

1 **Cover Page**

2 **Endoscopy and Cannulation as Non-Invasive Tools to Identify Sex and Monitor**
3 **Reproductive Development in *Arapaima gigas***

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14 Running head: New tools for monitoring reproduction in *Arapaima*.

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16 Keywords: broodstock management, gonadogenesis, Pirarucu, reproductive dysfunction,
17 micropyle.

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27 **Abstract**

28 The lack of tools for sex identification and assessment of gonadal development are hindering
29 our ability to study the reproductive dysfunction of *Arapaima gigas* in captivity. This study
30 initially aimed to validate a non-surgical endoscopy procedure to identify sex in juveniles and
31 assess stage of ovary development in female broodstock under field operational conditions.
32 Cannulation, assisted through the description of the genital anatomy, made ovarian biopsy
33 possible to describe oocyte development from primary growth to pre-ovulation, providing a first
34 classification scheme for oogenesis in the species including description of the micropyle
35 morphology using scanning electron microscopy. Cannulation was also successfully performed
36 without endoscopic guidance, which allowed monitoring of the ovarian development along the
37 reproductive season together with profiling of plasma sex steroids (17β -oestradiol (E_2) and 11-
38 ketotestosterone (11-KT) in females and males respectively). The monitoring of our study
39 population showed females paired with males in earhponds sexually matured and reached
40 oocyte maturation during the spawning season. However, since no spawning was recorded, eggs
41 had either been resorbed or released and not fertilized by the male. Plasma E_2 levels remained
42 high in females, as expected in an asynchronous species during the spawning season with
43 multiple batches of oocytes being recruited. Plasma 11-KT showed a tendency to decrease
44 suggesting a male reproductive dysfunction or the end of the reproductive season with a lack of
45 synchronisation between genders. In conclusion, endoscopy and cannulation are tools that can
46 be promptly applied to aid sex identification, assessment of reproductive function and overall
47 broodstock management in wild and captive stocks. These tools will greatly help future studies
48 looking at the effects of environmental, social and hormonal cues on reproductive development
49 with the aim to develop a spawning induction protocol for the species.

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55 **Body of text**

56 The Pirarucu *Arapaima gigas* is a dioic and iteroparous species without evident sexual
57 dimorphisms (Chu-Koo et al., 2009). Like salmonid and anguillid species, the ovary of *A. gigas*
58 lacks an external capsule (gynovarium), meaning mature oocytes are directly released into the
59 coelomatic cavity before reaching the gonopore at spawning (Grier et al., 2009; Godinho et al.,
60 2005; Colombo et al., 1984). However, the gonopore morphology and position have not been
61 described, which hindered the practice of cannulation (Núñez et al., 2011; Chu-Koo et al.,
62 2009). This is a major constraint to the study of reproductive function in the species for both
63 wild and captive stocks (Torati et al., 2016; Núñez et al., 2011), as assessment of reproductive
64 condition can only rely on sacrificing animals to sample gametes, which contrasts with
65 conservation efforts. Consequently, data on gonadal development, oocyte maturation, ovulation,
66 and spawning rhythms is still scarce (Godinho et al., 2005; Núñez and Duponchelle, 2009) as
67 for data on spawning rhythms. These gaps in knowledge have limited greatly progress made
68 over recent years to optimise reproduction of *A. gigas* in captivity.

69 *Arapaima gigas* breeding and spontaneous spawning have been reported in captivity in
70 many different farms across South America, however it remains uncontrolled and unpredictable.
71 Empirical evidence suggests that nutrition and rainfall act as proximate factors for the control of
72 final oocyte maturation and spawning (Núñez et al., 2011; Migaud et al., 2010). However, still
73 very little is known about the reproductive dysfunction of *A. gigas* in captivity. Current
74 practices for breeding the species consist of isolating pairs randomly into earthen ponds without
75 any scientific and validated assessment of sex and/or reproductive condition (Núñez et al.,
76 2011). Available methods to identify gender in *A. gigas* include color patterns, which display
77 large intra- and interpopulation variability, and can therefore be considered as unreliable (Chu-
78 Koo et al., 2009). Other methods to identify sex were investigated using genetic karyotyping
79 and bulked segregant analyses (Rosa et al., 2009; Almeida et al., 2013), however these were not
80 successful (Almeida et al., 2013). Hormonal analyses can be used to identify sex using the ratio
81 between plasma E₂ and 11-KT in maturing and also immature individuals, although reliability of
82 the technique may not be guaranteed (Chu-Koo et al., 2009). In addition, a vitellogenin enzyme

83 immune assay was developed and can be used to identify adult females (Dugue et al., 2008;
84 Chu-Koo et al., 2009). Alternatively, surgical laparoscopy was used to visualize gonads in
85 juveniles (Carreiro et al., 2011); however, in practice, this method is laborious, not fully
86 reliable, and very invasive with associated welfare concerns.

87 Recently, a non-surgical endoscopy procedure showed promising results that allowed
88 images to be taken from the ovary of adult female *A. gigas* (Torati et al., 2016), and the gross
89 inference of stage of ovarian development through the coloration of oocytes. Endoscopy has
90 been carried out recently in a proof-of-concept study on a limited number of adult *A. gigas*;
91 however, the method required further validation under field operational conditions before it can
92 be implemented into the industry (Torati et al., 2016). In addition, this method only provided
93 empirical observations as samples can not be obtained for further characterization of the oocytes
94 (e.g. diameter, histological analyses) without cannulation. Importantly, although the coelomatic
95 cavity could be visualized through the use of modern endoscopy apparatus, the morphology of
96 the urogenital papillae has not yet been described preventing the use of cannulation. Further
97 work is clearly required to address these limitations.

98 The aims of the current study were therefore: 1) to validate endoscopy described in
99 Torati et al. (2016) in adult females under field operational conditions and test the method for
100 the first time in juveniles (~10 kg) as a possible tool for early sex identification and assessment
101 of reproductive development, 2) to describe the anatomy of female urogenital papillae for
102 enabling collection of ovarian biopsy through cannulation and provide a detailed scheme of
103 oogenesis from primary growth to pre-ovulation, and 3) to use oocyte staging associated with
104 sex steroid analysis to monitor the reproductive function of a captive broodstock population.

105

106 **MATERIALS AND METHODS**

107 ***Fish, endoscopy and sampling procedures.***—From 20th to 21st Feb 2016, 20 immature juveniles
108 of *A. gigas* (19 months of age) held in the facilities of Embrapa Fisheries and Aquaculture
109 (Palmas-TO, Brazil), measuring 106.8 ± 4.6 cm in total length and weighing 10.2 ± 1.1 kg were

110 fasted for 24 hours, sacrificed and examined by endoscopy for sex determination and
111 morphological analyses of the urogenital papillae.

112 Adult broodstock females were studied in a farm located at Taipas-TO (12°09'40.79" S,
113 46°51'34.00" W), North Brazil. Broodstock were known to be over six years of age, and
114 reproduction had never been recorded in the study site. Twelve adult females (n=12, total
115 length: 166.6 ± 7.2 cm and body weight: 43.0 ± 5.6 kg), and twelve adult males (n=12, total
116 length: 159.6 ± 9.8 cm, body weight: 37.5 ± 6.0 kg) were monitored. Prior to each sampling,
117 fish were fasted for 24 hours, netted from earthen ponds, and kept contained outside on a soft
118 wet mat for approximately five to 10 minutes. Anaesthetics were not applied during sampling
119 because they have been shown to compromise welfare and resulted in mortalities due to air-
120 breathing behavior of *A. gigas* (Farrel and Randall, 1978). Fish breathing behavior was closely
121 monitored (breathing at regular intervals of four to six minutes). After each procedure, fish were
122 monitored until they return to normal breathing behavior.

