

**Analysis of gamete interactions, maternal, and paternal effects for improving hybrid
catfish aquaculture**

By

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Abstract

Hatchery techniques and technologies have evolved to make the large-scale production of channel catfish (*Ictalurus punctatus*) × blue catfish (*I. furcatus*) hybrids viable and profitable, but there are still reproductive roadblocks between the two species, leading to inconsistent fry production. Therefore, it is imperative to assess how gamete interactions, and maternal/paternal effects impact fertilization outcomes and offspring performance traits during the “critical” early life history stages. In this thesis, it was demonstrated that manual spawning practices can be improved and that gamete interactions can cause alterations in sperm performance for hybrid catfish and other cultured fish species. Environmental effects, parental effects, and interactions between them also control many traits early in life, which if linked to specific mRNA transcripts, may be a powerful mechanism to improve gamete and larval quality. Overall, this work has identified the importance of spawning strategies, females, males, and their mating compatibilities for proper broodstock selection.

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Chapter 1

General Introduction



1.1. Expansion of aquaculture worldwide

Aquaculture, defined briefly as the production of aquatic species in captivity, produces food and income for both developing and developed countries worldwide (Watson et al., 2016). As global fisheries continue to endure pressure from human exploitation and harvest, aquaculture has become essential to sustain wild populations while simultaneously meeting the continuously growing demand for fish. The harvest capacity of wild fisheries is limited by the reproduction and growth rates of the desired species, and yet even with knowledge of these factors, populations are continually being pushed beyond their sustainable limits (Smith et al., 2010). In the past two decades, the production yield generated by aquacultural fish farms has surpassed the capture of wild fish and continues to rise (Fig. 1.1). However, contributions by wild fisheries have remained relatively stable and have even seen a small decrease since 2016 despite the growing surplus of fish consumption (FAO, 2018).

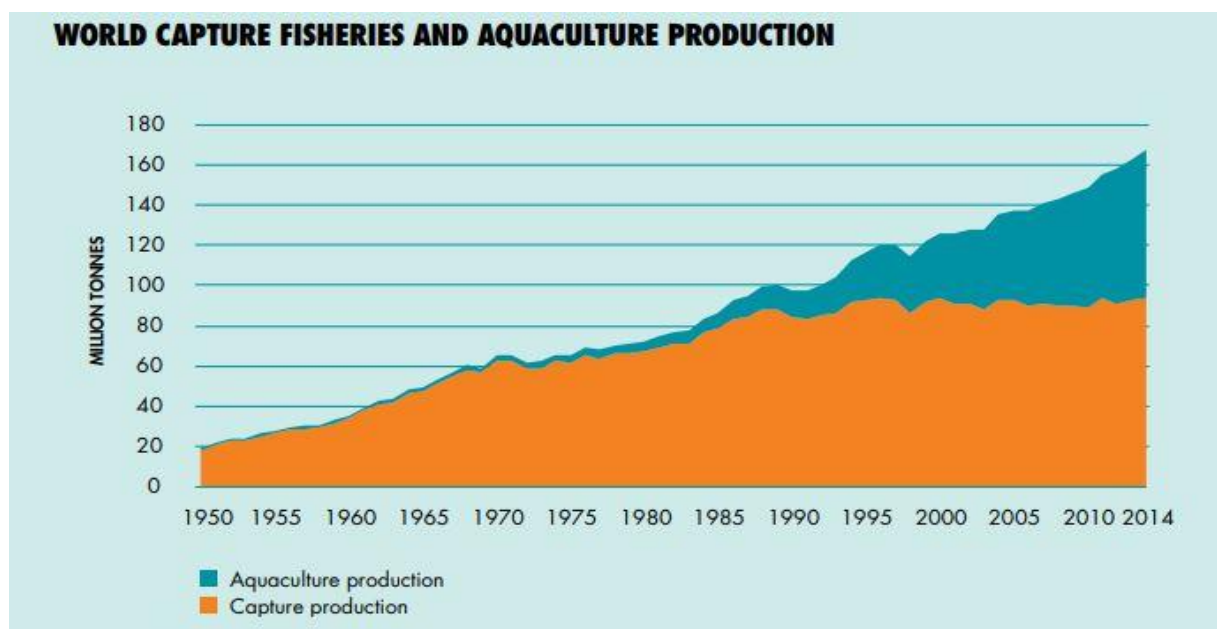


Fig. 1.1. Global contributions (in millions of tons) from aquaculture and by wild fisheries from 1950-2014 (FAO, 2016); <http://www.fao.org/3/a-i5555e.pdf>.

This is a clear indication that heightened global seafood consumption is due to aquaculture and not increased exploitation of wild populations. Of the world-wide aquaculture production of 80 million tons in 2016, the majority of the harvest was finfish (54 million), followed by mollusks (17 million), aquatic crustaceans (8 million), and other miscellaneous aquatic species (< 1 million; FAO, 2018).

Freshwater aquaculture was higher than that of marine in 2008, accounting for 60% of the world aquaculture production. This statistic is remarkable considering only 3% of the Earth's surface is freshwater and only a mere 0.3% of that is surface water (Bostock et al., 2010). Global freshwater fish production is dominated by various



Fig. 1.2. Several species of carp, tilapia, and catfish have been essential in establishing aquaculture historically and continues to dominate world aquaculture today (FAO 2017; 2019).

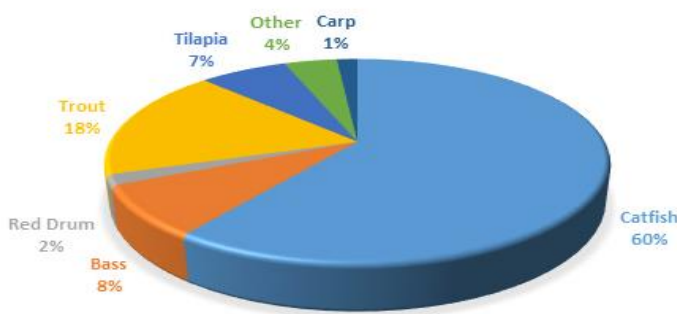
species of carp, tilapia, and catfish, which are collectively cultured in up to 100 different countries (FAO, 2018; Fig. 1.2). Regions of Asia (especially China) are the greatest contributors for freshwater aquaculture production at 89% with North and South America, Europe, and Africa making up the remaining 21%. (NOAA, 2019). Although contributions by the United States (ranked 16th in global production) are still small on this world stage, aquaculture has become well-established over the last 35 years and has potential to grow with continued innovative developments in recirculating aquaculture systems (RAS) and aquaponics technology (Jena et al., 2017). However, there are still significant challenges and margins for improvement in order to continue growth of this industry. Strengthening aquaculture depends on becoming competitive

on the international markets by maintaining and effectively managing aquatic resources and encouraging national policy development (USDA, 2018).

1.2. Aquaculture in the United States and the expanding hybrid catfish industry

There are five principal aquaculture fish species in the United States: catfish, trout, salmon, tilapia, and hybrid striped bass (USDA, 1995). Of these species, channel catfish, *Ictalurus punctatus*, took root in the southeastern states of Mississippi, Louisiana, Arkansas, and Alabama where the production environment is ideal for year-round growth (FAO, 2011). From the 1960s and 1970s, the channel catfish industry consisted of several independently operated farms, and profits were low due to lack of coordination between these farms and limited access to marketing outlets (Hargreaves, 2002). However, the channel catfish industry soon established itself as the largest aquaculture industry by production value by the 1980s (USDA, 1995). Characteristics of this ideal finfish species for

USA FOODFISH SALES BY SPECIES, 2013
Source: USDA NASS, 2013 Census of Aquaculture



— Fig. 1.3. USA aquaculture sales by species shows that catfish make up well over half of total aquaculture production, followed by trout and tilapia (USDA NASS, 2013 Census of Aquaculture).

pond aquaculture include manipulative human-controlled reproduction, efficient conversion of natural foods and formulated feeds, tolerance of low water quality, disease resistance, and tolerance of high stocking density (Bromage and Roberts, 1995; Teichert-Coddington et al., 1997). Channel catfish production increased steadily from the early 1980s until the early

2000s, but thereafter, has remained fairly constant with modest increase due to higher feed costs and international competition from Asia for cheaper imported fish (FAO, 2018). Due to these challenges, catfish farmers have been confronted with the demand to improve production practices and technologies as well as explore the potential benefits of selective breeding to market higher quality fish.

Success within any selection program begins with proper broodstock management (Bromage and Roberts, 1995). Selective breeding has been used within hatcheries to develop genetically superior fish for a variety of production traits (Gjedrem et al., 2012). In turn, this has led to the creation and maintenance of distinct strains with differential performance traits. Once isolated, strains of channel and blue catfishes have continuously been enhanced over generations to maximize genetic gain and have been crossbred with fish of different strains in efforts to stimulate hybrid vigor, with success of this occurrence based on the species (Dunham et al., 2000a). Another option for genetic enhancement is interspecific hybridization between two different species of catfish. Historically, this approach rarely results in a hybrid with higher aquaculture potential than the best-performing parent, but by analyzing the performance of 42 distinct hybrid crosses starting in the mid-1960s, only a single cross exhibited a remarkable advantage over both of the parent species, creating a milestone for aquaculture potential in the catfish industry (Dunham and Smitherman, 1983; Dunham, 2011). The channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *Ictalurus furcatus* ♂ hybrid soon became established in many farms in the Southern U.S. due to their superiority for culture in earthen ponds. Hybrids exhibit improved performance in regards to growth, disease resistance, dissolved oxygen tolerance, feed conversion, and harvestability compared to both parent species (Chatakondi et al., 2000; Brown et al., 2011; Dunham and

Masser, 2012; Perera et al., 2017). Drastic improvement for so many desirable traits in a single cross arguably makes the hybrid the greatest achievement in the field of genetic enhancement in aquaculture (Dunham and Masser, 2012). Due to increased profit yields and production efficiency, they now comprise up to 70% of the annual catfish harvest in the U.S. (Torrans and Ott, 2018).

Recent advances in embryo production technology have worked to make the large-scale production of hybrids viable and profitable (Lambert et al. 1999; Dunham et al. 2000b). In the early 2000s, approximately 1 million C × B hybrid fry were produced, but by 2011 that figure rose to a staggering 100 million with the aid of technological advances and commercial demand (Dunham and Masser, 2012). Improving artificial fertilization techniques has been a large contributor to the rising success (reviewed by Cabrita et al., 2008), altering techniques such as hormonal stimulation of broodstock (Su et al., 2013), sperm extraction/processing (Dunham and Masser, 2012), and sperm densities required to fertilize channel catfish eggs (Bart and Dunham, 1996). Environmental conditions for incubating eggs and fry including temperature (Small and Bates, 2001), salinity (Weirich and Tiersch, 1997), and pH (Mischke and Wise, 2008) have also been studied extensively. With continuous research on the baseline factors that mark success at the earliest production stages, the industry may continue to expect profit gains and overall industrial growth in the near future.

1.3. Limitations of hybrid catfish production and artificial fertilization

However plentiful the benefits of hybrid catfish are, there are additional efforts that must be taken into account in order to produce hybrid fry. Hormonal stimulation of females is a costly

and time-consuming process, requiring extra holding space and special handling techniques. Commercial availability of valuable blue catfish males is limited but remains a necessary investment for hybrid catfish production (Hu et al., 2011). Compared to channel catfish, blue catfish males have slower growth and maturity rates, reaching sexual maturity after 4-6 years (later than channel catfish males) and grow to larger sizes, requiring more feed resources.

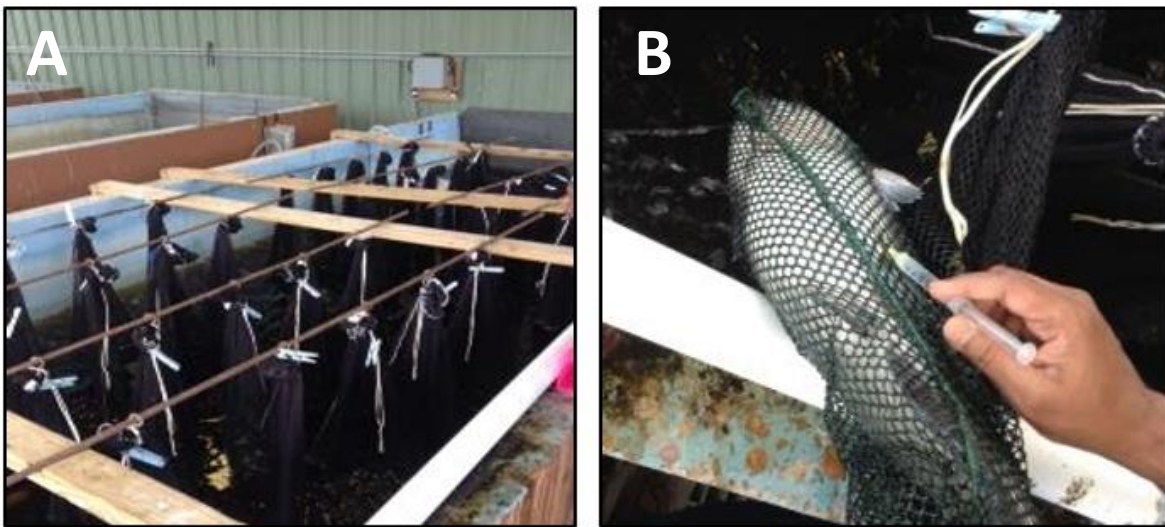


Fig. 1.4. Depiction of the initiation of spawning for female channel catfish. Females are held in soft mesh bags during hormonal stimulation (A). Typically, each fish is then manually administered intraperitoneal injections of luteinizing hormone-releasing hormone analogue, LHRHa (B). Ovulation response is checked routinely, and eggs are stripped immediately after detection to avoid over-ripening in the body cavity.

The major caveat is the lack of natural hybridization between channel and blue catfish due to natural barriers in the reproductive biology and time of spawning (Hu et al., 2011). For this reason, females are induced to spawn by the administration of hormones (Fig. 1.4). Common industry practice is to sacrifice the males and remove the testes for subsequent artificial fertilization (Argue et al., 2003; Hu et al., 2011). Unfortunately, these fish can only be used for a single year of spawning and for only a limited number of egg batches. Thus, each

individual male is highly valuable, and it is critical to initiate methods that efficiently utilize these indispensable gamete resources. As a result, it has also become important in all fields of aquaculture to study the effects of individual sperm and egg quality and their impacts on fertilization outcomes, early development, and recruitment output. Therefore, there is a margin to improve broodstock selection, allocating time and resources toward fish with better reproductive potential.

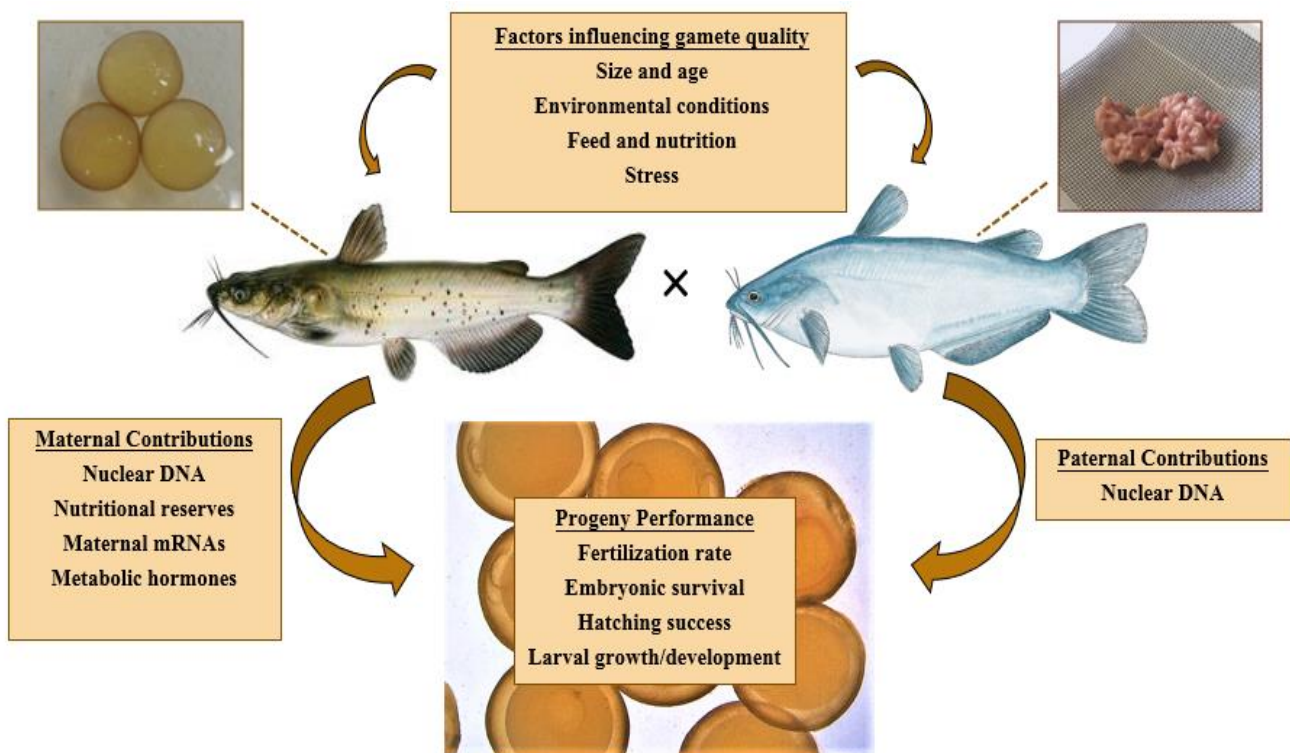


Fig. 1.5. Gamete quality of individual broodfish can be driven by a variety of factors, many of them environmental and can be manipulated in the hatchery environment. Both parents administer genetic material to the progeny. However, the female also contributes many additional necessary elements through the yolk, which sustain the embryos and fry through the early life history stages. Combined gamete quality from both parents may consequently affect progeny performance, which can be monitored at several time points during early development.

1.4. Reproductive biology and the importance of gamete quality in aquaculture

Knowledge of the reproductive biology of each cultured fish is essential to satisfy the specific requirements for that species, and as a result, maximize production efficiency in the hatchery environment. For both parents, environmental factors such as nutritional diet, stress levels, and water quality have respective effects on gamete quality and subsequent progeny performance and must be carefully monitored prior to and during the spawning season (reviewed by Bobe and Labbé, 2010) (Fig. 1.5). Gamete quality, defined as the capacity of eggs and sperm to fertilize and produce viable offspring (Bobe and Labbé, 2010), can be assessed in a variety of ways, but to date it remains challenging to determine prior to fertilization and remains neglected by the aquaculture industry (Migaud et al., 2013). For females, physical attributes, nutritional status, stress exposure, and egg maturation are also driving factors as to how many eggs can be produced and their overall quality (Bromage et al., 1992; Aegerter et al., 2005; Dunham, 2011). In catfish, specifically, egg quality has been closely linked to fertilization success (Dunham and Argue, 2000; Bosworth and Waldbiser, 2014). Egg quality also has lasting impacts on hatching rates, embryonic survival, and larval developmental stages since young fish are almost solely dependent on the nutritional value of the yolk until transition to the first feeding stages (Cabrita et al., 2008). It is possible to rank “good” and “bad” eggs by visual inspection using traits such as buoyancy in the water column and egg size (Brooks et al., 1997), and transparency and/or coloration (Bromage, 1995) (Fig. 1.6). Microscopically, it can also be done by observing embryonic survival and abnormalities during benchmark developmental stages (Migaud et al., 2013). However, reproductive success is not always reliant on these factors, which has encouraged research to look into molecular indices including gene expression of mRNAs

(Aegerter et al., 2005; Mommens et al., 2010), proteomics (Castets et al., 2012), and metabolomics (Samuelsson and Larsson, 2008).

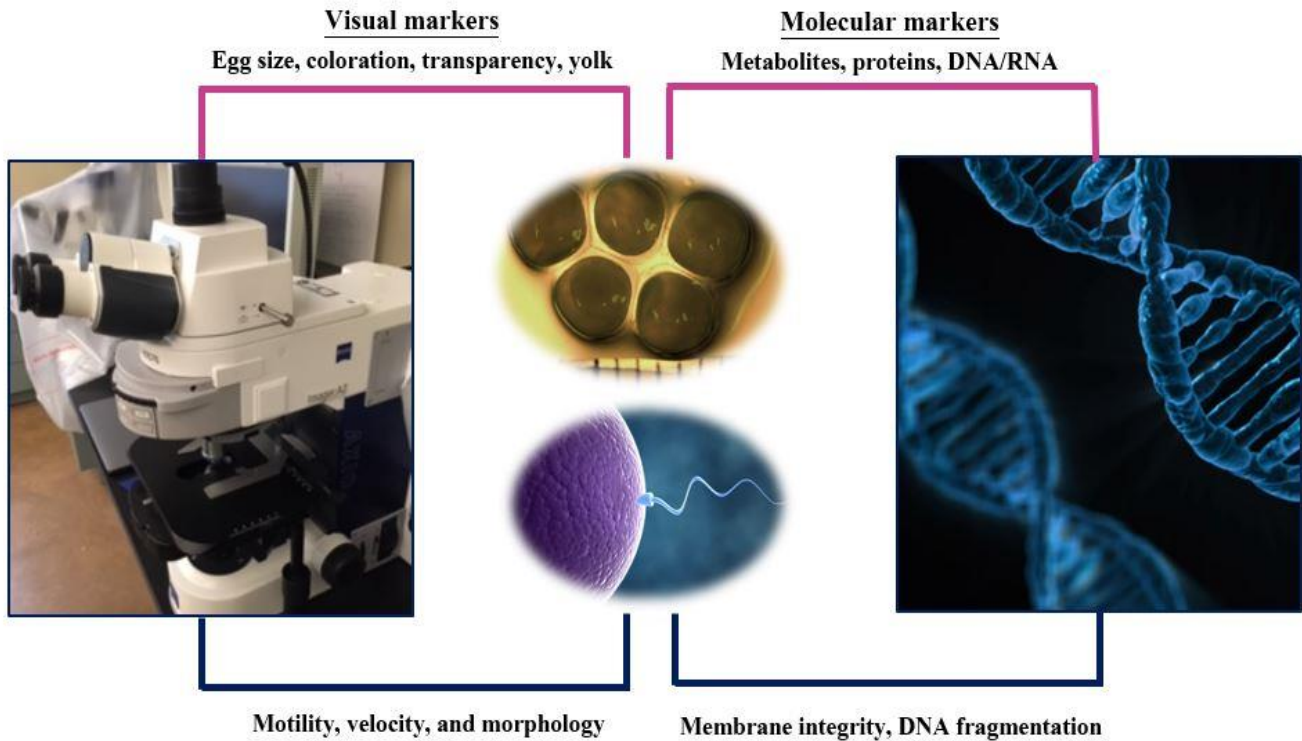


Fig. 1.6. Egg and sperm quality can be analyzed in many ways, either visually or with molecular markers. With advancements in DNA technology, it has also become possible to link gamete quality with gene expression and compounds important in intracellular pathways.

Although primary focus has historically been centered on egg quality, sperm from the male also varies in quantity and quality and also contributes to reproductive success, or lack of it (Rurangwa et al., 2004). This is particularly the case for species that do not readily release sperm by hand stripping (the blue catfish being one of several prime examples). Despite its recognition, however, pooling sperm from different males together for artificial fertilization is a common practice in hatcheries (Rideout et al., 2004). Therefore, hatcheries often forego assessing the quality of individual males and utilize pools that potentially have both high and low-quality males. Following this procedure, not all males fertilize equal amounts of eggs when differences

in gamete competitive ability exist (Bekkevold et al., 2002; Mjølnerød et al., 1998). Thus, fry produced may be dominated by the superior males, skewing paternity ratios within hatchery-reared cohorts. As studies reveal the importance of individual males (not just the females) has an impact on the next generation, it is becoming more important to isolate indicators of male sperm quality prior to fertilization.

Like eggs, male gametes may also be examined microscopically for motility parameters as estimates of overall quality (Fig. 1.6.). In general, the higher the percent motility and the faster the velocity, the better the fertilization potential (Gage et al., 2004). Other sperm quality estimators are also utilized based on morphological, biochemical and molecular/genetic characteristics (Bobe and Labbé, 2010). Motility can be estimated prior to fertilization with the aid of subjective assessment by an observer or with more advanced technology like computer assisted sperm analysis (CASA) software, which is steadily becoming more routine in assessing male fertility across fish species (Rurangwa et al., 2001). Interestingly, sperm motility and velocity can be directly affected by physical properties and biochemical composition of the activation environment, which can result in high variability in overall performance (Alavi and Cosson, 2005; Cosson et al., 2008).

A specific component of interest in the sperm activation solution is the presence/absence of female ovarian fluid (OF) that surrounds the egg batch (Fig. 1.7). This fluid has important functions as a chemo-attractant and for biochemical signaling for the sperm cells and has been shown to greatly influence swimming performance and fertility (Yanagimachi, 2013). Several studies have reported that ovarian fluid enhances sperm velocity, motility, and longevity (reviewed by Zadmajid et al., 2019). In some cases, ovarian fluid can also act as a platform for male × female interactions at the gametic level. On an evolutionary scale these interactions have

led to intra-specific sexual selection (also termed cryptic female choice; Thornhill, 1983) across the wide array of externally fertilizing fishes, caused by the unique and variable OF composition between females (Evans et al., 2012). The OF surrounding each female's eggs may select for specific genotypes or phenotypes (Alonzo et al., 2016) that give sperm from certain males a competitive advantage. Hence, the ability to control evolution at microscopic scales may be one of the most powerful selection pressures driving reproduction and consequent evolutionary processes (Rosengrave et al., 2009). Knowledge of these underlying gamete interactions may further our understanding of natural reproductive processes governing sperm performance, mating systems, and fertilization dynamics. Prior to this project, gamete interactions and their impacts on sperm performance have not yet been studied between channel and blue catfish. Overall, analyzing gamete quality and associated interactions between eggs and sperm is crucial, as it helps identify the most suitable broodfish for spawning.

1.5. Maternal and paternal effects on early ontogeny in fishes

Parental effects (a collective term referring to maternal and paternal components) and their importance in determining fitness of the next generation are widely recognized (Heath and Blouw, 1998). Of the two, maternal effects are more prominent during early development since females contribute valuable nutritional reserves, important metabolic hormones, and maternal RNAs through the yolk (Kerrigan, 1997; McCormick, 1998; 1999). In poikilotherms, the duration of maternal effects differs but corresponds to the rates of growth, development, and species-specific



Fig. 1.7. Ovarian fluid is secreted with the eggs and contains maternal compounds which have functions within the female reproductive tract. It also gives off chemical signals for sperm during spawning events. In catfish, volume and physical properties of the ovarian fluid dispelled with the egg batch may serve as an indicator of initial egg quality and ripeness.

reproductive strategies (Green, 2008). Overall, maternal effects and their respective impacts on early development have shown to have significant effects on fry performance. Although analyzed in many commercially important hatchery-reared species (primarily marine), there are still gaps remaining in the knowledge base for freshwater species such as the hybrid catfish. By determining that individual female quality impacts fry early in life when they are most susceptible to mortality, the industry can limit their selection to only fish that produce the best-quality eggs, basing this decision on traits proven to be reliable quality indicators.

Previously, paternal effects have been thought to be insignificant since males only contribute nuclear genetic material to the offspring (Heath et al., 1999). Additionally, hatchery practices that pool milt from multiple males for fertilization also eliminate the ability for paternal contributions

to be detected (Bekkevold et al., 2002; Rideout et al., 2004). However, more recent reports have found evidence that males also contribute to larval performance, although effects have not been shown for all larval morphology traits (Kroll et al., 2013). Although impacts of paternity are smaller than the maternal effects, evidence is growing to support that individual identity and quality of each male is of crucial importance during ELH stages (Siddique et al., 2017). The value of individual blue catfish males cannot be underestimated, and since they can only be used once, highlighting the importance of paternal effects will ensure only the worthwhile males are sacrificed for fertilization.

1.6. Objectives

In this project, the history and current status of hybrid catfish aquaculture was detailed, and the studies conducted were completed in order to address some of the remaining reproductive challenges between channel and blue catfish. Chapter 2 assesses the impacts of different sperm densities on hatching success in order to optimize resources partitioned from each male. Chapter 3 turns from the technical aspects of fertilization to assess the importance of gamete interactions (sperm and ovarian fluid) between males and females during sperm activation. Building from our detection of these interactions in catfish species, Chapter 4 is a meta-analysis and systematic overview designed to broaden our knowledge of gamete interactions between female ovarian fluid and sperm on sperm performance across all fish species and across different spawning environments. Chapter 5 implements a full-factorial breeding design between distinct male and female pairings conducted at two different temperatures. This study quantified maternal and paternal effects at each incubation temperature for embryonic developmental stages until the first

feeding stage to study how optimization of the rearing environment can also interact with underlying parentally derived effects. With the focus of this work leading to identifying indicators of broodstock quality, Chapter 6 examines molecular markers of egg quality during early embryonic development to identify if specific genes are differentially expressed between high-quality and low-quality egg batches.

Altogether, this research will contribute to improving hybrid catfish aquaculture but has wide implications for all hatchery-reared species. Optimizing artificial fertilization parameters like sperm densities needed to fertilize channel catfish eggs will lessen the demand for mature blue catfish males and future genetic cryo-banking repositories. Determining how maternal and paternal components influence important developmental traits like embryonic survival, hatch, rate of deformities, and morphology during early life history stages will improve incubation techniques and broodstock selection. This research will also provide preliminary findings on the effects of differentially expressed genes that have been associated with egg quality and embryogenesis for other cultured fish species, offering a genetic basis for egg quality assessment for hybrid catfish.

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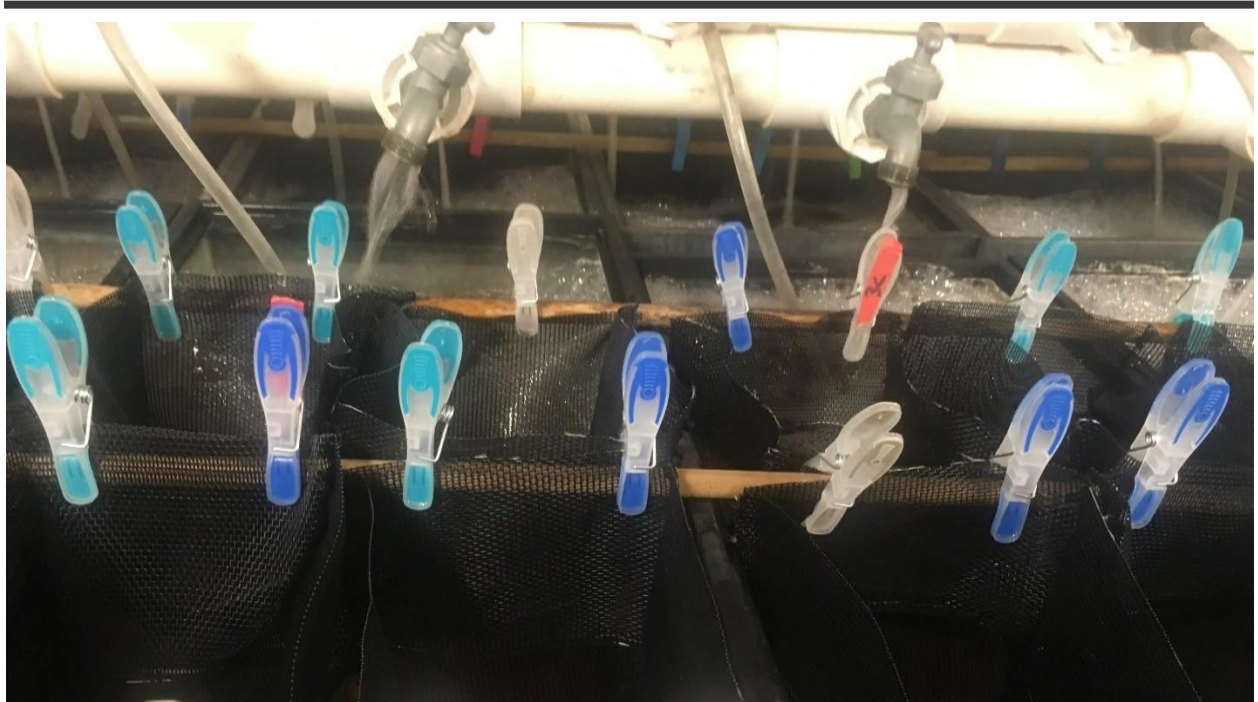
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Chapter 2

Impact of sperm to egg ratio on hatching success for channel catfish, *Ictalurus punctatus* ♀
× blue catfish, *I. furcatus* ♂ hybrid production



Myers, J.N., Nichols, Z., Abualreesh, M.H., El Hussein, N., Taylor, Z.G., Coogan, M., Gurbatow, J., Khoi, V., Zadmajid, V., Chatakondi, N.G., Dunham, R.A., Butts, I.A.E., Impact of sperm density on hatching success for channel catfish (*Ictalurus punctatus*) ♀ × blue catfish (*I. furcatus*) ♂ embryo production. To be submitted to *Aquaculture* in December 2019.

Abstract

Hybrid catfish, the progeny of channel catfish (*Ictalurus punctatus*) females × blue catfish (*I. furcatus*) males, are in high demand by the aquaculture industry due to their superiority for pond and raceway culture in the United States. Unfortunately, fry production can be a limiting factor due to a lack of natural hybridization between the two species and the necessity to sacrifice males for artificial fertilization. Therefore, it is essential to efficiently utilize gamete resources by using the minimum quantity of sperm that allows for the highest fry production from these valuable males. In this study, channel catfish eggs from 9 females were collected from two study different locations and fertilized with 6 different sperm to egg ratios ranging from $1.0 \times 10^3:1$ to $1.0 \times 10^5:1$. Embryos were then incubated under common environmental conditions until hatch. Average hatch success for the 9 females, using sperm to egg ratios between 1×10^3 and $1.0 \times 10^5:1$, ranged from 18.3% to 48.8%. Hatch for the lowest performing female ranged from 0% to 12.5%, while the highest ranged from 22.7 to 81.9% with much greater variability due to sperm density. Overall, there was a significant effect of sperm to egg ratio ($P < 0.0001$), such that hatch success increased from $5 \times 10^3:1$ to $1 \times 10^4:1$ sperm per egg. Thereafter, adding $>1 \times 10^4:1$ sperm per egg had no significant improvement on hatch success. Independently, both study locations indicated significant impacts of sperm to egg ratio ($P < 0.0001$) on hatch success, which ranged from 18.2% to 57.3% at location 1 and from 18.6% to 32.3% at location 2. For location 1, ratios higher than $1 \times 10^4:1$ sperm per egg yielded no increase in hatch, while this density threshold was lower for location 2, where no improvement at ratios higher than $5 \times 10^3:1$ were detected. Thus, lower sperm densities may be optimal under certain conditions. Maternal effects were responsible for up to 93.6% of the hatch variability,

indicating the importance of individual females for fry production. Based on these findings, we suggest that implementing the 1.0×10^4 :1 sperm to egg ratio will standardize hatch success for hybrid catfish production, while conservatively using the least amount of sperm and maximizing the hatching capacity for each blue catfish male.

2.1. Introduction

Artificial fertilization for hatchery production is a technique commonly implemented for fish species that do not achieve synchronous spawning in captivity due to reproductive isolation mechanisms or other physiological barriers (Chereguini et al., 1999). Hybrid catfish, the cross between channel catfish females (*Ictalurus punctatus*) and blue catfish males (*I. furcatus*) are in high demand by the aquaculture industry, but their production capacity is a limiting factor due to a lack of natural hybridization between the two species and the necessity to sacrifice males for *in-vitro* fertilization (IVF; Bart and Dunham 1996; Argue et al., 2003; Hu et al., 2011). In the United States, catfish farming accounts for nearly 70% of total freshwater aquaculture production (Torrans and Ott, 2018), in which hybrids constitute a growing percentage of the total production. Hybrid catfish possess several desirable traits for pond and raceway culture, such as faster growth, disease resistance, higher survival, and improved feed conversion efficiency (Chatakondi et al., 2000; Brown et al., 2011; Dunham and Masser, 2012; Perera et al., 2017). However, maintaining blue catfish males for hybrid production remains a costly and time-consuming endeavor for hatcheries, as blue catfish males reach sexual maturity later (after 4-6 years) than channel catfish males and can only be used once (Dunham et al., 1994). Therefore,

the efficient use of gamete resources by using the minimum amount of sperm necessary may serve to reduce the number sacrificed for artificial fertilization. This, in turn, will improve hatchery production and alleviate demands for mature males and cryopreserved sperm straws housed in genetic repositories (Hu et al., 2011; 2015).

The optimal sperm to egg ratio for fertility applications varies depending on the species and its associated reproductive physiology, specific mating strategies, and features of their gametes (Butts et al., 2009; 2012). For example, standard sperm to egg ratios in other hatchery-reared fishes were $>2 \times 10^5:1$ sperm per egg in wolfish, *Anarhichas lupus* (Moksness and Pavlov, 1996), 6×10^3 sperm per egg for turbot, *Scophthalmus maximus* (Suquet et al., 1995), $5 \times 10^4:1$ for sea lamprey, *Petromyzon marinus* (Ciereszko et al., 2000), and 1×10^5 for Atlantic cod, *Gadus morhua* (Butts et al., 2009), among others (reviewed in Butts et al., 2012). Even within the same species, different sperm densities have been recommended, due to differences in fertilization technique or initial gamete quality (Rincharde et al., 2005; Casselman et al., 2006). Sperm density, or specifically the male investment per ejaculate, can cause great changes in fertility outcomes and consequential hatch success (Casselman et al., 2006). Specifically, for hybrid catfish, Bart and Dunham (1996) reported that sperm to egg ratios lower than $5.0 \times 10^4:1$ caused a significant reduction in fertilization success, while densities of $1.25 \times 10^5:1$ to $1.2 \times 10^7:1$ yielded no difference in fertility. In a more recent review on hybrid catfish production, Dunham and Masser (2012) reported that the recommended sperm to egg ratio for fertilization of channel catfish eggs to be $1.2 \times 10^6:1$. Based on these prior findings, the optimal sperm to egg ratio currently used for artificial fertilization falls within a wide range and may exceed the actual sperm density required to maximize hybrid fry production.

