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Regular Article

Title: GnRHa implants and size pairing effects on plasma and cephalic secretion sex steroids in *Arapaima gigas*

Lucas S. Torati [a, b, *], John F. Taylor [b], Pedro E. C. Mesquita [c] and Hervé Migaud [b]

[a] EMBRAPA Fisheries and Aquaculture, 77022-000, Palmas-TO, Brazil;

[b] Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, Scotland, UK, j.f.taylor@stir.ac.uk (JFT), herve.migaud@stir.ac.uk (HM)

[c] Center of Research in Aquaculture Rodolpho von Ihering-CPA/DNOCS – Ombreira Direita, s/n. Pentecoste-CE, Brazil; pedro_mesquita@uol.com.br

* Corresponding author:
Lucas Simon Torati
EMBRAPA Fisheries and Aquaculture
Palmas-TO, Brazil (CEP 77022-000)
lucas.torati@embrapa.br
Tel.: +55 (63) 3229-7894

Highlights

- Effects of GnRHa implants and couple size pairing in *A. gigas*;
- Potency of mGnRHa demonstrated through sex steroid production;
- Cephalic secretion is a possible source of pheromones.

Abstract

Arapaima gigas, one of the world's largest freshwater fish, is considered an emerging species for aquaculture development in Brazil given its high growth rate and meat quality. However, the lack of reproductive control in captivity has limited the expansion of Arapaima farming. This study aimed to test the effects of hormonal induction using mGnRHa implants and size pairing on broodstock reproduction through the analyses of sex steroids. To do so, broodstock of different sizes (large, small or mixed) were paired and implanted. Plasma and cephalic secretion profiles of testosterone (T), 11-ketotestosterone (11-KT) and 17β-oestradiol (E_2) were analysed. Compared to control (non-implanted), implanted broodstock showed a significant increase in plasma 11-KT (large and small males) and T (large and mixed females) post GnRHa implantation. In females, a significant increase in plasma T levels was shown, however, E_2 remained unchanged after implantation. Despite the lack of clear spawning induction, this study showed the potency of GnRHa on sex steroid production regardless of pairing groups. Interestingly, significant correlations between blood plasma and cephalic secretion levels of 11-KT in males and T in females were observed, indicating the possible release of pheromones through the cephalic canals of *A. gigas*.

Keywords: Hormonal induction, lateral line, sex steroids, pheromones, Pirarucu, reproduction.

1. Introduction

Current knowledge on the biology of the Amazon Pirarucu *Arapaima gigas* (Schinz, 1822) remains scarce with regards to wild and captive populations, conservation and reproduction (Castello and Stewart, 2010; Du et al., 2019). The Pirarucu has been considered as an emerging species for aquaculture diversification in South America with strong market demand due to its growth potential and meat quality. *A. gigas* is an obligate air-breather species, and is one of the largest scaled freshwater fish in the world (Nelson et al., 2016), reaching more than 250 kg in the wild and with a growth potential of 10 kg+ within 12 months (Oliveira et al., 2012). However, achieving consistent spawning in captivity has remained the key challenge over the past decades that has prevented the expansion of the industry (Farias et al., 2015; Saint-Paul, 2017). Consequently, increased pressure on wild capture to meet market demands has resulted in *A. gigas* being placed on the CITES threatened list (Castello and Stewart, 2010). As such, there is a clear need to develop protocols to induce spawning in captivity and understand the factors influencing the reproductive success of *A. gigas*.

Arapaima gigas is gonochoristic and iteroparous with fish reaching first sexual maturity after three to five years of age (Godinho et al., 2005; Gurdak et al., 2019). In their Amazonian habitat, spawning occurs year round peaking in the rainy season from December to May (Castello, 2008; Núñez et al., 2011). Breeding pairs build nests in shallow flooded areas (c. 1-1.5 m depth) where mating, spawning and external fertilization occurs. After spawning, the pair guards the nest for approximately five days and parental care is performed by the male for approximately three months (Alcântara et al., 2019; Castello, 2008). During parental care the male's head and trunk becomes dorsally darkened providing camouflage for the offspring. Females can mate with several males and spawn multiple times during a reproductive season (Farias et al., 2015). This is made possible since ovarian development is asynchronous with several batches of vitellogenic oocytes recruited for maturation along the reproductive season (Godinho et al., 2005; Núñez et al., 2011). On the other hand, males have a tubular cord-like left testis, which after spermiation will still contain lobules of spermatozoa and semen, allowing multiple spermiation during a reproductive season in case parental care is interrupted (Godinho et al., 2005; Núñez and Duponchelle, 2009). Successful

reproduction of *A. gigas* in captivity is problematic, and to date, isolating pairs in earth ponds during the rainy season appears to stimulate reproduction in some cases although outcomes are unreliable and with very limited success (Lima, 2018; Núñez et al., 2011).

Reproductive dysfunction in fish reared in captivity is common. In most cases, spawning can be induced through the use of hormonal therapies (Mylonas et al., 2010; Mylonas and Zohar, 2001). While there are several hormones associated with stimulation of the brain-pituitary-gonad axis (BPG) to artificially induce oocyte maturation, ovulation/spermiation and spawning in fish, hypothalamic gonadotropin releasing hormone (GnRH) is considered the most potent, safe and reliable hormone to use (Mylonas et al., 2010). In fish hatcheries, GnRH analogues (GnRHa) are used to stimulate oocyte maturation and spermiation since they have an increased resistance to enzymatic cleavage compared to the native forms (Mylonas and Zohar, 2001). For asynchronous spawners such as *A. gigas*, the use of slow-release implants is preferred rather than multiple injections, as it promotes a sustained elevation in gonadotropins and reduces stress caused by repetitive handling (Mylonas et al., 2010). In Osteoglossidae and especially *A. gigas*, responsiveness of captive broodstock to GnRHa slow-release implants has not yet been examined.

Development of protocols to induce gonadal recruitment, gametogenesis and spontaneous spawning must consider technical limitations related to the biological features and reproductive strategy traits of a species (Mylonas et al., 2010). Varying widely among teleosts, paired mating systems are often associated with male body size or behavioural characteristics. To date, little is known about the social, behavioural and physiological factors controlling mating in *A. gigas* (Lima, 2018). This includes a lack of knowledge on bodyweight criteria for pairing fish in captivity, a critical component in the breeding success of many fish species (Lehtonen et al., 2015).

When breeding pairs are isolated in captivity, knowledge on mating preferences and success rates are unknown and potential drawbacks may also exist regarding male-female agonistic interactions resulting in unsuccessful matings (Morey et al., 2019). When pairing couples, gender identification in *A. gigas* is another key limitation as the species is not sexually dimorphic. Several techniques have been used to sex fish including colour patterns, laparoscopy to visualise the gonads or vitellogenin measurement to identify females but these can be unreliable, invasive or expensive

(Carreiro et al., 2011; Chu-Koo et al., 2009). Given the gonopore is not externally visible in *A. gigas*, cannulation to obtain ovarian biopsies is difficult and was only recently developed to monitor gonadal development in the species (Torati et al., 2019; Torati et al., 2016). Likewise, stripping of gametes for artificial fertilisation, routinely done in many other species is not suitable due to the species thick abdominal body wall preventing artificial stripping and collection of eggs and milt. In *A. gigas*, reproductive success in ponds cannot be confirmed through observation of spawning behaviour, oviposition, nor assessment of gonadal development. Proxy indicators of reproductive success such as a cessation of feeding behaviour and male darkening have been applied before (Fontenele, 1948, 1953; Monteiro et al., 2010) although these are not easily assessed and not always reliable. Given these limitations, the profiling of sex steroids following GnRHa induction becomes particularly important in this species to confirm its impact on the BPG axis directly associated with gametogenesis.

