1 **Title:** Evaluation of cGnRH IIa for Induction Spawning of Two Ornamental *Synodontis* Species

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Abstract

Efficacy, reliability, and safety are of principal concern for spawning aids used in reproduction protocols for aquaculture species. Ovaprim®, a salmon gonadotropin releasing hormone analog (sGnRH IIIa, D-Arg6-Pro9-Net, 20 µg/mL) and a dopamine antagonist (Domperidone, 10 mg/mL), is currently the preferred choice for induction spawning of ornamental fishes. However, this preparation may be unreliable or completely ineffective in some cases. cGnRH IIa (D-Arg6, Pro9-NHet) has garnered recent interest as an alternative GnRH subtype which offers increased biological activity, reliability, and may ultimately help to increase on farm productivity and expand the diversity of species able to be cultured. The objective of this study was to evaluate the efficacy of cGnRH IIa and Ovaprim® on various quantitative and qualitative measures of spawning performance in *Synodontis nigriventris* and *Synodontis eupterus*.

Four hormone doses were evaluated (50, 100, 200 µg/kg cGnRH IIa; 10 µg/kg sGnRH IIIa), each of which also contained an equal concentration of a dopamine antagonist (5 mg/kg domperidone). Fish were palpated to check for ovulation at 16, 20, and 24 hours post injection. Upon successful ovulation, eggs were manually stripped, weighed, and fertilized. A subsample was photographed under a dissecting microscope for subsequent determination of fertilization success and egg diameter. Hatching success for individual spawns was calculated from subsamples of fertilized eggs stocked into hatching containers.

Results indicated similar ovulation success among the four hormone treatments at the 16, 20, and 24 hour time periods. Total fecundity, fertilization, embryo diameter, hatching success, larval notochord length, and larval mortality did not vary significantly among treatments. These

results suggest that cGnRH IIa exhibits comparable spawning performance to the industry standard Ovaprim®, for induced spawning of *S. nigriventris* and *S. eupterus.*

Keywords: Induced spawning, *Synodontis*, gonadotropin releasing hormone, ornamental aquaculture

1. Introduction

Numerous fish species exhibit some 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 are 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 have proven to 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, pathogen introduction from less purified preparations is also 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, are more reliable options due to their known hormone concentration and decreased biosecurity concerns. 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

65 sequence, resulting in higher receptor binding affinity and resistance to enzymatic degradation
66 (Lovejoy et al. 1995; Mikolajczyk et al. 2003; Szabó et al. 2007; Podhorec and Kouril 2009).
67 More than 16 variants of the GnRH decapeptide have been identified in vertebrates with
68 two to three forms present in most vertebrate species (Millar 2003). Gonadotropin releasing
69 hormone system types I, II, and III have been previously identified in teleosts (Kah et al. 2007).
70 The hormone cGnRH II occurs ubiquitously among all teleosts. Conversely, sGnRH III is found
71 less universally but is specific to teleosts (Kah et al. 2007), making cGnRH II a desirable target
72 for development as a potential induced spawning aid in aquaculture. The cGnRH II variant is
73 highly conserved and has been found to be more potent, in terms of stimulation of luteinizing
74 hormone (LH) secretion, when compared to the hypophysiotropic sGnRH III form (Illing et al.
75 1999; Podhorec and Kouril 2009). Ovaprim® ([20 µg/mL sGnRH IIIa + 10 mg/mL
76 domperidone], Western Chemical Inc., Ferndale, WA) is a combination of a salmon GnRH
77 analog (D-Arg6-Pro9-Net) (Table 1) and a dopamine antagonist, suspended in a propylene glycol
78 solution and is commonly used in hormone induced spawning of ornamental fishes. As of March
79 2009, Ovaprim was added to the United States Food and Drug Administration (FDA) index of
80 legally marketed unapproved new animal drugs for minor species, allowing ornamental fish
81 farmers to purchase the drug directly. Although Ovaprim has been shown to successfully induce
82 FOM and ovulation in many species (Hill et al. 2005, 2009, Sahoo et al. 2005, 2007, DiMaggio
83 et al. 2013, 2014), members of the *Synodontis* genus have shown inconsistent responses to the
84 use of Ovaprim with variable ovulation rates (Hill et al. 2009).
85 Recently, there has been interest in exploring cGnRH IIa (D-Arg6, Pro-9-Net) as an
86 alternative drug for induced spawning due to its ubiquitous distribution across taxa and proven
87 potency in LH release (Forniés et al. 2003). The gonadotropin LH is essential to gonadal

