

Revised

1 **Production of live larvae following *in vitro* maturation of zebrafish oocytes**

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8 **Abstract**

9 This study aimed to assess the effects of carp pituitary extract (CPE), follicle
10 stimulating hormone (FSH) and luteinizing hormone (LH) on zebrafish oocyte
11 maturation and the ability of these mature oocytes to be fertilized and developed until
12 hatching. Stage III follicles were matured in eight treatments: five concentrations of
13 CPE (16, 32, 48, 64 and 80 µg/mL), one of FSH (0.5 µg/mL), one of LH (0.5 µg/mL),
14 or one combination of FSH (0.5 µg/mL) and LH (0.5 µg/mL). Maturation rates in CPE
15 treatments were 12.8% (16 µg/mL), 24.8% (32 µg/mL), 27.0% (48 µg/mL), 22.7% (64
16 µg/mL) and 9.6% (80 µg/mL); in FSH was 15.7% (0.5 µg/mL), in LH was 31.8% (0.5
17 µg/mL) and in FSH (0.5 µg/mL) combined with LH (0.5 µg/mL) it was 50.4%. *In vitro*
18 fertilization was performed in all treatments; however, only the treatment combining
19 FSH and LH resulted in fertilized oocytes. After maturation using FSH combined with
20 LH, the cleavage rate was 33.3% and hatching rate of live larvae was 20.0%. These
21 results showed that FSH combined with LH was effective in IVM of zebrafish oocyte.

22 Keywords: *in vitro* maturation; *Danio rerio*; ovarian follicles; carp pituitary; pituitary
23 gonadotropins.

24 **1. Introduction**

25 Maturation process, in teleost fish, is characterized by endocytosis reduction or
26 interruption, meiosis resumption, germinal vesicle breakdown (GVBD) and cortical
27 alveoli formation [1]. *In vitro* maturation (IVM) of early follicles provides an alternative
28 for generating mature oocytes; and a successful protocol would help to understand the
29 mechanism of regulating oogenesis and folliculogenesis. So far, only a few studies have
30 assessed the ability of *in vitro* matured fish oocytes to develop into hatching embryos
31 after fertilization [2-4]. However, these IVM protocols examined only the 17 α ,20 β -
32 dihydroxy-4-pregnen-3-one (DHP) as a maturation-inducing hormone.

33 As in other vertebrates, reproductive process in teleost is mainly regulated by the
34 brain-pituitary-gonad axis. Carp pituitary extract (CPE) is widely used to induce
35 spawning in fish farms, especially in developing countries. Studies have evaluated the
36 efficiency of CPE to induce oocyte maturation *in vivo* [5] and *in vitro* in fish species [6].
37 CPE contains both gonadotropins FSH and LH; and gonadotropins receptors are located
38 in the theca layer surrounding the follicle [7]. However, under inappropriate cultivation
39 conditions, the follicle epithelial surface may be impermeable to gonadotropin
40 hormones [8]. Therefore, medium composition, pH, osmolality and incubation period
41 are crucial for successful gonadotropin responsiveness.

42 Pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating
43 hormone (FSH), are among the major regulators during oocyte maturation. A study in
44 rainbow trout reported that follicular maturational competence acquisition was
45 associated to an increased expression of FSH receptor [9]. Fish gonadotropins are not
46 easily available; hence, hormones from mammalian sources have been suggested as the
47 alternative in fish research [10]. Some researchers have examined the effect of human
48 chorionic gonadotrophin (hCG) on IVM medium; they concluded that the hCG

49 treatment increased follicular diameter on early stage of zebrafish ovarian follicles [11]
50 and promoted the maturation of zebrafish stage III oocytes [12]. Currently, there has
51 been no reported usage of FSH or/and LH in IVM protocol of zebrafish follicles.

52 In this context, the present study aimed to assess the effects of CPE, FSH and
53 LH on oocyte maturation and the ability of these mature oocytes to be fertilized and
54 developed until hatching. *Danio rerio*, popularly known as zebrafish, was chosen for its
55 ability to produce a large number of fully grown oocytes and its popularity as a
56 vertebrate model organism for developmental studies.

57 **2. Materials and Methods**

58 *2.1 Zebrafish maintenance and ethical statement*

59 Mature zebrafish were kept in filtered and aerated 40 L fish tanks at $27\pm 2^{\circ}\text{C}$ (pH
60 7.2-7.4) with a light/dark cycle of 14/10 h. Fish were fed four times a day with Tetramin
61 dry flake fish food (Tetra, Germany).

