hybrid catfish embryos. Specifically, the research focuses on ncRNAs and mRNAs leading to an assessment of the blue catfish transcriptome including coding and noncoding RNAs. Important pathways and cellular functions were identified as well to add to the knowledge of molecular processes.

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Chapter 2

Identification and differential expression of small non-coding RNAs in testes and extracted testicular sperm of blue catfish, *Ictalurus furcatus*

2.1 Abstract

Catfish farming accounts for ~70% of US freshwater aquaculture production, where the channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus* ♂ hybrid constitutes >50% of harvest. Current technologies to produce hybrid embryos are labor intensive and require sacrifice of males that mature after 4-7 years. Catfish sperm are often of inadequate quality/quantity and do not necessarily give high fertility. MicroRNAs (miRNAs) are small non-coding RNAs (sRNAs) that regulate mRNAs at the post-transcriptional level, influencing almost all biological processes. Our objectives were to identify miRNAs, decipher differences in sRNAs from testes and extracted testicular sperm, compare differential expression of testes sRNAs between males, describe sequence length differences in sRNAs between testes and testicular sperm, and describe biological pathways for mapped miRNAs. Using mature blue catfish males [n = 5; 2.67 ± 0.72 kg (mean ± SD)], 174 known mature miRNAs were detected in both testes and sperm. All known miRNAs from sperm were detected in testes, and 5 novel miRNAs were unique to sperm, thus showing low tissue specificity. sRNAs with differential expression were clustered using DESeq2, revealing that testes and sperm exhibit different expression patterns. Sperm expressed more piwi-interacting RNA (piRNA, 33bp), whereas testes expressed more miRNA (22bp). KEGG enrichment for testes and testicular sperm showed that sRNAs were primarily involved in regulating genes involved with metabolism and endocytosis. This study describes sRNA profiles in *I. furcatus* male gonadal tissue and gametes, including core miRNAs that should be further explored as biomarkers.

2.2 Introduction

Sustainable aquaculture is a rapidly developing field, as it opens opportunities to limit land-use while providing a high-quality protein source (Waite et al., 2014; Thomas et al., 2021). Aquaculture has a global influence on public and environmental health, and therefore, pressure exists to develop efficient practices, beginning with reproduction of key aquaculture species (Boyd et al., 2020). Identifying biomarkers is advantageous for cultured fish with production gaps and inefficiencies, such as with channel catfish, *Ictalurus punctatus* × blue catfish, *I. furcatus* hybrids, the dominant catfish in aquaculture in the United States which currently constitutes >50% of total catfish production (Dunham and Masser, 2012; Dunham and Elaswad, 2018; Torrans and Ott, 2019). Production rates for hybrids (13,000 kg/ha) are routinely double that of channel catfish, with some even approaching 20,000 kg/ha using specialized intensive pond-based production (Brown et al., 2011; Bott et al., 2015; Kumar et al., 2018). Although profitable, the hybrid catfish industry suffers from a reproductive bottleneck because channel catfish and blue catfish do not naturally spawn together. As such, most hybrids are produced by in vitro fertilization (Dunham and Elaswad, 2018). Adding to the reproductive complications, males must be sacrificed for this procedure (Dunham, 1993). Therefore, two major concerns surrounding hybrid catfish hatchery production are the lack of natural spawning and the investment in males to obtain milt (Myers et al., 2020). Another impediment is tremendous variability in blue catfish testicular development, and thus, sperm production. Hence, a thorough understanding of the physiological and molecular mechanisms regulating testicular development and sperm quantity/quality may help explain some of the inherent male-to-male variability.

Gene regulatory networks that control physiological processes associated with reproduction are highly regulated by small noncoding RNAs (sRNAs), such as microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) (Govindaraju et al., 2012; Capra et al., 2017; Herráez et al., 2017; Watson et al., 2019). sRNAs are being used as biomarkers for a variety of applications, but currently, piRNA and miRNA are undergoing closer investigation for their applications in teleost fish (Zhang et al., 2020; Bhat et al., 2021). piRNAs and miRNAs both act in post-transcriptional gene silencing (MacFarlane and Murphy, 2010; Iwasaki et al., 2015). piRNAs are about 24 to 31 bp (base pairs) in length and have been shown to regulate gene silencing of retrotransposons in germ line cells of tongue sole, *Cynoglossus semilaevis* (Iwasaki et al., 2015; Zhang et al., 2020), where miRNAs are approximately ~22 bp and are well known post-transcriptional regulators of mRNAs in teleost fish (Bizuayehu and Babiak, 2014). While heavily involved in modulating gene regulation in mammals, many miRNAs are evolutionarily conserved within teleost fish lineages and have conserved functions (Desvignes et al., 2021). With an increasing amount of knowledge surrounding their impacts on reproductive pathways, studying miRNA profiles has become highly relevant for animal agriculture (Fatima and Morris, 2013; Gualtieri et al., 2020). There are a growing number of studies linking miRNAs to reproductive pathways in terrestrial animal species, such as bovine (Silva et al., 2019), boar (Dai et al., 2019), and chicken (Liu et al., 2018). These studies on miRNA have targeted processes that affect gametogenesis (Silva et al., 2019), gamete quality (Liu et al., 2018), and embryonic survival (Dai et al., 2019). In turn, miRNA expression in gametes have been linked to higher efficiency for in vitro fertility and offspring production (Silva et al., 2019; Salilew-Wondim et al., 2020).

Specific miRNA families such as miR-34a, let-7, miR-125, and miR-200 have been characterized in zebrafish, *Danio rerio* (Guo et al., 2017; Presslauer et al., 2017; Xiong et al., 2018). For example, the miRNA family (miR-34), despite the exact function being unknown, has been directly linked to sperm motility rates (Guo et al., 2017). Male zebrafish labeled "bad breeders" with poor sperm performance (motility, concentration, etc.) are characterized by the presence and expression levels of specific miRNA families such as miR-8 and miR-122 (Riesco et al., 2019b). These "bad breeders" also produced offspring with more developmental malformations. In addition, there has been work linking gonadal development in zebrafish to changes in abundance of miRNAs throughout sexual development (Presslauer et al., 2017). It is likely that results derived from zebrafish may also be expanded to include species of economic and ecological importance, as documented for salmonid *spp*. and Nile tilapia*, Oreochromis niloticus* (reviewed in Bhat et al., 2021).

In teleost fish, assessment of miRNAs in gamete tissue has highlighted their roles in regulating genes related to reproductive processes and as biomarkers of breeder quality (Bizuayehu et al., 2012; reviewed in Bhat et al., 2021). piRNAs are currently being used as a biomarker of breeding quality in bovine (Capra et al., 2017), which could be further expanded to fish as is currently being explored in tongue sole (*Cynoglossus semilaevis*) (Zhang et al., 2020). Identifying such biomarkers could be advantageous to aquaculture producers because sperm quality can be highly variable among individuals within species in the wild as well as under culture conditions (Robles al., 2019). This male-to-male variability can be caused by various intrinsic factors such as DNA, RNA, and epigenetic status and/or broodstock rearing conditions, ultimately impacting sperm performance during fertilization (Bobe and Labbé, 2010; Robles et al., 2017). Even with sperm exhibiting normal motility and morphology, fertility can still be low due to molecular defects like poor

plasma membrane integrity, or high levels of oxidative stress that are not apparent without complex analytical techniques (Cabrita et al., 2014). Sperm quality variation can cause major economic losses through low offspring production, especially when milt from selectively bred individuals must be discarded (Dunham and Elaswad, 2018; Myers et al., 2020). sRNAs are highly tissue specific and likely vary between gametes and gonadal tissue, although they have not been sufficiently characterized in important teleost aquaculture species. Thus, using sRNAs as indicators of reproductive potential and gamete quality could have huge ramifications for aquaculture.

The objectives of this study were to i) identify miRNAs in blue catfish male gonadal tissues; ii) decipher differences in sRNA from testes and extracted testicular sperm; iii) compare differential expression of testes sRNA between males; iv) describe sequence length differences in sRNA between testes and testicular sperm; and v) describe relevant biological pathways for mapped miRNA. Using molecular markers may improve selective breeding practices and produce better fish for hatchery production.

2.3. Materials and methods

2.3.1 Animal care and tissue sampling techniques

The fish used in this study, mature blue catfish males, were reared in 0.25-1.0 ha earthen ponds at the Auburn University E.W. Shell Fisheries Center (32.6524° N, 85.4860° W). Fish were fed a commercial pelleted catfish feed (Fish Belt Feed Mill, Moorhead, MS) daily until satiation. Selected males (n = 5) were euthanized by blunt force trauma to the head followed by pithing, in

conjunction with Institutional Animal Care and Use Committee specifications. Morphometric data was then collected for each male, where the mean weight (± SD) was 2.67 ± 0.72 kg and mean length (± SD) was 61.60 ± 4.50 cm. We aimed to assay gamete quality amongst the selected males by assaying sex steroid hormone levels, histology, sperm kinematics, sperm cell health, and initial sperm cell density. These specific metrics were chosen to obtain a broad overview of male and gamete quality from many different factors and to assess each individual male's reproductive quality.

To assay hormone levels, blood was drawn from the caudal vein and stored at 4°C for 12 to 24 h to allow red blood cells, white blood cells, and thrombocytes to separate from plasma. Plasma was then removed and centrifuged at 4ºC for 10 min at 4,000 RPM to further remove any red blood cells and white blood cells. Plasma was then stored at -20°C until further hormonal analysis could be completed (see *Section 2.3.3*).

For histology of testes and sRNA analysis, testes were dissected from each male and washed with HBSS (8 g/L NaCl, 0.4 g/L KCl, 0.16 g/L $CaCl_2 \times H_2O$, 0.2 g/L $MgSO_4 \times 7H_2O$, 0.12 g/L $Na_2HPO_4 \times 7H_2O$, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, 1 g/L glucose, pH 7.42, osmolality 305 mOsm/kg) (Tiersch et al., 1994). Approximately 2 g of testis from each male was randomly selected for histological analyses and preserved in 10% phosphate buffered formalin for a period of 12 weeks (see *Section 2.3.3*). To characterize the sRNAs in the testes, 0.5 g of tissue was collected in triplicate from each male and snap frozen in liquid nitrogen before being stored at -80°C (see *Section 2.3.2*). All remaining testes tissue was used to extract sperm cells for sperm quality analyses (see *Section 2.3.3*) and sRNA assessment. To further clean the testes tissue and prevent other tissue contamination during sperm extraction, blood vessels and peritoneum were manually dissected from remaining testes tissues. These cleaned testes tissues were once again

washed with HBSS (4ºC) before being macerated and strained through 200 µm mesh to release the milt. Extracted sperm cells were centrifuged at 4ºC for 10 min at 7,500 RPM. The supernatant was discarded, and sperm cells were snap frozen in liquid nitrogen and stored at -80°C for sRNA assessment.

2.3.2 sRNA sequencing and analysis

After being snap frozen, testes and aliquots of sperm were stored at -80°C until they were packed with dry ice and shipped to Novogene (Novogene Corporation, Durham, NC, USA). After extracting RNA from the shipped samples via an E.Z.N.A.® Total RNA Kit II (Omega Bio-Tek, Inc., Norcross, GA, USA), they were checked for quality. Two individual males with RNA integrity numbers (RIN) of 7.1 (Male 1) and 7.2 (Male 2) for sperm cells and 8.7 (Male 1) and 8.8 (Male 2) for testes tissue were used for individual comparisons of sperm and testes tissue. Males 3-5 initially had RIN values of 5.3, 6.8, and 6.9 for sperm RNA and were not used individually but were pooled in triplicate, in addition to the individual comparison. Similarly, testes samples (N = 5) were also used to generate three pooled samples with a RIN of 7.0 or higher. In the individual testes tissue samples from the five males, RINs ranged from 7.9-9.0. Pooled RNA was treated as 3 technical replicates for the rest of the analysis. RNA libraries were constructed using NEBNext® Multiplex Small RNA Library Prep Set. Once library construction was complete, sequencing was run on an Illumina sequencing instrument (Novoseq 6000, HWI-ST1276, Illumia, San Diego CA, USA). Quality control was performed on the raw reads using proprietary perl and python scripts, and Bowtie was used to map to the reference sequence (Langmead et al., 2009). As there is no published genome for blue catfish, the channel catfish

genome was used as a reference genome (v. IpCoco_1.2) (GenBank accession no. **NC_003489**). miRBase20.0, and a modified version of mirdeep2 was used to tag known miRNAs (Friedländer et al., 2012). Identification of novel miRNA sequences was performed using mirdeep2 software (Friedländer et al., 2012) along with miREvo software (Wen et al., 2012). miRNAs are grouped into families by their seed region, seed region being defined as the 2-7 nt from the 5' end of miRNA, which binds directly with messenger RNA (Olena and Patton, 2010). In our dataset, pre-miRNA hairpins were used to search miRbase's (v. 22.1) miRNA registry service, then categorized by their mature forms (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008; Kozomara and Griffiths-Jones, 2014; Kozomara et al., 2019).

miRNA expression levels were estimated using a transcript per million (TPM) approach with expression fold change for each miRNA calculated as the $\log_2$ ratio of the TPM value (Zhou et al., 2010). Differentially expressed miRNAs were identified using the DESeq2 package (v 1.8.3) in R, comparing between sperm and testes samples for individual samples (e.g., testis from Male 1 vs. sperm from Male 1) or the pooled samples (Love et al., 2014). Significant differentially expressed sRNAs were characterized using a Benjamini and Hochberg corrected *p*-value of 0.05. This was used as the threshold to differentiate for significantly expressed sRNAs and visualized using the pheatmap package in R (Love et al., 2014). A Pearson's correlation matrix and principal component analysis (PCA) was also generated to further quantify correlations in sRNA expression. A KEGG enrichment analysis was performed using the KEGG pathway database as described in (Kanehisa et al., 2008) through the KOBAS pipeline using 3 modules, kparser, blast2ko, and pathfind (Wu et al., 2006). Kparser parses the KO and KEGG gene datasets, Blast2ko annotates the new set of sequences, pathfind then identifies the frequent and enriched pathways within the sequences. The portion of the KOBAS pipeline, pathfind also calculates the false discovery rate

(FDR) using the GeneYS (v. 2.3) package in R (Mao et al., 2005). To compare enrichments between sperm and testes samples, a hypergeometric test and Fisher's exact tests were performed before the FDR correction.