88 maturation and steroidogenesis in fishes (Zohar and Mylonas 2001, Mylonas and Zohar 2007).
89 The cGnRH II variant has been identified as a potent LH releaser (Zohar and Mylonas 2001) and
90 has demonstrated superior ability to elicit LH secretion compared with other GnRH variants in a
91 number of species, including Goldfish *Carassius auratus*, Gilthead Seabream *Sparus aurata,* and
92 African Catfish *Clarias gariepinus* (Zohar et al. 1995; Illing et al. 1999; Bosma et al. 2000;
93 Podhorec and Kouril 2009). A comparative study which evaluated mGnRH Ia, cGnRH IIa and
94 sGnRH IIIa for spawning induction of Channel Catfish *Ictalurus punctatus* reported cGnRH IIa
95 yielded the highest efficacy, with an ovulation rate of 90.2% (Quiniou et al. 2014). Furthermore,
96 investigations with the Stinging Catfish *Heteropneustes fossilis* (Alok et al. 1999), Sharptooth
97 Catfish, *Clarias gariepinus* (Taufek et al. 2009), and Broadhead Catfish *Clarias microcephalus*
98 (Ngamvongchon et al. 1992) have demonstrated the efficacy of native cGnRH II to induce
99 ovulation in other Siluriform fishes. These results provide a strong impetus for further
100 examination of cGnRH IIa as an induced spawning aid for the ornamental aquaculture industry.
101 If effective, the use of cGnRH IIa could help to expand the diversity and yield of ornamental fish
102 being cultured today.
103 The genus *Synodontis* belongs to the specious Mochokidae family endemic to sub-
104 Saharan Africa and inhabit a wide variety of freshwater habitats from small creeks to large lakes
105 (Friel and Vigliotta 2006; Koblmüller et al. 2006). The 120 species of catfish in this genus
106 commonly are called “squeakers” due to the sound they make by rubbing their pectoral spines
107 when agitated (Friel and Vigliotta 2006; Koblmüller et al. 2006). In countries such as Benin,
108 members of the genus are a highly valued food fish and support a significant fishery (Lalèyè et
109 al. 2006). Their unique behavior, attractive markings, and relatively compact size has made the
110 *Synodontis* genus highly sought after in the ornamental aquarium trade (Friel and Vigliotta 2006;

111 Koblmüller et al. 2006). Moreover, with the underdeveloped state of ornamental fish export of
112 some African countries (Oben and Oben 2003) and relative mortality associated with wild
113 capture and transport of ornamental fish (Livengood and Chapman 2007), further research into
114 the culture practices for members of the genus *Synodontis* is warranted.
115 The objective of this study was to evaluate the effects of various dosages of cGnRH IIa
116 on parameters imperative to the successful culture of two ornamental *Synodontis* species. The
117 Feather Fin Squeaker *Synodontis eupterus* (Figure 1a) and Upside-Down Catfish *Synodontis*
118 *nigriventris* (Figure 1b) are two popular ornamental catfish species that are currently produced
119 using Ovaprim, the industry standard for induction spawning. Performance of cGnRH IIa was
120 compared to Ovaprim, and effects on ovulation, fecundity, fertilization success, hatch success, as
121 well as egg and larval morphometrics were assessed.
122 **2. Material and Methods**
123 Two distinct experiments were conducted evaluating the efficacy of cGnRH IIa as an
124 induced spawning aid for *S. nigriventris* and *S. eupterus*. The experimental design for both
125 catfish species was similar although minor deviations from standard methodologies occurred and
126 are detailed below.
127 *2.1 Experimental Design*
128 Sexually mature broodstock were obtained from a commercial ornamental fish producer
129 in Wimauma, Florida, USA 24 to 48 hours prior to injection. Fish were transported to the
130 University of Florida's Tropical Aquaculture Laboratory (UF-TAL), where males and females
131 were separated into respective 1,030 L concrete vats with a working volume of 380 L. Vats were
132 supplied with flow-through, degassed well water at 380 L/hour and supplemental aeration for the
133 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 by mouth 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 vitellogenic oocytes (Vtg3, Brown-Peterson et al. 2011) 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) (Table 1) 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