62 Experimental procedures and protocols described in this study were approved by
63 the Ethics Committee of our Institution (Federal University of Rio Grande do Sul).

64 *2.2 Collection of immature zebrafish oocytes at stage III*

65 Ovaries were collected from adult females anaesthetized with a lethal dose of
66 tricaine methane sulfonate (0.6 mg/mL) for 5 min and then decapitated. Ovaries were
67 placed into 90% Leibovitz L-15 medium (pH 9.0) containing 20% fetal bovine serum
68 (FBS) and 100 $\mu\text{g/mL}$ gentamycin. This solution was isosmotic with ovarian fluid (0.29
69 Osm/kg).

70 Follicles were separated by gentle pipetting of the ovaries and immature oocytes
71 at late stage (stage III) were collected. Only those follicles which displayed the

72 following characteristics were selected for culture: 1) 0.65–0.69 mm in diameter, 2)
73 intrafollicular oocyte with dark ooplasm and distinct germinal vesicle (visible under
74 transmitted light).

75 *2.3 Experiment 1 - In vitro maturation time*

76 The aim of Experiment 1 was to determine the length of maturation time.

77 IVM culture was an adaptation of a previous published protocol [3].

78 Stage III follicles were placed in wells of 12-well plates containing 4 mL of 90%
79 L-15 medium supplemented with 100 µg/mL gentamycin, 20% FBS and one of the
80 maturation induction treatments described below.

81 Carp pituitary extract (Danúbio®) was weighted, macerated and homogenized in
82 L-15 medium. CPE concentration treatments were 16, 32, 48, 64 and 80 µg/mL.

83 FSH concentration was 0.5 µg/mL; LH concentration was 0.5 µg/mL; and
84 finally the concentration of FSH combined with LH was 0.5 µg/mL each.

85 Ten follicles per well were cultured for 24 h at 27°C. The ability of stage III
86 follicles to mature (stage V) was assessed every two hours. Follicles were considered
87 mature when the germinal vesicle disappeared completely and oocytes became
88 translucent (GBVD). Immature oocytes remained opaque and with their germinal
89 vesicle intact.

90 *2.4 Experiment 2 – Effects of carp pituitary extract (CPE) and pituitary* 91 *gonadotropins (LH and FSH) on in vitro maturation and in vitro fertilization*

92 The aim of Experiment 2 was to assess the effects of CPE, LH and FSH on *in*
93 *vitro* maturation and *in vitro* fertilization of zebrafish stage III follicles.

94 IVM culture was the same previously described in Experiment 1.

95 According to the data obtained in Experiment 1, CPE treatments (16, 32, 48, 64
96 and 80 $\mu\text{g}/\text{mL}$) were cultured for 18 h and pituitary gonadotropin treatments (0.5 $\mu\text{g}/\text{mL}$
97 of FSH; 0.5 $\mu\text{g}/\text{mL}$ of LH; and 0.5 $\mu\text{g}/\text{mL}$ of FSH combined with 0.5 $\mu\text{g}/\text{mL}$ of LH)
98 were cultured for 20 h.

99 Maturation rate was assessed as described in Experiment 1.

100 Follicular membrane of matured follicles at stage V was removed with a pair of
101 delicate forceps, and the oocytes were put into a culture dish containing 90% L-15
102 medium (pH 9.0). Two zebrafish males were euthanized by decapitation following
103 lethal dose of tricaine methane sulfonate (0.6 mg/mL) for 5 min. Testes were collected
104 from each male and placed in phosphate buffered saline (PBS), where sperm was
105 obtained by tearing the testes with the use of forceps and a needle. Immediately after
106 collection, sperm was activated by suspending 100 μL of sperm in 2 mL of 90% L-15
107 medium containing 10 defolliculated oocytes. After 1 min, inseminated oocytes were
108 washed three times in 90% L-15 medium, and incubated at 27°C in 2 mL 90% L-15
109 medium (pH 9.0). Oocytes were observed under a microscope 2 h following
110 insemination to assess cleavage rate and once again 72 h after insemination for hatching
111 rate. Cleavaged embryos were considered fertilized.