2.3.3 Male reproductive performance indices

For a comprehensive assessment of male reproductive status and gamete quality, each individual was sampled for sex steroid hormones, testes histology, and sperm traits. Specific methods for each procedure are highlighted below.

Sex steroid hormones

Blood plasma samples were stored at -20°C until further hormonal analysis could be completed using testosterone (T, 582701, Cayman Chemical Company, Ann Arbor MI, USA) and 11-ketotestosterone (11-KT, 582751, Cayman Chemical Company, Ann Arbor MI, USA) ELISA kits. Blood plasma was assayed in triplicate for each male with an intra-assay variation of 19.1% for T and 16.8% for 11-KT, and an inter-assay variation of 44.1% and 14.2%, respectively. Relevant cross reactivity for the T assay includes: 19-nortestoserone (140%), 5α-dihydrotestosterone (27.4%), 5β-dihydrotestosterone (18.9%), methyltestosterone (4.7%), androstenedione (3.7%), 11-KT (2.2%), 5-androstenediol (0.51%), epi-testosterone (0.2%), progesterone (0.14%), testosterone enanthate (0.11%), androsterone (0.05%), androsterone sulfate (0.04%), testosterone sulfate (0.03%), DHEA sulfate (0.02%), estradiol (<0.01%), and testosterone glucuronide (<0.01%). Relevant cross reactivity for 11-KT includes: adrenosterone (2.9%), 4-androsten-11β,17β-diol-3-

one (0.01%), 5α-androstan-17β-ol-3-one (<0.01%), 5α-androsten-3β,17β-diol (<0.01%), and T (<0.01%). Readings were taken spectrophotometrically (Thermo Scientific Genesys 10S), where sensitivity (80% B/B_0) was 1.3 pg/mL, in the range of 0.78-1000 pg/mL, using 412 nm absorption for both sex steroid hormones.

Testes histology

Preserved tissue samples were processed at the Auburn University Pathobiology lab. Samples were gradually dehydrated using 70-100% ethanol solutions, before being embedded in paraffin and sectioned to 4-5 μm using a microtome. Staining was performed using hematoxylin and eosin. After slide preparation, digital images were taken using a Zeiss Imager.A2 microscope equipped with an Axio-cam 305 and Zen Pro v. 6.1 software (Zeiss, Oberkochen, Germany). For each male (N = 5), 3 slides were produced where 10 digital images were randomly taken per slide. Testes tissues were organized according to cell types (testicular somatic cells, spermatogonia, spermatocytes, spermatids, and sperm) and relative area fraction (F) was estimated (Tomkiewicz et al., 2011). Excluded areas had no tissue or were lumen. The area fractions of the different tissue types were estimated by placing a point grid (48 points) on the digital images using the ImageJ plugin "Analyze". Progression of spermatogenesis was then assessed using a spermatogenic maturity index (SMI) (Tomkiewicz et al., 2011). This index ranges from 0 for only testicular somatic cells to 1 if all germinal cells have transformed into sperm.

Cell concentration

Sperm from each male (N = 5) were counted under a Zeiss Imager.A2 using a 20× objective and improved Neubauer hemocytometer. Extracted testicular sperm from each male was first diluted 40 to 200-fold, depending on initial sperm densities, in a non-immobilizing medium (HBSS). Samples were homogenized for ~10 s, then 10 µL was pipetted onto the hemocytometer, in duplicate per each male. Sperm inside five 0.2 mm^2 squares were counted. To obtain average sperm concentration, diluted sperm in those five squares were summed and multiplied by 5 to estimate cells in the entire 5 × 5 grid. Sperm concentration in the 5 × 5 grid was then multiplied by 10^4 (total volume overlying the counting area) and then by the dilution factor to determine sperm concentration.

Activity analyses

Sperm activity traits (motility and velocity) were analyzed using Computer Assisted Sperm Analysis (CASA) software (CEROS II software, Hamilton Thorne Biosciences, Beverly MA, USA) according to Myers et al. (2020). In brief, sperm were activated in an 80 µm 2X-Cel chamber (Hamilton Thorne Biosciences, Beverly MA, USA) using distilled water supplemented with 0.5% bovine serum albumin (126609-10GM, Merck Millipore, Burlington MA, USA). We conducted 3 replicate activations per male, where video frames were analyzed at 10 s post-activation.

Viability analyses

Cell viability was assessed by flow cytometry following proprietary protocols in the Muse Count and Viability kit (Luminex, Austin TX, USA). The kit utilizes two fluorescent dyes, in which one is a membrane permeable fluorescent dye that stains all cells with a nucleus and distinguishes cells from debris in the sample. The second fluorescent dye only stains dead or dying cells that have lost membrane integrity, to distinguish dead and dying cells from healthy ones. First, cells were diluted in triplicate with HBSS ranging from 1×10^6 to 1×10^7 cells/mL, then 20 µL of cell suspension was mixed with 380 µL of Count and Viability Reagent in 1.7 mL microtubes. The solution was then incubated for 5 min in the dark at 22°C before being quantified in the Muse Guava Cell analyzer (Luminex, Austin TX, USA).

2.4. Results

To assess male reproductive status and gamete quality we used a series of reproductive indices, and the resulting descriptive statistics for the sex steroid hormones, testes histology (SMI), and sperm traits are shown in Table 2.1. Mean (± SEM) T and 11-KT were 0.917 ± 0.010 and 0.058 ± 0.001 ng/mL, respectively. Testicular development measured via SMI varied between 0.597 to 0.636 among the five males used in this study. Additionally, sperm motility had a mean of 61.06 (± 11.29%), with the velocity of cells averaging 183.99 ± 11.70 µm/s, and a viability assessment of 90.10 (± 1.60%) of the cell's membranes being viable. Sperm density was highly variable (ranged from 1.51×10^9 to 4.13×10^9) among males (CV = 33.1%).

sRNA sequencing yielded an average of 12,510,184 total raw reads across all samples (Table

2.2). Any reads containing poly-N, 5' adapter contamination, poly A or T or G or C, or any low-quality reads were removed. In addition, any reads that did not contain a 3' adapter or insert tag were removed, resulting in an average of 11,334,083 cleaned reads. Using clean reads as the input source, miRNAs were defined as having 21 or 22 bp, siRNA having 24 bp, and piRNA having 28-30 bp; all three groups were classified as sRNAs. Other small noncoding RNAs (i.e., rRNA, tRNA, snRNA, and snoRNA) were removed from the analyses based on their sequence from Rfam database matched reads. Mapping to the channel catfish genome resulted in an average of 5,313,252 mapped sRNAs and a 55.63% mapping efficiency. Additionally, there were 17,823 mapped unique sRNA reads.

In total, there were 174 known mature miRNAs found from the reference genome and 246 mature novel miRNAs predicted (Table 2.2). There were on average (± SD), more mapped mature novel miRNAs in the testes (197 ± 11) than sperm samples (154 ± 8). To briefly highlight, the five families with the most frequent occurrences of miRNA sequence detections include 11 miRNAs from the miR-10 family, 10 from the let-7 family, 6 from the miR-8 family, 5 from the miR-15 family, and 5 from the miR-27 family.

In the testes samples there were 157 ± 4 known mature miRNAs, and the sperm samples contained 137 ± 4. All known miRNAs from sperm samples were also detected in testes samples, and 5 novel predicted miRNAs were unique to the sperm samples (Fig 2.1). Testes samples from the 5 males shared 299 miRNAs (Fig 2.2), while the sperm samples from the 2 males shared 264 miRNAs. When testes and testicular sperm were compared, on an individual level and novel predicted miRNAs were included, there were 132 shared miRNAs in Male 1 and 139 in Male 2 (Fig 2.1b,c).

sRNAs with differential expression were clustered using DESeq2 clustering analysis, revealing

that testes and sperm showed different expression patterns (Fig 2.3). A Pearson's correlation matrix was then generated to further quantify correlations in sRNA expression between samples (Fig 2.4a). Testes samples from Males 1 to 5 had R^2 values ranging from 0.92 to 0.96, while the pooled sperm and testes samples had R^2 values ranging from 0.63 to 0.68. Next, a PCA was performed on all sRNAs, showing similar results, where the testes and sperm samples were distributed into two distinct groups where principal coordinate (PC) 1 explained 84.53% of the variation, PC 2 explained 11.1%, and PC 3 explained 1.93% (Fig 2.4b). Finally, when differentially expressed sRNAs from sperm and testes tissue were compared between Male 1, Male 2, and pooled males, there were 138 differentially expressed sRNAs shared among these three groups. Volcano plots were further used to visualize differential expression patterns between the pooled sperm and testes samples, where there were 105 significantly upregulated sRNAs and 115 significantly downregulated sRNAs (Fig 2.5a). When sRNAs were compared between sperm and testes from 2 individuals (Males 1-2), there were 109 and 108 significantly upregulated sRNAs, with 102 and 119 significantly downregulated sRNAs, respectively (Fig 2.5b, c).

Sequence length distributions of sRNAs in testes and sperm samples were different (Fig 2.6). Generally, sperm samples had shorter sequences, peaking at 22 nt, while testes samples had longer sequences peaking at 32-33 nt. Therefore, testes samples were skewed more towards miRNA, whereas sperm samples were skewed towards piRNA.

A KEGG enrichment was performed on pooled testes and sperm samples showing that detected sRNAs were primarily devoted to metabolism and secondly to endocytosis ($p < 0.05$, Fig. 2.7). In the enrichment, there were 50 significant pathways ($p < 0.05$) identified among pooled testes and sperm samples, as well as from individual testes and sperm samples from Male 1 and Male 2. When comparing pooled and individual samples (from Males 1-2), 15 out of the top 16 significant

pathways were similarly represented.

2.5 Discussion

Male reproductive indices, such as sex steroid hormones, testes histology, and sperm traits can be highly variable among individuals in the wild or in captivity (Butts et al., 2011; Abualreesh et al., 2020). This male-to-male variability is caused by various intrinsic factors and/or broodstock rearing conditions and ultimately impacts fertility and offspring performance (Bobe and Labbé, 2010; Cabrita et al., 2014). Here, we observed a similar phenomenon, where a high degree of male variability was detected for most of the measured reproductive indices. Despite this variation, our reported values fall within, or exceed ranges reported for blue catfish (Bart and Dunham, 1996; Hu et al., 2011; Myers et al., 2020). Therefore, the males used in this study had adequate reproductive quality and could be used for industry production.

Studying gamete RNAs for their potential as biomarkers has huge ramifications for aquaculture, where many species have reproductive bottlenecks that often lead to production inefficiencies, especially under captive settings (Butts et al., 2014; Abualreesh et al., 2020). As more sRNA profiles are investigated, especially miRNAs, they are being used as biomarkers for fertility and other reproductive performance metrics (Bhat et al., 2021). These biomarkers can be especially important for fish that undergo artificial fertilization as their gametes can be processed and then genetically selected to ultimately impact all subsequent stages in ontogeny (Robles et al., 2019). Specifically for blue catfish, a high degree of male-to-male variability in sperm quality has been reported, further justifying the need for selection of males with superior reproductive fitness (Myers et al., 2020). Therefore, in this study we aimed to characterize sRNA profiles of blue catfish

to provide a basis for future studies.

In this study, we identified 174 mapped mature miRNA, which falls within the range of mature miRNA found in teleost fish, ranging anywhere from 37 mature miRNA in Atlantic halibut, *Hippoglossus hippoglossus* to 255 mature miRNA in zebrafish (Bizuayehu and Babiak, 2014). Additionally, there were 17,823 mapped unique sRNAs, which is similar to values found in channel catfish, which yielded 25,538 mapped unique sRNAs (Xu et al., 2013). An important miRNA family that was conserved in all our samples was let-7, as would be expected as this family is found in almost all animals (Lee et al., 2016). In model organisms, such as *C. elegans*, this family has been shown to function as a heterochronic switch gene (Caygill and Johnston, 2008). Heterochronic switch genes regulate timing during the maturation processes, as is the case for Wuchang bream, *Megalobrama amblycephala*, where expression levels of let-7 in the ovary gradually increase with egg maturation, until a sharp decline is observed as eggs are ovulated (Lan et al., 2019). More specifically, *miR-let-7d* from within the let-7 family has also been correlated with spermatogenesis in Senegalese sole, *Solea sengalensis* (Riesco et al., 2019a). In our study, *miR-let-7d* was found in all males with relatively low sequence detection rates (<65 detections per sample), so it is unlikely to be a good candidate biomarker of male quality in blue catfish. The number of *miR-let7a* sequence detections were high among all the samples, which has also been expressed in mature gonads of zebrafish (Zhao et al., 2017). This result coupled with high SMI, sex steroid hormone levels, and sperm motility/velocity found in our selected males suggest that males used in this study were at a later stage in spermatogenesis. Despite this, there is currently not enough evidence to use the let-7 family of miRNAs as biomarkers in blue catfish. Thus, basic research on expression of miRNAs, like *miR-let7a,* during testicular development in blue catfish would help us decipher requirements for the spermatogenic process and may emerge as potential

biomarkers for male fertility.