157 peptide solutions were prepared. The carrier for all cGnRH IIa treatments consisted of the
158 dopamine antagonist, domperidone (Roadrunner Pharmacy, Phoenix, AZ), dissolved in
159 propylene glycol, yielding a final concentration of 10 mg/mL, equal to that of Ovaprim. Volumes
160 of 1000, 500, and 250 µL of the carrier was pipetted into three individual 100 µg cGnRH IIa
161 vials to achieve desired concentrations of 50, 100 and 200 µg/kg cGnRh IIa + 5 mg/kg
162 domperidone when administered at an injection volume of 0.5 µL/g of fish weight. Hormone
163 preparations were stored in a 4.0 ºC refrigerator and used within 12 hours. An additional vial of
164 propylene glycol was prepared alongside the cGnRH IIa treatments to serve as a negative
165 control. Ovaprim was used as a positive control at its stock concentration and administered at a
166 dosage of 10 µg/kg sGnRH IIIa + 5 mg/kg domperidone (0.5 µL/g). Broodstock availability
167 necessitated the use of temporal replicates. Six spawning trials were completed with three
168 replicates per treatment in each trial totaling 18 total replicates per treatment for *S. nigriventris.*
169 Two spawning trials were completed for *S. eupterus*. Due to broodstock availability an
170 unbalanced design was used for the *S. eupterus* experiment, with all cGnRH IIa treatments
171 having six replicates and the Ovaprim and propylene glycol controls having five.
172 All spawning preparations and controls were administered to female broodstock via a
173 single bolus intramuscular injection near the base of the dorsal fin using 100 µL Hamilton
174 gastight syringes (Hamilton Co., Reno, NV). Experimental injection regimes mirrored current
175 protocols used by the ornamental industry for production of *Synodontis* spp. The initial fish
176 weight from time of biopsy was used to calculate total volume of spawning aid to be injected.
177 Females were again anesthetized approximately 2 - 4 hours post biopsy in 150 mg/L buffered
178 tricaine solution prior to injection. Following injections, all fish were returned to their respective
179 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 plastic weigh boat and weighed to the nearest 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) container 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

202 Rafter Cell under a dissecting microscope. Embryos were photographed as previously described
203 for determination of fertilization success and egg morphometrics.
204 Hatching success was determined from the 50-embryo subsample approximately 44 hours
205 post fertilization. Sample cups which now contained hatched larvae were removed from the
206 hatching system and euthanized in a 300 mg/L tricaine solution. Larvae were photographed on a
207 Sedgewick Rafter Cell for morphometric analyses. Larvae that were hatched but atypical or dead
208 when observed were included in hatch success data however only live larvae that were
209 representative of typical development were used to determine notochord length. All images were
210 captured in this investigation using ProgRes® CapturePro v2.8.8 software by Jenoptik Optical
211 Systems (Jena, Germany) and analyzed using ImageJ v1.50i processing software (National
212 Institutes of Health, Bethesda, MD). Mortality of brood stock used in the experiment was
213 monitored and recorded for an additional 24 h after spawning.
214 *2.2 Water Quality*
215 Water quality parameters were monitored prior to and during spawning trials in the
216 broodstock holding and larval hatching systems. Dissolved oxygen (DO) and temperature were
217 measured using a YSI ProComm II meter (YSI Inc., Yellow Springs, OH). Total ammonia
218 nitrogen (TAN), nitrite-nitrogen, pH, hardness ($CaCO_3$), and alkalinity ($CaCO_3$) were measured
219 using standard colorimetric assays according to the manufacturers protocols (Hach Company,
220 Loveland, CO).
221 *2.3 Data Analysis*
222 Mean fertilization success, embryo diameter, hatching success, and larval notochord
223 length was calculated from the three sampling periods (16, 20, and 24 hours) for each individual
224 fish and used as a single data point for statistical analyses. Vitellogenic oocytes (Vtg3) from