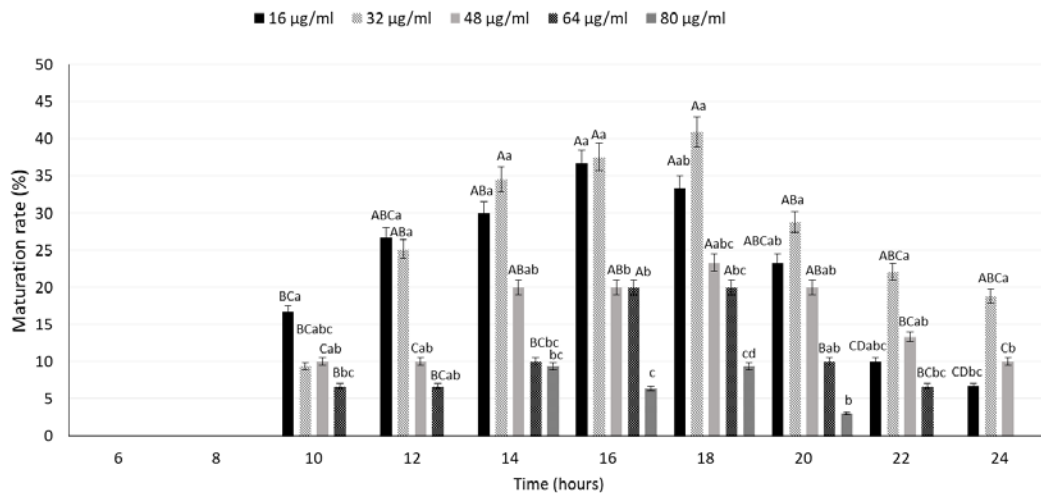
112 2.5 Statistical analysis

113 Experimental groups were composed by at least 100 oocytes in each group, and
114 the *in vitro* maturation and fertilization were done in triplicate. The distribution of the
115 data was evaluated and an abnormality was found even after transformation. The data
116 was analyzed using Chi-square and Kruskal-Wallis tests of the Statistical Analysis
117 System (SAS Institute version 9.0).

118 3. Results

119 3.1 Experiment 1 - In vitro maturation time

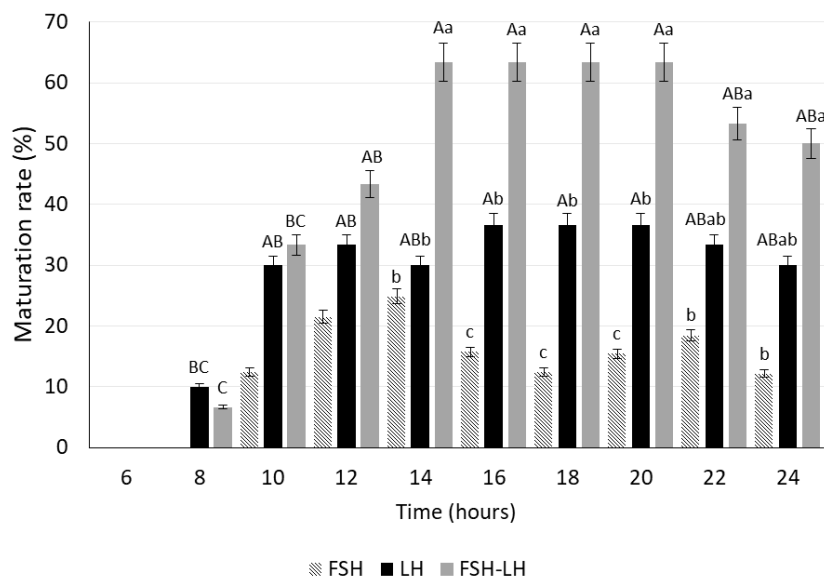
120 As shown in Figure 1, all CPE concentrations began oocyte maturation only 10 h
 121 after culture. The highest CPE maturation rate was observed in treatment 32 µg/mL at
 122 18 h of culture (40.9±4.5%, Figure 1). However, there was no difference among 14
 123 (34.5%), 16 (37.6%) and 18 h (40.9%) of IVM using 32 µg/mL of CPE. After 18 h of
 124 culture, all treatments presented oocyte degeneration. Oocyte degeneration was
 125 characterized by cellular content overflow, oocyte membrane deformity and rupture.



126
 127 **Fig. 1.** Average maturation rate of oocytes exposed to different carp pituitary extract
 128 (CPE) concentrations (16, 32, 48, 64 and 80 µg/mL; n=100 oocytes per group) at 6, 8,
 129 10, 12, 14, 16, 18, 20, 22 and 24 h of IVM at 27°C. Different lowercase letters indicate
 130 significant difference among treatments at the same time (hours) of culture (P<0.001).
 131 Different uppercase letters indicate significant difference among time (hours) of culture
 132 in the same treatment (P<0.001).

