EVALUATION OF INDUCED SPAWNING AND EMBRYO DISINFECTION PROTOCOLS FOR FOUR ORNAMENTAL FISH SPECIES

By

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To my daughter Mia
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EVALUATION OF INDUCED SPAWNING AND EMBRYO DISINFECTION PROTOCOLS FOR FOUR ORNAMENTAL FISH SPECIES

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Reproductive dysfunction is a common occurrence in production of numerous species of ornamental fish. Use of exogenous hormones may help overcome this impediment. Dosages of 50, 100, 200 µg/kg of the hormone cGnRH IIa were compared to the ornamental industry standard Ovaprim® (10 µg/kg sGnRHa) using standard metrics for spawning performance. Four commercially valuable ornamental species *Epalzeorhynchos bicolor, Epalzeorhynchos frenatum, Synodontis nigriventris* and *Synodontis eupterus* were used to assess spawning aid efficacy. All species exhibited similar ovulation performance across hormone treatments except for *E. frenatum* injected with Ovaprim (17% ovulation). Poor ovulation success excluded the *E. frenatum* Ovaprim treatment from subsequent analyses. In general, fertilization and hatch success were comparable for both analogs among species. Embryo and larval morphometric measurements were also similar among treatments across all four species. Taken together, these results suggest cGnRH IIa to be a safe and reliable option for induction spawning of ornamental species.

Prophylactic use of disinfectants on developing embryos of teleosts is a common practice in aquaculture used to mitigate disease transmission and increase hatch success.
Fertilized embryos from each of the four previously mentioned species were used to assess the safety of 15 minute static exposure to 100 mg/L iodine, 1,000 mg/L hydrogen peroxide and 1,500 mg/L formalin. Results indicated that iodine was detrimental to hatch of all species tested while formalin and/or hydrogen peroxide did not detrimentally affect hatch rate. Data generated from induced spawning and embryo disinfection studies will help to refine commercial production protocols for ornamental fish species.
CHAPTER 1
GENERAL INTRODUCTION

Internationally millions of people participate in keeping aquaria, making it amongst the most popular hobbies (Livengood and Chapman 2007). The origins of modern day aquarium keeping can be traced to the early 1850’s when a chemist named Robert Warrington added plants to a container which produced enough oxygen to sustain fish (Grier 2007). Intensive life support systems have since been developed allowing hobbyists the ability to accommodate numerous species at high densities creating a massive worldwide aquarium industry with a multi-billion-dollar economic impact. Currently the United States (US) is the largest importer of ornamental fish acquiring ~ 80 % of total imports from Asian countries and procuring ~ 13 % of total global exports (Monticini 2010). Conversely the US only accounts for 3.56 % of total value of global exports annually (Monticini 2010), highlighting the opportunity for expansion by the US into the international aquarium market. Florida is the largest producer of ornamental fish in the US growing most of the nation’s freshwater varieties (Livengood and Chapman 2007). In a 2017-2018 survey done by the American Pet Products Association (APPA), it was found that 12 % of the population in the United States keep either fresh or saltwater aquarium fish with the largest segment of owners being of “generation Y”, the youngest generation surveyed (APPA National Pet Owners Survey 2017). As keeping aquaria is becoming more popular with emerging generations, it is essential that ornamental producers innovate and increase efficiency of production to remain competitive in the global marketplace. Moreover, continued production of a cultured product helps to alleviate collection pressures on wild stocks,
some of which may already be strained due to factors such as climate change, loss of habitat, and other anthropogenic impacts.

Induction spawning is a common practice in the aquaculture industry. Manipulation of environmental cues such as photoperiod and temperature are common ways to initiate the required hormonal cascade (Figure 1-1) needed for gamete maturation and ovulation in fish (Rottmann et al. 1991a). In species that are less responsive to changing environmental cues, or in conditions where it may be impractical to attempt to replicate them, administration of exogenous hormones have been essential to the commercial development of a number of genera (Rottmann et al. 1991b). The benefits of inducing ovulation are great and have a number of applications including overcoming reproductive dysfunction, directed genetic selection, creation of novel hybrid species and streamlining labor associated with spawning. Reproductive dysfunction is a common occurrence in a variety of aquacultured species in captivity, particularly in ornamentals which can exhibit intricate reproductive strategies (Mylonas and Zohar 2007).

The cascade of reactions needed for suitable reproductive function of fish occurs within the HPG (hypothalamic gonadal pituitary) axis (Figure 1-1). The hormonal cascade is initiated by environmental stimuli which are interpreted by the brain and results in the release of gonadotropin releasing hormones (GnRH). These hormones stimulate the pituitary gland to release gonadotropins (GtH). The primary GtHs released by the pituitary are follicle stimulating hormone (FSH) and luteinizing hormone (LH) which directly stimulate the gonads to produce prostaglandins and steroids (Yanong 2017). These gonadotropins are critical to final oocyte maturation in females and
spermiation in males as steroids stimulate maturation of the egg and spermiation while prostaglandins aid in follicle rupture (Rottmann et al. 1991b). The neurotransmitter dopamine may act to inhibit the release of gonadotropins and can disrupt the completion of the cascade. (Figure 1-1) (Mikolajczyk et al. 2003).

In species that will not volitionally reproduce following environmental manipulations, exogenous gonadotropin releasing hormones (GnRHs), gonadotropins (GtHs) and pituitary extracts (PE) have all been successfully used to induce the hormonal responses needed for gamete maturation and ovulation (Rottmann et al. 1991b; Mikolajczyk et al. 2003; Mylonas and Zohar 2007; Podhorec and Kouril 2009). Fish in captive settings may spawn indiscriminately which does not allow for optimum harvestable yields or production efficiency. Induction spawning through the use of exogenous hormones allows producers to streamline the production process to achieve desired outcomes. Induced spawning reduces the uncertainty of volitional spawning, allows for the proper harvest and care of fertilized eggs and reduces the amount of labor needed through synchronization of large cohorts for spawning. When selecting for desired genetic traits, hormonal induced spawning eliminates the chance of unintended reproduction of broodfish (Hill et al. 2009). The production of hybrid catfish is an excellent example of where induction spawning has facilitated the production of a more robust and desirable product. The Channel Catfish *Ictalurus punctatus* X Blue Catfish *Ictalurus furcatus* hybrid is a sought-after combination in which milt from the Blue Catfish is used to fertilize Channel Catfish eggs. These hybrids cannot be produced through natural pond spawning and only occur through the use of induced spawning measures (Quiniou et al. 2014). The resulting hybrid offspring exhibit increased growth,
yield, processing efficiency and bacterial resistance compared to their parental counterparts (Quiniou et al. 2014). Hybrid ornamentals have also been produced and are common within the *Synodontis* catfish genus. These hybrid fish are desired as they are both visually appealing and are a novel commodity in the ornamental trade.

Endocrine manipulation for induction spawning of fish has been extensively used by the US aquaculture industry. In 1930, Bernardo Houssay induced ovulation in female fish by injecting ground pituitary glands from another species (Mylonas and Zohar 2000; Zohar and Mylonas 2001; Mikolajczyk et al. 2003; Yaron et al. 2009). The method was called hypophysation in which ground pituitary glands and pituitary extracts harvested from spawning fish such as carp could be administered to remedy reproductive dysfunction (Yaron et al. 2009). The desired effect of hypophysation can be linked to the GtH known as luteinizing hormone (LH) which is stored in the pituitary. Release of LH is essential for final oocyte maturation, spermiation and ovulation (Mylonas and Zohar 2000). Administering pituitary extracts bypasses the natural release of LH and provides the exogenous gonadotropins for direct stimulation of the gonad. Early hypophysation techniques had mixed outcomes as a result of inconsistencies of GtH content in the pituitaries due to size, sex, age or season in which the pituitaries were harvested (Zohar and Mylonas 2001). Recent improvements to the hypophysation method include increased biosecurity and standardized GtH content through purification using chromatographic separation (Zohar and Mylonas 2001). Although dated, the use of piscine GtH through hypophysation is still employed today due to its cost effectiveness and availability.
The use of hormones of non-piscine origin for spawning induction of fish began in the 1970’s when researchers experimented with hCG (human chorionic gonadotropin) (Zohar and Mylonas 2001). This gonadotropin is refined from the urine of pregnant women and is available in known clinical grade concentrations (Mylonas and Zohar 2007). The preparation has been successfully used in a number of species to induce ovulation (Shireman and Gildea 1989; Sahoo et al. 2007; Watson et al. 2009; DiMaggio et al. 2013; Elakkanai et al. 2017). The cause of hCG’s effectiveness is most likely related to the longer half-life of hormone compared to GtH’s of pituitary origin (Mylonas and Zohar 2007). As hCG can remain in circulation for prolonged periods of time, it allows a single dose to often be effective in inducing ovulation (Mylonas and Zohar 2007). Chorulon (Merk & Co., Kenilworth NJ) is a lyophilized and reconstituted preparation of hCG and is approved as a hormonal spawning aid for finfish by the US Food and Drug Administration (FDA). Although a potent source of LH, GtH’s are relatively large glycoproteins which may illicit an immune response by the fish making it less effective at inducing successful spawning with repeated use (Yaron et al. 2009). Moreover hCG alone may have minimal effectiveness as an induced spawning aid in particular species such as the Common Carp (Yaron et al. 2009) possibly as a result of an immunological reaction, dopaminergic inhibition, or species specificity to the hormone. However since gonadotropins act at the level of the gonad, they may be the best choice for induced spawning in circumstances where the fish’s pituitary may have low stores of endogenous LH.

Since its discovery in mammals in the early 1970’s and the advent of its analogs shortly after, gonadotropin releasing hormones (GnRHs) have been used as viable
induced spawning aids (Mylonas and Zohar 2007). This hormone works earlier in the hypothalamic pituitary gonadal axis (Figure 1-1) releasing endogenous gonadotropins from the pituitary that act on the gonad (Podhorec and Kouril 2009). There is a disparity in the exact number of isoforms of this decapeptide, however GnRH systems I, II and III have been identified in teleosts (Kah et al. 2007). The names of each variant originated from the first animal the peptide was isolated from with mGnRH I described first in mammals, cGnRH II in chicken, and sGnRH III in salmon. Most vertebrate species have two to three forms present throughout their body however sGnRH III is teleost specific while cGnRH II occurs ubiquitously throughout all vertebrate taxa (Millar 2003; Kah et al. 2007; Quiniou et al. 2014).

The use of GnRH and GnRHa’s have been favored as induced spawning aids over pituitary extracts and purified GtH’s for a variety of reasons. Shortly after the discovery of native GnRHs, analogs were created with amino acid substitutions in the decapeptide sequence that resist enzymatic degradation allowing the hormone to stay in circulation longer and exhibit increased bioactivity with a higher receptor binding affinity (Lovejoy et al. 1995; Mikolajczyk et al. 2003; Mylonas and Zohar 2007; Szabó et al. 2007; Podhorec and Kouril 2009). GnRH peptides are smaller in size than GtHs allowing them to evade the immunological refractory response that gonadotropins such as hCG can elicit (Zohar and Mylonas 2001). Since GnRH acts earlier in the hormonal cascade needed for final oocyte maturation (FOM), the response may be more natural than flooding the body with exogenous GtHs. Hormones such as growth hormone, insulin-like growth factors, prolactin and thyroid hormones may be stimulated and released following GnRH application and aid in FOM (Mylonas and Zohar 2007).
There are many isoforms of GnRH that have been identified, with hormones from systems I, II, and III currently being used in aquaculture. Dopamine is a neurotransmitter that negatively effects the release of GtHs from the pituitary by binding to receptors of GnRH (Mikolajczyk et al. 2003). GnRH hormone preparations often include a dopamine antagonist to prevent dopaminergic inhibition of the cascade needed for FOM. Ovaprim (Western Chemical Inc., Ferndale, WA) is a sGnRH IIIa preparation that includes domperidone as a dopamine antagonist and is the ornamental industry standard for induction spawning. Its status as indexed by the US Food and Drug Administration (FDA) as a legally marketed new animal drug for minor species allows it to be used for ornamental finfish brood stock and is available for producers to purchase directly from the manufacturer. While Ovaprim has been used in a variety of ornamental families with great spawning success, it has been met with variable success at FOM in families Mochokidae and Osteoglossidae with mean ovulation rates ranging from 0 – 44.1 ± 35.7 % (Hill et al. 2009).

Unlike sGnRH III, cGnRH II is found ubiquitously among all teleost species and therefore has garnered some interest as a new GnRH subtype for use by the aquaculture industry (Millar 2003; Quiniou et al. 2014). Fish exhibit all type II receptors which generally respond better to the highly conserved cGnRH II form (Kah et al. 2007). For the release of the gonadotropin LH, cGnRH II has been found in some species of fish to be the most potent of the endogenous form (Illing et al. 1999; Mylonas and Zohar 2000; Podhorec and Kouril 2009). When examining effectiveness of native GnRHs on ovulation and egg quantity produced in African Catfish *Clarias gariepinus*, cGnRH surpassed sGnRH and mGnRH in spawning performance (Szabó et al. 2007). In a
study comparing GnRH analogs as induced spawning aids, cGnRH IIa (D-Arg6,Pro9-Net) outperformed mGnRH Ia and sGnRH IIIa in spawning success in Channel Catfish *Ictalurus punctatus* yielding a mean ovulation rate of 90.2% (Quiniou et al. 2014). The cGnRH analog D-Arg6, Pro9-Net also produced the highest LH release in Sea Bass *Dicentrarchus labrax* compared with seven forms of GnRH including an sGnRHa variant (Forniés et al. 2003). The reported efficacy of cGnRH IIas as viable induced spawning aids supports further investigations to better describe its effect on parameters associated with production yields as well as additional species in which current preparations show decreased efficacy.

In addition to spawning success, disinfection of incubating embryos can help to increase yields of viable larvae per female spawned. The aquatic environment is home to a number of pathogens which may cause direct mortality or indirectly affect eggs by diminishing water quality during incubation. Fungi in the genus *Saprolegnia* are a significant problem, especially in freshwater trout operations, which can result in decreases in annual production from 20-40% (Forneris et al. 2003). There are a number of bacterial genera that can deleteriously affect the egg or newly hatched yolk sac larvae. Bacteria in the genus *Flexibacter* can penetrate the egg shell and infect the embryo while *Aermonas* and *Vibrio* may smother the surface the egg not allowing exchange of oxygen and metabolites or infect larvae immediately post hatch (Bergh 2000). Numerous protocols and products have been created to disinfect the egg surface from colonization of fungi or bacteria. Equipment such as mechanical filters, ozone generators and UV sterilizers may reduce the risk of pathogens, however the use of using chemotherapeutants by temporarily bathing eggs or treatment of the hatching
system is more common. Prophylactic treatment of embryos with chemical disinfectants to increase hatch success is a common practice in ornamental aquaculture. Chemicals used for egg disinfection include, hydrogen peroxide, glutaraldehyde, ozone, iodophors, formaldehyde, peracetic acid, bronopol, tannic acid, sodium chloride, copper sulfate (De Swaef et al. 2016) and malachite green (Sudova et al. 2007). Among the more common disinfectant chemicals used for prophylactic treatment of fish embryos are hydrogen peroxide, formalin and iodine due to their availability, effectiveness in treating a range of pathogens, and are legally approved or of low regulatory status. Hydrogen peroxide and formalin are both approved by the FDA as an antifungal treatment for eggs while iodine is listed as a low regulatory drug (Bowker et al. 2016). Efficacy of each treatment varies with concentration and exposure time between species. When used properly, egg disinfection can greatly increase hatch and survivability for the producer as well as lessen the chance of pathogen spread through vertical transmission.

Among the hundreds of species being traded in the ornamental fish industry, members of the family Mochokidae and Cyprinidae are very well represented. The Mochokidae family contains species of catfish known as “squeakers” which are endemic to sub-Saharan Africa (Friel and Vigliotta 2006). The most species rich genus in the family is the Synodontis catfish and are these specimens are highly sought after in the aquarium industry due to their physical appearance, size and interesting behavior (Koblmüller et al. 2006). Several species of the Synodontis genus are readily produced using induced spawning techniques while others are unresponsive (Hill et al. 2009). The Cyprinidae family includes > 2,010 species and > 210 genera and are native to North America, Africa, Europe and Asia (Podhorec and Kouril 2009). This anthropogenically
significant family contains the genus *Epalzeorhynchos* which are very prevalent in the ornamental fish industry. The Redtail Sharkminnow *Epalzeorhynchos bicolor* was thought to be extinct in its native range due to overharvesting for the aquarium trade (Vidthayanon 2013), however since the 1980’s has been heavily produced and considered a model for induction spawning methods (Hill et al. 2009). The relative high price and demand of the *Synodontis* and *Epalzeorhynchos* genera as well as their variability in response to current induction spawning hormones justifies research into optimization of spawning aid choice and production methods.

