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31 **Abstract**

32 The administration of exogenous hormones for induction spawning of captive fish
33 is a common practice throughout the finfish aquaculture industry. Induced spawning
34 protocols for ornamental cyprinids commonly rely on sGnRH IIIa, commercially available
35 as Ovaprim. In this study, the efficacy of an alternative spawning aid, cGnRH IIa, was
36 evaluated relative to Ovaprim, in two commonly cultured ornamental Cyprinids: the
37 Redtail Sharkminnow *Epalzeorhynchos bicolor* and the Rainbow Shark *E. frenatum*.
38 Broodstock of each species were injected with either a positive control (0.5 µL/g
39 Ovaprim), a negative control (propylene glycol), or one of three doses of cGnRH IIa (50,
40 100, or 200 µg/kg). Following spawning aid injection, ovulation success, fecundity,
41 fertilization success, embryo diameter, hatch success and larval notochord length were
42 evaluated. Ovulation success (82 ± 8%) was statistically similar to the positive control
43 group for all experimental doses of cGnRH IIa in *E. bicolor*, while 50 and 100 µg/kg
44 cGnRH IIa doses resulted in significantly higher ovulation success (100.0 and 83.0 %,
45 respectively) than the positive control (17.0%) in *E. frenatum*. All other parameters did
46 not vary significantly among treatments. These results indicate cGnRH IIa can be
47 successfully used as a spawning aid in both species.
48
49

50 **Key Words:** Induced spawning, ornamental, cGnRH IIa, *Epalzeorhynchos bicolor*,
51 *Epalzeorhynchos frenatum*
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55 **Introduction**

56 Successful spawning is among the many hurdles to overcome in aquaculture of a
57 new species, and is paramount to meet industry demands (DiMaggio, Broach, & Ohs,
58 2013). Reproductive dysfunction is commonplace in captivity, although its presentation
59 and severity may vary by species (Mylonas & Zohar, 2000, 2007; Podhorec & Kouril,
60 2009; Yaron et al., 2009). Addressing this impediment to culture often involves the use
61 of environmental manipulations or exogenous hormones (Rottmann, Shireman, &

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62 Chapman, 1991). In nature, environmental cues which signify the approach of
63 reproductive activity are perceived by the brain of the fish. This sensory data is
64 integrated by the hypothalamus which in turn generates and releases neuropeptides to
65 initiate a cascade of reactions resulting in mature gametes (Zohar & Mylonas, 2001).
66 The progression of this cascade is initiated following release of gonadotropin releasing
67 hormones (GnRH) from the hypothalamic region of the brain.

68 GnRH, which acts on the pituitary via direct innervation, stimulates the secretion
69 of gonadotropins (GtH) that act on the gonad resulting in the production of steroids and
70 prostaglandins (Rottmann et al., 1991). Since the discovery of the GnRH decapeptide
71 and its associated role in the HPG axis, a large body of research has focused on
72 optimization of dose, identification of active variants, and development of potent
73 analogues (Alok, Talwar, & Garg, 1999; Bosma, Rebers, Dijk, Willems, & Goos, 2000;
74 DiMaggio et al., 2013; DiMaggio, Broach, & Ohs, 2014; Forniés et al., 2003; Mikolajczyk
75 et al., 2003; Lovejoy et al., 1995; Mylonas, Hinshaw, & Sullivan, 1992; Ngamvongchon,
76 Rivier, & Sherwood, 1992; Podhorec & Kouril, 2009; Sahoo, Giri, & Sahu, 2005;
77 Prayogo, Wijayanti, Sulistyo, & Sukardi, 2016; Quiniou, Bosworth, Chatakondi, &
78 Oberle, 2014; Szabó, Radics, Barth, & Horváth, 2007; Taufek, Harmin, & Christianus,
79 2009; Zohar et al., 1995). One advantage of using GnRH is that it occurs earlier in the
80 hormonal cascade stimulating the release of endogenous stores of GtH and facilitating
81 the contribution of supplementary endocrine factors such as prolactin, thyroid
82 hormones, insulin like growth factors, and growth hormone (Podhorec & Kouril, 2009).
83 This approach is arguably more “natural” and preferred to flooding the gonad with
84 exogenous GtH, which may trigger an immunological response, thereby decreasing the
85 efficacy of the spawning aid over time (Zohar & Mylonas, 2001) .

86 The GnRH peptide contains 10 amino acids in sequence, the specific
87 arrangement of which can vary among species. In vertebrates there are 16 variants of
88 gonadotropin releasing hormones that belong to three distinct GnRH systems (Millar,
89 2003; Somoza, Miranda, Strobl-Mazzulla, & Guilgur, 2002). Most vertebrates have two
90 to three GnRH forms present in the body with hormones belonging to system II
91 occurring ubiquitously among taxa while system III hormones are teleost specific (Kah
92 et al., 2007). Variants within the systems are named after the organism they were first