123 Endoscopy procedures in juvenile and adult fish were performed according to Torati et
124 al. (2016) with the exception of the equipment used in the current study, which was a
125 cistoureteroscope (13 cm x 2.6—3.6 mm, 6 ° of angular field, 8—13.5 Charr operating sheath,
126 model 27030KA, Karl Storz Endoscopy, Tuttlingen, Germany). The main difference with the
127 uretero-roscope previously used in Torati et al. (2016) is that it is shorter and had better
128 maneuverability. A hydrophilic guidewire (B|Braum, Barcarena-Portugal) was used to inspect
129 the urogenital papilla region and search for the gonopore position (Suppl. video I). The
130 endoscope was equipped with a Telecam camera of 1 chip, a 50 watt Hi-Lux light source and a
131 15" LCD monitor (200450 01-PT, Tele Pack X, Karl Storz, Tuttlingen, Germany), and all
132 examinations were recorded for later analyses. Ovary visualisation was used to identify juvenile
133 females, and ovary color pattern was observed in adults to estimate overall developmental
134 condition. In sacrificed juveniles, a 4-mm-thick slice was dissected from the gonad median
135 region for histology. In adults, ovary biopsies were taken by inserting a flexible silicon tubing
136 catheter (3 mm internal diameter) into the gonopore and applying a gentle suction using a 25-ml
137 syringe. Collected gonadal samples were fixed in Bouin's solution (Sigma Aldrich, Saint Louis,

138 MO, USA) for 24 hours, washed in distilled water and stored in 70% ethanol until histological
139 analysis.

140 Broodstock were sampled three times during this study. Initially on 7th Jan 2016, fish
141 were tagged with a passive integrated transponder (AnimalTAG®, São Carlos, Brazil) inserted
142 in the dorsal muscle to allow individual identification in subsequent samplings. Blood (4 ml
143 maximum) was collected from the caudal vein of males and females using heparinized syringes
144 (BD Precisionglide, New Jersey, USA) flushed with 560 IU.ml⁻¹ heparin ammonium salt
145 solution (Sigma Aldrich, Saint Louis, MO, USA). Blood plasma was extracted after
146 centrifugation at 1200 g for 15 minutes and stored at -20 °C until steroid analysis. Sex was
147 determined with a vitellogenin enzyme immune assay kit (Acobiom, Montpellier, France)
148 developed specifically for *A. gigas* (Dugue et al., 2008). On 8th Jan 2016, females were
149 randomly paired with males into 250 m² breeding earthen ponds. By 16th March 2016, the 12
150 adult females were examined with endoscopy under practical operational field conditions (Fig.
151 1A-B), and cannulated to obtain biopsy samples (Fig. 1C). Adult males were not examined
152 under field conditions given prior results obtained for juvenile males showing the spermatic
153 duct can not be reached by endoscopy. On 17th May 2016, the cannulation biopsy procedure was
154 repeated on the same females without endoscopy guidance. No mortalities were recorded
155 following the procedure during the study.

156

157 **Gonad histology.**—Prior to histological analysis, all adult oocytes (n = 2,101) were
158 photographed on a scaled dish plate and individually measured for two perpendicular diameters
159 using ImageJ v. 1.49 software (Schneider et al., 2012). The oocyte volume was estimated using
160 the formula $V=1/6\pi OD^3$, where OD is the mean of the oocyte diameters (Jones and Simons,
161 1983). Measurements made before histological processing aimed to avoid effects of shrinkage
162 and membrane collapsing, which occur after oocyte dehydration in histological protocols
163 (Kennedy et al., 2011). Oocytes analysed for histology ranged in diameter from 354.5 to 2466.0
164 μm (n = 363 oocytes). Samples were gradually dehydrated in ethanol series (80—90—100%),
165 then infiltrated in glycol methacrylate resin and blocked following the manufacturer's protocol

166 (Leica Microsystems™, Wetzlar, Germany). Blocks were sectioned at 3–5 µm thick using a
167 rotary microtome (Leica RM 2235, Heidelberg, Germany). Alternatively, oocytes were also
168 infiltrated in paraffin, and sectioned in a Shandon Finesse microtome (Thermo Fisher Scientific,
169 Waltham, MA, USA) aiming to improve issues faced with oocyte crumbling during the
170 sectioning step. Slides were stained with hematoxylin – eosin (Fischer et al., 2008) for optical
171 microscopy.

172 Aiming to provide a detailed characterisation of oocyte development for *A. gigas*, we
173 adopted the staging terminology from Rhody et al. (2013) and Grier et al. (2018), in which
174 stages are indicated by uppercase letters (i.e. PG=primary growth), and steps within a stage by
175 lowercase letters (i.e. PGI = late step in primary growth stage). Gonadogenesis stages followed
176 classification of Núñez and Duponchelle (2009). Gonad sections were analyzed using an
177 Olympus BX51 microscope equipped with a Zeiss AxioCam MRc camera system. To describe
178 female reproductive condition, leading cohort oocyte diameter was defined as the mean of the
179 10 largest oocytes. For juvenile females, the leading cohort was measured from pictures taken
180 after histological sections using the 10 largest oocytes *per* juvenile female.

181

182 ***Micropyle analysis with scanning electron microscopy.***—Given egg release during sampling is
183 not common in *A. gigas*, non-fertilized eggs could only be kept frozen in liquid N₂ instead of
184 being fixed with appropriate fixatives for scanning electron microscopy analysis. Aiming to
185 prevent egg damage during sample thawing, eggs were thawed at room temperature while
186 immersed in a 0.5 M sucrose solution prepared with NaCl PBS buffer (pH 7.2), and then
187 immediately fixed in Karnovsky solution (Karnovsky, 1965) for five hours. Fixed eggs were
188 washed three times for 10 min in 0.1 M phosphate buffer. A second fixation (2 hours) was made
189 in 1% osmium tetroxide followed by a quick (2 sec) immersion in distilled water. A gradual
190 dehydration in ethanol was made, increasing 10% grade until 100% (15 min each grade). Eggs
191 were dried in a Critical Point K850 (Quorum Technologies, Lewes, UK) following equipment
192 protocol, then carefully mounted onto stubs, coated with gold, and observed in a Zeiss DSM
193 940A scanning electron microscope. Egg surface was visualized in 20 eggs. Given the

194 immovable position of eggs on stubs, a single micropyle was visualized in 5 eggs, and
195 measurements of micropylar cone diameter undertaken in 3 of them.

196

197 ***Steroid analysis.***—Plasma samples were thawed at room temperature, and extraction of sex
198 steroids made mixing 50 µl of plasma with 1 ml of ethyl acetate using 1 ml polypropylene assay
199 tubes. The mixture was vigorously vortexed and spun for 1 hour at room temperature (16°C)
200 using a rotary mixer, then centrifuged at 430 g for 10 min at 4°C. Levels of E₂ and 11-KT were
201 quantified in the blood plasma of females and males, respectively, using an enzyme-linked
202 immunosorbent assay (Cayman Chemical Inc., Michigan, USA) previously validated for *A.*
203 *gigas* (Chu-Koo et al., 2009). Just prior to assay, extracts were dried in a vacuum oven at 35°C
204 for 40 minutes. Given sample variability, dilutions in samples ranged from 1:5 to 1:15 (v:v) for
205 11-KT, and for E₂ samples had to be concentrated 6:1 (v:v). The manufacturer's protocol was
206 followed and microplates were read at 405 nm using a BioEasy microplate reader (Belo
207 Horizonte, Brazil). Steroid concentrations in blood were calculated from the assay value (pg.
208 tube⁻¹) corrected for the proportion of extract used in the assay and the volume of blood used for
209 extraction. Intra-assay coefficients of variation were 6.5 % and 1.7 % for 11-KT for E₂,
210 respectively.

211

212 ***Statistics.***—Statistical analyses were conducted in Minitab (version 17.3.1, Minitab, PA, USA).
213 One-way repeated measures ANOVA was used to compare steroid levels (ng.ml⁻¹) during the
214 study period. Paired t-tests were used to compare the leading cohort (mm) for each female
215 between biopsy samplings points (16th March and 17th May 2017). The level of significance was
216 set as $P \leq 0.05$, and values are presented as mean \pm SE.

217

218 **RESULTS**

219 ***Gonopore position, endoscopy and cannulation in females.***—Internally, the ovary was found
220 to be suspended in the coelomatic cavity by the mesovarium, while the intestines and urinary
221 ducts were located dorsally in relation to the ovary (Fig. 2A-B). Externally, the anus is found

222 anteriorly in relation to the urogenital papilla (Fig. 2C). The urogenital papilla is a single
223 opening and no evident sexual dimorphism was found on its external morphology (Fig. 2C).
224 Endoscope insertion, approximately through 4—5 mm of the female urogenital papilla pore, and
225 using a guiding wire inserted through the endoscope working channel, allowed identification of
226 a membranous septum dividing the gonopore access to the coelomatic cavity, which is ventral in
227 relation to the urinary opening (Fig. 2D). The septum recognition was key to visualizing the
228 gonopore and reaching the oviduct and coelomatic cavity where the internal organs and ovary
229 could be visualized (Fig. 2E). In practice, the urinary duct (Fig. 2F) was more easily assessed
230 depending on the angle of insertion if the septum was not noticed or inspected, especially
231 because the septum was normally found collapsed cranially enclosing the gonopore. In juvenile
232 males, neither a septum nor the spermatic duct opening could be observed after endoscopic
233 analyses.