In *A. gigas*, a cephalic secretion released from the sensorial cavities have been the subject of recent physiological investigations. This secretion has been reported to be enhanced during the reproductive period with potential roles in the parental care phase (Lüling, 1964). Its proteome and peptidome have been profiled recently, depicting hormones (*i.e.* prolactin, stanniocalcin), proteins and peptides potentially related to parental care and fish communication (Torati et al., 2017). Further, a transcriptome investigation surprisingly found male-specific gene expression in the sensorial cavities that "assigns both a fry-nutrition function and also a pheromone-type signaling functioning to local females" (Du et al., 2019). Another study found sex steroids (17α-hydroxyprogesterone) in the cephalic secretion suggesting their potential role as pheromones in the species (Amaral, 2009; Amaral et al., 2019).

The aims of the present study were to: 1) test the effects of GnRHa slow-release implants on sex steroid profiles measured in blood plasma and cephalic secretion, and 2) examine the effects of different size pairings on reproductive success and sex steroid profiles.

2. Materials and Methods

2.1. Experimental set up

This experiment was conducted at the Rodolpho von Ihering Station - DNOCS (3°48'09.54"S, 39°15'56.73"W) in Pentecoste-CE (Northeast Brazil). A total of 59 adult captive reared broodstock of approximately the same age (over six-year-old) had been previously held since 2013 in two large earth ponds, 8 females with 11 males in a 2300 m^2 pond and 19 females with 21 males in a 930 m^2 pond. No spontaneous spawnings are normally observed in these stocking ponds and fish density in the species.

The experiment started on January 21st 2014 (day 0), when broodstock were measured for bodyweight (BW) (± 0.1 kg), total length (TL) (± 0.1 cm), and Fulton's condition factor (K) calculated as $K=(BW\times100)/TL^3$ (Froese, 2006). Fish were photographed and implanted with a passive integrated transponder (PIT; AnimallTAG®, São Carlos, Brazil) in the dorsal muscle to allow individual identification. Each fish was sexed using a vitellogenin enzyme immune assay (EIA) kit (Acobiom, Montpellier, France) based on work of Dugue et al. (2008). Based on BW, each female was paired with a single male and pairs were allocated into 18 earthen ponds of 330 m^2 and depth of 1.95 ± 0.06 m (deepest point) (Fig. 1A). Four treatments were tested: control (handled as for other treatments but without placebo implant) with pairs of large fish (53.8 ± 3.3 kg; n=5 pairs), and three GnRHa implanted groups of mixed size fish: large (58.8 ± 5.3 kg; n=5 pairs), small (29.8 ± 5.0 kg; n=3 pairs) or mixed size pairs (large female: 56.1 ± 4.1 kg paired with small male: 21.5 ± 1.8 kg, n=5 pairs). Large breeding pairs (not implanted) were selected as controls for the experiment as they are representative of commercial practice in fish farms of *A. gigas*, and due to limited broodstock/earth ponds availability, it was not possible to include controls for other treatments. Average fish BW, TL and K among sex and treatments are presented in Table 1.

At day 62 post pairing and stocking into ponds, treated fish received a mGnRHa slow-release implant (Center of Marine Biotechnology, Baltimore, MD, USA) with a dose of 84.7 ± 8.7 $\mu g.kg^{-1}$ for females and 49.1 ± 6.7 $\mu g.kg^{-1}$ for males. Implants were made with ethylene-vinyl acetate polymer (EVAc) delivering desGly10, DAla6, Pro9-GnRH- NEthylamide for approximately 21 days (Mylonas et al., 2007). Each implant was inserted in the dorsal muscle using an implanter (Fig. 1B).

177 Fish were fed once a day *ad libitum* with 160 g floating balls made with a commercial ration
178 (38 % crude protein, Aquamix, Brazil) mixed with 10 % tilapia flesh (*Oreochromis niloticus*) (Fig.
179 1C). Water turbidity in the ponds hindered the possibility to directly observe spawnings, therefore
180 daily feed intake (i.e. number of floating balls consumed) per pair was recorded instead, and
181 cessation in feeding and/or pairs swimming at the same location for long periods were used as
182 proxy for mating and nest guarding behaviour (Fontenele, 1953). With such limitations to infer
183 reproductive activity in the species, effects of GnRHa implantation were restricted to hormonal
184 profiling.

185 This experiment occurred under natural photo-thermal regimes. Climatic data was obtained
186 from the National Institute for Space Research (INPE, bancodedados.cptec.inpe.br) and photoperiod
187 from the R package "StreamMetabolism" (R-Core-Team, 2016). During the study, air temperature
188 ranged from 23.8 to 30.5 °C, and photoperiod ranged from 11.9 to 12.3 hours of photophase.
189 Maximum daily rainfall recorded was 819 mm, and the last meaningful rain (204.8 mm) occurred at
190 day 112.

191

192 2.2. Sampling procedures

193 All broodfish were sampled for blood and cephalic secretion at pond allocation (day 0), GnRHa
194 implantation (day 62), two weeks post-implantation (day 76) and then monthly thereafter (days 111,
195 146 and 181). At each sample point, ponds were sampled in the same daily order between 6:00 and
196 10:00am. Prior to each sampling, fish were fasted for 24 hours. Fish were netted from the earth
197 ponds and kept contained in a cylinder-shaped net on a soft wet mat for approximately 5-10 minutes
198 maximum. Anaesthetics were not applied during sampling as anaesthesia has been shown to
199 compromise welfare and result in mortalities in *A. gigas* due to its air breathing behaviour (Farrel
200 and Randall, 1978). Fish breathing behaviour was closely monitored during each procedure
201 (breathing at regular intervals of 4-6 minutes). Fish were photographed to analyse colour patterns.
202 Approximately 4 ml of blood was sampled from the caudal vein using syringes (BD Precisionglide,
203 New Jersey, USA) flushed with 560 $IU.ml^{-1}$ heparin ammonium salt solution (Sigma Aldrich, Saint
204 Louis, MO, USA) (Fig. 1D). Plasma was collected by centrifugation at 1200 g for 15 minutes,

stored in cryovials and frozen in liquid nitrogen. Cephalic fluid (2-3 ml) was sampled from the dorsal most lateralis cavity of the preopercle using a sterile syringe carefully inserted underneath the dermis sensorial cavity (Fig. 1E), then immediately frozen in liquid nitrogen. Fish were then returned to the ponds and monitored until normal breathing behaviour returned. Due to unknown reasons, one female from the small group and one male from the mixed size group died following sampling on 13th May 2014 (day 111).

Samples were then transported to EMBRAPA research centre in Fortaleza (Brazil) and stored at -80 °C, and then shipped on dry ice to the University of Stirling (Stirling, Scotland) for analyses (Permit IBAMA/CITES n°14BR015849/DF and 14BR015850/DF). This research complied with the "Brazilian guidelines for the care and use of animals for scientific and educational purposes"–DBCA, it was granted approval from the National System for the Management of Genetic Heritage and Associated Traditional Knowledge – SISGen (AA4F2B0), and also by the Ethics Committee for the Use of Animals—CEUA of the National Research Center on Fisheries, Aquaculture and Agricultural Systems—CNPASA (specific protocol n°09).