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93 described in, with mGnRH I found initially in mammals, cGnRH II in chickens and
94 sGnRH III in salmon (Kah et al., 2007). With the discovery of GnRH came laboratory
95 synthesized analogs targeting substitutions of amino acid bonds that were prone to
96 enzymatic degradation (Lovejoy et al., 1995). Alterations at these specific positions
97 have made GnRH analogs (GnRHa) more potent as induced spawning aids by
98 increasing binding affinity and prolonging the duration of activity while in circulation
99 (Taufek et al., 2009; Zohar & Mylonas, 2001). The induction spawning drug known as
100 Ovaprim® ([20 μg/mL sGnRH IIIa + 10 mg/mL domperidone], Syndel, Ferndale, WA) is
101 an analog (D-Arg6-Pro9-Net) (Figure 1) of sGnRH III and has become the industry
102 standard in ornamental fish production due to its availability and efficacy in a range of
103 species (Hill et al. 2009). This hormone preparation was added to the United States
104 Food and Drug Administration (FDA) index of legally marketed unapproved new animal
105 drugs for minor species making it available for producers to purchase directly from the
106 manufacturer. The sGnRH IIIa hormone, administered in conjunction with a dopamine
107 antagonist, has been successfully used for induction spawning of Pinfish *Lagodon*
108 *rhomboides* (DiMaggio et al., 2013), Pigfish *Orthopristis chrysoptera* (DiMaggio et al.,
109 2014), Asian Catfish *Clarias batrachus* (Sahoo et al., 2005), African Catfish *Clarias*
110 *gariepinus* (Taufek et al., 2009), Channel Catfish *Ictalurus punctatus* (Quiniou et al.,
111 2014), Common Carp *Cyprinus carpio* (Yaron et al., 2009), and a variety of ornamental
112 species (Hill et al., 2009). Although sGnRH IIIa has successfully induced ovulation in
113 numerous species of ornamental (Hill et al., 2009), food (Quiniou et al., 2014; Sahoo et
114 al., 2005), and baitfish (DiMaggio et al., 2013, 2014), other GnRHa variants have been
115 administered with greater spawning efficacy (Quiniou et al., 2014). While Ovaprim is
116 currently the preferred choice for induction spawning of ornamental fishes, this
117 preparation may be unreliable or completely ineffective in some cases (Hill et al., 2009)
118 and investigations into additional spawning aids are warranted.

119 cGnRH II is known to be expressed in species from all vertebrate classes (King &
120 Millar, 1995). The highly conserved cGnRH IIa (D-Arg6-Pro9-Net) has garnered recent
121 interest as a potential alternative to sGnRH IIIa for induction spawning of fishes. The
122 cGnRH II variant has been found to stimulate the release of the gonadotropin,
123 luteinizing hormone (LH), in African Catfish *C. gariepinus* (Bosma et al., 2000) and

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124 Gilthead Sea Bream *Sparus aurata* (Zohar et al., 1995). Furthermore, spawning activity
125 was reported to be greater using cGnRH II as a spawning aid compared to other GnRH
126 types in African Catfish *C. gariepinus* (Szabó et al., 2007), Channel Catfish *I. punctatus*
127 (Quiniou et al., 2014), and European Seabass *Dicentrarchus labrax* (Forniés et al.,
128 2003). Further investigation into the efficacy of various cGnRH II analogues may
129 expand the number of species able to be cultured, while potentially producing greater
130 yields for species already in production.

131 The production volume of cyprinids in aquaculture eclipses many other families
132 of teleosts and induction spawning is a critical factor in their production. Within the
133 Cyprinidae family, the genus *Epalzeorhynchos* contains a limited number of highly
134 valuable ornamental fish species endemic to South East Asia (Elakkanai, Francis,
135 Ahilan, Jawahar, & Padmavathy, 2017; Kulabtong, Suksri, Nonpayom, & Soonthornkit,
136 2014). The Redtail Sharkminnow, *Epalzeorhynchos bicolor* (Figure 2A) and the
137 Rainbow Sharkminnow, *E. frenatum* (Figure 2B), are popular species within the
138 freshwater ornamental trade. Commercial spawning protocols for these species
139 commonly use Ovaprim, as strong dopaminergic inhibition of LH secretion is
140 characteristic of the Cyprinidae family (Chang, Cook, & Peter, 1983; Podhorec & Kouril,
141 2009; Trudeau, Sloley, Wong, & Peter, 1993). While both species have both been
142 shown to respond to exogenous hormone administration (da Silva Henriques, 2016; Hill
143 et al., 2005; Islami, Sudrajat, & Carman, 2017; Shireman & Gildea, 1989), a recent
144 survey of Ovaprim use in the ornamental industry by Hill et al. (2009) identified a 12%
145 failure rate for ovulation and an 8% mortality rate in *E. bicolor* administered this peptide
146 preparation. While the current ovulation rate of 88% in *E. bicolor* using Ovaprim is
147 laudable, the 8% mortality rate is a concern to industry as these fish may be spawned
148 multiple times in a season and the potential exists for significant reductions in captive
149 broodstock populations. Development of broodstock can be laborious and costly, and
150 any reduction in mortality would be welcome. To this end, there is a clear need to
151 explore alternative peptides which may increase ovulation success and decrease the
152 mortality rate of valuable brood animals.

153 The objective of this study was to evaluate the efficacy of various dosages of
154 cGnRH IIa (D-Arg6-Pro9-Net) on spawning performance in *E. bicolor* and *E. frenatum*.

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155 Data on successful ovulation, fecundity, fertilization, and hatching success, as well as
156 embryo and larval morphometrics were analyzed to assess effectiveness of drug
157 preparations among treatments. Ovaprim was used as a benchmark to compare
158 spawning performance of cGnRH IIa, as it is currently the drug of choice for producers
159 of these species.

160 **Materials and Methods**

161 All experiments were performed in compliance with the US National Research
162 Council's Guide for the Care and Use of Laboratory Animals, the US Public Health
163 Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the
164 Care and Use of Laboratory Animals under the University of Florida's Institutional
165 Animal Care and Use Committee protocol #201609460. Two experiments were carried
166 out during this study evaluating the efficacy of cGnRH IIa as an induced spawning aid
167 for *E. bicolor* and *E. frenatum*. The experimental procedures for both cyprinid species
168 were similar although minor modifications occurred and are noted in the methods below.