234 Out of 20 juveniles examined with endoscopy, 11 had the gonopore assessed and left
235 ovary observed after approximately 2.9 ± 2.7 minutes of examination. After gonad dissection
236 and histological analyses on sacrificed fish, these were confirmed to be females. Among the
237 remaining 9 fish whose gonopore could not be assessed, 7 were males and 2 were females
238 (Table 1). With this method, male identification remained indirect, and dependent on operator
239 ability to locate the septum to assess the gonopore, and also on fish morphological variability.
240 After histological analyses, the leading cohort in the ovary of juvenile females was classified at
241 the primary growth perinuclear step (PGpn), characteristic of immature fish (Stage I, Table 1).
242 Externally, the ovaries had a pale color and a foliaceous shape when visualized by endoscopy
243 (Fig. 2E).

244 Field endoscopy in adult females was successfully done without any difficulties (Fig.
245 1A-B). The coelomatic cavity of 12 adult females was assessed and the ovary observed after
246 approximately 1.24 ± 1.53 minutes (Table 2). Following endoscopy, a cannula was introduced
247 into the gonopore and oocyte biopsies were sampled (Fig. 1C). With the morphological
248 knowledge gained after endoscopic analyses, cannulation was repeated in the same females two
249 months later without endoscopic guidance. For each biopsy, from 3 to 206 oocytes were

250 obtained per individual female (mean of 87.5 ± 53.4) with a total of 2,101 oocytes staged and
251 measured over the two sampling times. Endoscopy and oocyte analyses combined showed
252 ovaries with a greenish pigmentation at Stage IV with leading cohort oocytes classified either at
253 the germinal vesicle migration stage (OMgvm, n=10) or ovulation (OV, n=1). One female had
254 a yellowish ovary at Stage II with leading cohort oocytes at the full-grown stage (SGfg, n=1)
255 (Suppl. Video I, Table 2).

256

257 ***Oocyte development, Primary growth stage.***—Multi-nucleoli oocytes (PGmn) were identified
258 by the presence of several nucleoli scattered within the germinal vesicle. Balbiani bodies were
259 present in the ooplasm, and oocyte diameter ranged from 32.2 to 113.1 μm ($72.0 \pm 17.3 \mu\text{m}$;
260 Fig. 3A). Oocytes at the PGmn step were present in all juvenile females analyzed, but not in
261 cannulated adults. In all juvenile females studied, the leading oocyte cohort was at the
262 Perinucleoli stage (PGpn). These oocytes were identified by the presence of several spherical
263 nucleoli positioned close to the inner germinal vesicle membrane. Balbiani bodies were still
264 present throughout the ooplasm. The diameter of PGpn oocytes ranged from 64.7 to 320.8 μm
265 ($105.2 \pm 103.9 \mu\text{m}$; Fig. 3B). The oil droplet step (PGod) was initiated with the appearance of
266 few oil droplets at the periphery of the ooplasm and cortical alveoli in the ooplasm. Cortical
267 alveoli were initiated centripetally with large yolk vesicles deposited in the circumnuclear area.
268 Oocytes at the PGod step were not observed in the juveniles analyzed, and were collected
269 through cannulation of all adult females. In PGod oocytes, the zona pellucida appeared
270 completely formed and visible, and oocyte diameter ranged from 354.5 to 599.0 μm ($473.4 \pm$
271 $82.1 \mu\text{m}$; Fig. 3C). At the cortical alveoli step (PGca), the multiple layers of cortical alveoli had
272 completely migrated towards the ooplasm periphery. Vesicles of glycoprotein were still present
273 although fewer and smaller compared to the PGod step. The diameter of PGca oocytes ranged
274 from 627.0 to 777.0 μm ($697.5 \pm 57.6 \mu\text{m}$; Fig. 3D).

275

276 ***Oocyte development, Secondary growth stage.***—The follicle layer was more evident lining the
277 zona pellucida from secondary growth onwards. Early secondary growth (SGe) oocytes were

278 distinguished from previous PGca step by a marked increase in the number of small yolk
279 globules (true yolk - lipovitellin and phosvitin) and oil droplets throughout the ooplasm. SGe
280 oocyte diameter ranged from 783.5 to 946.5 μm ($892.6 \pm 46.5 \mu\text{m}$; Fig. 4A). In the late
281 secondary growth (**SGI**), larger yolk globules became evident surrounding the germinal vesicle.
282 In SGI oocytes, the multiple layers of cortical alveoli became also more compacted lining the
283 zona pellucida, and oocyte diameter ranged from 1007.0—1111.5 μm ($1066.1 \pm 39.8 \mu\text{m}$; Fig.
284 4B). In full-grown oocytes (**SGfg**), an extensive accumulation of oil droplets was observed and
285 these were larger compared to SGI oocytes. Oil droplets were found between the nuclear region
286 and the cortical alveoli layer. At SGfg, oocytes reached their maximum diameter during
287 vitellogenesis ranging from 1139.5 to 1276.0 μm ($1214.9 \pm 52.9 \mu\text{m}$; Fig. 4C-D).

288

289 ***Oocyte development, Oocyte maturation and pre-ovulation steps.***—The germinal vesicle
290 started its migration towards the animal pole marking the start of the **OMgvm** stage. During
291 OMgvm, most of the oocyte hydration occurred, at a period when oocytes became greener when
292 observed externally with endoscopy or after biopsy. At this stage, oil droplets started to coalesce
293 becoming larger compared to the SGfg stage, and yolk became more fluid and the nucleo-
294 cytoplasmic ratio decreased. The diameter of OMgvm oocytes ranged from 1302.5 to 2354.0
295 μm ($1923.3 \pm 275.6 \mu\text{m}$; Fig. 5A-D). The ovulation (**OV**) step was observed in one female out
296 of 12, which released eggs during sampling. Ovulated oocytes measured from 2394.0 to 2466.0
297 μm ($2427.0 \pm 21.4 \mu\text{m}$) in diameter. Ovulated eggs are now surrounded by the chorion since
298 they detached from the follicular layer. In OV oocytes, the germinal vesicle has reached the
299 animal pole and its membrane has broken down (Fig. 6A). Scanning electron microscopy
300 analyses performed on unfertilized OV eggs revealed the presence of a single micropyle in *A.*
301 *gigas*, which has a series of radially arranged ridges leading into the micropylar canal, whose
302 diameter measured $11.3 \pm 0.02 \mu\text{m}$ (Fig. 6B-C).

303

304 ***Monitoring of the reproductive function of a captive broodstock.***—When the 12 couples were
305 first paired in earthponds on 7th January 2016, the cannulation technique had not been developed

306 yet. These females were first sampled on 16th March 2016, when 11 of them had their leading
307 cohort oocytes at OMgvm or OV (Table 2, Fig. 7). In comparison with the following sampling
308 undertaken on 17th May 2016, 9 out of these 11 females displayed reduced diameters of their
309 leading cohort oocytes ($P < 0.01$; Fig. 7), revealing ovarian development regressed. The ovary of
310 only a single female ($\text{♀}9$) showed advanced oocyte maturation during the monitoring period
311 ($P < 0.01$; Fig. 7).

312 Levels of E_2 remained steady throughout the rainy season (Fig. 8A, $P > 0.05$), ranging
313 from 0.6 to 16.0 ng.ml^{-1} (mean of $5.1 \pm 4.5 \text{ ng.ml}^{-1}$). In males, levels of 11-KT also remained
314 unchanged with a decreasing trend towards the end of the rainy season (Fig. 8B, $P > 0.05$). It
315 ranged from 4.5 to 59.0 ng.ml^{-1} (mean of $27.0 \pm 17.2 \text{ ng.ml}^{-1}$).

316

317 **DISCUSSION**

318 Methods to identify sex and assess fish reproductive development are key to any fish
319 broodstock management program, to understand potential reproductive dysfunctions and
320 validate hormonal therapies (Mylonas et al., 2010; Mylonas et al., 2017). In practice, such
321 methods vary according to the species morphology, ranging from external observations of the
322 abdomen and urogenital papilla intumescence (Carosfeld et al., 2003), biochemical analyses of
323 ovarian fluids (Johnson et al., 2014), cannulation and/or massaging out of oocytes and sperm
324 (Rhody et al., 2013; Martins et al., 2017) or through examinations with ultrasound, endoscopy,
325 boroscopy and laparoscopy instruments (Divers et al., 2009; Wildhaber et al., 2005; Kynard and
326 Kieffe, 2002; Albers et al., 2013; Filho et al., 2016). The description of the urogenital anatomy
327 is essential to perform cannulation and assess gonadal development (Siqueira-Silva et al., 2015;
328 Rasotto and Shapiro, 1998; Carlisle et al., 2000). In *A. gigas*, there is no evident secondary
329 sexual dimorphism in the urogenital papilla between sexes and cannulation so far has been
330 considered impossible (Chu-Koo et al., 2009). In this study, endoscopy examination in females
331 showed the urinary canal and the gonoduct reach a common aperture inside the urogenital
332 papilla, with their openings separated by a membranous septum. To observe and displace this
333 septum, a guiding wire was used through the endoscope working channel to assess the gonopore

334 and observe *in situ* the ovary. This was made possible both for pre-pubescent (juveniles) and
335 also adult females under farm conditions. Such analyses allowed a rapid and non-invasive
336 validation of the preliminary results described in Torati et al. (2016) with further sex assessment
337 possible in juveniles (10kg). New knowledge on the urogenital anatomy was translated into
338 practice through cannulation of adult *A. gigas* females and oocyte biopsies without endoscopic
339 guidance.