225 ovarian biopsies were enumerated and the percentage of oocytes exhibiting GVM was recorded.
226 Germinal vesicles that were observed to deviate from the initial central location in the egg were
227 categorized as migrating. Oocyte diameters (N = 33 ± 11 for *S. nigriventris* and N = 45 ± 8 for *S.*
228 *eupterus*) from each biopsy were measured with ImageJ. Mean pre-trial oocyte diameters as well
229 as percent GVM for each treatment were compared using an analysis of variance (ANOVA) to
230 confirm there were no significant differences among experimental populations. Ovulation data
231 were categorized into two levels, with successful ovulation = 1 and unsuccessful ovulation = 0.
232 A logistic regression was performed using the generalized linear model (GLM) function in the
233 base package of RStudio (family=binomial, V. 0.99.903 2015. RStudio: Integrated Development
234 for R. RStudio, Inc., Boston, MA), followed by an ANOVA to test for differences among the
235 categorical predictor variable (hormone treatment). A post hoc pairwise comparison was
236 performed using a Tukey Honestly Significant Difference test. The number of spawned eggs per
237 gram was calculated by enumerating a subset of egg samples from *S. nigriventris* (N = 8) and *S.*
238 *eupterus* (N = 6) with a known weight. This value was then used to calculate fecundity based on
239 the mass of eggs collected. Fecundity was then standardized by dividing the total number of eggs
240 spawned per individual by the initial pre-spawning weight of the fish. Fertilization success was
241 determined microscopically from a representative subsample of embryos (N = 189 ± 25 for *S.*
242 *nigriventris* and N = 101 ± 26 for S. *eupterus*). Up to 50 embryo diameters were measured using
243 methods previously described (N = 33 ± 15 for *S. nigriventris* and N = 9 ± 8 for *S. eupterus*).
244 Hatching success was calculated after correcting for individual fertilization rates from each
245 spawn. For *S. nigriventris*, mean larval notochord length was calculated from a total of N = 517
246 larvae from three trials across all treatments (N = 22 ± 9 larvae/spawn). For *S. eupterus*, mean
247 larval notochord length was calculated from a total of N = 250 larvae from two trials across all

248 treatments (N = 16 ± 8 larvae/spawn). Notochord length (NL) was defined as a measurement
249 from the most anterior portion of the head to the tip of the notochord.
250 All data were assessed for normality and homoscedasticity prior to statistical testing.
251 Data which satisfied these assumptions were evaluated using a one-way ANOVA. Data
252 transformations were used if assumptions of ANOVA were not met. Non-normal data was
253 analyzed using a one-way Kruskal-Wallis test. The propylene glycol negative control treatment
254 was not included in fecundity, fertilization success, fertilized egg diameters, hatching success or
255 larval length analyses due to a small sample size in *S. nigriventris* (N =2) for all response
256 variables and complete lack of data for *S. eupterus*. A *P*-value of ≤ 0.05 was considered
257 statistically significant for all analyses. Data are presented as mean ± standard deviation (SD).
258 All statistical analyses were performed using JMP Pro v13.0.0 (SAS Institute, Cary, NC) and
259 Program R v3.3.1 (R Core Team).
260 **3. Results**
261 For brevity, treatment groups will be referred to as follows for the remainder of the study: 50
262 µg/kg cGnRH IIa = 50, 100 µg/kg cGnRH IIa = 100, 200 µg/kg cGnRH IIa = 200, and Ovaprim
263 will be referred to as sGnRH IIIa.
264 *3.1 Synodontis nigriventris*
265 A total of 90 female *S. nigriventris* were injected over the course of the experimental
266 period, 72 of which were injected with cGnRH IIa or sGnRH IIIa. Mean TL of female fish used
267 in this experiment was 97 ± 8 mm with an initial weight of 23.92 ± 7.39 g. There were no
268 differences ($P = 0.126$) in the mean percentages of ovarian biopsies exhibiting GVM (88 ± 9%)
269 among all treatments. Mean biopsy oocyte diameter (1.222 ± 0.086 mm) across treatments was
270 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).

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 (Table 2).

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. 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 (Table 2). No mortality was recorded for *S. nigriventris* females during the 24 hour post trial observational period.

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

294 DO: 7.41 ± 0.16 mg/L. Mean water quality parameters recorded for the recirculating hatching
295 system were: TAN: 0.10 ± 0.11 mg/L, nitrite-nitrogen: 0.02 ± 0.05 mg/L, pH: 7.9 ± 0.3, hardness
296 ($CaCO_3$): 139.65 ± 42.46 mg/L, alkalinity ($CaCO_3$): 76.95 ± 17.93 mg/L, temperature; 27.11 ±
297 0.10 °C, and DO: 7.45 ± 0.07 mg/L.
298 *3.2 Synodontis eupterus*
299 A total of 28 female *S. eupterus* were administered injections over the course of the
300 experimental period, 23 of which were injected with cGnRH IIa or sGnRH IIIa. The mean TL of
301 female fish used in this experiment was 215 ± 28 mm with a mean initial weight of 165.86 ±
302 21.23 g. Comparisons of observed GVM in ovarian biopsies yielded no differences among
303 treatments ($P = 0.401$, 61 ± 21%). Mean biopsy oocyte diameter (1.242 ± 0.088 mm) across
304 treatments was also found to be similar ($P = 0.674$). Significant differences were detected in
305 ovulation success among treatments ($P = 0.025$); with the negative control yielding no successful
306 spawns, although the 200 and sGnRH IIIa treatments were not significantly different from the
307 negative control ($P \geq 0.108$). Comparable ovulation success was observed among hormonal
308 treatment groups and dosages ($P \geq 0.893$). Fish injected with the spawning aid treatments of 50
309 and 100 exhibited ovulation success of 83% while sGnRH IIIa injected fish resulted in 60%
310 success (Table 3).
311 Eggs collected from *S. eupterus* during this experiment numbered 1,337 ± 60 eggs/g of
312 spawned material. Fecundity was calculated to be of similar quantity among hormonal treatments
313 and dosages ($P = 0.519$), with mean number of eggs spawned per female's initial body weight
314 being 149 ± 54 eggs/g. Numerically, the highest value was obtained from the 200 treatment at
315 176 ± 38 eggs/g. Each spawning fish ovulated an average of 13.61 ± 11.35 g or 18,190 ± 15,181
316 total eggs. Analysis of mean fertilization success also yielded consistent results among spawning