2.3. Steroid analyses

Levels of testosterone (T) and 17β-oestradiol (E_2) in plasma and cephalic secretion were quantified in duplicate by radioimmunoassay (RIA), following methods developed by Duston and Bromage (1987). Tritiated radiolabels for T (GE Healthcare, UK) and E_2 (PerkinElmer, Boston, USA) were used with anti-T and anti-E_2 antisera (CER group, Marloie, Belgium). Radioactivity was measured using a Packard 1900 TR Liquid Scintillation Analyser (Pangbourne, UK). For analysis of 11-ketotestosterone (11-KT), an enzyme-linked immunosorbent assay (ELISA) kit was used (Cayman Chemical Inc., Michigan, USA) following manufacturer's protocol and microplates were read at 405 nm using an ELX808 reader (Biotek, Swindon, UK). T and E_2 RIA and 11-KT ELISA were validated for *A. gigas* through assay parallelism comparing serial dilutions of extracts to known concentrations of hormone standards as described in Sink et al. (2008). All assays have been validated in *A. gigas* prior to the analyses by confirming the parallelism between serial dilutions of plasma samples to the standard curve (F= 2.395; F= 0.434; F= 1.343 for T, E_2 and 11KT,

respectively; p >0.05; Supplementary Figure 1). The intra-assay and inter-assay coefficients of variation were 12.0 and 6.6 % for T (7 assays), 12.6 and 9.8 % for E_2 (7 assays) and 9.7 and 10.0 % for 11-KT (4 assays), respectively. Concentration of steroids in the blood or cephalic secretion were calculated from the value yielded in the assay ($pg.tube^{-1}$) corrected for: (a) proportion of extract added to the assay tube and (b) volume of blood or cephalic secretion used for extraction.

2.4. Statistical analysis

Statistical analyses were conducted with Minitab (version 17.3.1, Minitab, PA, USA). Parallelism between assay standard curves and serially diluted plasma samples were tested using F-test. Data on level change between implantation (day 62) and days 76 and 111 were not normally distributed (Kolmogorov-Smirnov test) even after transformations, a non-parametric Kruskal–Wallis one-way ANOVA and Dunn's pairwise post hoc tests were used to compare GnRHa effects between treatments. In order to describe time effects within treatments, Kolmogorov-Smirnov and Levene's tests were used to test normality and homogeneity assumptions, and then a one-way repeated measures ANOVA followed by Tukey post hoc tests were applied. Pearson Product Moment Correlations were calculated on log-transformed data, and used to correlate and compare steroid levels in blood plasma and cephalic secretion. Level of significance was set as $p \leq 0.05$ and data are presented as mean ± SEM unless stated otherwise.

3. Results

3.1. Reproductive behaviour following GnRHa implantation

No behavioural observations or reproductive activity (cease in feeding, nesting behaviour) were recorded in any of the experimental pairs during the 14 days following implantation. However, at day 146 (84 days post implantation - dpi), two of the large implanted pairs displayed nesting behaviour and one female from the small implanted pairs released eggs during the sampling.

3.2. Effects of GnRHa implants on plasma and cephalic sex steroid in males

3.2.1 Testosterone in males

Overall, no significant time effects were found in plasma T levels measured in males from control and small implanted pairs during the experimental study (Fig. 2A and C). In large pair groups, plasma levels increased by 2-fold (p <0.05) from 20.7 ± 5.8 to 41.4 ± 13.8 ng.ml^{-1} after 14 dpi on day 76 (Fig. 2B) and remained higher at 49 dpi. In mixed pair groups, plasma levels did not change post implantation however a significant (p <0.05) increase was observed prior implantation between day 0 and 76 (Fig. 2D). When comparing implanted treatments with control group at 14 and 49 dpi, plasma T level changes in relation to implantation (day 62) were not significantly different (Fig. 3A). However, change in plasma levels in large paired groups were significantly higher than small at both 14 and 49 dpi (Fig. 3A).

In the cephalic secretion, no time effects were observed in T levels from all groups (Fig. 2). A positive correlation between T levels in blood and cephalic secretion was found (r^2=0.33; p <0.001).

3.2.2. 11-ketotestosterone in males

Plasma 11-KT levels remained below 30 ng.ml^{-1} in males from the control pairs throughout the experimental study (Fig. 4A). Plasma 11-KT levels increased significantly (p <0.05) in males from large pairs (7.3-fold, from 13.6 ± 5.0 to 135.2 ± 30.0 ng.ml^{-1}) (Fig. 4B). In addition, changes in plasma levels at 14 dpi were significantly higher in large and small pairs than control (Fig. 3B). This increase was sustained at day 111 (49 dpi) in large pairs but not small, and returned to basal pre-implantation levels at day 146 (84 dpi) (Figures 4B-C and 3B).

In the cephalic secretion, 11-KT levels in control pairs increased 4-fold (from 63.8 pg.ml^{-1} to 252 pg.ml^{-1}) between day 0 (pair allocation into earthen ponds) and day 62 (implantation of GnRHa p<0.05, Fig. 4A). In large pairs, levels increased significantly (p <0.05) by 15-fold (from 162.4 ± 56.4 to 2433.1 ± 1722.3 pg.ml^{-1}) between implantation and day 76 (14 dpi), returning to pre-implantation levels from day 146 onwards (84 dpi) (Fig. 4B). In small size pairs, 11-KT levels increased significantly (p <0.05) from day 0 to day 76 (14 dpi, Fig. 4C). In mixed size pairs, no significant differences were seen over time although levels appeared to increase at 14 dpi (Fig. 4D).

Plasma T and 11-KT levels were positively correlated in males (r^2=0.62; p <0.001) (Fig. 5A). In addition, a significant positive correlation was found between 11-KT levels in blood plasma and cephalic secretion (r^2=0.74; p <0.001, Fig. 5B).

3.3. Effects of GnRHa implants on plasma and cephalic sex steroid in females

3.3.1 Testosterone in females

Plasma T levels remained below 40 ng.ml^{-1} in control pairs throughout the experimental study however levels appeared to increase slightly following implantation and then decreased significantly by the end of the study (day 181) (Fig. 6A). Levels increased significantly (p <0.05) in large (3.2-fold from 20.0 ± 14.0 to 64.7 ± 18.6 ng.ml^{-1}) and mixed size pairs (4.5-fold, from 12.4 ± 7.6 to 56.0 ± 27.2 ng.ml^{-1}) at 14 dpi (Fig. 6B-D and 3C). This increase was sustained at day 111 (49 dpi) in both groups and resumed to basal pre-implantation levels by the end of the study (Fig. 6B-D and 3C). In small size pairs, no time effects were seen (Fig. 6C and 3C).

No significant time effects were observed in female T levels measured in the cephalic secretion of control, small and mixed size pairs although levels appeared to increase in mixed pairs at 14 dpi and returned to pre-implantation basal levels by the end of the study (Fig. 6A, C and D). In large pair groups, T levels increased significantly (p <0.05) by 6-fold (from 429.4 ± 251.2 to 2544.0 ± 1489.4 pg.ml^{-1}) between implantation (day 62) and day 111 (49 dpi; Fig. 6B). A significant positive correlation was found between T levels in blood plasma and cephalic secretion in females (r^2=0.25; p <0.001; Fig. 5D).

3.3.2. 17β-oestradiol in females

No significant time effects were seen in E_2 levels measured in blood plasma and cephalic secretion along the study period for any of the GnRHa implanted groups (Fig. 7 and 3D). Plasma T levels showed a significant positive correlation with plasma E_2 levels (r^2=0.36; p <0.001, Fig. 5C). No correlation was found between E_2 levels in blood plasma and cephalic secretion (r^2=0.12; p =0.210).

4. Discussion

In recent years, a series of publications have studied the reproductive physiology of *A. gigas* reared in captive conditions or from the wild. These included the description of the adenohypophysis (Borella et al., 2009), the isolation of the pituitary gonadotrophic α-subunit hormone (Faria et al., 2013), follicle-stimulating hormone and luteinizing hormone β-subunit cDNAs (Sevilhano et al., 2017), the identification of gender through analysis of sex steroid, blood vitellogenin levels and colour patterns (Chu-Koo et al., 2009) and the description of the gametogenesis and gonadogenesis in both sexes (Godinho et al., 2005; Núñez and Duponchelle, 2009). However, the lack of reproductive control in *A. gigas* in captivity and the impact of hormonal induction on the control of gametogenesis and spawning have not yet been investigated despite being one of the most important challenge preventing the expansion of *A. gigas* aquaculture (Ferreira et al., 2020). This is largely explained by the constraints to reliably identify genders, assess reproductive condition *in vivo* through ovarian biopsy and source implants that can deliver hormonal dosage suitable for such large broodstock, which are all essential for developing hormonal induction protocols (Mylonas et al., 2010). In addition, sampling of adult broodstock from the wild or farms for research purposes has been prohibitive due to ecological and economic factors. Trying to overcome these limitations and capitalise on the recent validation of sex identification techniques (Chu-Koo et al., 2009; Dugue et al., 2008) and availability of suitable implants (Mylonas et al., 2007), this study described the combined effects of couple size pairing and GnRHa implantation on reproductive function of captive *A. gigas*. Although spawnings directly associated with the experimental manipulations could not be confirmed, results showed effects of the implants on sex steroid secreted in the blood and cephalic secretion. This confirmed that the hormonal induction protocol used stimulated the BPG axis, however without clear influence of size pairing.