340 The possibility to monitor reproductive function of *A. gigas* through cannulation was a
341 major breakthrough for the non-invasive study of the species reproduction, and this made it
342 possible to confirm an oocyte development scheme accounting for species-specific variations in
343 oocyte inclusions (i.e. cortical alveoli, oil globules) (Grier, 2012; Grier et al., 2009; Mañanós et
344 al., 2008). Previous studies performed on wild-caught fish have described the early stages of
345 oocyte development in *A. gigas* from sacrificial samplings (Godinho et al., 2005; Bazzoli and
346 Godinho, 1994) but no cytological details of the final oocyte maturation and ovulation stages
347 were reported, which are key developmental stages to study potential reproductive dysfunctions.

348 In the present study, a detailed description of oocyte development is presented for *A.*
349 *gigas*. Transition to oocyte maturation was seen in oocytes from 1.3 mm onwards, accompanied
350 by a color change from a yellow to greenish pattern due to yolk hydration (Núñez and
351 Duponchelle, 2009; Chu-Koo et al., 2009). At this stage, females are considered capable of
352 spawning and are often selected for hormonal manipulation in hatcheries (Mylonas et al., 2010).
353 Therefore, observation of ovary/oocyte color could be used as a good indicator of final oocyte
354 maturation for field study using either oocyte biopsies or endoscopy. During OM in *A. gigas*,
355 oocytes increase in diameter from 1.30 to 2.35 mm (size at pre-ovulation step). This increase is
356 within the typical 1—3 fold reported for other teleosts due to oocyte hydration (Cerdá et al.,
357 2007). At the pre-ovulation step, eggs have an adhesive gum layer, lack oil globules (which
358 normally provide buoyancy) and are highly hydrated. These properties help explain their benthic
359 nature when deposited in nests built in shallow areas (Castello, 2008). The present study
360 identified and described for the first time a single micropyle in fully mature eggs from *A. gigas*.
361 This is a characteristic of most Actinopterygii (Bartsch and Britz, 1997) although not described

362 previously for arapaimid species. This information will be useful in future applied investigations
363 on fertilization of *A. gigas* and comparative biology studies (Isaú et al., 2011) while also
364 supporting current taxonomic debates for *Arapaima* if biopsies can be obtained from other
365 species (Stewart, 2013b; Stewart, 2013a).

366 Previous works with wild caught *A. gigas* have reported up to six batches of eggs being
367 recruited and spawned during the reproductive season from October to May, with an inter-
368 spawning period of approximately 1 month and in some cases, spawning outside the rainy
369 season (Queiroz, 2000). In this study, analyses of ovarian biopsies in *A. gigas* showed most
370 females had oocytes ranging from primary growth (PGod) to final oocyte maturation (OMgvb),
371 typical of a species with asynchronous ovarian development (Godinho et al., 2005), where
372 several cohorts of developing oocytes subsequently undergo ovulation in successive batches
373 during the spawning season. It has been shown for species with asynchronous ovarian
374 development that plasma E₂ levels tend to increase and remain high during the spawning season
375 (Rinhard et al., 1993; Barcellos et al., 2001), when multiple oocyte batches are recruited
376 successively for final oocyte maturation, resulting in batch spawning. Likewise in this study,
377 levels of E₂ were found constantly high (cc. 5 ng.ml⁻¹) during the monitoring period if compared
378 to previous levels reported for *A. gigas* outside the reproductive period (Monteiro et al., 2010).

379 Regarding the possible reproductive dysfunction in *A. gigas* reared in captivity, this
380 study for the first time demonstrated that females kept with a single male in earthen ponds
381 undergo final oocyte maturation, meaning that they have been recruited into a reproductive
382 cycle. Dysfunction leading to the lack of apparent spawning is due to a failure at the ovulation
383 stage (females) or a lack of spawning synchronization between sexes. For females, the leading
384 cohort of oocytes seen at March 16th – 2016 was either released and not fertilized in the nests or
385 resorbed in the ovary since at May 17th – 2016 most of the screened females showed signs of
386 atresia. For males, it is not possible to apply endoscopy and cannulation given the gauge and
387 unknown position of the spermiduct opening, and further studies are therefore required. For this
388 reason, steroid analyses are still key to infer reproductive condition. Our results on levels of 11-
389 KT in males showed a tendency to decrease (approx. 2 folds) from 7th Jan – 2016 to 17th May –

390 2016, suggesting males were reaching the end of the spawning season (start of the drought
391 period in North Brazil). Follow up studies should monitor reproductive function over a longer
392 timeframe and with a higher time resolution to better describe oocyte recruitment,
393 spermatogenesis and spawning rhythms in *A. gigas* while developing non-invasive methods to
394 monitor male spermatogenesis.

395 In conclusion, this study advanced our knowledge of the reproductive biology of *A.*
396 *gigas* in captivity, with novel data on gonad anatomy and ovarian development. With the
397 information provided herein, endoscopy and cannulation are clearly useful tools that can be
398 promptly applied for sex identification and monitoring of reproductive function in wild and
399 captive stocks. As such, the deployment of these tools should help to better understand the
400 environmental, social and hormonal cues that are required to promote or induce spawning in this
401 commercially valuable species. In addition, while no spawning and/or mating could be clearly
402 observed, in all females under investigation, oocytes obtained by biopsy were at final oocyte
403 maturation or ovulation stages. This confirmed that the difficulty to breed *A. gigas* is not due to
404 reproductive dysfunctions in females during oogenesis but rather at spawning, either due to
405 atresia post-ovulation (lack of spawning), males not spermiating, or a lack of synchronization
406 between sexes. Further testing and optimisation of hormonal therapies should be done to
407 promote spawning in *A. gigas* together with investigations into male reproductive development
408 and behavior. Finally, the study showed eggs of *A. gigas* have a single micropyle, whose
409 morphology was described for the first time in an arapaimid species, which could prove useful
410 not only for systematic/taxonomic purposes, but also for potential future studies on fertilisation
411 processes in basal teleost species.

412

413 **DATA ACCESSIBILITY**

414 Supplemental information is available at <https://www.copeiajournal.org/XXX>.

415

416 **ACKNOWLEDGEMENTS**

417 Authors thank Karl Storz, Strattner and Ana Paula Varges for providing the endoscopy system
418 used, and Celli Muniz and Laura Satiko Okada Nakaghi for scanning electron microscopy
419 analyses performed on eggs of *Arapaima*. This research complied with the “Brazilian guidelines
420 for the care and use of animals for scientific and educational purposes”—DBCA being approved
421 by the Ethics Committee for the Use of Animals—CEUA of the National Research Center on
422 Fisheries, Aquaculture and Agricultural Systems—CNPASA (specific protocol n°09). It has
423 also been approved by the National System for the Management of Genetic Heritage and
424 Associated Traditional Knowledge – SISGen (AA4F2B0).