169 *Experimental Design*

170 Reproductively competent broodstock were obtained from a commercial
171 ornamental aquaculture producer in Plant City, Florida, USA. Fish were transported to
172 the University of Florida's Tropical Aquaculture Laboratory (UF-TAL) where males and
173 females were separated into individual 1,030 L concrete tanks with an operational
174 volume of 380 L. During the experiment, vats were maintained with degassed flow-
175 through well water at 380 L/hour with supplementary aeration. All brood fish were
176 anesthetized in a 150 mg/L tricaine methanesulfonate (MS-222, Western Chemical Inc.,
177 Ferndale, WA) solution buffered with 300 mg/L sodium bicarbonate prior to
178 determination of total length (TL) and weight. Sexual maturity and gonadal development
179 were evaluated using minor pressure applied to the male's coelom to confirm the
180 presence of flowing milt. For anesthetized females, a silicone tube (0.76 mm inside
181 diameter and 1.65 mm outside diameter) was inserted into the oviduct after which
182 modest suction was applied to obtain an ovarian biopsy. The oocyte sample was
183 transferred to a Sedgewick Rafter Counting Cell and photographed under a dissecting
184 microscope with image capture capabilities. Fish selected for this study exhibited
185 secondary stage vitellogenic oocytes with ≥ 50 percent germinal vesicle migration

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186 (GVM). Bright and dark field digital photomicrographs were obtained for determination
187 of vitellogenic oocyte diameters as well as rates of GVM. Female fish used in the
188 experiment were individually placed into 19 L buckets (working volume 9.77 L) with
189 holes drilled along the circumference to permit exchange of water and placed within two
190 concrete tanks. Throughout the duration of the experiment, buckets were covered with a
191 lid except for removal during hormone administration or intermittent checks for
192 ovulation. A 120 L plastic tub was used to hold male fish. The tub was floated in a vat
193 near the female fish and holes drilled in the perimeter of the container allowed for water
194 exchange. During a pilot study, temperature, dissolved oxygen (DO), total ammonia
195 nitrogen (TAN), nitrite-nitrogen, pH, hardness ($CaCO_3$), and alkalinity ($CaCO_3$) were
196 tested in water originating from the buckets and surrounding vat area and revealed no
197 significant variation.
198 The cGnRH IIa (D-Arg6, Pro-Net, Figure 1) spawning aid used in this experiment
199 was synthesized by Genscript Labs (Piscataway, NJ) and shipped and stored in a
200 lyophilized state. The peptide was aliquoted into individual vials each containing 100 µg
201 of the hormone analogue and stored in a -80.0 ºC freezer prior to use. Fresh cGnRH IIa
202 peptide solutions were prepared for each spawning trial using methods described
203 herein. A carrier solution was prepared that consisted of the dopamine antagonist,
204 domperidone (Roadrunner Pharmacy, Phoenix, AZ) dissolved in propylene glycol,
205 yielding a final concentration of 10 mg/mL; equivalent to that of Ovaprim. Volumes of
206 1000, 500, and 250 µL of the carrier was pipetted into three individual 100 µg cGnRH
207 IIa vials to achieve desired concentrations of 50, 100, and 200 µg cGnRh IIa + 5 mg
208 domperidone when administered per kg fish weight at an injection volume of 0.5 µL/g.
209 Once mixed, experimental spawning aids were stored at 4.0ºC and used within 12 h. A
210 negative control treatment of pure propylene glycol was prepared alongside the cGnRH
211 IIa treatments. A positive control treatment of Ovaprim was used at its stock
212 concentration and administered at a dose of 10 µg/kg sGnRH IIIa + 5 mg/kg
213 domperidone (0.5 µL/g). Temporal replicates were utilized as dictated by broodstock
214 availability. In each trial a minimum of one replicate from each of the five experimental
215 treatments was included. An unbalanced design was used for the *E. bicolor* experiment
216 due to accessibility of broodstock. Six spawning trials were completed with 15 total

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217 replicates for Ovaprim, 50, and 100 µg/kg cGnRh IIa + 5 mg/kg domperidone
218 treatments. The additional experimental treatments of propylene glycol had 14
219 replicates while the 200 µg/kg cGnRh IIa + 5 mg/kg domperidone had 16 for *E. bicolor.*
220 Two spawning trials were completed for *E. frenatum,* with 3 replicates per treatment in
221 each trial. For brevity, the treatment groups 50, 100, 200 µg/kg cGnRH lla + 5 mg/kg
222 domperidone and Ovaprim will be referred to as 50, 100, 200 and sGnRH IIIa
223 respectively for the remainder of this study.
224 Experimental spawning treatments were administered to female broodstock via
225 intramuscular injections near the base of the dorsal fin using 100 µL Hamilton gastight
226 syringes (Hamilton Co., Reno, NV). Injection regimes incorporated in this study
227 emulated protocols currently used by the ornamental industry for *Epalzeorhynchos* spp.
228 production. The weight of females from time of biopsy was used to calculate a total
229 injection volume for the spawning aid or control. The total volume of drug to be
230 administered was divided between a 10% priming and 90% resolving injection which
231 were administered 6 h apart. All fish were individually anesthetized in a 150 mg/L
232 buffered tricaine solution before treatments were dispensed. Following injections,
233 broodfish were returned to their corresponding holding containers and allowed to
234 recover. To ensure spermiation at time of female ovulation, all males were injected with
235 0.5 µL/g Ovaprim immediately after female resolving injections. For both species male
236 fish were returned to the floating tubs within the vat post administration of spawning
237 drug. Approximately 10 - 12 h elapsed between ovarian biopsy and treatment injections.
238 Observations for ovulation success occurred hourly from 3 – 9 h post resolving
239 injection for *E. bicolor* and from 3 – 10 h post resolving injection for *E. frenatum*. During
240 ovulation sampling periods, female fish were netted out of their holding buckets and
241 light pressure was applied to the coelom to observe if any eggs were expelled. Female
242 fish were placed back into the treatment bucket and left undisturbed until the next
243 sampling time if ovulation did not occur. Females which expressed eggs after coelomic
244 palpation were anesthetized (150 mg/mL buffered tricaine) blotted dry and weighed.
245 Ovulated eggs were expelled into a plastic weigh boat and subsequently weighed to the
246 nearest 0.001 g. Spawns that weighed less than 0.2 g were not used in this experiment
247 and female fish were quickly returned back to their corresponding bucket. A single male