Two miR-200 family sequences, miR-200b and miR-200c, were detected in all samples, but our samples were lacking miR-200a, as found in zebrafish. In zebrafish, the miR-200 family is involved with the innervation of dopamine producing neurons in the brain and sperm motility (Choi et al., 2008; Xiong et al., 2018). More specifically, dopamine is an antagonist to reproductive hormones and is inhibited with the production of gonadotropin during the spawning season of fish (Peter et al., 1988). In channel catfish females, a dopamine antagonist injection has shown to increase spawning frequency (Silverstein et al., 1999). However, no work has linked dopamine regulation, especially in terms of genetic control to reproduction in blue catfish; thus, this is worthy of future investigation. Additionally, sperm motility is extremely important for fertilization rates, and can be manipulated by knocking out miR-200 genes (miR-200a and miR-200b) on the 23rd chromosome of zebrafish (Xiong et al., 2018). Thus, further study into the miR-200 family would provide insights into the endocrinology of blue catfish and sperm function.

In this study, we deciphered differences in sRNA between testes tissue and sperm cells, where sRNA expression and correlation expression patterns showed a high degree of variability. In zebrafish, miRNA expression is very tissue (Wienholds et al., 2005) and cell (Chen et al., 2005) specific, which coincides with our findings. However, when miRNAs were identified, only 5 novel miRNAs were unique to sperm cells vs. testes tissue, therefore showing low tissue specificity. When testes samples from 5 different males were compared, they shared 299 miRNAs, and when testes and sperm samples from 2 of those 5 males were compared, there were 264 miRNAs in common, meaning that there were many miRNAs in common between and within the males.

In addition to changes in sRNA expression, one of the main differences we observed between sRNAs in testes tissue and sperm cells were differences in the length of RNA sequences, and

therefore the type of sRNA present. Sequence length for the sperm cells was more closely aligned with piRNA, which are longer than miRNAs and have a single stranded precursor (Iwasaki et al., 2015). piRNAs are germ-cell specific RNAs that bind to the piwi clade of proteins involved in target gene regulation, and some piRNAs have been discovered to have inhibitory effects on gene expression (Zhang, 2009). On the other hand, sequence lengths for testes samples were more closely aligned with miRNA (Bizuayehu and Babiak, 2014). Therefore, both sequence length peaks align with the length of sRNAs that influence gene expression, despite falling into two different categories.

The current study also described relevant biological pathways for mapped sRNA. Here, the KEGG enrichment showed that there was significance in the cellular pathways devoted to cell metabolism and endocytosis. Cellular metabolism produces adenosine triphosphate (ATP), which is important for sperm energetics as large stores of ATP in the midpiece are required for sperm to remain motile (reviewed in Kowalski and Cejko, 2019). Endocytic processes are important for future embryo health (Bökel and Brand, 2014) and are related to sperm maturation (Jones et al., 2013). More specifically, endocytosis is a key factor in maintaining surface area of embryos during development (Covian-Nares et al., 2008). Additionally, it plays a role in spermiogenesis acting mainly in Sertoli cells, as demonstrated by Stanley and Lambert (1985). Thus, further analysis of these pathways would lead to a better understanding of how ATP is used within sperm cells, as there are reserved ATP stores at the end of the motility period (Dzyuba et al., 2017).

2.6 Conclusions

This study investigated a suite of characterized and novel sRNAs in testes and sperm for an economically important aquaculture species, the blue catfish, and identified many areas for future study. Several miRNAs exhibit tissue type-specific expression patterns, where some are important for maintenance of the undifferentiated state of spermatogenesis (Riesco et al., 2019b), others for induction of differentiation (Yu et al., 2015), and others have an important regulatory role for sperm function (Guo et al., 2017). Most of these miRNAs and their functions have been identified using zebrafish before being discovered in other fish species. Moving forward these characterized miRNAs can be used in further studies for their role in regulating biological processes relevant for aquaculture sustainability, including reproduction. Overall, our results show that sperm and testes showed different miRNA expression, but with low male to male variability. Thus, future study is needed to determine the use of miRNAs as a viable candidate for biomarkers in catfish reproduction.

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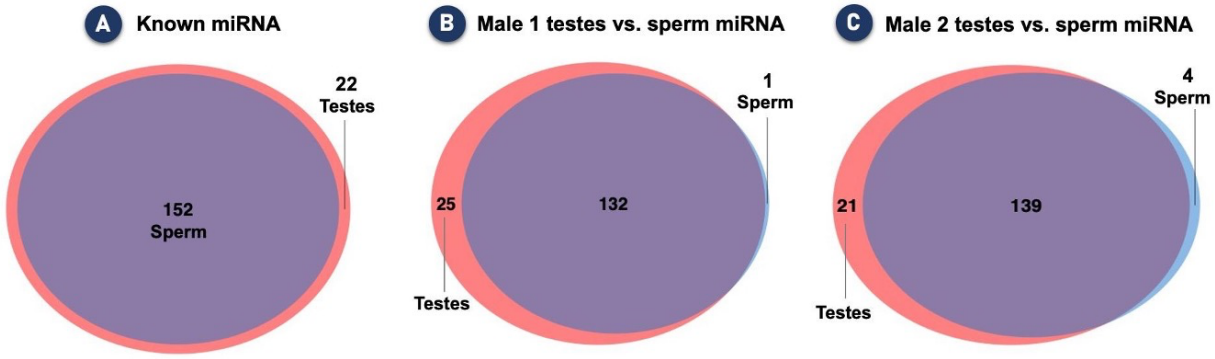


Fig 2.1 Individual differences in known miRNA in sperm and testes tissue among five blue catfish (*Ictalurus furcatus*) males. (A) Known miRNAs in all five males. In panels B and C a single individual was used to compare tissue types, including novel predicted miRNAs that were found.

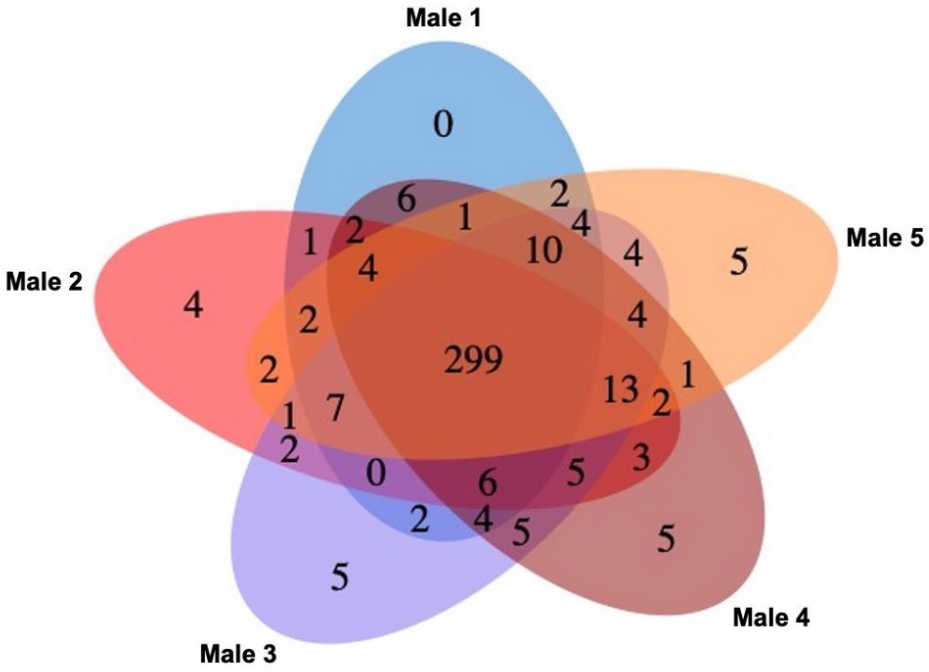


Fig 2.2 Individual variation in testes miRNAs. Known and predicted miRNAs in testes tissue for five blue catfish (*Ictalurus furcatus*) males.

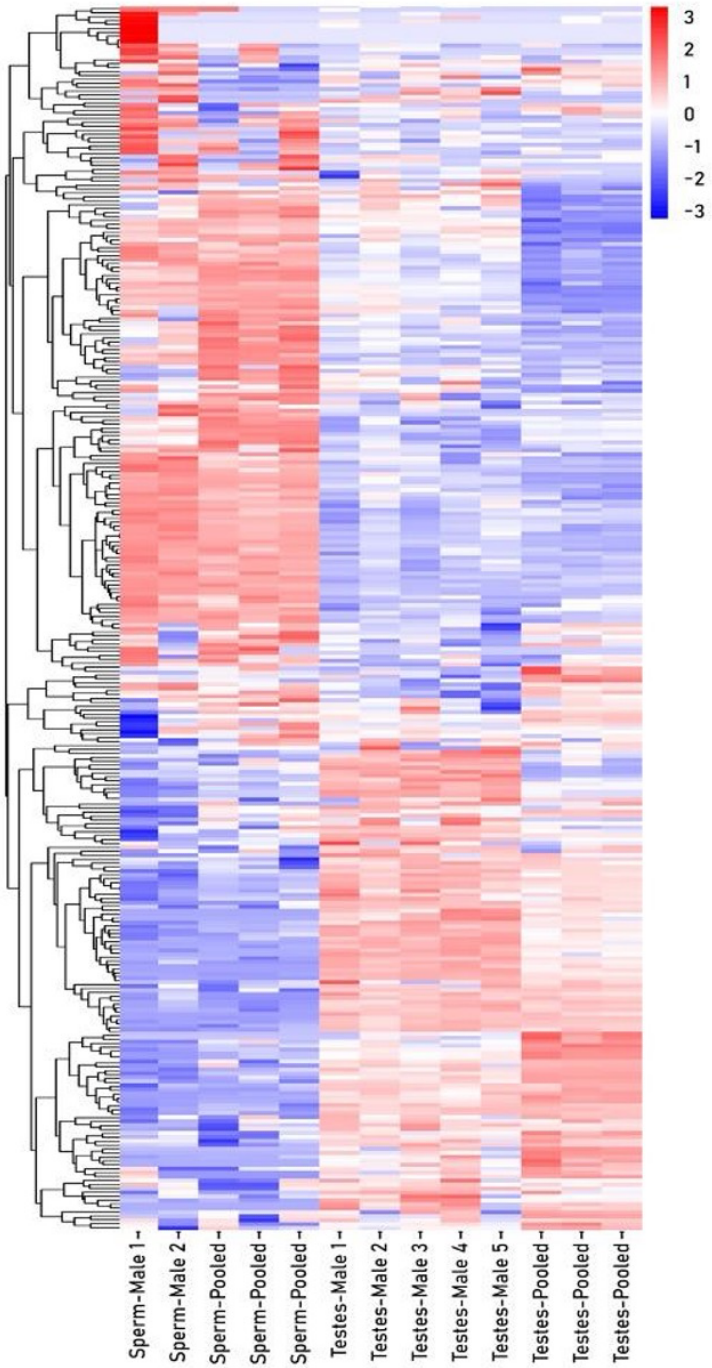


Fig 2.3 Differential sRNA expression in blue catfish (*Ictalurus furcatus*) five males. Each row represents expression values for each differentially expressed sRNA found within the sample (columns). The blue toned blocks relate to down regulated sRNAs, and the red tones represent up regulated sRNAs.

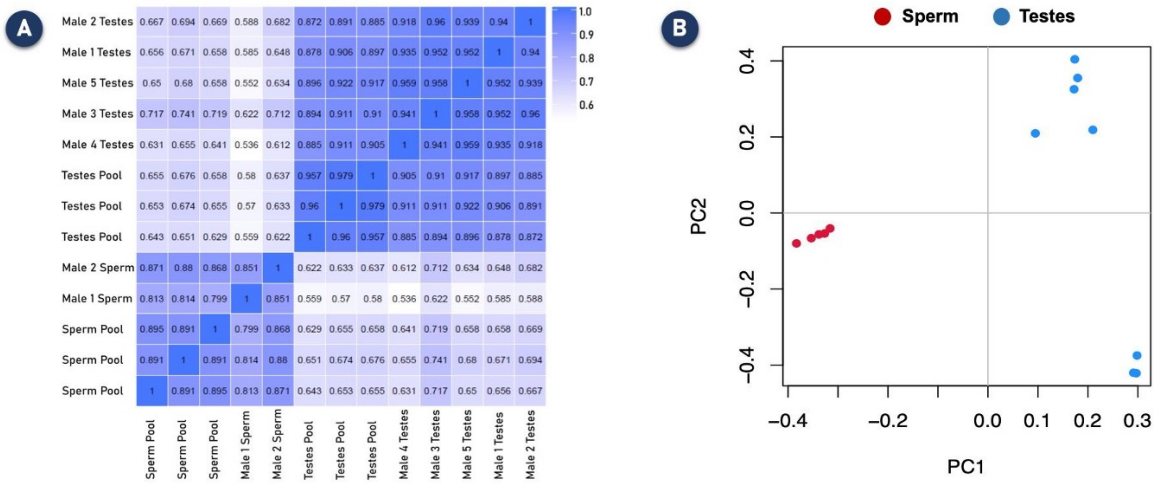


Fig 2.4 Sperm and testes tissues from five blue catfish (*Ictalurus furcatus*) show different sRNA profiles. (A) Pearson's correlation (expressed as R^2) of sRNA profiles between all samples. (B) Principal component analysis of transcripts per million for all sRNAs in each sample. Blue dots represent the testes samples, and red dots represent the sperm samples. Five males were used to form the pools for the pooled testes and sperm samples, as well as used for individual comparison in both A and B panels.