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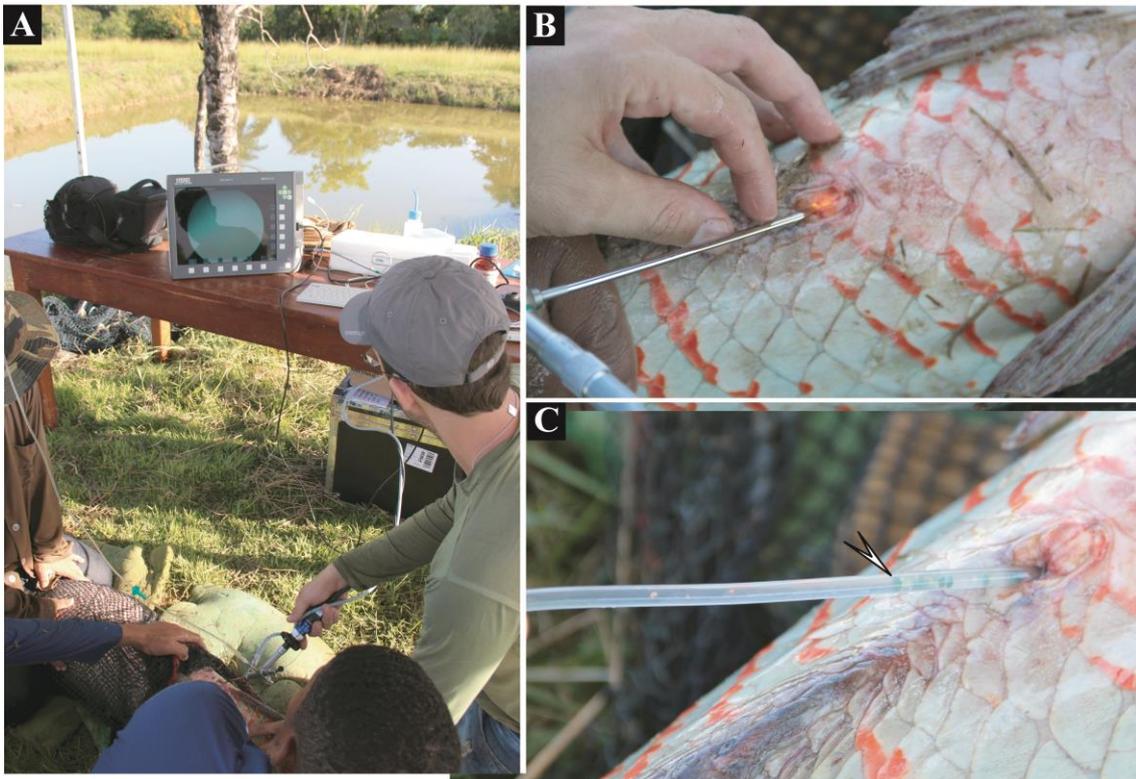
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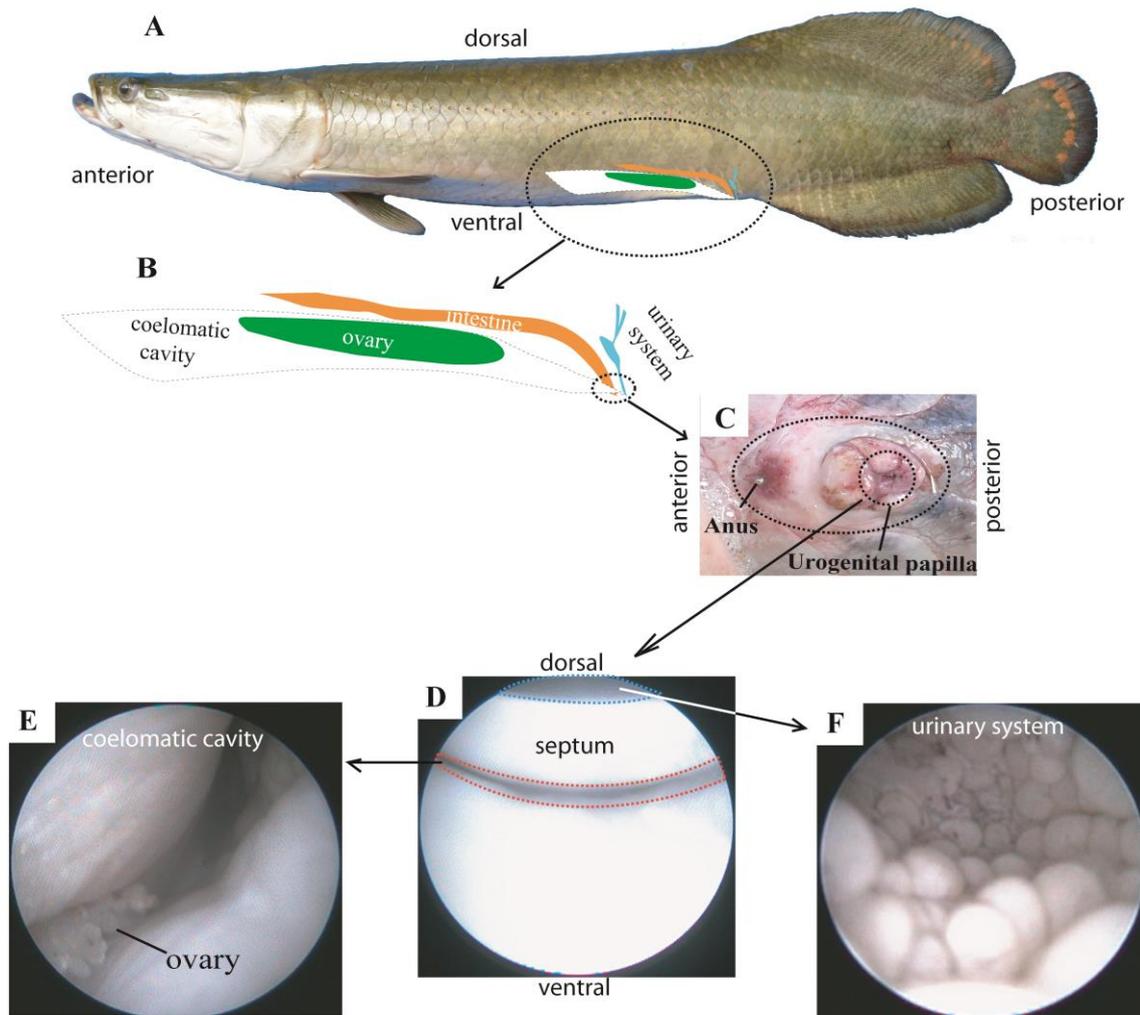
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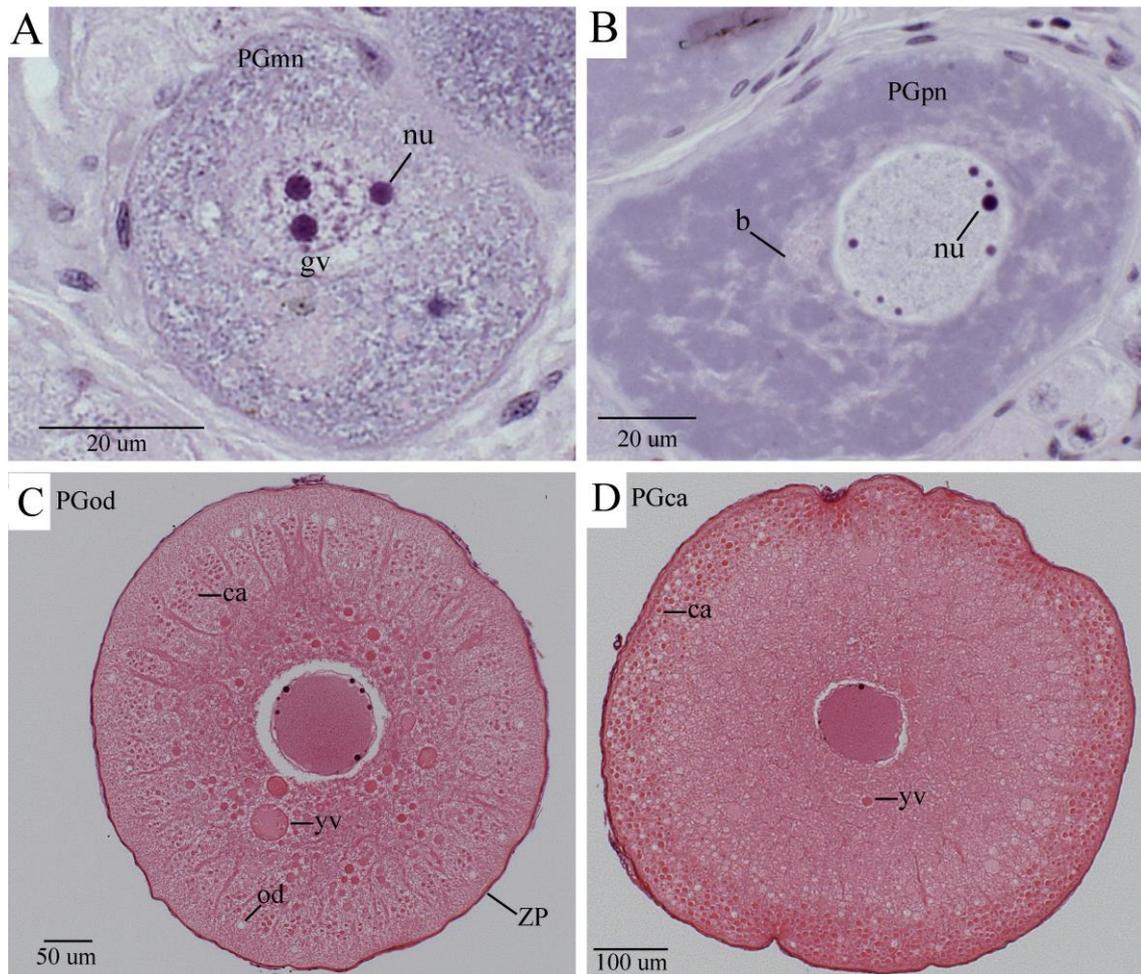
578 **Figure 1.** (A) Details of field endoscopy procedure in adult females of *Arapaima gigas*. (B)
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580 Detail of angulation used to access the gonopore and coelomatic cavity during field endoscopy
581 procedure, which allowed observation of the ovary. (C) Detail of a flexible silicon cannula
582 inserted through the gonopore into the coelomatic cavity, suctioning greenish oocytes (arrow).