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248 was used to fertilize each collected spawn. Prior to fertilization, males were
249 anesthetized, blotted dry, milt was hand stripped, and collected in a 1 mL syringe. To
250 fertilize the eggs, milt was added to the weigh boat containing the eggs and carefully
251 mixed. Water (20 mL) from the hatching system was then added to the weigh boat to
252 activate the sperm and the mixture was allowed to fertilize for 30 seconds. A subsample
253 of embryos was then collected from the weigh boat for further analyses. A fertilized egg
254 subsample (N=50) was chosen haphazardly from each spawn and stocked into floating
255 150 mL screen bottomed (50 µm) containers in the recirculating hatching system. The
256 remaining embryos were stocked into 1 L suspended screen bottom (400 µm)
257 containers also housed within the hatching system. A subsample was collected from the
258 1 L floating containers 1.5 - 2 h post fertilization and visualized on a Sedgewick Rafter
259 Cell using a dissecting microscope. Digital photographs were taken of the embryos for
260 subsequent determination of fertilization success and egg morphometrics.

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

272 Fecundity was standardized by calculating the number of eggs spawned
273 (determined by weight) divided by the respective female fish's weight to obtain the
274 number of eggs per gram body weight. Mean fertilization success, embryo diameter,
275 hatching success, and larval notochord length were calculated for each individual
276 replicate. The percentage of secondary vitellogenic oocytes exhibiting GVM was
277 recorded following analysis of digital photomicrographs of ovarian biopsies. Mean egg
278 diameters (N = 44 ± 10 for *E. bicolor* and N = 49 ± 2 for *E. frenatum*) from each biopsy

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279 were measured from digital images. A subset of egg samples from *E. bicolor* (N = 10)
280 and *E. frenatum* (N = 5) each with a known weight, were used for gravimetric estimation
281 of egg quantity and subsequent estimations of fecundity. A representative subsample of
282 embryos (N = 72 ± 19 for *E. bicolor* and N = 62 ± 20 for *E. frenatum*) was
283 microscopically examined to determine fertilization success for each replicate. Up to 50
284 embryo diameters were measured for each replicate using methods previously
285 described (N = 49 ± 2 for *E. bicolor* and N = 48 ± 3 for *E. frenatum*). Hatch success was
286 calculated after correcting for individual spawn fertilization rates. For *E. bicolor*, mean
287 larval NL was calculated from a total of N = 398 larvae from four trials across all
288 treatments with successful hatch (N = 11 ± 4 larvae/spawn). For *E. frenatum*, mean
289 larval NL was calculated from a total of N = 142 larvae from two trials across all
290 treatments with successful hatch (N = 12 ± 4 larvae/spawn). Larval NL was measured
291 from the most anterior portion of the head to the tip of the notochord.
292 *Water Quality*
293 Water quality parameters were analyzed before and during spawning trials in the
294 broodstock holding and larval hatching systems. Dissolved oxygen (DO) and
295 temperature were measured using a YSI ProComm II meter (YSI Inc., Yellow Springs,
296 OH). Total ammonia nitrogen (TAN), nitrite-nitrogen, hardness ($CaCO_3$), alkalinity
297 ($CaCO_3$), and pH were measured using standard colorimetric assays according to the
298 manufacturer's protocols. (Hach Company, Loveland, CO).
299 *Data Analysis*
300 Normality and homoscedasticity were assessed for all data prior to statistical
301 testing. One-way ANOVA was used to evaluate data which satisfied these assumptions.
302 If assumptions of ANOVA were not met, data transformations were used or a one-way
303 Kruskal-Wallis test was used to analyze non-normal data. Analysis of Variance (ANOVA)
304 or Kruskal-Wallis tests were used to compare mean pre-trial oocyte diameters as well
305 as percent GVM for each treatment and confirmed there were no significant differences
306 among experimental populations prior to the study. The negative control treatment of
307 propylene glycol was not included in fecundity, fertilization success, embryo diameter,
308 hatching success, or larval length analyses due to complete lack of data for these
309 metrics for both species. For *E. frenatum* the Ovaprim treatment was not included in

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310 statistical analyses for all spawning metrics besides ovulation success and morality
311 status due to small sample size (N = 1). Ovulation data was categorized into two levels,
312 with successful ovulation = 1 and unsuccessful ovulation = 0. A logistic regression was
313 performed using the generalized linear model (GLM) function in the base package of
314 RStudio (family = binomial, V. 0.99.903 2015. RStudio: Integrated Development for R.
315 RStudio, Inc., Boston, MA), followed by an ANOVA to test for differences among the
316 categorical predictor variable (hormone treatment). A post hoc pairwise comparison was
317 performed using a Tukey Honestly Significant Difference test. A *P*-value of ≤ 0.05 was
318 considered significant for all statistical analyses. Mortality data was analyzed using
319 similar methods to evaluating ovulation success, with live fish = 1 and dead fish = 2. All
320 data are presented as mean ± standard deviation. All statistical analyses were
321 performed using JMP Pro v13.0.0 (SAS Institute, Cary, NC) and RStudio.