The current experiment was carried out during the rainy season when spawning has been previously reported at the experimental site (Rebouças et al., 2014), and therefore females were expected to be recruited into reproduction. Although the observation of two spawnings prior to the start of the experiment (pairs excluded from the study) suggested some broodstock were recruited into reproduction before implantation, no spawning nor reproductive behaviour associated with nest building or mating (e.g. reduced feeding, alteration in swimming and air breathing patterns) were

observed in the control and implanted pairs post hormonal induction. A possible explanation for the lack of spawning could be that females at the time of implantation were at an early stage of oogenesis and therefore follicular cells were not responsive to gonadotropin (LH and FSH) stimulation. The lack of spontaneous spawning following the hormonal stimulation contrasts with previously published results obtained in other asynchronous spawners like Senegalese sole (*Solea senegalensis*) which released eggs after 4 dpi (Guzmán et al., 2009), Meagre (*Argyrosomus regius*) which spawned after 2-3 dpi (Mylonas et al., 2013) or the Atlantic Bluefin Tuna (*Thunnus thynnus*) which spawned after 6 dpi (Rosenfeld et al., 2012). A possible explanation for the lack of spawning following hormonal implantation in *A. gigas* could be a lack of appropriate stimulation of the mating behaviour in the species which involves nest building, courtship and parental care. This has been reported for other species and in such cases egg collection through stripping is often necessary (Mylonas and Zohar, 2001). If this was the case, the implants could have induced oocyte maturation and spawning in females which then released eggs without fertilization or lacking parental care provision. Alternatively, such reproductive dysfunction can either be hormonal with possible involvement of other GnRH forms playing a neuromodulation role on the reproductive behaviour (Okubo and Nagahama, 2008), or behavioural due to a lack or reduction of pheromonal signaling and reproductive synchronization among partners. In such cases, a treatment with only GnRHa might not be enough to induce oocyte maturation, ovulation and spawning despite the observed impact on 11-KT (males) and T (females). Indeed, this explanation finds support in the unchanged E_2 levels in all implanted females post-implantation. Another factor that could explain the lack of spawnings is a potential dopaminergic inhibition of gonadotrophin production and release (Dufour et al., 2010; Dufour et al., 2005), however this has not been investigated yet in *A. gigas*. Strong dopaminergic inhibition have been reported in the Japanese eel *Anguilla japonica* (Ohta et al., 1997) and male Senegalese sole (*Solea senegalensis*) (Guzmán et al., 2011), and in such cases additional treatments with dopamine antagonists (i.e. pimozide, domperidone) were required to induce spawning (Mylonas et al., 2010).

In species where GnRHa induction was reported to be successful, plasma sex steroid levels usually peak a few days after the implantation as reported in Atlantic Bluefin Tuna (*Thunnus*

365 *thynnus*) (Rosenfeld et al., 2012), Senegalese sole (*Solea senegalensis*) (Guzmán et al., 2011) and
366 yellowtail flounder (*Pleuronectes ferrugineus*) (Larsson et al., 1997). In this study, a window of 14
367 days (days 62-76) was given between implantation and the following sampling point to minimise
368 handling stress which is known to disrupt reproduction in many species and monitor potential
369 mating and breeding behaviour in *A. gigas*. By day 76 (14 dpi), plasma T levels were increased
370 significantly in females from large and mixed implanted pairs contrasting with the lack of plasma E_2
371 response. The lack of increase in circulating E_2 suggests either an enzymatic deficiency in
372 cytochrome P450 aromatase (P450arom) activity converting precursor T into E_2 in the granulosa
373 cells of the oocytes, or the timing of the sampling did not have the resolution to detect an E_2 peak
374 that is usually slightly phase shifted from the T increase. However, while E_2 main role during
375 oogenesis is to stimulate hepatocytes to produce vitellogenin that accumulates in the oocytes during
376 vitellogenesis (Lubzens et al., 2010), it has also been suggested to play a role during final oocyte
377 maturation (FOM) and ovulation (OV). While plasma E_2 levels during vitellogenesis can remain
378 high during a prolonged window (from weeks to months in iteroparous spawners), increase during
379 FOM and OV can also be transient (hours to days depending on species) (Lubzens et al., 2010).
380 Therefore, in future experiments, sampling schedule should be adapted to confirm this hypothesis
381 and alternatively, *in vitro* experiments could be performed studying other sex steroids or hormone
382 like compounds involved in the later stages of oogenesis such as the maturation inducing steroids
383 (MIS) or prostaglandins.

384 In fish hatcheries, problems with male reproduction are less common than for females and
385 generally are associated with a reduced sperm volume and quality (Migaud et al., 2013; Mylonas et
386 al., 2017). Since possible dysfunctions in male *A. gigas* are unknown, this study evaluated the
387 effects of a lower GnRHa dose (49.1 ± 6.7 $\mu g.kg^{-1}$) compared to females, but intended to increase
388 chances of reproduction by synchronizing the pairs and also evaluate GnRHa impact on male BPG
389 axis. Given that T is the main precursor of 11-KT, levels of both androgens co-vary during most of
390 the reproductive season (Mylonas and Zohar, 2001) with 11-KT being considered as the key
391 hormone peaking during spermatogenesis and declining prior to the spermiation period (Mylonas
392 and Zohar, 2001; Schulz et al., 2010). In the present experiment, a significant correlation between T

and 11-KT was found suggesting a positive impact of the GnRHa implants on the BPG axis. Stripping of males after implantation was attempted without success due to the thick abdominal body wall of *A. gigas* and the specific anatomy of the urogenital system of the species, and it was therefore difficult to infer possible impacts of the hormonal induction on spermiation or milt volumes. When compared to control males, all implanted groups showed a significant increase in 11-KT levels post GnRHa implantation (14 dpi). These results suggest the lack of observed spontaneous spawning post implantation was unlikely related to male reproductive dysfunction.

During the present study, one female spawned while being sampled (from small pair groups) after 84 dpi and outside the rainy season. During the same period, nest building behaviour was observed in two other implanted pairs (large) with the male displaying an apparent darkened external pigmentation. The link between these reproductive events and the GnRHa treatments is unclear, however spawning of *A. gigas* outside the rainy season is rare especially on the studied site. Analysis of steroid profiles for these three pairs clearly showed GnRHa contributed to stimulate the BPG axis and possibly spermatogenesis/vitellogenesis. The observation of ripe females spawning while being sampled has already been anecdotally reported during samplings of Pirarucu on farms (pers. comm.), suggesting the involvement of stress factors in the induction of spawning in *A. gigas*. Spawning induced by stress are common among other fish species (Schreck et al., 2001) but so far have not been documented for *A. gigas*. These novel observations suggest artificial fertilisation could be feasible in *A. gigas* especially after the recent development of endoscopy and cannulation to monitor female ovary development (Torati et al., 2019; Torati et al., 2016). However, artificial fertilisation in *A. gigas* will require further characterisation of the unusual male gonadal anatomy especially regarding the position of the gonopore in the genital papilla, to develop non-invasive protocols for milt collection as done in *Clarias* spp. (Idahor, 2014).