The objective of this work was to provide a more recent assessment of the sperm to egg ratio necessary to maximize hatch success under controlled hatchery conditions, utilizing modern artificial fertilization techniques that address advancements in gamete quality and industry protocols. We hypothesize that hatching success will increase when more sperm are incorporated into an artificial spawning or IVF environment, but the benefit of adding more sperm will diminish as the total number of sperm cells approaches the number required to fertilize all available ova.

2.2. Materials and methods

2.2.1. Broodstock holding conditions and initiation of spawning

All experiments were carried out at 2 respective study locations: the E.W. Shell Fisheries Center and the Fish Genetics Research Unit at Auburn University, Auburn, AL (32.6524° N, 85.4860° W; hereafter, location 1) and the USDA Thad Cochran National Warm Water Aquaculture Center in Stoneville, MS, USA (33.4240° N, 90.9151° W; hereafter, location 2). In total, 6 fertilization trials were conducted at location 1 and 3 trials were conducted at location 2. For both locations, mature female channel catfish were collected from 0.25-1.0 ha aquacultural ponds and transported to flow-through raceway tanks provided with diffused aeration. At location 1, holding temperatures were between 28-30 °C and pH was 6.8-7.2. Prior to experimentation, fish were fed Special Formulation pelleted catfish feed (Fish Belt Feed Mill, Moorhead, MS) daily until satiation. At location 2, holding temperatures were similar (27-30 °C)

with water pH between 8.2-8.5. Fish were fed 3 times a week at 3% body weight with a pelleted 35% protein catfish feed. Females were held in soft mesh bags until ovulation. Female channel catfish were induced to spawn with two intraperitoneal injections of luteinizing hormone-releasing hormone analogue (LHRHa, Syndel USA, Ferndale, WA) using a priming dose of 20 µg/kg female body weight and a resolving dose of 80 µg/kg body weight 12 h later. At 36 h post-injection, fish were checked routinely every 4-6 h for ovulation by visually inspecting the mesh bags for the adherence of released eggs.

2.2.2. Sperm collection and quality assessment

Mature blue catfish males ($n = 18$; mean \pm SEM length = 70.1 ± 8.7 cm, weight = 5.1 ± 2.3 kg) were selected and processed following industrial protocols. In this design, a total of 6 sperm pools each consisting of 3 males were crossed with each female. Testes were dissected and removed from each male using forceps and surgical scissors. Peritoneum and blood vessels were removed manually, the testes were rinsed with 0.9% saline solution, and then macerated through a fine mesh strainer into 50 mL centrifuge tubes. Collected milt was then diluted with saline solution at 8-10 mL/g of testes weight. Sperm samples were immediately stored in refrigerated conditions (4° C) for computer assisted sperm analyses (CASA) within 6 h of collection.

Sperm traits were analyzed using CEROS II software (Hamilton Thorne Biosciences, Beverly MA, USA). Sperm were activated in an 80 µm 2X-CEL chamber (Hamilton Thorne Biosciences, Beverly MA, USA) with distilled water (pH = 8, 22° C) supplemented with 0.5%

BSA to prevent the cells from sticking to the slide. There were 3-5 replicate activations per male. Sperm velocity (curvilinear velocity, VCL; $\mu\text{m/s}$) is regarded as a key determinant of fertility in fishes (Lahnsteiner et al. 1998; Gage et al., 2004; Linhart et al., 2005) and was therefore assessed to ensure adequate sperm quality for each male pool. VCL of motile sperm at 10 s post-activation was used to ensure all sperm pools had similar fertilization capacity and that hatch results were not due to differences in male quality. VCL ranged from 126.9 to 180.6 $\mu\text{m/s}$ with no significant differences between sperm pools ($P > 0.05$).

Milt was pooled from 3 males, and sperm density was quantified using a Neubauer hemocytometer. Cells were counted at 20 \times magnification after a 1:40 dilution with 0.9% saline. Counts of five squares (1 mm^2) were inspected. The mean of the counts for each milt pool was calculated, and then the average of these values was used to determine the actual sperm cells/mL. Milt was then diluted into 1.5 mL microcentrifuge tubes to obtain 6 sperm to egg ratios: 1×10^3 , 5.0×10^3 , 1.0×10^4 , 2.5×10^4 , 5.0×10^4 , and 1.0×10^5 :1. Based on prior results and our goal of determining the minimum amount of sperm needed to maximize hatch success, we did not test ratios higher than 1.0×10^5 :1. The final volume per tube was standardized to 1.0 mL using 0.9% saline with 4 replicates for each ratio.

2.2.3. Egg collection and quality determination

Once ovulation was detected, females (mean SEM length = 57.5 ± 1.3 cm, weight = 2.2 ± 0.1 kg) were anaesthetized with 200 ppm MS-222 (tricaine methanesulphonate; Argent Laboratories Inc., Redmond, WA, USA) buffered with 400 ppm sodium bicarbonate to minimize

stress during handling. The urogenital pore was wiped dry with a towel, and eggs were released by gently squeezing the abdominal area toward the vent. Special care was taken to avoid contamination with urine, blood, or feces. Eggs were hand-stripped into round steel pans (30 cm diameter) lined with Crisco vegetable lipid extract to reduce adherence to the surface. Egg density was estimated by weighing and counting ~5 g of eggs with two replicate counts to obtain the total number of eggs per gram. The mean for the 9 females was 51.2 eggs/g and ranged from 40 to 70 eggs/g. A positive linear relationship has been detected between ovarian fluid pH of stripped channel catfish eggs and hatch success (Chatakondi and Torrains, 2012). Thus, ovarian fluid pH was measured after stripping using a handheld pH meter (Symphony B10P, VWR, Radnor, PA). Based on this criteria, all females (n = 9, ranged from 7.6 to 8.0) were deemed as being of “high-quality”, as ovarian fluid pH was >7.4 (Chatakondi and Torrains, 2012).

2.2.4. Artificial fertilization

Mean (\pm SEM) aliquots of 250 ± 10 eggs (5g), were weighed out according to each female’s calculated egg density (see Section 2.3) and transferred into 100 mL plastic cups lined with a thin layer of Crisco vegetable lipid extract. Sperm solutions for each ratio were pipetted directly onto the eggs, cups were gently swirled for 5 s, and the sperm and egg mixtures were allowed to incubate for 2 min. Each cup was then activated with 5 mL of hatchery water (27.5 to 28.5 °C) supplemented with 6 g/L Fuller’s Earth (Mesh 100-200; MP Biomedicals, Santa Ana, CA, USA) to reduce clumping of the eggs. Cups were incubated for another 2 min with

continuous manual stirring to ensure sufficient gamete contact and then transferred to incubation aquaria.

2.2.5. Embryo and fry incubation

Eggs and embryos were held in similar conditions at both experimental facilities, utilizing recirculation aquaculture systems (RAS) equipped with artificial lighting and a simulated natural photoperiod (12 h light/12 h dark). Dissolved oxygen was >7.4 mg/L and temperature was held between 27.5 to 28.5° C during the holding period. Each tank was provided with continuous water flow of 3-5 L/min. Eggs and embryos were held ~10 cm below the surface of the water in mesh screen baskets (18 × 16 × 14 cm) incubated within larger aquaria (60 × 30 × 32 cm). Ammonia and nitrate levels were kept < 0.05 mg/L, pH between 7.0-7.5, and hardness between 60-75 ppm. General water quality was monitored daily using freshwater aquarium test kits and oxygen levels with a YSI ProODO meter (YSI Inc., Yellow Springs, OH, USA). Dead eggs were identified visually by their white, opaque color or enlarged size and removed daily to minimize risk of fungal and bacterial infections. Dead eggs that were adhered to fertilized eggs were left undisturbed to avoid damage to the fertilized eggs.

2.2.6. *Statistical analyses*

Data were analyzed using SAS statistical software (SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro–Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT). A-posteriori analyses performed on fixed effects were constructed using Tukey's HSD multiple comparisons procedure.

Hatch success data were analyzed using several different approaches. First, data from all females were analyzed together to get an overall effect and then analyzed independently by location (i.e. Auburn University is location 1 and USDA is location 2). For each of these models, hatch success was analyzed using a mixed-model factorial ANOVA (PROC MIXED) with the sperm to egg ratio (fixed factor), and female (random factor) main effects, as well as the corresponding sperm to egg ratio \times female interaction (random factor). A-posteriori analyses were not performed on random effects. Instead, percent variability for these random factors were constructed using variance components according to Littell et al. (1996), Messina and Fry (2003), and Fry (2004). Secondly, hatch success was analyzed for each individual female using a series of one-way ANOVA models, where female was considered a fixed factor. Thirdly, for each female, the coefficient of variation ($CV = \text{standard deviation (SD)} / \text{mean} \times 100$) was calculated for each sperm to egg ratio. Thereafter, the female was considered the replicate and a one-way ANOVA was used to compare the CV (or between-female variability) between the sperm to egg ratios. Finally, curvilinear regression was used to determine the relationship between sperm density and hatch success. To determine the model of best fit for the data, relationships between sperm density and fertilization/hatch success were assessed in previous

studies and appropriate models were examined. The quadratic and logarithmic models were constructed using the PROC REG statement following methods by McDonald (2014). Visual interpretation of the shape of the curve as well as the highest r^2 value determined the model of best fit. All values are expressed as means \pm SEM, and alpha was set at 0.05 for main effects and interactions.

2.3. Results

Overall average hatch success with sperm to egg ratios between 1×10^3 and $1.0 \times 10^5:1$, ranged from 18.3% to 48.8% (Fig. 2.1). Hatch success increased significantly from 5×10^3 to $1 \times 10^4:1$ sperm per egg ($P < 0.0001$). Thereafter, adding $>1 \times 10^4:1$ sperm per egg caused no significant improvement in hatch success (Fig. 2.1A). Of the two random effects, variation in hatch due to female was highly significant ($P < 0.0001$) and explained the largest amount of variance in the model (variance component = 93.6%), while the sperm to egg ratio \times female interaction was non-significant ($P > 0.05$, variance component = 1.2%).

Independently, both locations indicated significant impacts of sperm to egg ratio ($P < 0.0001$) on hatch success, which ranged from 18.2% to 57.3% at location 1 (Fig. 2.1B) and from 18.6% to 32.3% at location 2 (Fig. 2.1C). For location 1, ratios higher than $1 \times 10^4:1$ sperm per egg yielded no increase in hatch. This density threshold was lower for location 2, where no improvement at ratios higher than $5 \times 10^3:1$ were detected. At location 1, the individual female effect was significant ($P < 0.0001$), accounting for up to 68.7% of the variation in hatch due to random effects. At location 2, the female variance component was even higher (91.2%), while no

significant sperm to egg ratio \times female interaction was detected for either location (variance components of 5.4% and 1.7%, respectively). For 8 of the 9 females, the effect of sperm to egg ratio on hatch was significant ($P < 0.05$), and there was observable variation across the ratio gradient (Fig. 2.1D-L). Hatch for the lowest performing female ranged from 0% to 12.5% while the highest ranged from 22.7 to 81.9% with much greater variability due to sperm density. For females 1, 2, 8, and 9, the $5 \times 10^3:1$ sperm to egg ratio was optimal, while for females 3, 4, 6, and 7 the $1 \times 10^4:1$ was optimal. Female 5 had relatively high hatch at all ratios (ranging from 40-62%), and there were no differences between sperm densities. The coefficient of variation did not significantly differ across the sperm to egg ratio gradient ($P = 0.155$), such that the lower ratios had the same variability as the higher ones.

The logarithmic model had the highest r^2 value (0.230 for logarithmic vs. 0.217 for quadratic) and was deemed as the most representative relationship between sperm to egg ratio and hatch success ($y = -25.24 + 6.52 \cdot \ln(\text{abs}(x))$; $r^2 = 0.230$; $P = 0.002$; Fig. 2.2).

2.4. Discussion

Fish populations, both captive and wild-caught, are dependent on high-quality gametes for production of valuable offspring. The importance of maternal effects on the reproductive potential of fish has gained much support in research circles while studies on paternal (or sperm) effects have been underrepresented (e.g. Siddique et al. 2017). At first inspection this makes sense as sperm seems to be unlimited in supply and the limited production of larger eggs make them more valuable. However, hatcheries, knowingly or not, have been concerned about sperm

quality ever since aquaculture began since hatcheries often pool milt from multiple males in an attempt to ensure high fertility. Hybrid catfish hatcheries are no exception. Therefore, quantifying the optimal sperm to egg ratio needed for artificial fertilization protocols is essential to achieve maximum production efficiency and is often highly species-specific (Butts et al., 2009; Beirão and Ottesen, 2018). This is especially critical for hybrid catfish production, as blue catfish testes must be surgically removed, crushed, and diluted to make fertilization solutions. Therefore, minimizing the amount of sperm required to maximize hatch will advance artificial reproductive technologies by utilizing gamete resources more conservatively. Here, by using hatch success as an indicator, our results showed that sperm to egg ratio has a significant impact on overall production of hybrid catfish fry and that previously, an excess amount of sperm has been utilized for artificial fertilization.

For freshwater species like blue catfish, sperm are only active for a short window of time (Billard and Cosson, 1992; Alavi and Cosson, 2004; Alavi et al., 2007). Thus, when sperm density is too low, the cells have a smaller chance of locating and penetrating the micropyle, resulting in low fertility and consequently lower hatch success (Beirão and Ottesen, 2018). On the other hand, above a certain threshold there is no additional gain by using higher proportions of sperm per egg (Butts et al., 2014). In many cases, fertilization success plateaus after a certain density is reached (Rinchard et al., 2005; Casselman et al., 2006; Butts et al., 2009; 2012; 2014; Beirão and Ottesen, 2018). Previously, Bart and Dunham (1996) reported that the mean fertilization rate of channel catfish eggs using different densities of blue catfish sperm was highly variable. They found a significant reduction in hatch success with sperm to egg ratios lower than $5.0 \times 10^4:1$ and highest success with ratios between $1.25 \times 10^5:1$ to $1.2 \times 10^7:1$, with no discernible difference beyond the lower threshold. Additionally, Dunham and Masser (2012)

recommended using a ratio of $1.2 \times 10^6:1$ for artificial fertilization. Our results showed that hatch success increased by 30% from the lowest to highest sperm to egg ratio and that at higher densities, more sperm were able to effectively fertilize each egg batch. Based on data from all 9 females, lower hatch success was detected at $1.0 \times 10^3:1$ and $5.0 \times 10^3:1$ sperm per egg. However, at one of our study locations (and for 4 individual females) there was no improvement in fertility at ratios higher than $5.0 \times 10^3:1$, and for one female there was no difference across the sperm density gradient. Thus, lower sperm densities may be optimal for certain gamete combinations, which deserves further exploration. Nevertheless, we suggest that hatcheries should conservatively implement the $1.0 \times 10^4:1$ sperm to egg ratio to maximize hatching capacity for each blue catfish male.

In the present study, most of the remaining variability in hatch success (not due to sperm density) was due to maternal factors since hatch success varied significantly between females. Hatch success in our study was analogous to the wide reported values for hybrid catfish between 10-85% (Dunham et al., 2000; Phelps et al., 2007; Gima et al., 2014; Silverstein et al., 1999; Small and Chatakondi, 2006; Chatakondi and Torrans, 2012). In general, for many species subjected to artificial spawning, hatch success fluctuates due to a variety of factors such as initial gamete quality (Chereguini, 1999; Bobe and Labbé, 2010), fertilization techniques (Phelps et al., 2007), and egg incubation conditions (Small and Chatakondi, 2006). In catfish, specifically, hatch success depends not only the quantity of sperm but also the quality of the eggs (present study). Even within the same species, variation in sperm to egg ratios exist due to the above factors and their associated interactions (Rinchard et al., 2005; Casselman et al., 2006; Butts et al., 2012).

Maternal effects are attributable to natural differences in female egg quality (Bobe and Labbé, 2010; Butts et al., 2014), which may be due to physical differences among individuals such as age, size, and condition at the time of ovulation (Dunham, 2011). For example, differences in egg ripeness (the amount of time that unfertilized eggs have been retained in the body cavity after ovulation) occurs between females, and quality can deteriorate rapidly if the eggs are not expelled readily (Kamler, 2005; Ochokwu et al., 2015). Furthermore, some females produce egg batches that are also more inclined to harbor bacterial and fungal growth during incubation, which can cause deleterious losses in hatch success (Small and Chatakondi, 2006). All of these factors provide evidence that hatch from specific females may be variable and not only reflect the sperm density used to fertilize them but also the quality of gametes. Thus, higher amounts of sperm may be needed to fertilize a single egg when the quality of gametes is poor. A possible explanation for this observation provided by Suquet et al. (1995) is that fertility may be linked to the decrease in sperm potential due to an unfavorable fertilization environment created by each female (Fauvel et al., 1993). This is likely caused by the ovarian fluid dispelled with each egg batch (Fauvel et al., 1993; Lahnsteiner et al., 1995). Physical and biochemical properties (i.e. osmolality, viscosity, pH, ion composition) of the ovarian fluid are unique for each female and have been shown to interact with sperm from specific males via biochemical signaling (reviewed Zadmajid et al., 2019). Properties of the female ovarian fluid, thus, can be assessed as indicators of egg quality. For example, prior research has shown that low ovarian fluid pH led to a reduction in hatch success for channel catfish (Chatakondi and Torrains, 2012). By using females with low ovarian fluid pH, perhaps lower hatch success data would have been observed unrelated to the sperm density treatments. Based on the work by Chatakondi and Torrains (2012), in in this study, ovarian fluid pH was between 7.6 and 8.0 and was confirmed as

an indicator that all females were of similar quality at the time of stripping. Attributes such as osmolality and ion composition were not assessed in this study but may have been responsible for some of the underlying female variability, emphasizing the importance of identifying these specific indicators of egg quality. Overall, this also highlights the need to select high-quality females as broodstock in order to obtain higher hatch egg batches but perhaps also to provide more favorable fertilization conditions for sperm. To account for the differences created by maternal variability, efforts should continue to be invested in advancing channel catfish broodstock condition and identifying determinants of egg quality (i.e. maternal mRNA and genetic factors; Bobe and Labbé, 2010; Rozenfeld et al., 2016) in order to select the best females for reproduction (Migaud et al., 2003).

Lastly, based on our current findings and those reported in the literature, we created a simple model to predict how many eggs an individual male can fertilize using the current sperm to egg ratio implemented by the industry and the one we propose (1×10^4 :1 sperm per egg). Following our methods, macerated testes from an average blue catfish male (age = 5-9 years, weight = 5.3 kg, testes weight = 10.0 g; Bart and Dunham, 1996; Dunham et al., 2000; Kristanto et al., 2009; Dunham and Masser, 2012) would typically be diluted with 7-10 mL of diluent/g of testes weight. Based on our results, this sperm solution would have an average density of 0.76×10^8 to 1.09×10^8 sperm/mL. Therefore, using 1.25×10^5 :1 as the minimum published sperm to egg ratio for hybrid catfish (Bart and Dunham, 1996), 1 mL of this sperm solution could fertilize between 610 to 872 eggs, while our minimal recommended sperm to egg ratio (1.0×10^4 :1) could fertilize between 7,600 to 10,900 eggs (up to a 12 fold increase in productivity). This model demonstrates that the catfish industry can utilize a lower sperm to egg ratio to rapidly

increase production efficiency while conserving blue catfish males and their precious gamete resources.

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Figures

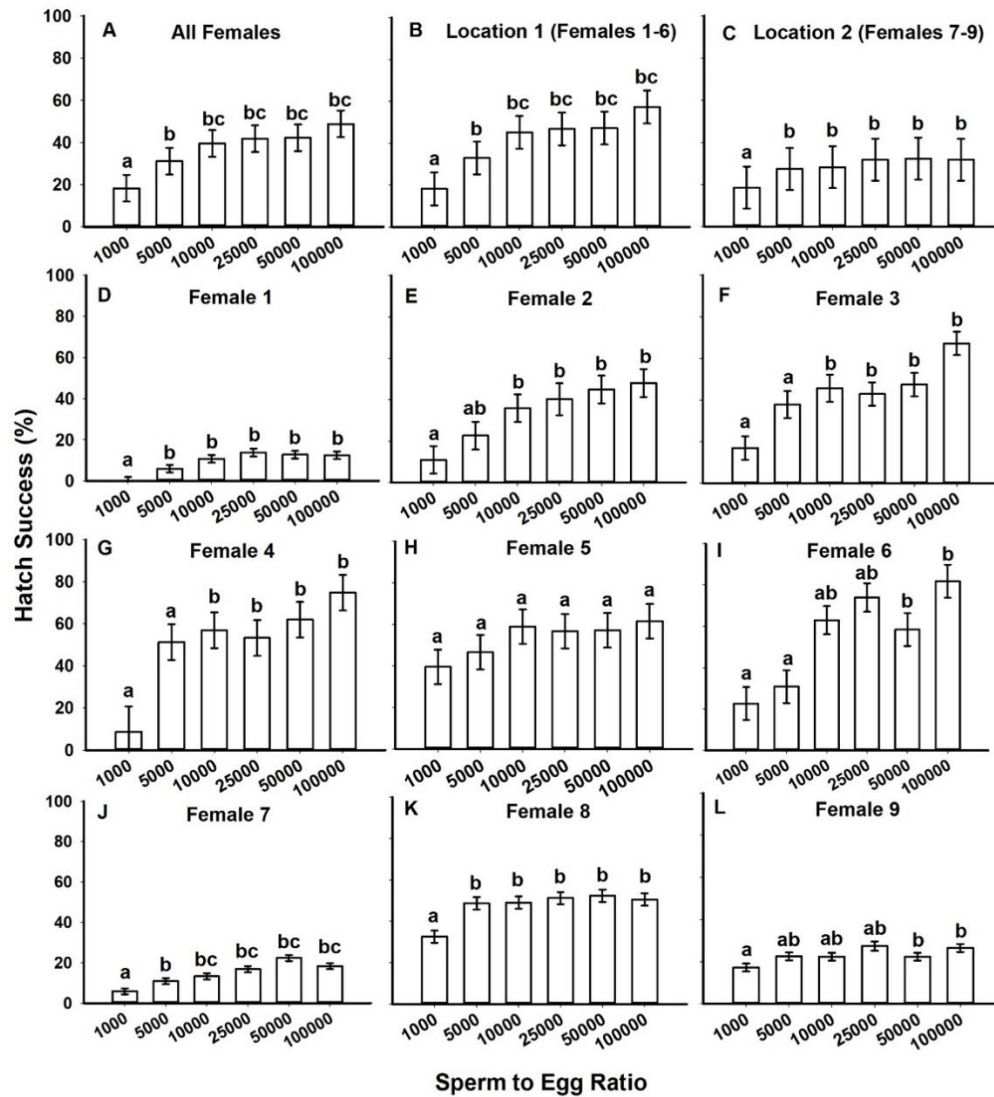


Fig. 2.1. Mean hatch success for hybrid channel catfish (*Ictalurus punctatus*) ♀ × blue catfish (*I. furcatus*) ♂ embryos using sperm to egg ratios ranging from $1.0 \times 10^3:1$ to $1.0 \times 10^5:1$ were analyzed using a mixed-model factorial ANOVA. Results are shown for all females (A), by location [E.W. Shell Fisheries Center and the Fish Genetics Research Unit at Auburn University, Auburn, AL as location 1 (B) and USDA Thad Cochran National Warm Water Aquaculture Center in Stoneville, MS, USA as location 2 (C)], and by individual female (n = 9; D-L). Sperm

to egg ratios with different letters are significantly different ($P < 0.05$) by Tukey's HSD tests.

Bars represent least squared means for hatch success \pm SEM.

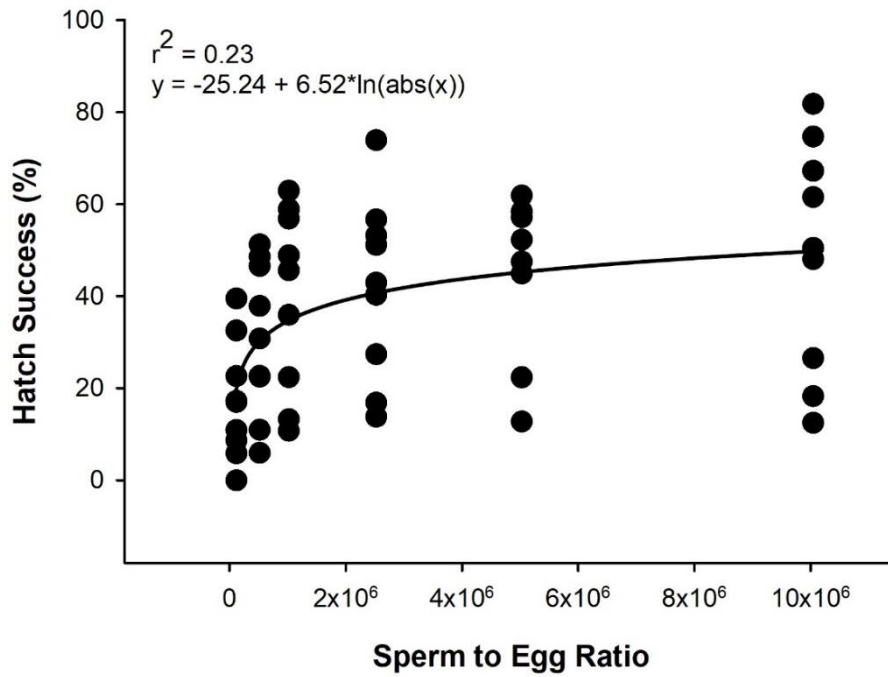
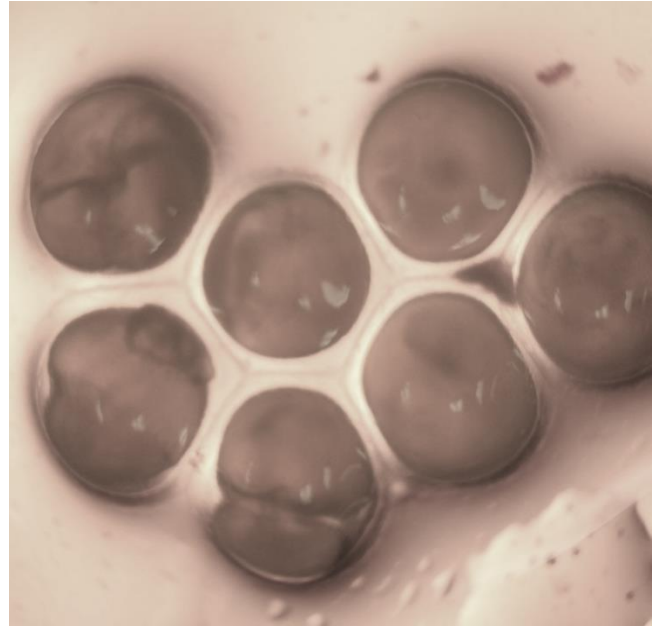
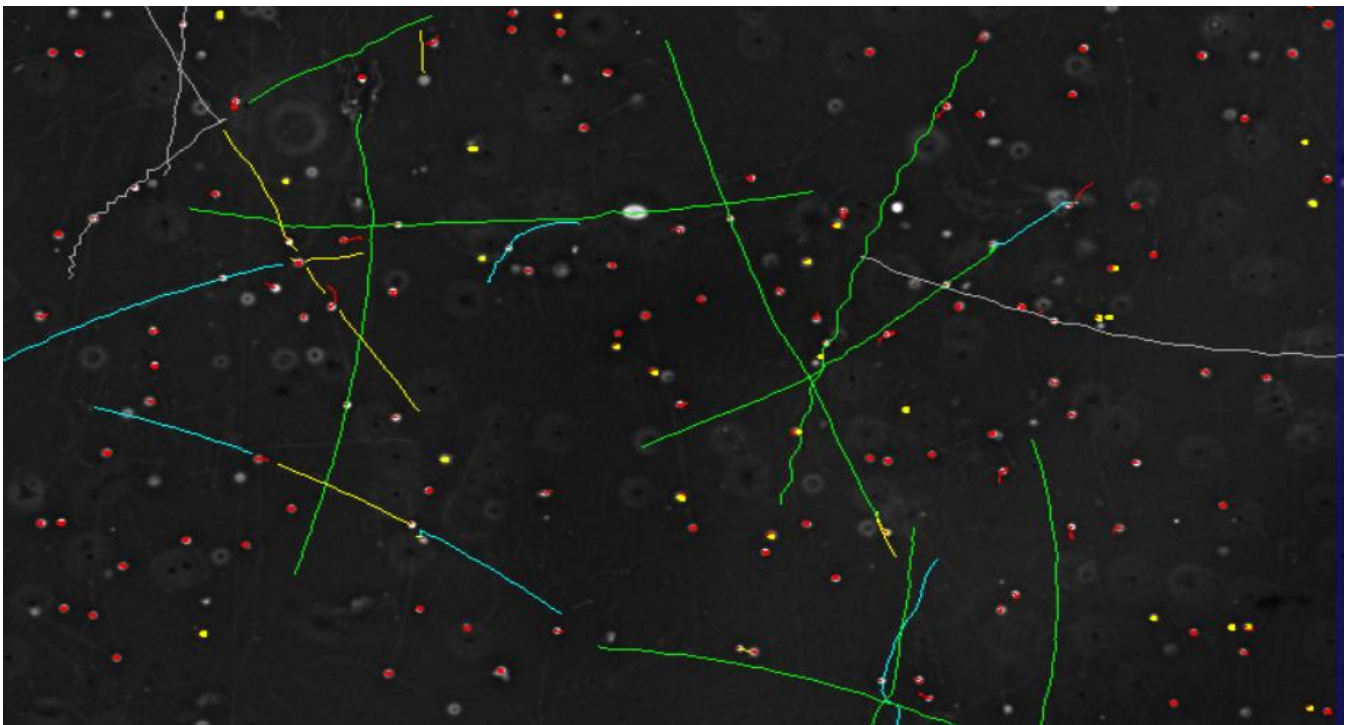


Fig. 2.2. Relationship between sperm density and hatching success for hybrid catfish (*Ictalurus punctatus*) ♀ × blue catfish (*I. furcatus*) ♂ embryo production. These data fit a logarithmic equation, where each point represents hatch success for a single female (n = 9 in total) at each sperm to egg ratio.



Chapter 3

Channel catfish ovarian fluid differentially enhances blue catfish sperm performance



Myers, J.N., Bradford, A.J., Hallas, V.S., Lawson, L.L., Pitcher, T.E., Dunham, R.A., Butts, I.A.E. Channel catfish ovarian fluid differentially enhances blue catfish sperm performance. Under Review, *Theriogenology*.

Abstract

Ovarian fluid (OF) has been shown to alter the swimming behavior of fish sperm, making it a determining factor for fertility and a potential mechanism for cryptic female choice. With the expansion of the channel × blue hybrid catfish industry, it is becoming essential to understand the magnitude of these gametic interactions so that the industry can maximize fertility outcomes. This study was conducted to address the following: 1) activate blue catfish sperm with/without channel catfish OF to determine impacts on sperm performance and 2) assess if sperm behaves differently when activated in the OF from individual females. Sperm (n = 4 males) were activated without OF (control) and with OF from unique females (n = 6), creating 24 experimental crosses. Sperm motility (%) and velocity (VCL) were later analyzed using computer assisted sperm analyses software. For velocity, OF effects were significant for each female and time when compared to the control. OF did not have an impact on motility for any females at 10 s and 20 s post-activation but became apparent at 30 s. In all cases, OF treatments always induced increased longevity, and male × female interactions were highly significant, suggesting that channel catfish OF differentially enhances blue catfish sperm performance. This information has important implications for developing biotechnological techniques to preserve gametes and successfully fertilize and rear embryos in an artificial environment, understanding recruitment variability and recovery strategies for threatened and/or endangered freshwater species, and developing biophysical models that quantitatively describe the fluid mechanics of sperm propulsion in aquatic media.

3.1. Introduction

Fish sperm are dormant in the male reproductive tract, and activation of sperm occurs after release from the genital pore into an aquatic environment (reviewed in Alavi and Cosson, 2006). The duration of sperm motility varies widely among fishes, though generally it is shorter in freshwater than marine species (~1-2 min) (Kime et al., 2001; Alavi et al., 2007). During this critical window, sperm swimming velocity and motility are imperative for successful fertilization because each sperm cell has limited time to locate an egg and subsequently penetrate the micropyle to achieve fertilization (Rurangwa et al., 2001). For externally fertilizing fishes, sperm motility strongly depends on the physical and biochemical properties of the activation environment, including but not limited to temperature, pH (Alavi and Cosson, 2005), osmolality and ionic composition (Alavi and Cosson, 2006), and viscosity (Turner and Montgomerie, 2002). Notably, the presence of ovarian fluid (OF) may considerably alter these parameters, affecting sperm swimming behavior and fertility outcomes (Hatef et al., 2009; Butts et al., 2012; Jia et al., 2015; Lehnert et al., 2017).

OF accumulates before ovulation and is stored inside the coelomic cavity with the eggs (Aegerter and Jalabert, 2004). When the egg batch is released during spawning, this maternal fluid adheres closely to the outer membrane of the egg surface. In addition to OF having important biological functions, prior studies have shown that it may alter sperm behavior and swimming trajectories both positively and negatively. In freshwater fishes, many studies have reported enhanced sperm performance (i.e. percent motility, velocity, longevity) with OF present in the activation media. Many of these cases demonstrated extended longevity, higher swimming velocities, and increased percent motility (Turner and Montgomerie, 2002; Urbach et al. 2005;

Diogo et al., 2010; Galvano et al., 2013). From these results, sperm may be affected because of the ability of OF to act as a chemoattractant that stimulates biochemical signaling and inter-gametic communication. Evidence of chemotaxis has been found for a diverse array of freshwater and marine species, but how this occurs is still a topic of investigation (Kholodnyy et al., 2019). It is hypothesized that factors such as specific herring sperm-activating proteins (HSAPs) and sperm motility initiating factors (SMIFs), enzymes, metabolites, and ions are involved in these processes, depending on the OF concentration and sperm proximity to the egg surface (Oda et al., 1995; Lahnsteiner, 2000; Rosengrave et al., 2009).

Not only does the presence/absence of OF impact sperm performance and fertility but its properties also vary across/within species and even by individual females (Johnson et al., 2014). This may be due to intrinsic differences in female quality, egg ripeness, or differences in OF composition. It remains contested exactly how OF enhances sperm motility and behavior, but the physical and biochemical properties are candidate factors, as they alter the activation micro-environment (Cosson, 2004; Elofsson et al., 2006). Ovarian fluid is unique in its viscosity, pH, osmolality, organic constituents (i.e. proteins, metabolites, enzymes), and ionic composition (reviewed by Zadmajid et al., 2019). When all these different factors are incorporated in the activation media, the OF becomes a platform for chemical signaling and male \times female interactions. For example, in chinook salmon, *Oncorhynchus tshawytscha*, the ions Ca^{2+} and Mg^{2+} in the OF strongly regulate sperm motility (Rosengrave et al., 2009). Additionally, Na^+ , Cl^- , and K^+ have also been identified as important ions affecting motility and that OFs with different concentrations of these ions altered sperm performance (Morisawa, 1994; Dietrich et al., 2007; İnanan and Oğretmen, 2015). Some of these chemical constituents have been shown to

improve energy production and duration by influencing adenine triphosphate (ATP) metabolism in sperm, fueling motility (Perchee et al., 1995; Turner and Montgomerie, 2002).

Differing OF composition between females (which may be related to increased ATP efficiency and other drivers of sperm motility) creates a unique fertilization micro-environment and allows intra-specific sperm selection at the gametic level (Evans et al., 2012). This selection, termed “cryptic female choice” (Thornhill, 1983) dictates that females select for specific genotypes or phenotypes that increases genetic quality of their progeny and give sperm from certain males a competitive advantage (Alonzo et al., 2016). Hence, the ability to control reproductive success at microscopic scales may be one of the most powerful selection pressures driving reproduction and subsequent evolutionary processes (Rosengrave et al., 2009). Previous studies have explored male × female interactions and have found remarkable variability in sperm swimming behavior of specific males when sperm was activated in OF from different females (Urbach et al., 2005; Rosengrave et al., 2008). Evolutionarily, these changes in gamete behaviors may have developed to prevent the mating of closely related individuals in some fishes (Yeates et al., 2013). Alternatively, sperm containing nuclear DNA of more related genotypes (both intraspecific and interspecific) are sometimes favored as well (Butts et al., 2012). These interactions, however important in swaying fertility outcomes, are yet to be explored for certain cultured fishes.

Hybrid catfish, the cross between channel catfish, *Ictalurus punctatus*, females and blue catfish, *Ictalurus furcatus*, males now account for a large percentage of total US aquaculture production (Torrans and Ott, 2018). However, because reproductive isolating mechanisms between the two species prevent natural spawning, *in-vitro* fertilization is applied in which sperm and eggs are stripped and mixed together manually. With hybrid catfish aquaculture steadily

growing, it has become essential to maximize fertility outcomes during artificial fertilization. One way of accomplishing this is studying gamete interactions during the short window of contact in order to understand the overarching mechanisms of fertility and improve artificial fertilization techniques. As mentioned previously, the activation period for many freshwater species (including blue catfish) is very short when analyzed by typical sperm assessment methods (described by Fauvel et al., 2010). However, previous quantifications of sperm activity for this species have not simulated natural conditions because they lacked OF in the activation media. The ability for OF to interact with sperm and possibly enhance its performance indicates that the fertilization capacity for each male may be much greater than what has been previously observed because OF may lengthen the sperm activity window. The impacts of cryptic female choice found in other species give reasons to believe that specific male/female pairs may also lead to differential sperm behavioral interactions in relation to sperm motility, velocity, and longevity metrics (Urbach et al., 2005; Dietrich et al., 2008).