The objective of this study was to develop protocols which would increase spawning success as well as hatch success in several ornamental fish species using cGnRH IIa and embryo disinfection techniques. Spawning induction and embryo disinfection techniques focused on the Upside-Down Catfish *Synodontis nigriventris*, Featherfin Squeaker *Synodontis eupterus*, Redtail Sharkminnow *Epalzeorhynchos bicolor* and Rainbow Shark *Epalzeorhynchos frenatum*. Induction spawning regimes for this study mirrored commercial practices utilizing Ovaprim (sGnRH IIIa) however varied concentrations of cGnRH IIa were also investigated in an attempt to identify an effective dose of the novel hormone. Ovulation success, fecundity, fertilization success, embryo diameter, hatch success and broodstock mortality were analyzed to gauge the performance of cGnRH IIa in relation to Ovaprim. Embryos collected from spawning experiments with each species were subjected to disinfection protocols using iodine, hydrogen peroxide or formalin solutions to observe effects on hatch success. As there is a lack of published information on disinfection protocols of ornamental fish embryos, data generated from these experiments will be used to recommend species specific
disinfection protocols. Results from this study may help to increase the number of taxa currently produced by the aquaculture industry as well as aid in optimizing yields for ornamental species currently in production.
Figure 1-1. Simplification of the hypothalamic pituitary gonadal (HPG) axis. GnRH: gonadotropin releasing hormone LH: luteinizing hormone FSH: follicle stimulating hormone
EVALUATION OF cGnRH IIa FOR INDUCTION SPAWNING OF TWO ORNAMENTAL Synodontis SPECIES

Introduction

Numerous fish species exhibit a form of reproductive dysfunction in captivity, the most common being failure to undergo final oocyte maturation (FOM) in females or production of low quality or reduced volumes of milt in males (Mylonas and Zohar 2000). The inability to provide appropriate environmental stimuli in a captive setting has been recognized as the primary cause of reproductive failure of fish (Rottmann et al. 1991b). Spawning induction is a common alternative for obtaining viable gametes needed for captive reproduction (Sahoo et al. 2007). Manipulation of environmental factors such as water temperature and photoperiod can trigger hormonal cascades needed for gametic maturation, however, responses to these artificial cues may be species specific and reproductive success may be variable (DiMaggio et al. 2014). In circumstances where environmental alterations fail to stimulate ovulation and spermiation, induced spawning methods which employ the administration of exogenous hormones may be a reliable substitute (Rottmann et al. 1991a). There have been numerous advances in the development of spawning aids from the early 1900’s until present day. Crude pituitary extracts harvested from spawning fish were initially used but variation can exist in hormone content depending on the size, age and season in which the fish was harvested. Additionally, concerns regarding pathogen introduction with less purified preparations were also recognized as a significant shortcoming (Zohar and Mylonas 2001). Use of isolated gonadotropins (GtHs) which stimulate the gonad directly, and native gonadotropin releasing hormones (GnRHs) which act higher on the
hypothalamic pituitary gonadal axis, have gradually increased owing to the known hormone concentration and decreased biosecurity concerns associated with these hormone preparations. More recently, synthetic gonadotropin releasing hormone analogs (GnRHas) have become the standard for hormone induced spawning as these preparations demonstrate increased potency due in part to amino acid substitutions in their sequence resulting in higher receptor binding affinity and resistance to enzymatic degradation (Lovejoy et al. 1995; Mikolajczyk et al. 2003; Szabó et al. 2007; Podhorec and Kouril 2009).

More than 16 variants of the GnRH decapeptide have been identified in vertebrates with two to three forms present in most vertebrate species (Millar 2003). Gonadotropin releasing hormone system types I, II, and III have been previously identified in teleosts (Kah et al. 2007). The hormone cGnRH II occurs ubiquitously among all teleost whereas sGnRH III is found less universally but is specific to teleosts (Kah et al. 2007), making cGnRH II a desirable target for development as a potential induced spawning aid in aquaculture. The cGnRH II variant is highly conserved and has been found to be more potent, in terms of stimulation of luteinizing hormone (LH) secretion, when compared to the hypophysiotropic sGnRH III form (Illing et al. 1999; Podhorec and Kouril 2009). Ovaprim® ([20 µg/mL sGnRH IIIa + 10 mg/mL domperidone], Western Chemical Inc., Ferndale, WA) is a combination of a salmon GnRH analog (D-Arg6-Pro9-Net) (Figure 2-1) and a dopamine antagonist, suspended in a propylene glycol solution and is commonly used in hormone induced spawning of ornamental fishes. As of March 2009, Ovaprim was added to the FDA index of legally marketed unapproved new animal drugs for minor species, allowing ornamental fish
farmers to purchase the drug directly. Although Ovaprim has been shown to successfully induce FOM and ovulation in many species (Hill et al. 2005, 2009, Sahoo et al. 2005, 2007, DiMaggio et al. 2013, 2014), members of the Synodontis genus have shown inconsistent responses to the use of Ovaprim with variable ovulation rates (Hill et al. 2009).

Recently there has been interest in exploring cGnRH IIa (D-Arg6, Pro-9-Net) as an alternative drug for induced spawning due to its ubiquitous distribution across taxa and proven potency in LH release (Forniés et al. 2003). The gonadotropin LH is essential to gonadal maturation and steroidogenesis in fishes (Zohar and Mylonas 2001, Mylonas and Zohar 2007). The cGnRH II variant has been identified as a potent LH releaser (Zohar and Mylonas 2001) and has demonstrated superior ability to elicit LH secretion compared with other GnRH variants in a number of species, including Goldfish Carassius auratus, Gilthead Seabream Sparus aurata and African Catfish Clarias gariepinus (Zohar et al. 1995; Illing et al. 1999; Bosma et al. 2000; Podhorec and Kouril 2009). A comparative study which evaluated mGnRH Ia, cGnRH IIa and sGnRH IIIa for spawning induction of Channel Catfish Ictalurus punctatus reported cGnRH IIa yielded the highest efficacy as an induced spawning treatment with an ovulation rate of 90.2% (Quiniou et al. 2014). Furthermore, investigations with the Stinging Catfish Heteropneustes fossilis (Alok et al. 1999), Sharptooth Catfish, Clarias gariepinus (Taufek et al. 2009), and Broadhead Catfish Clarias microcephalus (Ngamvongchon et al. 1992), have demonstrated the efficacy of native cGnRH II at induced ovulation in other Siluriform fishes. These results provide a strong impetus for further examination of cGnRH IIa as an induced spawning aid for the ornamental
industry. If effective, the use of cGnRH IIa could help to expand the diversity and yield of ornamental fish being cultured today.

The genus *Synodontis* belongs to the specious Mochokidae family endemic to sub-Saharan Africa and inhabit a wide variety of freshwater habitats from small creeks to large lakes (Friel and Vigliotta 2006; Koblmüller et al. 2006). The 120 species of catfish in this genus commonly are called “squeakers” due to the sound they make rubbing their pectoral spines when agitated (Friel and Vigliotta 2006; Koblmüller et al. 2006). In countries such as Benin, members of the genus are a highly valued food fish and support a significant fishery (Lalèyè et al. 2006). Their unique behavior, attractive markings and relatively compact size has made the *Synodontis* genus highly sought after in the ornamental aquarium trade (Friel and Vigliotta 2006; Koblmüller et al. 2006). Moreover, with the underdeveloped state of ornamental fish export of some African countries (Oben and Oben 2003) and relative mortality associated with wild capture and transport of ornamental fish (Livengood and Chapman 2007), further research into the culture practices for members of the genus *Synodontis* is warranted.

The objective of this study was to evaluate the effects of various dosages of cGnRH IIa on parameters imperative to the successful culture of two ornamental *Synodontis* species. The Upside-Down Catfish *Synodontis nigriventris* and the feather fin squeaker *Synodontis eupterus* are two popular ornamental catfish species that are currently produced using Ovaprim, the industry standard for induction spawning. Performance of cGnRH IIa was compared to Ovaprim, and effects on ovulation, fecundity, fertilization success, hatch success, as well as egg and larval morphometrics were assessed.
Methods

Two distinct experiments were conducted evaluating the efficacy of cGnRH IIa as an induced spawning aid for *S. nigriventris* and *S. eupterus*. The experimental design for both catfish species was similar although minor deviations from standard methodologies occurred and are detailed below.

Experimental Design

Sexually mature broodstock were obtained from a commercial ornamental fish producer in Wimauma, Florida, USA. Fish were transported to the University of Florida’s Tropical Aquaculture Laboratory (UF-TAL) where males and females were separated into respective 1,030 L concrete vats with a working volume of 380 L. Vats were supplied with flow-through, degassed well water at 380 L/hour and supplemental aeration for the duration of the experiment. All fish were individually anesthetized in 150 mg/L tricaine methanesulfonate (MS-222, Western Chemical Inc., Ferndale, WA) solution buffered with 300 mg/L sodium bicarbonate and then measured for total length (TL) and weight. To ascertain sexual maturity, slight pressure was applied to the male’s coelom to confirm the presence of flowing milt. Females appeared to have slightly distended and softer coeloms. A silicone tube (1.47 mm inside diameter and 1.96 mm outside diameter) was inserted into the oviduct after which light suction was applied to acquire an ovarian biopsy. The sample was placed on a Sedgewick Rafter Counting Cell for scale and then observed under a dissecting microscope with digital image capture capabilities. Only fish displaying ≥ 50 percent germinal vesicle migration (GVM) of secondary stage vitellogenic oocytes were chosen for this study. Digital photomicrographs were taken in both bright and dark field for subsequent determination
of GVM rates as well as diameters of vitellogenic oocytes. Experimental females were separated into individual 19 L buckets (working volume 9.77 L) with holes drilled along the circumference to allow for water passage and floated within two concrete vats. Buckets remained covered with a lid for the duration of the experiment except during hormone administration or periodic checks for ovulation. Male *S. nigriventris* were held in a single 120 L plastic tub with holes drilled around the perimeter and floated in a vat near the female fish. Male *S. eupterus* were segregated into individual 38 L tanks upon arrival. Temperature, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite-nitrogen, hardness (CaCO$_3$), and alkalinity (CaCO$_3$) were tested both in the covered buckets and surrounding vat area during a pilot study and revealed no significant variation.

Lyophilized cGnRh IIa (D-Arg6, Pro-Net) used in this experiment was synthesized by Genscript Labs (Piscataway, NJ). The peptide was individually packaged in 100 µg vials and stored in a -80.0 ºC freezer until use. For each spawning trial, fresh cGnRH IIa peptide solutions were prepared. The carrier for all cGnRH IIa treatments consisted of the dopamine antagonist, domperidone (Roadrunner Pharmacy, Phoenix, AZ), dissolved in propylene glycol, yielding a final concentration of 10 mg/mL, equal to that of Ovaprim. The use of heat applied in a warm water bath along with periodic vortexing aided in homogenously mixing the lyophilized domperidone into solution with propylene glycol. Volumes of 1000, 500 and 250 µL of the carrier was pipetted into three individual 100 µg cGnRH IIa vials to achieve desired concentrations of 50, 100 and 200 µg/kg cGnRh IIa + 5 mg/kg domperidone when administered at an injection volume of 0.5 µL/g of fish weight. Hormone preparations were stored in a 4.0
ºC refrigerator and used within 12 hours. An additional vial of propylene glycol, was prepared alongside the cGnRH IIa treatments to serve as a negative control. Ovaprim was used as a positive control at its stock concentration and administered at a dosage of 10 µg/kg sGnRH IIIa + 5 mg/kg domperidone (0.5 µL/g). Broodstock availability necessitated the use of temporal replicates. A minimum of two replicates from each of the five experimental treatments was included in each trial. Six spawning trials were completed with three replicates per treatment in each trial totaling 18 total replicates per treatment for *S. nigriventris*. Two spawning trials were completed for *S. eupterus*. Due to broodstock availability an unbalanced design was used for the *S. eupterus* experiment, with all cGnRH IIa treatments having six replicates and the Ovaprim and propylene glycol controls having five.

All spawning preparations and controls were administered to female broodstock via a single bolus intramuscular injection near the base of the dorsal fin using 100 µL Hamilton gastight syringes (Hamilton Co., Reno, NV). Experimental injection regimes mirrored current protocols used by the ornamental industry for production of *Synodontis* spp. The initial fish weight from time of biopsy was used to calculate total volume of spawning aid to be injected. Females were again anesthetized approximately 2 - 4 hours post biopsy in 150 mg/L buffered tricaine solution prior to injection. Following injections, all fish were returned to their respective holding containers and allowed to recover. All males were injected with 0.5 µL/g Ovaprim immediately after female injections to ensure spermiation at time of female ovulation. Male *S. nigriventris* were returned to the floating tub within the vat, while during the *S. eupterus* trial, males were
individually housed in 38 L flow through glass aquariums to minimize injury due to aggression post administration of spawning drug.

Periodic observations for ovulation success occurred at 16, 20, and 24 hours post drug administration. At these sampling times female fish were netted out of their holding buckets and light pressure was applied to the coelom to observe if any eggs were expelled from the oviduct. If ovulation had not occurred, females were placed back into the individual holding buckets and left undisturbed until the next sampling time. If eggs were readily expelled, ovulating females were anesthetized (150 mg/mL tricaine) blotted dry to remove excess water and weighed. Ovulated eggs were then stripped into a previously tared plastic weigh boat and weighed to the 0.001g. Spawns smaller than 0.2 g were not recorded and females were promptly returned back to holding buckets. For each collected spawn a single male fish was anesthetized, blotted dry, and milt was stripped and collected in a 1 mL syringe. Milt was added to the weigh boat to fertilize the eggs and gently mixed with a feather. Following mixing, 20 mL of water from the hatching system was then added to the weigh boat to activate the sperm and the mixture was allowed to sit undisturbed for 30 seconds. After the elapsed time a subsample of embryos was removed from the weigh boat for subsequent analyses. From the subsample, 50 eggs were chosen at random and stocked into a 150 mL screen bottomed (50 µm) container floated within the recirculating hatching system. The remaining embryos were stocked into a 1 L floating screen bottom (400 µm) containers and also housed within the hatching system. At 1.5 - 2 h post fertilization, a random subsample from the 1 L floating container was collected and viewed on a Sedgewick
Rafter Cell under a dissecting microscope. Embryos were photographed as previously described for determination of fertilization success and egg morphometrics.

Hatching success was determined from the 50 embryo subsample approximately 44 hours post fertilization. Sample cups which now contained hatched larvae were removed from the hatching system and euthanized in a 300 mg/L tricaine solution. Larvae were photographed on a Sedgewick Rafter Cell for morphometric analyses. Larvae that were hatched but atypical or dead when observed were included in hatch success data however only live larvae that were representative of typical development were used to determine notochord length. All images were captured in this investigation using ProgRes® CapturePro v2.8.8 software by Jenoptik Optical Systems (Jena, Germany) and analyzed using ImageJ v1.50i processing software (National Institutes of Health, Bethesda, MD). Mortality of brood stock used in the experiment was monitored and recorded for an additional 24 h after spawning.

**Water Quality**

Water quality parameters were monitored prior to and during spawning trials in the broodstock holding and larval hatching systems. Dissolved oxygen (DO) and temperature were measured using a YSI ProComm II meter (YSI Inc., Yellow Springs, OH). Total ammonia nitrogen (TAN), nitrite-nitrogen, pH, hardness (CaCO$_3$), and alkalinity (CaCO$_3$) were measured using standard colorimetric assays according to the manufacturers protocols. (Hach Company, Loveland, CO).

**Data Analysis**

Mean fertilization success, embryo diameter, hatching success and larval notochord length was calculated from the three sampling periods (16, 20, and 24 hours).
for each individual fish and used a single data point for statistical analyses. Secondary vitellogenic oocytes from ovarian biopsies were enumerated and the percentage of oocytes exhibiting GVM was recorded. Oocyte diameters (N = 33 ± 11 for *S. nigriventris* and N = 45 ± 8 for *S. eupterus*) from each biopsy were measured with ImageJ. Mean pre-trial oocyte diameters as well as percent GVM for each treatment were compared using an analysis of variance (ANOVA) to confirm there were no significant differences among experimental populations. Ovulation data was categorized into two levels, with successful ovulation = 1 and unsuccessful ovulation = 0. A logistic regression was performed using the generalized linear model (GLM) function in the base package of RStudio (family=binomial, V. 0.99.903 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA), followed by an ANOVA to test for differences among the categorical predictor variable (hormone treatment). A post hoc pairwise comparison was performed using a Tukey Honestly Significant Difference test. The number of spawned eggs per gram was calculated by enumerating a subset of egg samples from *S. nigriventris* (N = 8) and *S. eupterus* (N = 6) with a known weight. This value was then used to calculate fecundity based on the mass of eggs collected. Fecundity was then standardized by dividing the total number of eggs spawned per individual by the initial pre-spawning weight of the fish. Fertilization success was determined microscopically from a representative subsample of embryos (N = 189 ± 25 for *S. nigriventris* and N = 101 ± 26 for *S. eupterus*). Up to 50 embryo diameters were measured using methods previously described (N = 33 ± 15 for *S. nigriventris* and N = 9 ± 8 for *S. eupterus*). Hatching success was calculated after correcting for individual fertilization rates from each spawn. For *S. nigriventris*, mean larval notochord length was calculated from a
total of \( N = 517 \) larvae from three trials across all treatments (\( N = 22 \pm 9 \) larvae/spawn). For \textit{S. eupterus}, mean larval notochord length was calculated from a total of \( N = 250 \) larvae from two trials across all treatments (\( N = 16 \pm 8 \) larvae/spawn). Notochord length (NL) was defined as a measurement from the most anterior portion of the head to the tip of the notochord.