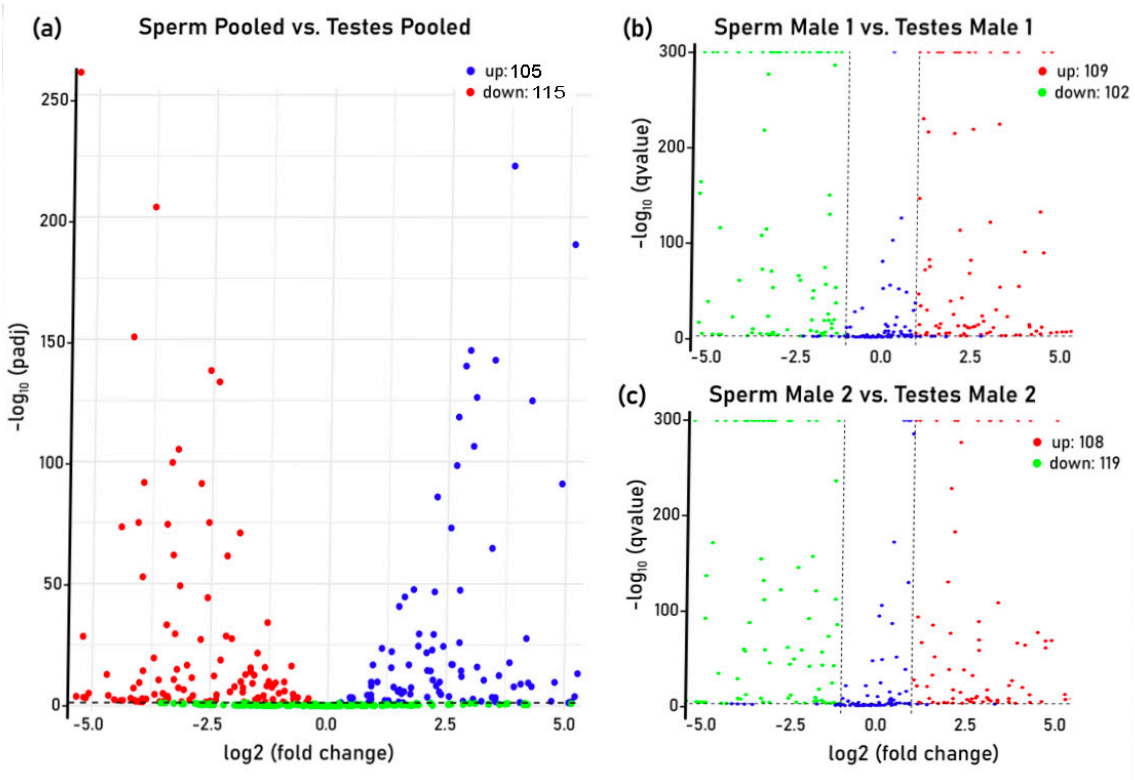


Fig 2.5 Volcano plots showing differential expression in five blue catfish (*Ictalurus furcatus*) males. Pooled samples were used for panel (A), and two individual males were used in (B) and (C) for comparison of tissue types.

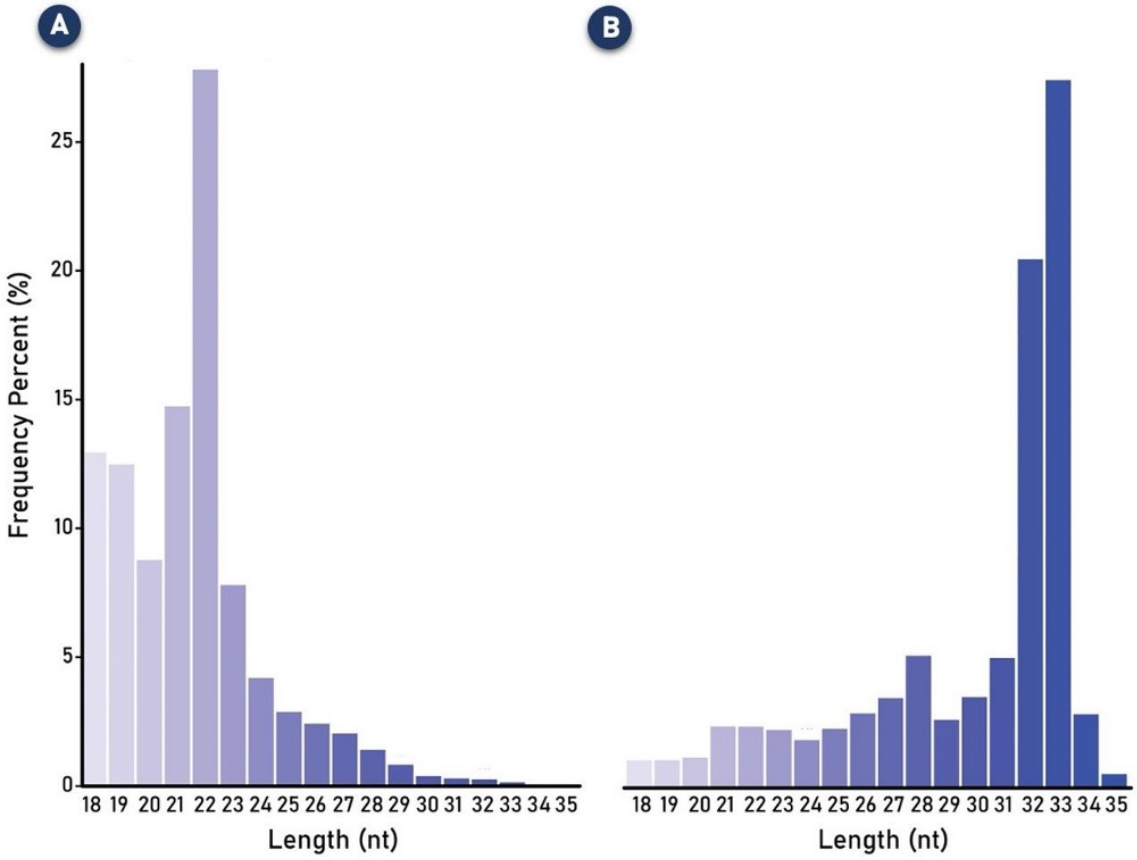


Fig 2.6 Sequence length distributions for sRNA in testes (A) and sperm (B). The length of sRNAs on the left panel are from a pooled testes tissue of five blue catfish (*Ictalurus furcatus*) males and on the right panel are from a pooled sperm sample of two males.

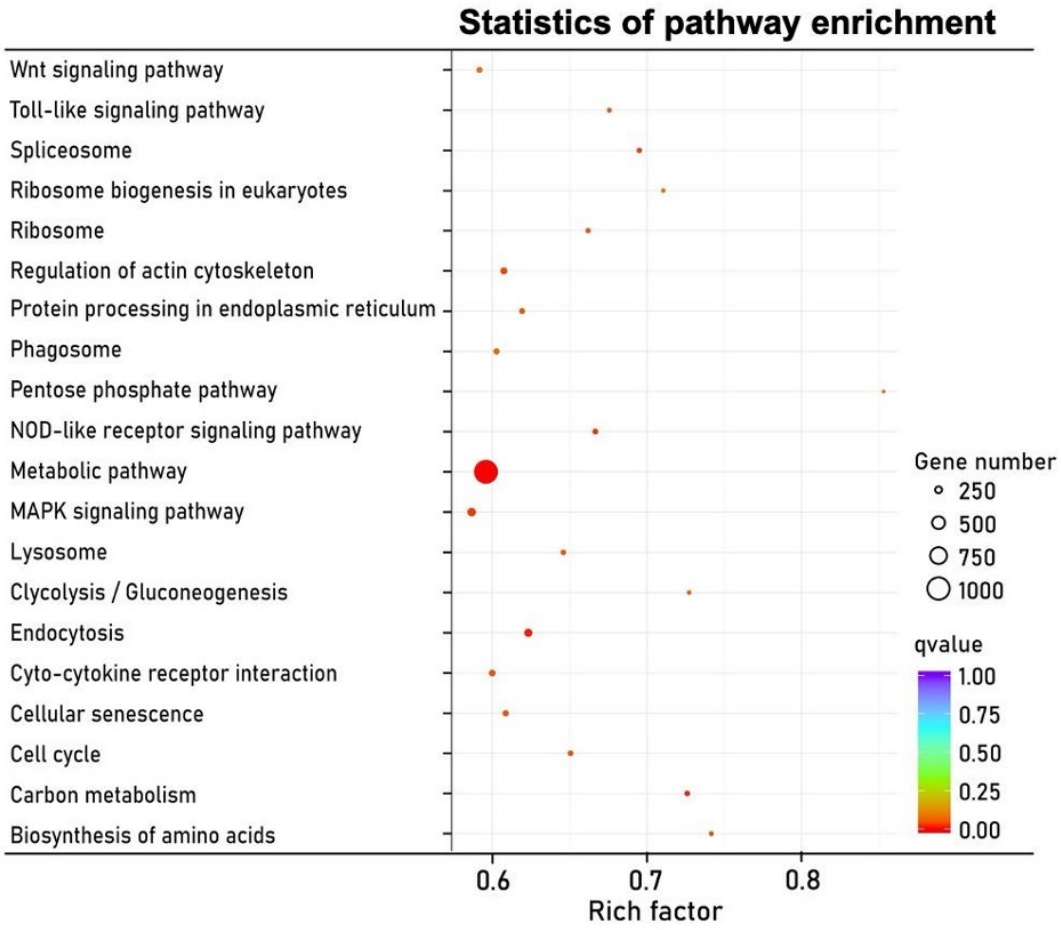


Fig 2.7 KEGG enrichment analysis identifies sRNAs that regulate different pathways in sperm and testes tissue. KEGG analysis with pooled sample from the sperm and testes tissue samples of five blue catfish (*Ictalurus furcatus*) males. Larger circles have more genes associated with that pathway, and qvalue is denoted by color. All of the listed pathways are statistically significant ($p < 0.05$).

Table 2.1 Quantitative characteristics of blue catfish (*Ictalurus furcatus*) male reproductive indices. The sample size (N), mean, standard deviation (SD), standard error (SEM), coefficient of variation (CV), minimum (Min), and maximum (Max) values are reported for each male reproductive index.

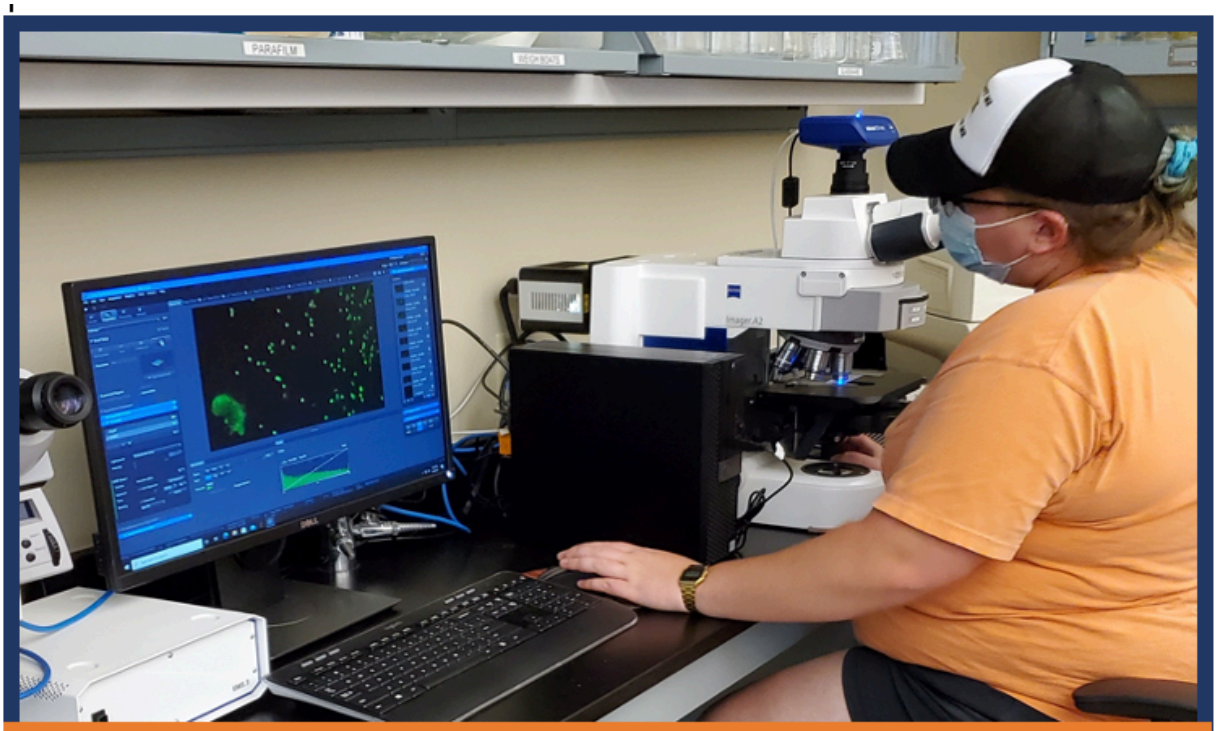
Male reproductive index	N	Mean	SD	SEM	CV	Min	Max
Sex steroid hormones							
Testosterone (ng/mL)	5	0.917	0.023	0.010	2.5	0.901	0.957
11-Ketotesosterone (ng/mL)	5	0.058	0.002	0.001	2.6	0.057	0.060
Testes histology							
SMI	5	0.617	0.016	0.007	2.6	0.597	0.636
Sperm analysis							
Motility (%)	5	61.06	25.25	11.29	41.4	22.75	93.20
Curvilinear velocity (μm/s)	5	183.99	26.15	11.70	14.2	155.68	212.46
Viability (%)	5	90.10	3.59	1.60	4.0	84.47	92.53
Sperm density (cells/mL)	5	3.06×10^{9}	1.01×10^{9}	4.53×10^{8}	33.1	1.51×10^{9}	4.13×10^{9}

SMI = sexual maturity index (Tomkiewicz et al., 2011)

Table 2.2 Sequencing metrics from five blue catfish *(Ictalurus furcatus)* testes and extracted testicular sperm.