The objectives of this study are to determine 1) if sperm motility, swimming velocity, and longevity of blue catfish are impacted by the presence of channel catfish OF and 2) if sperm from specific males behave differently when activated in the OF from individual females (to identify gamete interactions and potential mechanisms of cryptic female choice). By addressing these specific questions, we hope to highlight the importance of simulating natural spawning conditions when making deductions about male fertility potential. With little known on exactly how OF alters sperm performance and with no previous research done for ictalurid catfishes, it is crucial to identify if male \times female interactions impact fertilization. Our conclusions can be applied to hybrid catfish aquaculture while simultaneously progressing current knowledge of how these processes occur from both aquacultural and ecological perspectives.

3.2. Materials and Methods

3.2.1. Ovarian fluid collection

All fish used for this experiment originated from agricultural research ponds at the EW Shell Fisheries Center at Auburn University, AL, USA. Female channel catfish ($n = 6$; mean \pm SEM weight = 0.87 ± 0.06 kg) were transferred to $260 \times 72 \times 50$ cm flow-through holding tanks and held in soft mesh bags. Temperatures were 28-30 °C, pH was between 6.8-7.2, and water flow was 1.5-2 L/s. Prior to collection, fish were fed once daily with 35% protein pelleted catfish feed until satiation. Females were initiated to spawn with 2 intraperitoneal injections of luteinizing hormone-releasing hormone analogue (priming dose of 20 μ g/kg female body weight and a resolving dose of 80 μ g/kg), LHRHa, (Syndel, Ferndale, WA, USA) following protocols by Dunham and Masser (2012). Starting at 36 h post-injection, fish were checked routinely every 4-6 h for ovulation. Fish were removed from the bags when eggs were seen adhered to the mesh. At that time, they were sedated in tanks with 200 ppm MS-222 (tricaine methanesulphonate; Argent Laboratories Inc., Redmond, WA, USA) buffered with 400 ppm sodium bicarbonate to stabilize the pH and minimize stress during handling. Care was taken to dry the vent to avoid contamination from urine or feces. The eggs were stripped from the body cavity and filtered through a 1 mm mesh screen over a 250 mL beaker to separate the OF from the eggs. The volume of the collected OF was small (~0.5 mL per female) and was therefore pipetted from the bottom of the beaker and transferred to 1.5 mL microcentrifuge tubes. To remove egg debris, blood, and other residue, OF was centrifuged (Mikro 200, VWR, Radnor, PA) at 4° C at 5,000

rpm for 10 min (Hatef et al., 2009), and only the supernatant was used for experiments. The OF pH was obtained directly by a B10P Benchtop pH meter (VWR, Radnor, PA, USA). Osmolality of the fluid was obtained from each individual using a Vapro 5600 osmometer (Wescor Inc, Logan, UT, USA), and the mean was taken of two replicates. A summary of the OF properties for each female are provided in Table 3.1.

3.2.2. Sperm collection

Blue catfish males ($n = 5$; mean \pm weight = 3.53 ± 0.18 kg) were euthanized following industrial protocols, and testes were dissected from the body cavity using forceps and surgical scissors. The whole testes were rinsed with 1X PBS, drained, and then finely macerated through a mesh screen. For microscopic examination, it is recommended to use a predilution step in a medium that does not initiate motility in order to reduce sperm solution viscosity and better enable it to mix with the activation media (Billard and Cosson, 1992; Cosson, 2004). Therefore, collected milt was further diluted to 8-10 mL/g of testes weight in non-activating 1X PBS to $\sim 6.5 \times 10^7$ sperm/mL, which is standard for use in artificial fertilization of catfish (Dunham and Masser, 2012). Sperm solutions were kept at 4° C, and all trials were conducted within 18 h of processing to ensure there was no degradation of sperm quality/viability due to prolonged storage time.

3.2.3. CASA sperm motility assessment

Sperm samples from all males were assessed to ensure sufficient quality immediately after collection using computer-assisted sperm analysis (CASA) and CEROS II software (Hamilton Thorne Biosciences, Beverly MA, USA) equipped with a 10 × magnification negative phase objective and AX10 Lab.A1 microscope (Carl Zeiss Meditec Inc., CA, USA). Sperm (< 0.1 μL) was activated in an 80 μm 2X-CEL chamber and a 22 × 22 mm glass coverslip (Hamilton Thorne Biosciences, Beverly MA, USA). Each treatment was done in triplicate. Videos were taken at 10 s post-activation and every 10 s afterwards until cessation, defined as the point when motility dropped to < 5%. Percent motility and curvilinear velocity (VCL) are commonly used indicators of sperm performance (Kime et al., 2001; Gage et al., 2004; Linhart et al., 2005) and were analyzed for each activation. CASA sperm detection parameters were optimized on the CEROS II software based on the recommended default settings and after manual adjustments. Camera settings were as follows: images were taken at with a capture speed of 60 frames per second, exposure was set at 4 milliseconds, camera gain at 300, and the integration time at 500 milliseconds. For cell detection, cells were tracked with sizes between 1-8 μm, minimum cell brightness was set at 45, and the photometer range of the illumination fields were between 20-30. Each recorded video frame was checked manually for tracking accuracy. Sperm tracks were removed from analyses if the software incorrectly combined crossing tracks of multiple sperm, split the track of a single sperm, or if a cell exited the observation window before being adequately tracked (Butts et al., 2013).

3.2.4. *Experimental design*

Sperm were activated without OF (control) and with OF from 6 unique females. All trials were conducted at room temperature. For the control solution, 15 μ L of distilled water was used (pH = 8, osmolality = 6 ± 1.3). For the treatments, OF was diluted with an equal volume of the control solution to get a concentration of 50%. OF percentages as little as 5-10% (Beirão et al. 2014, 2015; Galvano et al. 2013; Makiguchi et al., 2016) and up to 100% (Dietrich et al. 2008; Yeates et al., 2003; İnanan and Öğretmen, 2015) have been commonly used to assess sperm performance across fish species depending on the species and the amount of fluid expelled with each egg batch. The amount of OF encountered by sperm varies in the activation environment, whether it be natural or artificial fertilization (reviewed by Zadmajid et al., 2019). Due to this study being the first of its kind on blue catfish sperm interacting with channel catfish OF and with our objective of identifying if OF enhances sperm performance, we conducted preliminary testing with 25% OF and also 50% OF. We observed no discernable differences between the two concentrations and therefore used 50% OF, following the methods of previous studies (Turner and Montgomerie, 2002; Rosengrave et al., 2009; Lehnert et al., 2017). A full-factorial design was implemented by crossing 4 males and 6 females, resulting in 24 unique sperm-OF combinations. For each trial, sperm from each male was activated in 15 μ L of the diluted OF solution from each female, and videos were taken starting at every 10, 20, and 30 s. Because longevity outlasted the control in the OF treatments, videos were further obtained at 40 and 60 s and then at every 15 s afterwards until cessation.

3.2.5. Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were evaluated for normality (Shapiro–Wilk test) and homoscedasticity (plot of residuals vs. predicted values) to ensure they met model assumptions. VCL data were $\log_{(10)}$ transformed and motility data were arcsine square root transformed to meet these assumptions. Alpha was set at 0.05 for testing main effects and interactions. Variance components (% of overall variation due to random effects) were constructed using the restricted maximum likelihood (REML) method. Least squared means (LSMs) and standard errors were reported for all motility and velocity data.

We utilized two different statistical approaches to address our objectives. First, we wanted to assess if OF has an impact on sperm performance. Here, we ran a series of one-way ANOVA models at each post-activation time (10, 20, and 30 s) to compare sperm motility and velocity ($n = 4$ replicate males) when sperm were activated with (from $n = 6$ females) and without (control) OF. A posteriori analyses were performed using Dunnett's multiple comparisons method where sperm activated with each female OF was compared to the control. Second, motility and velocity were analyzed using a full-factorial ANOVA with post-activation time as a fixed factor and male, female, and all associated interactions as random effects. With this mixed model, denominator degrees of freedom for all F-tests were approximated using the Kenward-Roger procedure. Variance components (VCs) were constructed as percentages to represent the overall variability in the data due to each random effect. To test for significant variability among VCs greater than zero in the PROC MIXED model, likelihood ratio statistics were generated (Littell et al., 1996) from the -2[Res]tricted log-likelihood estimate of the full model and then with each

VC held to 0 using the PARMS statement. The probabilities were halved to account for the one-tailed probability, and the significance level (p-value) for each random VC was obtained (Littell et al., 1996; Messina and Fry, 2003; Fry, 2004).

3.3. Results

OF did not have an impact on percent motility for any of the females at 10 s ($P = 0.450$; Fig. 3.1A) and 20 s post-activation ($P = 0.483$; Fig. 3.1B). However, a significant effect became apparent as motility for the control treatment neared cessation ($P < 0.0001$; Fig. 3.2) where improvements in motility were detected for three of the six females. Mean sperm motility was 2-3 times higher at 30 s for all females (Fig. 3.1C). For velocity, OF had notable impacts on sperm performance for each female and time when compared to the control (Fig. 3.1D-F). Specifically, at 10 s post-activation, sperm velocity between the control and OF treatments was improved by as much as 61% ($P = 0.0187$; Fig. 3.1D), while at 20 and 30 s improvements in velocity were as high as 53% when compared to the control ($P < 0.0001$; Fig. 3.1E-F).

To explore potential evidence for cryptic female choice, we ran a series of mixed model ANOVAs. Here, as expected, both sperm motility and velocity were significantly impacted by time ($P < 0.0001$). Control treatments, in general, showed the highest motility at 10 s post-activation but then decreased at each time point until cessation, which never outlasted 30 s (Fig. 3.3). In all cases, OF treatments always induced increased longevity beyond that of the control. Sperm activated in OF had a much longer activity window, and decreases in motility were less defined, often remaining constant for multiple time points. Additionally, motility significantly

varied between each male ($P = 0.011$; $VC = 21\%$) and there was a highly significant male \times female interaction ($P < 0.0001$; $VC = 26\%$), showing that channel catfish OF differentially enhances blue catfish sperm performance (Fig. 3.3, Table 3.2). For example, sperm from Male 1 lasted 210 s with OF from Females 4 and 6 but only lasted 105 to 120 s when activated in OF from Females 3 and 5, respectively. For sperm velocity, the interaction term was also highly significant and accounted for 23.1% of the variability (Table 3.2), and no other random effects were significant ($VC = \leq 4\%$). Similar to motility, control treatments had the highest sperm velocity at 10 s that gradually decreased at 20 and 30 s post-activation, but this effect was mitigated with OF from all females (Fig 3.4). There were also large differences between females within each male, such that in one example, sperm from Male 4 swam for 75 s in OF from Females 3 and 5 but up to 135 and 150 s for Females 1 and 2, respectively (Fig. 3.4B). Similar cases of different longevity between females can be observed for the other males in this study.

3.4. Discussion

With hybrid catfish aquaculture steadily growing, it has become essential to understand gamete interactions and the mechanisms of fertility that may help to improve artificial fertilization. This study was the first of its kind to analyze the impacts of channel catfish OF on blue catfish sperm performance, simulating spawning conditions by incorporating OF in the sperm activation media. When sperm were activated without OF, motility duration was remarkably short (~30 s), which is characteristic of freshwater species (Billard and Cosson, 1992; Cosson, 2004). Interestingly, inclusion of 50% OF in the activation media enhanced sperm performance considerably beyond 30 s, highlighting a phenomenon that has been observed in

several other hatchery-reared fishes (Lahnsteiner et al., 2002; Hatef et al., 2009; Rosengrave et al., 2009).

Previously, reports of sperm motility from the extracted testes of blue catfish encompass a wide range with reported values between 26-69% (Lang and Riley, 2003; Hu, 2011). We observed sperm motility on the lower end of this spectrum for blue catfish sperm (<30%), but our value fell within the motility ranges previously reported for this species. Highly variable motility percentages may be due to the necessary lethal methods of extraction and processing as well as the status of gonadal development. It is not surprising, then, that motility is lower compared to fish in which manual, non-lethal stripping of mature sperm cells can be employed. Thus, for species with low initial sperm quality (such as blue catfish), incorporating OF may be even more essential since it enhances critical sperm performance traits and increases the fertilization capacity of each male.

Female OF had a remarkable role in enhancing sperm motility and velocity, both of which are positively correlated with fertility (Gage et al., 2004). It was hypothesized that these parameters would be higher in OF than in water alone based on reports from previous studies. Differences in sperm motility were observed as time progressed and as the control neared cessation. Some studies have shown improvements in sperm performance with OF immediately after sperm activation (Butts 2012; 2017). Alternatively, there are also accounts of delayed responses in which sperm traits were not immediately improved but exhibited greater longevity (Hatef et al., 2009; Elofsson et al., 2003), corresponding to our own findings for motility. Although motility did not show immediate positive effects (improvements observed at 30 s), the differences between the control and OF treatments were immediate for velocity. Freshwater sperm, powered by ATP stored in the cells prior to activation, use up their energy reserves

rapidly after activation, causing motility and velocity to decline as sperm lose propulsive power (Cosson et al., 1999; Butts et al., 2010). For the control, this happens within 30 s, at which there are few sperm cells left exhibiting movement. However, when OF was present, many more cells surpassed this time frame and were still actively swimming at higher speeds, perhaps due to factors of the OF that create a more favorable environment for the sperm (Fauvel et al., 1993). Future research on identifying these sperm-enhancing factors presents the next step to understanding the complex underlying mechanisms of fertility.

In this study, it was evident that male × female interactions altered sperm motility and velocity and that longevity within each male was highly variable depending on the specific female's OF. In some cases, motility duration from one female was almost double than that of another (120 s as compared to 210 s). These results offer the first clear evidence of gamete interactions between channel catfish and blue catfish since sperm responded more favorably to the cues of specific OFs better than others. Thus, it is imperative to look beyond the scope of individual maternal and paternal effects and consider them together. Gamete interactions and consequent sexual selection has been observed in several other externally fertilizing species including Arctic charr, *Salvelinus alpinus* (Urbach et al., 2005), chinook salmon (Rosengrave et al., 2009), lake trout, *Salvelinus namaycush* (Butts et al., 2012), ocellated wrasse, *Symphodus ocellatus* (Alonzo et al., 2016), and zebrafish, *Danio rerio* (Spence and Smith, 2006), among others. Most claims supporting sexual selection stem from fish species with similar reproduction strategies in the wild, in which males and females gather and release massive amounts of gametes freely into the water column. For fish that spawn in large aggregations, gametes endure high levels of competition. Only sperm that reaches the micropyle first can achieve fertilization, making the ability for eggs to attract different sperm types a remarkably powerful selective force

(Stockley et al., 1997; Birkhead and Møller, 1998). However, this phenomenon has also been identified in species that undertake internal fertilization such as the guppy, *Poecilia reticulata* (Pilastro et al., 2004; Gasparini et al., 2011). The common theme to the development of gamete interactions appears to be some degree of male competition. In nature, cryptic female choice may select for unrelated males, serving as an evolutionary tool to prevent inbreeding (Gasparini et al., 2011), but it may also select in the other direction in favor of more related genotypes to avoid breaking up co-adapted gene complexes (Butts et al., 2012; Yeates et al., 2013). Future hatchery research should analyze genetic relatedness and/or phenotypes of individual fish to discover which direction is selected for and what effects it has on the magnitude of gamete interactions. However it occurs, the ways in which gamete interactions alter fertility, hatch, and offspring production in hybrid catfish hatcheries should also be explored.

Variation in physical and biochemical properties of the OF between females alter the activation micro-environment and sperm behavior (Cosson, 2004). Compositions of ions, proteins, and other components have been previously quantified in fishes, but such detailed analyses have not been assessed for channel catfish OF. Knowledge of these factors may establish links to specific OF characteristics that can be tested prior to spawning and how they can be used as indicators of egg quality, thus, improving selection of females for reproduction. From the genetic standpoint, it has been hypothesized that sexual selection in fishes occurs through major histocompatibility complex (MHC) genes (Lehnert et al., 2016). Supporting this claim, Yeates et al. (2008) found that Atlantic salmon, *Salmo salar*, fertility promoted males with similar MHC genes over males that were more genetically distinct. In another example, fertilization success was positively correlated with specific MHC class II genes in chinook salmon (Gessner et al., 2017). For catfish and other teleosts, these genes are yet to be fully

explored and provide promising areas for future molecular analyses as it relates to fertility for optimizing fry production.

Some studies have also documented differential sperm performance between low and high OF concentrations (Woolsey et al., 2006; Kanuga et al., 2012), and in many cases they share a positive relationship. As mentioned previously, ovarian fluid percentages as little as 5-10% and as high as 100% have been commonly used in sperm motility studies. Some species including Atlantic cod, *Gadus morhua*, and rainbow trout, *Oncorhynchus mykiss*, produce large amounts of the OF relative to the total egg volume (10-30%) (Wojtczak et al., 2008; Beirão et al., 2015) while others produce considerably less. In accordance with the latter, we observed that channel catfish produce small volumes of OF with their eggs, although this may also be affected by small female size and that fish were spawned later in the season. Our results show that although the volume is small, sperm swimming behavior is still significantly affected within close proximity to the OF. We were cognizant of the effects different OF concentrations could have, leading us to conduct exploratory trials prior to experimentation with 25% OF and 50%. We observed that sperm activity was still predominantly higher than the control and that OF acted as a chemoattractant/stimulant similarly at both concentrations. The OF concentration in the activation environment changes with distance, increasing as sperm approach the egg surface and the micropyle (Litvak and Trippel, 1998; Poli et al., 2019). The concentration would be higher around the micropyle than in the surrounding solution, making it likely that sperm encounter a flexible range of concentrations during fertilization events. With more experimentation, the optimal concentration range of the OF can be determined. Activation solutions containing it can be then be created artificially for utilization for male sperm quality assessments and for hybrid catfish spawning in order to further standardize hatchery operations.

3.5. Conclusions

Overall, our results show that female channel catfish OF differentially enhances behavior of blue catfish sperm. From this study, we have contributed valuable information that confirms the importance of female OF for fertility, although there are still questions to be answered regarding the underlying mechanisms behind sperm \times OF interactions. On a broader scale perspective, this knowledge can be applied to improve aquaculture for hybrid catfish but also for other species whose production relies on artificial fertilization. Finally, these results have added to the growing knowledge base of the overarching mechanisms of reproduction in freshwater fishes and how gamete interactions have transcended across the diverse array of teleosts present today.

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Figures

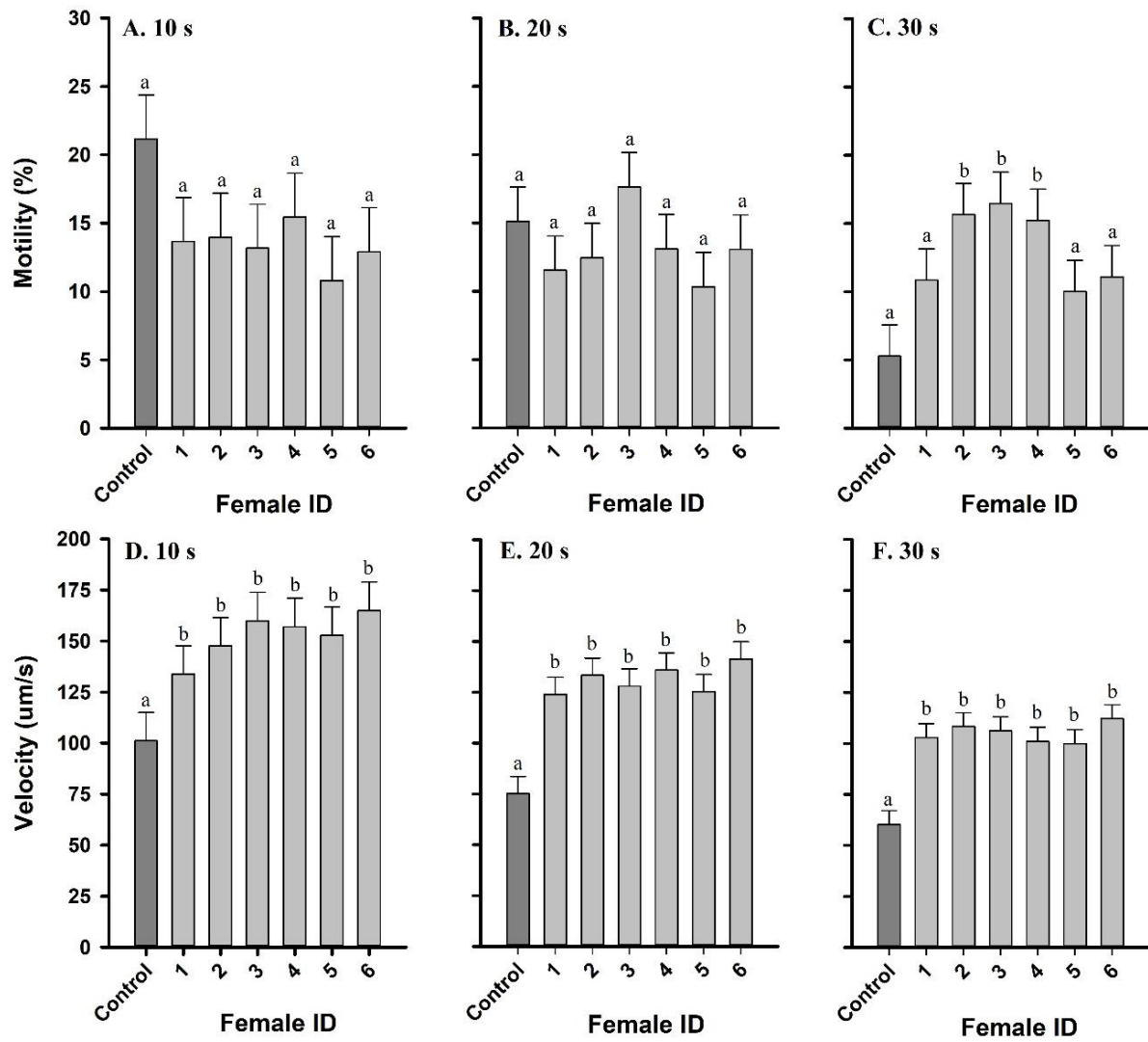


Fig. 3.1. Percent motility and velocity (VCL) of blue catfish, *Ictalurus furcatus*, sperm activated without ovarian fluid (control) and in ovarian fluid from channel catfish, *I. punctatus*, females (n = 6) at 10, 20, and 30 s post-activation. Each bar represents the least square means of the 4 males + SEM. Letters represent a significant difference from the control as determined from the Dunnett's test at alpha = 0.05.

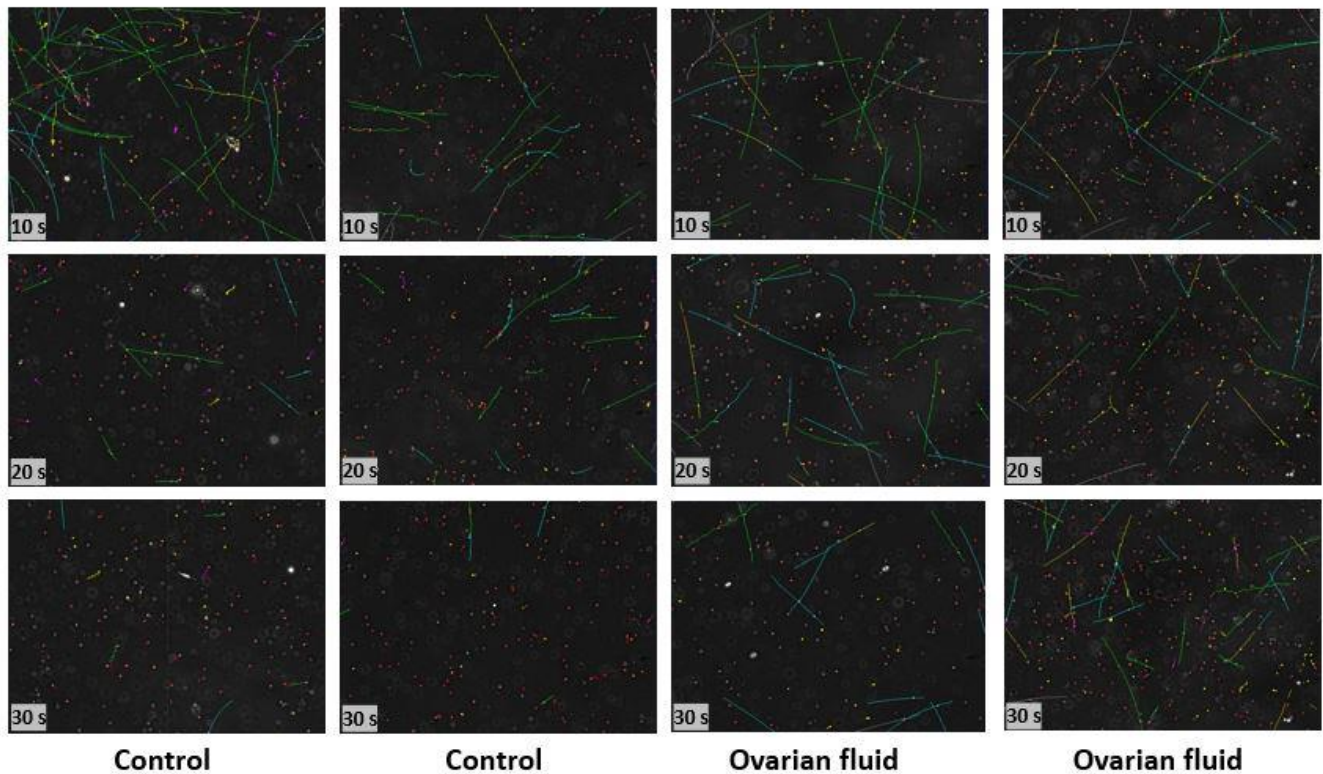


Fig. 3.2. Still frames obtained from CASA sperm analysis software of blue catfish, *Ictalurus furcatus*, sperm taken at 10 s, 20 s, and 30 s post-activation in control solutions without ovarian fluid and with ovarian fluid from channel catfish, *I. punctatus*, females. Individual sperm cells were tracked for 1 s at a rate of 60 frames/s at each time. Cells were classified by the computer tracking software based on movement speeds during each capture frame. Rankings are listed from the most to least active as follows: blue = progressive movement, green = standard motile movement, yellow/purple = slow movement, and red = static/immotile. White tracks represent cells that were removed due to errors in tracking accuracy.

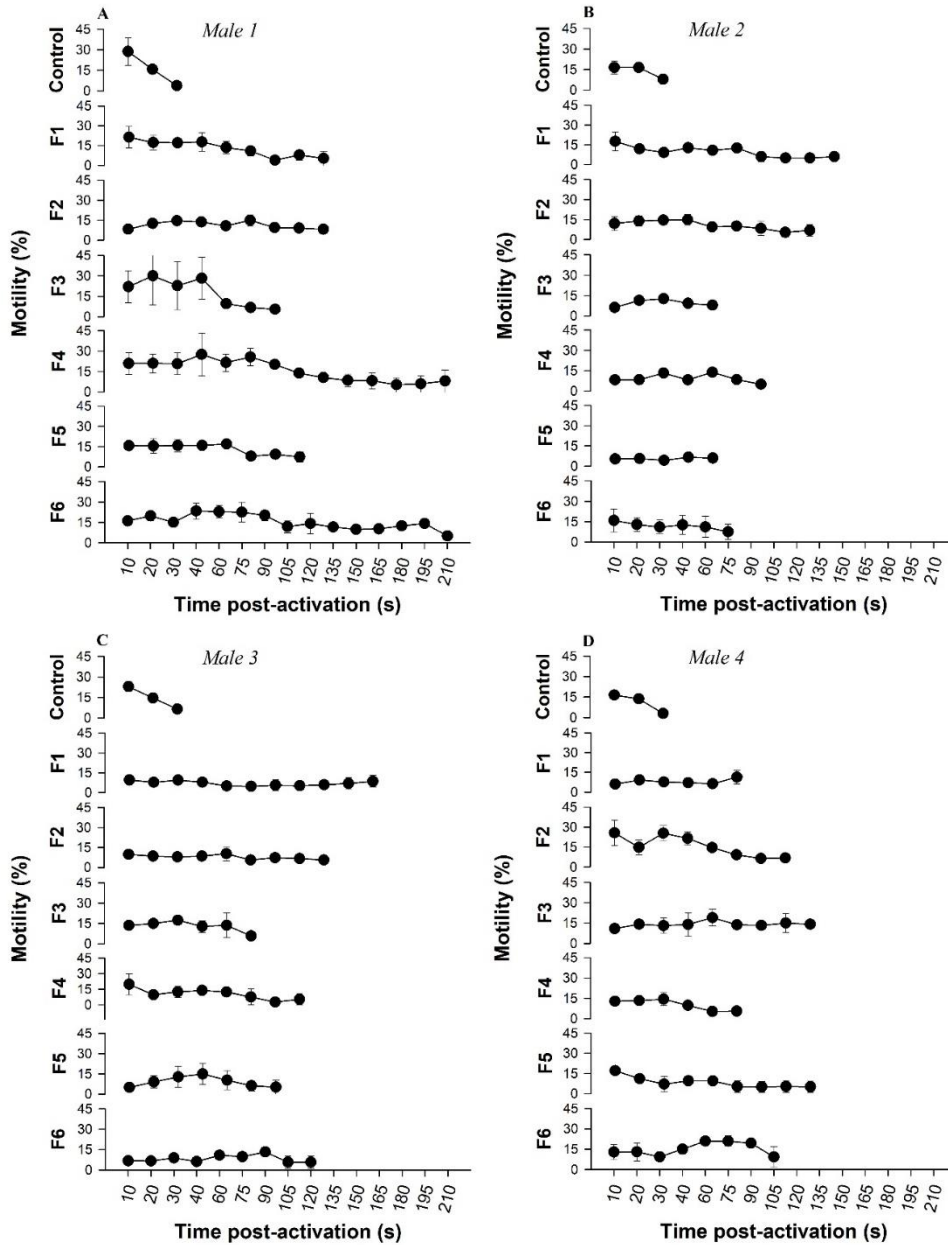


Fig. 3.3. Sperm longevity for each blue catfish, *Ictalurus furcatus*, male activated without ovarian fluid (control) and in ovarian fluid from channel catfish, *I. punctatus*, females (n = 6). There were 24 crosses in total, and male × female interactions were responsible for differences in longevity observed between pairs. Each point represents the motility (%) ± SEM at each time until cessation, defined as the point in which sperm motility dropped to < 5%.

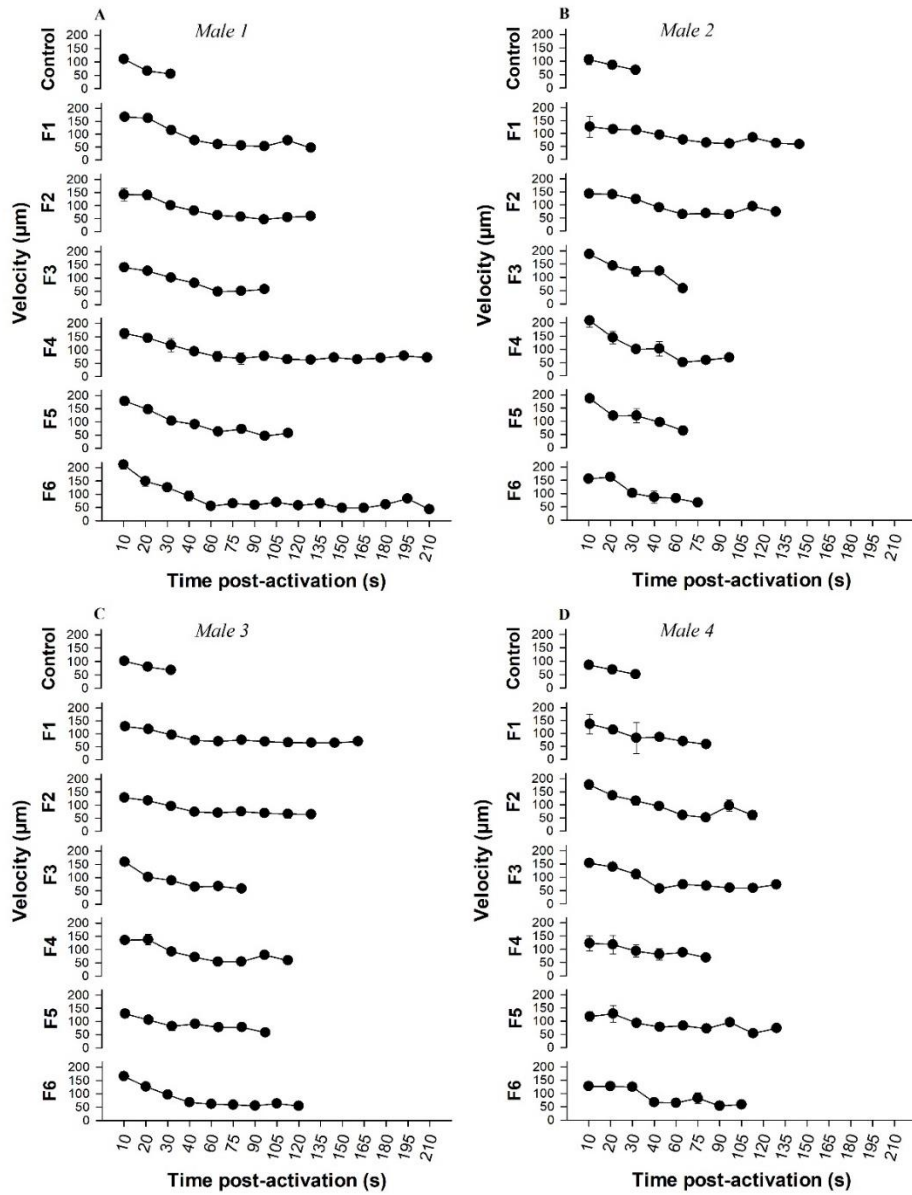


Fig. 3.4. Sperm longevity for each blue catfish, *Ictalurus furcatus*, male activated without ovarian fluid (control) and in ovarian fluid from channel catfish, *I. punctatus*, females (n = 6), with 24 crosses total. Male × female interactions were largely responsible for the differences in longevity between specific pairs. Each point represents the velocity (VCL) ± SEM at each time until cessation, defined as the point in which sperm motility dropped to < 5%.

Table 3.1. Ovarian fluid properties of the individual channel catfish, *Ictalurus punctatus*, females used in this study (n = 6), including pH and osmolality taken from the fluid during egg collection.

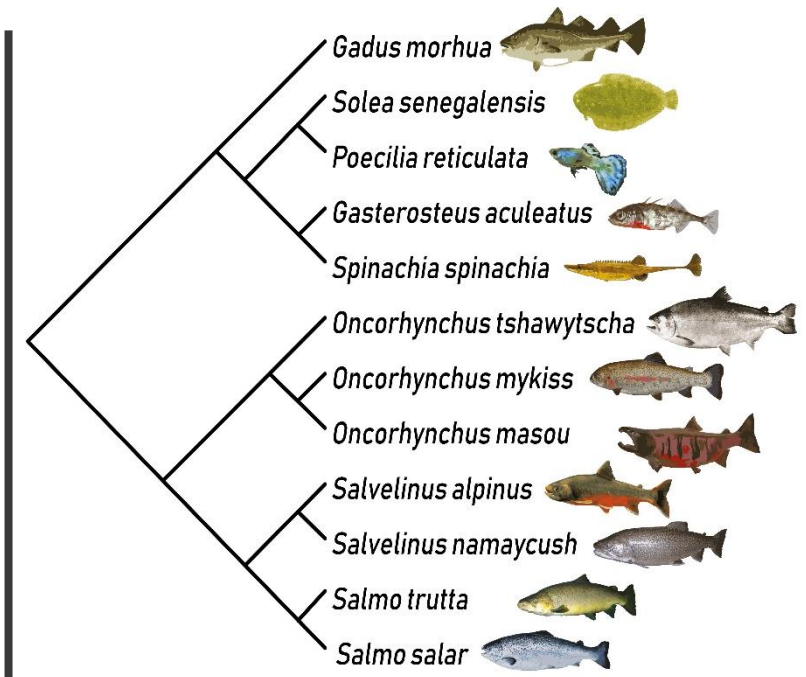
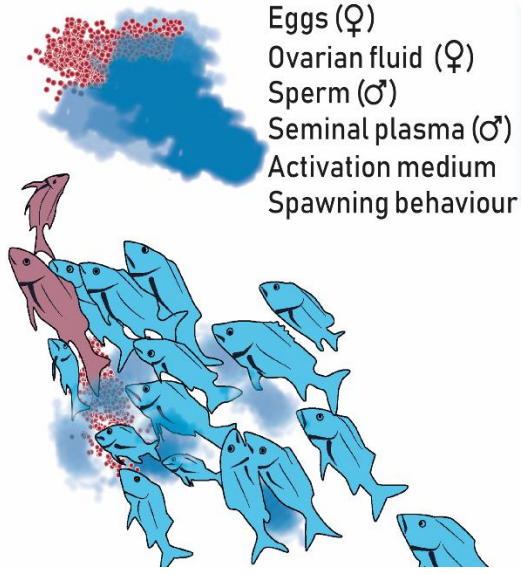
Female ID	Weight (kg)	Ovarian Fluid	
		pH	Osmolality (mOsm/kg)
1	0.9	8.5	241
2	0.94	8.2	227
3	0.86	7.3	221
4	0.98	8.1	171
5	0.63	8.1	229
6	0.96	7.9	243

Table 3.2. Restricted Maximum Likelihood (REML) variance component (VC) percentages from the mixed model factorial ANOVA for sperm motility (%) and velocity (VCL) of blue catfish, *Ictalurus furcatus*, (n = 4) crossed with ovarian fluid from channel catfish, *I. punctatus*, females (n = 6). Variance components of the random effects were classified into female, male, interactions terms, and residual error. Significant variance components are denoted by * at alpha = 0.05.