All data was assessed for normality and homoscedasticity prior to statistical testing. Data which satisfied these assumptions were evaluated using a one-way ANOVA. Data transformations were used if assumptions of ANOVA were not met. Non-normal data was analyzed using a one-way Kruskal-Wallis test. The propylene glycol negative control treatment was not included in fecundity, fertilization success, fertilized egg diameters, hatching success or larval length analyses due to a small sample size in \textit{S. nigriventris} (\( N = 2 \)) for all response variables and complete lack of data for \textit{S. eupterus}. A \( P \)-value of \( \leq 0.05 \) was considered statistically significant for all analyses. Data is presented as mean \( \pm \) standard deviation (SD). All statistical analyses were performed using JMP Pro v13.0.0 (SAS Institute, Cary, NC) and R v3.3.1 (R Core Team).

**Results**

For brevity, the treatment groups 50, 100, 200 \( \mu \)g/kg cGnRH IIa + 5 mg/kg domperidone cGnRH IIa and Ovaprim will be referred to as 50, 100, 200 and sGnRH IIIa respectively for the remainder of this study.

**\textit{Synodontis nigriventris}**

A total of 90 female \textit{S. nigriventris} were injected over the course of the experimental period, 72 of which were injected with cGnRH IIa or sGnRH IIIa. Mean TL
of female fish used in this experiment was 97 ± 8 mm with an initial weight of 23.92 ± 7.39 g. No differences ($P = 0.126$) were found among all treatments when the mean percentages of ovarian biopsies exhibiting GVM (88 ± 9%) were compared. Mean biopsy oocyte diameter (1.222 ± 0.086 mm) across treatments was also found to be similar ($P = 0.181$). Ovulation success of the propylene glycol treatment was significantly less than all other experimental treatments tested ($P < 0.001$) however, no differences were found in success of ovulation among hormonal treatment groups ($P > 0.267$). Fish injected with spawning aid treatments of 100 and 200 had 100% ovulation success while 83% sGnRH IIIa injected fish spawned (Table 2-1).

Eggs collected from *S. nigriventris* during this experiment averaged 1,291 ± 88 eggs/g of spawned material. Fecundity (eggs/g bodyweight), was found to be similar in quantity among hormonal treatments and dosages ($P = 0.168$), with mean number of eggs spawned per gram of the female’s initial body weight being 200 ± 106. Numerically, the highest fecundity value was observed from the 50 treatment at 228 ± 25 eggs/g. Each spawning fish ovulated an average of 3.67 ± 2.08 g translating to 4,688 ± 2,799 eggs. Analysis of mean fertilization success also yielded similar results among spawning hormones ($P = 0.491$), with a range of 66 ± 23% – 76 ± 16% and the highest mean fertilization success (76 ± 16%) observed in the 100 treatment.

Embryo diameters did not vary significantly among treatments ($P = 0.133$) with sGnRH IIIa injected fish having the largest diameters at 1.409 ± 0.066 mm and 50 treated fish with the smallest embryonic diameters at 1.360 ± 0.066 mm. Hatch success was also comparable among treatments ($P = 0.735$). The highest hatching success (35 ± 6%) occurred in both the 50 and 100 treatments and lowest (26 ± 7%) in the sGnRH
IIIa injected fish. Analysis of mean larval notochord length data yielded similar sizes among treatments \((P = 0.670)\) with a range of only 0.1 mm. The longest larval notochords occurred in the 50 treatment with mean notochord length of 3.520 ± 0.075 mm while smallest larvae occurred in the 100 and sGnRH IIIa groups with average lengths of 3.407 ± 0.208 mm and 3.407 ± 0.372 mm, respectively (Table 2-2). No mortality was recorded for *S. nigriventris* females during the 24 hour post trial observational period.

**Water Quality**

Mean water quality parameters recorded for the experimental flow through systems were: TAN 0.10 ± 0.11 mg/L, nitrite-nitrogen 0.00 ± 0.00 mg/L, pH 8.0 ± 0.0, hardness (CaCO\(_3\)) 456.00 ± 17.66 mg/L, alkalinity (CaCO\(_3\)) 182.40 ± 8.83 mg/L, temperature 25.28 ± 0.12 °C and DO 7.41 ± 0.16 mg/L. Mean water quality parameters recorded for the recirculating hatching system were: TAN 0.10 ± 0.11 mg/L, nitrite-nitrogen 0.02 ± 0.05 mg/L, pH 7.9 ± 0.3, hardness (CaCO\(_3\)) 139.65 ± 42.46 mg/L, alkalinity (CaCO\(_3\)) 76.95 ± 17.93 mg/L, temperature 27.11 ± 0.10 °C and DO 7.45 ± 0.07 mg/L.

**Synodontis eupterus**

A total of 28 female *S. eupterus* were administered injections over the course of the experimental period, 23 of which were injected with cGnRH IIa or sGnRH IIIa. The mean TL of female fish used in this experiment was 215 ± 28 mm with a mean initial weight of 165.86 ± 21.23 g. No differences \((P = 0.401)\) were found among all treatments when the mean percentages of ovarian biopsies exhibiting GVM (61 ± 21%) were compared. Mean biopsy oocyte diameter (1.242 ± 0.088 mm) across treatments was
also found to be similar \((P = 0.674)\). Significant differences were detected in ovulation success among treatments \((P = 0.025)\); with the negative control yielding no successful spawns, although the 200 and sGnRH IIIa treatments were not significantly different than the negative control \((P \geq 0.108)\). Comparable rates of ovulation success occurred among hormonal treatment groups and dosages \((P \geq 0.893)\). Fish injected with the spawning aid treatments of 50 and 100 exhibited ovulation success of 83\% while sGnRH IIIa injected fish resulted in 60\% success.

Eggs collected from *S. eupterus* during this experiment numbered 1,337 ± 60 eggs/g of spawned material. Fecundity was calculated to be of similar quantity among hormonal treatments and dosages \((P = 0.519)\), with mean number of eggs spawned per female's initial body weight being 149 ± 54 eggs/g. Numerically, the highest value was obtained from the 200 treatment at 176 ± 38 eggs/g. Each spawning fish ovulated an average of 13.61 ± 11.35 g translating to 18,190 ± 15,181 total eggs. Analysis of mean fertilization success also yielded consistent results among spawning hormones \((P = 0.864)\) with a range of 74 ± 23\% – 83 ± 7\% and the highest mean fertilization rate (83 ± 7\%) observed in the 100 treatment.

Mean embryo diameters were similar among experimental treatment groups and the positive control \((P = 0.791)\) with embryos from 200 injected fish having the largest diameters at 1.691 ± 0.115 mm and sGnRH IIIa treated fish exhibiting the smallest embryonic diameters of 1.611 ± 0.232 mm. Hatch success was also comparable among the treatments \((P = 0.943)\). The highest hatching success of 46 ± 8\% occurred in the 100 treatment and the lowest of 36 ± 26\% was observed in the 200 treated fish. Evaluation of mean larval notochord length revealed similar larval size among
treatments ($P = 0.387$). The largest larval notochord lengths occurred in the 50 treatment with a mean length of $3.833 \pm 0.208$ mm while smallest larvae occurred in the 200 treatment at $3.486 \pm 0.354$ mm. No mortality was recorded for $S. eupterus$ females during the 24 hour post trial observational period

**Water Quality**

Mean water quality parameters observed in the broodstock flow through systems were: TAN $0.0 \text{ mg/L}$, nitrite-nitrogen $0.0 \pm 0.0 \text{ mg/L}$, pH $8.0 \pm 0.0$, hardness (CaCO$_3$) $436.05 \pm 12.10$ mg/L, alkalinity (CaCO$_3$) $188.1 \pm 0.0$ mg/L, temperature $25.35 \pm 0.49 \degree\text{C}$ and DO $8.41 \pm 0.53$ mg/L. Mean water parameters recorded for the recirculating hatching system were: TAN $0.0 \pm 0.0 \text{ mg/L}$, nitrite-nitrogen $0.0 \pm 0.0 \text{ mg/L}$, pH $8.0 \pm 0.0$, hardness (CaCO$_3$) $119.70 \pm 24.18$ mg/L, alkalinity (CaCO$_3$) $76.95 \pm 12.09$ mg/L, temperature $25.30 \pm 0.42 \degree\text{C}$ and DO $8.80 \pm 0.01$ mg/L.

**Discussion**

Analysis of pretrial mean oocyte diameter and percent germinal vesicle migration among treatments indicated no significant differences for both the $S. nigriventris$ and $S. eupterus$ ($P \geq 0.126$) experiments. These results indicate all treatment test subjects were equal in reproductive condition prior to injection, reducing the chance of type I error. The dose of cGnRH IIa or Ovaprim did not significantly affect ovulation rate in either species examined ($P \geq 0.267$). The comparable performance in ovulation success among all hormonal treatments suggests all concentrations of drug administered were equally as potent, irrespective of species. The protracted batch spawning behavior of the two species examined in this investigation did not allow for interpretation of the optimum latency period based on the experimental design. Ovulation frequency at 16,
20, and 24 hours was not an accurate predictor of fecundity. Partial spawns that were repeatedly collected from individuals during sampling periods were likely the result of incomplete or prolonged FOM; evidenced by single spawns which yielded more oocytes than some females which spawned multiple times during experimental trials. Despite a single bolus injection of the spawning hormone, final oocyte maturation in both species appeared to be relatively protracted and occurred over an 8 hour window. Prolonged retention of oocytes in the coelomic cavity following ovulation can degrade egg quality. Periodic checks for ovulation is a common practice in induced spawning to ensure ovulated eggs are promptly stripped and fertilized to reduce the chance of over ripening and reduced viability of oocytes. Data from both experiments illustrate a high degree of success and predictability of the hormone preparations, it was notable that 100% ovulation was recorded in two dosages of cGnRH IIa in *S. nigriventris* and 83% in two dosages in *S. eupterus* 24 hours post drug administration (Table 2-1 & 2-2). When strip spawning Japanese Flounder (*Limanda yokohamae*) eggs can be released over a period of 5 days with over-ripening occurring three to five days post ovulation (Hirose et al. 1979) increasing the chances for reduced egg quality. The reliability of cGnRH IIa, synchronizing up to 100% of fish to ovulate in a predictable manner, aids in the efficiency of induced spawning protocols, streamlining labor and ensuring eggs are of good quality. Fertilization and hatching success appeared to be independent of spawning hormone choice or dose. While no statistical differences were elucidated among response variables for the spawning aids evaluated; when considering ovulation, fertilization and hatch data collected for both species over the course of this
investigation, the 100 treatment is recommended for induction spawning applications with these species.

In ornamental aquaculture production the number of individuals and the effort to capture brood stock being spawned can be less than in food fish aquaculture allowing producers the ability to check for ovulation more frequently without exerting copious amounts of labor. It is unclear why ovulation was observed in the *S. nigriventris* negative control; however, it has been observed that capture and handling of gravid fish can inadvertently result in ovulation and spawning (C. Watson, personal communication). While 100% of *S. nigriventris* treated with 100 and 200, only 11% of *S. nigriventris* treated with the propylene glycol ovulated, once again illustrating the effectiveness of cGnRH IIa for induction spawning in *S. nigriventris*. The *S. eupterus* produced similar results with high rates of ovulation (up to 83%) occurring in hormonally treated groups and no ovulation in propylene glycol treated fish. The functional role of the conserved cGnRH II variant with fishes was previously unclear with some species exhibiting relatively constant levels of the hormone (Somoza et al. 2002). The results of this experiment and other similar studies suggest a strong correlation between administration of exogenous cGnRH II and initiation of FOM and ovulation, further supporting the purported hypophysiotropic role for this variant (Ngamvongchon et al. 1992; Alok et al. 1999; Szabó et al. 2007; Taufek et al. 2009; Quiniou et al. 2014). No mortality was observed 24 hours post injection in *S. eupterus* or *S. nigriventris* trials indicating a high degree of safety for all drug preparations used in this study. Mortality associated with induction spawning protocols can vary dependent upon species as well as hormone choice and administration route. Hill et al. (2009) reported a wide range of
mortalities (0 – 35.7%) for various ornamental species following induction spawning protocols using sGnRH IIIa. Interestingly, a mortality rate of 0% was recorded for all Mochokids evaluated by Hill et al. (2009) which mirrored results obtained in this investigation. Cause of death was postulated by Hill et al. (2009) to result from poor water quality or egg binding during the induction spawning procedure. Provision of appropriate water quality and high mean ovulation success in hormonally treated fish, *S. nigriventris* (94 ± 8%) and *S. eupterus* (73 ± 12%), likely aided in the reduced treatment associated mortality observed in the current study.

Additional investigations into dose response are needed to recommend an optimal cGnRH IIa hormone concentration which maximizes efficacy and safety while minimizing the economic investment. Dosages of cGnRH IIa for this experiment were chosen based on the success reported by Quiniou et al. (2014), effectively inducing ovulation of Channel Catfish *Ictalurus punctatus* at 100 µg/kg. This study utilized the previously mentioned dose as well as a higher (200 µg/kg) and lower (50 µg/kg) dose that bracketed the concentration cGnRH IIa that was shown to be effective. Experimental results did not yield an observable dose response among the cGnRH IIa treatments, with no lower or upper limit identified for spawning effectiveness. Reducing the amount of drug administered would decrease the cost per unit and allow more fish to be spawned using the same amount of drug. Studies involving Turbot (*Scophthalmus maximus*), Brown Trout (*Salmo trutta*) and Pigfish (*Orthopristis chrysoptera*) have found that high doses of GnRHa in similar concentrations used in this experiment, may have negative effects in spawning success and oocyte quality (Mylonas et al. 1992; Mugnier et al. 2000; Mylonas and Zohar 2000; DiMaggio et al. 2014). It remains unclear why
excessive amounts of GnRHa may adversely impact spawning efficacy, however, it is hypothesized that this response may be linked to rapid oocyte maturation and ovulation resulting in overripened oocytes (Hirose et al. 1979; Mugnier et al. 2000; Mylonas and Zohar 2000) or desensitization of the pituitary and a subsequent reduction of LH secretion (DiMaggio et al. 2013). Although comparable to other treatment groups for all metrics measured, the highest concentration of the cGnRH IIa drug at 200 µg/kg produced some of the numerically largest fecundities of both species. This result is in direct contrast to research completed in Asian Catfish *Clarias batrachus* which found the largest administered dose of sGnRH IIIa (40 µg/kg) underperformed compared to lower concentrations of the drug, especially in measures of fecundity (Sahoo et al. 2005). It is possible that the range of effectiveness of cGnRH IIa is more broad than sGnRH IIIa allowing producers more leeway when estimating a proper concentration for effective induction spawning of new species.

Analogs are synthetic forms of native GnRHs that have substitutions in the amino acid sequence and can be defined as agonistic or antagonistic. For the purposes of induction spawning we are most concerned with the agonistic varieties which aid in the release of GtHs rather than suppress them. The substitutions in these decapeptides frequently occur between amino acids 5-6, 6-7 and 9-10 as the native forms of GnRH experience rapid enzymatic deterioration from cleavage at position 6 (Magon 2011). The longer the GnRH is active in the organism the longer it may elicit the desired reproductive response. Analogs may also increase binding affinity to pituitary GnRH receptors making them more potent than their native forms (Zohar and Mylonas 2001). The cGnrH II analog of (D-Orn6) has been demonstrated to induce 100 % ovulation in
the African Catfish *Clarias gariepinus* using a single injection (Szabó et al. 2007). In a study with European Seabass *Dicentrarchus labrax*, two isoforms of cGnRH IIa were tested for their potency in LH release. The analog (D-Arg6, Pro9 Net) resulted in the greatest amount of LH release among the cGnRH groups tested, more potent than the cGnRH IIa (D-Ala 6, Pro9 Net) peptide. Additionally, the analog (D-Ala6, Pro9 Net) also performed comparably yielding a response six times the potency of native cGnRH forms (Forniés et al. 2003). A study evaluating spawning success with several isoforms of GnRH analogs in Channel Catfish *Ictalurus punctatus* revealed the greatest success was achieved with the cGnRH II analog of (D-Arg6, Pro9 Net) with a mean ovulation of 90.2% (Quiniou et al. 2014). Multiple studies have been carried out with the cGnRH II (D-Arg6, Pro9 Net) analog which have examined resulting LH release or progression of FOM culminating in ovulation (Lovejoy et al. 1995; Szabó et al. 2007; Quiniou et al. 2014). Results from these investigations helped to shape the choice of analog and concentrations that were evaluated in the current research. Although ovulation success of 100% in *S. nigriventris* and 83% in *S. eupterus* was achieved for some concentrations of cGnRH IIa (D-Arg6, Pro9 Net) in this experiment, it would be prudent to further examine additional isoforms of GnRH’s moving forward, as species can respond differently to native forms as well as analogs.