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 593 **Figure 2.** (A) Lateral view of juvenile *Arapaima gigas* depicting the coelomatic cavity (white),
 594 left ovary (green), intestine (orange) and urinary system (blue). (B) Amplification of coelomatic
 595 area where the left ovary lies (green), including relative position of the intestine and urinary
 596 system. (C) Picture depicting the relative position of the anus (anterior) in relation to the
 597 urogenital papilla (posterior). D. Endoscopic image at the opening (aprox. 0.5cm) of the
 598 urogenital papilla depicting the septum (*) which separates the paths into the coelomic cavity
 599 (ventral; red-dashed) or the urinary system (dorsal, blue-dashed). (E) Endoscopic image of the
 600 coelomatic cavity showing the pale-colored-ovary of a juvenile immature female. (F)
 601 Endoscopic image depicting the internal appearance of the urinary bladder.

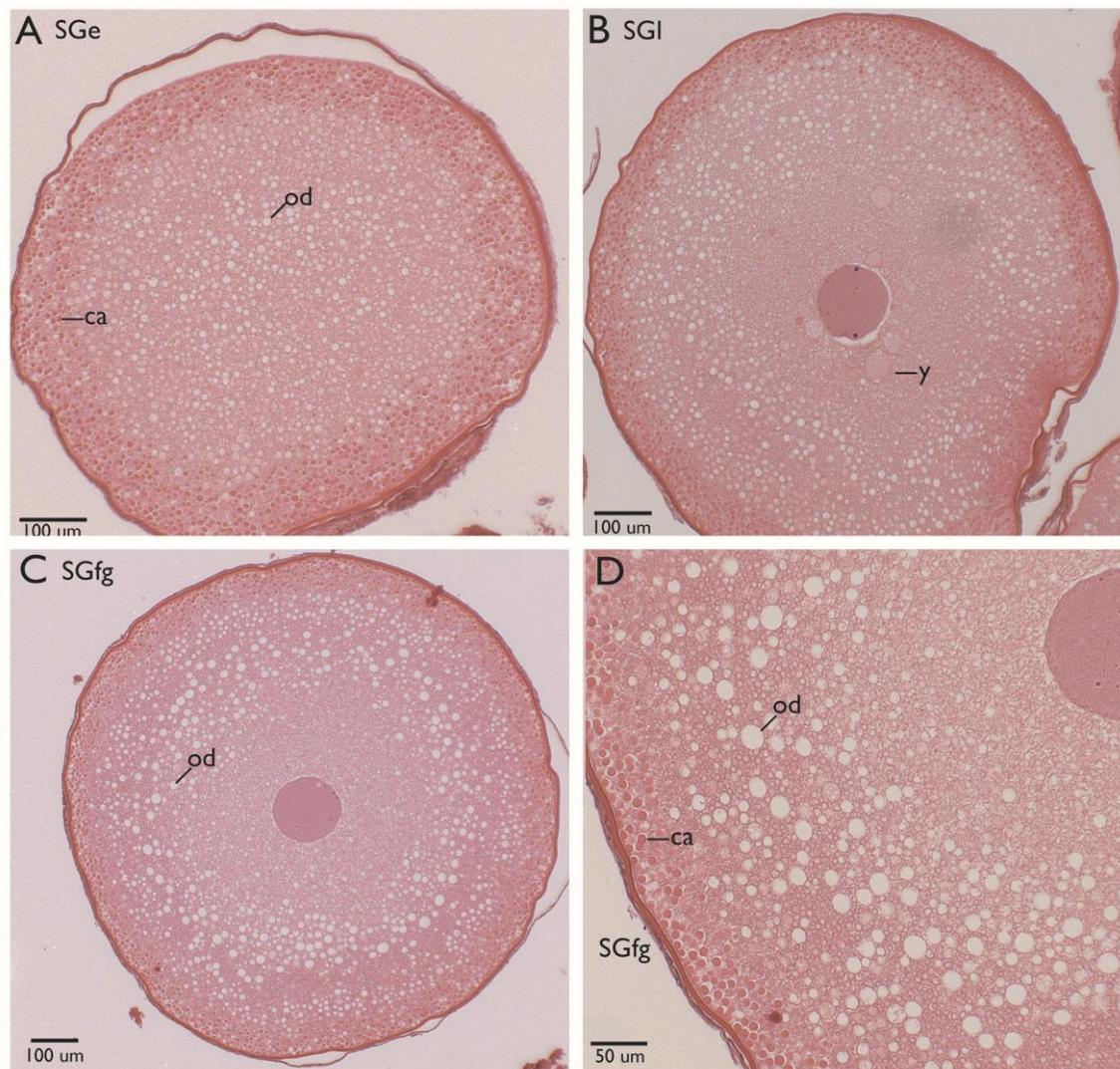
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 606 **Figure 3.** Light micrographs of primary growth (PG) step in oocytes of *Arapaima gigas*. (A)
 607 Multiple nucleoli stage (PGmn), depicting several nucleoli inside the germinal vesicle.
 608 Hematoxylin – eosin, Bar=20μm. (B) Perinucleoli (PGpn) stage, nucleoli at the internal margins
 609 of the germinal vesicle and Balbiani bodies scattered throughout the ooplasm. Hematoxylin –
 610 eosin, Bar=20μm. (C) Oil droplets stage (PGod), depicting early appearance of oil droplets at
 611 the ooplasm periphery, large vesicles of glycoprotein yolk and cortical alveoli. Hematoxylin –
 612 eosin, Bar=50μm. (D) Cortical alveoli stage (PGca) depicting the cortical alveoli layer migrated
 613 towards ooplasm periphery and yolk vesicles. Hematoxylin – eosin, Bar=100μm. b = Balbiani
 614 body, gv = germinal vesicle, nu = nucleoli, ca = cortical alveoli, od = oil droplet, yv = yolk
 615 vesicle, ZP = zona pellucida.