322 **Results**

323 *Epalzeorhynchos bicolor*

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

336 Unfertilized eggs averaged 2,486 ± 155 eggs/g of material spawned for *E.*
337 *bicolor*. Fecundity (eggs/g bodyweight) was determined to be consistent among all
338 treatments of cGnRH IIa and sGnRH IIIa (*P* = 0.967) with a relatively narrow range of
339 440 ± 95 to 466 ± 128 eggs/g bodyweight for the 200 and 50 treatments, respectively
340 (Table 1). The mean recorded mass of eggs per spawn per female was 4.47 ± 3.22 g or

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341 11,123 ± 8,000 eggs. Mean fertilization success was high (>90%) for all hormone
342 treatments throughout the experimental period with no significant differences detected
343 (P = 0.267) (Table 1).

344 Embryo diameters, were found to be relatively uniform among all spawning
345 treatments (P = 0.572) with the largest difference of only 0.280 mm observed between
346 the 100 and sGnRH IIIa treatments (Table 1). While the highest hatch success was
347 observed in the 50 treatment (62 ± 25%), all hormonal treatments performed similarly
348 for this metric (P = 0.299). Larval morphometric analysis indicated comparable NL
349 among cGnRH IIa and sGnRH IIIa treatments upon hatch (P = 0.478). Mean NL values
350 differed by only 0.101 mm with a range of 3.266 ± 0.155 - 3.365 ± 0.131 mm.
351 Broodstock mortality at 24 hours was affected by the experimental drug injected (P =
352 0.048) however post hoc pairwise analysis did not demonstrate differences in mortality
353 rate among treatments ($P \geq 0.073$). The greatest losses occurred in the 200 treatment
354 group, where 5 out of the 16 fish died.

355 Mean water quality parameters recorded for the experimental flow through
356 systems were: TAN 0.09 ± 0.11 mg/L, nitrite-nitrogen 0.00 ± 0.00 mg/L, pH 8.00 ± 0.00,
357 hardness ($CaCO_3$) 473.91 ± 36.56 mg/L, alkalinity ($CaCO_3$) 195.43 ± 13.45 mg/L,
358 temperature 25.30 ± 0.14 °C and DO 7.92 ± 0.20 mg/L. Mean water quality parameters
359 recorded for the recirculating hatching system were: TAN 0.06 ± 0.10 mg/L, nitrite-
360 nitrogen 0.01 ± 0.03 mg/L, pH 7.86 ± 0.24, hardness ($CaCO_3$) 131.91 ± 16.27 mg/L,
361 alkalinity ($CaCO_3$) 78.17 ± 13.45 mg/L, temperature 25.28 ± 1.18 °C and DO 8.09 ± 0.52
362 mg/L.

363 *Epalzeorhynchos frenatum*

364 Over the course of the study 30 female *E. frenatum* were administered
365 experimental treatments. A total of 24 of those fish were injected with sGnRH IIIa or
366 cGnRH IIa. The mean total length of females utilized during the experiment was 131 ± 9
367 mm with a pretrial weight of 21.31 ± 2.85 g. Percentage of oocytes undergoing GVM
368 was comparable among females in all treatments prior to experimentation (P = 0.670). A
369 mean total of 97 ± 2% of oocytes obtained from ovarian biopsies demonstrated GVM
370 and had a mean diameter of 1.033 ± 0.099 mm. Oocyte diameters were similar among
371 treatments prior to injection (P = 0.888). *E. frenatum* treated with sGnRH IIIa were

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372 excluded from statistical tests comparing fecundity, fertilization success, hatch success,
373 embryo diameter, and larval notochord length due to a small sample size (N = 1).
374 Reported data are mean values from a single replicate and should be interpreted as
375 such. Ovulation success varied significantly among treatments ($P < 0.001$) with the 50
376 and 100 treatments outperforming the sGnRH IIIa (Table 2). Mean ovulation success of
377 100% was reported for female *E. frenatum* injected with the 50 treatment (Table 2).

378 Unfertilized eggs averaged 2,453 ± 186 eggs/g of spawned material for *E.*
379 *frenatum*. Fecundity was comparable among all treatments of cGnRH IIa ($P = 0.594$)
380 with a mean range of 346 ± 77 to 421 ± 142 eggs/g bodyweight for fish in the 200 and
381 50 treatments, respectively (Table 2). The mean recorded mass of an ovulated spawn
382 per female was 3.48 ± 1.14 g or 8,544 ± 2,789 eggs. Mean fertilization success was
383 high (>95%) for all cGnRH IIa hormone treatments throughout the experimental period
384 with no significant differences detected ($P = 0.418$, Table 2). Embryo diameters, hatch
385 success, NL, and broodstock mortality were similar among cGnRH IIa treatments ($P \geq$
386 0.470, Table 2).

387 Mean water quality parameters observed in the broodstock flow through systems
388 were: TAN 0.00 ± 0.00 mg/L, nitrite-nitrogen 0.00 ± 0.00 mg/L, pH 8.00 ± 0.00,
389 hardness ($CaCO_3$) 461.70 ± 0.00 mg/L, alkalinity ($CaCO_3$) 213.75 ± 12.09 mg/L,
390 temperature 24.35 ± 0.21 °C, and DO 7.75 ± 0.35 mg/L. Mean water parameters
391 recorded for the recirculating hatching system were: TAN 0.00 ± 0.00 mg/L, nitrite-
392 nitrogen 0.00 ± 0.00 mg/L, pH 8.00 ± 0.00, hardness ($CaCO_3$) 111.15 ± 12.09 mg/L,
393 alkalinity ($CaCO_3$) 76.95 ± 12.09 mg/L, temperature 25.40 ± 0.42 °C, and DO 7.50 ±
394 0.07 mg/L.