Delivery methods of GnRH can vary depending on species, size and reproductive strategy of the fish being induced. Spawning hormones have been successfully administered using a myriad of delivery systems and injections schedules including single injection (Quiniou et al. 2014), multiple injections (Shireman and Gildea 1989), intramuscular injections (DiMaggio et al. 2014), intracoelomic injections (also
referred to as intraperitoneal) (Clemens and Grant 1965), intra-pericardial cavity injections (Kouril et al. 1986), intravenous injections (Mikolajczyk et al. 2003), ovarian lavage (Watson et al. 2009), sustained release implants (Mugnier et al. 2000) and even diffusion over gills (Hill et al. 2005). In species which exhibit synchronous egg development, a single LH surge is often adequate to induce ovulation; conversely, if a species exhibits asynchronous ovarian development, a sustained release delivery of exogenous hormones may be the more appropriate route (Podhorec and Kouril 2009).

The amount of stress associated with the capture and application of hormones also should be taken into consideration. If it is problematic to harvest and subdue brood stock, the hormone administration regime which involves the least amount of handling may be the most suitable treatment. Ovulation of Asian catfish *Clarias batrachus* has been successfully induced with a single dose of sGnRH IIIa (Sahoo et al. 2005) while successful ovulation has also been reported using priming (20%) and resolving (80%) doses with Channel Catfish using cGnRH IIa (Quiniou et al. 2014). Time to apparent ovulation can vary greatly among species. During this period elevated levels of LH must be maintained for successful ovulation and can be achieved with multiple GnRHa treatments administered over a protracted period (Mylonas and Zohar 2000). The single injection regime utilized in this study emulated commercial protocols commonly implemented by producers of these species. Although a high degree of success was achieved using these spawning protocols, a future assessment that evaluates the effect of a staggered priming and resolving dose of cGnRH IIa on spawning efficacy is warranted.
The time period between administration of hormonal therapy and ovulation is commonly referred to as the latency period. Elucidation of this time period is critical and may be influenced by factors such as temperature and choice of spawning aid. Due to limitations with broodstock availability, latency period was unable to be evaluated in the current experiment. A study design similar to the one implemented by Sahoo et al. (2005) for Asian Catfish *Clarias batrachus*, would be advantageous for determination of latency period in *Synodontis* spp. and should be further pursued as results could help to further optimize commercial production protocols for these economically valuable ornamental species. Additionally, it should be noted that hatching success and notochord lengths reported in this study may have been adversely impacted from added handling associated with the experimental sampling procedure. While empirically valid, these results may not be truly indicative of outcomes one might expect in a commercial setting and should be interpreted as such.

The impact that siluriformes have on an assortment of industries within aquaculture is immense, the range of which extends from food fish to ornamentals. Streamlining captive culture may help to increase production and reduce pressure on wild conspecifics, meet the demand of flourishing markets and increase profitability for producers. To the author’s knowledge this experiment represents the most comprehensive study to date describing hormone induced spawning of *Synodontis* catfish. Data from this work suggests that cGnRH IIa exhibits hypophysiotropic activity in *Synodontis* spp. and performs as well as Ovaprim in induction spawning of *S. nigriventris* and *S. eupterus*. Although not statistically significant, the 100 µg/kg cGnRH IIa + 5 mg/kg domperidone treatment generally yielded values that were numerically the
highest for most response variables in both experiments (Table 2-1 & 2-2). Due to its comparable efficacy in pertinent spawning criteria, reliability, safety, and broad range of effective concentrations, cGnRH IIa appears to be a practical induced spawning option. Further investigations which explore optimization of dosages, alternative hormone analogs and additional administration regimes may help to improve the performance of cGnRH IIa as a viable alternative to currently available spawning preparations. A number of spawning aids are approved for use through the investigational new animal drug (INAD) program allowing them to be administered with data collection and enrollment in the plan. Although these drugs are not yet formally approved by the FDA, studies such as the current one provide valuable data which may contribute to a formal INAD exemption. Development of an INAD project or proceeding directly to drug indexing for cGnRH IIa may be warranted as a growing body of literature has demonstrated its effectiveness as an induced spawning aid.
Table 2-1. Mean ± SD ovulation success, fecundity (eggs per gram body weight), fertilization success, fertilized oocyte diameter, hatch success and notochord length for *Synodontis nigriventris* administered one of three concentrations of cGnRH IIa, sGnRH IIIa (positive control) or propylene glycol (negative control). All GnRHa treatments included the dopamine antagonist domperidone at a concentration of 5 mg/kg. Different superscript letters within a column denotes statistically significant differences ($P \leq 0.05$). The symbol * indicates descriptive data that was not included in statistical analyses due to a limited sample size ($N = 2$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ovulation (%)</th>
<th>Eggs Per Gram Body Weight</th>
<th>Fertilization Success (%)</th>
<th>Embryo Diameter (mm)</th>
<th>Hatch Success (%)</th>
<th>Notochord Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylene Glycol</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>130 ± 136*</td>
<td>64 ± 2*</td>
<td>1.377 ± 0.004*</td>
<td>48 ± 6*</td>
<td>3.415 ± 0.033*</td>
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<tr>
<td>sGnRH IIIa 10 µg/kg</td>
<td>83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>160 ± 27</td>
<td>75 ± 22</td>
<td>1.409 ± 0.066</td>
<td>26 ± 7</td>
<td>3.407 ± 0.372</td>
</tr>
<tr>
<td>cGnRH IIa 50 µg/kg</td>
<td>94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>228 ± 25</td>
<td>70 ± 20</td>
<td>1.360 ± 0.066</td>
<td>35 ± 6</td>
<td>3.520 ± 0.075</td>
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<tr>
<td></td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>181 ± 25</td>
<td>76 ± 16</td>
<td>1.363 ± 0.068</td>
<td>35 ± 6</td>
<td>3.407 ± 0.208</td>
</tr>
<tr>
<td></td>
<td>200&lt;sup&gt;a&lt;/sup&gt;</td>
<td>227 ± 25</td>
<td>66 ± 23</td>
<td>1.385 ± 0.060</td>
<td>33 ± 6</td>
<td>3.483 ± 0.252</td>
</tr>
</tbody>
</table>
Table 2-2. Mean ± SD ovulation success, fecundity (eggs per gram body weight), fertilization success, fertilized oocyte diameter, hatch success and notochord length for *Synodontis eupterus* administered one of three concentrations of cGnRH IIa, sGnRH IIIa (positive control) or propylene glycol (negative control). All GnRHa treatments included the dopamine antagonist domperidone at a concentration of 5 mg/kg. Different superscript letters within a column denotes statistically significant differences ($P \leq 0.05$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ovulation (%)</th>
<th>Eggs Per Gram Body Weight</th>
<th>Fertilization Success (%)</th>
<th>Embryo Diameter (mm)</th>
<th>Hatch Success (%)</th>
<th>Notochord Length (mm)</th>
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<tr>
<td>Propylene Glycol</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>sGnRH IIIa</td>
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<tr>
<td>10 µg/kg</td>
<td>60&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>157 ± 69</td>
<td>81 ± 15</td>
<td>1.611 ± 0.232</td>
<td>40 ± 23</td>
<td>3.677 ± 0.333</td>
</tr>
<tr>
<td>cGnRH IIa</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>50 µg/kg</td>
<td>83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120 ± 63</td>
<td>79 ± 18</td>
<td>1.689 ± 0.094</td>
<td>45 ± 34</td>
<td>3.833 ± 0.208</td>
</tr>
<tr>
<td>100 µg/kg</td>
<td>83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>151 ± 48</td>
<td>83 ± 7</td>
<td>1.632 ± 0.119</td>
<td>46 ± 8</td>
<td>3.576 ± 0.249</td>
</tr>
<tr>
<td>200 µg/kg</td>
<td>67&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>176 ± 38</td>
<td>74 ± 23</td>
<td>1.691 ± 0.115</td>
<td>36 ± 26</td>
<td>3.486 ± 0.354</td>
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<tr>
<td><strong>Mammal</strong> (mGnRH I)</td>
<td>pGlu-</td>
<td>His-</td>
<td>Trp-</td>
<td>Ser-</td>
<td>Tyr-</td>
<td>Gly-</td>
</tr>
<tr>
<td><strong>Salmon</strong> (sGnRH III)</td>
<td>pGlu-</td>
<td>His-</td>
<td>Trp-</td>
<td>Ser-</td>
<td>Tyr-</td>
<td>Gly-</td>
</tr>
<tr>
<td><strong>Salmon Analog</strong> (sGnRH IIIa)</td>
<td>pGlu-</td>
<td>His-</td>
<td>Trp-</td>
<td>Ser-</td>
<td>Tyr-</td>
<td>DArg-*</td>
</tr>
<tr>
<td><strong>Chicken</strong> (cGnRH II)</td>
<td>pGlu-</td>
<td>His-</td>
<td>Trp-</td>
<td>Ser-</td>
<td>His-</td>
<td>Gly-</td>
</tr>
<tr>
<td><strong>Chicken Analog</strong> (cGnRH IIa)</td>
<td>pGlu-</td>
<td>His-</td>
<td>Trp-</td>
<td>Ser-</td>
<td>His-</td>
<td>DArg-*</td>
</tr>
</tbody>
</table>

Figure 2-1. Amino acid sequence of mammalian GnRH, the two GnRH analogs used in this experiment alongside their native forms. The symbol * signifies alterations of amino acid sequence from native GnRH forms and their analogs.
CHAPTER 3
EVALUATION OF cGnRH IIa FOR INDUCTION SPAWNING OF TWO ORNAMENTAL _Epalzeorhynchos_ SPECIES

Introduction

Among the many hurdles to overcome in aquaculture, successful spawning of fish is paramount to meet industry demands (DiMaggio et al. 2013). Reproductive dysfunction is commonplace in captivity although its presentation and severity may vary by species (Mylonas and Zohar 2000, 2007; Podhorec and Kouril 2009; Yaron et al. 2009). Addressing this impediment to culture often involves the aid of environmental manipulations or use of exogenous hormones (Rottmann et al. 1991b). In settings where it may be too difficult to alter photoperiod, temperature, water quality, tidal effects, lunar cycles and other critical environmental variables, administration of exogenous hormones to obtain viable gametes may be the most appropriate or only option (Rottmann et al. 1991a). In nature, environmental cues which signify the approach of reproductive activity are perceived by the brain of the fish. This sensory data is integrated by the hypothalamus which in turn generates and releases neuropeptides to initiate a cascade of reactions resulting in mature gametes (Zohar and Mylonas 2001). Briefly, the progression of this cascade is initiated following release of gonadotropin releasing hormones (GnRH) from the hypothalamic region of the brain. GnRH, which acts on the pituitary via direct innervation, stimulates the secretion of gonadotropins (GtH) that act directly on the gonad resulting in the production of steroids and prostaglandins (Rottmann et al. 1991b). The succession of events occurring in the hypothalamic pituitary gonadal (HPG) axis is similar in both male and female organisms, diverging only with respect to what cell types of the gonad are effected and final sex hormones that are produced (Mylonas and Zohar 2000). Much of the early attempts to
induce this cascade focused on introduction of GtHs through the use of pituitary extracts and homogenates acquired from slaughtered fish (Mylonas and Zohar 2007). These crude extracts not only posed a risk with respect to biosecurity and disease transmission but moreover, were difficult to dose and yielded inconsistent results as the amount of active hormone concentration was unknown (Rottmann et al. 1991b). Since the discovery of the GnRH decapeptide and its associated role in the HPG axis, a large body of research has focused on optimization of dose (Mylonas et al. 1992; Ngamvongchon et al. 1992; Alok et al. 1999; Mikolajczyk et al. 2003; Sahoo et al. 2005; Taufek et al. 2009; DiMaggio et al. 2013, 2014), identification of active variants (Ngamvongchon et al. 1992; Zohar et al. 1995; Alok et al. 1999; Illing et al. 1999; Bosma et al. 2000; Forniés et al. 2003; Szabó et al. 2007; Podhorec and Kouril 2009; Taufek et al. 2009; Quiniou et al. 2014; Prayogo et al. 2016), and development of potent analogues (Ngamvongchon et al. 1992; Lovejoy et al. 1995; Zohar et al. 1995; Alok et al. 1999; Forniés et al. 2003; Szabó et al. 2007; Podhorec and Kouril 2009). One advantage of using GnRH is that it occurs earlier in the hormonal cascade stimulating the release of endogenous stores of GtH and facilitating the contribution of supplementary endocrine factors such as prolactin, thyroid hormones, insulin like growth factors and growth hormone (Podhorec and Kouril 2009). This approach is arguably more "natural" and preferred to flooding the gonad with GtHs, as repeated use of heterologous gonadotropins may trigger an immunological response thereby decreasing the efficacy of the spawning aid over time (Zohar and Mylonas 2001).

The GnRH peptide contains 10 amino acids in sequence, the specific arrangement of which can vary among species. In vertebrates there are 16 discovered
variants of gonadotropin releasing hormones that belong to three distinct GnRH systems (Somoza et al. 2002; Millar 2003). Most vertebrates have two to three GnRH forms present in the body with hormones belonging to system II occurring ubiquitously among taxa while system III hormones are teleost specific (Kah et al. 2007). Variants within the systems are named after the organism they were first described in, with mGnRH I found initially in mammals, cGnRH II in chickens and sGnRH III in salmon (Kah et al. 2007). With the discovery of GnRH came laboratory synthesized analogs targeting substitutions of amino acid bonds that were prone to enzymatic degradation (Lovejoy et al. 1995). Alterations at these specific positions have made GnRH analogs (GnRHα) more potent as induced spawning aids by increasing binding affinity and prolonging the duration of activity while in circulation (Zohar and Mylonas 2001; Taufek et al. 2009). The induction spawning drug known as Ovaprim® ([20 µg/mL sGnRH IIIa + 10 mg/mL domperidone], Western Chemical Inc., Ferndale, WA) is an analog (D-Arg6-Pro9-Net) (Figure 3-1) of sGnRH III and has become the industry standard in ornamental fish production due to its availability and efficacy in a range of species (Hill et al. 2009). This hormone preparation was added to the United States Food and Drug Administration (FDA) index of legally marketed unapproved new animal drugs for minor species making it available for producers to purchase directly from the manufacturer. The sGnRH IIIa hormone, administered in conjunction with a dopamine antagonist, has been successfully used for induction spawning of Pinfish *Lagodon rhomboides* (DiMaggio et al. 2013), Pigfish *Orthopristis chrysoptera* (DiMaggio et al. 2014), Asian Catfish *Clarias batrachus* (Sahoo et al. 2005), African Catfish *Clarias gariepinus* (Taufek et al. 2009), Channel Catfish *Ictalurus punctatus* (Quiniou et al. 2014), Common Carp
Cyprinus carpio (Yaron et al. 2009) and a variety of ornamental species (Hill et al. 2009). Although sGnRH IIIa has successfully induced ovulation in numerous species of ornamental (Hill et al. 2009), food (Sahoo et al. 2005; Quiniou et al. 2014) and baitfish (DiMaggio et al. 2013, 2014), other GnRHa variants have been administered with greater spawning efficacy (Quiniou et al. 2014). While Ovaprim is currently the preferred choice for induction spawning of ornamental fishes, this preparation may be unreliable or completely ineffective in some cases (Hill et al. 2009) and investigations into additional spawning aids are warranted.

The highly conserved cGnRH IIa (D-Arg6-Pro9-Net) has garnered recent interest as a potential alternative to the hypophysiotropic sGnRH IIIa for induction spawning of fishes. The cGnRH II variant has been found to stimulate the release of the gonadotropin, luteinizing hormone (LH), in African Catfish Clarias gariepinus (Bosma et al. 2000) and Gilthead Sea Bream Sparus aurata (Zohar et al. 1995). Furthermore, induction spawning activity was reported to be greater using cGnRH II as a spawning aid compared to other GnRH types in African Catfish Clarias gariepinus (Szabó et al. 2007), Channel Catfish Ictalurus punctatus (Quiniou et al. 2014), and European Seabass Dicentrarchus labrax (Forniés et al. 2003). Further investigation into the efficacy of various cGnRH II analogues may expand the number of species able to be cultured while also potentially producing greater yields for species already in production.