Variance component	Motility %VC	Velocity %VC
Female	0.00	0.00
Male	21.02*	4.00
Female x Male	25.74**	23.07**
Time x Female	0.00	0.00
Time x Male	0.00	3.04
Time x Female x Male	0.00	0.14
Residual Error	53.24	69.75

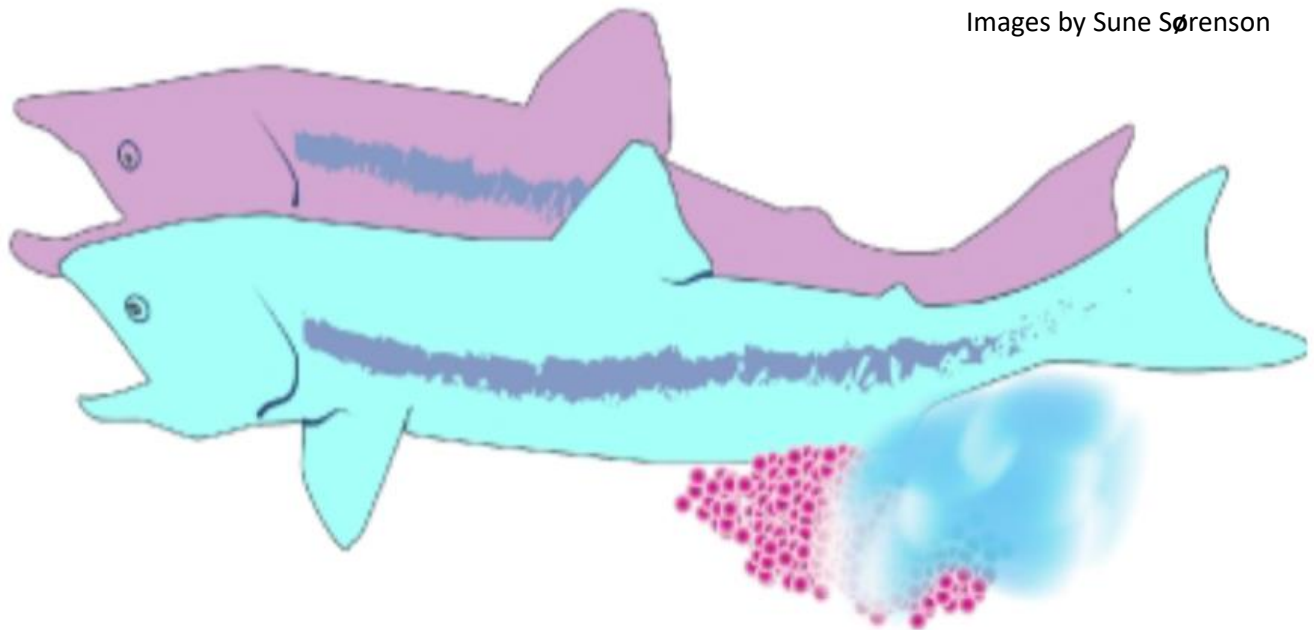
* P < 0.05; ** P < 0.0001

Spawning micro-environment



Chapter 4

Associations between ovarian fluid and sperm swimming trajectories in marine and freshwater fishes: A meta-analysis



Myers, J.N., Zadmajid, V., Senior, A., & Butts, I.A.E. Quantifying ovarian fluid impacts on sperm performance in fish: A meta-analysis. Submitted to Reviews in Fisheries and Aquaculture: in review.

Abstract

Marine and freshwater spawning environments present fish sperm with unique challenges, but for both, gametes often signal prior to contact via biochemical interactions through maternally derived compounds (i.e. eggs and ovarian fluid; OF). For example, when OF is incorporated into the fertilization environment, sperm have been observed to exhibit changes in swimming trajectories (e.g. motility and velocity). However, it remains unclear whether the presence of this OF consistently improves sperm performance. Our objectives were to determine the overall effect of OF on sperm performance using meta-analysis. We searched published literature for studies comparing sperm motility and/or velocity in the presence and absence of OF. For each study, we calculated the log response ratios (lnRR), in which positive values indicate improved sperm performance in the presence of OF. For motility, the overall effect size was non-significant (lnRR = 0.09, CL = -0.06, 0.24), whereas velocity was positively affected by OF (lnRR = 0.10; CL = 0.04, 0.17), indicating an 11% increase in velocity. When segregated by environment, for freshwater species there was a significant positive effect of the OF on velocity (lnRR = 0.18, CL = 0.07, 0.29), which translated to an improvement of 20%. In contrast, we detected no OF effects on velocity for marine species (lnRR = -0.01, CL = -0.02, 0.01). There is evidence that OF improves sperm performance, although spawning environment and/or taxonomic factors are likely to moderate these sperm-OF interactions. Together, these results further our understanding of natural reproductive processes governing sperm performance, mating systems, and fertilization dynamics.

4.1. Introduction

Fish are the largest group of living vertebrates with ~33,500 species (www.fishbase.org), all of which exhibit diverse reproductive strategies (Patzner, 2008; Smith and Wootton, 2016). Such diversity is fascinating for comparative biology in which many fish species have been appointed as biological models for various scientific disciplines. The spawning environment is often identified as a cause of variation in reproductive biology and specifically how gametes interact. Sustainable management of fish populations, both *in situ* and within a production setting, requires an intimate working knowledge of gamete biology and the factors affecting fertilization (Andrews and Kaufman, 1994; Mylonas et al., 2010). Domestication and commercial culture for many important fish species are impeded by difficulties with either low fertilization or other production roadblocks during the “critical” early life history stages (i.e. egg to young juveniles). Therefore, increased knowledge of gamete biology is likely to make a significant contribution to species under investigation, as well as in fish reproduction overall. During the last decade, however, renewed attention has focused on gamete interactions not only within a single species/group but also across different phylogenetic lineages. As a result of this effort, knowledge of gamete quality and fertilization dynamics for a variety of fish species (both cultured and wild) has increased, but still many questions remain to be resolved. It is fascinating to clarify the diversity among different gametes, their physiology, and what evolutionarily enabled them to adapt to environmental change. Now being aware of the necessity to provide an integrated view of sperm and oocyte communication mechanisms, the present study has been entirely devoted to shed light on the impact of maternal components (e.g. ovarian fluid (OF)) on sperm performance in two distinct groups of teleosts: marine and freshwater spawners.

4.1.1. An overview of fertilization dynamics across fish taxa

Fish ontogeny initiates with fertilization, or more precisely, with egg activation. Generally, in teleosts, eggs and sperm develop separately within male and female sexes. However, in some cases (e.g. hermaphrodite species), individuals exhibit maturation of both parts of the ovotestis simultaneously, making them both male and female from a functional viewpoint (Coward et al., 2002). Fertilization in its broadest sense refers to the steps leading to and resulting in the fusion of the nuclei of the male and female gametes to form a diploid zygote (Kunz, 2004). Many of the processes that happen during teleost fertilization seem similar to those seen in marine invertebrates and mammalian eggs. During the process of fertilization, sperm make essential contributions as transmitters of paternal genetic material and as initiators of key intracellular signaling processes within the oocyte with consequences for egg activation and development (Whitaker and Swann, 1993; Babin et al., 2007). However, unlike mammalian sperm, sperm of most teleosts do not possess an acrosome, although some non-teleost fishes do, such as lamprey and sturgeon (Hirohashi and Yanagimachi, 2018). Thus, acrosomal lytic enzymes (e.g. hyaluronidase and trypsin-like enzyme) are not involved in gamete interactions for these fishes. Furthermore, for fish sperm, motility hyperactivation of the flagellum (i.e. asymmetrical flagellar bending based on increased intracellular Ca^{2+}) has not been extensively reported compared to mammalian sperm in which capacitated sperm exhibit large-amplitude beats (Yanagimachi, 1972,1988; Cooper, 1986).

In teleosts, fertilization can occur either inside (internally) or outside (externally) of the female reproductive tract. In oviparous species eggs are ovulated from the ovarian follicles into the ovarian lumen or peritoneal cavity, usually following completion of the first meiotic division. In

these species, once the mature eggs are promoted to complete the metaphase of the second maturation (meiotic) division, they are released into the external aquatic environment with associated OF and are then subsequently fertilized (Fig. 4.1). Afterward, embryonic development takes place outside of the maternal body cavity (Coward et al., 2002; Kunz, 2004). External fertilization is by far the most common reproductive strategy in teleosts (Patzner, 2008). In teleosts with internal fertilization (e.g. live-bearing fish such as swordtails and platyfish of the genus *Xiphophorus*), males transfer the sperm packed (usually by modified anal fin) into spermatophores to the female gonadoduct. Afterward, the sperm may be retained for up to several months after insemination (Huang et al., 2004) and acquire fertility within the female (Iwamatsu, 2000). In these species, sperm are motile in OF and enter the ooplasm of intrafollicular oocytes in order to induce egg activation via the cortical reaction within the ovary (Iwamatsu, 2000). Generally, internal fertilizers that give birth to live offspring are sub-classified as ovoviparous (eggs are incubated in a modified oviduct of the female) or viviparous (eggs develop in the ovary or the uterus after internal fertilization) (Coward et al., 2002).

In both external and internal fertilizing teleosts, sperm-egg fusion occurs between the microvilli of the sperm entry site on the oocyte surface and the plasma membrane of the apical region of sperm head and gradually advances toward the end of the sperm tail (Iwamatsu, 2000). Similar to sea urchin and frog eggs, in some fish species a single transient of Ca^{2+} is triggered during egg activation for development to proceed normally (Stricker, 1999). This results in various modifications of the cytochemistry and ultrastructure, such as chorion separation from the vitelline membrane and formation of the perivitelline space (Babin et al., 2007). The process continues with chorion hardening, which serves as an efficient mechanical barrier to protect embryonic development from environmental peril (Hart, 1990). However, the hardening process can change

either by substances released from the egg following fertilization or the activation medium. Eggs (particularly for freshwater species), can lose their fertilization capacity very rapidly (Yamamoto, 1961) due to plugging of the micropyle (Kunz, 2004), and thus, a sperm cell must locate and pass through it quickly (Yanagimachi et al., 2017). Collectively, the “capacity to fertilize” varies across fish taxa and this variation has been attributed largely to genetic variability, intra-testicular aging of sperm and post-ovulatory ageing of eggs, seasonality of spawning, breeding state, and differences in reproduction strategies (Scott and Baynes, 1980; Coward et al., 2002).

4.1.2. Sperm activation, swimming trajectories, and fertilization success

In teleosts, sperm exhibit a remarkable variety of adaptations. Sperm cells are cast out from the soma into an external environment to spend their pre-fertilization lives fundamentally as free-living organisms, and they have adapted to specific fertilization environments depending on the species (Pitnick et al., 2009). Generally, marine and freshwater environments each present sperm with unique challenges, causing differences in morphology, kinematic characteristics (i.e. motility, velocity, linearity), and biological processes (i.e. ATP usage). Differing activation mechanisms are also seen between these habitats (Suquet et al., 1994; Cosson, 2004). In externally fertilizing fishes, it is common knowledge that sperm cells are immotile in the testes until activation is triggered upon release into the ambient environment (Morisawa et al., 1983), yet there is tremendous variation in how activation occurs. The major signals that may activate the motility of released or ejaculated sperm are changes in concentrations of inorganic (e.g. CO₂, H⁺, K⁺, Na⁺, Ca²⁺) and organic compounds. In addition, non-chemical factors such as osmolality and temperature are involved in triggering sperm motility (Alavi and Cosson, 2005). For marine

teleosts sperm become motile once they are released into hypertonic environments, but for freshwater species activation occurs adversely in hypotonic environments (Alavi and Cosson, 2005; Alavi et al., 2007; Morisawa, 2008). Interestingly, in some species such as medaka, *Oryzias latipes*, motility of sperm can be achieved by hypertonic, isotonic, or hypotonic solutions, depending on if the fish is acclimatized to a freshwater or seawater environment (Yang and Tiersch, 2009). This phenomenon has also been observed in tilapia, *Oreochromis mossambicus* which can reproduce in both freshwater and seawater. The sperm of freshwater-acclimated tilapia exhibit motility only in hypotonic conditions, but the sperm of seawater-acclimated tilapia are motile in both hypertonic and hypotonic conditions (Morita et al., 2004). Typically, sperm from marine species have longer longevity than those of freshwater species likely due to differences in the physical properties of the activation media and reproductive strategies of each spawning type (Kime et al., 2001). Interestingly, species with internal fertilization exhibit much longer windows of motility. The viviparous guppy, for instance, displays longevity in seminal plasma for periods up to 48 h (Billard, 1978). One common feature across species seems to be the importance of sperm swimming speed during each activity period; the average sperm velocity being 140 $\mu\text{m/s}$ (ranging from 70-220 $\mu\text{m/s}$) for marine and 135 $\mu\text{m/s}$ (ranging from 50-250 $\mu\text{m/s}$) for freshwater species (Browne et al. 2015).

Sperm quality in fishes can be assessed in many ways, but regardless of spawning type, sperm motility and velocity are viewed as primary determinants of reproductive success. As such, these traits are commonly used to assess male gamete quality and fertilization potential (Gage et al., 2004; Rurangwa et al., 2004; Gallego and Asturiano, 2018; 2019). Typically, sperm with higher velocity and motility have the advantage of reaching the micropyle within a shorter window of time (Cosson et al., 2008). Increased sperm velocity and motility, thus, provide a crucial advantage

for fish that spawn in highly competitive environments. However, in internally fertilizing species with prolonged sperm that are retained in the female reproductive tract, sperm migration toward the ovum may be influenced more by the female reproductive tract than sperm swimming behavior directly (Pizzari and Parker, 2009). Accordingly, in many species, studies have reported positive correlations between sperm motility and fertilization success (Lahnsteiner et al., 1998; Casselman et al., 2006; Gallego et al., 2017), and similarly, reductions in sperm motility result in decreased reproductive success (Burness et al., 2004; Casselman et al., 2006). Like motility, higher rates of fertilization and paternity were also achieved with higher sperm velocities in both marine and freshwater species, such as Atlantic salmon, *Salmo salar* (Gage et al., 2004), bluegill, *Lepomis macrochirus* (Burness et al., 2004), Atlantic cod, *Gadus morhua* (Rudolfson et al., 2008), and green swordtail, *Xiphophorus helleri* (Gasparini et al., 2010). Within the motile portion of an ejaculate, variation in sperm velocity could impact fertility potential of individual sperm cells (Pizzari and Parker, 2009).

4.1.3. How do sperm and oocytes communicate before fertilization events?

Fish species have evolved a diversity of strategies to maximize their chances of reproductive success and a wide range of processes that lead up to fertilization (Coward et al., 2002; Smith and Wootton, 2016). Fertilization depends on successful interactions between eggs and sperm during spawning. As such, there has always been keen interest in gamete interactions, specifically how the oocyte, sperm, and secretion of maternally-derived substances from the female reproductive tract cooperatively impact fertilization success. However, the specific mechanisms underlying these interactions remain elusive for most species. It seems this complex chemical dialogue

between eggs and sperm provides the scope for female-induced sperm recognition and selection, ensuring that only a small subset of sperm cells have the opportunity to reach the fertilization site (Fitzpatrick and Lüpold, 2014). Sperm are usually produced and released more abundantly than eggs (e.g. billions of spermatozoa during spawning) yet sperm-egg fusion occurs at a ratio of 1:1. Therefore, sperm are always subject to strong selection or competition (Parker and Pizzari, 2010; Parker and Lehtonen, 2014; Matsuzaki et al., 2018). In order to fully understand how competition occurs, we must first answer a question: How do sperm find the egg? Sperm ejaculated into either the female genital tract or external environment do not likely reach the oocyte by coincidence, and the fact that very few succeed in making their way to the oocyte points to sperm guidance factors. Once sperm cells are activated, they must compete to be the first to locate an oocyte and find the entry site on the egg plasma membrane (Yanagimachi et al., 2013; Browne et al., 2015). In order to do this, they must first interact with either the female's reproductive tract or maternal components in the vicinity of the micropyle such as OF. This fluid acts as a guide by stimulating sperm to undergo numerous physiological, biochemical, morphological, and behavioral modifications (Oda et al., 1995; Coward et al., 2002; Yanagimachi et al., 2013). Accumulating evidence from different taxa show that both the female reproductive tract and oocyte secrete many factors such as sperm-activating peptides (e.g. polypeptides and glycoprotein), amino acids, small molecules, yolk lipids such as polyunsaturated fatty acid (PUFA), and nitric oxide which can act as chemo-attractants that "direct" sperm into the micropylar canal (Creech et al., 1998; Kubagawa et al., 2006; Cherr et al., 2008; Han et al., 2010; Yanagimachi et al., 2013; 2017). These substances are also known to aid in sperm chemotaxis, as initially described by Dan (1950) in medusan, *Spirocodon saltatrix*. Specifically, the matrix and the jelly coat encompassing the egg envelope (i.e. chorion) varies in different species and generally displays a prominent role in sperm-egg

detection (Iwamatsu, 2000). The swarming of numerous sperm cells in or near the micropyle during fertilization gives evidence that the egg chorion may release substance(s) (e.g. sperm motility-initiating factors) that attract homologous sperm toward the egg (Hart, 1990). Apparently, those sperm cells moving along the surface of the egg chorion have a higher chance of entering the micropylar canal than those swimming freely and randomly in the aqueous environment (Yanagimachi et al., 2013). In this scenario, the strongest evidence comes from studies with various fish species but also in mammals and invertebrates (Iwamatsu, 2000; Browne et al., 2015; Cosson, 2015; Yanagimachi et al., 2013; 2017; Yoshida and Yoshida, 2011; 2018). For example, in teleosts, sperm motility initiating factors (SMIFs), a type of glycosylated peptide located in the egg chorion have been shown to play a role in triggering sperm motility. In Pacific herring, *Clupea pallasii*, diffusible sperm-activating proteins called HSAPs (herring sperm activating proteins) which are structurally related to trypsin inhibitor like proteins, as well as SMIF and tightly linked with the micropyle region, affect sperm motility by inducing Ca^{2+} and Na^+ efflux, membrane depolarization of sperm (Vines et al., 2002; Cherr et al., 2008), or by interacting with prolyl endopeptidase on the surface of the sperm tail (Yoshida et al., 1999). HSAPs were previously purified from egg-conditioned seawater by gel filtration and isoelectric focusing chromatography by Oda et al., (1995), who later identified the sperm-activating factor as probably an OF constituent attached to mature ovulated eggs (Oda et al., 1998). Additionally, in fathead minnow, *Pimephales promelas*, it was shown that nitric oxide synthases by the chorion of the oocyte can enhance sperm motility and velocity (Creech et al., 1998). Furthermore, unfertilized eggs of flounder and salmon also have a species-specific glycoprotein that binds to the surface of the chorion in the outer opening of the micropyle (Yanagimachi et al. 2013). Interestingly, there is evidence that oocyte PUFAs are sperm attractant precursors and components of phospholipids necessary to create a

fluid membrane environment (Han et al., 2010). Therefore, oocyte PUFAs can regulate spermatozoa guidance by impacting sperm motility and velocity. For instance, analysis of *fat-2* and *fat-3* genes showed that oocyte PUFAs are a direct requirement for sperm-oocyte communication, in which sperm velocity and fertility in a *fat* mutants animal (e.g. nematode) changed by direct PUFA microinjection into the gonad (Kubagawa et al., 2006). Whether PUFAs function in sperm-oocyte interactions within the teleost reproductive tract or maternal components remains unknown.

Collectively, changes in the swimming patterns of teleost sperm toward an egg may have something in common with the “chemotactic” movement of invertebrate sperm to reach an egg as well as hyperactivation of mammal sperm before fertilization. However, sperm-oocyte communication in fish is quite different from other phyla, largely due to the diverse environments where gamete encounters take place, whether it be inside the female reproductive tract or immersed in huge volumes of maternal components mixed with water. Furthermore, the variability of gamete interactions across fish taxa may also partly be due to different motility and morphology traits required for each fertilization environment and for penetration of the chorion (Browne et al., 2015).

4.1.4. Does ovarian fluid impact sperm swimming behavior and fertility?

OF, the substance containing maternal compounds, is secreted by female ovarian epithelial cells before or at the start of ovulation (Aegerter and Jalabert, 2004). For the majority of external fertilizers, eggs are ovulated into ovarian cavities and spawned with an abundance of OF (the quantity of OF is variable across species) out of the genital pore through the short oviduct together with the cumulus-oocyte complex (Iwamatsu, 2000). Previous models of gamete biology and

sperm performance as well as methods of assessing male sperm quality in the laboratory have largely focused on aqueous solutions that lack OF. As such, these studies do not necessarily represent the broader chemical environment in which sperm are likely to have evolved and may also overlook the effects caused by each female's unique micro-environment. In cases where sperm motility, longevity, and velocity are improved by OF, fertilization potential of these males may then be underestimated. Sperm motility and velocity can be directly affected by physical properties of the activation environment (Kime and Tveiten, 2002; Alavi and Cosson, 2005; Cosson et al., 2008; Browne et al., 2015). Experimental protocols have been employed over the years to assess the importance of sperm-OF interactions (reviewed by Zadmajid et al., 2019). Results have shown that when maternally-derived compounds are incorporated in the fertilization environment, sperm exhibited increased motility and velocity in several fish species (Yoshida and Nomura, 1972; Turner and Montgomerie, 2002; Rosengrave et al., 2009a; Butts et al., 2012; Galvano et al., 2013; Geßner et al., 2017). For example, in the brown trout, *Salmo trutta fario*, incorporating OF into the activation environment enhanced sperm longevity to over 5 min and improved fertilization success (Lahnsteiner, 2002). In several other salmonids, sperm velocity was also elevated when cells were activated in OF as compared to water alone (Gage et al., 2004; Butts et al., 2012; Rosengrave et al., 2016). Furthermore, in internally fertilizing teleosts such as ocean pout, *Macrozoarces americanus* (Yao and Crim, 1995) and spotted wolffish, *Anarhichas minor* (Kime and Tveiten, 2002), sperm remained motile for 24 to 48 h in OF and became immotile on contact with seawater. An interesting example of the influence of OF on sperm swimming behavior has been reported by Elofsson et al., (2003b) with the three-spined stickleback, *Gasterosteus aculeatus*: a teleost found inhabiting fresh, brackish, and marine waters. In this species, OF prolonged sperm longevity from both fresh and brackish-water for up to 7 and 10 h, respectively,

with some sperm found to be active for up to 24 h. Furthermore, in some species, OF enhanced sperm fertility more than in other activation media. For instance, in salmonids (e.g. lake trout, *Salmo trutta* and Atlantic salmon, *Salmo salar*) when sperm was diluted in water, they had completely lost their fertilizing capacity at 40 s post-activation, while those in Ringer's solution showed 15.4% fertility after 2 min. Those diluted in OF fertilized 78.8% of the eggs after 5 min (Ginsburg, 1963). However, there are a few contradicting cases that have shown that OF had a limited or negative impact on sperm performance (Wojtczak et al., 2007; İnanan and Öğretmen, 2015; Kleppe et al., 2018).

Although the mechanisms by which OF enhances and/or inhibits sperm performance are not fully understood, OF encompasses an array of physical (i.e. color, volume, viscosity, pH, and osmolality), biochemical (e.g. Na⁺, Cl⁻, K⁺, Mg²⁺, Ca²⁺, proteins) and organic constituents (metabolites and enzymes) that are known to influence sperm swimming trajectories (Turner and Montgomerie, 2002; Woolsey et al., 2006; Wojtczak et al., 2007; İnanan and Öğretmen, 2015; Alonzo et al., 2016). Potentially, components of the OF can also influence ATP metabolism, thus increasing sperm longevity and velocity (Turner and Montgomerie, 2002). In mammals, OF called follicular fluid has been proposed to induce chemotaxis in sperm, changing sperm swimming behavior in several mammals such as humans (Ralt et al., 1991), mice (Oliveira et al., 1999), rabbit (Fabro et al., 2002), and bovine (Gil et al., 2008).

Collectively, this complex chemical dialogue between OF and sperm provides the scope for sperm recognition by the female (Fitzpatrick and Lüpold, 2014), thereby offering a mechanism for sexual selection *via* cryptic female choice and potentially altering the dynamics of sperm competition (Gasparini and Pilastro, 2011; Butts et al., 2012; Makiguchi et al., 2016; Lehnert et al., 2017). For example, the ejaculates of various males may co-occur around a set of ova at the

time of gamete interaction, resulting in intense sperm competition and selection (Parker 1970,1984,1998; Pizzari and Parker, 2009). Such competition among individual males is now recognized as one of the most powerful evolutionary forces influencing reproductive outcomes for most taxa (Parker, 1970; Birkhead and Pizzari, 2002; Pizzari and Parker, 2009; Kelly and Jennions, 2011; Firman et al., 2017).

4.1.5. Motivation for meta-analysis on sperm-ovarian fluid interactions

Generalities about the magnitude and relevance of OF-sperm interactions across fish species are currently difficult to make. One dilemma lies in the variation of observed results among studies, which have made it difficult to draw broad conclusions across species. In such cases, meta-analysis provides a quantitative approach to systematically assess the degree and causes of heterogeneity among the results of individual studies and to derive an estimate of the magnitude of any overall biological effect (Huque, 1988; Haidich, 2010). Therefore, our objectives were to determine whether OF has an overall effect on sperm performance traits across fish species that spawn in freshwater and marine environments. We did this by systematically analyzing the current published studies on the effects of the OF on sperm motility (%) and velocity ($\mu\text{m/s}$). For each study, we calculated the log response ratio (lnRR) between control groups (without OF) and treatment groups (with OF). We collated these effect size statistics using random effects meta-analytic models to determine the direction and strength of any overall relationships and to discuss potential causes for heterogeneity among the results reported by all studies.

4.2. Methods

4.2.1. Literature search and data extraction

Studies were collected systematically following PRISMA's best-practice protocols in three databases: Web of Science, Google Scholar, and Academic Search Premier. Research articles were searched from inception until 10 August 2018. We collected studies on the effects of OF on primary determinants of male reproductive success in fishes: motility and velocity (Lahnsteiner et al., 1998; Gage et al., 2004; Linhart et al., 2005). Keywords used were “ovarian fluid”, “sperm”, “sperm and/or motility”, “sperm and/or velocity”, “sperm and/or competition”, “sperm and/or performance”, and “cryptic female choice.” To be included, studies must:

(i) Be performed on a fish species.

(ii) Report data on sperm motility and/or velocity; acceptable measures of sperm velocity included average path velocity (VAP), curvilinear velocity (VCL), and straight-line velocity (VSL), with the majority of studies (80%) reporting VCL. Due to collinearity between the different measures of velocity, only one measure of velocity was selected when more than one was reported in a study. 40% of studies reported more than one measure of velocity, but each of these studies reported VCL, therefore it was selected as the primary measure of velocity when available. As for the studies that did not report VCL, VAP was chosen as the primary measure. These measurements were analyzed collectively with VCL as a single unit of velocity (all expressed in $\mu\text{m/s}$) for this analysis.

- (iii) Include treatment groups, wherein one group or more consisted of activated sperm in the presence of OF and an equivalent control group of sperm was activated in a media designed to reflect the natural environment without OF (i.e. a full control group).
- (iv) Analyze motility and velocity within the first 0-15 s post-activation, which is the most critical window for fertility in fishes (Iwamatsu et al., 1991; Fleming, 1996; Hoysak and Liley, 2001; Yeates et al., 2007). Data was collected only within this interval to investigate the impacts during the most influential period of sperm activation and fertility.
- (v) Report all of the following (or data from which they can be derived) for all treatment groups; means, standard deviations (SDs), and sample sizes.

After first screening by title and abstracts and then by full text for the aforementioned criteria, there were 19 studies included in the meta-analyses (Fig. 4.2; Table 4.1). From each study, we collected data on means, SDs, and sample sizes in treatment and control groups from tables, text, and figures. The studies gathered used subtly different designs, where replication was designed to capture/control different sources of variation. For example, some studies pool the OF from several females and compare the performance of sperm from individual males in presence/absence of this fluid, meaning replicates capture among-male variation. In other cases, the sperm of males was pooled and tested against the fluid of individual females, thus capturing among-female variation. Nevertheless, in all cases the relative effect of OF on mean sperm function in each group is comparable among all studies.

Where data were reported in figures, they were extracted manually using WebPlotDigitizer v.3.9 (WebPlotDigitizer, Austin, TX, USA). When multiple data points were reported within the critical 0-15 s window, we extracted all data points, and subsequently collated as described below.

For any missing data, we attempted to contact the authors, and thereafter, their inputs were included. We also collected data on moderator variables (study-specific factors), which might be expected to influence the sign or magnitude of the OF effects on sperm traits. Those variables were: (i) taxonomic data on the study species (families), (ii) whether fish spawned in fresh or saltwater environments, and (iii) OF concentration.

4.2.2. Effect size calculations

For meta-analysis, the results of different studies must be combined via effect sizes that are on a common scale, which summarize the sign and magnitude of the effect reported in each study. Where studies report data on traits measured in different units (e.g. measures of sperm velocity) a standardized, unitless effect size is required. Here, we used the log response ratio (lnRR), which is the natural logarithm ratio of the means in the two groups. We calculated the lnRR (and its associated sampling variance; s^2_{\lnRR}) using the “escal” function, in the package *metafor* (Viechtbauer, 2010) in R Studio v.1.1.383 (RStudio, Boston, MA, USA). R Studio was used for all analyses, unless otherwise stated. For ecological/evolutionary meta-analyses such as ours, the lnRR and also the standardized mean difference (SMD) are both commonly used (Nakagawa and Santos, 2012; Senior et al., 2016a). We used the lnRR rather than the SMD as the latter is standardized in units of pooled standard deviation (SD). As discussed above, the studies synthesized here have slightly different designs which capture different sources of variation, rendering the SDs of different studies incomparable (although the means remain comparable). Unlike the SMD, the interpretation of effect magnitude for lnRR is not affected by the SD of the study. Effect sizes were calculated such that positive values indicate that the measure of interest is

greater in the presence of OF. To aid interpretation, in places we back transform the overall estimates of the lnRR, yielding the estimated difference ratio of the means between control and treatment groups.

For studies where data were reported over multiple time points, all means and SDs reported within the 0-15 s window were combined into a single measurement following Higgins and Green (2011). In cases where there were more than one treatment group compared to the same control group (such as OF from two different fish populations), the sample size of the control group was divided by the total number of treatment groups to ensure the n in the control group was not over-represented.

Elofsson et al., (2003b) conducted trials on freshwater and marine sticklebacks within the same species (*Gasterosteus aculeatus*). Additionally, Yeates et al., (2013) conducted independent experiments within the same study on two different fish species: trout, *Salmo trutta*, and Atlantic salmon. As such, data points for each species were treated as separate samples for both studies. It is to note that the study also employed the use of conspecific OF (within the same species) as well as heterospecific OF (from different species) as an independent variable. For our analyses, only data for conspecific OF was assessed to maintain consistent with other studies. Additionally, within-study divisions were made, such that studies analyzing >1 OF concentration were treated separately due to their potential to cause variations in effect size and to minimize within-study variation. In such cases, again, the n of the control group was still divided by the number of treatment groups before pooling the data, as stated previously. Sperm has been shown to behave differently depending on whether OF is present in high or low concentrations in the activation environment (Diogo et al., 2010; Beirão et al., 2014). Therefore, after separation by OF

concentration, data points were then processed using the method described above. In total we were left with 24 pairwise effect sizes derived from 19 published studies.

4.2.3. Statistical analysis

Effect sizes were analyzed using random-effects meta-analytic models (REMAs), which allow us to: 1) estimate the overall sign, magnitude, and statistical significance of effects, and 2) estimate the degree of heterogeneity among the effects. REMAs were considered more applicable than fixed-effect meta-analysis (FEMA), as REMAs do not assume that all effect sizes are drawn from the same statistical population (Nakagawa et al., 2017) and therefore allow for heterogeneity among the effect sizes reported by different studies. In the current case, heterogeneity is likely to be present as we have data drawn from several different species (Senior et al., 2016a). REMAs were implemented using the ‘rma’ function in *metafor* and estimated *via* restricted maximum-likelihood estimate (REML) (Viechtbauer 2010). Outcomes were measures of lnRR, and sampling variance was specified as $s^2_{\ln RR}$. REMA (as opposed to fixed-effects meta-analysis) and related multi-level meta-analysis are generally the most applicable methods because they allow for heterogeneity, which is expected in almost all biological datasets (Gurevitch and Hedges, 1999). Heterogeneity may be attributable to many sources, but in studies in which each effect size comes from a different species or biological system, inter-specific differences are expected to impact the results (Senior et al., 2016a). Separate REMAs were implemented for lnRR of motility and velocity. We considered overall estimates of effect with 95% confidence limits (CL) not spanning zero statistically significant. Heterogeneity was quantified using the Q test (Cochran 1954), which provides a test for the presence of statistically significant (i.e. non-zero when $p < 0.05$)

heterogeneity. We also reported I^2 values, which measure the percentage of variance among effect sizes due to true heterogeneity (Huedo-Medina et al., 2006) (i.e. not due to sampling variance). Values of I^2 are somewhat arbitrarily defined, but 25, 50 and 75%, are typically taken to represent low, medium, and high heterogeneity, respectively (Higgins and Thompson, 2002), although in a multi-species study such as ours estimates of around 85-90% are common (Senior et al., 2016a).

An additional source of heterogeneity and non-independence in multi-species analyses such as ours is phylogenetic covariance, wherein more closely related species may be expected to more closely represent one another owing to a shared evolutionary history (Harvey and Pagel, 1991; Pagel, 1999; Nakagawa and Santos, 2012). The effects of such shared evolutionary history can be quantified as $I^2_{\text{Phylogeny}}$ (the percentage of among-effect size variation due to shared evolutionary history; similar to Pagel's λ ; (Pagel 1999)) using phylogenetic multi-level meta-analysis (PMLMA; (Nakagawa and Santos, 2012; Hadfield and Nakagawa, 2010)). PMLMAs are an extension of REMAs, which allow for multiple sources of heterogeneity (such as correlated evolutionary history) to be modeled as random effects in a very similar manner to generalized linear-mixed models, as are commonly used in the analysis of primary data (Nakagawa and Santos, 2012; Hadfield and Nakagawa, 2010). A phylogeny was created for the species included in the analysis using the interactive Tree of Life (iTOL; <http://itol.embl.de/>), which generated a tree based on data from the National Center for Biotechnology Information (NCBI) taxonomy (Fig. 4.3). The phylogeny was converted to an ultra-metric format following Grafen's method (Grafen, 1989) with $\rho = 1$, and an associated covariance matrix for effect sizes under a Brownian motion model of evolution was included in the PMLMA as a random effect using `rma.mv` function. From the estimated variance components $I^2_{\text{Phylogeny}}$ was calculated following Nakagawa and Santos (2012).

To explore whether the recorded moderator variables (see *Literature Search and Data Extraction* above) explained heterogeneity, we first fitted separate REMAs for each spawning type (freshwater vs marine) to get independent overall effect sizes for each. We then tested for differences between spawning types using random-effects meta-regression (REMR), again implemented using the ‘rma’ function, as well as for linear effects of OF concentration. Effects of moderators were determined to be significant when based on the Q -test for moderators ($p < 0.05$).

Publication bias occurs when processes of publication systematically cause the under/over-representation of studies with specific outcomes relative to the total pool of studies performed. This creates meta-analyses based on the published literature but generates biased estimates (Møller and Jennions, 2001). A common occurrence is when there is a tendency for studies with significant and/or large effects to have a higher rate of submission and acceptance (Egger et al., 1997; Rothstein et al., 2006). In addition to visually analyzing funnel plot symmetry, here we use a trim-and-fill analysis, as described by Duval and Tweedie (2000), to estimate the number of potentially ‘missing’ effects and any potential bias in the overall estimated effect caused by the exclusion of those studies. Trim and fill analyses were implemented using the ‘trimfill’ function in *metafor*.

4.3. Results

4.3.1. Motility

Data for motility was collected from 14 different studies, yielding a total of 18 effect sizes (Fig. 4.4). Of these, 12 were for freshwater and 6 for marine species. The results of the REMA for the full analysis gave an overall effect size of $\ln RR = 0.09$ (CL = -0.06, 0.24), which did not deviate

significantly from 0. There was high heterogeneity between studies ($Q = 301$, $df = 17$, $p < 0.001$; $I^2 = 97.7\%$), matching previous meta-analytic results for sperm motility in which $I^2 = 92.5\%$ (Senior et al. 2016b). The PMLMA estimated that total heterogeneity was not attributed to shared evolutionary history ($I^2_{\text{Phylogeny}} < 0.01$).

When analyzed individually by spawning environment, freshwater (12 effect sizes) and marine (6 effect sizes) had overall $\ln\text{RR} = 0.13$ (CL = -0.09, 0.36) and $\ln\text{RR} = -0.02$ (CL = -0.06, 0.02), respectively ($I^2_{\text{Fresh}} = 98.5\%$, $I^2_{\text{Marine}} = 0\%$), perhaps indicating sperm for freshwater species exhibited a greater positive response to OF than for marine species. However, a direct comparison using REMR detected no statistically significant difference between groups ($Q_{\text{Moderator}} = 0.26$, $df = 1$, $p = 0.61$). REMR for OF concentration also had no significant effect on effect magnitude ($Q_{\text{Moderator}} = 0.84$, $df = 1$, $p = 0.36$). The estimated slope of the relationship was effectively zero (slope for 1% increase in OF = -0.002, CL = -0.006, 0.002).

Publication bias was inspected visually by analyzing asymmetry in the funnel plot (Fig. 4.5a). Most of the studies reported either had no effect or small-sized effects in the positive direction, but there were some negative effects (primarily from the marine studies) that balanced the distribution to the left. There were 3 outliers outside the confidence interval region, but neither of them had justifiable reasons to be excluded from the analysis. The trim and fill method, however, did not estimate any studies to be missing from the left side, and results of the REMR remained unchanged with this modification.

4.3.2. Velocity

Data for velocity was collected from 16 different studies, yielding a total of 20 effect sizes (Fig. 4.6). Of these, 12 were for freshwater and 8 for marine species. Across all studies, velocity was positively affected by OF. For the full analysis REMA, the lnRR was 0.10 (0.04, 0.17), which when back-transformed equates to a ratio of 1.11, suggesting velocity is increased by 11% in the presence of OF. Again, however, heterogeneity was high ($Q = 100$, $df = 19$, $p < 0.001$; $I^2 = 91.0\%$) between studies. The PMLMA detected a low degree of phylogenetic heterogeneity ($I^2_{\text{Phylogeny}} = 31.4\%$), suggesting a mild correlation in effect among closely related species.