317 hormones (P = 0.864) with a range of 74 ± 23% – 83 ± 7% and the highest mean fertilization rate
318 (83 ± 7%) observed in the 100 treatment (Table 3).
319 Mean embryo diameters were similar among experimental treatment groups and the
320 positive control (P = 0.791) with embryos from 200 injected fish having the largest diameters at
321 1.691 ± 0.115 mm. Hatch success was also comparable among the treatments (P = 0.943). The
322 highest hatching success of 46 ± 8% occurred in the 100 treatment and the lowest of 36 ± 26%
323 was observed in the 200 treated fish. Evaluation of mean larval notochord length revealed similar
324 larval size among treatments (P = 0.387) with a range of only 0.3 mm (Table 3). No mortality
325 was recorded for *S. eupterus* females during the 24 hour post trial observation period.
326 Mean water quality parameters observed in the broodstock flow through systems were:
327 TAN: 0.0 mg/L, nitrite-nitrogen: 0.0 ± 0.0 mg/L, pH: 8.0 ± 0.0, hardness ($CaCO_3$): 436.05 ±
328 12.10 mg/L, alkalinity ($CaCO_3$): 188.1 ± 0.0 mg/L, temperature: 25.35 ± 0.49°C, and DO: 8.41 ±
329 0.53 mg/L. Mean water parameters recorded for the recirculating hatching system were: TAN:
330 0.0 ± 0.0 mg/L, nitrite-nitrogen: 0.0 ± 0.0 mg/L, pH: 8.0 ± 0.0, hardness ($CaCO_3$): 119.70 ±
331 24.18 mg/L, alkalinity ($CaCO_3$): 76.95 ± 12.09 mg/L, temperature: 25.30 ± 0.42°C, and DO:
332 8.80 ± 0.01 mg/L.
333 **4. Discussion**
334 The dose of cGnRH IIa or Ovaprim did not significantly affect ovulation rate in either
335 species examined. The comparable performance in ovulation success among all hormonal
336 treatments suggests all concentrations of drug administered were equally as potent, irrespective
337 of species. The repeated spawning behavior of the two species examined in this investigation did
338 not allow for interpretation of the optimum latency period based on the experimental design.
339 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 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 (Tables 1 & 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 promoting egg viability. 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. Analysis of pretrial mean oocyte diameter and percent germinal vesicle migration among treatments yielded no significant differences for both the *S. nigriventris* and *S. eupterus* experiments. These results indicate all treatment test subjects were equal in reproductive condition prior to injection.

363 It is unclear why ovulation was observed in the *S. nigriventris* negative control; however,
364 it has been observed that capture and handling of gravid fish can spontaneously result in
365 ovulation and spawning (C. Watson, personal communication). While 100% of *S. nigriventris*
366 ovulated when treated with 100 and 200, only 11% ovulated when treated with the propylene
367 glycol; once again illustrating the effectiveness of cGnRH IIa for induction spawning in this
368 species. *S. eupterus* produced similar results with high rates of ovulation (up to 83%) occurring
369 in hormonally treated groups and no ovulation in propylene glycol treated fish. The functional
370 role of the conserved cGnRH II variant in fishes was previously unclear with some species
371 reported to exhibit relatively constant levels of the hormone (Somoza et al. 2002). The results of
372 this experiment and other similar studies suggest a strong correlation between administration of
373 exogenous cGnRH II and initiation of FOM and ovulation, further supporting the purported
374 hypophysiotropic role for this variant (Ngamvongchon et al. 1992; Alok et al. 1999; Szabó et al.
375 2007; Taufek et al. 2009; Quiniou et al. 2014).
376 No mortality was observed 24 hours post injection in *S. eupterus* or *S. nigriventris* trials
377 indicating a high degree of safety for all drug preparations used in this study. Mortality
378 associated with induction spawning protocols can vary dependent upon species as well as
379 hormone choice and administration route. Hill et al. (2009) reported a wide range of mortalities
380 (0 – 35.7%) for various ornamental species following induction spawning protocols using
381 sGnRH IIIa. Interestingly, a mortality rate of 0% was recorded for all Mochokids evaluated by
382 Hill et al. (2009) which mirrored results obtained in this investigation. Cause of death was
383 postulated by Hill et al. (2009) to result from poor water quality or egg binding during the
384 induction spawning procedure. Provision of appropriate water quality and high mean ovulation