Members of the Cyprinidae family are arguably the most economically important fishes in food and ornamental aquaculture. The Common Carp C. carpio was the first domesticated fish dating back nearly 2000 years ago in ancient Rome; moreover, the Goldfish Carassius auratus was used for ornamental purposes as early as ~1200 A.D.
in China (Balon 2004). The sheer volume in which cyprinids are aquacultured eclipses many other families of teleosts and induction spawning is a critical factor in production. Within the Cyprinidae family, the genus *Epalzeorhynchos* contains a limited number of highly valuable ornamental fish species endemic to South East Asia (Kulabtong et al. 2014; Elakkanai et al. 2017). The Redtail Sharkminnow, *Epalzeorhynchos bicolor*, is a popular species within the freshwater ornamental trade and was so greatly pursued that for nearly 50 years was believed to be extinct in their native range due to mass collection accompanied by habitat destruction and pollution (Vidthayanon 2013; Kulabtong et al. 2014). Commercial spawning protocols for this species commonly use Ovaprim, as strong dopaminergic inhibition of LH secretion is characteristic of the Cyprinidae family (Chang et al. 1983; Trudeau et al. 1993; Podhorec and Kouril 2009). While this species and its congener the Rainbow Sharkminnow, *E. frenatum*, have both been shown to respond to exogenous hormone administration (Shireman and Gildea 1989; Hill et al. 2005; Henriques 2016; Islami et al. 2017), a recent survey of Ovaprim use in the ornamental industry by Hill et al. (2009) identified a 12% failure rate for ovulation and an 8% mortality rate in *E. bicolor* administered this peptide preparation. While the current ovulation rate of 88% in *E. bicolor* using Ovaprim is laudable, the 8% mortality rate is a concern to industry as these fish may be spawned multiple times in a season and the potential exists for significant reductions in captive broodstock populations. As development of broodstock can be laborious and costly, any reduction in observed mortality would be welcome. These statistics clearly indicate the need to explore alternative peptides which may increase ovulation success and decrease the mortality rate of valuable brood animals.
The objective of this study was to evaluate the efficacy of various dosages of cGnRH IIa (D-Arg6-Pro9-Net) on spawning performance in Redtail Sharkminnows *E. bicolor* and Rainbow Sharkminnows *E. frenatum*. Data on successful ovulation, fecundity, fertilization and hatching success, as well as embryo and larval morphometrics was analyzed to assess effectiveness of drug preparations among treatments. Ovaprim was used as a benchmark to compare spawning performance of cGnRH IIa as it is currently the drug of choice for producers of these species.

**Methods**

Two distinct experiments were carried out during this study evaluating the efficacy of cGnRH IIa as an induced spawning aid for *E. bicolor* and *E. frenatum*. The experimental procedures for both cyprinid species were similar although minor modifications occurred and are noted in the methods below.

**Experimental Design**

Reproductively competent broodstock were obtained from a commercial ornamental aquaculture producer in Plant City, Florida, USA. Fish were transported to the University of Florida’s Tropical Aquaculture Laboratory (UF-TAL) where males and females were separated into individual 1,030 L concrete vats with an operational volume of 380 L. During the experiment, vats were maintained with degassed flow-through well water at 380 L/hour with supplementary aeration. All brood fish were anesthetized in a 150 mg/L tricaine methanesulfonate (MS-222, Western Chemical Inc., Ferndale, WA) solution buffered with 300 mg/L sodium bicarbonate prior to determination of total length (TL) and weight. Sexual maturity and gonadal development was evaluated using minor pressure applied to the male’s coelom to confirm the
presence of running milt. Females appeared grossly to have somewhat enlarged and softer coeloms. While anesthetized, a silicone tube (0.76 mm inside diameter and 1.65 mm outside diameter) was inserted into the oviduct after which modest suction was applied to obtain an ovarian biopsy. The oocyte sample was transferred to a Sedgewick Rafter Counting Cell for scale and then visualized under a dissecting microscope with image capture capabilities. Fish selected for this study exhibited secondary stage vitellogenic oocytes with ≥ 50 percent germinal vesicle migration (GVM). Bright and dark field digital photomicrographs were obtained for determination of vitellogenic oocyte diameters as well as rates of GVM. Female fish used in the experiment were individually placed into labeled 19 L buckets (working volume 9.77 L) with holes drilled along the circumference to permit exchange of water and placed within two concrete vats. Throughout the duration of the experiment, buckets were covered with a lid except for removal during hormone administration or intermittent checks for ovulation. A 120 L plastic tub was used to hold male fish. The tub was floated in a vat near the female fish and holes drilled in the perimeter of the container allowed for water exchange. During a pilot study, temperature, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite-nitrogen, pH, hardness (CaCO$_3$) and alkalinity (CaCO$_3$) were tested in water originating from the buckets and surrounding vat area and revealed no significant variation.

The cGnRh IIa (D-Arg6, Pro-Net, Figure 3-1) spawning aid used in this experiment was synthesized by Genscript Labs (Piscataway, NJ) and shipped and stored in a lyophilized state. The peptide was aliquoted into individual vials each containing 100 µg of the hormone analogue and stored in a -80.0 ºC freezer prior to use. Fresh cGnRH IIa peptide solutions were prepared for each spawning trial using
methods described herein. A carrier solution was prepared that consisted of the dopamine antagonist, domperidone (Roadrunner Pharmacy, Phoenix, AZ) dissolved in propylene glycol, yielding a final concentration of 10 mg/mL; equivalent to that of Ovaprim. To homogenously dissolve the lyophilized domperidone into solution with propylene glycol, heat applied in a warm water bath along with periodic vortexing was used on the vials for dilution. Volumes of 1000, 500 and 250 µL of the carrier was pipetted into three individual 100 µg cGnRH IIa vials to achieve desired concentrations of 50, 100 and 200 µg/kg cGnRh IIa + 5 mg/kg domperidone when administered at an injection volume of 0.5 µL/g. Once mixed, experimental spawning aids were stored at 4.0 ºC and used within 12 hours. A negative control treatment of pure propylene glycol was prepared alongside the cGnRH IIa treatments. A positive control treatment of Ovaprim was used at its stock concentration and administered at a dose of 10 µg/kg sGnRH IIIa + 5 mg/kg domperidone (0.5 µL/g). Temporal replicates were utilized as dictated by broodstock availability. In each trial a minimum of one replicate from each of the five experimental treatments was included. An unbalanced design was used for the E. bicolor experiment due to accessibility of broodstock. Six spawning trials were completed with 15 total replicates for Ovaprim, 50 and 100 µg/kg cGnRh IIa + 5 mg/kg domperidone treatments. The additional experimental treatments of propylene glycol had 14 replicates while the 200 µg/kg cGnRh IIa + 5 mg/kg domperidone had 16 for E. bicolor. Two spawning trials were completed for E. frenatum, with 3 replicates per treatment in each trial.

Experimental spawning treatments were administered to female broodstock near the base of the dorsal fin via intramuscular injections using 100 µL Hamilton gastight
syringes (Hamilton Co., Reno, NV). Injection regimes incorporated in this study emulated protocols currently used by the ornamental industry for *Epalzeorhynchos* spp. production. The weight of females from time of biopsy was used to calculate a total injection volume for the spawning aid or control. The total volume of drug to be administered was divided between a 10% priming and 90% resolving injection which were administered 6 hours apart. All fish were individually anesthetized in a 150 mg/L buffered tricaine solution before treatments were dispensed. Following injections, broodfish were returned to their corresponding holding containers and allowed to recover. To ensure spermiation at time of female ovulation, all males were injected with 0.5 µL/g Ovaprim immediately after female resolving injections. For both species male fish were returned to the floating tubs within the vat post administration of spawning drug. Approximately 10 - 12 hours elapsed between ovarian biopsy and treatment injections.

Observations for ovulation success occurred hourly from three to nine hours post resolving injection for *E. bicolor* and from three to ten hours post resolving injection for *E. frenatum*. During ovulation sampling periods, female fish were netted out of their holding buckets and light pressure was applied to the coelom to observe if any eggs were expelled. Female fish were placed back into the treatment bucket and left undisturbed until the next sampling time if ovulation did not occur. Females which expressed eggs after coelomic palpation were anesthetized (150 mg/mL tricaine) blotted dry and weighed. Ovulated eggs were expelled into a tared plastic weigh boat which was subsequently reweighed to the nearest 0.001 g. Spawns that weighed less than 0.2 g were not used in this experiment and female fish were quickly returned back
to their corresponding bucket. A single male was used to fertilize each collected spawn. Prior to fertilization, males were anesthetized, blotted dry, milt was hand stripped and collected in a 1 mL syringe. To fertilize the eggs, milt was added to the weigh boat containing the eggs and carefully mixed with a feather. Water (20 mL) from the hatching system was then added to the weigh boat to activate the sperm and the mixture was allowed to incubate for 30 seconds. A subsample of embryos was then collected from the weigh boat for further analyses. A fertilized egg subsample (N=50) was chosen at random from each spawn and stocked into 150 mL screen bottomed (50 µm) containers suspended in 0.5 cm thick foam board. The apparatus contained holes to snugly fit sample cups partially submersed in water and was floated in the recirculating hatching system. The remaining embryos were stocked into 1 L suspended screen bottom (400 µm) containers also housed within the hatching system. A random subsample was collected from the 1 L floating containers 1.5 - 2 hours post fertilization and visualized on a Sedgewick Rafter Cell using a dissecting microscope. Digital photographs were taken of the embryos for subsequent determination of fertilization success and egg morphometrics.

The subsample of 50 embryos was used to determine hatching success at approximately 24 h post fertilization. Sample cups which now contained hatched larvae were removed from the hatching system and euthanized in a 300 mg/L buffered tricaine solution. A sample of larvae were photographed on a Sedgewick Rafter Cell to calculate larval notochord length (NL). Hatched larvae that were atypical or dead when viewed were included in hatch success data however only live larvae that were representative of normal development were used to determine NL. All images were captured in this
study using ProgRes® CapturePro v2.8.8 software by Jenoptik Optical Systems (Jena, Germany) and analyzed using ImageJ v1.50i processing software (National Institutes of Health, Bethesda, MD). Broodstock mortality was monitored and recorded for an additional 24 hrs post spawning.

**Water Quality**

Water quality parameters were analyzed before and during spawning trials in the broodstock holding and larval hatching systems. Dissolved oxygen (DO) and temperature were measured using a YSI ProComm II meter (YSI Inc., Yellow Springs, OH). Total ammonia nitrogen (TAN), nitrite-nitrogen, hardness (CaCO$_3$) and alkalinity (CaCO$_3$) and pH were measured using standard colorimetric assays according to the manufacturers protocols. (Hach Company, Loveland, CO).

**Data Analysis**

Fecundity was standardized by calculating the number of eggs spawned divided by the respective female fish weight acquired during biopsy sampling to obtain the number of eggs per gram body weight. Mean fertilization success, embryo diameter, hatching success and larval notochord length were calculated for each individual replicate. The percentage of secondary vitellogenic oocytes exhibiting GVM was recorded following analysis of digital photomicrographs of ovarian biopsies. Mean egg diameters (N = 44 ± 10 for *E. bicolor* and N = 49 ± 2 for *E. frenatum*) from each biopsy were measured in ImageJ from catalogued digital images. Analysis of Variance (ANOVA) or Kruskal-Wallis tests were used to compare mean pre-trial oocyte diameters as well as percent GVM for each treatment and confirmed there were no significant differences among experimental populations prior to the study. To allow for gravimetric
estimation of egg quantity, a subset of egg samples from *E. bicolor* (N = 10) and *E. frenatum* (N = 5) each with a known weight, were enumerated and used to calculate a mean number of eggs/g. This value was then used to calculate fecundity based on the mass of eggs collected from each female. A representative subsample of embryos (N = 72 ± 19 for *E. bicolor* and N = 62 ± 20 for *E. frenatum*) was microscopically examined to determine fertilization success for each replicate. Up to 50 embryo diameters were measured for each replicate using methods previously described (N = 49 ± 2 for *E. bicolor* and N = 48 ± 3 for *E. frenatum*). Hatch success was calculated after correcting for individual spawn fertilization rates. For *E. bicolor*, mean larval NL was calculated from a total of N = 398 larvae from four trials across all treatments with successful hatch (N = 11 ± 4 larvae/spawn). For *E. frenatum*, mean larval NL was calculated from a total of N = 142 larvae from two trials across all treatments with successful hatch (N = 12 ± 4 larvae/spawn). Larval NL was measured from the most anterior portion of the head to the tip of the notochord.

Normality and homoscedasticity were assessed for all data prior to statistical testing. One-way ANOVA was used to evaluate data which satisfied these assumptions. If assumptions of ANOVA were not met, data transformations were used. A one-way Kruskal-Wallis test was used to analyze non-normal data. The negative control treatment of propylene glycol was not included in fecundity, fertilization success, embryo diameter, hatching success or larval length analyses due to complete lack of data for these metrics for both species. For *E. frenatum* the Ovaprim treatment was not included in statistical analyses for all spawning metrics besides ovulation success and mortality status due to small sample size (N = 1). Ovulation data was categorized into
two levels, with successful ovulation = 1 and unsuccessful ovulation = 0. A logistic regression was performed using the generalized linear model (GLM) function in the base package of RStudio (family = binomial, V. 0.99.903 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA), followed by an ANOVA to test for differences among the categorical predictor variable (hormone treatment). A post hoc pairwise comparison was performed using a Tukey Honestly Significant Difference test. A $P$-value of $\leq 0.05$ was considered significant for all statistical analyses. Mortality data was analyzed using similar methods to evaluating ovulation success, with live fish = 1 and dead fish = 2. All data are presented as mean ± standard deviation. All statistical analyses were performed using JMP Pro v13.0.0 (SAS Institute, Cary, NC) and R v 3.3.1 (R Core Team).

**Results**

For brevity, the treatment groups 50, 100, 200 µg/kg cGnRH IIa + 5 mg/kg domperidone and Ovaprim will be referred to as 50, 100, 200 and sGnRH IIIa respectively for the remainder of this study.

*Epalzeorhynchus bicolor*

Over the course of the study 75 female *E. bicolor* were subjected to experimental treatments. A total of 61 of those fish were injected with sGnRH IIIa or cGnRH IIa. The mean total length of females treated during the experiment was 121 ± 18 mm with a pretrial weight of 23.75 ± 13.69 g. The percentage of oocytes undergoing GVM was comparable among females in all treatments prior to experimentation ($P = 0.619$). A mean total of 95 ± 4% of oocytes obtained from ovarian biopsies demonstrated GVM and had a mean diameter of 1.148 ± 0.1329 mm. No disparities in oocyte diameter were
determined among treatments prior to injection ($P = 0.390$). Ovulation success was affected by treatment with the propylene glycol treatment resulting in the lowest observed ovulation success ($P < 0.001$). Rates of ovulation were comparable among sGnRH IIIa and cGnRH IIa treatments ($P \geq 0.532$) with a range of 73 – 93% success. The highest observed ovulation success was recorded in the 50 treatment (Table 3-1).

Unfertilized eggs averaged 2,486 ± 155 eggs/g of material spawned for *E. bicolor*. Fecundity (eggs/g bodyweight) was determined to be consistent among all treatments of cGnRH IIa and sGnRH IIIa ($P = 0.967$) with a relatively narrow range of 440 ± 95 to 466 ± 128 eggs/g bodyweight for the 200 and 50 treatments, respectively (Table 1). The mean recorded mass of an ovulated spawn per female was 4.47 ± 3.22 g or 11,123 ± 8,000 eggs. Mean fertilization success was high (>90%) for all hormone treatments throughout the experimental period with no significant differences detected ($P = 0.267$) (Table 3-1).

Embryo diameters, were found to be relatively uniform among all spawning treatments ($P = 0.572$) with the largest difference of only 0.280 mm observed between the 100 and sGnRH IIIa treatments (Table 3-1). While the highest hatch success was observed in the 50 treatment (62 ± 25%), all hormonal treatments performed similarly for this metric ($P = 0.299$). Larval morphometric analysis indicated comparable NL among cGnRH IIa and sGnRH IIIa treatments upon hatch ($P = 0.478$). Mean NL values differed by only 0.101 mm with a range of 3.266 ± 0.155 - 3.365 ± 0.131 mm. Broodstock mortality at 24 hours was affected by the experimental drug injected ($P = 0.048$) however post hoc pairwise analysis did not demonstrate differences in mortality.
rate among treatments \((P \geq 0.073)\). The greatest losses occurred in the 200 treatment group where 5 out of the 16 fish injected died.

**Water Quality**

Mean water quality parameters recorded for the experimental flow through systems were: TAN \(0.09 \pm 0.11\) mg/L, nitrite-nitrogen \(0.00 \pm 0.00\) mg/L, pH 8.00 \(\pm 0.00\), hardness \((\text{CaCO}_3)\) 473.91 \(\pm 36.56\) mg/L, alkalinity \((\text{CaCO}_3)\) 195.43 \(\pm 13.45\) mg/L, temperature 25.30 \(\pm 0.14\) °C and DO 7.92 \(\pm 0.20\) mg/L. Mean water quality parameters recorded for the recirculating hatching system were: TAN \(0.06 \pm 0.10\) mg/L, nitrite-nitrogen \(0.01 \pm 0.03\) mg/L, pH 7.86 \(\pm 0.24\), hardness \((\text{CaCO}_3)\) 131.91 \(\pm 16.27\) mg/L, alkalinity \((\text{CaCO}_3)\) 78.17 \(\pm 13.45\) mg/L, temperature 25.28 \(\pm 1.18\) °C and DO 8.09 \(\pm 0.52\) mg/L.