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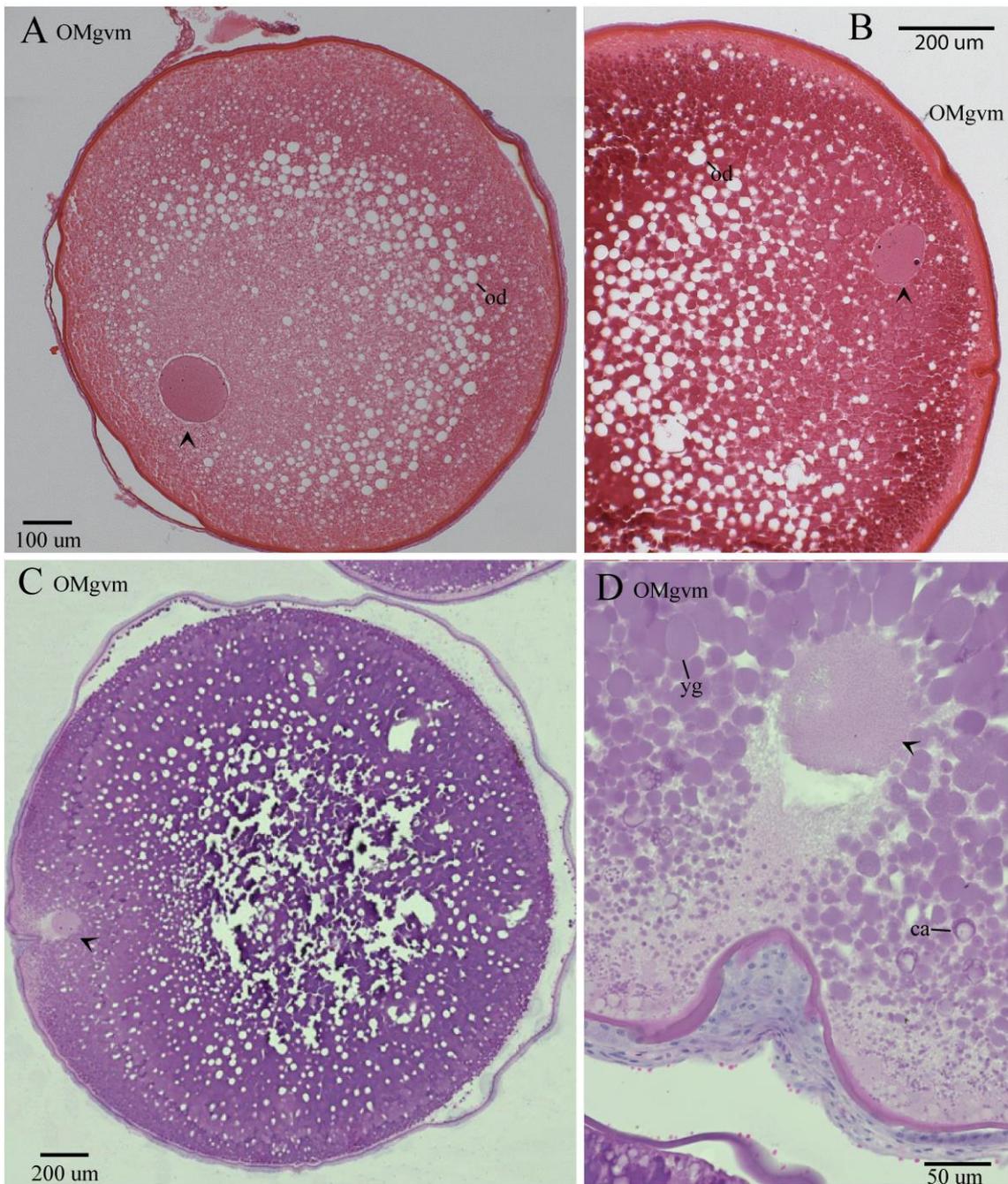
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 619 **Figure 4.** Light micrographs of secondary growth (SG) step in *Arapaima gigas*. (A) Early
 620 secondary growth (SGe) depicting wide layer of cortical alveoli at the ooplasm periphery and
 621 increase in oil droplet number. Hematoxylin – eosin, Bar=100μm. (B) Late secondary growth
 622 (SGI) depicting appearance of (true) yolk globules at the central region. Hematoxylin – eosin,
 623 Bar=100μm. (C) Full-grown (SGfg) oocyte depicting enlarged oil droplets and a thin layer of
 624 cortical alveoli. Hematoxylin – eosin, Bar=100μm. (D) Magnification of the peripheric area of a
 625 SGfg oocyte depicting the thin layer of cortical alveoli and appearance of oil droplets.
 626 Hematoxylin – eosin, Bar=20μm. ca = cortical alveoli, od = oil droplet, y= yolk globules.

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 630 **Figure 5.** Light micrographs of oocyte maturation (OM) step in *Arapaima gigas*. (A) Early
 631 migration of germinal vesicle towards the animal pole, depicting oil droplets coalescing.
 632 Hematoxylin – eosin, Bar=100μm. (B) Germinal vesicle closer to the oocyte periphery and with
 633 a reduced nucleocytoplasmic ratio. Hematoxylin – eosin, Bar=200μm. (C) Germinal vesicle
 634 close to micropyle area at the maximum hydration volume observed prior to germinal vesicle
 635 breakdown. Hematoxylin – eosin, Bar=200μm. (D) Magnification of the periphic area of a
 636 OMgvm oocyte depicting the migrated germinal vesicle close to the micropyle area.

637 Hematoxylin – eosin, Bar=50 μ m. Arrow = germinal vesicle, od = oil droplet, yg = yolk
638 granules, ca = cortical alveoli.