For freshwater species there was a statistically significant positive effect of OF (lnRR = 0.18, CL = 0.07, 0.29). Back transforming the overall estimate indicates a mean ratio of 1.20, suggesting OF increases velocity by 20% in freshwater species. The effect sizes within freshwater species were, however, heterogeneous ($I^2_{\text{Fresh}} = 86.7\%$). In contrast, we detected no effect of OF on velocity in marine species, and there was no heterogeneity among effect sizes (lnRR = -0.01, CL = -0.02, 0.01, $I^2_{\text{Marine}} = 0\%$). A direct comparison among groups using REMR detected a statistically significant difference in the effect of OF on velocity between marine and freshwater species ($Q_{\text{Moderator}} = 7.7$, $df = 1$, $p < 0.01$). REMR of OF concentration did not detect a significant impact on effect sizes across studies ($Q_{\text{Moderator}} = 1$, $df = 1$, $p = 0.32$) and had a negligible slope (slope for 1% increase in OF = 0.002, CL = -0.001, 0.004).

Publication bias was not detected for velocity (Fig. 4.5b). The shape of the funnel plot suggested that there are two outlying large positive effects beyond the confidence interval region. However, when the trim and fill function was applied, there were no studies estimated to be

missing from the left side, and the results were unchanged with this modification. Thus, we concluded that our results were not overestimated or misinterpreted due to publication bias.

4.4. Discussion

The receptivity of teleost gametes is rather short, and thus, fertilization through the egg micropylar canal must occur rapidly (Yanagimachi et al., 2017). Upon sperm contact with an activation media, sperm cells become vigorously motile until cessation but can be altered by maternally-derived fluids expelled with each egg batch. Results from our meta-analysis show that the presence of OF has an impact on sperm swimming trajectories and that this impact varies between spawning types (i.e. freshwater or marine). More specifically, for freshwater species there was a positive effect of OF on sperm velocity, but no effect was detected for marine species.

Generally, all but a few studies reported positive effects for sperm velocity. For that reason, studies that generated negative effect sizes are further considered here in detail. Beirão et al. (2014,2015) concluded that OF inhibited sperm performance in Atlantic cod, contradictory to the findings of Litvak and Trippel (1998) on the same species. Litvak and Trippel (1998) reported increased sperm motility and swimming speeds in OF. However, Beirão et al., (2014, 2015) reported that the control (seawater without OF) yielded better sperm performance despite female origin (captive vs. wild or native vs. foreign population) or OF concentration used (5 or 25%). The authors attributed their findings to differences in methodology, such as using previously frozen OF samples instead of freshly collected samples as in the prior study, which could have altered some of its physical properties. There could have also been changes in osmolality and pH of the OF between experiments that was not tested for and potential mechanisms of cryptic female choice

among the different treatment populations. Whether the results are due to one or all these factors remains unclear. In this example and in all studies considered, we need to be cognizant of all sperm activating factors (i.e. temperature, pH, osmolality; reviewed by Alavi et al., 2008) that may impact performance outcomes.

Heterogeneity is almost always expected in ecological studies of this sort, and we statistically tested for a number of moderators using meta-regression. This approach is commonly used to explain sources of the high heterogeneity in meta-analyses and confirms the robustness of the results (Nakagawa and Santos, 2012). We explored the potential impacts of spawning type (freshwater vs marine), OF concentration, as well as shared evolutionary history (Hadfield and Nakagawa, 2010). Differences in life-history patterns, evolutionary aspects, gamete biology, and reproductive strategies across species within considerably different evolutionary lineages introduced the potential for heterogeneity within our analysis. In our case, our groups comprised of popular, hatchery-reared species of economic value (for freshwater species in particular). This resulted in most of our data originating from salmonids. We detected evidence for mild correlations (~30%) among related species in the effects of OF on sperm velocity but not for motility. It would be interesting to determine if representation from more family groups of different origins would heighten or diminish these phylogenetic correlations as more data becomes available.

Our analysis did not detect any significant effect of OF concentration on overall effect size strength, but reports from within individual studies indicate that this effect can be quite apparent. Interestingly, in some fish species, OF causes either a negative or neutral impact on sperm performance (Wojtczak et al., 2007; İnanan and Öğretmen, 2015; Kleppe et al., 2018), which may be partly due to OF concentration. Negative relationships may be observed with high OF concentrations because of inhibition of the osmotic mechanisms needed for sperm activity and

increased viscosity of the activation media (Beirão et al., 2014), but the opposite effect has also been documented. In one example, sperm velocity was enhanced at 50% OF but inhibited at 5% (Turner and Montgomerie, 2002). In another example, there was no difference in sperm performance between 5% and 25% OF (Beirão et al., 2014), indicating that the differences in concentrations may not have been high enough to observe an inhibition effect. Our inability to detect significance for OF concentration as a moderator is likely due to such conflicting reports between studies, fish species, and the inconsistencies between the concentrations that were compared.

We can discuss further that in our analysis, represented taxonomic groups comprised primarily of salmonids, in which high positive effect sizes have been consistently reported. The spawning behavior of salmonids may have encouraged sperm to react positively to OF over the course of evolutionary history. Males and females gather in huge aggregations to spawn in heavily crowded areas, introducing fierce male competition at the gametic level (Mjølnørød et al., 1998). Therefore, sperm must have high motility and velocity -traits that were enhanced by OF in the studies we analyzed. One reason may be due to the high OF levels expelled with the eggs compared to other families, which can be 10-30% volume relative to the egg mass (Lahnsteiner et al., 1999; Wojtczak et al., 2007). This has been shown to have a necessary function in enhancing fertility for this family and may reflect the strong responses exhibited by sperm during activation (Urbach et al., 2005; Galvano et al., 2013).

Another source of variation may be related to the population origin of the fish, and/or genetic relatedness between populations (Beirão et al., 2014,2015). This hypothesis is clearly validated in distinct populations of Atlantic cod, where OF from southern origin females had greater inhibiting effects on sperm performance for northern males than those from their native population,

indicating a preference for mates within the same population (Beirão et al., 2015). There is evidence that fish OF composition shows intra-specific variation, particularly with respect to the constituents known to influence sperm behavior (Lahnsteiner et al., 1995; Wojtczak et al., 2007; Rosengrave et al., 2009b). Therefore, OF composition between females of each population likely resulted in differential sperm performance among genetically diverged populations and acted as a method of cryptic female choice in some marine species such as Atlantic cod. Further evidence of intraspecific variation in sperm-OF interactions was also indicated in freshwater species. For example, sperm from genetically unrelated males was estimated to be 10% more competitive in fertilization trials with OF than their relatives in guppies (Gasparini and Pilastro, 2011). This relationship shows the opposite effect by selecting against more genetically similar individuals. Within the same population individuals are not affected by reproductive isolation, so it may serve to prevent inbreeding at the gametic level. It has been proposed that cryptic female choice may be a significant selection mechanism responsible for this variation within and across populations (Gasparini and Pilastro, 2011; Mautz et al., 2013; Yeates et al., 2013). Whether or not effect size estimations in our analysis were due to population-level mate preference certainly warrants further study. Due to a small number of studies testing such effects, OF from distinct intra-specific populations was not examined as a source of heterogeneity in our meta-analysis but certainly may have significant within-study impacts.

To fully explain the patterns in our results, we must be aware of the many different properties that distinguish each activation environment. Freshwater and saltwater environments each present unique challenges that sperm must overcome to reach the egg and achieve fertilization. One notable difference is that the ions that comprise the OF micro-environment may be altered by the osmolality of the external environment (Hirano et al., 1978; Elofsson et al., 2006). Thus, sperm

are responding to different biochemical cues. For instance, freshwater and saltwater differ tremendously by ion composition, which is shown to alter sperm performance, especially in freshwater fishes (Alavi and Cosson, 2006). Therefore, it is possible that sperm of freshwater fishes show higher positive responses when activated in OF because maternal ions that stimulate sperm activity are more easily detected in a low-ion environment (Elofsson et al., 2006; Rosengrave et al., 2009a). This effect would not be so apparent in marine species due to high amounts of ions already present that interfere with the sperm's ability to distinguish and respond to the OF micro-environment.

Furthermore, the timing of activation trials could have led to some significant results going undetected. In some cases, sperm may show a delayed response that occurs beyond 0-15 s post activation. For example, OF did not yield a positive response until 30 s post-activation for Caspian brown trout, *Salmo trutta caspius* (Hatef et al., 2009). Furthermore, in sticklebacks (in which sperm motility can last several minutes), there were positive responses for sperm velocity activated in seawater after 45 min (Elofsson et al., 2003a) and after 60 min post-activation (Elofsson et al., 2003b). With activation times in our analysis limited to the first 0-15 s, any delayed responses were not considered, but it is unlikely that they are relevant to a male's fertilization success (Casselman et al., 2006). To illustrate the importance of immediate fertilization, a 2 s delay of sperm release caused significant reductions of paternity in Atlantic salmon (Yeates et al., 2007). Furthermore, in sockeye salmon, *Oncorhynchus nerka*, 80% of fertilization was achieved within first 5 s of sperm-egg mixing (Hoysak and Liley, 2001) These results suggest that sperm swimming speed is an important trait of male gamete quality, and even small variations in the timing of gamete interactions could have consequences for fertility. Thus, improvements in sperm

performance due to OF after the critical first 15 s may not have substantial biological impacts on fertilization outcomes.

Lastly, our analysis also points to the unavoidable differences in methodology between studies that are not held constant (i.e. methods of sperm extraction/processing, origin of gametes, and activation techniques) that may have contributed underlying effects. Subtle differences such as sperm density, seminal plasma and OF pH and composition, and dilution rates varied between studies and were sometimes not even reported, which could have altered sperm swimming behavior. OF concentration also ranged widely from low to high amounts across studies. Although methodology was not analyzed statistically due to a small number of studies for both traits, it was still likely to be a large source of remaining unexplained heterogeneity in our analysis. Future studies may confirm or expand off our interpretation of the results as more data becomes available and methodology becomes more consistent.

4.5. Conclusions

Our meta-analysis showed that sperm velocity was enhanced by the presence of female OF for species spawning in freshwater environments. However, no such relationship could be concluded for the marine species in our analyses. We determined that spawning environment can have significant impacts on the magnitude and direction of the observed effects and also that results are subject to vary based on phylogeny of the fish species. We are confident that our investigation was a reliable method that allowed for representative quantification of the overall true effect and exploring sources of variability across studies from different statistical populations. We limit our interpretation to the range of species on which data are available, comprising primarily of species

sharing similar phylogeny and lineage. With more research done on OF as a factor to impact fertility across a wider range of species, it may serve to broaden the understanding of natural processes that govern sperm performance, reproductive success across fish species, and fertilization techniques in hatcheries.

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Figures

Spawning micro-environment

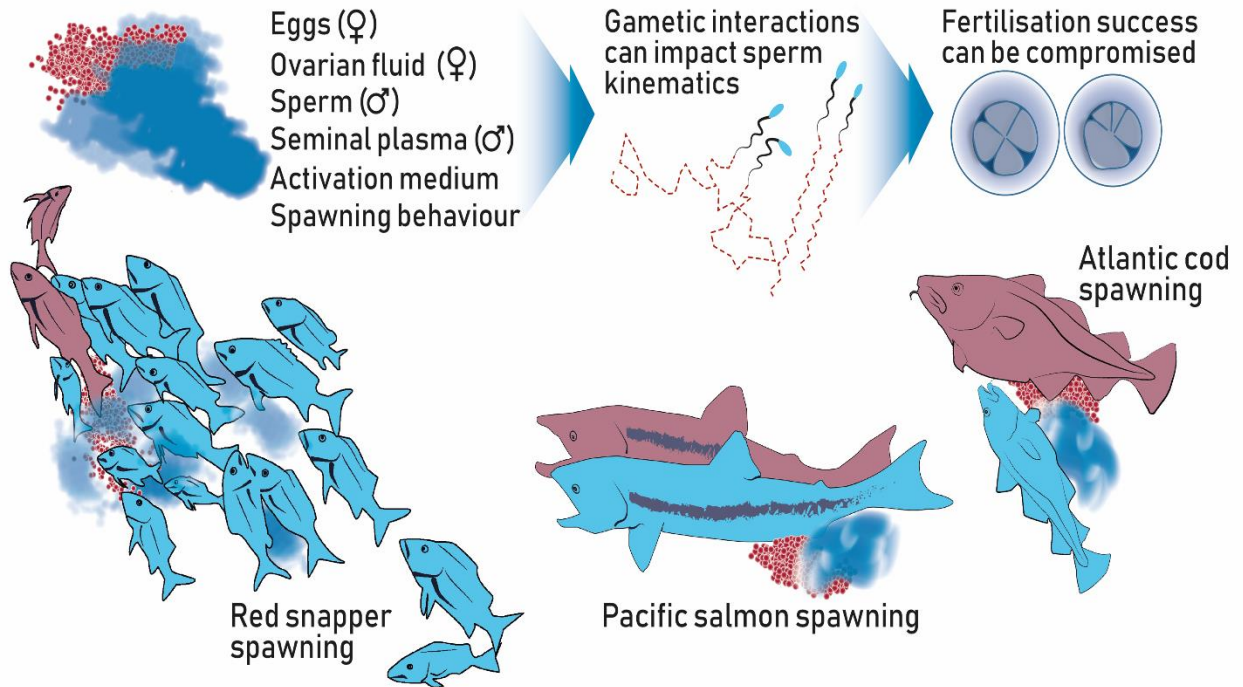


Fig. 4.1. Realistic representation of spawning dynamics for various fish species (e.g. Atlantic cod *Gadus morhua*, Pacific salmon *Oncorhynchus* spp., and Red snapper *Lutjanus campechanus*). In each scenario, females create unique fertilization micro-environments by expelling their distinct ovarian fluid (OF) along with an egg batch. In turn, these maternally-derived substances can potentially impact the outcome of a fertilization event by modifying sperm trajectories. Image designed by Sune Sørensen.

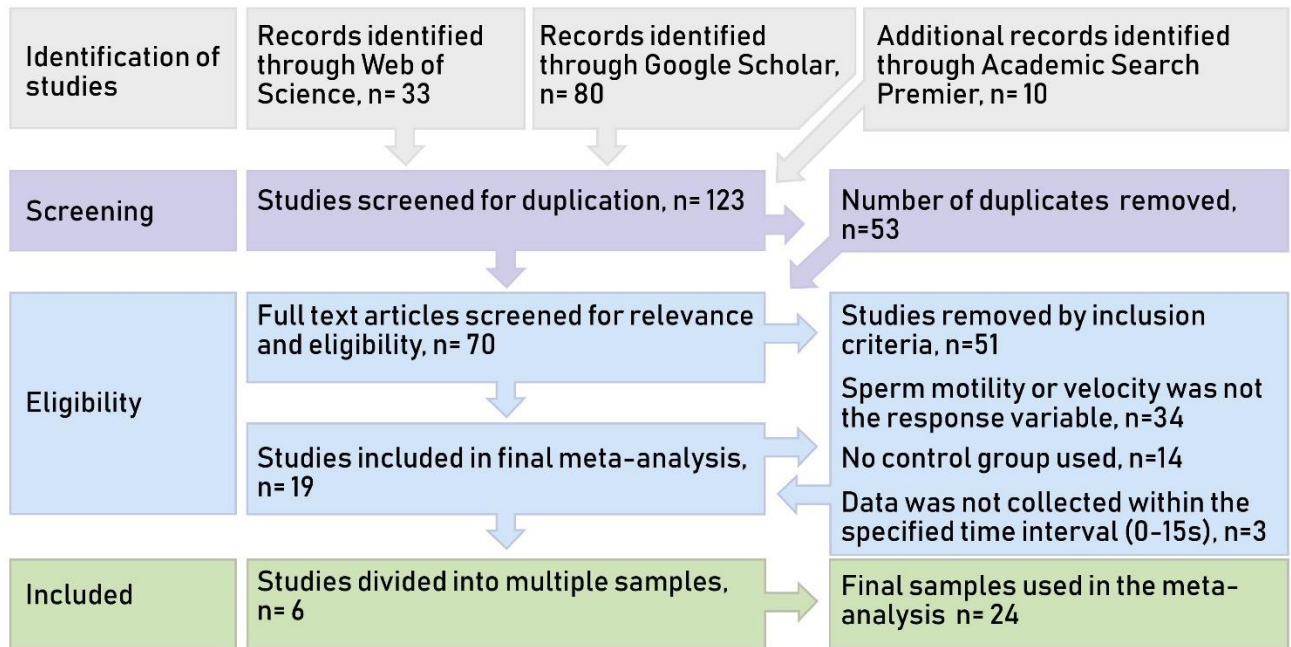


Fig. 4.2. PRISMA flow diagram describing the initial search, screening, and final inclusion of studies for this meta-analysis on the effects of ovarian fluid on sperm performance. Number of studies is indicated by “n” in each box.

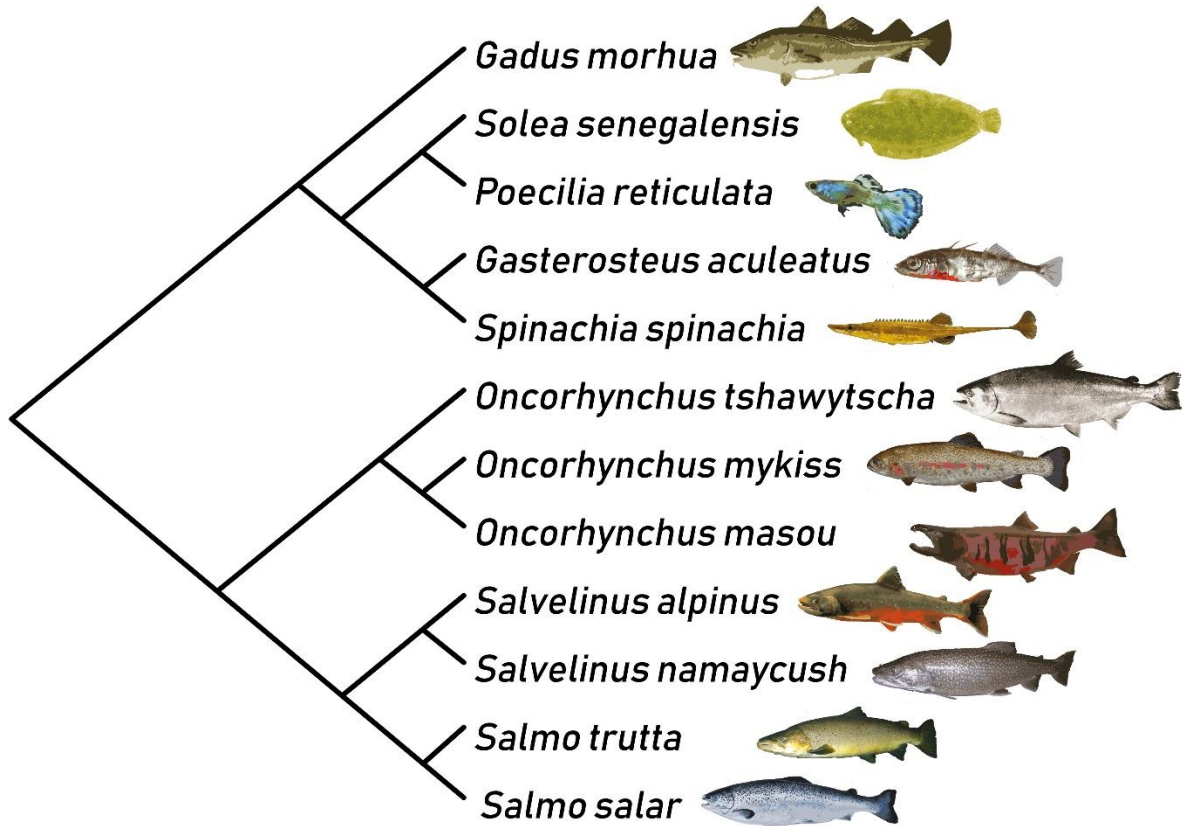


Fig. 4.3. Genetic relatedness phylogeny diagram for the fish species included in the meta-analysis using the interactive Tree of Life-based on the National Center for Biotechnology Information (NCBI) taxonomy. Note that many species shared closely related phylogenies (consisted primarily of salmonids).

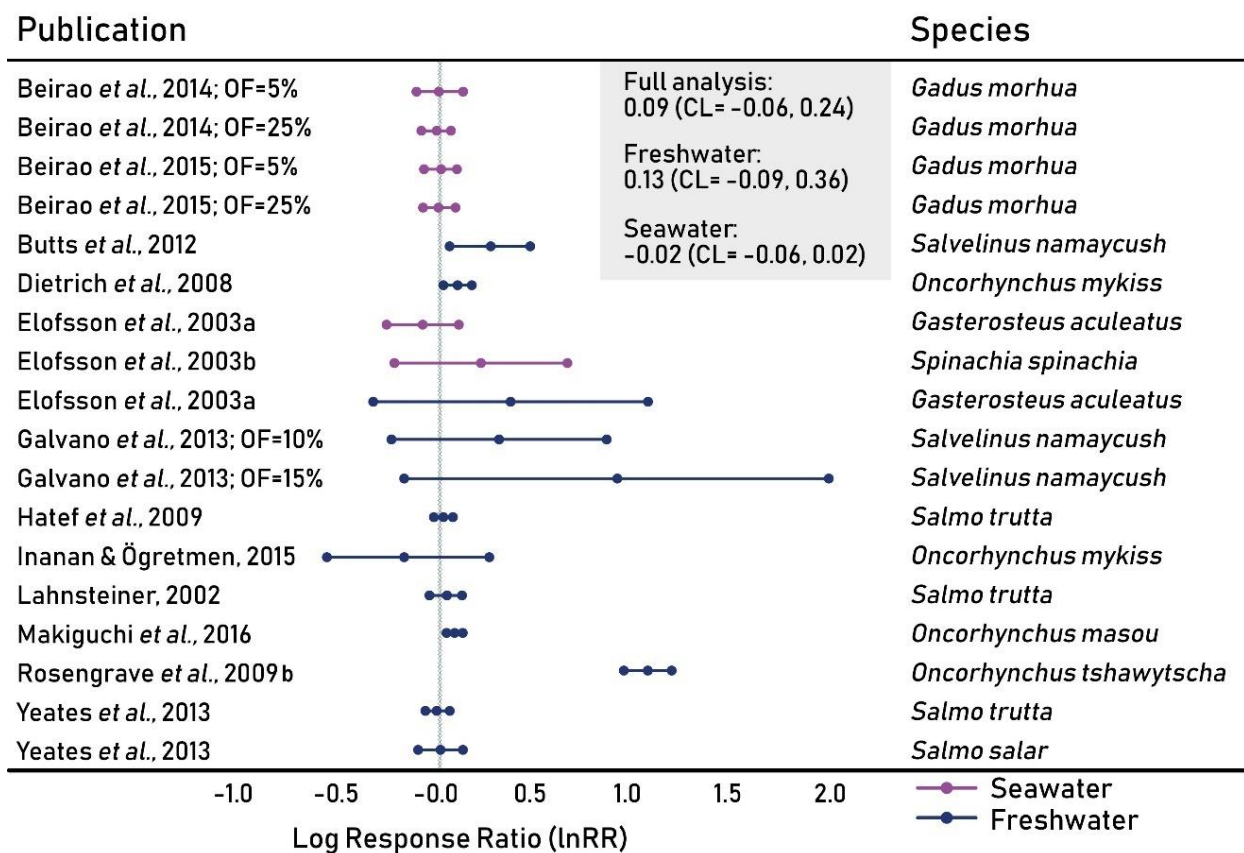


Fig. 4.4. Forest plot for the effects of ovarian fluid (OF) on sperm motility (n = 18 effect sizes). Study ID is given on the left and species of fish used in each is listed on the right. Effect sizes are given as log response ratios (lnRR) with 95% confidence limits for all species. Effect sizes with confidence intervals that encompass 0 are not statistically significant.

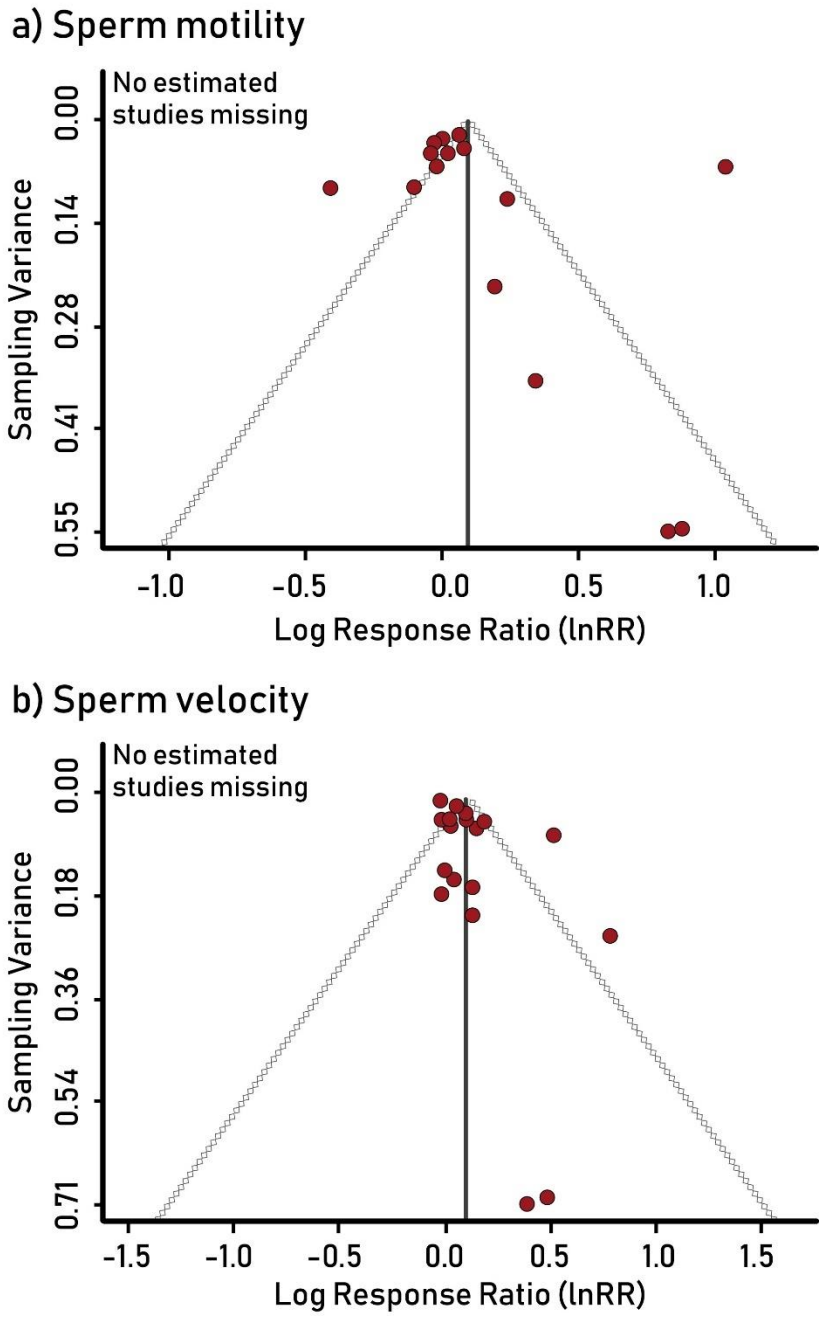


Fig. 4.5. Funnel plots constructed to verify the presence of publication bias for each trait for (a) sperm motility and (b) sperm velocity. Dashed lines represent meta-analytic mean of the lnRR as estimated by the REMA analysis. Due to funnel asymmetry around the means, the number of studies estimated to be missing from a given side was assessed with the ‘trimfill’ function.

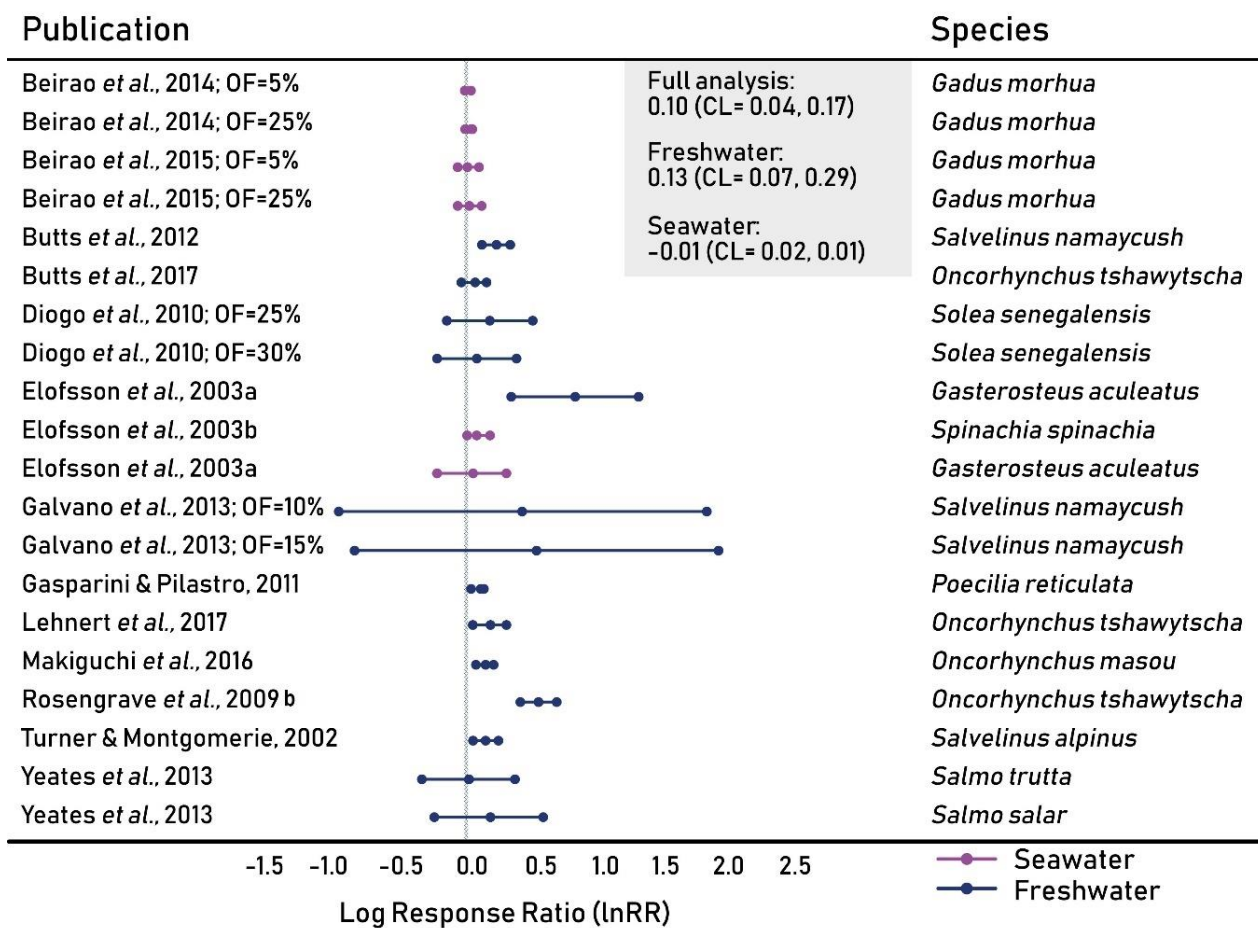


Fig. 4.6. Forest plot for the effects of ovarian fluid (OF) on sperm velocity ($n = 20$ effect sizes). Study ID is given on the left and species of fish used in each is listed on the right. Effect sizes are given as log response ratios (lnRR) with 95% confidence limits for all species. Effect sizes with confidence intervals that encompass 0 are not statistically significant.

Tables

Table 4.1. Final published studies (n = 19) that met our final inclusion criteria and were included for meta-analysis. Studies either reported sperm motility, velocity, or both as response variables. Additional information on spawning habitat and % ovarian fluid (OF) used was collected to assess their respective effects on the RMA. Studies indicated by a and b are separate studies within the same year, and duplicate letters represent multiple samples within the same study (one for freshwater and one for marine). Studies with multiple species or OF concentrations listed were also divided into independent samples for the analysis.

Publication	Species	Spawning habitat	OF (%)	Data reported
Beirão <i>et al.</i> , 2014	<i>Gadus morhua</i>	Seawater	5, 25	Motility, velocity
Beirão <i>et al.</i> , 2015	<i>Gadus morhua</i>	Seawater	5, 25	Motility, velocity
Butts <i>et al.</i> , 2012	<i>Salvelinus namaycush</i>	freshwater	20	Motility, velocity
Butts <i>et al.</i> , 2017	<i>Oncorhynchus tshawytscha</i>	freshwater	40	Velocity
Dietrich <i>et al.</i> , 2008	<i>Oncorhynchus mykiss</i>	freshwater	100	Motility
Diogo <i>et al.</i> , 2010	<i>Solea senegalensis</i>	Seawater	25, 50	Velocity
Elofsson <i>et al.</i> , 2003b	<i>Spinachia spinachia</i>	Seawater	25	Motility, velocity
Elofsson <i>et al.</i> , 2003a	<i>Gasterosteus aculeatus</i>	Seawater	25	Motility, velocity
Elofsson <i>et al.</i> , 2003a	<i>Gasterosteus aculeatus</i>	freshwater	25	Motility, velocity
Galvano <i>et al.</i> , 2013	<i>Salvelinus namaycush</i>	freshwater	10, 15	Motility, velocity
Gasparini & Pilastro, 2011	<i>Poecilia reticulata</i>	freshwater	60	Velocity
Hatef <i>et al.</i> , 2009	<i>Salmo trutta</i>	freshwater	100	Motility
Inanan & Ögretmen, 2015	<i>Oncorhynchus mykiss</i>	freshwater	100	Motility
Lahnsteiner, 2002	<i>Salmo trutta</i>	freshwater	100	Motility, velocity
Lehnert <i>et al.</i> , 2017	<i>Oncorhynchus tshawytscha</i>	freshwater	50	Velocity
Makiguchi <i>et al.</i> , 2016	<i>Oncorhynchus masou</i>	freshwater	10	Motility, velocity
Rosengrave <i>et al.</i> , 2009b	<i>Oncorhynchus tshawytscha</i>	freshwater	50	Motility, velocity
Turner & Montgomerie, 2002	<i>Salvelinus alpinus</i>	freshwater	50	Velocity
Yeates <i>et al.</i> , 2013	<i>Salmo salar</i> , <i>Salmo trutta</i>	freshwater	100	Motility, velocity



Chapter 5

Genetic architecture of early life history traits for channel catfish, *Ictalurus punctatus* ♀ ×
blue catfish, *I. furcatus* ♂ hybrid production



Myers, J.N., Chatakondi, N., Dunham, R.A., Butts I.A.E. 2019. Genetic architecture for early life history traits for channel catfish, *Ictalurus punctatus* ♀ x blue catfish, *I. furcatus* ♂ hybrid production. *Aquaculture*: <https://doi.org/10.1016/j.aquaculture.2019.734436>.

Abstract

Hybrid catfish, the progeny of channel catfish, *Ictalurus punctatus*, females × blue catfish, *I. furcatus*, males, are in high demand by the aquaculture industry due to their superiority for pond culture. Unfortunately, fry production is a limiting factor due to lack of natural hybridization between the species and the necessity to sacrifice males for artificial fertilization. In this study, we used a quantitative genetic breeding design to assess genetic, environmental, and genotype by environment interactions to detail the genetic architecture of fitness during the “critical” early life history (ELH) stages. Males and females were crossed using a full-factorial design, creating 20 unique families. Offspring from each family were split into 2 temperature-controlled environments based on conditions that mimic early (26.6 °C) and late (32.2 °C) seasonal temperatures. Embryonic survival, hatch success, larval morphology, and deformities were quantified at hatch, mid-yolk sac transition, and swim-up stages of early development. Variation in early performance traits (calculated as variance components, VC) were partitioned to maternal/paternal effects as well as parental x environmental interactions, analyzed across and within temperatures. Embryonic survival ranged from 45 to 93% by 120°-hours post-fertilization and was not impacted by temperature. Maternal effects were responsible for large amounts of variation (VC = 51.0%), and paternal effects also became apparent during later stages but in smaller quantities (VC = ~7%). Temperature significantly impacted hatch success, in which hatch decreased at 32.2 °C (from 40% to 32%). Therefore, we conclude that temperatures at the start of the spawning season yield higher hatch success. Maternal effects were highly significant (VC = 65%), and there were also significant paternal effects (VC = 12%) with wide family variation (ranging from 14 to 71%). The deformity rate increased from 3.6% at 26.6 °C to 6.0% at 32.2 °C, but variation was only significant for the maternal effects. For morphology, fry reared

at 32.2 °C had smaller body sizes at each developmental stage. Maternal variation across morphology traits ranged widely from 9 to 80% and was highest for yolk area. Paternal effects/interactions ranged from 0–29%. Genotype by environment interactions were also observed for morphology traits since values for VCs differed within each temperature. This information showed the importance of environmental effects, parentage, and their associated interactions, which by isolating indicators of male/female quality, can be used to develop parameters for broodstock selection. Results can also be applied to improve incubation conditions for hybrid catfish during the crucial ELH stages.

5.1. Introduction

In the southern United States, catfish farming in Mississippi, Arkansas, Alabama, and Texas accounts for nearly 70% of total US freshwater aquaculture production, in which the channel catfish, *Ictalurus punctatus* ♀ × blue catfish ♂ hybrid constitutes >50% of the harvest (Torrans and Ott, 2018). Hybrid progeny produced by this cross are superior for pond culture as they exhibit improved growth, disease resistance, feed conversion, and harvestability (Chatakondi et al., 2000; Brown et al., 2011; Dunham and Masser, 2012; Perera et al., 2017), making them highly valuable for aquaculture. Although the hybrid industry has seen sustained growth, there are still challenges associated with inconsistent production of hybrid catfish fry. As such, research protocols are exploring ways to improve this dilemma.