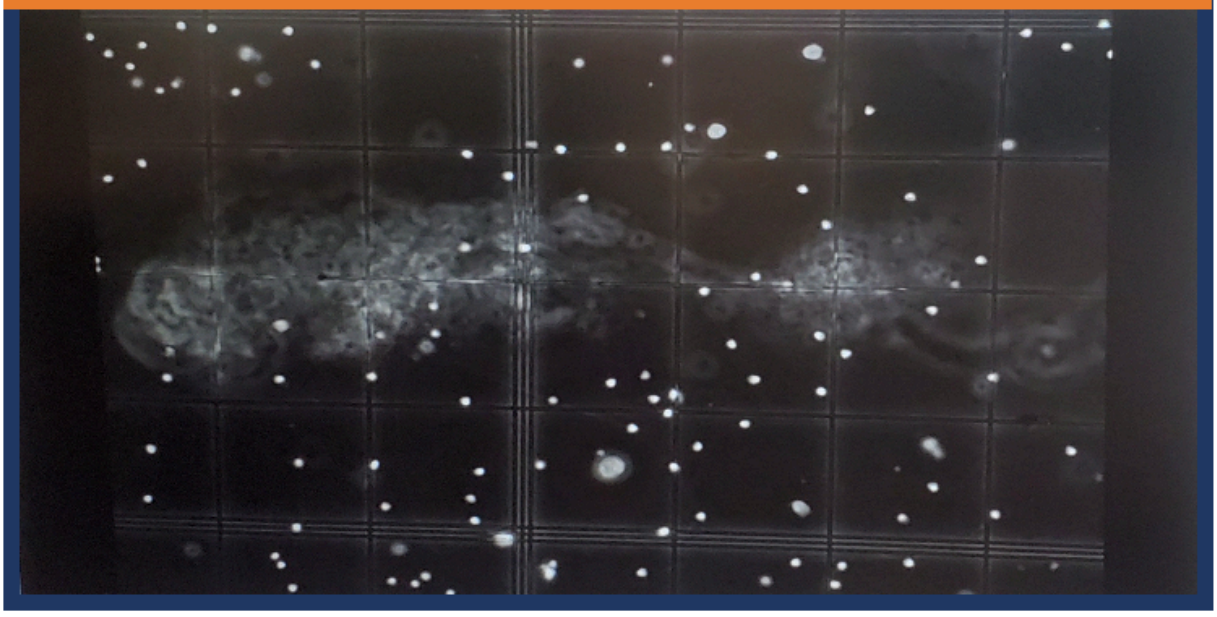
Sample ID	Total reads	Clean reads	% Clean reads	Total sRNA	Mapped sRNA	% Mapped sRNA	sRNAs aligned to exon	sRNAs aligned to intron	Mature Known miRNA	Mature Novel miRNA
Sperm Male 1	12,088,181	11,473,615	94.92%	10,714,859	5,469,920	51.05%	133,719	151,791	133	159
Sperm Male 2	12,673,328	12,295,531	97.02%	11,311,306	6,038,931	53.39%	253,775	199,255	143	164
Sperm Pool	10,585,629	10,337,461	97.66%	9,780,265	5,095,604	52.10%	147,507	156,675	137	145
Sperm Pool	9,560,902	9,261,520	96.87%	8,543,481	4,536,579	53.10%	141,335	146,697	139	148
Sperm Pool	10,231,294	9,979,596	97.54%	9,480,700	5,182,368	54.66%	142,506	153,137	136	154
Testes Male 1	11,243,870	11,070,330	98.46%	10,479,154	5,678,388	54.19%	289,491	521,612	160	193
Testes Male 2	12,911,260	12,623,743	97.77%	11,492,838	6,310,053	54.90%	266,542	446,726	157	193
Testes Male 3	13,522,560	13,266,221	98.10%	13,241,446	7,218,712	54.52%	284,298	521,095	161	200
Testes Male 4	11,250,741	10,903,196	96.91%	9,478,319	5,054,065	53.32%	245,027	406,235	161	209
Testes Male 5	15,163,818	14,916,898	98.37%	11,706,201	6,578,730	56.20%	262,805	531,272	161	210

Testes Pool	12,762,691	7,981,426	62.54%	3,497,992	2,175,226	62.18%	110,862	170,872	149	177
Testes Pool	15,581,841	13,116,464	84.18%	9,641,479	5,956,903	61.78%	229,034	396,424	156	206
Testes Pool	15,056,277	10,117,078	67.20%	6,109,385	3,776,802	61.82%	162,996	268,800	154	188
AVERAGE	*12,510,184*	*11,334,083*	*91.35%*	*9,652,110*	*5,313,252*	*55.63%*	*205,377*	*313,122*	*150*	*180*



Chapter 3

Molecular biomarkers for speedy sperm in an economically important fish



3.1 Abstract

Intraspecies variability in sperm quality is common among cultured species, therefore there is an urgent need to establish reliable molecular biomarkers to screen for males with high quality sperm. The channel catfish (*Ictalurus punctatus*) ♀ × blue catfish (*I. furcatus*) ♂ hybrid, which accounts for >50% of United States catfish production. Blue catfish sperm cannot be collected by non-lethal methods, and thus, males must be sacrificed to determine reproductive quality. We characterized important sperm traits and ranked blue catfish males based on velocity of their sperm. Of the 43 males characterized, testicular tissue from 4 individuals with low velocity and 4 with high velocity were sequenced for sRNAs and mRNAs. No differentially expressed sRNAs were detected between the two performance groups, suggesting regulatory roles of sRNAs are not directly related to sperm quality in *I. furcatus*. However, we identified two differentially expressed mRNA transcripts between low and high velocity groups, *aqp3a* and *ly97.3*, both upregulated in the high velocity group compared to the low velocity group. Overall, molecular biomarkers were identified in testicular tissues for an important aquaculture species that are linked to gamete quality and relevant biological pathways are described.

3.2 Introduction

Fish reared in captivity often exhibit reproductive dysfunction (Zohar and Mylonas, 2001). Historically, research on egg quality has been the focus for mitigating reproductive dysfunction (Sullivan et al., 2015; Reading et al., 2018). However, recently it has been established that males contribute to reproductive success beyond solely delivering the haploid genome (Siddique et al., 2017). In aquaculture production facilities, there are complex interactions between genetic, environmental, and physiological factors that cause intraspecies variability in sperm quality; therefore, there is an urgent need to establish consistent and accurate biomarkers for sperm quality (Rurangwa et al., 2004). Biomarkers can be advantageous to aquaculture managers (Cabrita et al., 2014), as they allow managers to exclude individuals likely to have "poor" quality sperm, thus improving production yield and optimizing efficiency.

Sperm quality is assessed by its capacity to fertilize an egg, followed by the ability to produce viable offspring (Bobe and Labbé, 2010). The most used sperm quality biomarkers are motility and velocity, but these kinematic parameters only assess one portion of the cell's attributes - the ability to move toward the egg (Gallego and Asturiano, 2019). As such, motility alone does not assess molecular defects that also contribute to sperm quality, such as DNA damage or high levels of oxidative stress (Cabrita et al., 2014). New emerging tools for the assessment of sperm quality go beyond microscopy and include work on a molecular level to include stress markers, antioxidant status, DNA damage, lipid peroxidation, mitochondria dysfunction, and genome analysis (Cabrita et al., 2014).

RNA transcripts can be predictors of sperm quality (Herráez et al., 2017). While RNA transcripts can be interpreted as "snapshots in time", validated transcriptomic biomarkers can be

reliable, accurate, and efficient as biomarkers (Qian et al., 2014; Herkenhoff et al., 2018). Though sperm are transcriptionally inactive, studies in mammals have shown residual messenger RNAs (mRNAs) from spermatogenesis that have been proven to have roles in early embryonic development and ultimately impact fertility (Ostermeier et al., 2002; Lalancette et al., 2008; García-Herrero et al., 2011; Johnson et al., 2011). Non-coding (ncRNAs) are involved in post-translational regulation of genes associated with spermatogenesis and oogenesis (Robles et al., 2019). microRNAs (miRNAs) are short ncRNAs that play key roles in regulation of gene expression networks and show potential to be used as sperm quality biomarkers (Bizuayehu and Babiak, 2014; O'Brien et al., 2018). mRNAs have also been investigated for use as biomarkers for reproductive quality in aquaculture species (Sullivan et al., 2015; Myers et al., 2020a). Because sperm quality leads to high variability in reproductive success and latent effects in larvae of fish aquaculture species (Bobe and Labbé, 2010), investigating molecular biomarkers, including the potential for either mRNA or short RNA (sRNA) biomarkers, associated with sperm quality are urgently needed to optimize efficiency in aquaculture (Herráez et al., 2017).

Hybrid catfish (channel catfish, *Ictalurus punctatus* ♀ × *I. furcatus* ♂) are economically important accounting for more than 50% of total catfish production in the United States (Torrans and Ott, 2018). These hybrids are ideal for aquaculture, due to improved feed conversion rates (Green and Rawles, 2010), greater tolerance of potentially lethal levels of low oxygen (Dunham et al., 1983), and increased disease resistance (Wolters et al., 1996; Arias et al., 2012) among other factors, when compared to channel catfish. The hybrid catfish industry has seen growth, however, reproductive bottlenecks that delay production and reduce efficiency remain. Hybrids rarely mate without human intervention due to behavioral incompatibility, preferences in spawning environments, or other factors (Dunham and Masser, 2012). In addition to this, there

are two specific dysfunctions that affect blue catfish, a disjunct in age to maturity for parental species, inability to strip sperm. To further explain, blue catfish have long generation intervals, as males do not produce mature sperm until 4 to 7 years of age (Graham, 1999). Further, their sperm cannot be collected by non-lethal methods, and thus require removal and maceration of the testes (Bart and Dunham, 1990), meaning that males can only be used once for breeding. This contrasts with female channel catfish that mature at 2 to 4 years of age and have eggs that can be readily stripped for in-vitro fertilization (IVF) (Dunham et al., 1999). Thus, females can be reused over multiple spawning seasons, whereas each male is a large investment and therefore creates a bottleneck in reproduction. To help alleviate this bottleneck, sperm cryopreservation protocols have been developed on a commercial scale to make sperm available when females are in peak spawning condition (Bart et al., 1998; Hu et al., 2011, 2014).

Unfortunately, this cryogenic technology has rarely adopted by the industry, and furthermore a high degree of variability in fresh ("neat") or cryopreserved sperm parameters has been reported between males. At present, our current lack of understanding of the underlying mechanisms involved in sperm function prevents progress in this area. Therefore, it is paramount to investigate molecular processes associated with sperm quality to improve offspring performance and predict fertility via biomarkers. The objectives of this study were to identify molecular biomarkers in testicular tissues that are linked to gamete quality by comparing differential expression between males with low-velocity and high-velocity sperm. We also aimed to describe biological pathways relevant to these molecular biomarkers.

3.3 Materials and Methods

3.3.1 Initial sampling techniques

Mature blue catfish males (N = 43) were acquired from Jubilee Farms (Indianola, MS), and transported to the E.W. Shell Fisheries Center in Auburn, Alabama, USA (32.6524° N, 85.4860° W). These fish were housed in six, 0.25-acre earthen ponds, where each pond held ~7 fish while being sampled, temperature ranged between 19.9°C to 25.4°C and dissolved oxygen was maintained > 6 mg/L using a floating pond surface aerator. Salinity of ponds was < 2 ppm, alkalinity and hardness ranged between 50 to 80 mg/L, and pH varied between 7.5 and 8.6. Each male was collected by seining before being euthanized according to Institutional Animal Care and Use Committee guidelines. Blood was immediately drawn from the caudal vein post-euthanasia and stored at 4°C. Morphometric data was then collected for each male, including weight (mean ± SD; 5.19 kg ± 1.01 kg) and length (mean ± SD; 74.63 cm ± 7.72 cm). Testes were then dissected from each male and washed with Hank's balanced salt solution (HBSS; 8 g/L NaCl, 0.4 g/L KCl, 0.16 g/L $CaCl_2 \times H_2O$, 0.2 g/L $MgSO_4 \times 7H_2O$, 0.12 g/L $Na_2HPO_4 \times 7H_2O$, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, 1 g/L glucose, pH 7.42, osmolality 305 mOsm/kg)(Tiersch et al., 1994). For each male, a small section of testis (~1 g) was randomly taken from the whole tissue for histological analysis (*Section 3.3.2*), while another testis sample (~0.5 g) was removed, snap frozen, and stored at -80°C for molecular analysis (*Section 3.3.3*). The remaining testicular tissue was used to extract sperm cells for assessment of male reproductive indices (*Section 3.3.2*).