Sex steroids were also analysed in the cephalic secretion released from the head of *A. gigas* males and females. There is very limited available data on the biochemical nature of this fluid in the cephalic canals of the lateral line system in teleosts (Coombs et al., 2014). This cephalic fluid in *A. gigas* is known by the Amazonian indigenous as the “Pirarucu milk”, given its whitish colour especially during the parental care phase. However, the role(s) of this substance in the biology of

the species is still unknown. In a recent study performed on wild specimens, steroids (T, E_2 and 17α-hydroxyprogesterone) were detected in the cephalic secretion (Amaral, 2009; Amaral et al., 2019), with levels of 17α-hydroxyprogesterone higher in maturing females. In the present experiment, positive correlations between plasma and cephalic secretion steroid levels were observed for 11-KT and T. This strongly supports the release of steroids as pheromones through the cephalic canals and the circulatory system. Interestingly, no correlation was found for E_2. Since T is also converted by aromatase into E_2 in the brain (Forlano et al., 2001), the cerebrospinal fluid could be hypothesized as a possible source of E_2 for the cephalic secretion, as a dual source (blood plasma / cerebrospinal fluid) would explain the lack of correlation observed between plasma and cephalic secretion levels of E_2. Given that the lateral line in osteoglossids is an opened system, and the cephalic secretion is released externally, results support the hypothesis that the cephalic fluid could play an important role in pheromonal signaling. Further investigations are needed to characterise the nature and role(s) of this cephalic secretion in *A gigas*.

5. Conclusions

This study showed for the first time the impact of slow-release GnRHa implants on the pituitary-gonad axis of *A. gigas*, eliciting significant increases in T and 11-KT levels in females and males, respectively. Couples paired with different sizes showed similar responses to GnRHa in terms of steroid levels, but impact on mating and spawning could not be assessed properly. Lack of correlation between T and E_2 levels in blood plasma of females suggests a reduced activity in aromatase P450 in the species and/or some dopaminergic inhibition on gonadotroph cells in the pituitary. Interestingly, positive correlations between plasma and cephalic secretion steroid levels suggest a link between the anterior lateral line and the circulatory systems. This is a possible new route of pheromone release in a teleost species.

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H.M.; supervision, H.M.; project administration, L.S.T.; funding acquisition, L.T. All authors have read and agreed to the published version of the manuscript.

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620

621 **Figure Captions**

622 **Figure 1.** *Arapaima gigas* experimental details. (**A**) Site indicating earthen ponds used for pair
623 allocation (image from http://www.google.com/earth/index.html; accessed at 13.10.16); (**B**)
624 GnRHa slow-release implant and fish implantation in dorsal muscle; (**C**) Feed pellet offered to
625 fish during the trial; (**D**) Sampling of blood from the caudal vein; (**E**) Sampling of cephalic
626 secretion from preopercle cavity.

627 **Figure 2.** Testosterone (T) levels in plasma ($ng.ml^{-1}$) and cephalic secretion ($pg.ml^{-1}$) in
628 *Arapaima gigas* males from day 0 (couple pairing and stocking into ponds) to day 181 (119
629 days post GnRHa implantation) in the four experimental groups. (**A**) Control pairs (n = 5); (**B**)
630 Large pair implanted groups (n = 5); (**C**) Small pair implanted groups (n = 3); and (**D**) Mixed
631 pair implanted group (n = 5). Values are presented as mean ± SEM. Lowercase superscripts
632 denote time effect in blood plasma levels (p <0.05). Arrows indicate GnRHa implantation time
633 at day 62.

634 **Figure 3.** Post-implantation changes in plasma sex steroid levels ($ng.ml^{-1}$) at days 76 and 111
635 (14 and 49 days post implantation, respectively) expressed as relative changes to levels
636 recorded on implantation day (0 dpi). (**A**) Male Testosterone (T); (**B**) Male 11-ketotestosterone
637 (11-KT), (**C**) Female testosterone and (**D**) Female 17β-oestradiol (E_2). Data presented as mean
638 ± SEM. Different uppercase letters denote statistical difference among groups at a given time
639 (p <0.05). **Control** - fish pairs not implanted (53.8 ± 3.3 kg; n=5); **Large** – fish pairs (58.8 ±
640 5.3 kg; n=5) implanted with GnRHa; **Small** - fish pairs (29.8 ± 5.0 kg; n=3) implanted with
641 GnRHa; and **Mixed** - large female (56.1 ± 4.1 kg) paired with a small male (21.5 ± 1.8 kg,
642 n=5) implanted with GnRHa.

643 **Figure 4.** Levels of 11-ketotestosterone (11-KT) in the plasma ($ng.ml^{-1}$) and cephalic secretion
644 ($pg.ml^{-1}$) in males of *Arapaima gigas* from day 0 (couple pairing and stocking into ponds) to
645 day 181 (119 days post GnRHa implantation) in the four experimental groups. (**A**) Control
646 pairs (n = 5); (**B**) Large implanted pairs (n = 5); (**C**) Small implanted pairs (n = 3); and (**D**)
647 Mixed size implanted groups (n = 5). Values are presented as mean ± SEM. Lowercase and

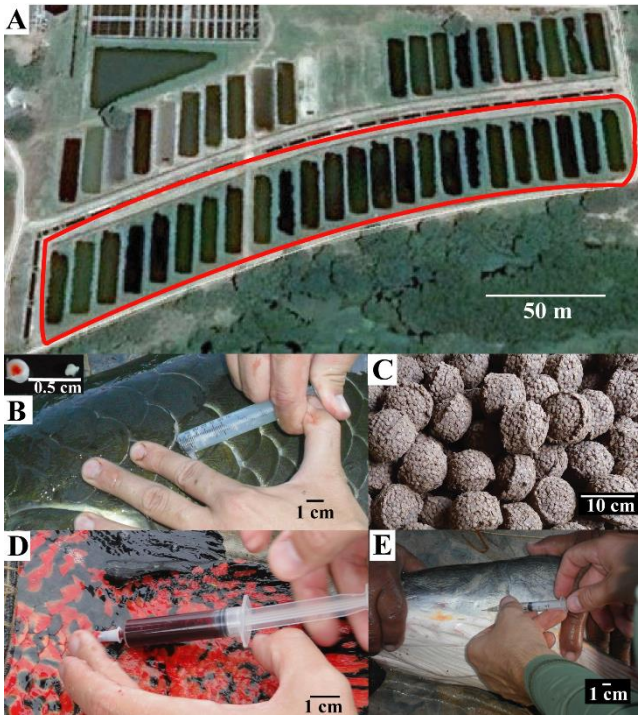
uppercase superscripts denotes time effects in blood plasma and cephalic secretion levels, respectively (p <0.05). Arrows indicate GnRHa implantation time at day 62.

Figure 5. Sex steroid correlations in male and female *Arapaima gigas* broodstock. (**A**) Correlation between male testosterone (T) and 11-ketotestosterone (11-KT) levels (ng.ml^{-1}) in blood plasma; (**B**) Correlation between male 11-KT levels in blood plasma (ng.ml^{-1}) and cephalic secretion (pg.ml^{-1}); (**C**) Correlation between female Testosterone (T) and 17β-oestradiol (E_2) levels (ng.ml^{-1}) in blood plasma and (**D**) Correlation between female T levels in blood plasma (ng.ml^{-1}) and cephalic secretion (pg.ml^{-1}). Pearson Product Correlation Coefficients were calculated on log-transformed data.