133 A significantly higher maturation rate (63.3±3.33%) was observed in oocytes
 134 that were cultured using the combination of FSH and LH at 14, 16, 18 and 20 h (Figure
 135 2). This result indicated that FSH and LH act synergistically in stimulating the

136 maturation of stage III oocytes. Maturation process started after 8 h of culture, and the
 137 maturation rate started to decrease after 20 h (Figure 2).



138

139 **Fig. 2.** Average maturation rate of oocytes exposed to pituitary gonadotropins
 140 treatments (0.5 µg/mL of FSH; 0.5 µg/mL of LH; and 0.5 µg/mL of FSH combined
 141 with 0.5 µg/mL of LH; n=100 oocytes per group) for 24 h of IVM at 27°C. Different
 142 lowercase letters indicate significant difference among treatments at the same time
 143 (hours) of culture (P<0.001). Different uppercase letters indicate significant difference
 144 among time (hours) of culture in the same treatment (P<0.001).

145 *3.2 Experiment 2 – Effects of carp pituitary extract (CPE) and pituitary*
 146 *gonadotropins (LH and FSH) on in vitro maturation and in vitro fertilization*

147 Table 1 shows maturation rate of oocytes exposed to different CPE
 148 concentrations (16, 32, 48, 64 and 80 µg/mL) after 18 h of culture; however, there was
 149 no significant difference among CPE treatments. There was no success in *in vitro*
 150 fertilization.

151 **Table 1.** Embryo development of zebrafish oocytes exposed to different concentrations
 152 of carp pituitary extract (CPE) during maturation.

Treatment	Number of Oocytes	Number of Matured Oocytes (% per Oocyte) ¹	Number of Cleaved Embryos (% per Oocyte)	Number of Live Larvae (% per Oocyte)	Percentage of Live Larvae / Cleaved
CPE 16 $\mu\text{g/mL}$	103	13 (12.8 \pm 4.0%)	0	0	0
CPE 32 $\mu\text{g/mL}$	111	27 (24.8 \pm 3.1%)	0	0	0
CPE 48 $\mu\text{g/mL}$	104	29 (27.0 \pm 2.8%)	0	0	0
CPE 64 $\mu\text{g/mL}$	100	23 (22.7 \pm 3.9%)	0	0	0
CPE 80 $\mu\text{g/mL}$	108	10 (9.6 \pm 2.6%)	0	0	0

153 ¹Not significant (P > 0.001)

154 As shown in Table 2, maturation rate in the treatment using the combination of
 155 FSH and LH was significantly higher (50.4 \pm 1.93%) when compared to the treatments
 156 using each hormone separately (FSH 15.7 \pm 1.46%, LH 31.8 \pm 1.88%). Only oocytes
 157 matured using the combination of FSH and LH were able to fertilize and hatch.
 158 Cleavage rate was 33.3 \pm 2.7% and hatching rate of live larvae was 20 \pm 0.4%.

159 **Table 2.** Embryo development of zebrafish oocytes exposed to pituitary gonadotropins
 160 (LH and FSH) during maturation.

Treatment	Number of Oocytes	Number of Matured Oocytes (% per Oocyte) ¹	Number of Cleaved Embryos (% per Oocyte)	Number of Live Larvae (% per Oocyte)	Percentage of Live Larvae / Cleaved
FSH	127	20 (15.75 \pm 1.46%) ^c	0	0	0
LH	129	41 (31.78 \pm 1.88%) ^b	0	0	0
FHS+LH	147	74 (50.40 \pm 1.93%) ^a	49 (33.3 \pm 2.7%)	29 (20.0 \pm 0.4%)	59.4

161 ¹Different letters indicate significant difference among treatments in the same column
 162 (P < 0.001).