395 **Discussion**

396 The successful implementation of induced spawning protocols in the ornamental
397 aquaculture industry requires the identification of multiple spawning aids to
398 accommodate the highly diverse taxa and hormone forms represented. Results of this
399 study indicate equivalent or superior efficacy for cGnRH IIa compared to the established
400 standard spawning aid, sGnRH III, with respect to all parameters evaluated for both *E.*
401 *bicolor and E. frenatum*. This provides evidence for an alternative spawning aid that can
402 be used to induce spawning in ornamental Cyprinids.

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403 Analysis of oocyte diameter and percent GVM obtained from biopsy samples
404 indicated all test subjects were of similar reproductive condition prior to administration of
405 hormonal therapies for both *E. bicolor* and *E. frenatum* ($P \geq 0.390$). The comparable
406 condition of the brood fish helped to exclude these factors from confounding results and
407 reduced the chance of type I error. Ovulation success varied significantly among
408 treatments for both *E. bicolor* and *E. frenatum* ($P < 0.05$). Ovulation success ranged
409 from 0 - 93% for *E. bicolor* in this experiment (Table 1). Pairwise analyses indicated,
410 significant differences in mean ovulation success between the propylene glycol negative
411 control and all other hormonal treatments ($P < 0.05$, Table 1). Conversely, similar
412 ovulation performance was observed for *E. bicolor* among all hormonal therapies ($P \geq$
413 0.532). These results suggest that all dosages of cGnRH IIa and sGnRH IIIa tested in
414 this investigation were equally effective at inducing ovulation for *E. bicolor*. In *E.*
415 *frenatum*, ovulation success among all treatments ranged from 0 -100% with a range of
416 17 - 100% among all treatments administered hormones (Table 2). sGnRH IIIa and
417 propylene glycol injected *E. frenatum* exhibited statistically similar performance ($P =$
418 0.918) with no recorded ovulation in the negative control and successful ovulation by
419 only one fish in the sGnRH IIIa. Ovulation success was comparable among all dosages
420 of cGnRH IIa ($P \geq 0.471$), while 50 and 100 treatments varied when compared to
421 ovulation success of sGnRH IIIa treated fish ($P \leq 0.021$). Post hoc analysis also
422 indicated ovulation success between the 200 and sGnRH IIIa injected fish to be similar
423 ($P = 0.122$), however this may be a result of small experimental sample size resulting in
424 type II error. The lack of ovulation response demonstrated by *E. frenatum* following
425 sGnRH IIIa administration was surprising as this spawning aid is commonly used for
426 successful reproduction of this species by ornamental fish producers. A survey of
427 sGnRH IIIa reported induced ovulation of 75% in *E. frenatum* females (Jeffrey E. Hill et
428 al., 2009) while 100% ovulation was achieved by researchers utilizing the same dose of
429 0.5 µl/g (Islami et al., 2017). Differences in hormone dosage or gonad stage may
430 explain the discrepancy in ovulation rate observed between this study and commercial
431 production scenarios, however, this experiment followed dosing guidelines (0.5 µl/g)
432 recommended by the manufacturer. The dosages of cGnRH IIa used in this study were
433 chosen due to the high success of ovulation induction Quiniou et al. (2014) recorded for

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434 Channel Catfish *I. punctatus* at 100 µg/kg. This experiment bracketed the verified 100
435 µg/kg concentration of cGnRH IIa between an upper and lower limit of 50 µg/kg and 200
436 µg/kg. High ovulation success, ranging from 67-100%, was achieved in *E. frenatum* that
437 were hormonally induced with all doses of cGnRH IIa.

438 The number of eggs per gram bodyweight was comparable across all hormonal
439 treatments for both species ($P \geq 0.594$). Interestingly, the 50 treatment for both *E.*
440 *bicolor* and *E. frenatum* had numerically the highest rate of ovulation (93% and 100%,
441 respectively) and also the highest standardized mean number of spawned oocytes (466
442 ± 147 and 421 ± 142, respectively). While egg production data for both species across
443 all cGnRH IIa treatments was statistically comparable, further investigations which
444 examine lower doses are justified as the lowest dose of 50 in both species resulted in
445 the maximum values recorded for fecundity and ovulation success. It is possible that
446 cGnRH IIa doses lower than those used in this experiment, may yield superior results
447 for response variables of interest and may reduce costs. As postulated in a previous
448 study by DiMaggio et al. 2013, doses of spawning aids (GnRH) in excess of species
449 specific requirements may result in desensitization of the pituitary manifesting in
450 reduced LH secretion. Luteinizing hormone is directly responsible for oocyte maturation
451 and ovulation (Mylonas & Zohar, 2007) making any impediment to its release
452 detrimental to spawning performance.

453 The addition of dopamine antagonists in hormonal spawning therapies aids in
454 blocking the neurotransmitter dopamine which has inhibitory effects on the progression
455 of hormonal cascades within the HPG axis. Particular species are more affected by
456 dopaminergic inhibition than others; LH release in channel catfish has been found to be
457 relatively unaffected by dopamine (Quiniou et al., 2014) while members of the cyprinid
458 family generally exhibit a more pronounced inhibitory effect on LH in response to
459 dopamine (Chang et al., 1983; Podhorec & Kouril, 2009; Yaron et al., 2009). In a study
460 using both *E. bicolor* and *E. frenatum*, greater rates of ovulation success were achieved
461 with the addition of a dopamine antagonist (reserpine) to the hormone LHRHa
462 (luteinizing hormone releasing hormone analog) (Shireman & Gildea, 1989). It is likely
463 that the ovulation rates observed across all cGnRH IIa doses in both species was
464 positively impacted by the inclusion of domperidone in the carrier solution. While the

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465 concentration of domperidone used in this study was selected to mirror that of Ovaprim,
466 further investigations which evaluate efficacy and optimal dosing regimens of various
467 dopamine antagonists would be of great interest and could help to further induced
468 spawning protocols for these two valuable cyprinid species.