385 success in hormonally treated fish, *S. nigriventris* (94 ± 8%) and *S. eupterus* (73 ± 12%), likely
386 aided in the reduced treatment associated mortality observed in the current study.
387 Additional investigations into dose response are needed to recommend an optimal
388 cGnRH IIa hormone concentration which maximizes efficacy and safety while minimizing the
389 economic investment. It is entirely plausible that observed responses resulting from sGnRH IIIa
390 and cGnRH IIa could indeed be due to differences in administered concentrations. Dosages of
391 cGnRH IIa for this experiment (100 µg/kg) were chosen based on the success reported by
392 Quiniou et al. (2014), effectively inducing ovulation of Channel Catfish *Ictalurus punctatus*.
393 This study utilized the previously mentioned dose as well as a higher (200 µg/kg) and lower (50
394 µg/kg) dose that bracketed the concentration of cGnRH IIa that was shown to be effective.
395 Endogenous levels of gonadotropin releasing hormones in fishes can vary due to factors such as
396 species, season, gonadal stage, health status, etc. Extrapolation of naturally occurring
397 endogenous GnRH concentrations to guide administration of GnRH analogues may be complex
398 as GnRH analogues exhibit increased biological activity when compared with native forms.
399 However, previous research has demonstrated GnRH (sbGnRH, cGnRH II, sGnRH)
400 concentrations in whole pituitaries to range from 0.1 to >40.0 ng/pituitary for species such as the
401 Striped Bass, *Morone saxatilis* (Holland et al. 2001), European Sea Bass, *Dicentrarchus labrax,*
402 L. (Rodriguez et al. 2000), and Goldfish *Carassius auratu*s (Yu et al. 1991). Results in this
403 experiment did not yield an observable dose response among the cGnRH IIa treatments, with no
404 lower or upper limit identified for spawning effectiveness. Reducing the amount of drug
405 administered would decrease the cost per unit and allow more fish to be spawned using the same
406 amount of drug. Studies involving Turbot (*Scophthalmus maximus*), Brown Trout (*Salmo trutta*),
407 and Pigfish (*Orthopristis chrysoptera*) have found that high doses of GnRHa, similar to

concentrations used in this experiment, may have negative effects on 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 broader 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

431 the African Catfish *Clarias gariepinus* using a single injection (Szabó et al. 2007). In a study
432 with European Seabass *Dicentrarchus labrax*, two isoforms of of cGnRH IIa were tested for
433 their potency in LH release. The analog (D-Arg6, Pro9 Net) resulted in the greatest amount of
434 LH release among the cGnRH groups tested, more potent than the cGnRH IIa (D-Ala 6, Pro9
435 Net) peptide. Additionally the analog (D-Ala6, Pro9 Net) also performed comparably yielding a
436 response six times the potency of native cGnRH forms (Forniés et al. 2003). A study evaluating
437 spawning success with several isoforms of GnRH analogs in Channel Catfish *Ictalurus punctatus*
438 revealed the greatest success was achieved with the cGnRH II analog of (D-Arg6, Pro9 Net) with
439 a mean ovulation of 90.2% (Quiniou et al. 2014). Multiple studies have been carried out with the
440 cGnRH II (D-Arg6, Pro9 Net) analog which have examined resulting LH release or progression
441 of FOM culminating in ovulation (Lovejoy et al. 1995; Szabó et al. 2007; Quiniou et al. 2014).
442 Results from these investigations helped to shape the choice of analog and concentrations that
443 were evaluated in the current research. Although ovulation success of 100% in *S. nigriventris*
444 and 83% in *S. eupterus* was achieved for some concentrations of cGnRH IIa (D-Arg6, Pro9 Net)
445 in this experiment, it would be prudent to further examine additional isoforms of GnRH's
446 moving forward, as species can respond differently to native forms as well as analogs.
447 Delivery methods of GnRH can vary depending on species, size, and reproductive
448 strategy of the fish being induced. Spawning hormones have been successfully administered
449 using a myriad of delivery systems and injections schedules including single injection (Quiniou
450 et al. 2014), multiple injections (Shireman and Gildea 1989), intramuscular injections (DiMaggio
451 et al. 2014), intracoelomic injections (also referred to as intraperitoneal) (Clemens and Grant
452 1965), intra-pericardial cavity injections (Kouril et al. 1986), intravenous injections
453 (Mikolajczyk et al. 2003), ovarian lavage (Watson et al. 2009), sustained release implants