3.3.2 Reproductive performance indices

Before performing sperm extraction, blood vessels and peritoneum were manually dissected from testes tissue to prevent contamination. Testes were washed again with HBSS and macerated before being strained through 200 μm mesh to separate sperm cells from testes tissue. Using the extracted sperm, our aim was to assess reproductive status and gamete quality. Here, each male (N = 43) was initially sampled for testes histology, sex steroid hormones, and sperm traits. Specific methods for each procedure are highlighted below.

Cell concentration

To assess initial sperm density for each male, cells were counted under a Zeiss Imager.A2 using a 20× objective and improved Neubauer hemocytometer. To prepare samples for counting, they were diluted 200-fold using a non-immobilizing medium (HBSS) and homogenized for ~10 s. In duplicate for each male, 10 μL of the prepared dilution was pipetted onto the hemocytometer. To obtain average sperm concentration, sperm cells inside five 0.2 mm^2 squares on the hemocytometer were counted, then summed and multiplied by five to estimate the cells within the entire 5 × 5 grid. Concentrations from the 5 × 5 grid were then multiplied by the total volume overlying the counting area (10^4), and further multiplied by the dilution factor to determine sperm concentration.

Activity analyses

Computer Assisted Sperm Analysis (CASA) software was used to quantify sperm activity traits (CEROS II software, Hamilton Thorne Biosciences, Beverly MA, USA) according to Myers et al. (2020). To summarize the protocol used, sperm were placed in an 80 µm 2X-Cel chamber (Hamilton Thorne Biosciences, Beverly MA, USA) and activated with distilled water supplemented with 0.5% bovine serum albumin (126609-10GM, Merck Millipore, Burlington Massachusetts, USA). Three replicate activations were performed per male, and video frames were analyzed at 10 s post-activation. Curvilinear velocity (VCL) and motility were determined using CASA software. As sperm velocity is typically the primary determinant of fertilization success (Lahnsteiner et al., 1998; Gage et al., 2004; Linhart et al., 2005), the 43 males were ranked based on mean VCL and then separated them into low and high velocity groups (Fig 1a). Initially, 8 males were in each group (low and high velocity) and (N = 16) were submitted to Novogene for molecular analysis (*Section 3.3.3*) (Novogene Corporation, Durham NC, USA).

Statistical analysis of performance indices

Data were analyzed using SAS statistical software (v.9.4; SAS Institute Inc., Cary, NC, USA). Normality of the residuals was tested using a Shapiro-Wilk test and homogeneity of variance was tested using plots of residuals vs. predicted values. When necessary, data were arcsin square root or $\log_{10}$ transformed to meet appropriate model assumptions. Paired t-tests were used to determine differences in performance groups per each reproductive index, using a P-value of < 0.05 for

significance cutoff. Tukey's HSD was used for post-hoc testing, and alpha was set at 0.05. The reported values are least-squares means ± standard error.

Testes histology

Testes were stored in 10% buffered formalin solution for a period of 4 months. Samples were then transported to the Scott-Ritchey Research Center at the Auburn University College of Veterinary Medicine, where the following was conducted by a licensed technician. Tissue samples were dried using ethanol solutions increasing from 70 to 100%, then embedded in paraffin. Samples were sectioned to 4-5 μm before being stained using hematoxylin and eosin and mounted onto glass slides. Digital images of the slides were taken using a Zeiss Imager.A2 microscope equipped with an Axio-cam 305 and Zen Pro v. 6.1 software (Zeiss, Oberkochen, Germany). From the digital images, Image J (v. 1.53o) was used to apply an 80-point grid to the images using the plugin *Analyze*. Progression of spermatogenesis was assessed using spermatogenic maturity index (SMI) as described (Tomkiewicz et al., 2011) where tissue was organized by cell type (testicular somatic cells, spermatogonia, spermatocytes, spermatids, and spermatozoa) and relative area fraction was then estimated.

Sex steroid hormones

After the blood samples were stored for at least 12 h at 4°C, plasma was separated from the total blood sample. The plasma was then further refined by centrifuging at 4,000 rpm for 10 min, while maintaining 4°C. The supernatant was stored at -20°C until analysis could be completed using testosterone (T, 582701, Cayman Chemical Company, Ann Arbor Michigan, USA) and 11-

ketotestosterone ELISA kits (11-KT, 582751, Cayman Chemical Company, Ann Arbor Michigan, USA). Blood plasma was assayed in triplicate per male. Relevant cross reactivity for the T assay includes: 19-nortestoserone (140%), 5 α-dihydrotestosterone (27.4%), 5β-dihydrotestosterone (18.9%), methyltestosterone (4.7%), androstenedione (3.7%), 11-KT (2.2%), 5-androstenediol (0.51%), epi-testosterone (0.2%), progesterone (0.14%), testosterone enanthate (0.11%), androsterone (0.05%), androsterone sulfate (0.04%), testosterone sulfate (0.03%), DHEA sulfate (0.02%), estradiol (<0.01%), and testosterone glucuronide (<0.01%). Relevant cross reactivity for 11-KT includes: adrenosterone (2.9%), 4-androsten-11β,17β-diol-3-one (0.01%), 5α-androstan-17β-ol-3-one (<0.01%), 5α-androsten-3β,17β-diol (<0.01%), and T (<0.01%). Results were taken spectrophotometrically (Cytation 3, Biotek), where sensitivity (80% B/B_0) was 1.3 pg/mL, in the range of 0.78-1000 pg/mL, using 412 nm absorption for both sex steroid hormones.

3.3.3 RNA sample processing

After dissection and washing with HBSS, testes were flash frozen and stored at -80°C (N = 16; *Section 3.3.2*) before shipping to Novogene for further processing and analysis. Total RNA was extracted from testes, and samples with RNA integrity numbers (RINs) ranging from 7.5-9.3 were further processed (N = 8; 4 from the high velocity group and 4 from the low velocity group; see *Section 3.3.2* for selection criteria). From here these 8 samples were divided for processing separately using either sRNA protocols or mRNA protocols.

sRNA sequencing and bioinformatic analysis

An initial 3 μg total RNA per sample (N = 8 with 4 from the high velocity group, and 4 from the low velocity group) was used to construct the sRNA library, via NEBNext® Multiplex Small RNA Library Prep Set. cDNA was synthesized using M-MuLV Reverse transcriptase (RNase H^-), and PCR amplification was done using LongAMP Taq 2X Master Max, SR primer for Ilumina and index (X) primer. The products of PCR amplification were purified on 8% polyacrylamide gel at 100V for 80 min, followed by the recovery and elution of DNA fragments between 140 bp and 160 bp. Library quality was assessed using an Agilent Bioanalyzer 2100, using DNA high sensitivity chips. Index-coded samples were clustered using a cBot Cluster Generation System with a TruSeq SR Cluster Kit v3-cBot-HS. Sequencing was then run on an Illumina sequencing instrument (Novoseq 6000, HWI-ST1276, Illumia, San Diego CA, USA) where 50 bp single-end reads were generated.

After sequencing, quality control was performed via Novogene's proprietary perl and python scripts, where clean data were obtained by removing reads with ploy-N or ploy A/T/G/C, 5' adaptor contaminates, those missing 3' adapter or insert tags, or low-quality (Qscore ≤ 5 making up > 50% of reads). Cleaned reads were mapped to the channel catfish genome (v. IpCoco_1.2) using Bowtie, as blue catfish lack a published genome (Langmead et al., 2009). Mapped sRNA tags from miRBase20.0 were used to look for known miRNA (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008, 2006 Kozomara and Griffiths-Jones, 2011, 2014; Kozomara et al., 2019). Novel miRNA sequences were identified using mirdeep2 (Friedländer et al., 2012) and srna-tools-cli (Moxon et al., 2008).

To estimate sRNA expression levels, a transcript per million (TPM) approach was used, where the expression fold change for each sRNA was calculated as the $\log_2$ratio of the TPM (Zhou et al., 2010). The DESeq2 package (v 1.8.3) in R was used to identify differentially expressed sRNAs between the two performance groups (high and low velocity), and a Benjamini and Hochberg corrected p-value of 0.05 was used as a cutoff to determine statistical significance (Love et al., 2014). A rank-based Gene Ontology enrichment analysis was done in R, using a Mann-Whitney U (MWU) approach, to test whether each GO term is significantly enriched for up or down regulated genes (Wright et al., 2015).

mRNA sequencing and bioinformatic analysis

From total RNA (N = 8 with 4 from the high velocity group and 4 from the low velocity group), mRNA was purified using poly-T oligo-attached magnetic beads and cDNA was synthesized. Final cDNA libraries were quality controlled using Qubit and real-time PCR before sequencing was performed on an Illumia platform (Novoseq 6000, HWI-ST1276, Illumia, San Diego CA, USA). Index-coded samples were clustered using a cBot Cluster Generation System with a TruSeq SR Cluster Kit v3-cBot-HS. Sequencing was then performed on an Illumina sequencing instrument (Novoseq 6000, HWI-ST1276, Illumia, San Diego CA, USA) where 150 bp paired-end reads were generated. Raw reads were processed through fastp, which removed reads containing adapter, poly-N sequences, or otherwise low-quality reads (Qscore ≤ 5 making up > 50% of reads) from the raw data (Chen et al., 2018). The *I. punctatus* genome (v. IpCoco_1.2) was used as a reference genome, and cleaned reads were mapped using HISAT2 software (Kim et al., 2019). Transcript isoforms were assembled using Stringtie (Pertea et al.,

2015), followed by gffcompare to distinguish known and novel genes (Pertea and Pertea, 2020).

Gene expression was quantified using a reads per kilobase of exon model (RPKM) (Van Verk et al., 2013). Using the same statistical cutoffs as the sRNA analysis, the R package DESeq2 was used for differential expression analysis between the high and low velocity groups. A KEGG enrichment analysis was performed using R's clusterProfiler package to determine statistical enrichment of differentially expressed genes in KEGG pathways (Yu et al., 2012). The R package clusterProfiler was also used in the Gene Ontology (GO) enrichment analysis, where gene length bias was corrected, and GO terms with a corrected P-value of < 0.05 were considered significant.

3.4 Results

3.4.1 Male Reproductive Indices

The reproductive quality of the males used in this study were assessed using a series of reproductive indices, such as sperm kinematics, cell density, spermatogenic maturity index (SMI), and blood plasma hormone levels. Males were ranked using mean VCL speeds and separated into low and high velocity groups (Fig 3.1a). The sperm kinematic measures (motility and VCL) were significantly different ($P < 0.05$) between the two performance groups (Fig 3.1b,c), whereas testosterone ($P = 0.76$) and SMI ($P = 0.80$) were not significantly different between low and high velocity performance groups.

3.4.2 mRNA

mRNA sequencing yielded an average of 32,340,520.6 raw reads per sample (Table 3.1). Reads with adaptor contamination, reads with uncertain nucleotides accounting for >10% of their content, and reads with low quality nucleotides (Qscore ≤ 5) making up >50% of reads were removed leaving an average of 30,591,122 cleaned reads for all samples. Mapping to the channel catfish genome (v. IpCoco_1.2) resulted in a 70.07% mapping efficiency.

Using a Benjamini and Hotchberg corrected P-value of 0.05, there were two significant DEGs found between the low velocity and high velocity group, *aqp3a* and *ly97.3* (Fig 3.2a). For both DEGs, gene expression was upregulated in the high velocity group compared to the low velocity group (Fig 3.3). Using a rank-based approach to GO enrichment analysis, there were significant terms upregulated in the high velocity group related to biological processes such as cellular amide metabolism and organonitrogen compound biosynthesis (Fig 3.4a). In addition, the cellular components of cytoplasm and ribonucleoprotein (Fig 3.4b), and those related to molecular functions such as structural components, oxidoreductase, and electron transfer were significantly upregulated in the high velocity group, and G-protein beta/gamma subunit complex binding was upregulated in the low velocity group (Fig 3.4c). KEGG enrichment between the low velocity and high velocity groups identified two significant terms: mitogen-activated protein kinase (MAPK) signaling pathway, and neuroactive ligand-receptor interaction, both of which were upregulated in the high velocity group (Fig 3.5).

2.4.3 sRNA sequencing

sRNA yielded an average of 15,419,585.5 reads per sample (Table 3.1). Reads with >50% of their bases having a Qscore ≤5, reads with >10% uncertain nucleotides, reads with 5' primer contamination, reads lacking 3' primer or insert tag, and reads with poly A, T, G, or C were removed for an average of 15,151,396.3 cleaned reads across all samples. Mapping these cleaned reads to the reference genome resulted in a mapping efficiency of 61.43%. There were 165 total mapped mature miRNAs, 162 from the low velocity group and 160 from the high velocity group, and a mean of 9,145,901 total mapped sRNA. There were no differentially expressed miRNAs ($P < 0.05$) when the two performance groups were compared (Fig 3.2b).