**Epalzeorhynchos frenatum**

Over the course of the study 30 female *E. frenatum* were administered experimental treatments. A total of 24 of those fish were injected with sGnRH IIIa or cGnRH IIa. The mean total length of females utilized during the experiment was 131 \(\pm 9\) mm with a pretrial weight of 21.31 \(\pm 2.85\) g. Percentage of oocytes undergoing GVM was comparable among females in all treatments prior to experimentation \((P = 0.670)\). A mean total of 97 \(\pm 2\)% of oocytes obtained from ovarian biopsies demonstrated GVM and had a mean diameter of 1.033 \(\pm 0.099\) mm. No distinctions in oocyte diameter were detected among treatments prior to injection \((P = 0.888)\) with overall size being 1.033 \(\pm 0.099\) mm. sGnRH IIIa treated fish for *E. frenatum* were excluded from statistical tests comparing fecundity, fertilization success, hatch success, embryo diameter and larval notochord length due to a small sample size \((N = 1)\). Reported data are mean values
from a single replicate and should be interpreted as such. Ovulation success was affected by treatment administered ($P < 0.001$). Similar ovulation success was noted among the cGnRH IIa treatments ($P \geq 0.471$), between sGnRH IIIa and the propylene glycol negative control treatment ($P = 0.918$), and between sGnRH IIIa and 200 ($P = 0.122$). Mean ovulation success of 100% was reported for female *E. frenatum* injected with the 50 treatment (Table 3-2).

Unfertilized eggs averaged $2,453 \pm 186$ eggs/g of spawned material for *E. frenatum*. Fecundity (eggs/g bodyweight) was comparable among all treatments of cGnRH IIa ($P = 0.594$) with a mean range of $346 \pm 77$ to $421 \pm 142$ eggs/g bodyweight for fish in the 200 and 50 treatments, respectively (Table 3-2). The mean recorded mass of an ovulated spawn per female was $3.48 \pm 1.14$ g or to $8,544 \pm 2,789$ eggs. Mean fertilization success was high (>95%) for all cGnRH IIa hormone treatments throughout the experimental period with no significant differences detected ($P = 0.418$) (Table 3-2).

Embryo diameters, were found to be relatively similar among all spawning treatments ($P = 0.484$) with the largest difference of only $0.355$ mm observed between the 100 and 50 treatments (Table 3-2). Although the highest hatch success occurred in the 200 treatment ($52 \pm 29\%$) all cGnRH IIa hormonal treatments performed similarly across this metric ($P = 0.488$). Larval morphometric analysis indicated comparable NL among cGnrH IIa treatments at hatch ($P = 0.470$) (Table 3-2). Treatment or dose of spawning aid injected did not affect rate of broodstock mortality 24 hours post spawning ($P = 0.567$).
**Water Quality**

Mean water quality parameters observed in the broodstock flow through systems were: TAN 0.00 ± 0.00 mg/L, nitrite-nitrogen 0.00 ± 0.00 mg/L, pH 8.00 ± 0.00, hardness (CaCO$_3$) 461.70 ± 0.00 mg/L, alkalinity (CaCO$_3$) 213.75 ± 12.09 mg/L, temperature 24.35 ± 0.21 °C and DO 7.75 ± 0.35 mg/L. Mean water parameters recorded for the recirculating hatching system were: TAN 0.00 ± 0.00 mg/L, nitrite-nitrogen 0.00 ± 0.00 mg/L, pH 8.00 ± 0.00, hardness (CaCO$_3$) 111.15 ± 12.09 mg/L, alkalinity (CaCO$_3$) 76.95 ± 12.09 mg/L, temperature 25.40 ± 0.42 °C and DO 7.50 ± 0.07 mg/L.

**Discussion**

Analysis of oocyte diameter and percent GVM obtained from biopsy samples indicated all test subjects were of similar reproductive condition prior to administration of hormonal therapies for both *E. bicolor* and *E. frenatum* (*P* ≥ 0.390). The comparable condition of the brood fish helped to exclude these factors from confounding results and reduced the chance of type I error. Ovulation success varied significantly among treatments for both *E. bicolor* and *E. frenatum* (*P* < 0.05). Ovulation success ranged from 0 - 93% for *E. bicolor* in this experiment (Table 3-1). Pairwise analyses indicated, significant differences in mean ovulation success between the propylene glycol negative control and all other hormonal treatments (*P* < 0.05, Table 3-1). Conversely, similar ovulation performance was observed for *E. bicolor* among all hormonal therapies (*P* ≥ 0.532). These results suggest that all dosages of cGnRH IIa and sGnRH IIIa tested in this investigation were equally effective at inducing ovulation for *E. bicolor*. In *E. frenatum*, ovulation success among all treatments ranged from 0 -100% with a range of
17 - 100% among all treatments administered hormones (Table 3-2). sGnRH IIIa and propylene glycol injected *E. frenatum* exhibited statistically similar performance \((P = 0.918)\) with no recorded ovulation in the negative control and successful ovulation by only one fish in the sGnRH IIIa. Ovulation success was comparable among all dosages of cGnRH IIa \((P \geq 0.471)\), while 50 and 100 treatments varied when compared to ovulation success of sGnRH IIIa treated fish \((P \leq 0.021)\). Post hoc analysis also indicated ovulation success between the 200 and sGnRH IIIa injected fish to be similar \((P = 0.122)\), however this may be a result of small experimental sample size resulting in type II error. The lack of ovulation response demonstrated by *E. frenatum* following sGnRH IIIa administration was surprising as this spawning aid is commonly used for successful reproduction of this species by ornamental fish producers. A survey of sGnRH IIIa reported induced ovulation of 75% in *E. frenatum* females (Hill et al. 2009) while 100% ovulation was achieved by researchers utilizing the same dose of 0.5 µl/g (Islami et al. 2017). Differences in hormone dosage or gonad stage may explain the discrepancy in ovulation rate observed between this study and commercial production scenarios (M. DiMaggio personal communication), however this experiment followed dosing guidelines (0.5 µl/g) recommended by the manufacturer. The dosages of cGnRH IIa used in this study were chosen due to the high success of ovulation induction Quiniou et al. (2014) recorded for Channel Catfish *Ictalurus punctatus* at 100 µg/kg. This experiment bracketed the verified 100 µg/kg concentration of cGnRH IIa between an upper and lower limit of 50 µg/kg and 200 µg/kg. High ovulation success, ranging from 67-100%, was achieved in *E. frenatum* that were hormonally induced with all doses of cGnRH IIa.
The number of eggs per gram bodyweight was comparable across all hormonal treatments for both species \( (P \geq 0.594) \). Interestingly, the 50 treatment for both \textit{E. bicolor} and \textit{E. frenatum} had numerically the highest rate of ovulation (93% and 100%, respectively) and also the highest standardized mean number of spawned oocytes (466 ± 147 and 421 ± 142, respectively). While egg production data for both species across all cGnRH IIa treatments was statistically comparable, further investigations which examine lower doses are justified as the lowest dose of 50 in both species resulted in the maximum values recorded for fecundity and ovulation success. It is possible that cGnRH IIa doses lower than those used in this experiment, may yield superior results for response variables of interest. As postulated in a previous study by DiMaggio et al. 2013, doses of spawning aids (GnRH) in excess of species specific requirements may result in desensitization of the pituitary manifesting in reduced LH secretion. Luteinizing hormone is directly responsible for oocyte maturation and ovulation (Mylonas and Zohar 2007) making any impediment to its release detrimental to spawning performance.

The addition of dopamine antagonists in hormonal spawning therapies aids in blocking the neurotransmitter dopamine which has inhibitory effects on the progression of hormonal cascades within the HPG axis. Particular species are more affected by dopaminergic inhibition than others; LH release in channel catfish has been found to be relatively unaffected by dopamine (Quiniou et al. 2014) while members of the cyprinid family generally exhibit a more pronounced inhibitory effect on LH in response to dopamine (Chang et al. 1983; Podhorec and Kouril 2009; Yaron et al. 2009). In a study using both \textit{E. bicolor} and \textit{E. frenatum}, greater rates of ovulation success were achieved with the addition of a dopamine antagonist (reserpine) to the hormone LHRHa
(luteinizing hormone releasing hormone analog) (Shireman and Gildea 1989). It is likely that the ovulation rates observed across all cGnRH IIa doses in both species was positively impacted by the inclusion of domperidone in the carrier solution. While the concentration of domperidone used in this study was selected to mirror that of Ovaprim, further investigations which evaluate efficacy and optimal dosing regimes of various dopamine antagonists would be of great interest and could help to further induced spawning protocols for these two valuable cyprinid species.

Fertilization success among hormonal treatments was consistently high (> 90%) with no significant differences detected among performance in all treatments \( P \geq 0.267 \). Mean aggregated fertilization rates of 91 ± 11% were documented for *E. bicolor* and 97 ± 6% for *E. frenatum*. Spawning drugs administered to both species in this experiment resulted in adequate oocyte development. The high fertilization success and low variation in embryo sizes observed among hormone treatments \( P \geq 0.484 \) for both species in this study is indicative of a proven induction spawning protocol. Hatch success, although similar across treatments in *E. bicolor* and *E. frenatum* \( P \geq 0.299 \), was considerably lower and more variable than aggregated mean fertilization rates with pooled mean hatch rates across hormonal treatments calculated at 48 ± 32% and 36 ± 31%, respectively. The experimental design used in this study called for a subsample of embryos to be segregated for determination of hatch. This manual removal of fertilized eggs could have increased prehatch mortality as developing embryos are sensitive to handling and can be prone to mechanical injury (Small and Chatakondi 2006). Although hatching success during both experiments appeared to be somewhat reduced when compared to those delineated by Islami et al. (2017), similar results across all
treatments suggest hatching success to be independent of the spawning aids and doses examined in this study. Commercial production practices for *Epalzeorhynchos* spp. may yield hatch success superior to those reported herein as commercial protocols typically involve less handling and manipulation of developing embryos.

Female broodstock mortality associated with treatment varied significantly in *E. bicolor* \( (P = 0.048) \) but not *E. frenatum* \( (P = 0.567) \). In *E. frenatum* only two out of the total 24 fish injected with spawning aids died within 24 h of treatment. These individuals belonged to the sGnRH IIIa and 200 treatments. Tukey post hoc analysis of *E. bicolor* did not clarify disparities in rate of mortality of the logistic model \( (P \geq 0.073) \). Within the 24 hours post resolving injection 31% of *E. bicolor* treated with 200 died while the next highest rate occurred in sGnRH IIIa injected fish at 20%. Similar frequencies of mortality were observed by Hill et al. (2009) in a survey of sGnRH IIIa with mortality rates in *E. bicolor* of 35.7% and 14.8% in *E. frenatum*. Administration of spawning aids via injection can compromise the epithelium of broodfish, potentially resulting in opportunistic bacterial or fungal infections and osmoregulatory disturbance. Alternative experimental methods for administration of spawning hormones have been used for *E. frenatum* with comparable ovulation success. The hormone sGnRH IIIa was dissolved in solution with dimethyl sulfoxide (DMSO) and applied directly to the gills of *E. frenatum* with 78% ovulation success and no mortalities in contrast to the 100% ovulation and 18% morality reported following delivery of the spawning aid via intramuscular injection (IM) (Hill et al. 2005). Furthermore, Hill et al. 2009 postulated that observed mortality in the survey of Ovaprim was largely believed to be attributed to fish handling, injection procedure and diminished water quality in holding systems. Mortality in the current study was likely due
to prolonged handling or damage to the epithelium as a result of strip spawning practices. Investigations which explore additional application routes for cGnRH IIa would be prudent to diminish or eliminate mortality of valuable brood fish.

Spawning performance of cGnRH IIa was found to be comparable to sGnRH IIIa in all variables investigated in this study for *E. bicolor*. Results from the *E. frenatum* experiment suggest the 50 and 100 µg/kg cGnRH IIa treatments were superior to 10 µg/kg sGnRH IIIa with respect to ovulation success. Based on data from this study we can recommend a dose of 50 µg/kg cGnRH IIa for induction spawning of the two *Epalzeorhynchos* species evaluated. Lack of clear dose response supports the use of the minimum concentration tested. Due to satisfactory observations in relevant spawning criteria and broad range of effective concentrations, cGnRH IIa appears to be a viable option for induced spawning of *E. bicolor* and *E. frenatum*. Additional examinations of effective dosages and alternative application routes may help to improve the efficacy of cGnRH IIa as a spawning aid for the aquaculture industry. Enrolling cGnRH IIa in an investigational new animal drug (INAD) program or proceeding directly to drug indexing may be justified as this study and a number of others have demonstrated its effectiveness as an induced spawning aid.
Table 3-1. Mean ± SD ovulation success, fecundity (eggs per gram body weight), fertilization success, fertilized oocyte diameter, hatch success and notochord length for *Epalzeorhynchos bicolor* administered one of three concentrations of cGnRH IIa, sGnRH IIIa (positive control) or propylene glycol (negative control). All GnRHa treatments included the dopamine antagonist domperidone at a concentration of 5 mg/kg. Different superscript letters within a column denotes statistically significant differences \( (P \leq 0.05) \).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ovulation (%)</th>
<th>Eggs Per Gram Body Weight</th>
<th>Fertilization Success (%)</th>
<th>Embryo Diameter (mm)</th>
<th>Hatch Success (%)</th>
<th>Notochord Length (mm)</th>
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<tbody>
<tr>
<td>Propylene Glycol</td>
<td>0(^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>sGnRH IIIa</td>
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<tr>
<td>10 µg/kg</td>
<td>73(^a)</td>
<td>457 ± 187</td>
<td>96 ± 7</td>
<td>2.602 ± 0.165</td>
<td>44 ± 32</td>
<td>3.266 ± 0.155</td>
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<tr>
<td>cGnRH IIa</td>
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<tr>
<td>50 µg/kg</td>
<td>93(^a)</td>
<td>466 ± 128</td>
<td>91 ± 8</td>
<td>2.360 ± 0.146</td>
<td>62 ± 25</td>
<td>3.365 ± 0.131</td>
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<tr>
<td>100 µg/kg</td>
<td>80(^a)</td>
<td>460 ± 147</td>
<td>92 ± 14</td>
<td>2.322 ± 0.158</td>
<td>42 ± 35</td>
<td>3.343 ± 0.171</td>
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<tr>
<td>200 µg/kg</td>
<td>81(^a)</td>
<td>440 ± 95</td>
<td>90 ± 12</td>
<td>2.334 ± 0.152</td>
<td>42 ± 35</td>
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Table 3-2. Mean ± SD ovulation success, fecundity (eggs per gram body weight), fertilization success, fertilized oocyte diameter, hatch success and notochord length for *Epalzeorhynchos frenatum* administered one of three concentrations of cGnRH IIa, sGnRH IIIa (positive control) or propylene glycol (negative control). All GnRHα treatments included the dopamine antagonist domperidone at a concentration of 5 mg/kg. Different superscript letters within a column denotes statistically significant differences (*P* ≤ 0.05). An * indicates descriptive data that was not included in statistical analyses due to a limited sample size (N = 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ovulation (%)</th>
<th>Eggs Per Gram Body Weight</th>
<th>Fertilization Success (%)</th>
<th>Embryo Diameter (mm)</th>
<th>Hatch Success (%)</th>
<th>Notochord Length (mm)</th>
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<tr>
<td>Propylene Glycol</td>
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<tr>
<td>sGnRH IIIa 10 µg/kg</td>
<td>17&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>241*</td>
<td>100*</td>
<td>3.914*</td>
<td>68*</td>
<td>3.375*</td>
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<td>421 ± 142</td>
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<td>2.806 ± 0.695</td>
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<td>83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>390 ± 90</td>
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<td>26 ± 15</td>
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<td>67&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>346 ± 77</td>
<td>98 ± 3</td>
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<td>52 ± 29</td>
<td>3.422 ± 0.171</td>
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<td>100 µg/kg</td>
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<td>346 ± 77</td>
<td>98 ± 3</td>
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<td>His-</td>
<td>Trp-</td>
<td>Ser-</td>
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<td>Ser-</td>
<td>Tyr-</td>
<td>DArg-*</td>
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<tr>
<td>Chicken Analog (cGnRH IIa)</td>
<td>pGlu-</td>
<td>His-</td>
<td>Trp-</td>
<td>Ser-</td>
<td>His-</td>
<td>DArg-*</td>
</tr>
</tbody>
</table>

Figure 3-1. Amino acid sequence of mammalian GnRH and the two GnRH analogs used in this experiment. The symbol * signifies alterations in amino acid sequence from their native forms.
CHAPTER 4
EVALUATION OF THREE EMBRYO DISINFECTANTS FOR OPTIMIZATION OF HATCHING IN FOUR FRESHWATER ORNAMENTAL FISH SPECIES

Introduction

The aquatic environment is home to numerous pathogens that can negatively impact the production capability of aquaculture operations. Arguably one of the most vulnerable periods of an aquatic organism’s life is the embryonic stage, inclusive of incubation and hatching. At this stage the organism is sessile and susceptible to fungal and bacterial infections due to its immature immune system and innate defenses (Liu et al. 2014). During the embryonic stage, the surface of the egg is the only way the organism may exchange gases and metabolites needed for proper development. Fungi or bacteria, which can rapidly proliferate in the culture environment, may attach to the surface of developing embryos decreasing permeability and potentially smothering the growing larvae. Dead embryos can quickly diminish water quality parameters for fellow conspecifics incubating nearby. Fouled water quality from decaying eggs can create an antagonistic environment which can manifest in further mortalities of incubating embryos or newly hatched larvae. In an attempt to mitigate mortality and reduce the abundance of potentially pathogenic microbes associated with embryos and the incubation environment, commercial aquaculture hatcheries commonly employ a variety of strategies to increase survival and hatching of embryos which can range from the use of chemical disinfectants to implementation of targeted biosecurity practices.