(Mugnier et al. 2000), and even diffusion over the gills (Hill et al. 2005). In species which exhibit synchronous egg development, a single LH surge is often adequate to induce ovulation. Conversely, in a species with asynchronous ovarian development, a sustained release delivery of exogenous hormones may be the more appropriate route (Podhorec and Kouril 2009). The degree of stress associated with capture and hormone application should also 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 *C. 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 as the natural release of GnRH in vertebrates is pulsatile in nature (Dellovade et al. 1998).

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 *C. 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 procedures. 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 expanding markets, and increase profitability for producers. To the authors' 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 (Tables 1 & 2). Due to its comparable efficacy in pertinent spawning criteria, reliability, safety, and broad range of effective concentrations, cGnRH IIa appears to be to be a practical option for induction spawning. 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 option 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.

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Figure Captions

Figure 1. A) Feather Fin Squeaker *Synodontis eupterus* and B) Upside-Down Catfish *Synodontis nigriventris*.

519 Table 1. Amino acid sequences of mammalian GnRH and the two GnRH analogs used in this experiment alongside their native forms

520 (Adapted from Ngamvongchon et al. 1992). The symbol * signifies alterations in amino acid sequence from native GnRH forms.

	1	2	3	4	5	6	7	8	9	10	
Mammal (mGnRH I)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Arg-	Pro-	Gly	NH2
Salmon (sGnRH III)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Trp-	Leu-	Pro-	Gly	NH2
Salmon Analog (sGnRH IIIa)	pGlu-	His-	Trp-	Ser-	Tyr-	DArg-*	Trp-	Leu-	Pro-	Net*	
Chicken (cGnRH II)	pGlu-	His-	Trp-	Ser-	His-	Gly-	Trp-	Tyr-	Pro-	Gly	NH2
Chicken Analog (cGnRH IIa)	pGlu-	His-	Trp-	Ser-	His-	DArg-*	Trp-	Tyr-	Pro-	Net*	

521
522

523 Table 2. Mean ± SD ovulation success, fecundity (eggs per gram body weight), fertilization success, fertilized oocyte diameter, hatch
524 success, and notochord length for *Synodontis nigriventris* administered one of three concentrations of cGnRH IIa, sGnRH IIIa
525 (positive control), or propylene glycol (negative control). All GnRHa treatments included the dopamine antagonist domperidone at a
526 concentration of 5 mg/kg. Different superscript letters within a column denotes statistically significant differences ($P \leq 0.05$). The
527 symbol * indicates descriptive data that was not included in statistical analyses due to a limited sample size (N = 2).

Treatment	Ovulation (%)	Eggs Per Gram Body Weight	Fertilization Success (%)	Embryo Diameter (mm)	Hatch Success (%)	Notochord Length (mm)
Propylene glycol	11[b]	130 ± 136*	64 ± 2*	1.377 ± 0.004*	48 ± 6*	3.415 ± 0.033*
<u>sGnRH IIIa</u>						
10 µg/kg	83[a]	160 ± 27	75 ± 22	1.409 ± 0.066	26 ± 7	3.407 ± 0.372
<u>cGnRH IIa</u>						
50 µg/kg	94[a]	228 ± 25	70 ± 20	1.360 ± 0.066	35 ± 6	3.520 ± 0.075
100 µg/kg	100[a]	181 ± 25	76 ± 16	1.363 ± 0.068	35 ± 6	3.407 ± 0.208
200 µg/kg	100[a]	227 ± 25	66 ± 23	1.385 ± 0.060	33 ± 6	3.483 ± 0.252

528

529

530 Table 3. Mean ± SD ovulation success, fecundity (eggs per gram body weight), fertilization success, fertilized oocyte diameter, hatch
531 success, and notochord length for *Synodontis eupterus* administered one of three concentrations of cGnRH IIa, sGnRH IIIa (positive
532 control), or propylene glycol (negative control). All GnRHa treatments included the dopamine antagonist domperidone at a
533 concentration of 5 mg/kg. Different superscript letters within a column denotes statistically significant differences ($P \leq 0.05$).