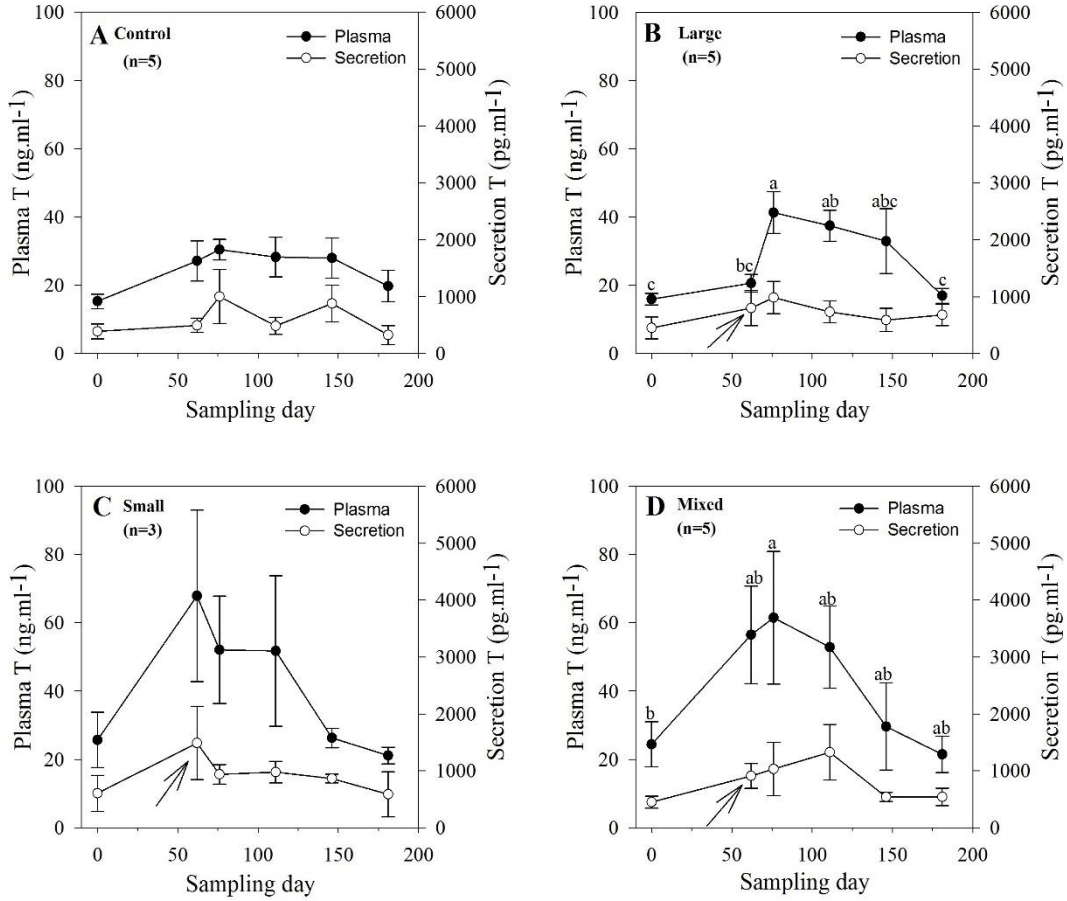
Figure 6. Levels of testosterone (T) in the plasma (ng.ml^{-1}) and cephalic secretion (pg.ml^{-1}) in females of *Arapaima gigas* from day 0 (fish pairing and stocking into ponds) to day 181 (119 days post GnRHa implantation) in the four experimental groups. (**A**) Control pairs (n = 5); (**B**) Large implanted pairs (n = 5); (**C**) Small implanted pairs (n = 3); and (**D**) Mixed size implanted pairs (n = 5). Values are presented as mean ± SEM. Lowercase and uppercase superscripts denotes time effects in blood plasma and cephalic secretion levels, respectively (p <0.05). Arrows indicate GnRHa implantation time at day 62.

Figure 7. Levels of 17β-oestradiol (E_2) in the plasma (ng.ml^{-1}) and cephalic secretion (pg.ml^{-1}) in females of *Arapaima gigas* from day 0 (fish pairing and stocking into ponds) to day 181 (119 days post GnRHa implantation) in the four experimental groups. (**A**) Control pairs (n = 5); (**B**) Large implanted pairs (n = 5); (**C**) Small implanted pairs (n = 3); and (**D**) Mixed size implanted pairs (n = 5). Values are presented as mean ± SEM. Arrows indicate GnRHa implantation time at day 62.

Figure 1.



674 **Figure 2.**



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677 **Figure 3.**

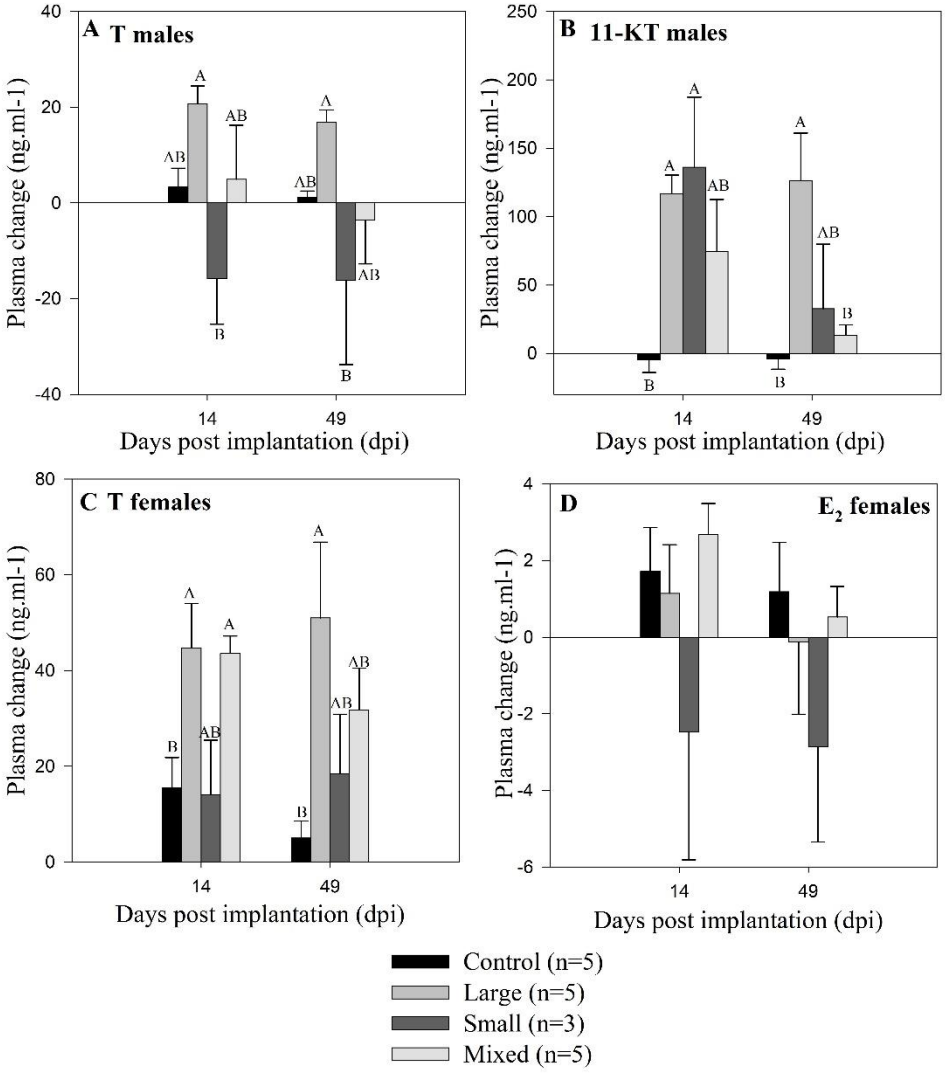

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Figure 4.