Knowledge of optimal environmental conditions for newly hatched fry is essential to maximize yields during early development (Lambert and Dutil, 2001; Shan et al., 2008). A

crucial step in accomplishing this is understanding larval development, growth, and survival during the early life history stages (ELH; from embryonic stages to first feeding) when fry mortality rates are highest and most variable (Houde, 1997). This stage is commonly coined the “critical period” (Hjort, 1914) and has been widely recognized in the field of aquaculture, especially for marine fishes (Sifa and Mathias, 1987). In the hatchery environment, larval mortality peaked during this crucial time for species that spawn in saltwater such as Japanese eel, *Anguilla japonica* (Kurokawa et al., 2008) and Baltic cod, *Gadus morhua callarias* L. (Voss et al., 2001) but also for those that spawn in freshwater including walleye, *Sander vitreus*, (Li and Mathias, 1982) and brown trout, *Salmo trutta* (Elliott, 1989). Regardless of the species, both commercial and research scale hatcheries depend on maximizing larval performance and survival during ELH stages and must be cognizant of the factors that influence it.

Catfish species, specifically, exhibit differential growth and survival of newly-hatched fry due to physical properties of the rearing environment such as photoperiod (Appelbaum and McGeer, 1998), pH (Mischke and Wise, 2008), salinity (Britz and Hecht, 1997; Weirich and Tiersch, 1997), and water temperature (Small and Bates, 2001; Green and Fisher, 2004), all of which can be manipulated in hatcheries. Perhaps the most important of these factors is incubation temperature as it controls traits such as metabolic activity, developmental rate, time of hatch, survival, and rate of larval deformities (Small and Bates, 2001; Georgakopoulou et al., 2007). Temperature of the incubation environment can range widely in the wild and within hatcheries, and tolerance to these extremes is often species specific (reviewed by Burt et al., 2011).

In catfish hatcheries, female channel catfish are induced to spawn in spring/early summer when seasonal water temperatures range from 23 to 30 °C, with the optimal being ~26–27 °C

(Wellborn, 1990; Chapman, 2000). Therefore, early spawning females are subjecting their progeny to different temporal conditions in the spring than encountered later in the summer, which may have lasting effects. The impacts of temperature on early development and survival of cultured and wild fish larvae have been widely studied across taxa (reviewed by Blaxter, 1992). As rearing temperature increases so does metabolic activity, causing embryos to mature faster and hatch sooner (perhaps even prematurely) with the opposite effect observed as the temperature decreases (Holt et al., 1981; Puvanendran et al., 2015; Bian et al., 2016; Falk-Petersen et al., 2019). Survival and hatch success have also been shown to decrease when reared in suboptimal conditions (Geffen et al., 2006; Falk-Petersen et al., 2019). The percentage of environmentally-induced larval deformities is also driven by temperature-growth relationships with higher numbers of deformities observed when temperatures drifted outside non-optimal ranges for a species (Nagano et al., 2007; Dionísio et al., 2012). Since rearing temperature determines how long the embryos stay in the egg before hatching, size traits including egg size, size at hatch, yolk sac area, and yolk utilization efficiency are commonly affected. These traits determine how quickly fry reach the first feeding stage and how susceptible they are to mortality during this time (Politis et al., 2014).

Parental effects (a collective term referring to maternal and paternal components) and their importance in determining fitness of individual progeny are widely recognized (Heath and Blouw, 1998). Of the two, maternal effects are more prominent during early development since females contribute valuable nutritional reserves, important metabolic hormones, and maternal RNAs through the yolk (Kerrigan, 1997; McCormick, 1998, McCormick, 1999). Growth and viability are influenced by intrinsic maternal factors corresponding to size, age, and condition of the female (Morehead et al., 2001; Dunham, 2011). Egg size and quality (nutritional composition

of the yolk) both contribute to the progeny's fitness such that larger, older females may produce larger eggs of better quality than younger females (Siraj et al., 1983; Vallin and Nissling, 2000). Therefore, progeny produced by these higher quality females may have an early competitive advantage at hatch over others (Dunham, 2011). In cultured salmonids, the positive relationship between body size of the female and size of fry at hatch is a well-documented maternal effect (Heath and Blouw, 1998), with reports of increased hatching success and larval survival as well (Beacham and Murray, 1985; Beacham, 1988; DeMarch, 1991; Berg et al., 2001). In poikilotherms, the duration of maternal effects corresponds to the rates of growth and development (Green, 2008), which is primarily driven by temperature. For instance, maternal effects were detected several months post hatch for the cold-water Chinook salmon, *Oncorhynchus tshawytscha* (Heath et al., 1999) and for only 30 days in channel catfish (Reagan Jr and Conley, 1977). Of the two species, channel catfish spawn in much warmer environments, resulting in faster development and rapid disappearance of maternal influence. Overall, understanding the relationships between females and their respective effects on early development are important for efficient fry production.

Previously, paternal effects have been thought to be insignificant since males only contribute nuclear genetic material to the offspring (Heath et al., 1999). Additionally, hatchery practices that pool milt from multiple males for fertilization also eliminate the ability for paternal contributions to be detected (Bekkevold et al., 2002; Rideout et al., 2004). However, more recent reports have found evidence that males also contribute to larval performance, although effects have not been shown for all larval morphology traits (Kroll et al., 2013). Paternal effects have been identified as significant variance components (VC) for phenotypic variation in cultured species including Atlantic herring, *Clupea harengus* (Høie et al., 1999a; Høie et al., 1999b),

winter flounder, *Pseudopleuronectes americanus* (Butts and Litvak, 2007a; 2007b), brown trout (Vøllestad and Lillehammer, 2000), haddock, *Melanogrammus aeglefinus* (Rideout et al., 2004), and Atlantic cod (Kroll et al., 2013), European eel, *Anguilla anguilla* (Benini et al., 2018), and especially in species that exhibit parental care provided by the male before and after hatch (Green and McCormick, 2005). Although impacts of paternity are smaller than the maternal effects, evidence is growing to support that individual identity and quality of each male is of crucial importance during ELH stages (Siddique et al., 2017).

Responses to environmental conditions and parentage during early development have been widely investigated, but the link between the two is still poorly understood. This interaction simply means that the effect distributions of parentage may not be equal across different environmental conditions. Several accounts have linked incubation temperature \times parental interactions to embryonic and larval development, indicating that effects due to family identity on progeny phenotype are based on qualities of the incubation environment (Burt et al., 2011; Dahlke et al., 2016). In two specific cases, genetic \times temperature interactions accounted for 5% and 60% of variance in phenotypic traits during development in pink salmon, *Oncorhynchus gorbuscha*, and chum salmon, *Oncorhynchus keta* (Beacham, 1988, Beacham, 1990), showing that high variability exists for the strength of this interaction. Given the evidence of genetic \times environmental interactions across studies, consideration of parentage is essential, and its lasting effects on development and ELH should not be overlooked in aquaculture practices (Burt et al., 2011). To our knowledge, strength of the variance components for thermal and parental effects (as well as associated interactions) has not been analyzed for the commercially important hybrid catfish.

In this study, we used a quantitative genetic breeding design to assess parental effects, temperature effects of incubation and rearing environment, and their associated interactions to determine how these factors collectively influence hatching success, embryonic survival, larval morphology/growth, and deformities in hybrid catfish fry during early life stages. Together, this will allow us to identify the impacts of individual families as well as the optimal temperature regime that may enhance current hybrid catfish production techniques.

5.2. Materials and methods

5.2.1. Broodstock holding conditions and initiation of spawning

All experiments were carried out at the Thad Cochran National Warm Water Aquaculture Center in Stoneville, MS, USA (33.4240° N, 90.9151° W) in May 2018 (IACUC approved protocol #FY17-008). Mature 4-year-old female channel catfish of the ‘Delta select’ strain ($n = 4$) were obtained from facility aquaculture ponds and transported to 10,000 L holding tanks. Length and weight ranged from 57 to 60 cm and 2.3–2.8 kg, respectively. Gravid catfish were placed in individual soft mesh bags that were suspended in a concrete raceway (water temperature 26–27 °C, pH = 8.6 and dissolved oxygen = 6.8 mg/L) following the methods described by Chatakondi (2014). Each fish was administered two intraperitoneal injections of luteinizing hormone-releasing hormone analogue, LHRHa (Syndel USA, Ferndale, WA, USA) with a priming dose of 20 µg/kg and a resolving dose of 80 µg/kg 15 h later. Ovulation response

was checked every 3 h, beginning 26 h after the resolving dose until adherence of ovulated eggs to the bag was observed.

5.2.2. Egg collection and determination of osmolality and pH

Once ovulation was detected, females were anaesthetized with 200 ppm MS-222 (tricaine methanesulphonate; Argent Laboratories Inc., Redmond, WA, USA) buffered with sodium bicarbonate to minimize stress during handling. The urogenital pore was wiped dry, and eggs were released by gentle squeezing of the abdominal area toward the vent. Special care was taken to avoid contamination with urine, blood, or feces. Eggs were hand-stripped into plastic bowls, lined with Crisco vegetable lipid extract to reduce adherence to surface of the containers. Total egg mass was weighed to the nearest ± 0.1 g. Ovarian fluid pH was measured with an electrode using a HI9321 pH meter (Hanna Instruments, Ann Arbor, MI, USA). The pH of stripped eggs ranged from 7.0 to 8.4. Aliquots of eggs were weighed (~ 5 g) and counted to estimate egg density (eggs/g) for each female from an average of two counts.

5.2.3. Sperm collection

Mature D and B strain of blue catfish males ($n = 5$, age = 6–7 years, length ranged from 75 to 90 cm, weight ranged from 3.5 to 5 kg) were selected for use in this study. After euthanasia, testes were dissected from each fish using forceps and surgical scissors. The average

testes weight was 8.2 g but ranged from 7.5 to 8.8 g. Peritoneum and blood vessels were removed manually. Testes were then rinsed with 0.9% saline solution and macerated through a fine mesh strainer into plastic 50 mL centrifuge tubes. Sperm solution was diluted with saline solution at ~10 mL/g testes weight with 1X PBS. Sperm samples were stored in refrigerated conditions (4 °C) for ~24 h for sperm quality assessment and fertilization. Sperm density for each male was quantified with a Thermo Scientific Genesys 20 spectrophotometer (Fisher Scientific, Madison, WI, USA) at 546 nm based on methods previously described by Chatakondi and Davis (2013). For spectroscopic measurements, saline solution (0.85%) served as a blank solution, and serial dilutions of sperm solutions were prepared with 10^7 , 5×10^6 , 2.5×10^6 , 1.25×10^6 and 0.625×10^6 cells/mL⁻¹. A linear relationship between sperm concentration and absorbance was observed ($r^2=0.96$, $P < .05$), and this calibration was repeated 4 times to confirm the relationship. Sperm solutions for each male were then standardized by adjusting to the density to $2.0-3.0 \times 10^7$ by dilution in saline.

5.2.4. Sperm quality assessment

Sperm traits were analyzed using CEROS II software (Hamilton Thorne Biosciences, Beverly MA, USA). Motility videos were obtained at 10 s post-activation, with 3–5 replicate activations per male. Velocity ($\mu\text{m/s}$) is regarded as a key determinant of fertility in fishes (Lahnsteiner et al., 1998; Gage et al., 2004; Linhart et al., 2005) and was therefore assessed to ensure adequate sperm quality for each male.

5.2.5. Artificial fertilization

Males ($n = 5$) and females ($n = 4$) were crossed in a full factorial design, resulting in 20 hybrid families in triplicate. To incorporate the effects of rearing temperature on each family, the experiment was conducted at two different rearing temperatures simulating conditions encountered early and late in the spawning season (26.6 °C and 32.2 °C), resulting in a total of 120 baskets of fry. Aliquots of ~500 eggs were measured out by weight, according to each female's egg density, into 10 mL plastic cups lined with a thin layer of Crisco vegetable lipid extract to reduce sticking. Standardized sperm solution (0.25 mL) was micro-pipetted directly onto the eggs. Cups were immediately swirled to stimulate gamete contact and allowed to incubate for 3–5 min. They were then activated with 10 mL hatchery water supplemented with 6 g/L Fuller's Earth (used to reduce clumping of the eggs), swirled again, and allowed to incubate for ~5 additional minutes before transferring them into incubation aquaria for the duration of the experiment.

5.2.6. Embryo and fry incubation

Aquaria for fertilized eggs were assembled in an on-site indoor facility with separate recirculating systems used for each rearing temperature. Eggs from each cup were randomly distributed into each basket. Embryos were held approximately 10 cm below the surface in mesh screen baskets (4 × 8 × 12.5 cm) incubated within 20-L polycarbonated aquaria, which was part of the 'aquarium rack' system described in detail by Small (2006). Each rack system consisted of

twelve aquaria provided with flow-through water at 7.5 L/min and diffused air (to saturation) with a common water-treatment system consisting of mechanical filtration, biofiltration, and temperature control. Two of the four rack systems were supplied with 26.6 °C water of identical quality, and the remaining two rack systems were supplied with 32.2 °C (± 1.0 °C). Ammonia levels and nitrate levels were kept at 0–0.05 mg/L, hardness at between 56 and 60 ppm, and dissolved oxygen between 6.2 and 7.0 ppm. General water quality was monitored daily using Hach FF-2 water test kits with a digital titration system (Hach, Colorado, USA) and oxygen levels with a YSI model 58 DO meter with a 550A probe (YSI, Yellow Springs, OH, USA) to ensure they remained within acceptable levels.

5.2.7. Embryonic survival

Developmental rate of embryos and fry during the ELH stages is primarily driven by temperature, with faster growth and development expected at higher temperatures (Blaxter, 1992). In catfish, time to hatch decreases considerably as temperature increases (Small and Bates, 2001). Thus, sampling was done in degree-hours so that fry from both temperatures were at approximately the same level of development at each sampling point. At 48°-hours, all unfertilized/dead eggs were removed to minimize risk of fungal infections. The number of viable eggs remaining was used as our initial estimate of embryonic survival. Dead eggs were identified visually by their white, opaque color and/or enlarged size, counted, and removed with forceps at 72, 96, and 120°-hours until hatch. Sampling during the embryonic stages was conducted with minimal disturbance to the embryos as not to induce stress.

5.2.8. *Hatch success*

Hatch success was determined upon termination of the experiment and calculated as the total number of hatched fry (including those removed for sampling) divided by the initial number of eggs.

5.2.9. *Classification of deformities*

Deformed fry were identified visually and classified into the following groups: pericardial (edema), head (deformed head shape), eye (no eye/abnormal eye), yolk (abnormal lobe shape), tail (bent, curled, or missing), and spine (bent curvature). Rate of deformities (%) was calculated as the total deformed fry sampled/total fry sampled per basket.

5.2.10. *Larval morphology*

Once >50% hatch was reached, ~12 individuals were randomly sampled from each basket at three time points coinciding with natural developmental stages: hatch (~130°-hours), mid-yolk sac transition (~160°-hours), and before first feeding when >50% of fry swam up to the surface (~180°-hours). Fry were euthanized prior to handling with 15 mM MS-222 buffered with sodium bicarbonate (Close et al., 1996). Digital images were obtained at 10× magnification using a Meiji Techno RZ objective microscope (Meiji Techno America, CA, USA) equipped with a

DSLR Canon E03 Rebel T3i camera (Canon USA, Inc., NY, USA). All morphology measurements were extracted using WebPlotDigitizer v.3.9 (WebPlotDigitizer, Austin, TX, USA). Total length (distance from tip of snout to fork of tail), notochord length (distance from tip of snout to tip of notochord), yolk sac area, eye diameter, jaw length, myotome height (body height measured immediately posterior to anus), and body area (body area excluding finfold area and yolk sac) were obtained for each individual. Yolk utilization efficiency (YUE) was calculated as the increase in length from hatch to the swim-up stage divided by the corresponding decrease in yolk area (Hardy and Litvak, 2004).

5.2.11. Statistical analysis

All data was analyzed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were evaluated for normality (Shapiro–Wilk test) and homoscedasticity (plot of residuals) to ensure they met model assumptions. Data were \log_{10} or arcsine square root transformed to meet these assumptions when necessary. Alpha was set at 0.05 for testing main effects and interactions. Traits were analyzed independently at each sampling point. Least squared means (LSMs) and VCs (% of overall variation due to random effects) were constructed using the restricted maximum likelihood (REML) method with the SAS PROC MIXED statement. The factorial mating design measures the variance of the male (sire) and female (dam) effects and the sire x dam interaction effects (Comstock and Robinson, 1952), allowing us to infer the VCs in terms of combining ability as an additional interpretation of maternal/paternal random effects. Paternal and maternal effects are equivalent to general

combining ability (GCA) for sire and dam, respectively, whereas the paternal \times maternal interaction is equivalent to specific combining ability (SCA) (Hallauer and Miranda Filho, 1988). Hereafter, dam and sire GCA as well as SCA will be referred to as maternal effects, paternal effects, and maternal \times paternal interactions, respectively. Our objective was to analyze the results at each time point and to obtain distributions of the random effects within and across temperatures. Thus, we utilized two different model approaches as described below.

5.2.12. Variation across temperatures

Each trait was analyzed using a multi-factorial ANOVA (with fixed effect of temperature and random effects including maternal/paternal effects and all interactions terms) based on the means for each basket. Denominator degrees of freedom for all F-tests were approximated using the Kenward Roger procedure (Spilke et al., 2005). A posteriori analyses performed on fixed effects were constructed using Tukey's multiple comparisons method. To test for significant variability among VCs greater than zero in the PROC MIXED model, likelihood ratio statistics were generated (Littell et al., 1996) from the $-2[\text{Res}]$ tricted log-likelihood estimate of the full model and then with each VC held to 0 using the PARMs statement. The probabilities were halved to account for the one-tailed probability and obtain the significance level for each VC (Littell et al., 1996; Messina and Fry, 2003; Fry, 2004).

5.2.13. Variation within temperature

Separate PROC GLM models were used to analyze the following random effects at each temperature: maternal, paternal, and maternal \times paternal interactions. VCs were generated directly for the model using the PROC VARCOMP statement.

5.3. Results

5.3.1. Embryonic survival

Results from the full model showed that temperature did not impact embryonic survival from 48 to 120°-hours post-fertilization ($P > .05$; Fig. 5.1). VCs from the full model revealed that maternal effects were responsible for up to 51% of the variation. Paternal effects also became significant ($P < .05$) at the two later stages of embryonic development (96 and 120°-hours), accounting for ~7% of the variation (Table 5.1). Results by temperature increased the magnitude of the maternal effects (53–69%; Table 5.1). Paternal effects continued to appear during the later embryonic stages at 6–7%. At 26.6 °C, embryonic survival by 120°-hours post-fertilization was highly variable, ranging from 53 to 89% between the 4 females (Fig. 5.2a). There was also within-female variability driven by individual males, with differences ranging from 8 to 14%. At 32.2 °C there was higher parental variation, spanning 45–93% across females and 9–22% across males (Fig. 5.2b). Highest male variability was observed for the worst-performing female

(Female 3) and was lowest for the best performing female (Female 4) at 32.2 °C, but this trend was not observed at 26.6 °C. Thus, temperature of the rearing environment caused differential ranges for the observed distribution of the parental effects. Mortality was highest from 48 to 72°-hours for all families, in which afterward survival was consistent until hatch.

5.3.2. Hatch success

Temperature had a significant impact on hatch success ($P = .031$; Fig. 5.3a), with values of 40% at 26.6 °C and a reduction of 32% at 32.2 °C. Maternal effects were highly significant (VC = 65%; $P = .004$), and paternal effects also contributed up to 12% ($P = .001$; Table 5.1). In the analysis by temperature, there were strong maternal effects (68%) as well as lesser paternal effects (11 and 20%, respectively). Both maternal and paternal effects caused wide variation in hatch, which ranged from 16 to 71% at 26.6 °C and 14 to 48% at 32.2 °C (Fig. 5.4a). At 26.6 °C, the maternal \times paternal interaction term was significant ($P < .05$; VC = 4%) but was undetected at the higher temperature.

5.3.3. Yolk utilization efficiency

Mean YUE was 1.47 at 26.6 °C and 1.71 at 32.2 °C, but this difference due to temperature was not statistically significant ($P = .154$). There was a significant maternal \times paternal interaction (VC = 12%) observed ($P = .035$), and the other random effects were very

small or undetected (Fig. 5.3b, Table 5.2). By temperature, the size of the interaction term increased (up to 24%) at 26.6 °C but decreased to only 6% at 32.2 °C, showing a sharp reduction in the VC distribution depending on the temperature. Family variation ranged from 1.04–2.34 at 26.6 °C and 1.20–3.33 at 32.2 °C (Fig. 5.4b). Neither maternal nor paternal effects were found to be statistically significant.

5.3.4. Rate of deformities

The mean deformity rate increased from 3.6% at 26.6 °C to 6.0% at 32.2 °C (Fig. 5.5a). However, the impact of temperature was not statistically significant ($P = .101$). Total counts for each deformity class increased (except spinal deformities) at the higher temperature (Fig. 5.5b). Of each type, heart and tail deformities occurred most frequently (up to 45 observations at 32.2 °C), which was 2–3 times higher than what was observed at 26.6 °C. VCs for the maternal effects were significant (14%, $P = .002$), but paternal effects and interactions did not contribute to the variation (Table 5.2). Results by temperature left the results virtually unchanged, with maternal effects still accounting for approximately 13%, although results were only significant for 32.2 °C ($P = .027$). The worst-performing female for hatch and embryonic survival (Female 3) also had the highest number of deformities while the best female (Female 4) had considerably less (Fig. 5.4c).

5.3.5. Larval morphology

Results of the full model showed that temperature impacted several morphometric indices at all developmental stages with significant differences detected for total length, notochord length, yolk area, and body area (Fig. 5.6; $P < .05$). Fry, in general, had shorter lengths and smaller overall body size when reared at 32.2 °C. No differences were detected for jaw length at either stage or for eye diameter and myotome height at hatch or the mid-yolk sac transitional stage. Some delayed effects were observed such that during the swim-up stage, differences were detected for eye diameter and myotome height, in which they become smaller at 32.2 °C. These findings then coincided with the results for total length, notochord length, and body area by the swim-up stage. Another delayed effect observed was that yolk sac area was larger at 32.2 °C despite being smaller at the earlier stages.

Maternal effects varied widely but accounted for up to 80% of the random effects variation (for the maternally-derived yolk) and were consistently significant for total length, notochord length, and yolk area. They were highest for the maternally derived yolk area at hatch ($P = .001$; VC = 80%), which decreased to 63% by the swim-up stage ($P = .008$; Table 5.3). Across traits, the extent of the paternal effects remained nearly constant over time, with a small increase from 5 to 8% between hatch to the swim-up stage. However, paternal effects did not prove to be significant for any trait ($P > .05$). The maternal \times temperature interaction was significant for eye diameter, jaw length, and body area ($P < .05$). VC values were highest for jaw length at hatch (31%) and decreased to only 3% by the swim-up stage. However, for eye

diameter and body area, values of the VCs remained unchanged at each stage. Although maternal effects were high for yolk area, the interaction term was detected in only trace amounts (0–4%), indicating that almost all random variation for yolk area was proportioned into the maternal effect. Paternal \times temperature interactions were also detected across traits, rising from 5% at hatch to 12%, although this interaction was only significant for notochord length during the mid-yolk sac stage ($P = .047$). No traits showed this interaction to be the primary source of variation. The maternal \times paternal interaction was detected in trace amounts for most traits. It was significant for the yolk area at the swim-up stage ($P = .002$), coinciding with the results for YUE in which the interaction was also observed ($P = .035$).

Results by temperature indicated that maternal effects still had a strong influence on morphology traits (Table 5.4.). Unlike the results described above, they were significant for every trait for at least one or more stages, especially for yolk area ($P < .001$). For yolk area, maternal effects dropped over time, but these results were not universal across both temperatures, changing from 80% to 56% at 26.6 °C and 80% to 74% at 32.2 °C. VCs for the paternal effects were higher than those generated from the full model, accounting for up to 16% of the variation at 26.6 °C and 14% at 32.2 °C. There was no apparent increase over time for either temperature. Paternal effects were significant at hatch for eye diameter and myotome height at 26.6 °C and at the mid-yolk sac stage for total length, notochord length, and jaw length at 32.2 °C ($P < .05$). More variation was proportioned into the maternal \times paternal interaction, which was newly detected for jaw length and myotome height at 26.6 °C and remained for yolk area (~7%) at 32.2 °C ($P < .05$).

5.4. Discussion

The present study revealed that temperature had a significant impact on several performance traits in hybrid catfish during ELH stages. Incubation temperatures simulating conditions encountered later in the spawning season (32.2 °C) were responsible for lower hatch and smaller body sizes of the fry than conditions simulating early seasonal temperatures (26.6 °C). Parental effects were evident for almost all traits (as quantified through VCs/combining abilities), especially the maternal effects, which have been well-documented across species to have major impacts during early developmental stages (Heath and Blouw, 1998; Green, 2008). Maternal effects were most apparent for embryonic survival, hatch, and yolk sac area. All of these traits have been correlated with initial female quality (DeMarch, 1991; Berg et al., 2001). There was large variation in performance between the four females in this study, but some of these differences were also attributable to paternal effects and specific maternal × paternal interactions. Paternal effects (previously undervalued in catfish hatcheries due to pooling of milt from multiple males for fertilization) also impacted larval performance traits, although in smaller quantities, indicating it has important lasting impacts that cannot be underestimated. The extent of variation attributable to parental effects varied between temperatures, and thus, the degree their expression varies in response to the suitability of the incubation/rearing environment.

Critical temperature extremes at which embryonic development is negatively affected or ceases altogether have been documented across species (Kucharczyk et al., 1997; Falk-Petersen et al., 2019). In this study, embryonic survival was unaffected by temperature, which may account for the fact that female channel catfish spawn over a wide range of temperatures. In

ponds, females spawn naturally when temperatures fall within 21–29 °C and depending on the weather patterns for a given year, temperatures increase to the point that spawning decreases and eventually stops by late summer (Lang et al., 2003). Therefore, embryos need to be adapted to develop properly in such variable environmental conditions. Our results showed that mortality was highest between 48 and 72°-hours post-fertilization and was observed across both temperatures. This period just after gastrulation is a natural occurrence that often coincides with high embryonic mortality (Geffen et al., 2006).

Lower hatch success at sub-optimal temperatures also occurs in many cultured fishes and sensitivity is species-specific (Hokanson et al., 1973; Laurel et al., 2008; Kurokawa et al., 2008). Unlike embryonic survival, hatch was negatively affected at higher rearing temperatures in this study, indicating that 32.2 °C is exceeding the optimal incubation temperature for hybrid catfish. Our hatch success was comparable to results from Broussard Jr. and Stickney (1981), in which hatch for channel catfish was roughly 50% when eggs were incubated at 32 °C (compared to our hatch of 32%). The authors remarked that their hatch success was lower because of the sub-optimal incubation environment. It can then be expected that hatch would continue to decrease at more extreme temperatures. Other accounts have confirmed that hatch and survival of channel catfish is severely impacted by incubation temperatures <21 °C but that normal development occurs between 21 and 26 °C (Small and Bates, 2001). Accordingly, it is typically recommended that hatcheries incubate developing embryos between 25 and 28 °C (Steeby and Avery, 2005). Therefore, progeny would not typically be subjected to such extreme thermal conditions as analyzed in this study unless reared late during the season. Hatcheries that allow water temperatures to reach these levels could see a drastic decrease in hatch fry yield.

Due to faster rates of development and metabolic activity, it has been shown that yolk is utilized faster and more efficiently as rearing temperature increases (Klimogianni et al., 2004; Sund and Falk-Petersen, 2005). Changes in YUE have been detected for temperature differences as small as 2 °C (Hardy and Litvak, 2004). Nevertheless, thermal conditions that approach the upper critical thermal limits may be dangerous to young fry. In relation to the “critical period” concept, warmer temperatures cause yolk reserves to be depleted faster, making larvae switch to external feeding sooner as the yolk sac diminishes. This may lead to starvation and higher mortality if it occurs prematurely (Politis et al., 2014). However, hybrid catfish did not show major differences in YUE across temperatures, indicating that conditions did not exceed the extremes necessary to observe this effect. We can then determine from our results that fry may be able to utilize their yolk sac with similar efficiency across the range of rearing temperatures they would typically experience in the hatchery setting.

Rate of deformities is another trait that corresponds to quality of the rearing environment and increases as thermal conditions become unfavorable (Nagano et al., 2007; Dionísio et al., 2012; Puvanendran et al., 2015). Deformity rates remain relatively low when conditions are kept at or near optimal conditions (Kurokawa et al., 2008). In a two-year study on channel catfish, deformity rate was compared across strains and families with an overall mean deformity rate of 11.3% for one year and 1.5% the following year (Broussard Jr and Stickney, 1981). The authors determined it was due to differences in water quality (especially high ammonia concentrations the first year), indicating the importance of maintaining optimal rearing environment conditions. Our estimated deformity rates fell in the middle of this range (3–6%), although temperature did not significantly impact these results. Deformity rates this small account for little reduction in fry

production, and hatcheries would not anticipate damaging losses when incubation temperatures are kept within acceptable levels.

Morphometric traits develop retrospective to relative growth rates and are influenced by temperature during the early developmental period (Lindsey, 1988; Imre et al., 2002). On a broad scale, larger body size of fry is positively correlated to swimming performance and consequently, improved feeding success (Drost, 1987; Gallego, 1994). Typically, increased metabolic activity during ELH stages leads to earlier time of hatch and smaller lengths at hatch (Pepin et al., 1997; Benoît and Pepin, 1999). In this study, larval length at each stage during early ontogeny was smaller at the higher incubation temperatures, confirming these previous reports. Length indices for jaw and eye diameter exhibited too little variation to detect any differences. Additionally, yolk sac was smaller at the higher temperature, coinciding with the results of Martell et al. (2005). Yolk sac size was smaller at hatch but not being utilized faster than at the lower temperature, keeping it consistently smaller over time. Besides the accelerated developmental rate, another contributing factor to smaller body size may be premature hatch (i.e. when fry are induced to hatch sooner as conditions become unfavorable). This has been observed in cases where oxygen levels drop below acceptable levels (Czerkies et al., 2001), a response that is exacerbated as temperature increases (Small and Wolters, 2003; Martin et al., 2004).

Parental effects have been widely investigated across fish species and were prevalent for almost all ELH traits in this study. Maternal contributions are generally considered more influential than paternal effects (Rideout et al., 2004) since initial egg quality is essential for the production of viable offspring (Trippel et al., 1997). For instance, in European eel, *Anguilla anguilla*, VCs for maternal effects were comparable to our own results, with values of 61.7% for embryonic survival, 88.1% for hatching success, and 62.8% for larval deformities (Benini et al.,

2018). In catfish, specifically, maternal effects have been closely linked to fertilization success and larval growth (Dunham and Argue, 2000; Bosworth and Waldbieser, 2014). A previous report quantifying variance components in progeny of specific crosses determined that VC estimates for fry growth were significantly large for maternal effects, intermediate for paternal effects, and small for maternal \times paternal interactions (Bosworth and Waldbieser, 2014). Our results agreed that maternal effects contributed significantly to the progeny, accounting for most of the random effects variation and that this effect diminishes over time as the environmental effects become more prominent. In channel catfish, the maternal effect duration is estimated to last up to 30 days (Reagan Jr. and Conley, 1977), but it is clear by the differential embryonic survival and hatch between females in our study that these effects are detectable almost instantly after fertilization. It can be concluded that quality of individual females greatly influences offspring performance and must be put into careful consideration by catfish hatcheries. Therefore, industry improvements can be made by ensuring proper broodstock preparation/care and by limiting selection genetically to only females that produce the best eggs, which will help to increase hybrid fry production and overall efficiency.

In addition to maternal effects, paternal effects and interactions between males and females are also recognized as crucial factors influencing larval fitness (Butts and Litvak, 2007a, Butts and Litvak, 2007b; Green, 2008; Butts et al., 2009; Burt et al., 2011, Curley et al., 2011). In our study, paternal effects accounted for less than maternal effects but still showed that individual males impact progeny performance especially for hatch and embryonic survival. These paternal effects were detected as early as 96^o-hours post-fertilization. Our results confirm what has previously been found in tropical clownfish (Green and McCormick, 2005), *Leuciscus idus*, and Northern pike, *Esox Lucius* (Siddique et al., 2017) in which paternity

interacted with maternity to affect traits during embryonic development and after hatching. However, in Atlantic cod, significant paternal effects were detected 8 days post-hatch after the yolk-sac was depleted (Kroll et al., 2013), likely due to the lower metabolic rate characteristic of a cold-water species. Similar to maternal effects, expression of paternal effects appears to be species specific. In contrast to Atlantic cod, hybrid catfish express strong effects of parentage very early during ELH stages. There was evidence that paternal effects play an essential role early during development and that over time, they would become more prevalent beyond the ELH stages we observed, demonstrating the undeniable importance of individual males.

Lastly, our study showed that parental components have differential lasting effects based on temperature and overall quality of the incubation/rearing environment. Although these effects were only detected in trace amounts across temperatures, comparison of the parental effects and differences in combining abilities between temperatures indicated noticeable variation exists. From our data, we concluded that maternal effects appear to be stronger at 26.6 °C for YUE and some of the morphometric traits. Likewise, paternal effects and interactions increased for hatch and for some of the morphometric traits at the lower temperature, so effects of individual parentage may be more apparent at optimal incubation temperatures. At suboptimal temperatures, more variation may be driven by environmental effects, overshadowing and further decreasing the parental influence. Our results have proven that there is significant evidence of parental \times environmental interactions for hybrid catfish, which has also been confirmed across several other species (primarily salmonids) for traits including embryonic development, morphology, and larval growth (Beacham, 1990; Heath et al., 1993; Hebert et al., 1998). In one exceptional case, ELH traits were all heavily influenced by parental \times temperature interactions in tropical clownfish, which was more than the independent maternal effects (up to 30% of total

variation; Green and McCormick, 2005). The interaction between paternity and temperature was also shown in Atlantic cod (Dahlke et al., 2016). The influence of paternity itself was highly significant and explained between 44 and 56% of the variation in hatch success in a series of half-sibling families, and additionally, different families varied in performance across different incubation temperatures. With their results and those we have demonstrated in this study, research has begun to highlight the importance of these interactions. Therefore, it has been strongly recommended that temperature be fully considered in association with parent-progeny relationships in order to accurately quantify all aspects of variation during early development (Burt et al., 2011). By more efficiently partitioning variation from all possible sources (whether they are genetic or environmental) we continue to broaden our understanding on how these factors interact to create phenotypic differences during the “critical” ELH stages.

5.5. Conclusions

Together, this information demonstrated the importance of environmental effects, parentage, and their associated interactions during early developmental stages of cultured fishes. Results of this study can be applied to improve incubation conditions for long-term sustainable development of hybrid catfish and promote the industry to isolate indicators of male and female quality to improve broodstock selection. With progeny production so dependent on initial egg quality, hand selecting individuals for spawning with the best eggs may decrease the variability gap between best-performing and worst-performing females. Additionally, due to differential performance by individual males, we recommend selecting high-quality individuals and that

pooling sperm during fertilization should be avoided by hatcheries as it masks potential underlying paternal effects. Due to the high value of blue catfish males and the length of time it takes for them to mature, future research should then focus on isolating the factors that make a good donor so that low-quality males are not sacrificed needlessly. Cryogenic genetic repositories can then be optimized to preserve gametes from these high-quality fish for future fertility applications.

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Figures

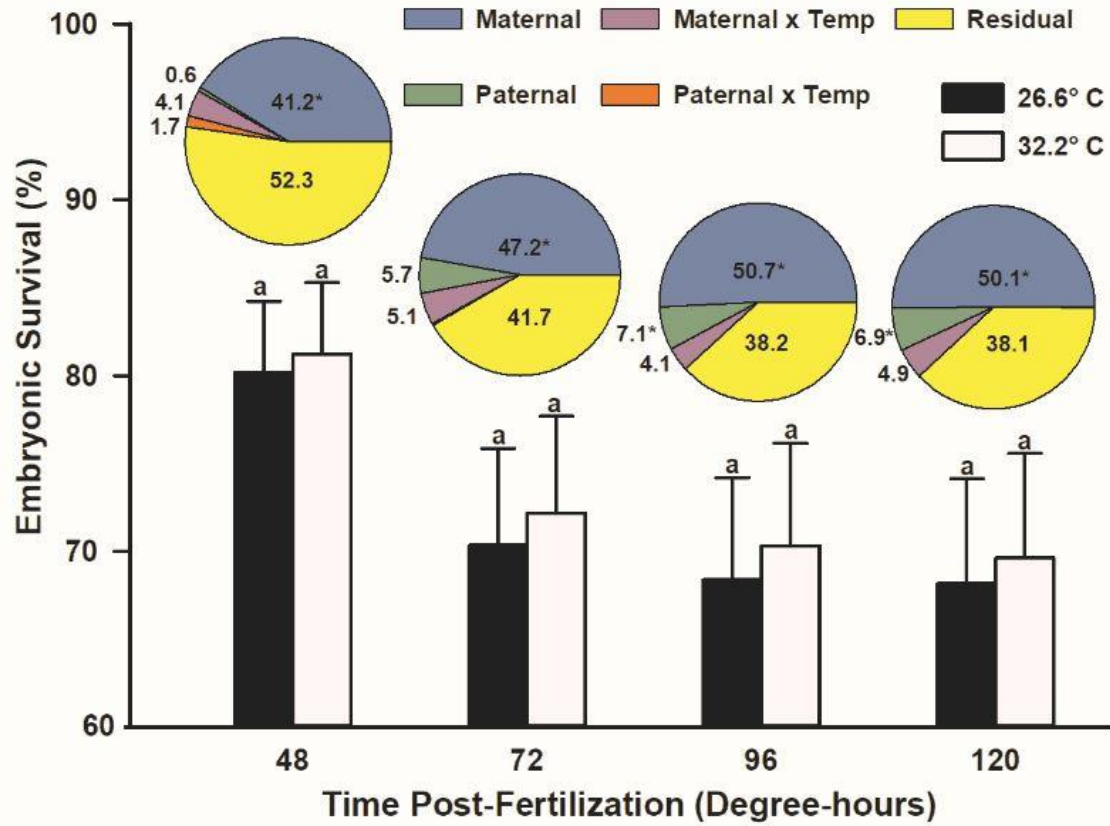


Fig. 5.1. Embryonic survival of hybrid catfish (*I. punctatus* × *I. furcatus*) embryos from 48 to 120°-hours post-fertilization. Error bars indicate means + SEM at each temperature with dissimilar letters representing significant differences at alpha = 0.05. Pie charts indicate the variance component (VC) distribution (%) for the random effects in the full model analysis and significant VCs are denoted by *.