469 Fertilization success among hormonal treatments was consistently high (> 90%)
470 with no significant differences detected among performance in all treatments ($P \geq$
471 0.267). Mean aggregated fertilization rates of 91 ± 11% were documented for *E. bicolor*
472 and 97 ± 6% for *E. frenatum.* Spawning drugs administered to both species in this
473 experiment resulted in adequate oocyte development. The high fertilization success and
474 low variation in embryo sizes observed among hormone treatments ($P \geq 0.484$) for both
475 species in this study is indicative of a proven induction spawning protocol. Hatch
476 success, although similar across treatments in *E. bicolor* and *E. frenatum* ($P \geq 0.299$),
477 was considerably lower and more variable than aggregated mean fertilization rates with
478 pooled mean hatch rates across hormonal treatments calculated at 48 ± 32% and 36 ±
479 31%, respectively. The experimental design used in this study called for a subsample of
480 embryos to be segregated for determination of hatch. This manual removal of fertilized
481 eggs could have increased prehatch mortality as developing embryos are sensitive to
482 handling and can be prone to mechanical injury (Small & Chatakondi, 2006). Although
483 hatching success during both experiments appeared to be somewhat reduced when
484 compared to those delineated by Islami et al. (2017), similar results across all
485 treatments suggest hatching success to be independent of the spawning aids and
486 doses examined in this study. Commercial production practices for *Epalzeorhynchos*
487 spp. may yield hatch success superior to those reported herein as commercial protocols
488 typically involve less handling and manipulation of developing embryos.

489 Female broodstock mortality associated with treatment varied significantly in *E.*
490 *bicolor* ($P = 0.048$) but not *E. frenatum* ($P = 0.567$). In *E. frenatum* only two out of the
491 total 24 fish injected with spawning aids died within 24 h of treatment. These individuals
492 belonged to the sGnRH IIIa and 200 treatments. Post hoc analysis of *E. bicolor* data did
493 not clarify disparities in rate of mortality of the logistic model ($P \geq 0.073$). Within the 24 h
494 post resolving injection, 31% of *E. bicolor* treated with 200 died while the next highest
495 rate occurred in sGnRH IIIa injected fish at 20%. Similar frequencies of mortality were

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496 observed by Hill et al. (2009) in a survey of sGnRH IIIa with mortality rates in *E. bicolor*
497 of 35.7% and 14.8% in *E. frenatum.* Administration of spawning aids via injection can
498 compromise the epithelium of broodfish, potentially resulting in opportunistic bacterial or
499 fungal infections and osmoregulatory disruption. Alternative experimental methods for
500 administration of spawning hormones have been used for *E. frenatum* with comparable
501 ovulation success. The hormone sGnRH IIIa was dissolved in solution with dimethyl
502 sulfoxide (DMSO) and applied directly to the gills of *E. frenatum* with 78% ovulation
503 success and no mortalities in contrast to the 100% ovulation and 18% morality reported
504 following delivery of the spawning aid via intramuscular injection (IM) (Hill et al., 2005).
505 Furthermore, Hill et al. 2009 postulated that observed mortality in the survey of Ovaprim
506 was largely believed to be attributed to fish handling, injection procedure, and
507 diminished water quality in holding systems. Mortality in the current study was likely due
508 to prolonged handling or damage to the epithelium as a result of strip spawning
509 practices. Investigations which explore additional application routes for cGnRH IIa
510 would be prudent to diminish or eliminate mortality of valuable brood fish.
511 Spawning performance of cGnRH IIa was found to be comparable to sGnRH IIIa
512 in all variables investigated in this study for *E. bicolor*. Results from the *E. frenatum*
513 experiment suggest the 50 and 100 µg/kg cGnRH IIa treatments were superior to 10
514 µg/kg sGnRH IIIa with respect to ovulation success. Based on data from this study we
515 can recommend a dose of 50 µg/kg cGnRH IIa for induction spawning of the two
516 *Epalzeorhynchos* species evaluated. Lack of clear dose response supports the use of
517 the minimum concentration tested. Due to satisfactory observations in relevant
518 spawning criteria and broad range of effective concentrations, cGnRH IIa appears to be
519 a viable option for induced spawning of *E. bicolor* and *E. frenatum*. Additional
520 examinations of effective dosages and alternative application routes may help to
521 improve the efficacy of cGnRH IIa as a spawning aid for the aquaculture industry.
522 Enrolling cGnRH IIa in an investigational new animal drug (INAD) program or
523 proceeding directly to drug indexing may be justified as this study and a number of
524 others have demonstrated its effectiveness as an induced spawning aid.
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535 **Data Availability**

536 Data may be made available by request to interested parties. For inquiries,
537 please email Dr. Matthew DiMaggio at mdimaggi@ufl.edu.

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Figure Captions

Figure 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.

Figure 2. A) Redtail Sharkminnow *Epalzeorhynchos bicolor* B) Rainbow Sharkminnow *Epalzeorhynchos frenatum*. Photos courtesy of the Florida Tropical Fish Farms Association Photographer Dr. Harry Grier.

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Tables

Table 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 ($P \leq 0.05$).