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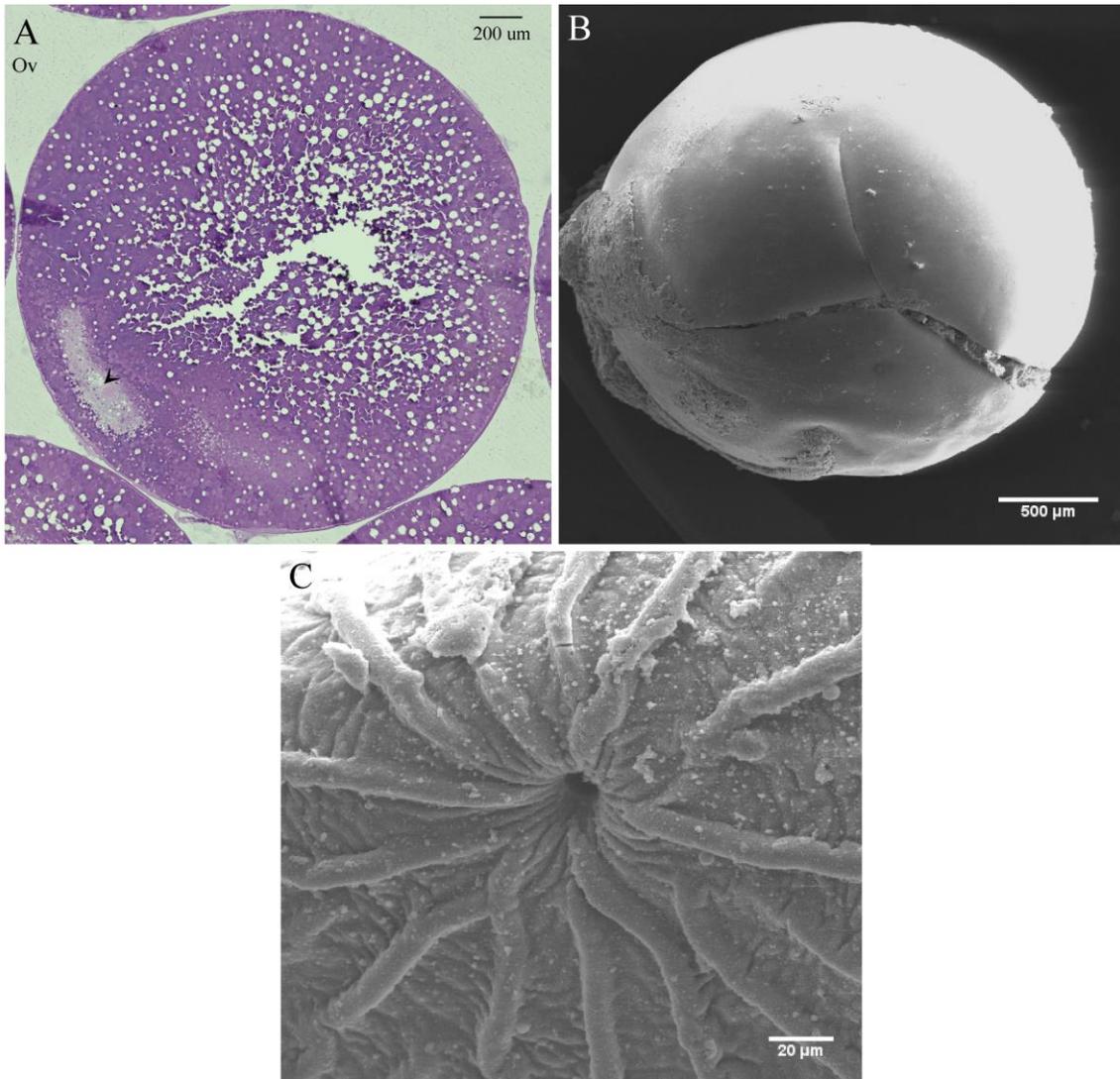
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664 **Figure 6.** Non-fertilized eggs of *Arapaima gigas* at the post-ovulatory step (OV). (A). Light
 665 micrograph depicting region where germinal vesicle broke down (arrow). Hematoxylin – eosin.
 666 Bar=200μm. (B) Scanning electron microscopy depicting egg surface, Bar=500μm. (C) Details
 667 of the concentric ridges external to themicropyle. Bar=20μm.
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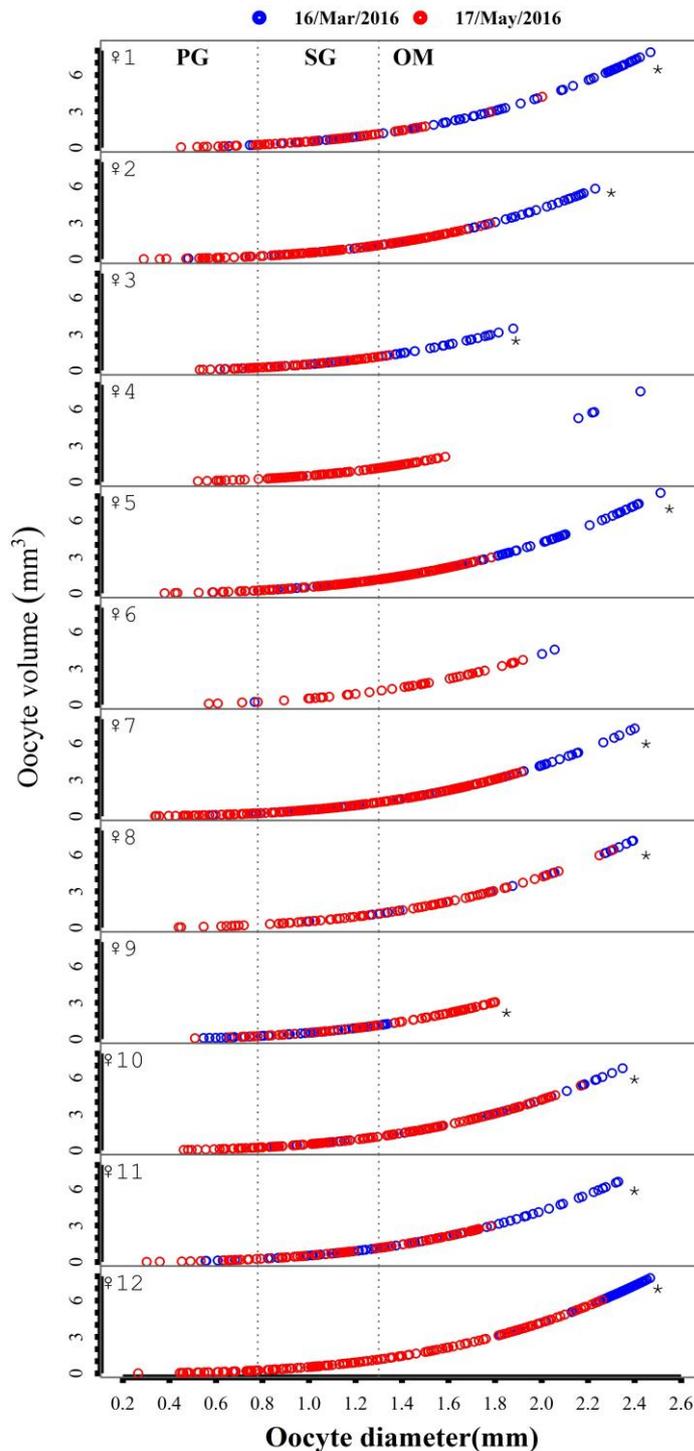
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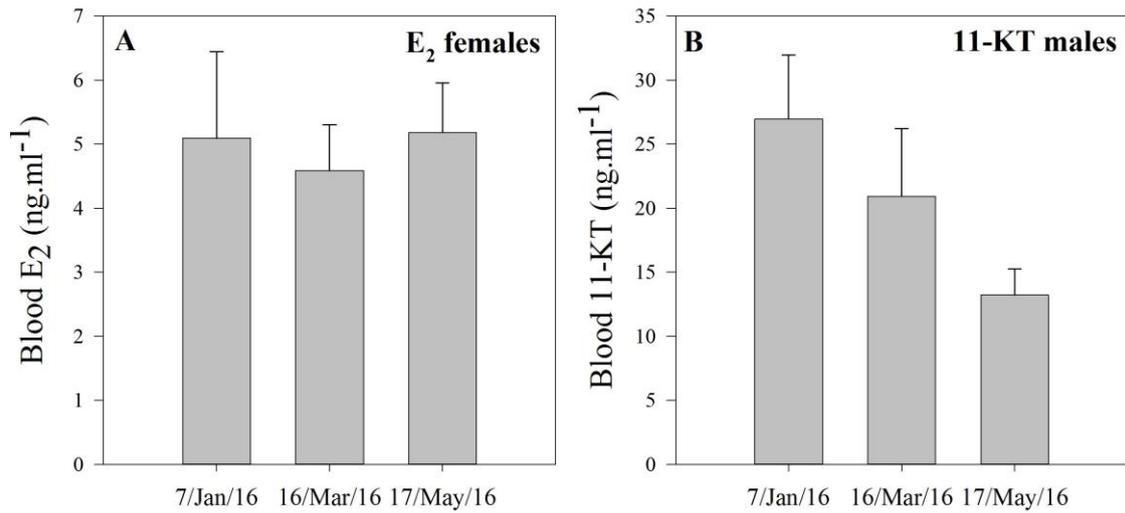
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677 **Figure 7.** Oocyte diameter (mm) vs volume (mm³) for 12 adult females of *Arapaima gigas*
 678 sampled on 16th March (blue) and 17th May (red) 2016. Dashed line indicates stage of oocyte
 679 development for a given oocyte diameter range (PG = primary growth; SG = secondary growth
 680 and OM = oocyte maturation). * indicate significant difference in the leading cohort between
 681 different sampling dates.



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Figure 8. Plasma sex steroid levels in *Arapaima gigas* broodstock. No significant time effects

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were seen ($P > 0.05$). (A) 17 β -oestradiol (E₂) in females (12 individuals). (B) 11-ketotestosterone

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(11-KT) in males (12 individuals).

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702 **Table 1.** Endoscopy procedure in juveniles of *Arapaima gigas*. Fish body weight (kg), total length (cm), time elapsed to observe ovary (minutes), gender
 703 confirmation after histology, diameter and stage of leading cohort (μm) and ovary stage *according to Núñez and Duponchelle (2009). PGpn – Primary
 704 growth perinucleoli.

Juvenile n°	Body Weight	Total Length (cm)	Procedure time (min)	Gender	Leading Cohort	Leaging Cohort Stage	Ovary Stage *
1	12.1	110.5	4.0	Female	220.9	PGpn	I
2	9.5	107.0	2.6	Female	161.9	PGpn	I
3	10.2	107.2	1.6	Female	159.1	PGpn	I
4	12.2	100.0	0.3	Female	208.9	PGpn	I
5	10.1	107.0	—	Male	—	—	—
6	8.9	106.0	—	Male	—	—	—
7	10.2	107.5	—	Male	—	—	—
8	11.7	112.5	1:6	Female	215.4	PGpn	I
9	9.8	106.0	1.1	Female	191.2	PGpn	I
10	11.6	115.0	7.1	Female	167.4	PGpn	I
11	10.6	109.5	—	Male	—	—	—
12	9.9	107.7	—	Male	—	—	—
13	9.8	106.0	No access	Female	209.2	PGpn	I
14	10.9	112.0	—	Male	—	—	—
15	8.9	101.5	9.0	Female	206.1	PGpn	I
16	9.6	103.0	—	Male	—	—	—
17	11.3	110.0	0.7	Female	220.1	PGpn	I
18	9.6	98.5	2.3	Female	246.5	PGpn	I
19	10.4	110.5	1.9	Female	229.5	PGpn	I
20	8.0	98.5	No access	Female	170.3	PGpn	I

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708 **Table 2.** Endoscopy procedure in adult broodstock females of *Arapaima gigas*. Females are numbered as presented in Figure 7, and differently from Table 1
709 which correspond to examinations in juveniles. Female body weight (kg), total length (cm), time elapsed to observe ovary (minutes), ovary predominant color,
710 diameter and stage of leading cohort (μm) and ovary stage *according to Núñez and Duponchelle (2009). OMgvm = Oocyte maturation germinal vesicle
711 migration, SGfg = Secondary growth full-grown, OV = Ovulation.

Adult female n°	Body Weight (kg)	Total Length (cm)	Procedure time (min)	Ovary color	Leading Cohort	Leading	Ovary Stage *
					(μm)	Cohort Stage	
1	37.0	155.0	1.3	green	2391.5	OMgvm	IV
2	31.0	156.0	0.7	green	2152.5	OMgvm	IV
3	45.0	162.0	1.2	green	1740.9	OMgvm	IV
4	-	-	0.6	green	2256.4	OMgvm	IV
5	48.5	175.0	0.7	green	2399.5	OMgvm	IV
6	41.0	167.0	0.6	green	2064.5	OMgvm	IV
7	48.0	173.0	0.5	green	2232.6	OMgvm	IV
8	51.0	175.0	0.7	green	2299.1	OMgvm	IV
9	41.0	160.0	5.8	yellow	1324.0	SGfg	II
10	45.0	168.0	2.1	green	2225.1	OMgvm	IV
11	42.0	166.0	0.5	green	2258.0	OMgvm	IV
12	44.0	171.0	0.3	green	2435.4	OV	IV

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