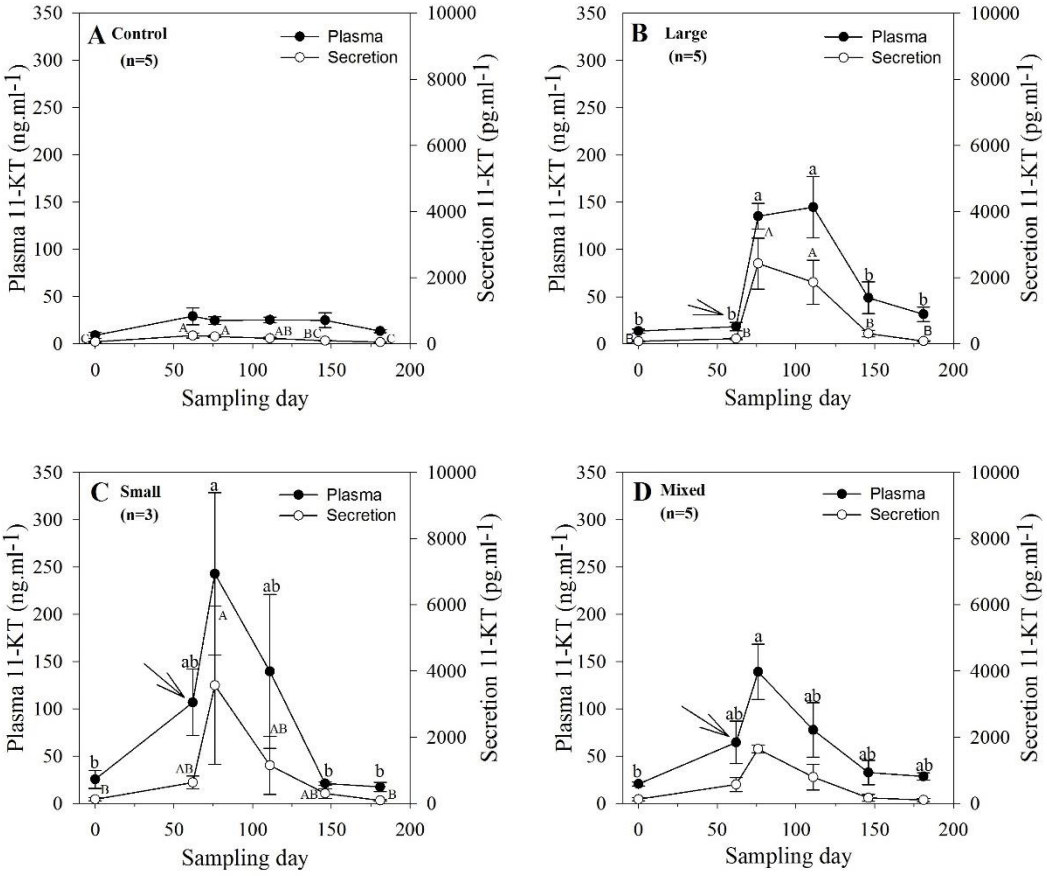
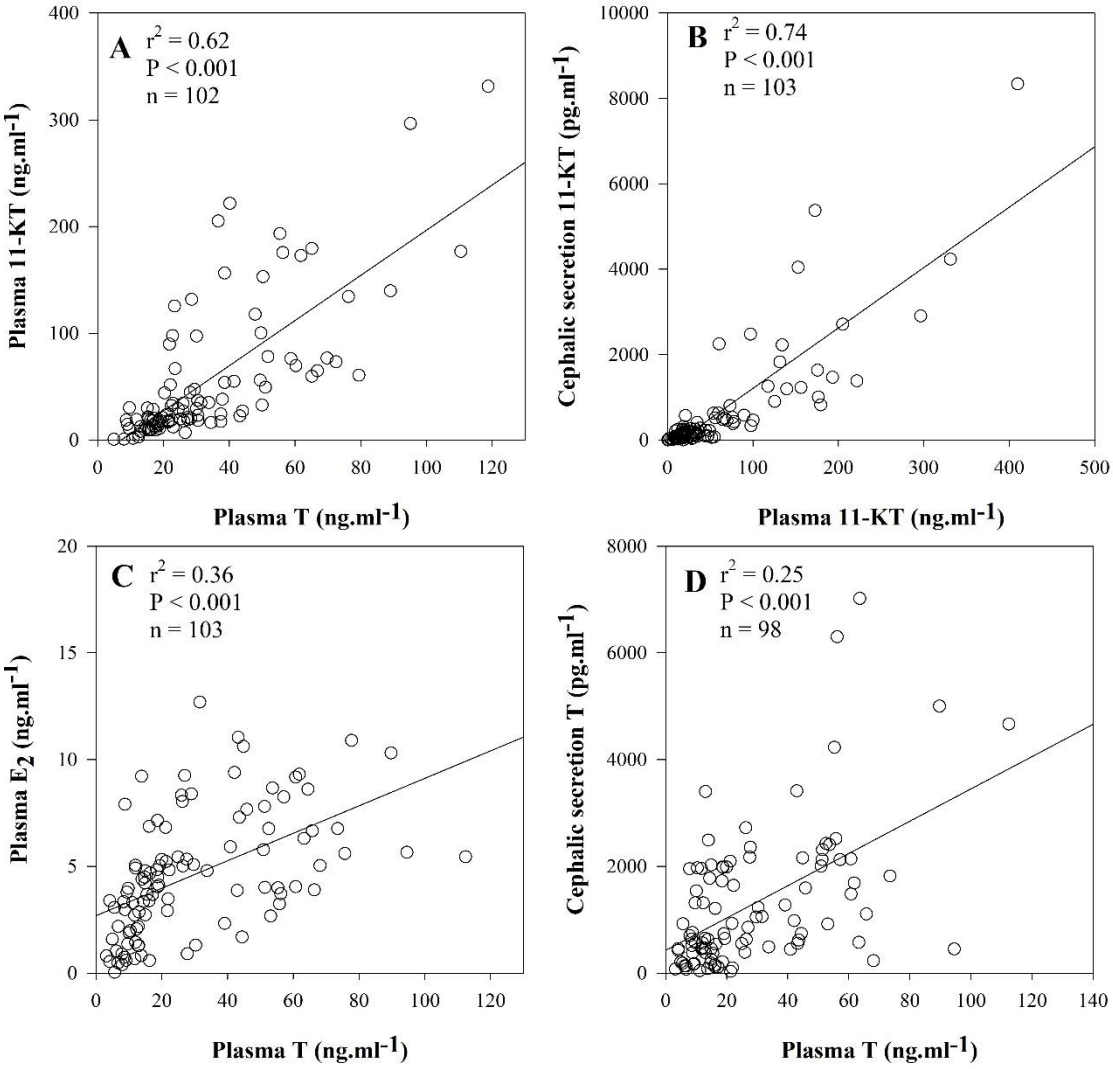
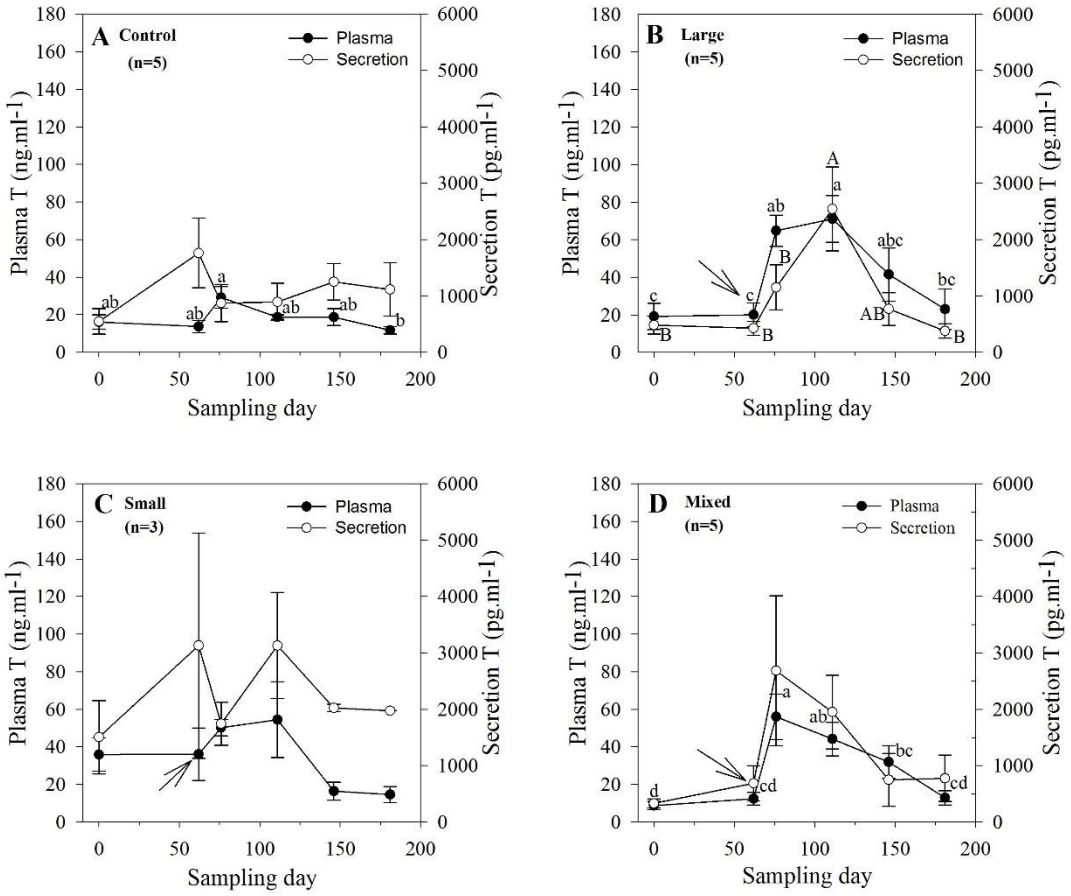