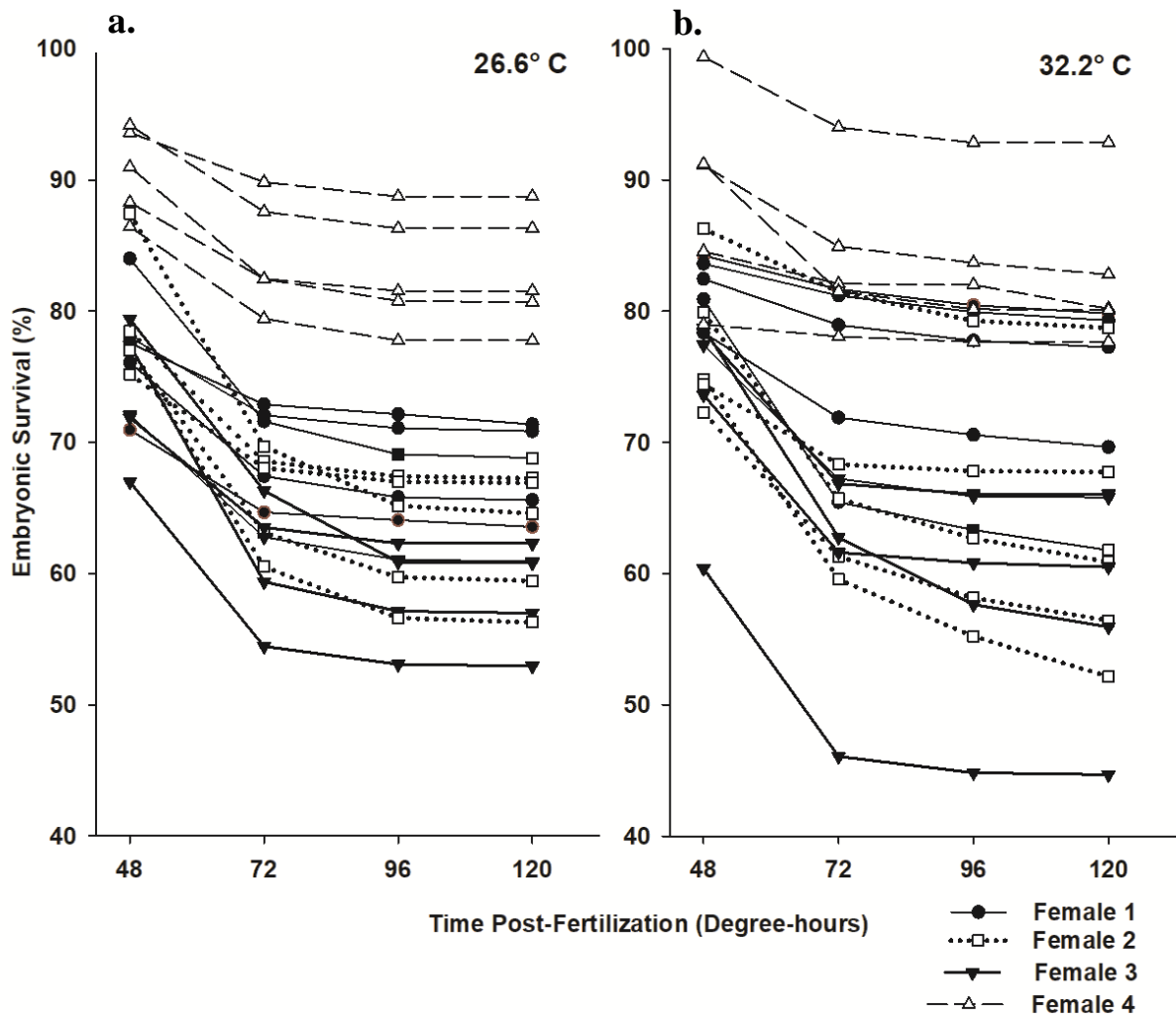


Fig. 5.2. Embryonic survival of hybrid catfish embryos (*I. punctatus* × *I. furcatus*) from 48 to 120°-hours post-fertilization at 26.6 °C (a) and 32.2 °C (b). Each line is the mean + SEM for each of the 20 families. Identical lines represent different males nested within the same female.

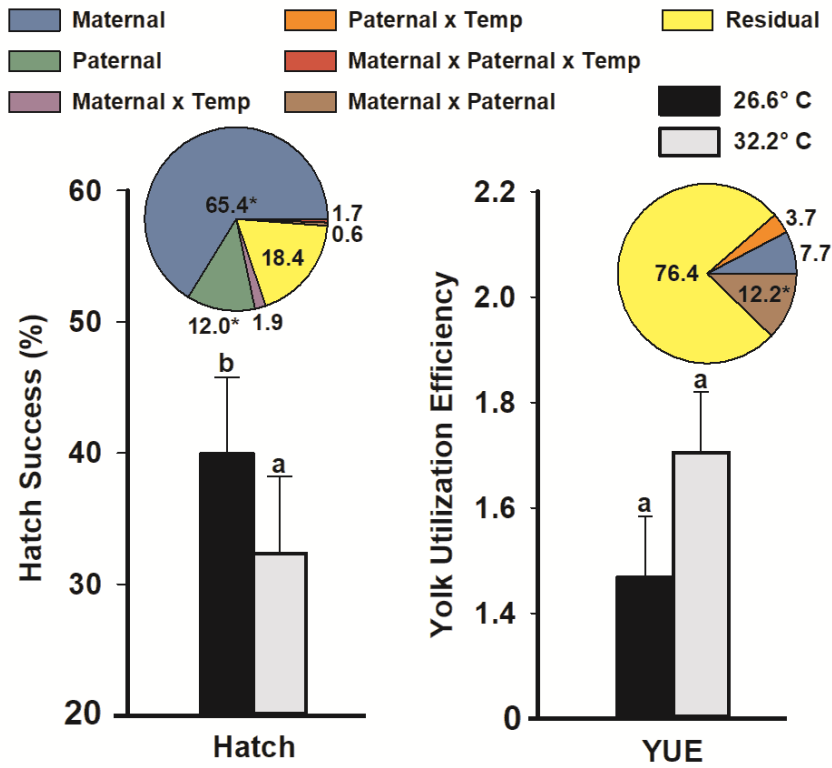


Fig. 5.3. Hatch success and yolk utilization efficiency (YUE; decrease in yolk area from swim-up stage/initial yolk area) for hybrid catfish fry (*I. punctatus* × *I. furcatus*). Error bars indicate means + SEM at each temperature with dissimilar letters representing significant differences at alpha = 0.05. Pie charts indicate the variance component (VC) distribution (%) for the random effects in the full model analysis, and significant VCs are denoted by *.

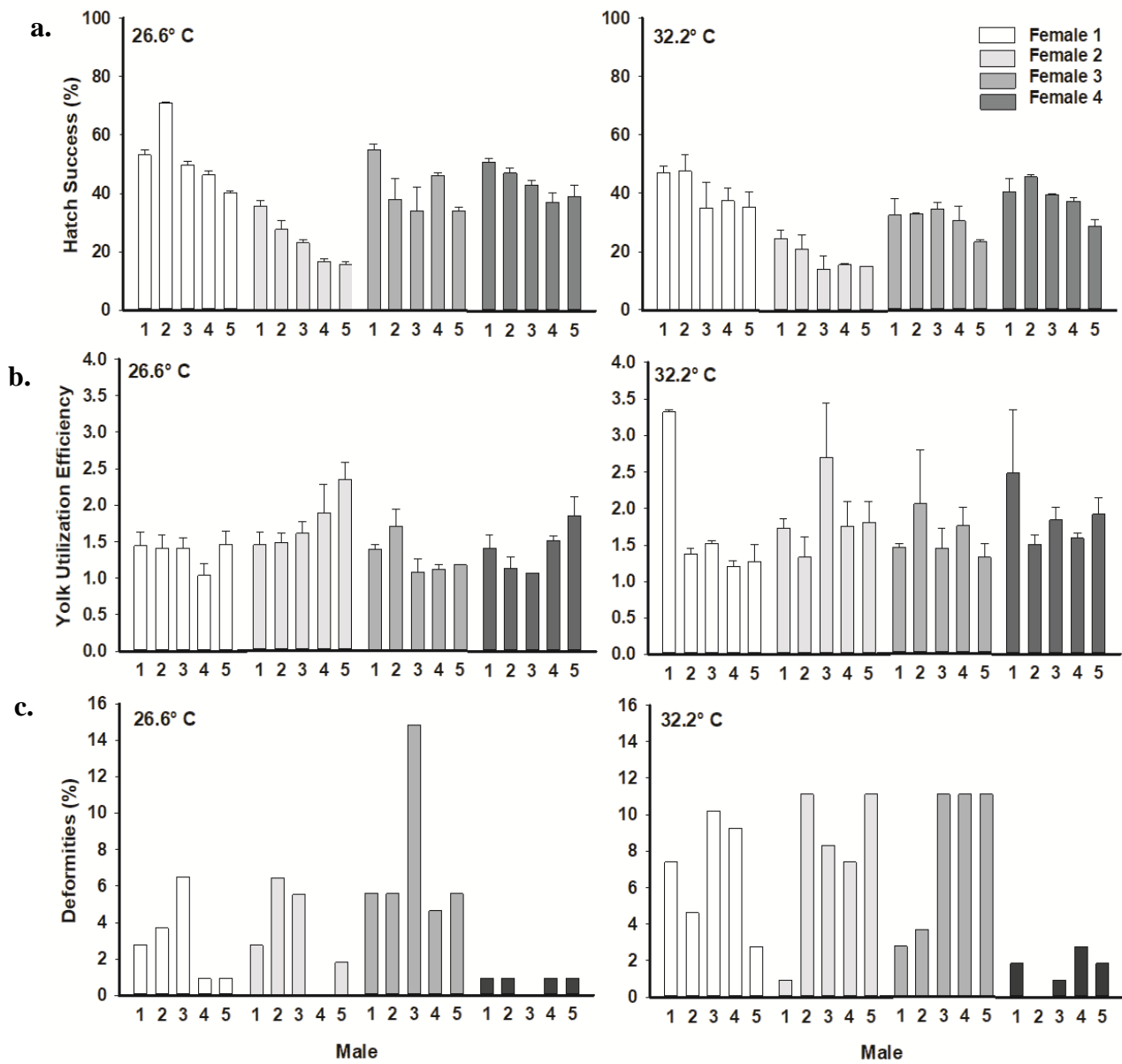


Fig. 5.4. Means + SEM for hatch success (a), yolk utilization efficiency (YUE; b), and deformity rate (c) for each of the 20 families of hybrid catfish (*I. punctatus* × *I. furcatus*) at 26.6 °C and 32.2 °C. Females 1–4 are denoted by bar color, and 1–5 indicates males within each female.

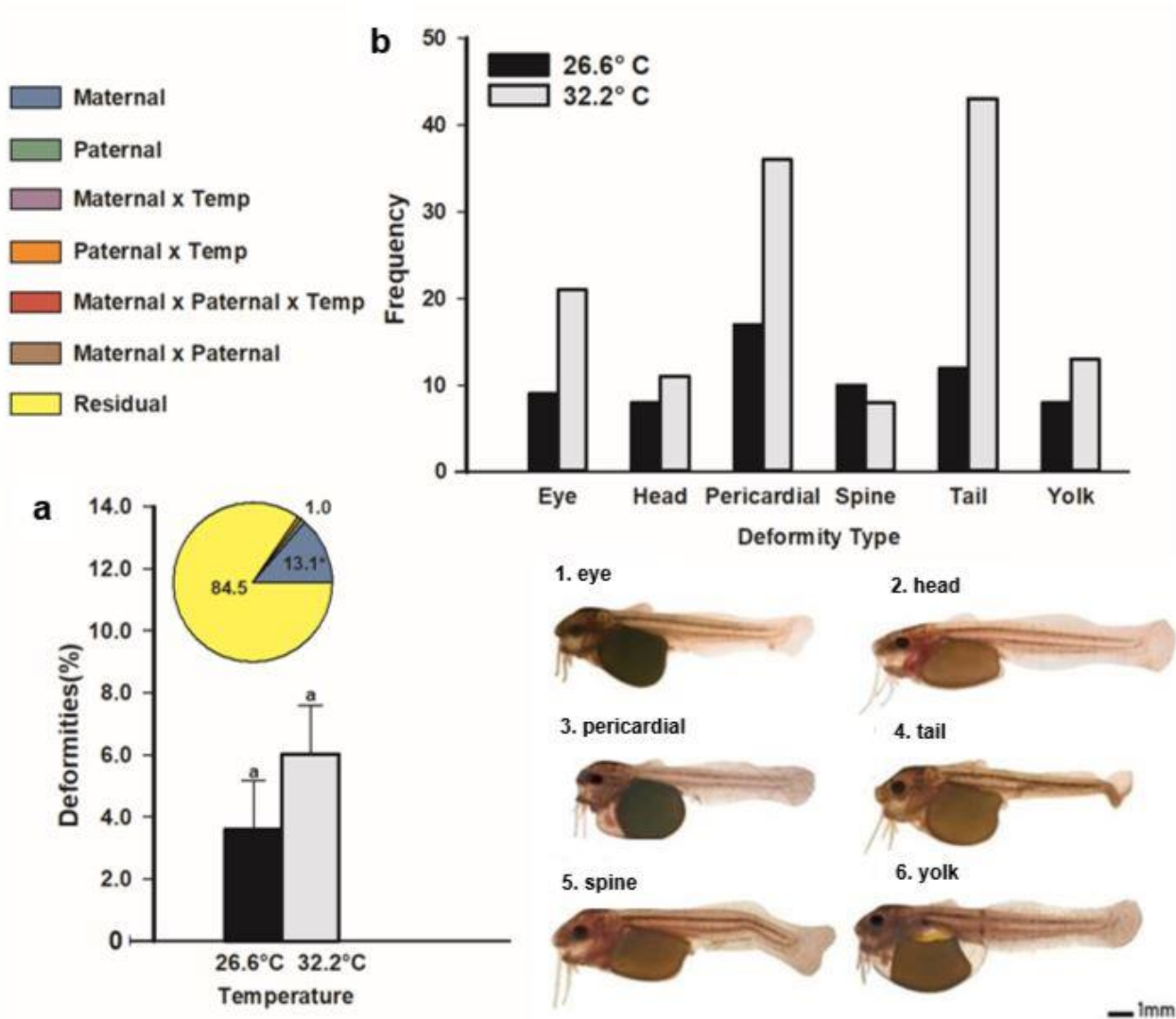


Fig. 5.5. Deformities (%) for hybrid catfish fry (*I. punctatus* × *I. furcatus*) observed at hatch, mid-yolk sac, and swim-up stages collectively. Error bars indicate means + SEM at each temperature with dissimilar letters representing significant differences at alpha = 0.05 (a). The pie charts indicate the variance component (VC) distribution (%) for the random effects across temperatures, and significant VCs are denoted by *. Individuals were assigned to six defined classes (1-6), and frequency of occurrences for each deformity class was calculated for each temperature (b).

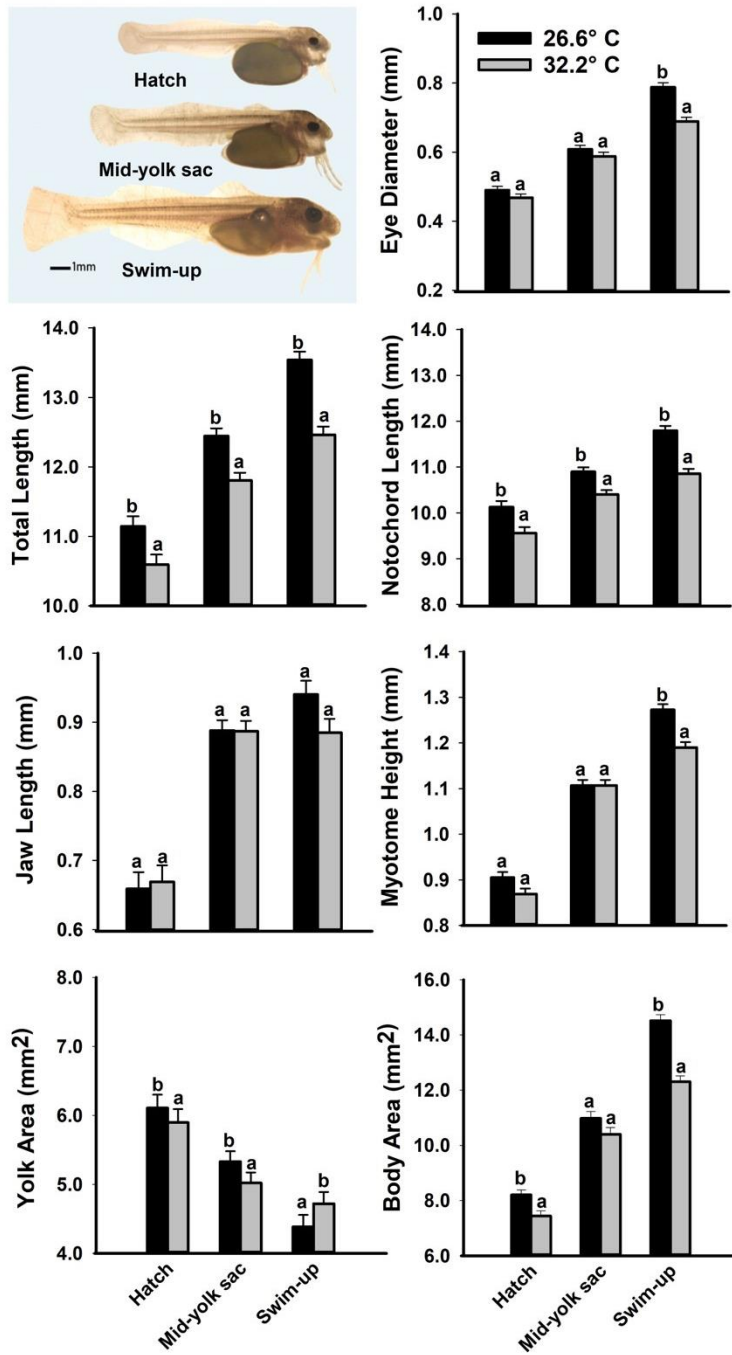


Fig. 5.6. Morphology of hybrid catfish (*I. punctatus* × *I. furcatus*) fry at three early development stages: hatch, mid-yolk sac, and swim-up. Bars indicate least-squared means + SEM at each temperature with dissimilar letters representing significant differences at alpha = 0.05.

Table 5.1. Restricted Maximum Likelihood (REML) variance components (expressed as %) for hybrid catfish (*I. punctatus* × *I. furcatus*) embryonic survival at 48, 96, 72, and 120°-hours post-fertilization and hatch success for the full model and at each temperature. Maternal variance, paternal variance, and their interaction are equivalent to dam and sire general combining ability (GCA) and specific combining ability (SCA), respectively. Significant variance components are denoted by * at alpha = 0.05.

	Random Effect	48h	72 h	96 h	120 h	Hatch Success
Full Model	Maternal (GCA)	41.23*	47.23*	50.73*	50.06*	65.43**
	Paternal (GCA)	0.61	5.71	7.08*	6.86*	11.97**
	Maternal x Temperature	4.11	5.13	3.96	4.94	1.94
	Paternal x Temperature	1.72	0.26	0.00	0.00	0.00
	Maternal x Paternal (SCA)	0.00	0.00	0.00	0.00	1.69
	Maternal x Paternal x Temperature	0.00	0.00	0.00	0.00	0.58
	Residual Error	52.32	41.68	38.23	38.14	18.40
26.6° C	Maternal (GCA)	53.12***	60.62***	63.00***	63.85***	68.54***
	Paternal (GCA)	4.08	7.47**	7.98***	7.61***	19.84***
	Maternal x Paternal (SCA)	0.00	0.00	0.00	0.00	4.18*
	Residual Error	42.82	31.91	29.02	28.54	7.45
32.2° C	Maternal (GCA)	53.60***	60.50***	61.82***	62.05***	68.07***
	Paternal (GCA)	1.60	4.74	6.38*	6.31*	10.69**
	Maternal x Paternal (SCA)	6.77	1.37	0.04	0.00	0.00
	Residual Error	38.03	33.39	31.76	31.64	21.24

* P<0.05 **P<0.01 *** P<0.001

Table 5.2. Restricted Maximum Likelihood (REML) variance components (expressed as %) for hybrid catfish (*I. punctatus* × *I. furcatus*) yolk utilization efficiency (YUE; increase in length from hatch divided by decrease in yolk area) and deformity rate for the full model and at each temperature. Maternal variance, paternal variance, and their interaction are equivalent to dam and sire general combining ability (GCA) and specific combining ability (SCA), respectively. Significant variance components are denoted by * at alpha = 0.05.

	Random Effect	YUE	Deformities
Full Model	Maternal (GCA)	7.69	13.69*
	Paternal (GCA)	0.00	1.00
	Maternal x Temperature	0.00	0.00
	Paternal x Temperature	3.68	0.79
	Maternal x Paternal (SCA)	12.19*	0.00
	Maternal x Paternal x Temperature	0.00	0.00
	Residual Error	76.44	84.52
26.6° C	Maternal (GCA)	15.60	13.22
	Paternal (GCA)	0.92	3.65
	Maternal x Paternal (SCA)	23.81*	0.00
	Residual Error	59.67	83.14
32.2° C	Maternal (GCA)	0.00	13.20*
	Paternal (GCA)	1.41	0.26
	Maternal x Paternal (SCA)	6.60	0.00
	Residual Error	91.99	86.54

* P<0.05 **P<0.01 ***P<0.001

Table 5.3. Full model Restricted Maximum Likelihood (REML) variance components (expressed as %) for hybrid catfish fry (*I. punctatus* × *I. furcatus*) morphology traits (TL = total length, NL = notochord length, JL = jaw length, ED = eye diameter, MH = myotome height, YA = yolk sac area, BA = body area) for the full model, measured at hatch, mid-yolk sac transition, and swim-up stages of early development. Maternal variance, paternal variance, and their interaction are equivalent to dam and sire general combining ability (GCA) and specific combining ability (SCA), respectively. Significant variance components are denoted by * at alpha = 0.05.

Stage	Random Effect	TL (mm)	NL (mm)	JL (mm)	ED (mm)	MH (mm)	YA (mm ²)	BA (mm ²)
Hatch 130 degree-hours	Maternal (GCA)	21.50*	26.95*	6.34	8.38	0.00	79.56**	0.36
	Paternal (GCA)	2.60	3.80	1.63	1.66	5.03	0.00	1.21
	Maternal × Temperature	12.97*	11.06*	31.39**	15.09*	8.52	0.68	16.61*
	Paternal × Temperature	5.06	4.84	5.80	0.00	0.00	0.15	4.51
	Maternal × Paternal (SCA)	0.64	0.14	0.00	1.08	0.00	2.18	0.91
	Maternal × Paternal × Temperature	0.00	0.00	3.44	7.80	16.77*	0.08	4.34
	Residual Error	57.24	53.21	51.40	65.99	69.68	17.35	72.06
Mid-yolk sac 160 degree-hours	Maternal (GCA)	31.33***	32.95*	4.92	17.59	0.00	67.67**	10.92
	Paternal (GCA)	0.00	0.00	4.40	4.92	3.85	0.29	3.76
	Maternal × Temperature	0.00	0.85	15.03*	11.44*	11.16*	3.74	13.26*
	Paternal × Temperature	6.39	8.05*	0.00	0.00	0.00	0.00	0.00
	Maternal × Paternal (SCA)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Maternal × Paternal × Temperature	0.24	0.30	0.00	0.00	0.00	1.15	0.00
	Residual Error	62.03	57.85	75.65	66.05	84.99	27.14	72.06
Swim-up 180 degree-hours	Maternal (GCA)	22.22*	34.87***	9.93	12.82	17.11	63.28**	5.56
	Paternal (GCA)	7.82	5.31	2.89	0.75	0.00	0.00	0.73
	Maternal × Temperature	5.76	0.00	3.16	19.15*	1.66	3.32	18.57*
	Paternal × Temperature	0.00	0.00	11.73	0.00	0.00	0.65	0.00
	Maternal × Paternal (SCA)	0.00	0.46	7.49	0.00	0.34	7.22**	0.00
	Maternal × Paternal × Temperature	0.00	2.78	2.08	3.16	1.04	0.00	4.68
	Residual Error	64.20	56.58	62.73	64.11	79.85	25.54	70.47

* P<0.05 **P<0.01 ***P<0.001

Table 5.4. Restricted Maximum Likelihood (REML) variance components for each temperature (expressed as %) for hybrid catfish fry (*I. punctatus* × *I. furcatus*) morphology (TL = total length, NL = notochord length, JL = jaw length, ED = eye diameter, MH = myotome height, YA = yolk sac area, BA = body area) at each incubation temperature, measured at hatch, mid-yolk sac transition, and swim-up stages of early development. Maternal variance, paternal variance, and their interaction are equivalent to dam and sire general combining ability (GCA) and specific combining ability (SCA), respectively. Significant variance components are denoted by * at alpha = 0.05

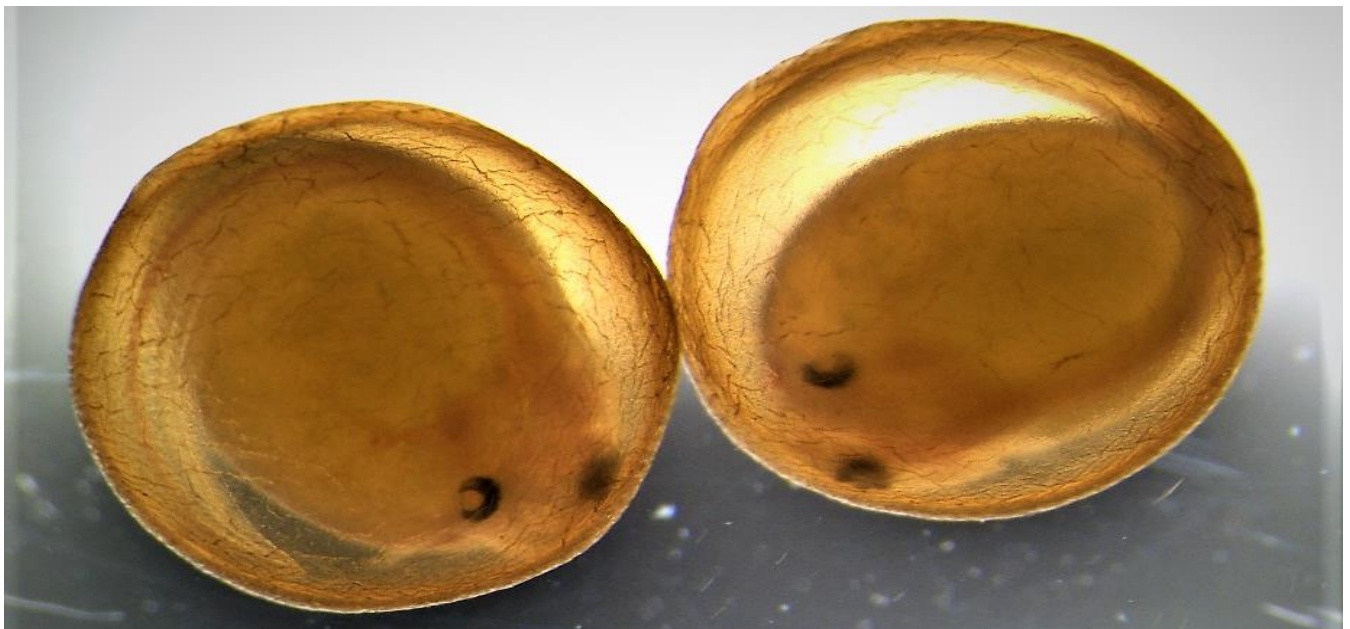
Temperature	Stage	Random Effect	TL (mm)	NL (mm)	JL (mm)	ED (mm)	MH (mm)	YA (mm ²)	BA (mm ²)
26.6° C	Hatch 130 degree-hours	Maternal (GCA)	52.34***	55.90***	51.77***	44.79***	7.31	80.34***	30.44*
		Paternal (GCA)	3.04	5.93	0.23	0.00	0.00	0.00	0.00
		Maternal x Paternal (SCA)	6.86	3.87	1.54	0.00	18.05*	0.61	10.10
		Residual Error	37.75	34.30	46.47	55.21	74.64	19.05	59.46
	Mid-yolk sac 160 degree-hours	Maternal (GCA)	21.72***	29.93**	21.98**	31.78***	12.94**	75.22***	26.31***
		Paternal (GCA)	2.07	3.76	1.56	4.23*	1.63*	1.07	0.20
		Maternal x Paternal (SCA)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		Residual Error	76.21	66.32	76.47	63.99	85.43	23.70	73.49
	Swim-up 180 degree-hours	Maternal (GCA)	39.28**	36.88**	17.41	35.04**	17.69	55.79***	26.23*
		Paternal (GCA)	0.00	0.00	16.00	0.00	0.00	1.96	0.00
		Maternal x Paternal (SCA)	6.11	14.33	21.13*	2.47	13.77	8.74	2.61
		Residual Error	54.61	48.78	45.46	62.48	68.54	33.51	71.17
Hatch 130 degree-hours	Maternal (GCA)	24.02**	25.31**	25.73**	13.63	9.31	80.40***	9.48	
	Paternal (GCA)	10.31*	10.88*	13.69*	1.96	6.80	0.57	9.98	
	Maternal x Paternal (SCA)	0.00	0.00	4.75	14.23	15.64	3.92	6.54	
	Residual Error	65.67	63.80	55.83	70.19	68.25	15.12	74.00	
Mid-yolk sac 160 degree-hours	Maternal (GCA)	31.10**	30.44**	17.61**	27.16**	9.90*	64.68***	22.74**	
	Paternal (GCA)	8.86	10.33	4.51	3.35	0.09	0.00	5.31	
	Maternal x Paternal (SCA)	0.00	0.00	0.00	0.00	0.00	7.49*	0.00	
	Residual Error	60.05	59.23	77.88	69.49	90.01	27.83	71.96	
Swim-up 180 degree-hours	Maternal (GCA)	31.64**	32.22**	4.25	29.61**	18.25*	74.68***	22.82*	
	Paternal (GCA)	0.29	4.31	1.52	0.00	0.00	0.00	2.91	
	Maternal x Paternal (SCA)	2.84	0.00	0.00	5.09	0.00	4.04	4.91	
	Residual Error	65.22	63.47	94.23	65.30	81.75	21.28	69.36	

*P<0.05 **P<0.01 *** P<0.001



Chapter 6

Analysis of specific mRNA gene expression profiles as markers of egg and embryo quality for hybrid catfish aquaculture



Myers, J.N., Dyce, P.W., Chatakondi, N.G., Gorman, S., Quineau, S., Su, B., Dunham, R.A., Butts, I.A.E. Analysis of specific mRNA gene expression profiles as markers of egg and embryo quality for hybrid catfish aquaculture.

Abstract

High variability in egg quality of spawning females exists in individuals due to a variety of factors and can be challenging to assess. In some hatchery species, egg quality and generation of viable embryos is correlated to abundances of specific mRNAs. Channel catfish females show considerable variability in egg quality, causing inconsistencies in hybrid fry production. The objectives of this study were to examine mRNAs linked to egg and embryo quality and determine expression between low-hatch and high-hatch eggs batches through development (0, 24, 48, and 96 hours post-fertilization; HPF). RNA was extracted from eggs/embryos of nine females (n = 4 high quality, n = 5 low quality) and q-pCR was used to quantify relative mRNA abundances. The transcripts assessed in this study perform critical cellular functions, including Tubulin β (*tubb*), Cathepsin D (*ctsd*), Cathepsin Z (*ctsz*), Cyclin B (*ccnb1*), Exportin-1 (*xpo1*), and Ring finger protein 213 (*rnf123*). In this study, percent neurulation and hatch success were significantly less in the low-hatch group than the high-hatch group ($P < 0.01$). Relative gene expression of all transcripts was up-regulated in the high-hatch group and peaked at 48 HPF (neurulation stage), indicating the importance of higher gene expression at this threshold to normally progress until hatch. Due to lack of expression during earlier stages, maternally-derived mRNAs for these genes do not seem to impact early embryonic development. By pairing these molecular markers with physical indicators of egg quality, it may enhance hybrid catfish aquaculture by eliminating problems associated with poor egg quality.

6.1. Introduction

The importance of egg quality (or egg competence), defined as the capacity for eggs to be fertilized and develop into viable offspring (Bobe & Labbé, 2010), has been widely studied across hatchery-reared fishes (Lahnsteiner et al., 2008; Migaud et al., 2013). Nevertheless, challenges still remain in estimating egg quality prior to fertilization due to lack of clear biomarkers (Migaud et al., 2013, Źarski et al., 2017). Despite best efforts to optimize reproduction, egg incubation, and larval performance in captivity, inconsistencies in production are still created by high variations in egg quality from individual females (Myers et al., 2019). Some of the variability is caused by environmental variables the females experience in captivity prior to and at the time of spawning (Lambert et al., 1999; Cabrita et al., 2008). Additionally, hatchery spawning techniques may also be responsible, and egg viability may be compromised by differential responses to hormone administration (Mugnier et al., 2000; Mylonas et al., 2003; Ibarra-Castro and Duncan, 2007), stress to the broodfish during handling (Shreck et al., 2001), mechanical damage to eggs during stripping (Dunham et al., 2000), exposure to microbial diseases (Small and Chatakondi, 2006), and egg over-ripening in the body cavity (Samarin et al., 2011; Ma et al., 2015).

Since maternal contributions are very influential in determining reproductive outcomes, traditional approaches have been to use physical characteristics of females to estimate egg quality because of their known relationships to fecundity, egg size, and embryonic/larval performance (reviewed by Green, 2008). However, identifying the best broodfish by visual indicators alone may not be the most reliable option because traits can vary widely among species and by hatchery conditions (Ciereszko et al., 2009). Several biological/biochemical indicators associated with egg quality have also been identified, some of which are ovarian fluid composition (Zadmajid et al.,

2019), fatty acid levels (Henrotte et al., 2010), and post-ovulatory aging (Aegerter et al., 2004; 2005; Phelps et al., 2007). In many fish species, the maternally derived yolk acts as the primary energy reserve from initiation of development to the first exogenous feeding stages (Kerrigan, 1997). Yolk content, its nutritional value, and other corresponding factors attributable to the female have been shown to influence the size of fry at hatch, age at first feeding, starvation tolerance, and growth/survival through early life history (ELH) stages (Maruyama et al., 2003; Berkeley et al., 2004; Kamler, 2005; Donelson et al., 2008). Due to its undeniable importance, physical and biochemical properties of the yolk and ovarian fluid have become important predictors of egg quality (Cabrita et al., 2008).

Interestingly, recent discoveries on egg quality across teleost species have shown that up and down regulation of specific maternally inherited messenger RNA (mRNA) transcripts are linked to high and low egg quality and consequent larval success (Desvignes et al., 2011; Rozenfeld et al., 2016; Źarski et al., 2017). Transcription of mRNAs that code for essential amino acids and proteins during early development by the zygote is a complex process requiring extreme accuracy and precision (Sullivan et al., 2015). A diverse array of mRNA transcripts with critical cellular functions such as regulation of transcription, immune function, protein activity, cell proliferation, apoptosis, and cell signaling are linked to egg competence in teleost fishes (reviewed in Sullivan et al., 2015). These transcripts have been identified in species such as rainbow trout, *Onchorynchus mykiss* (Aegerter et al., 2004; 2005), zebrafish, *Danio rerio* (Desvignes et al., 2011), gilthead sea bream, *Sparus aurata* (Fernández et al., 2012), Atlantic halibut, *Hippoglossus hippoglossus* (Mommens et al., 2010; 2014), Atlantic cod, *Gadus morhua* (Rise et al., 2014), European eel, *Anguilla anguilla* (Rozenfeld et al., 2016), and European sea bass, *Dicentrarchus labrax* (Źarski et al., 2017), some of which are also found in the hybrid catfish genome.

Initially, eggs depend solely on inherited maternal mRNAs, and production of their own gene products does not take place until the breakdown of the maternal transcripts during the mid-blastula transition (Tadros and Lipshitz, 2009). Afterward, the embryo becomes self-sufficiently governed by zygotic transcription, producing the necessary gene transcripts endogenously from the DNA template of the maternal genome (Pelegri, 2003), the timing of which is species-specific (Schier, 2007; Tadros and Lipshitz, 2009). The transcriptome profile before the embryo transitions to producing its own mRNAs, thus, acts as a “summary” of oogenesis and the overall contributions derived from the female (Chapman et al., 2014). During subsequent developmental stages, abundance of such transcripts can vary widely, but differences have been shown in relative gene expression between high and low quality eggs, confirming that mRNAs can be used as genetic indicators of egg competence. Studying transcriptomic profiles within the egg/zygote is another emerging method to find molecular markers of egg quality at the genetic scale, especially since prior studies have been conducted on only a small number of cultured species (Bobe and Labbé, 2010; Chapman et al., 2014). Of these species, few molecular studies have been done for ictalurid catfishes or specifically, hybrid catfish, despite their economic value and elevated aquaculture status (see Peterson et al., 2005; 2019). Hybrid catfish, the cross between channel catfish, *Ictalurus punctatus*, females and blue catfish, *I. furcatus*, males currently dominate aquaculture production in the United States due to their superiority for hatchery culture compared to either parent species (Dunham and Masser, 2012). Channel catfish females exhibit unpredictable variability in egg quality that greatly influences hatch success and fry produced from a given spawn, highlighting the need to find molecular markers to explain some of this variability at the genetic level.