Treatment	Ovulation (%)	Eggs Per Gram Body Weight	Fertilization Success (%)	Embryo Diameter (mm)	Hatch Success (%)	Notochord Length (mm)
Propylene Glycol	0^{b}	-	-	-	-	-
sGnRH IIIa						
10 μg/kg	73^{a}	457 ± 187	96 ± 7	2.602 ± 0.165	44 ± 32	3.266 ± 0.155
cGnRH IIa						
50 μg/kg	93^{a}	466 ± 128	91 ± 8	2.360 ± 0.146	62 ± 25	3.365 ± 0.131
100 μg/kg	80^{a}	460 ± 147	92 ± 14	2.322 ± 0.158	42 ± 35	3.343 ± 0.171
200 μg/kg	81^{a}	440 ± 95	90 ± 12	2.334 ± 0.152	42 ± 35	3.349 ± 0.115

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Table 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 GnRHa treatments included the dopamine antagonist domperidone at a concentration of 5 mg/kg. Different superscript letters within a column denotes statistically significant differences ($P \leq 0.05$). An * indicates descriptive data that was not included in statistical analyses due to a limited sample size (N = 1).

Treatment	Ovulation (%)	Eggs Per Gram Body Weight	Fertilization Success (%)	Embryo Diameter (mm)	Hatch Success (%)	Notochord Length (mm)
Propylene Glycol	0^{b}	-	-	-	-	-
sGnRH IIIa						
10 µg/kg	17^{bc}	241*	100*	3.914*	68*	3.375*
cGnRH IIa						
50 µg/kg	100^{a}	421± 142	95 ± 9	2.806 ± 0.695	35 ± 42	3.566 ± 0.133
100 µg/kg	83^{a}	390 ± 90	100 ± 0	3.161 ± 0.245	26 ± 15	3.407 ± 0.202
200 µg/kg	67^{ac}	346 ± 77	98 ± 3	3.072 ± 0.301	52 ± 29	3.422 ± 0.171

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	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 Analog (sGnRH IIIa)	pGlu-	His-	Trp-	Ser-	Tyr-	DArg-*	Trp-	Leu-	Pro-	Net*	
Chicken Analog (cGnRH IIa)	pGlu-	His-	Trp-	Ser-	His-	DArg-*	Trp-	Tyr-	Pro-	Net*	

Figure 1.

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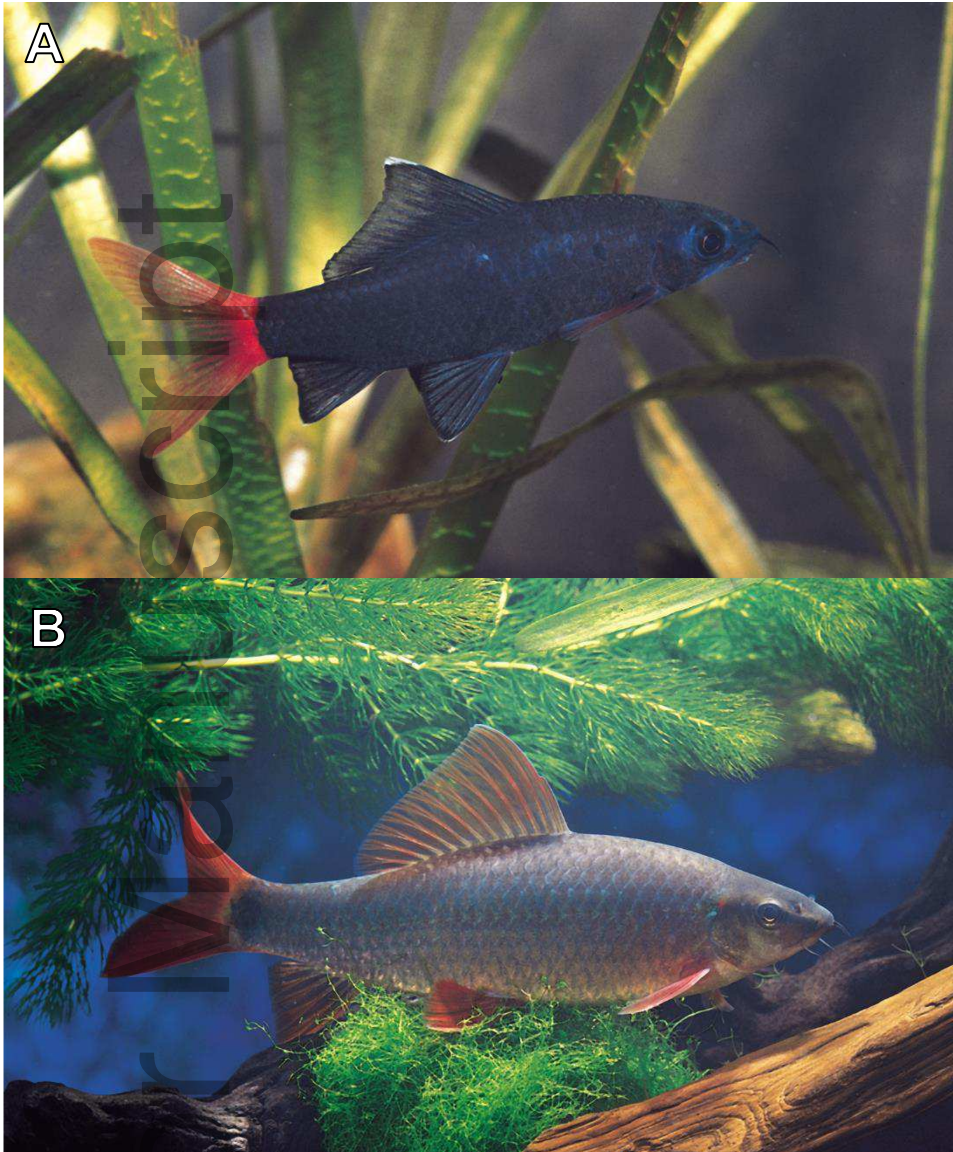

Figure 2.

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