163 **4. Discussion**

164 This is the first study that assesses the effects of FSH and LH on *in vitro*
165 maturation (IVM) of stage III zebrafish ovarian follicles. The results showed that stage
166 III zebrafish ovarian follicles could be matured *in vitro* for 20 h containing 0.5 µg/mL
167 of FSH and 0.5 µg/mL of LH. In addition, FSH combined with LH was the only
168 treatment that resulted in oocytes fertilized and hatched. However, the time required (20
169 h) for maturation using FSH and LH was longer when compared to results published
170 using DHP (1 µg/mL), when stage III follicles matured into stage V in just 4.5 hours
171 [3,4]. DHP is a specific hormone of fish in the maturation phase; therefore, a quicker
172 action on oocyte maturation is expected. However, the cleavage (34%) and hatching
173 (20%) rates obtained in the present study are lower than the cleavage (45%) and
174 hatching (33%) rates obtained with DHP by Seki et al. (2008) [3]. Although the
175 maturation conditions applied in our study were similar to those used by Seki et al.
176 (2008) [3], the serum source used in it was different. Furthermore, these authors
177 reported that the addition of 0.5% bovine serum albumin (BSA) to a maturation medium
178 increased significantly cleavage and hatching rates to 70% and 63%, respectively.
179 Culture media are usually supplemented with different types of serum, such as fetal
180 bovine serum (FBS), estrous cow serum (ECS) or bovine serum albumin (BSA). Serum
181 is an essential component of cell culture methodology, providing complex biological
182 molecules, for example, hormones, growth factors, attachment factors, in addition to
183 several low molecular weight nutrients. In the present study, maturation medium was
184 supplemented with 20% of FBS unlike Seki et al. (2008) [3], whom supplemented the
185 medium with 0.5% of BSA. A previous study demonstrated that FBS is a superior
186 serum supplement compared to the often used 0.8% BSA for *in vitro* maturation (IVM)
187 of bovine and hamster oocytes [13]. However, IVM medium containing FBS increased
188 total lipid content and resulted in higher lipid accumulation in oocytes when compared

189 to media with BSA [14]. Furthermore, Del Collado et al. (2016) [14] showed that
190 lowering the FBS concentration to 5% reduces lipid accumulation in embryos without
191 adversely affecting oocyte maturation and embryo development. Therefore, we suggest
192 that the addition of low serum concentrations, such as 0.5% BSA or 5% FBS, instead of
193 20% FBS, to the IVM medium containing FSH and LH should be tested in order to
194 improve maturation, cleavage and hatching rates.

195 The proposal to use CPE was due to its easy handling and storage, since it does
196 not need refrigeration and its dosage is readily calculated by the gonad weight. A
197 previous study using pituitary extract on IVM of *Dicentrarchus labrax* oocytes obtained
198 a maturation rate of 89% [6]. In the present study, the best maturation rate using CPE
199 was 40.9% (32 µg/ mL at 18 h). The difference between the studies could be due to
200 variation among pituitary extracts. Therefore, the disadvantage of using CPE is the large
201 difference in pituitary samples from the same batch, since they are obtained from
202 different donors. Thus, the hormone concentration may vary among batches. Our data
203 showed that the pituitary concentration used in IVM was dose-dependent, as reported by
204 Sorbera et al. (1999) [6].

205 As in mammals, cytoplasmic maturation may occur asynchronously with nuclear
206 maturation. The oocyte requires a longer cytoplasmic maturation for the acquisition of
207 competence to be fertilized and hatch *in vitro*. In teleost, there are two different
208 gonadotropins FSH and LH [15]. FSH level is high during oocyte growth and LH level
209 is high in the maturation stage [16, 10]. Since fish gonadotropins are not easily
210 available, hormones from mammalian sources have been proposed to fish maturation
211 [11]. In order to evaluate the performance of hormones in maturation, the fertilization
212 and hatching rates were determined in Experiment 2. Only oocytes cultured for 20 h
213 using the combination of FSH (0.5 µg/mL) and LH (0.5 µg/mL) were able to fertilize

214 and hatch. According to Kwok et al. (2005) [10] both FSH and LH receptors were
215 abundantly expressed in zebrafish gonads. However, these authors also showed that
216 bovine FSH stimulated both zebrafish FSH and LH receptors over the high
217 concentration range (1–4 µg/mL), whereas bovine LH was effective in stimulating LH
218 receptor at low concentrations (0.005–5 µg/mL). Therefore, we believe that increasing
219 the FSH concentration from 0.5 to 1 µg/mL or higher could improve the maturation
220 rates of zebrafish oocytes. It is necessary to investigate other concentrations of FSH and
221 LH and the interaction between them.

222 The results obtained from the present study showed that FSH combined with LH
223 is effective on IVM of zebrafish oocyte and suggests that these hormones could be
224 applied to IVM of other teleost. Furthermore, the developed protocol may be used as a
225 tool for understanding the mechanism of oogenesis and folliculogenesis regulation in
226 fish. Further studies are needed to design and improve maturation and fertilization
227 medium for *in vitro* culture of fish oocytes, since this is an area of study little explored
228 in fish species.

229 **Acknowledgement**

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234 **Author's contributions**

235 Conception and design of the study: LAS, LSM, TZ, DPS. Acquisition of data:
236 LAS, LSM. Analysis and interpretation of data: LAS, LSM, RBR, LRE. Drafting the

237 article or revising it critically for important intellectual content: LAS, LSM, TZ, RBR,
238 DPS. All authors read and approved the final version to be submitted.

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