The objectives of this study were to examine specific mRNA genes linked to egg quality and embryo viability in other fish species and determine if they were differentially expressed

between low-hatch and high-hatch hybrid catfish egg batches. The transcripts assessed in this study were genes that perform critical cellular functions during embryonic development such as protein degradation, intracellular transport, and early cell cycling. Specific targets were Tubulin β (*tubb*), Cathepsin D (*ctsd*), Cathepsin Z (*ctsz*), Cyclin B (*ccnb1*), Exportin-1 (*xpo1*), and Ring finger protein 213 (*rnf123*), of which there were two variants, with Ribosomal 18S RNA (*18S*) as the reference gene. Due to changing levels of these transcripts before and after the maternal to zygotic transition (MZT), mRNAs were quantified during various times during the embryonic development period (0, 24, 48, and 96 hours post-fertilization (HPF)). This will, in turn, determine if expression levels of these specific molecular markers linked to egg quality in other fishes are also reliable for hybrid catfish. By pairing these markers with physical indicators of egg quality, it may serve to enhance aquaculture production for this highly-cultured and profitable fish.

6.2. Materials and Methods

6.2.1. Broodfish

All experiments were conducted at the USDA-ARS Warm Water Aquaculture Center in Stoneville, MS, USA and at Auburn University, AL from June to October 2019. Mature female channel catfish (n = 12) were obtained from earthen aquaculture ponds and transported to 10,000 L holding tanks. Gravid females were placed in individual soft mesh bags that were suspended in a concrete raceway. Each fish was administered two intraperitoneal injections of luteinizing hormone-releasing hormone analogue, LHRHa (Syndel USA, Ferndale, WA, USA) with a priming dose of 20 $\mu\text{g}/\text{kg}$ and a resolving dose of 80 $\mu\text{g}/\text{kg}$ 15 h later. Ovulation response was

checked every 4 h, beginning 26 h after the resolving dose until adherence of ovulated eggs to the bag was observed. Testes from blue catfish males were dissected and sperm solutions were made following methods by Myers et al. (2019). For fertilizations, a unique sperm pool from 3 males was crossed with each female.

6.2.2. Artificial fertilization and embryo incubation

For each female, eggs were hand-stripped into plastic bowls lined with Crisco vegetable lipid extract to reduce adherence to surface of the containers. As initial assessment of egg quality, ovarian fluid pH and osmolality were measured from the whole egg batch. Ovarian fluid pH ranged from 7.4 to 7.9 and osmolality from 249 to 269. Fertilization procedures followed previous methods by Myers et al. (2019). Aliquots of ~500 eggs (~10 g) were measured out by weight, and 1 mL of the pooled sperm solution was micro-pipetted directly onto the eggs. A total of five replicates were incubated from each female, each in separate mesh baskets.

Aquaria for fertilized eggs were assembled in a flow-through system in an on-site indoor rearing facility. Each system was provided with constant water flow and diffused air, and water quality was assessed daily. For incubation, temperature was kept between 27-30 °C, pH between 8.2-8.5, ammonia levels and nitrate levels at 0–0.05 mg/L, hardness between 56-60 ppm, and dissolved oxygen to saturation. Embryos were sampled at four specific time points during the embryonic developmental window: 0, 24, 48, and 96 hours post-fertilization (HPF) (Fig. 6.1). For all sampling times, only eggs that showed signs of developmental progress were selected so that differences in mRNA expression were attributable to differences in the embryos and not from the low-hatch samples consisting of more nonviable eggs. Neurulation % at 48 hours post-

fertilization (HPF) was obtained from subsamples of each basket (40-50 eggs), which has been used as a conservative estimate of fertilization success and embryo viability in catfish (Tiersch et al., 1994). Neurulated embryos can be identified visually by the emergence of skeletal development and movement within the egg at this stage. At this time point, unfertilized/dead eggs were removed to minimize risk of fungal infections to the remaining viable embryos. Hatch success was determined upon termination of the experiment and calculated as the total number of hatched fry (minus embryos removed during sampling) divided by the initial number of eggs for each basket. The Pearson correlation test revealed that percent neurulation and hatch success were positively correlated ($r = 0.85$). Both traits were used to select the classification groups from the averages of the replicate egg batches within each female (Table 6.1). Egg batches from each female were categorized into two experimental groups: high-hatch (>30% hatch and >50% neurulation, $n = 4$) and low-hatch (<20% hatch and near or <50% neurulation, $n = 5$).

6.2.3. Extraction of total RNA and cDNA synthesis

Egg/embryo samples (20 eggs per sample) to be used for RNA extraction were chosen from 9 females that showed extremes in hatch variability. Samples from females yielding intermediate hatch success were discarded from molecular analysis. For each sample, eggs were thoroughly homogenized in 3 mL TRIzol reagent (Invitrogen, Cergyponoise, France) with a Mini Bead Mill Homogenizer (VWR, Radnor, PA). Total RNA was extracted with the PureLink RNA Purification Kit according to the manufacturer's instructions (Invitrogen, Cergyponoise, France) and resuspended in 35 μ L of nuclease-free water. Total RNA was quantified by a Qubit 2.0 fluorometer and Nanodrop ND-1000 (NanoDrop Technologies, Rockland, DE, USA).

Quantity was deemed acceptable for downstream applications with a concentration >20 ng/ μ L (Rozenfeld et al, 2016). Additionally, RNA quantity and purity were measured with UV absorbance at 260 and 280 nm with the Nanodrop to ensure that samples were free of contaminants (e.g. DNA, proteins, leftover extraction reagents). Samples with 280/260 values ≥ 1.9 and 260/230 values ≥ 1.2 were used for downstream applications, as suggested by <http://biomedicalgenomics.org/index.html>. Extracted RNA (1000 ng) was used to generate cDNA using q-script cDNA Supermix, following the manufacturer's recommendations (Quantabio, Beverly, MA). Synthesis of cDNA was carried out in three steps with the following qPCR parameters: initial denaturation for 5 min at 25 °C, reverse transcription for 30 min at 42 °C, and termination incubation for 5 min at 85 °C.

6.2.4. *Target mRNAs*

Transcripts that have been identified as indicators of egg and embryo quality across other fish species were queried using the published GeneBank accession numbers (Sullivan et al., 2015). A total of 31 were searched, of which 23 had an exact match in the channel catfish genome. The same target mRNAs were queried within the channel catfish genome by the name of the gene in the National Center for Biotechnology Information (NCBI) nucleotide database. Channel catfish was used as the model species for sequence design due to the availability of the entire genome. For the transcripts that were identified in channel catfish, unique primer sequences were designed from the GeneBank accession numbers with Primer BLAST comparisons. All primers were designed with amplification size ranging from 100 to 200 base pairs. All primers were ordered from Eurofins Genomics and are listed in Table 6.2. Primer functionality, efficiency, and

optimal concentration were confirmed prior to Real-Time PCR. In order to estimate a suitable dilution of the cDNA samples for qPCR and to test the efficiency of the PCR reaction, standard dilution curves were performed for each primer pair. Primers used for experiments had efficiency percentages ranging between 90-110%.

6.2.5. Real-Time PCR

Real-time PCR was performed using a CFX96 Real-Time System (BioRad, Hercules, CA). Technical duplicate PCR reactions were performed using PerfeCTa SYBR green FastMix (Quantabio). Each reaction well contained 1 μ L of 10 nmol forward and reverse primer templates, 5 μ L of SYBR green, and 1 μ L nuclease-free water. All cDNA was diluted to 5X concentration, and 2 μ L of the diluted product were used for each well, resulting in a total of 10 μ L per reaction. Because the number of samples and genes in this study exceeded the capacity of one PCR plate, *18S* was run with each sample on each plate as a calibrator. Real-time PCR reactions were carried out as follows: incubation was carried out for 30 s at 95 °C and for 10 s at 95 °C. Amplification was performed using the following cycle: 95 °C for 10 s; 58 °C for 20 s; 40 times. Afterward, melting curves were obtained using the following protocol: 5 s holding followed by a 0.5 °C rise starting at 65 °C.

6.2.6. Statistical Analysis

All statistical analyses were conducted in R Studio v.1.1.383 (RStudio, Boston, MA, USA). Student's t-test was used to confirm the statistical significance of the differences in neurulation and hatch success between the low-hatch and high-hatch groups. Real-Time PCR data was extracted using the Bio-Rad CFX Manager Software. For each sample, the two replicate Ct values (Cycle threshold) were averaged. Relative abundances of all cDNAs were calculated for each sample from the averaged Ct values using the $\Delta\Delta\text{Ct}$ method, with the $\Delta\Delta\text{Ct}$ values representing the relative gene expression in the high-hatch egg batches compared to the control (low-hatch group). The mean relative mRNA abundances were directly compared between the low-hatch and high-hatch groups using a series of student's t-tests. This method was done at each time point (0, 24, 48, and 96 HPF). Up- or down-regulation of a gene was considered significantly different from zero at $\alpha = 0.05$.

6.3. Results

There was a drastic reduction in the percent of embryos that reached the neurulation stage ($P < 0.01$) in the low-hatch group when compared to the high-hatch group (averages of $25.0\% \pm 3.8$ and $70.3\% \pm 3.1$, respectively). Hatch success was also significantly less in the low-hatch group than the high-hatch group ($P < 0.0001$), with average hatch success of $6.4\% \pm 0.9$ and $36.9\% \pm 2.4$, respectively.

In the unfertilized eggs (0 HPF), there were no significant differences in gene expression between low-hatch and high-hatch egg batches for each of the studied transcripts (*tubb*, *ctsd*, *ctsz*,

rnf123X1, *rnf123X2*, *ccynb1*, and *xpo-1*; Fig. 6.1). Progressing through development, no differences in expression were detected at 24 HPF, indicating that embryos were developing similarly in both groups through the early stages before segmentation and organogenesis of the embryo. At the neurulation stage (48 HPF), all the mRNA transcripts peaked in expression and were significantly up-regulated in the high-hatch group ($P < 0.05$ for all genes). The effect of this up-regulation diminished by the late period of embryonic development (96 HPF), in which afterwards there were no differences between egg groups.

6.4. Discussion

Low egg quality of channel catfish has been problematic for hybrid catfish aquaculture since the necessary assisted reproduction protocols reduce egg and embryo viability (Phelps et al., 2007), decreasing fry production and overall hatchery efficiency (Chatakondi and Torrans, 2012). In this study, we found that there was significant up-regulation in eggs with higher neurulation and hatch success for mRNA transcripts coding for seven unique genes (*tubb*, *ctsd*, *ctsz*, *rnf123X1*, *rnf123X2*, *ccynb1*, and *xpo-1*) at 48 HPF. These molecular targets have been linked to egg quality and developmental competence in other fish species (Sullivan et al., 2015). The difference in gene expression suggests that the abundance of these mRNA transcripts is linked to critical cellular functions that ensure embryo viability and survival of hybrid catfish until hatch.

Across animal taxa, Tubulin β is an essential building block of cytoskeleton microtubules that control processes of cell division and embryonic development (Aegerter et al., 2005). Because of its importance in regulating intracellular processes, prior results have shown that poor quality eggs have significantly lower abundances of tubulin β . For European eel, tubulin β was expressed

in lower levels in no-hatch eggs at 30 HPF but not before, drawing the conclusions that tubulin β levels are significant during development and that maternally-derived mRNA transcripts (before MZT) do not have an impact for this species (Rozenfeld et al., 2016). For rainbow trout, lower levels of tubulin β were also correlated to poor-quality eggs (Aegerter et al., 2005). Unlike for European eel, the differences were observed at the time of ovulation when gene abundances are fully controlled by the maternally derived mRNA transcripts. In this study, results were similar to Rozenfeld et al. (2016) in that tubulin β was also expressed in higher amounts for high-hatch eggs compared to low-hatch eggs, and this effect occurred at 48 HPF after the transition to zygotic transcription.

Several cathepsin enzymes have been identified in eggs and embryos (Carnevali et al., 2001), which vary in activity levels and time of peak expression and have been thought to be correlated with normal embryonic development (Sire et al., 1994). Cathepsin D is an aspartic protease within the lysosomes that functions in intracellular protein degradation and processing of enzyme activators and inhibitors (Diment et al., 1989). Cathepsin D levels were positively correlated with enzyme activity and yolk sequestration during vitellogenesis in rainbow trout, providing evidence that this gene could be used as a marker for egg quality (Retzek et al., 1992; Brooks et al., 1997). Palomino et al. (2017) confirmed that cathepsin D levels were highest during vitellogenesis and diminished in the ovulated eggs after fertilization. However, other reports have concluded that the activity of cathepsin D (as well as other cathepsin genes) may still be expressed during early embryonic development (Fernández et al., 2012; Lanes et al., 2013). Cathepsin D may be a reliable indicator of female quality, but its role during embryonic development is less clear. Brooks et al. (1997) observed that cathepsin D exists in developing embryos but that levels remain insignificant until gastrulation, which was confirmed by Aegerter et al. (2005) for rainbow trout.

This study found existence of cathepsin D with a peak in expression after gastrulation and initiation of organogenesis of the embryo. Carnevali and colleagues (1999) provided evidence of its potential role as an egg quality marker, showing that cathepsin D was higher in sinking (presumed high quality) eggs than floating eggs (presumed low quality) for gilthead sea bream. This study confirmed such findings, such that high quality eggs were up-regulated for Cathepsin D compared to poor quality eggs. Therefore, it can be concluded that higher cathepsin D expression is beneficial during embryogenesis, likely performing necessary roles like regulating yolk proteins to ensure proper embryonic development (Carnevali et al., 2001).

Cathepsin Z functions similarly to cathepsin D as a lysosomal enzyme and is also present in eggs and embryos during development, previously showing peak expression at the blastula stage (Fernández et al., 2012). Unlike cathepsin D, higher expression of cathepsin Z has been previously linked to poor-quality eggs, the reason of which is still unclear (Aegerter et al., 2005). To the best of our knowledge, Cathepsin Z as an mRNA marker for fish eggs has only been evaluated in rainbow trout, making it an interesting target. This study contrasted the results of Aegerter et al. (2005), showing that cathepsin Z was up-regulated in high quality eggs. However, the previous study generated their results only at ovulation, using post-ovulatory ageing as the treatment factor to separate the high and low quality treatment groups. Thus, the expression of this gene was not examined throughout the whole developmental period. It is possible up-regulation may have occurred later during embryonic development if eggs were sampled beyond ovulation, especially if a gene peaks in expression after the MZT.

Ring finger protein 123, one of many genes located in the RING domain, is coded by *mf123*. Little is known about the exact significance of the gene, but it has roles in mediating protein-protein interactions and is also conserved across phylogenetic groups (RefSeq, 2018). This

study examined expression of ring finger protein 213 because of previous work on Atlantic halibut that showed it was down-regulated in low quality oocytes (Mommens et al., 2014). Our study showed the opposite effect, in which higher expression at 48 HPF of both variant types was associated with higher hatch. Further analyses on this gene during the whole embryonic development window and in other species would clarify the true relationship between *rnf123* and egg quality or if such a relationship can be determined.

Cyclins play important roles in the reproductive development of eukaryotes, and oocyte maturation is strongly regulated by cyclin B (Hirai et al., 1992; Visudtiphole et al., 2009). Fish that spawn in captivity (including channel catfish) commonly encounter dysfunction during final oocyte maturation. Abundances of cyclin B assist in creating mature oocytes by initiating cells to progress beyond the arrested M phase in the ovary (Zhou et al., 2002), justifying it as an important target for female quality. It has also been examined in embryos because of its involvement in early mitotic divisions. Maternal transcription of cyclin B and other cyclin proteins diminishes after fertilization in order to allow phase progression through subsequent developmental checkpoints (Chapman et al., 2014), although this is not the case for all cyclins (Mathavan et al., 2005). Accordingly, Aegerter et al. (2004) found that higher abundances of cyclin B had no significant effect on embryonic survival (hatch success was not assessed) but caused higher percentages of larval malformations. Cyclin B was detected throughout embryonic development of hybrid catfish but with no indication that levels of this gene diminished with time post-fertilization. Cyclin B was up-regulated in high-hatch egg batches, concluding that for this gene, the maternally derived mRNAs did not affect egg or embryo quality. Once gene expression peaked at 48 HPF, higher levels of Cyclin B produced by zygotic transcription may become beneficial to the embryo. Possible explanations for the persistence of cyclin B after oocyte maturation may be continued

functions in mitotic cell divisions and interactions with highly-regulated cyclin-dependent kinases (Brown et al., 1999).

Exportin-1 assists in the nuclear export of proteins, rRNA, and some mRNAs from the nucleus and also regulates mitotic divisions (Kudo et al., 1997; Köhler et al., 2007). Curiously, low expression of Exportin-1 caused arrested embryonic development during neurulation in amphibians (Callanan et al., 2000), but to our knowledge, only one other study examined this gene in fish. Mommens et al. (2010) found that hatch success increased with higher levels of exportin-1 for Atlantic halibut. This study confirmed these findings, indicating that specific Exportin-1 levels may be required to ensure embryonic survival past the neurulation stage. Hence, this gene could be used as a marker of egg quality for hybrid catfish embryos and perhaps other species as well.

Peak gene expression may occur during blastulation, gastrulation, or later stages depending on the species and its highly specific developmental rates (Mathavan et al., 2005). In a previous study, no differences in relative mRNA abundances were detected in hybrid embryos before gastrulation (Peterson et al., 2005). Our results also show no evidence that maternal gene products govern the early embryonic stages for hybrid catfish eggs and instead, up-regulation was observed in high-hatch eggs at 48 HPF when the embryos undergo segmentation and organogenesis - a period termed the neurulation stage. During this critical time, there are significant peaks in expression levels of essential developmental genes, and perhaps embryos with higher expression progress forward and overcome the neurulation threshold. Past this stage, survival of embryos under normal incubation conditions remains fairly constant (Myers et al., 2019). There were no differences in gene expression at 96 HPF, drawing the conclusion that the embryos from both low

and high quality egg groups that survived neurulation to the next time point were the ones sampled for molecular analysis. Because of this, all samples had similar mRNA abundances.

6.5. Conclusions

It is evident that the seven mRNA transcripts analyzed in this study are linked to embryo viability for hybrid catfish and that their expression peaks at the neurulation stage (48 HPF) of embryonic development. There are likely many more genes that have not yet been isolated but may also be used as molecular markers to distinguish high and low quality embryos. Overall, knowledge of mRNA abundances and their influences on egg quality are still elementary and has only been assessed in a handful of the wide array of cultured fish species. The timing of peak gene expression, whether it is directly after fertilization or when embryos incorporate their own transcription, also raises more questions as to what roles each gene plays during embryogenesis. Emerging transcriptome studies would further efforts to find differentially expressed mRNAs on a genome-wide scale, offering new insights on other genes potentially related to egg quality and embryonic development. By pairing these markers with physical indicators of egg quality, it may serve to enhance hybrid catfish aquaculture by eliminating problems associated with the incubation of poor egg quality eggs and inconsistent embryo production.

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Figures

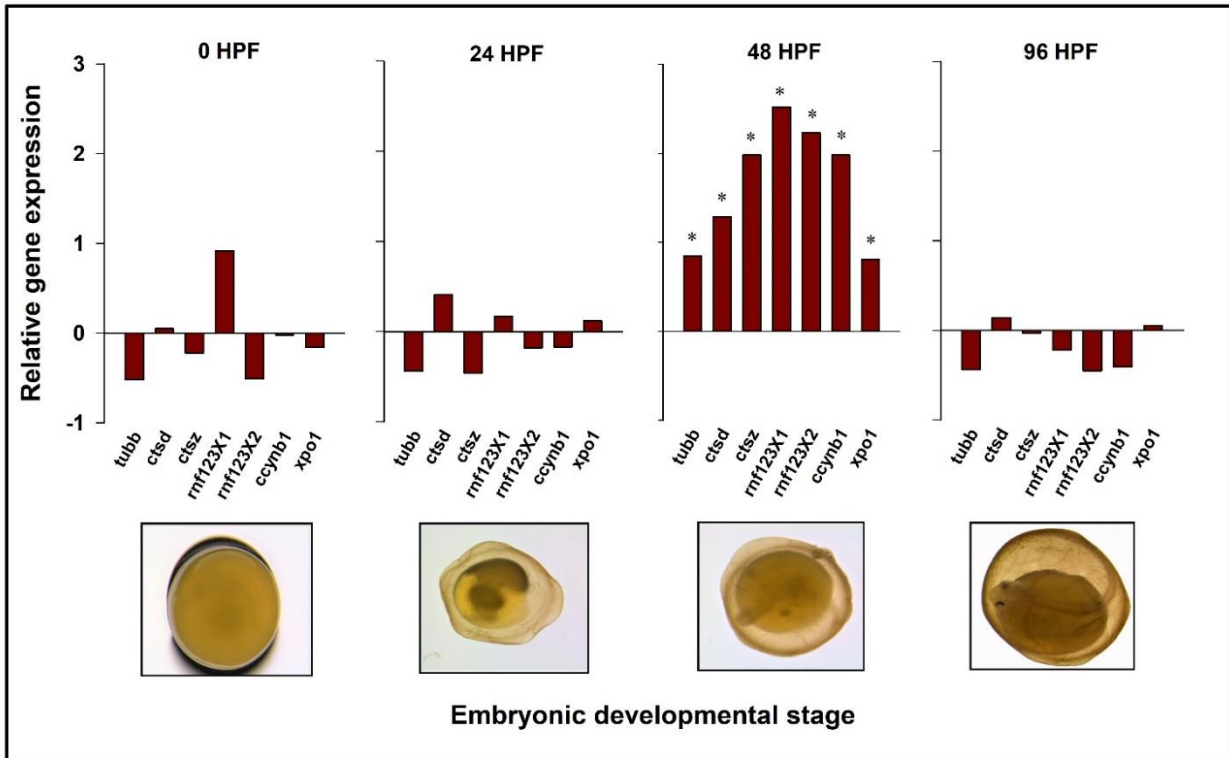


Fig. 6.1. Relative gene expression of high-hatch hybrid catfish eggs compared to a low-hatch control group for seven target mRNAs related to egg quality and embryonic development (*tubb*, *ctssd*, *ctssz*, *rnf123X1*, *rnf123X2*, *ccynb1*, and *xpo-1*). Hybrid catfish embryos were photographed at various times during embryonic development at 27-30 °C, given in hours post-fertilization (HPF). At 0 HPF, the egg is opaque and yellow with no other defining features. After fertilization occurs, eggs are hydrated and expand in size, and first cell divisions begins to occur. A visible dark mark at 6 HPF within the egg reveals the accumulation of cytoplasm at the animal pole. By 24 HPF, the egg membrane clears, and the cap-like blastodisc can be clearly observed. By 48 HPF, gastrulation, epiboly, segmentation, and organogenesis are underway, and elongated embryos can be seen moving without the aid of microscopy. Their bodies are still firmly wrapped around the yolk,

which takes up most of the internal egg space. By 96 HPF, the eyes are black and well developed, and the frequency and range of movement within the egg have increased. The yolk diminishes in size as it is consumed by the embryo. The hatching window begins soon after at ~120 HPF, depending on incubation conditions.

Tables

Table 6.1. Average percent neurulation at 48 hours post-fertilization (HPF) and hatch success of hybrid catfish egg batches for the females showing highest variability in hatch. Eggs from high-hatch females (n = 4) were classified using an arbitrary scale, defined as having both neurulation >50% and hatch >30% and low-hatch females (n = 5) as having neurulation near or < 50% and hatch < 20%.

Group	Neurulation (%)	Hatch (%)
	58.2	33.1
	67.9	31.6
High-Hatch	81.1	50.5
	81.2	32.3
	51.7	12.3
	33.2	5.2
Low-Hatch	5.6	1.0
	26.4	9.8
	7.8	2.4

1 Table 6.2. Transcripts of mRNA targets, biological functions, Genebank accession numbers, and primer sequences used for Real-Time
 2 PCR primers.

3

Transcript	Abbrev.	Gene function	GeneBank #	Forward Sequence	Reverse Sequence
18S Ribosomal RNA	<i>18S</i>	ribosomal protein (reference gene)	AF021880	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG
Tubulin β	<i>Tubb</i>	development of cytoskeleton	XM_017468721	GTGCGGTAACCAGATCGGA	TGGTACCAGGCTCCAAGTCG
Cathepsin D	<i>Ctsd</i>	protein degradation	NM_001257110	GCTCCTGGTTCTTGCTGCAT	GAGTTCCTCCACAGATCGGC
Cathepsin Z	<i>Ctsz</i>	protein degradation	XM_017481874	TAACTCCTGGGGTGAGCCAT	GGGTCCCACATACATGCAGTC
Cyclin B	<i>ccnb1</i>	early cell cycling	XM_017459889	CATGCGCGCCATCCTTATTG	TAGCGGTTACACCAACCAGC
Ring finger protein 123X1	<i>rnf123X1</i>	Protein-protein interactions	XM_017489382	CTGTGGCCGTTCTTAACCC	CCCACACTGACTGCAGAACT
Ring finger protein 123X2	<i>rnf123X2</i>	Protein-protein interactions	XM_017489390	TCAAAGCAGAAGCTGTGTCAATA	GTCATCATACTGATGCCATTCTC
Exportin-1	<i>xpo1</i>	intracellular transport	XM_017476032	GGCGACCATCGTGAACAAAC	AGTGGGAATTCCTACTGCCTGC



Chapter 7

General overview and perspectives



7.1. Impacts of fertilization technique, parental effects, gamete interactions, and molecular indices on the early life history stages of fishes

7.1.1. Sperm to egg ratio

Our research has shown that the current density of blue catfish sperm used to fertilize channel catfish eggs implemented by the industry is more than what is actually required (conservative estimate of 10×10^4 :1 sperm per egg vs. $\sim 1.2 \times 10^6$). This represents a staggering 1,000-fold decrease in sperm density that can successfully be used for fertilization solutions and that by reducing the sperm to egg ratio, each male can fertilize up to 12 times more channel catfish eggs while maintaining consistent hatch success. After exceeding 1×10^4 :1 sperm per egg, there was no evident increase in hatch success. Thus, by standardizing this ratio to our minimum suggested amount, precious gamete resources from valuable males can be used more conservatively, requiring less males to be sacrificed needlessly and improving overall spawning productivity within hatcheries.

7.1.2. Gamete interactions

Within the evolving world of aquaculture, gamete quality has been receiving considerably more attention as parental effects have proved to be important input factors for offspring production. For externally fertilizing fishes, interactions between sperm and the ovarian fluid micro-environment are powerful microscopic selection mechanisms that are not to be underestimated due to their ability to alter sperm swimming trajectories and fertility outcomes

(Zadmajid et al., 2019). The results from this project showed that blue catfish sperm have higher motility, velocity, and duration and movement in the presence of channel catfish ovarian fluid (OF) and that it is a critical component of the fertilization environment. Potential mechanisms for sexual selection were also identified between the two species because sperm from each male behaved differently in the OF of specific females, showing higher and lower pair-wise compatibilities. As a forethought, is imperative to add OF to sperm when activating it under the microscope to assess male quality, otherwise sperm performance and male fertility potential can be greatly underestimated.

Part of this research looked beyond the scale of gamete interactions occurring in ictalurid catfishes and used a meta-analytic approach to examine the effects of OF on sperm performance on many species available across the published literature. Results of this meta-analysis showed that OF caused significant changes in sperm swimming behavior (i.e. velocity, trajectories, motility) but that these effects vary between species and by spawning environment. Positive OF effects (improved sperm velocity) were more evident in species that reproduce in freshwater (such as our fish of interest, the blue catfish), but no significant conclusions could be drawn for marine species as a whole. Thus, there are many factors that contribute to the strength of these gamete interactions to be aware of that haven't been thoroughly explored due to the relative newness of this research field and small numbers of studies drawing effect size from these factors. Such factors include spawning strategies, reproductive physiology, and evolutionary history. Currently, the available literature falls short of fully representing even a fraction of these diverse groups for these categories, making it challenging to make broad scale inferences across taxa, but we are confident that our results are consistent for the taxonomic families included in this analysis. As previously mentioned, it is important to create an optimal fertilization

environment for the artificial mixing of gametes. With this research, we have provided additional evidence of how maternal \times paternal interactions occur before the zygote develops (during the fertilization event itself) and how OF can be a powerful selective force to influence gamete quality and cryptic potential sexual selection.

7.1.3. Maternal and paternal effects

Parental effects, divided into maternal and paternal components, were apparent in hybrid catfish during their crucial early developmental stages, impacting traits such as embryonic survival, hatching rate, deformities, and fry morphology. The degree of these contributions varied based on the quality of the incubation and fry rearing environments (tested in this study by contrasting a higher, suboptimal temperature experienced late in the spawning season to a lower, less extreme temperature characteristic of early in the season) during the early life history (ELH) stages. When analyzing maternal effects, paternal effects, and all associated interactions between them, maternal contributions accounted for the most variability for each trait, especially embryonic survival and hatch success. This was anticipated since females are responsible for providing genetic material but also the valuable yolk reserves that sustain embryos and larvae early in life (Rideout et al., 2004). Initial egg quality has been reviewed extensively by the literature, and it is known to have substantial impacts on fertilization and subsequent reproduction potential. Paternal effects were not equivalent in magnitude to the maternal effects but still present, having significant impacts on embryonic survival as early as 72 hours post-fertilization and the subsequent developmental stages afterward. Some variability in larval morphology was also contributed by the male, and there were maternal \times paternal interactions

observed for some of the morphology traits as well as yolk utilization efficiency (YUE). This suggests that besides just being aware of individual male and female qualities as separate entities, reproduction in hatcheries is also dependent on specific fish pairings and must also be considered collectively.

7.1.4. Molecular indices of egg quality

Assessing egg quality prior to fertilization can be challenging due to a lack of clear biomarkers (Migaud et al., 2013, Źarski et al., 2017). High variations in egg quality exist in individual females due to environmental factors but have also been shown to be linked to relative abundances of specific mRNAs. Seven mRNA transcripts analyzed in this study (*tubb*, *ctsd*, *ctsz*, *rnf123X1*, *rnf123X2*, *ccynb1*, and *xpo-1*) are linked to embryo viability for hybrid catfish. Expression of all these genes is up-regulated in high-hatch egg batches compared to low-hatch and peak at the neurulation stage of embryonic development. Overall, knowledge of mRNA abundances and their influences on egg quality is still elementary, and very little has been done for ictalurid catfishes. Transcriptome studies may further efforts to find differentially expressed mRNAs on a genome-wide scale, which will potentially offer new insights on other genes related to egg quality and embryonic development. So far, molecular markers of egg and embryo quality have not yet been utilized in hatcheries for commercial production due to emerging technologies still being relatively new and with limited applicability, which will hopefully change as more genes are confirmed to be important genetic markers. By pairing these markers with physical

indicators of egg quality, it may serve to enhance hybrid catfish aquaculture by eliminating problems associated with the incubation of poor egg quality eggs and inconsistent fry production.

7.2. Implications for hybrid catfish aquaculture

7.2.1. Optimizing artificial fertilization protocols

Hybrid catfish aquaculture has soared in the southern U.S. since its establishment and has overtaken production of channel catfish alone over the past decades (Towers, 2014). With added pressure from international countries (especially in Asia), improvements in the industry now rely on advancing reproduction techniques and technologies to maximize fish cultivation efficiency in order to remain competitive on the global aquaculture market. A critical strategy for improvement involves optimizing artificial fertilization techniques within the hatchery since channel and blue catfish fail to reproduce naturally. Mastering broodstock handling techniques and providing the optimal fertilization environment for gamete contact and mixing is essential for successful spawning. In controlled conditions, altering the sperm density per egg batch can have notable impacts, as using too little sperm leads to poor fertilization turnout (as shown in Casselman et al., 2006) and using too much is a waste of gamete resources for no additional gain (Butts et al., 2009). We demonstrated with our new proposed sperm to egg ratio that previously, the predicted amount of sperm used for fertilization was excessive. Overall, optimizing this ratio will serve to improve hatchery production for this valuable fish. We also demonstrated the importance of reassessing various aspects of artificial fertilization protocols that can be improved over time as technologies advance. For any fish bred and maintained in captivity, optimizing the

sperm to egg ratio is one of the critical first steps for establishing successful hatchery protocols, and our results stress the need to reassess these protocols over time. With future advancements in hatchery care, broodstock handling, gamete quality, and artificial spawning, perhaps the proposed sperm density can be reduced even more.

7.2.2. Confirming the importance of parentage

Genetic enhancement and selective breeding are the foundation for the development of highly valuable strains, parental lines, and families (Dunham, 2011). Aquaculturists have invested extensive levels of research in how to select the best fish that produce better broods that exhibit heterosis, which can be seen in parental crosses possessing commercially desirable traits such as faster growth and disease resistance (Masser and Dunham, 1998; Fjalestad, 2005). These studies on combining abilities identify sources of heredity, quantify additive genetic gain, and explore the nature of genetic variances that exist in breeding populations (Deng et al., 2010; Yadav et al., 2010). In our case, it was possible to rank best and worst-performing families by utilization of a full-factorial breeding design between specific male and female individuals. Performance of these families also varied between two temperatures simulating different incubation environments that may be encountered in the hatchery. Further research should examine how distributions of these parental effects would be altered at temperatures even more extreme. Other important environmental quality indicators (i.e. pH, water hardness, ammonia levels) can also be tested to determine if they also interact with the parental factors during early development. By exhibiting the significance of both parents during ELH stages, research may be expanded to examine the magnitude of these effects beyond the first feed and into the juvenile

stages. Other traits beyond what was used in this study (such as larval growth indices) may also show parental variation and are worth further research attention.

7.2.3. Improving broodfish selection

Focusing on the parental effects governing progeny production demonstrates that proper selection of channel catfish females and blue catfish males ensures that the fish chosen produce high quality gametes for the next generation. For females, this is bringing together all our knowledge to answer a daunting question: what factors identify good eggs and how can females be selected that reflect these qualities? For males we can raise the same question only with a modification: what factors make the best spermatazoa and how can it be improved for artificial fertilization? Strategies for accomplishing this task involve linking existing physical determinants of fish quality to gamete quality and even to molecular/genetic indices. The latter is an area of research that is still new for most aquaculture species including catfish but is brimming with potential.

Currently, quality of both parents can be vaguely estimated the fish's appearance and overall physical condition. For females, factors such as properties of the ovarian fluid and the ovulated eggs can also be measured. During stripping, appearance of the eggs can vary drastically between "good" and "bad" eggs, as overripe eggs can have different coloration, viscosity, and adherence (Steeby and Avery, 2005). From our observations, poor-quality eggs were sometimes observed to be expelled with copious amounts of fluid and blood contaminants. Specific components of the eggs and surrounding fluid such as ionic composition, osmolality, and organic constituents (metabolites, proteins) and their effects on egg quality have not yet been

thoroughly investigated for channel catfish, which provides a promising area of future discoveries. In many species, specific mRNAs have been identified that are directly linked to egg quality (Aegerter et al., 2004; 2005; Bonnet et al., 2007), and only limited work in these areas has been looked at in hybrid catfish (Peterson et al., 2005; Small et al., 2008) prior to this work. Beyond just identifying a few important transmittable RNAs, research is turning to analyses of the whole transcriptome, providing a less targeted method of finding differentially expressed genes throughout the early stages of development (Traverso et al., 2012). To paint the picture accurately in all its complexity, transcriptome analyses have also been paired with proteome and miRNAome profiles to capture the potential interactions between these mechanisms in how they influence the welfare of eggs and developing zygotes (Schier and Giraldez, 2006; Cerdà et al., 2008). Thus, we will further our understanding of which transcripts are transferred to the embryos specifically through the female and which are important after the zygotes transition to their own transcription. With our current knowledge, what makes up a “transcriptionally competent egg” remains rather rudimentary (Sullivan et al., 2015). Although still limited to highly technical lab studies, commercial hatcheries may benefit from these research endeavors in the future once these molecular analyses become more easily assessable for use in broodstock selection.

Blue catfish sperm quality has not received the attention it deserves despite the commercial value of this species and of each mature male. Examination of sperm kinetics, morphology, intracellular composition, and genetic transcripts within the sperm cells and the surrounding seminal plasma would reveal underlying causes of male quality at the microscopic level (reviewed by Fauvel et al., 2010). Like the determinants of egg quality discussed above, there are still gaps remaining in what makes a “good sperm” for this species. Best-performing

sperm from quality males can also go beyond a single fertilization event if cryopreservation techniques are taken advantage of. Long-term preservation of gametes has been successful but is still being developed for use in commercial hatcheries (Bromage and Roberts., 1995; Cabrita et al., 2010). In the future, genetic repositories may be the answer since they are used to preserve sperm from superior males and their valuable genes. Thus, cryopreservation increases effective population sizes of breeding populations and maintains genetic diversity (Lang and Riley, 2003). Cryopreservation has already been widely successful for terrestrial domestic livestock (Tiersch et al., 2007) but has also broken ground for both marine and freshwater fish species as well (Cabrita et al., 2010; Suquet et al., 2010). Blue catfish sperm has also been successfully cryo-preserved in recent years (Lang et al., 2003; Hu et al., 2011; 2014). Unfortunately, cryopreservation of eggs has not yet proven successful due to their vulnerability to withstand such extreme freezing temperatures, but having this selective power on just one side of the parental spectrum can still be a powerful force. Hatcheries that fully take advantage of these maturing techniques for broodstock selection may gain an upper hand in producing the best hybrid progeny.

7.3. Concluding remarks

In light of recent progress through previous achievements and the contributions here presented, it is apparent that hatchery research has come a long way but still has challenges to overcome in order to ensure a proper, sustainable future. We may never fully uncover every aspect of a fish's remarkably complex reproductive biology, and many questions remain in how to fully take advantage of it within the hatchery, but this research has helped make such a goal more attainable for the economically valuable hybrid catfish. By pursuing the research questions

that have arisen from this research and those that will inevitably be come about as progress ensues, scientists and hatchery managers alike may continue to see improvements in fish culture. This progress will be seen at the microscopic level as the overwhelming importance of reproductive mechanisms, gametes, and development during the most sensitive early life stages are recognized to their full potential.

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