Treatment	Ovulation (%)	Eggs Per Gram Body Weight	Fertilization Success (%)	Embryo Diameter (mm)	Hatch Success (%)	Notochord Length (mm)
Propylene glycol	0^{b}	-	-	-	-	-
<u>sGnRH IIIa</u>						
10 µg/kg	60^{ab}	157 ± 69	81 ± 15	1.611 ± 0.232	40 ± 23	3.677 ± 0.333
<u>cGnRH IIa</u>						
50 µg/kg	83^{a}	120 ± 63	79 ± 18	1.689 ± 0.094	45 ± 34	3.833 ± 0.208
100 µg/kg	83^{a}	151 ± 48	83 ± 7	1.632 ± 0.119	46 ± 8	3.576 ± 0.249
200 µg/kg	67^{ab}	176 ± 38	74 ± 23	1.691 ± 0.115	36 ± 26	3.486 ± 0.354

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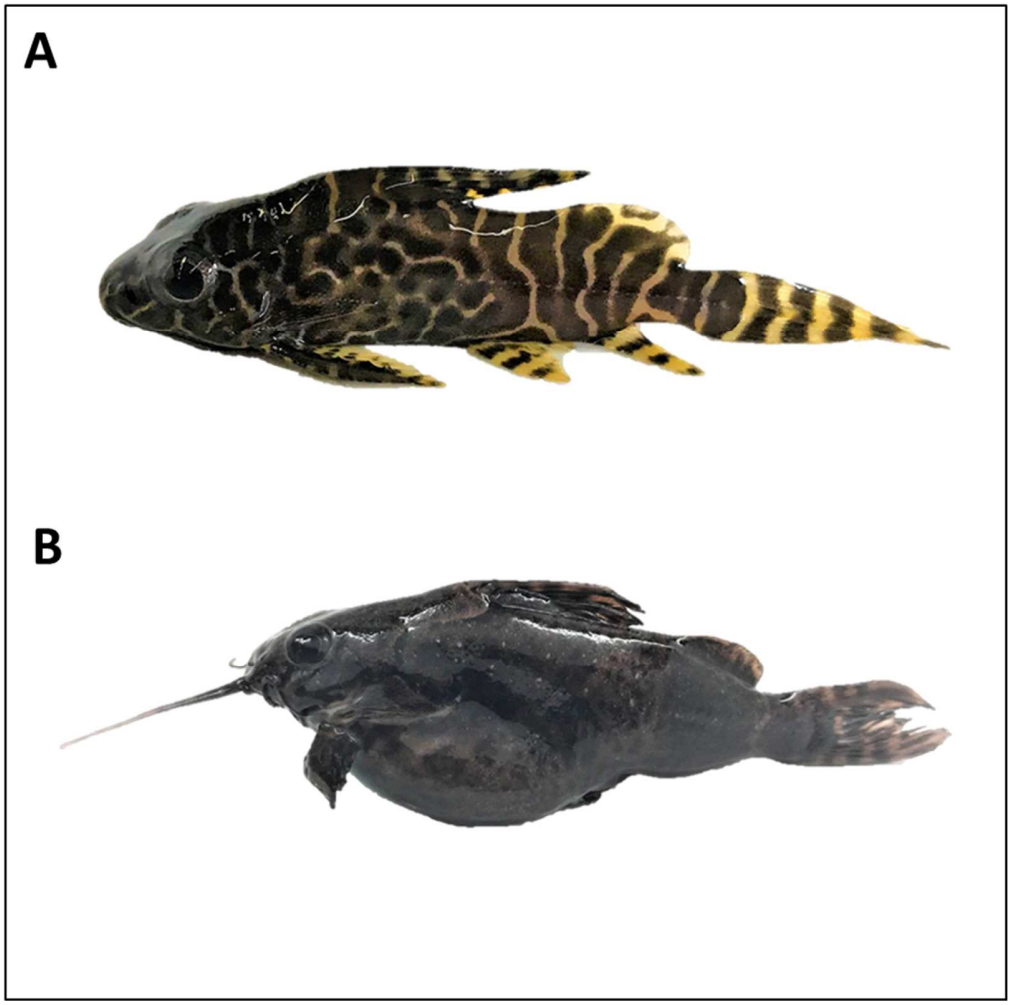

Figure 1.

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