Bacteria occur ubiquitously in the aquatic environment and can be pathogenic or mutualistic in the development of a fish (Bergh 2000). The majority of pathogenic bacteria which may negatively affect teleosts can be broadly categorized in four groups: ulcer forming or systemic gram-negative bacteria, external gram-negative bacteria,
systemic gram-positive bacteria and slow-growing acid fast bacteria (Roberts et al. 2009). The gram negative bacteria in the genus *Flexibacter* and *Flavobacterium* are of high concern for adversely impacting fish production and have been reviewed extensively (Hansen et al. 1992; Pavlov and Moksness 1993; Bergh 2000; Sudova et al. 2007; Wagner et al. 2008; De Swaef et al. 2016). Virtually any surface can be colonized by bacteria in an aquatic environment including the mucosal lining of fish eggs and skin (Oppenheimer 1955; Hansen and Olafsen 1989). The chorion contain glycoproteins which aid in the substrate adhesion of microorganisms (Hansen and Olafsen 1989). Most disease causing bacteria are secondary opportunistic pathogens that take advantage of a compromised immune system as a result of a stressed host, however some may be obligate or primary pathogens (Roberts et al. 2009). Poor environmental conditions are the most common stressor and can suppress a fish’s immune system or create a habitat in which pathogens may flourish (Roberts et al. 2009). Bacteria in the genus *Flexibacter* may also be able to penetrate the egg chorion and infect the forming embryo inside (Bergh 2000). Egg ulceration caused by *Flexibacter sp.* bacteria has been demonstrated to result in premature hatching in common Wolfish, *Anarhichas lupus* (Pavlov and Moksness 1993). Asphyxiation of the developing embryo from bacterial overgrowth is a more typical concern in a hatchery setting (Hansen et al. 1992; De Swaef et al. 2016).

Fungi and fungal spores are common in the hatchery environment and can be present in both fresh or saltwater and inhabit a wide range of temperatures (Monticini 2010). Fungi heavily impact aquaculture with the most influential species occurring in the family *Saprolegniaceae* (Noga 1993). *Saprolegnia* fungi exhibit complex lifecycles
reproducing both sexually or asexually and can also generate flagellated or hooked zoospores (Noga 1993; Liu et al. 2014). Unable to photosynthesize, fungi most often feed saprophytically consuming organics and dead substrates (Monticini 2010). Fungal infections in fish are usually secondary and superficial which cause mortalities through disruption in osmoregulation by damaging epithelium (Roberts et al. 2009). On fish eggs it is believed that mortality is caused from breaching the chorionic membrane from hyphal growth thus causing osmoregulatory difficulties for the embryo (Liu et al. 2014). Colonization of fungi usually begins in non-viable or unfertilized eggs and spreads to the developing healthy embryos (Monticini 2010). In trout hatcheries, saprolegniasis of eggs has been reported to impair production by 20-40% (Forneris et al. 2003).

There is a wide assortment of disinfectant chemicals and associated protocols used to treat teleost eggs. The level of suitability varies with the pathogen the producer is attempting to prevent or eliminate as well as the tolerance of the embryo to therapeutic levels of the disinfectant, which in many cases can be species specific. A myriad of chemotherapeutants have been used for disinfection of fish embryos including, hydrogen peroxide, glutaraldehyde, ozone, iodophors, formaldehyde, peracetic acid, bronopol, tannic acid, sodium chloride, copper sulfate (De Swaef et al. 2016), malachite green (Sudova et al. 2007) and methylene blue (Chambel et al. 2014). Efficacy of the chosen chemical can vary greatly and may be dependent on species, contact time, stage of embryo development, water parameters, as well as application methods and concentration of the disinfectant. Many commercial aquaculture production protocols prophylactically treat the surface of eggs with disinfectants through immersion baths of the chemical in solution or by treating the entire hatching system
during incubation (Wagner et al. 2008). Application methods for these chemicals may differ contingent on the environment and production unit being treated as disinfectants may indiscriminately destroy beneficial bacteria, plants or invertebrates (Forneris et al. 2003; Monticini 2010). It is also essential to check the legalities of the chemical therapeutants one desires to use as drug approval status may vary among regulatory bodies dependent on the final use of the aquatic product.

Among the more common disinfectant chemicals used in prophylactic treatment of fish embryos are hydrogen peroxide, formalin and iodine. This is partially due to their over the counter availability, effectiveness in treating a range of pathogens, and legally approved or low regulatory status. The mechanisms in which chemotherapeutants eliminate organic pathogens vary. Hydrogen peroxide oxidizes components essential to the cell such as cell membrane lipids, proteins and DNA through the use of hydroxyl free radicals (McDonnell and Russell 1999). Formaldehyde, the active ingredient in formalin, disables pathogens through alkylation of sulfhydryl and amino groups as well as nitrogen atoms on purines (Rutala and Weber 2008). Iodine destroys microbes through halogenation or oxidation by binding to the cell wall of the microorganism, in this process iodine turns into iodide (Katharios et al. 2007). Hydrogen peroxide and formalin are approved by the U.S. Food and Drug Administration (FDA) to be used as fungicides on fish eggs, however drugs containing iodine are currently listed for use as low regulatory priority (Bowker et al. 2016). A number of drugs may be effective in treating a range of pathogens while only being approved to target one (De Swaef et al. 2016). For extra-label use of disinfectants, consultation with a licensed veterinarian is
required as they have the authority to advise and prescribe alternative applications of
drugs (Nickum et al. 2004).

The objective of this study was to identify if prophylactic treatment of embryos
using formalin, hydrogen peroxide and iodine will improve hatching success of the
Upside-Down Catfish *Synodontis nigriventris*, Featherfin Squeaker *Synodontis
eupterus*, Redtail Shankminnow *Epalzeorhynchos bicolor* and Rainbow Shankminnow
*Epalzeorhynchos frenatum* when administered as an immersion bath. As there is a
dearth of published data on efficacy of embryo disinfection methods for ornamental
species, results from this experiment will aid in identification or effective hatching
protocols for four economically significant ornamental fish species

**Methods**

**Experimental Design**

For each species (*S. nigriventris, S. eupterus, E. bicolor E. frenatum*) a separate
experiment was conducted to evaluate the effect of three embryo disinfectant protocols
on hatching success. Eggs used in this study were obtained from hormone induced
spawning trials using gonadotropin releasing hormone analogs (GnRHa’s). *E. frenatum*
and *E. bicolor* were spawned using similar methods. Briefly, both *Epalzeorhynchos*
species received a 10% priming dose and a 90% resolving dose of the GnRHa drug
preparation staggered 6 hours apart at 0.5 µL/g fish’s bodyweight. The *S. eupterus* and
*S. nigriventris* were also spawned using analogous protocols. Both *Synodontis* catfish
received a single dose of the GnRHa drug preparation at 0.5 µL/g fish’s bodyweight.
Spawning aids in all species were administered as an intramuscular injection at the
base of the dorsal fin. Ovulation occurred 3-10 hours post resolving injection for the
*Epalzeorhynchos* species while ovulation was observed at 16-24 hours post injection for both *Synodontis* species. At time of ovulation, females were stripped of oocytes and fertilized with milt from a single male. Each spawn was then placed into a 1 L floating screen bottom (400 µm) container and incubated within a recirculating system until subsamples were to be collected. Embryo samples used for subsequent experiments resulted from spawns collected from at least two individual females and were homogenized approximately 1.5 hours post fertilization and pooled prior to use.

Three experimental disinfectant solutions and one control group containing system water were evaluated for each species. Disinfectant solutions and concentrations evaluated were 1,500 mg/L formalin (formalin [37 % formaldehyde], Thermo Fisher Scientific, Waltham, MA), 100 mg/L iodine (10 % povidone-iodine buffered solution [1 % available iodine] Thermo Fisher Scientific, Waltham, MA), and 1,000 mg/L hydrogen peroxide (3% hydrogen peroxide, Equate, Bentonville AR). Concentrations of each disinfectant were chosen based on FDA approved aquaculture drug guidelines provided by the American Fisheries Society Fish Culture Section (Bowker et al. 2016) as well as commercial recommendations made by the approved product supplier. Duration of embryo contact time for the disinfectant was standardized among treatments (15 minutes) according to FDA guidelines for dosing of the approved drugs (hydrogen peroxide and formalin) for finfish eggs and previous studies with Lake Trout *Salvelinus namaycush* (Rach et al. 2005) and Rainbow Trout *Oncorhynchus mykiss* (Amend 1974; Schreier et al. 1996). Solutions were prepared by mixing a predetermined quantity of disinfectant into respective containers containing 1 L of hatching water to achieve the treatment concentrations previously stated. New
disinfectant solutions were made the day of each trial for each of the four species examined. Each treatment contained 10 replicates, with each replicate comprised of 50 embryos that were stocked into 150 mL (40 µm) screen bottom cups. Embryos were transferred from a homogenized pool to replicate cups using 1 mL transfer pipettes. Screen bottomed containers which included embryos, were nested within an additional 150 mL sample container filled with 80 mL of the respective disinfectant solutions or hatching water (control) and allowed to remain static for 15 minutes. Post disinfectant immersion, screened sample containers were transferred to a recirculating system where the containers were incubated until hatching. The recirculating system was 1,120 L and comprised a gravity fed header tank, 175 L sump, bubble wash bead filter and styrofoam float to suspend the cups. Hatch success was evaluated within 24 hours post fertilization for the *Epalzeorhynchos* species and 36 hours for *Synodontis* species due to natural differences in incubation time. Prior to enumeration, sample cups were placed into a 300 mg/L tricaine methanesulfonate solution (MS-222, Western Chemical Inc., Ferndale, WA) to euthanize larvae. Larvae were then enumerated grossly using transmitted light for increased contrast. Larvae that appeared to be successfully hatched but deceased prior to euthanasia were considered as hatched.

**Water Quality**

Water quality parameters for broodstock holding and hatching systems were tested prior to and during spawning trials to minimize environmental differences in system conditions on embryos. Dissolved oxygen (DO) and temperature were measured using a YSI ProComm II meter (YSI Inc., Yellow Springs, OH). Total ammonia nitrogen (TAN), nitrite-nitrogen, pH, hardness (CaCO₃) and alkalinity (CaCO₃)
were measured using standard colorimetric assays according to the manufacturers protocols (Hach Company, Loveland, CO).

Data Analysis

A single experimental egg disinfection trial was completed for every species. Each trial had a total of 10 replicates per experimental treatment (N=4) with the exception of *E. bicolor* having 9 replicates for the hydrogen peroxide, formalin and control treatments due to damaged specimen cups. Hatch success for each replicate was calculated by dividing total larvae present in treatment cup by the amount of stocked eggs per cup (50). A Shapiro-Wilk test was used to assess normality while a Levene’s test was conducted to evaluate equality of variances among treatments. Distributions of hatch success data for all species violated assumptions of normality and homoscedasticity. Non-normal hatch success data was analyzed using a one-way Kruskal-Wallis statistical test as well as the Wilcoxon method with a Holm correction for post hoc pairwise comparisons among treatments. Coefficient of variation (CV) values (CV = [Standard deviation / mean] x 100) were generated for each treatment to examine degree of variance. A *P*-value of ≤ 0.05 was considered statistically significant.

Statistical analyses were performed using JMP Pro v13.0.0 (SAS Institute, Cary, NC). Percent values are rounded to the nearest whole number and are represented as means ± standard deviation.

Results

Hatch Success

In each species hatch success was significantly affected by egg disinfectant (*P* < 0.001). In *E. frenatum* the highest mean hatch success was observed in the control (24
± 9%) and the hydrogen peroxide treated embryos (22 ± 9%) and did not differ significantly ($P = 0.382$, Table 4-1). Formalin treated embryos yielded a hatch success (10 ± 9%) approximately half that of the control and hydrogen peroxide treatments while the iodine treatment resulted in 100% mortality (Table 4-1). For *E. bicolor* both formalin and hydrogen peroxide treated embryos exhibited hatching success comparable to that of the control treatment ($P = 0.742$ and 0.252, respectively) although formalin performed significantly better than hydrogen peroxide ($P < 0.001$, Table 4-1). Iodine exposure once again resulted in complete mortality for replicates evaluated with this species.

Hydrogen peroxide and control treatment groups produced similar hatching results (40 ± 9% and 36 ± 6%) for *S. nigriventris* embryos ($P = 0.566$); with hydrogen peroxide performing significantly better than both formalin and iodine exposure protocols (Table 4-1). *E. frenatum* had the highest hatch rates amid the formalin (36 ± 14%) and control treatments (40 ± 12%) ($P = 0.569$, Table 4-1). The hydrogen peroxide (26 ± 8%) and iodine (1 ± 1%) treatments resulted in embryo hatching success significantly less than what was recorded for the control group during this experiment (Table 4-2). Levene’s test for each species indicated variance was unequal among mean hatch success values obtained from each treatment ($P \leq 0.015$). No apparent trends in the coefficients of variation were observed among disinfectants, however calculated values elucidated treatments within species that performed with greater consistency (Table 4-2).

**Water Quality**

Mean (± SD) water quality parameters recorded for the flow through spawning systems for all experiments were: TAN 0.0 ± 0.0 mg/L, nitrite-nitrogen 0.0 ± 0.0 mg/L,
pH 8.0 ± 0.0, hardness (CaCO\(_3\)) 440.33 ± 32.37 mg/L, alkalinity (CaCO\(_3\)) 192.38 ± 8.55 mg/L, temperature 25.23 ± 0.51 °C and DO 7.90 ± 0.62 mg/L. Water quality parameters recorded for the recirculating hatching system for each experiment are depicted in Table 4-3.

**Discussion**

When prophylactically treating embryos, dosage of disinfectant, application duration and species can greatly affect the hatch success. Among the four species in this investigation, the variability in hatch success of each chemical tested underscores the need to identify the most appropriate drug and application protocol for successful disinfection of embryos. Our studies indicate that 1,000 mg/L hydrogen peroxide applied to embryos in a static immersion for 15 minutes does not detrimentally effect hatch success when compared to the control treatment (\(P \geq 0.252\)) in *E. frenatum*, *E. bicolor* and *S. nigriventris*. Results also show that while concentrations of 1,500 mg/L formalin did not confer any increase in hatching success, this disinfectant may be applied for the designated 15 minute duration to prophylactically treat embryos without adverse effects on hatch rate (\(P \geq 0.053\)) in *E. bicolor*, *S. eupterus* and *S. nigriventris*.