3.5 Discussion

Molecular biomarkers were identified in testicular tissue that are linked to gamete quality and describe biological pathways relevant to these biomarkers. However, before the molecular biomarkers could be assayed, we needed to determine if the males used in this study were of adequate quality. Male-to-male variability in reproductive quality is common across aquatic taxa and ultimately impacts offspring performance (Bobe and Labbé, 2010). This variability is caused by a variety of individual-male specific factors (i.e., epigenetics, nuclear genetic contributions (Cabrita et al., 2014; Barreto et al., 2019) and/or broodstock rearing conditions and ultimately impacts sperm performance (Bobe and Labbé, 2010). The velocity of sperm cells post-activation is typically the primary determinant of fertilization success (Lahnsteiner et al., 1998; Gage et al.,

2004; Linhart et al., 2005), and therefore males with significantly different low and high sperm velocity were chosen to determine molecular biomarkers. For sperm kinematic measures, our motility and VCL measures fell within the range or exceeded previously reported blue catfish values (Bart and Dunham, 1996; Hu et al., 2011; Myers et al., 2020b; Myers et al., 2020a), and testosterone also followed this pattern (Hettiarachchi et al., 2020). For SMI, neither the low nor high velocity groups were statistically different meaning that they were in the same reproductive state. Additionally, SMI was high (on a scale of 0-1) (Tomkiewicz et al., 2011) meaning a high proportion of cells were spermatozoa and spermatids, meaning males were in late stages of spermiogenesis. Therefore, we can conclude that the males used in this study were reproductively sound and acceptable for research and industry production.

By examining molecular differences underlying sperm quality, no statistically significant differentially expressed sRNAs were found between low and high velocity males. sRNAs function as post-transcriptional gene expression regulators (Bizuayehu and Babiak, 2014) and their expression can be influenced by developmental stage, environmental factors, or physiological condition (Qian et al., 2014). As a result, sRNAs have been used as biomarkers for reproductive performance in humans (Vashisht and Gahlay, 2020) and domestic livestock species (Fatima and Morris, 2013). Additionally, using zebrafish (*Danio rerio*) as a model species, different suites of sRNAs were expressed in different reproductive quality groups (Xiong et al., 2018). This evidence led us to believe that sRNA expression would differ between our low and high velocity groups as well; however, we found this was not the case. In teleost fish, though sRNAs have been shown to be important for sex steroid hormone synthesis and function as well as in regulating gonadal development, their role in other reproductive functions is not well known, as reviewed in Bhat et al. (2021). More robust data in the functional implications of sRNAs and validation are needed to

improve breeding protocols and potential biomarkers for aquaculture species if they are to follow the same advancements made in other organisms.

In our mRNA assay of DEGs in testes extracted from males with low and high velocity sperm, we identified two differentially expressed transcripts were identified. One of these is the gene *aqp3a* or aquaporin-3a, which we found to be upregulated in males with high velocity sperm. Aquaporins (AQPs) are membrane-bound water channels that facilitate water transport and small solutes between cells (Roche and Törnroth-Horsefield, 2017). Because of their ability to transport water, AQPs are important for osmoregulation, and their expression responds to environmental salinity (Giffard-Mena et al., 2007). AQPs are present in germ cells for mammals (Huang et al., 2006) as well as fish (Zilli et al., 2009), where they are known to impact oocyte hydration and sperm motility in gilt-head bream (*Sparus aurata*) (Fabra et al., 2005), as well as the fertilization process and embryo development in the sea urchin (*Paracentrotus lividus*) (Amaroli et al., 2013). AQPs are expressed in male germ cells, testis, spermatic ducts, and spermatozoa where they play a key role in sperm cell hydration and impact the release of gametes and their successful activation (Monsang et al., 2019). Therefore, as *aqp3a* was found to be upregulated in the males with high sperm velocity and it plays a vital role in sperm cell hydration and could be used as a biomarker for sperm quality in blue catfish.

Blue catfish males with high velocity sperm upregulate *ly97.3* otherwise known as *ly2.3*. Importantly, *ly2.3* is upregulated in zebrafish when challenged with lipopolysaccharide (LPS), therefore it is implicated to play a role in immune defense mechanisms (Wang et al., 2016). LPS is an endotoxin of gram-negative bacteria (Sahnoun et al., 2017) and is known to cause issues in spermatogenesis due to inflammation, as well as sperm maturation and therefore final quality (Li et al., 2016). Further, in humans (Yu et al., 2013), rabbits (Collodel et al., 2012), and boar (Okazaki

et al., 2010) LPS causes inflammation and results in motility loss for their sperm. Therefore, our findings that blue catfish males with better sperm quality significant upregulate *ly97.3* suggests that these individuals may be better able to mount immune responses against gram-negative bacteria, preventing downstream negative consequences on spermatogenesis. These results suggest that the *ly97.3* gene, along with *aqpa3a*, are high-quality candidates for biomarkers of male reproductive performance.

Testis from low and high quality males also exhibited differences in expression of broad functional categories of genes. Males with high velocity sperm showed GO enrichment upregulation of terms such as cellular amide metabolism, organonitrogen compound biosynthesis, cytoplasm and ribonucleoprotein structural components, oxidoreductase, and electron transfer. Oxidoreductase and electron transfer are important for cell metabolism, especially in relation to its series of oxidation-reduction reaction, and proper function of cell metabolism is important for normal sperm function (Peña et al., 2021). Blue catfish males with low velocity sperm significantly upregulated G-protein beta/gamma-subunit complex binding. In southern flounder (*Paralichthys lethostigma*), sperm is activated by the progestin hormone 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) at the site of membrane progestin membrane receptor alpha (mPRα, or Paqr7) which is coupled to a stimulatory olfactory G protein (G_{olf}) (Tubbs et al., 2011). For two teleost fish, Atlantic croaker (*Micropogonias undulatus*) and southern flounder, pathways stimulating sperm motility occur both through the alpha subunit (via Acy) and through the beta-gamma subunit (Egfr pathway, Pi3K-Akt pathway) of the G-protein, and inhibition of any of these three pathways reduces sperm hypermotility which is associated with increases in fertilization success (Tan et al., 2019). An upregulation of the beta/gamma subunit in low velocity sperm suggests a compensatory mechanism and/or an explanation for poor functioning of those sperm.

KEGG enrichment analysis showed upregulation of both MAPK signaling, and neuroactive ligand-receptor interaction in individuals with high velocity sperm. MAPKs are a family of oxygen sensing enzymes (Zhu et al., 2013). In zebrafish they have been found to mediate oxidative or anti-oxidative stress pathways (Shi and Zhou, 2010), and partially due to their high unsaturated fatty acid content sperm cells are very sensitive to oxidative stress (Sandoval-Vargas et al., 2021) thus, making the pathways function integral to sperm quality. Additionally, the MAPK cascade has been proven to be involved with spermatogenesis, sperm capitation, and acquisition of motility (Sun et al., 2000). As for the neuroactive ligand receptor interaction pathway, it consists of a group of neuroreceptor genes including dopamine receptors, and functions in environmental information processing (Ji et al., 2018). In another fish species, Przewalskii's naked carp (*Gymnocypris przewalskii*), changes in day length led to the upregulation of this pathway, and possibly caused GnRH signaling and steroid hormone synthesis (Tian et al., 2019). Thus, the significant pathways or terms from the enrichment processes are important for different aspects of reproduction and warrant further investigation in this species for solving reproductive dysfunction.

3.6 Conclusion

In this study, we present two candidates for biomarkers (*ly97.3* and *aqp3a*) in blue catfish testis that are significantly associated with high velocity sperm performance. Utilizing these biomarkers in the future will include laparoscopic scopes and minimally invasive surgery to screen testis tissue for *ly97.3* and *aqp3a* expression levels in live blue catfish to predict their reproductive quality non-lethally. Laparoscopic scopes have been used successfully for sexual determination (Moccia et al., 1984; Ortenburger et al., 1996; Hernandez-Divers et al., 2004; Bryan et al., 2007; Hurvitz et al.,

2007) and, with further investigation could be a good match for our needs to determine quality of gonad without sacrificing the fish. This finding is incredibly important for aquaculture producers, as it will reduce unnecessary sacrifice of sexually mature fish and reduce profit losses that occur because of low-quality males being used for breeding. Additionally, in describing the pathways significant to our enrichment processes, we identified future areas for study in the reproductive improvement of the economically important male blue catfish.

3.7 References

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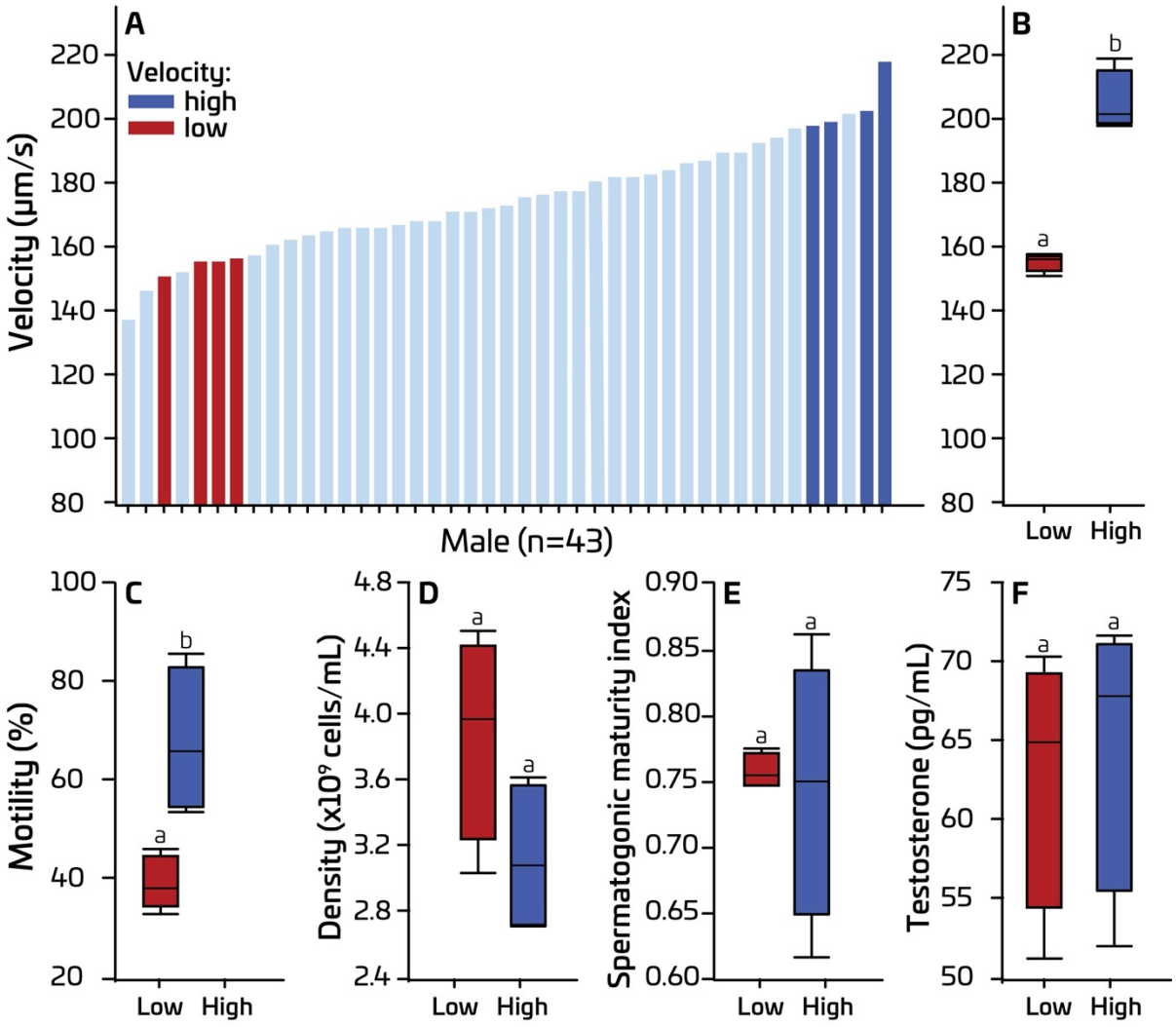

Fig 3.1 (a) Curvilinear velocity for 43 blue catfish (*Ictalurus furcatus*) males' sperm at 10 s post activation. Highlighted bars establish performance groups. (b,c,d,e,f). The boxplots show the results of each assay (motility, initial sperm density, SMI, and testosterone) that is identified on the Y-axes, with the letter on top of the boxes denoting significantly different results ($p < 0.05$) among groups (low or high velocity) using a t-test.