Given the broad diversity among extant fishes it is reasonable to expect the effects of chemotherapeutants on developing embryos to be species specific. Although iodine produced poor hatch success for the four ornamental species tested in this trial, previous literature has reported increased hatching success at higher concentrations and/or longer application times using this chemical (Amend 1974; Khodabandeh and Abtahi 2006; Chambel et al. 2014). In a study completed on Common Carp *Cyprinus carpio*, eggs infected with *Saprolegnia* fungus were treated with 200 mg/L iodine twice.
daily on a flush system and hatch rate was reported to have increased by 27% over the control group following this intervention (Khodabandeh and Abtahi 2006). Furthermore, concentrations of 100-500 mg/L iodine administered as a 10 minute bath were demonstrated to be the most effective treatment in reducing the number of colony forming units (CFU) on Rainbow Trout *Oncorhynchus mykiss* embryos when compared with formalin, hydrogen peroxide and salt (Wagner et al. 2008). Conversely, in Black Sea Turbot *Psetta maxima* embryos treated with concentrations of 100-3,000 mg/L iodine for 10 minutes, all had lower hatching success and higher rates of deformity than untreated embryos (Aydin 2011). A study using the ornamental species *Gymnocorymbus ternetzi, Danio rerio* and *Pterophyllum scalare* treated embryos with 1, 5, and 15 mg/L iodine for a duration of 24 hours and observed greater or equal performance in hatch success compared to the control group (Chambel et al. 2014). These results suggest an increased exposure time with a substantially reduced concentration may be a more viable approach for embryo disinfection using iodophors; especially in sensitive ornamental species. Moreover, in the same study marginally higher concentrations of iodine (25 mg/L) resulted in 0% survival exhibiting a low safety margin for the drug (Chambel et al. 2014). Although our experimental concentration of 100 mg/L iodine and contact time of 15 minutes mirrored protocols used in state hatcheries for salmonid eggs (Wagner et al. 2008) and successful toxicity studies done on Rainbow Trout embryos (Amend 1974), the observed embryo mortality in all our tested species supports the contention that the development of overarching guidelines for embryo disinfection may be inappropriate and emphasizes the need to formulate species specific protocols (Chambel et al. 2014; De Swaef et al. 2016).
Embryos in this trial were incubated in a recirculating aquaculture system of low stocking density, with low organics and high filtration rates. In general, these environmental conditions are not conducive to rapid growth of bacteria and fungi as environmental requirements may be limiting. In contrast, in a production setting, producers often stock systems with higher quantities of embryos and larvae and may not monitor or adjust water quality parameters as often as in a laboratory experimental setting. Higher organic loads which result from higher stocking densities can also influence the activity and thus the toxicity of administered disinfectants. In a study focused on salmonid egg disinfection, a volume ratio of 1:1, egg : 100 mg/L iodine solution was used, at the end of one hour the amount of iodine left in solution was not able to be measured (Chapman and Rogers 1992). A more pronounced decrease in iodine concentration was also observed when eggs that were used for a study were not rinsed and contained both milt and ovarian fluid (Chapman and Rogers 1992). Results from this study highlight an important consideration when administering chemicals to systems with substantial organic loads. The ratios of embryos to iodine solution used in our experiment were possibly much higher than even the highest treatment (1:4) tested by Chapman and Rogers (1992). The greater quantity of active iodine in relation to egg volume in the current experiment could have had deleterious effects on embryos, translating to poor hatch rate regardless of duration of treatment (Table 4-1).

Further considerations when selecting a disinfectant are safety to humans, water parameters, and developmental stage of the embryo when treating with chemotherapeutants. Although iodine appeared toxic to our study species embryos at the given concentration and duration of treatment (Table 4-1), handling of the chemical
by the applicator is comparatively safe. Iodine has been reported to be used as a germicide in human drinking water at concentrations as high as 20 mg/L with no ill effects (Punyani et al. 2006); moreover, iodine in the environment readily sublimes (NIOSH 2015). Safety while using hydrogen peroxide varies with concentration. When using 35% hydrogen peroxide interactions with the user may be caustic and proper skin and eye protection should be used (Rach et al. 2005) while 3% hydrogen peroxide can be used as an oral mouth wash, the concentration of 1,000 mg/L hydrogen peroxide used in this study could be considered quite safe. Peroxide is often given preference based on environmental safety, the byproducts of hydrogen peroxide reacting are water and oxygen (Yanong 2011). Formaldehyde (formalin), although an FDA approved treatment for finfish eggs is also carcinogenic (Roberts et al. 2009). While working with formaldehyde, the Occupational Safety and Health Administration (OSHA) set permissible exposure limits of airborne formalin at 8 hours of 0.75 ppm and 15 minutes for concentrations of 2 ppm (OSHA 2011). Storage of formalin should be cool however if temperatures drop to below 40º F, paraformaldehyde is formed; furthermore, contact with the white precipitate is extremely toxic and can kill fish (Francis-Floyd 1996).

Water quality parameters may further affect the efficacy or toxicity of the chemotherapeutic agent in the aquatic environment. Iodine becomes more toxic to eggs below pH of 6 and becomes a less effective disinfectant in environments with a pH of 8 (Torgersen and Håstein 1995). Hatching in all experimental trials for this study occurred in pH ranges of 7.5-8.0 (Table 4-3). Hydrogen peroxide may become more toxic at higher temperature and will decrease the pH of the water being treated (De Swaef et al. 2016). This investigation occurred in a temperature-controlled laboratory and water
used for the experiments contained sufficient amounts of CaCO₃ to account for fluctuations in pH (Table 4-3). When applied to water, formalin removes dissolved oxygen and also increases in toxicity with elevated temperatures (Francis-Floyd 1996). No supplemental aeration was used in the treatment baths but concentrations were administered at FDA recommended levels and did not appear to decrease hatch rate among all species tested as formalin provided comparable hatch rates (P ≥ 0.531) to the control treatment (Table 4-1).

Toxicity of the disinfectant on fish embryos can change depending on developmental stage. The rate at which fish embryos develop is highly variable. In all species used in this study hatch occurred 36 hours post fertilization while salmonids can take up to 7 weeks to hatch (Hinshaw 1990). A large body of literature exists which focuses on egg disinfection of salmonids as a result of them being in the vulnerable egg state for such an extended duration. A study examining the effect of iodine disinfection of *Epinephelus coioides* determined that grouper embryos handled the iodine treatment better at the eyed stage compared to the cleavage stage (Tendencia 2001). A 30 percent increase in hatch rate was observed when non-eyed Rainbow Trout embryos were treated with 566 mg/L of hydrogen peroxide for 15 minutes every other day (FDA 2007). Non-eyed Common Carp infected embryos were treated until they became eyed with sodium chloride, formalin and iodine, all of which increased hatch rate when compared to the control treatment (Khodabandeh and Abtahi 2006). Embryos selected for this experiment were in late cleavage or early morula stage of development. While the ornamental species evaluated here all produced altricial larvae, it may be possible
to achieve alternative hatch success results with delaying disinfection of embryos until later stages of maturity.

The uses of chemical disinfectants in aquaculture are widespread and have a range of applications. In a production setting the majority of chemotherapeutant use is prophylactic and aimed towards disease management and mortality mitigation; however, disinfectants may also be used to reduce the horizontal transmission of pathogens between conspecifics and facilities by sanitizing egg surfaces (Torgersen and Håstein 1995). Disinfection procedures are also used to create axenic or gnotobiotic larvae for model organisms in research facilities (De Swaef et al. 2016). To best prescribe a disinfectant and concentration it is essential to know desired results of drug application as axenic levels of disinfection may compromise hatch success; moreover, there is evidence that commensal bacteria of the eggs surface may help to mitigate pathogens (Liu et al. 2014).

Based on the results obtained from this experiment, it is recommended to use a dose of 1,500 mg/L formalin administered as an immersion bath for 15 minutes to prophylactically treat *E. bicolor* eggs. Using this method, the highest hatch success of 78 ± 5 % was achieved in this species and the lowest variability in performance was also observed. Although the hatch success between the control and formalin treated fish was similar (*P* = 0.742) the coefficients of variation differed greatly with a CV of 46.8 in the control group and a CV of 6.9 in the formalin treated embryos (Table 4-2). Drugs which have lower CV values may be chosen by the producer to yield a more consistent response of prophylactic treatment. Although 1,000 mg/L hydrogen peroxide exposure did not outperform the control treatment in *E. frenatum* and *S. nigriventris*, use of this
drug may be beneficial in aquatic environments with reduced water quality or known pathogens when incubating embryos of these species. The 1,500 mg/L formalin exposure treatment is suggested to treat *S. eupterus* embryos as it did not detrimentally effect hatching and may be advantageous in a commercial production setting. The dose of 100 mg/L iodine at 15 minute immersion is not recommended to treat embryos of the four ornamental species used in this study. Further species-specific investigations which focus on disinfectant concentration and contact time are urged to develop empirically based recommendations that are both safe for the developing embryo and may prophylactically address pathogenic microbes which can adversely impact commercial hatching protocols.
Table 4-1. Mean hatch success percentages ± standard deviations for embryos from four species of ornamental fish exposed to four disinfectant chemicals. Disinfectant concentrations and exposure times were as follows: 1,500 mg/L formalin [15 min], 100 mg/L iodine [15 min], 1,000 mg/L hydrogen peroxide [15 min], and hatching water [15 min]. Different letters within columns denote statistically significant differences among treatment groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E. frenatum</th>
<th>E. bicolor</th>
<th>S. eupterus</th>
<th>S. nigriventris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62 ± 29&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>40 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iodine</td>
<td>0 ± 0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>22 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formalin</td>
<td>10 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36 ± 14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>30 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 4-2. Levene’s test P values and coefficient of variation values among treatments for each species. The – symbol signifies 0 hatch success.

<table>
<thead>
<tr>
<th>E. frenatum</th>
<th>E. bicolor</th>
<th>S. eupterus</th>
<th>S. nigriventris</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0.010</td>
<td>&lt;0.001</td>
<td>0.015</td>
</tr>
<tr>
<td>Control</td>
<td>36.2</td>
<td>46.8</td>
<td>30.4</td>
</tr>
<tr>
<td>Iodine</td>
<td>–</td>
<td>–</td>
<td>161.0</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>39.7</td>
<td>35.9</td>
<td>32.1</td>
</tr>
<tr>
<td>Formalin</td>
<td>88.4</td>
<td>6.9</td>
<td>40.3</td>
</tr>
</tbody>
</table>

Table 4-3. Water parameters recorded for recirculating hatching system the day of each experimental run. TAN (total ammonia nitrogen), nitrite-nitrogen, hardness (CaCO<sub>3</sub>), alkalinity (CaCO<sub>3</sub>) and DO (dissolved oxygen) are measured as mg/L while temp (temperature) data was recorded in Celsius (°C).

<table>
<thead>
<tr>
<th>Species</th>
<th>TAN</th>
<th>Nitrite-Nitrogen</th>
<th>pH</th>
<th>Hardness</th>
<th>Alkalinity</th>
<th>Temp</th>
<th>DO</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. nigriventris</td>
<td>0.0</td>
<td>0.0</td>
<td>8.0</td>
<td>136.8</td>
<td>68.4</td>
<td>27.0</td>
<td>7.5</td>
</tr>
<tr>
<td>S. eupterus</td>
<td>0.0</td>
<td>0.0</td>
<td>8.0</td>
<td>136.8</td>
<td>85.5</td>
<td>25.0</td>
<td>8.8</td>
</tr>
<tr>
<td>E. frenatum</td>
<td>0.0</td>
<td>0.0</td>
<td>8.0</td>
<td>102.6</td>
<td>68.4</td>
<td>25.7</td>
<td>7.6</td>
</tr>
<tr>
<td>E. bicolor</td>
<td>0.0</td>
<td>0.0</td>
<td>7.5</td>
<td>119.7</td>
<td>68.4</td>
<td>25.4</td>
<td>8.3</td>
</tr>
</tbody>
</table>
CHAPTER 5
CONCLUSION

Among the numerous biological challenges faced in aquaculture, consistent and reliable production of high quality eggs and larvae may be the most daunting. Many species of fish in a captive environment exhibit forms of reproductive dysfunction that can vary in degree with the most common being failure of final oocyte maturation (FOM). Manipulating environmental variables may successfully initiate hormonal cascades essential to gametogenesis and oocyte maturation. In cases in which environmental manipulation is unrealistic or ineffective, use of exogenous hormones can be an effective way of obtaining gametes fundamentally needed for culture of the desired species. Embryo incubation represents an additional bottleneck that can hinder the production of viable larvae due to numerous pathogens that may interrupt this process. Prophylactically treating embryos with chemical disinfectants is a common practice used by the ornamental aquaculture industry to mitigate disease transmission and increase hatching success. Investigations into induced spawning and embryo disinfection are valuable as results can help to increase the efficiency of the ornamental aquaculture industry by increasing production yields to meet market demands.

Four ornamental species (Redtail Sharkminnow *Epalzeorhynchos bicolor*, Rainbow Sharkminnow *Epalzeorhynchos frenatum*, Upside-Down Catfish *Synodontis nigriventris* and Featherfin Squeaker *Synodontis eupterus*) were selected to assess the efficacy of cGnRH IIa (D-Arg6-Pro9-Net) as an induced spawning aid compared to Ovaprim® (sGnRHa [D-Arg6-Pro9-Net]), the industry standard. Concentrations of 50, 100, and 200 µg/kg cGnRH IIa + 5 mg/kg domperidone were tested alongside of the standard Ovaprim dose (10 µg/kg sGnRH IIIa + 5 mg/kg domperidone). Performance of
spawning aids was evaluated based on metrics such as ovulation success, fecundity, fertilization success, hatch success, egg morphology, larval morphology and mortality of broodstock.

Ovulation success was found to be similar among cGnRH IIa treatments and Ovaprim for all species tested with the exception of *E. frenatum*. For this species only 17% of the Ovaprim treated individuals spawned thus excluding this spawning treatment from further inclusion in analyses. Fecundity measured in eggs per gram body weight was also found to be comparable across all spawning aids for all species tested. The mean eggs spawned per gram female’s body weight ± SD were 456 ± 136 for *E. bicolor*, 391 ± 108 for *E. frenatum*, 200 ± 106 for *S. nigriventris* and 149 ± 54 for *S. eupterus*. Fertilization success was not significantly affected by spawning aid treatment in all species tested with highest rates occurring in *E. frenatum* at 97 ± 6% and lowest occurring in *S. nigriventris* at 72 ± 20%. Observed hatching success in each species was comparable among spawning aids and was nearly half of the calculated mean fertilization values for each hormonal treatment among the four species. Reduced hatch success may have been a result of mechanical trauma to the developing embryos from additional handling. Producers may expect higher reported values than those in from this investigation when using typical spawning protocols. Furthermore, no differences were observed in both egg and larval morphology among cGnRH IIa and Ovaprim induced fish across the four species evaluated. Broodstock mortality following hormonal treatments was not observed in the synodontid species and negligible among treatments in *E. frenatum*. For *E. bicolor* mortality was affected by the experimental drug injected with the greatest losses occurring in the 200 µg/kg cGnRH IIa treatment.
Fertilized embryos from the hormone induced spawning trials were used to conclude a series of egg disinfection experiments for each of the four species. Efficacy of iodine, formalin and hydrogen peroxide treated eggs was assessed based on observed hatching success. Concentrations of 100 mg/L iodine, 1,000 mg/L hydrogen peroxide and 1,500 mg/L formalin were administered via 15 minute static bath to prophylactically treat embryos. Iodine was found to be the least viable choice at the concentration and duration applied as nearly all species tested exhibited 0% hatch. Experimental results from *E. bicolor*, *E. frenatum* and *S. nigriventris*, suggest that 1,000 mg/L hydrogen peroxide does not adversely affect hatch success. In *E. bicolor*, *S. eupterus* and *S. nigriventris*, results illustrate that concentrations of 1,500 mg/L formalin may be prophylactically administered to embryos without negative effects on hatch rate. Results from this study suggest a dose of 1,500 mg/L formalin administered as an immersion bath for 15 minutes to be preferred for prophylactic treatment of *E. bicolor* embryos. Using this concentration and application time, the highest hatch success (78 ± 5 %) and lowest variability in performance was achieved in this species. Furthermore, the disinfectant treatment of 1,000 mg/L hydrogen peroxide solution also administered as a 15 minute bath, may be beneficial in aquatic environments with reduced water quality or known pathogens when incubating *E. frenatum* and *S. nigriventris*. The 15 minute 1,500 mg/L formalin exposure is recommended to treat *S. eupterus* embryos as it did not detrimentally effect hatching and may be advantageous in a commercial production setting. The 15 minute, 100 mg/L iodine exposure was found to be inappropriate for use with embryos of the four ornamental species used in this study.
Further investigation is needed to recommend an optimal species specific dose of cGnRH IIa, however results currently indicate equivalent spawning performance to the industry standard, substantiating the viability of this new spawning aid as a potential alternative. Data analyzed from embryo disinfection studies suggested possible improvement of hatch rates of ornamental species with prophylactic formalin or hydrogen peroxide treatments at concentrations and durations explored in these investigations, however disinfectant efficacy may be governed by species specific effects. Additional research into the use of cGnRH IIa for induction spawning may expand the current number of species able to be captively bred. Taken together, results from these investigations have contributed to the improvement of induced spawning and embryo disinfection practices for *E. bicolor, E. frenatum, S. nigriventris* and *S. eupterus*. This information may be applied in a broader context with potential benefits to the culture of other species of cyprinids and synodontids as well as to the ornamental industry as a whole.
REFERENCES


Francis-Floyd, R. 1996. Use of formalin to control fish parasites. College of Veterinary Medicine, Institute of Food and Agriculture Sciences 77:7–9.


BIOGRAPHICAL SKETCH

Michael J. Sipos was born in Passaic, NJ and moved to Fort Myers, FL when he was 8 years old. From an early age he developed a love for animals, specifically herps and fish. Throughout his life Michael was an avid hobbyist in aquaria, keeping several aquariums and dabbling in aquaculture. He attended the University of Florida and graduated in 2013 with a B.S. in zoology and minors in both anthropology and fisheries & aquatic sciences. Soon after, Michael became a fisheries biologist for the University of Florida researching barotrauma and release mortality of red and gag grouper as well as studying the effects of the Deepwater Horizon oil spill on gamefish species. Michael left his position at the university to further his education and obtained a Master of Science degree in fisheries and aquatic sciences in the summer of 2018.