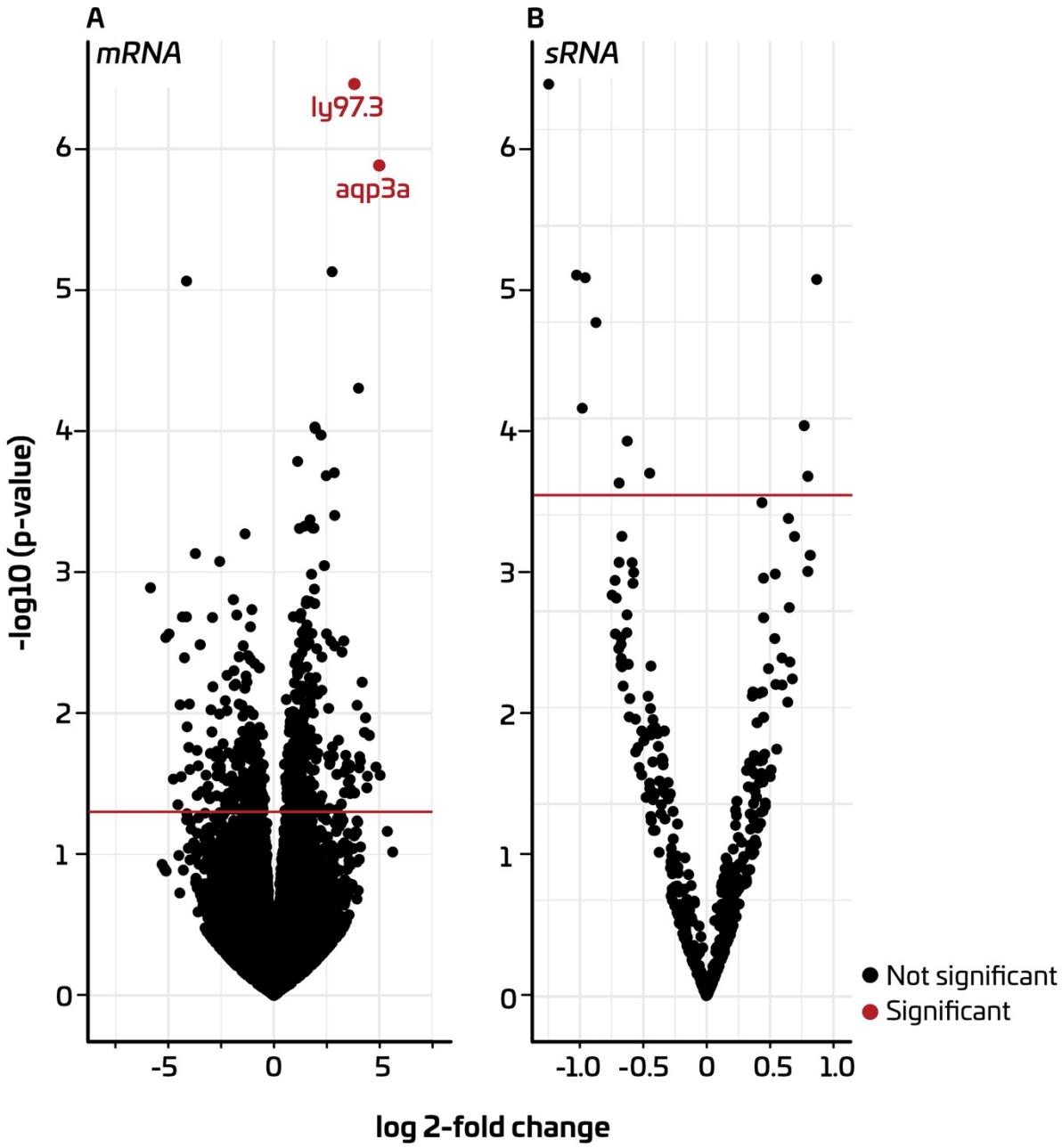

Fig 3.2 Differential expression of mRNAs (a) and sRNAs (b) in eight blue catfish (*Ictalurus furcatus*) males. The red dots signify significantly expressed genes and the black are nonsignificant.

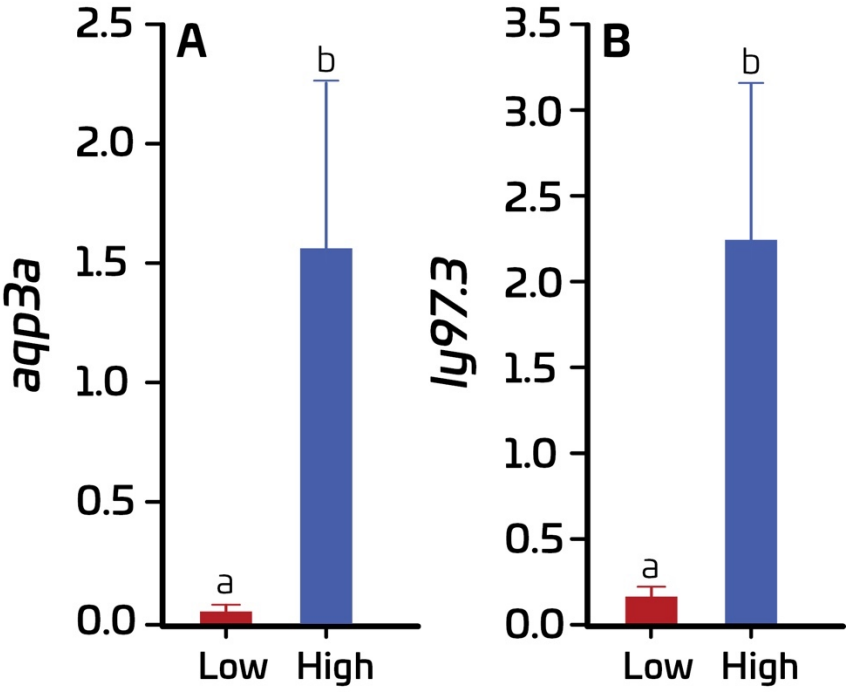

Fig 3.3 Gene expression levels for two genes (aqp3a and ly97.3) upregulated in eight blue catfish (*Ictalurus furcatus*) within a high velocity sperm performance group, compared to low velocity performance group.

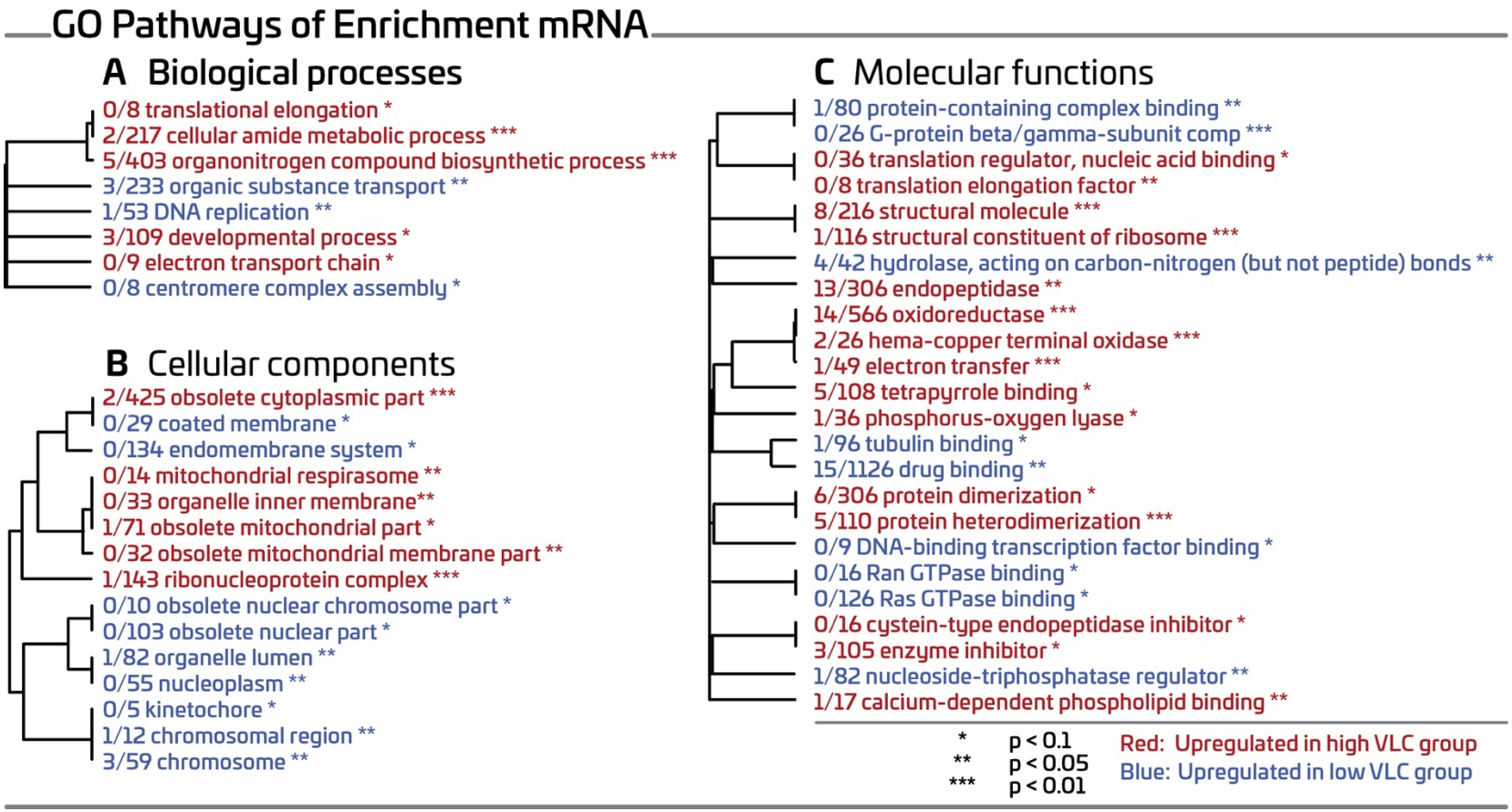

Fig 3.4 Gene ontology (GO) terms from rank based GO analysis for mRNAs in eight blue catfish (*Ictalurus furcatus*) males testes tissue. Levels of significance indicated by asterisks, and color (red for high velocity, blue for low velocity) represents the performance group it is upregulated in.

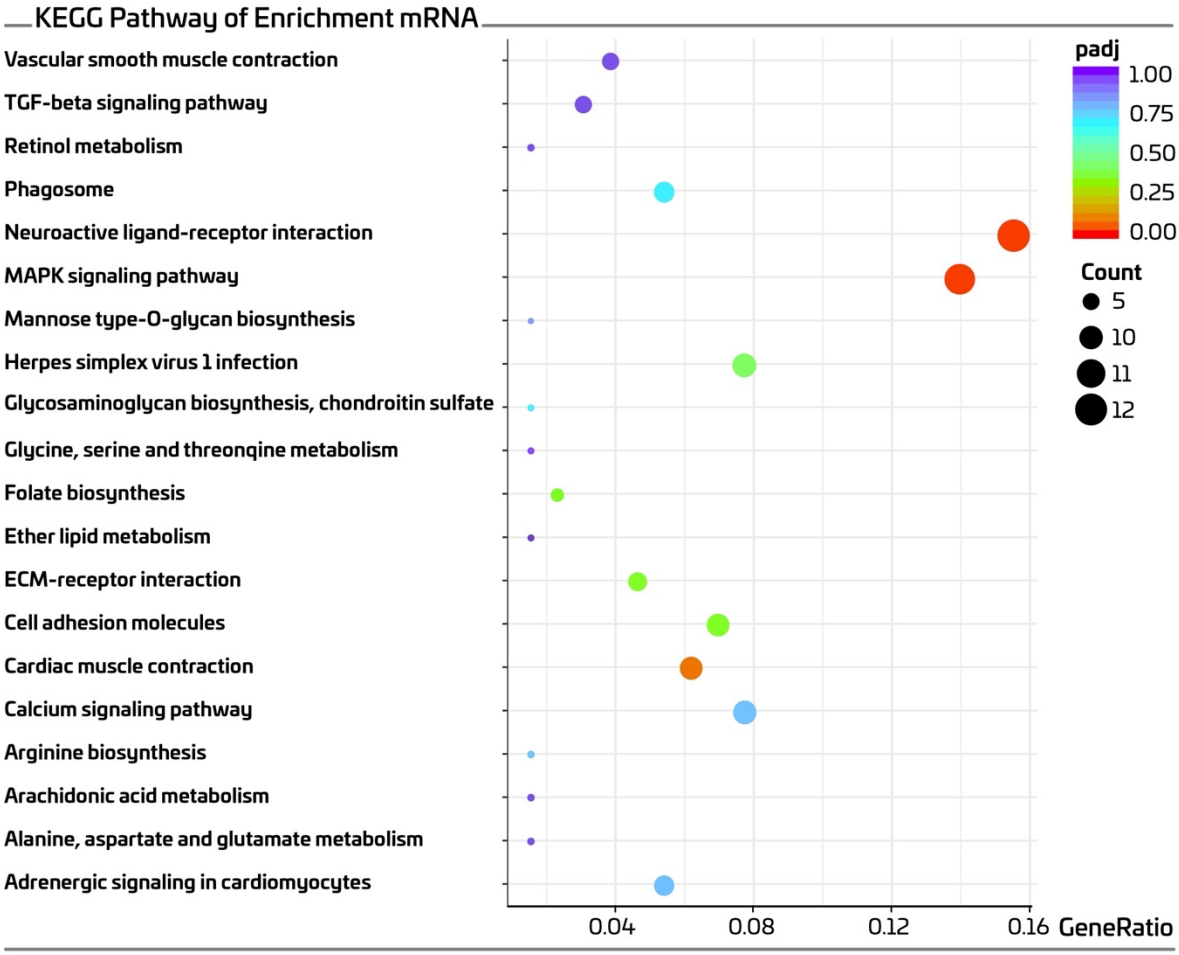

Fig 3.5 KEGG enrichment analysis identifies mRNAs that regulate different pathways. KEGG analysis with two performance groups based on curvilinear velocity of their sperm. Four blue catfish (*Ictalurus furcatus*) males testes tissue were used per group. Larger circles have more genes associated with that pathway, and qvalue is denoted by color. All listed pathways are statistically significant ($p < 0.05$).

Group Name	Mean Raw Reads mRNA	Mean Cleaned Reads mRNA	Mean Total Reads sRNA	Mean Cleaned sRNA	Mean Mature Known miRNA
High VCL	32,973,539.50 (± 2,924,089.09)	31,465,102.25 (± 3,409,853.70)	15,026,380.75 (± 1,569,337.40)	14,754,693 (± 1,564,701.96)	162
Low VCL	31,707,501.75 (± 1,183,570.41)	29,717,141.75 (± 1,259,963.52)	15,812,790.25 (± 2,389,041.44)	15,548,099.50 (± 2,329,921.96)	160
Total	32,340,520.63 (± 2,173,184.33)	30,591,122 (± 2,556,631.84)	15,419,585.5 (± 1,917,881.89)	15,151,396.25 (± 1,885,640.23)	165

Table 3.1 RNA sequence (both mRNA and sRNA) mapping information from 8 total blue catfish (*Ictalurus furcatus*) males, with four individuals per group.