A Control
(n=5)
B Large
(n=5)
C Small
(n=3)
D Mixed
(n=5)
Plasma
Secretion
Plasma 11-KT (ng.ml-1)
Secretion 11-KT (pg.ml-1)
Sampling day

Figure 5.


A
r² = 0.62
P < 0.001
n = 102
Plasma 11-KT (ng.ml-1)
Plasma T (ng.ml-1)
B
r² = 0.74
P < 0.001
n = 103
Cephalic secretion 11-KT (pg.ml-1)
Plasma 11-KT (ng.ml-1)
C
r² = 0.36
P < 0.001
n = 103
Plasma E2 (ng.ml-1)
Plasma T (ng.ml-1)
D
r² = 0.25
P < 0.001
n = 98
Cephalic secretion T (pg.ml-1)
Plasma T (ng.ml-1)

686 **Figure 6.**



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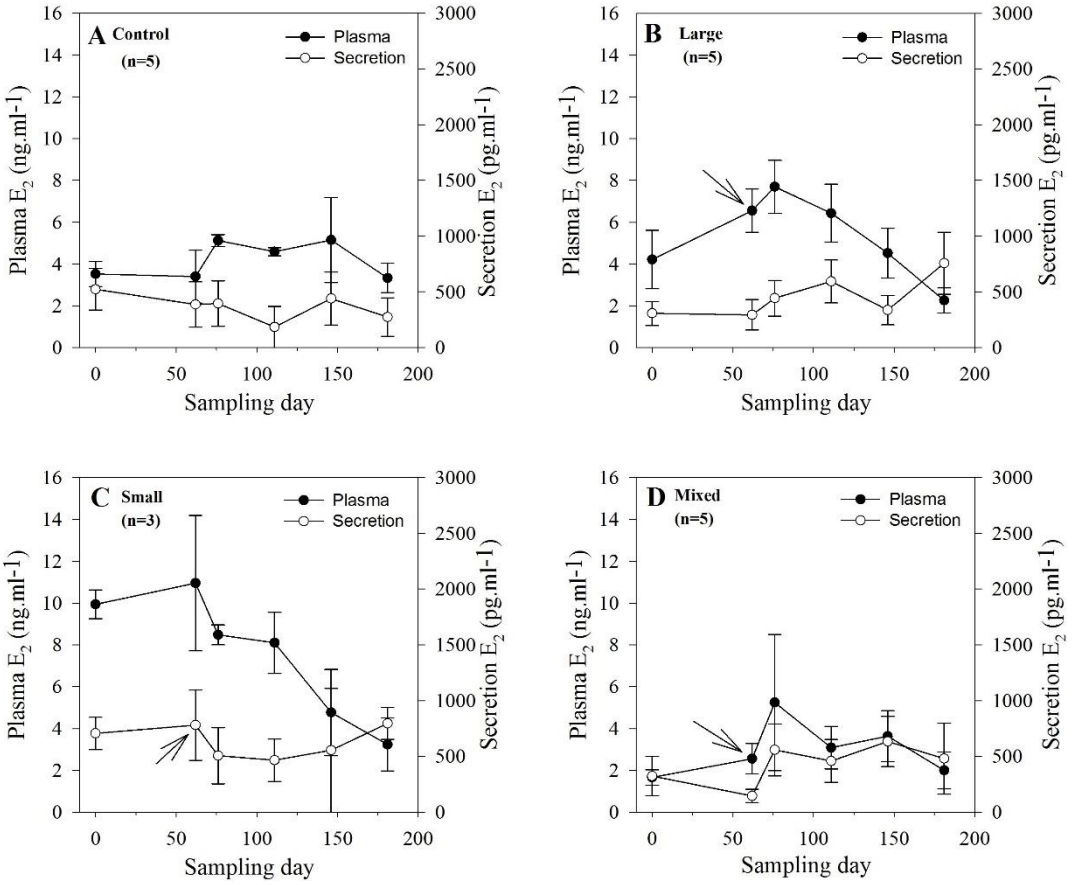
689 **Figure 7.**



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692 **Table 1.** Bodyweight (BW, kg), total length (TL, cm) and Fulton's condition factor (K) in
693 control, large, small and mixed size-pairings. Values are presented as mean (± SD).
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	Sex	BW (kg)	TL (cm)	K
Control	Male	54.1±3.6	185.8±2.	0.85±0.08
	Female	53.6±3.4	186.6±4.	0.82±0.02
Large	Male	57.4±3.8	188.8±3.	0.85±0.04
	Female	60.2±6.7	189.2±5.	0.89±0.09
Small	Male	26.7±2.5	143.0±0.	0.91±0.06
	Female	33.0±5.2	157.0±8.	0.85±0.06
Mixed	Male	21.5±1.8	134.8±3.	0.88±0.06
	Female	56.1±4.1	187.0±2.	0.86±0.05

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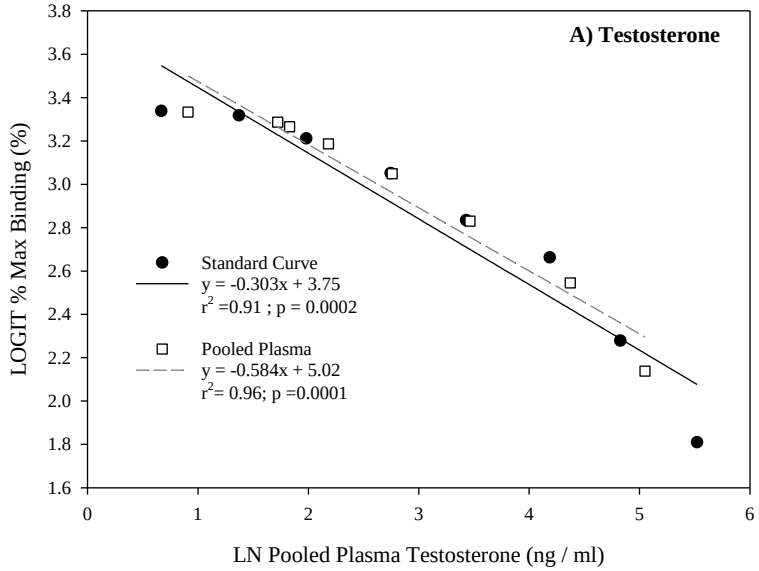

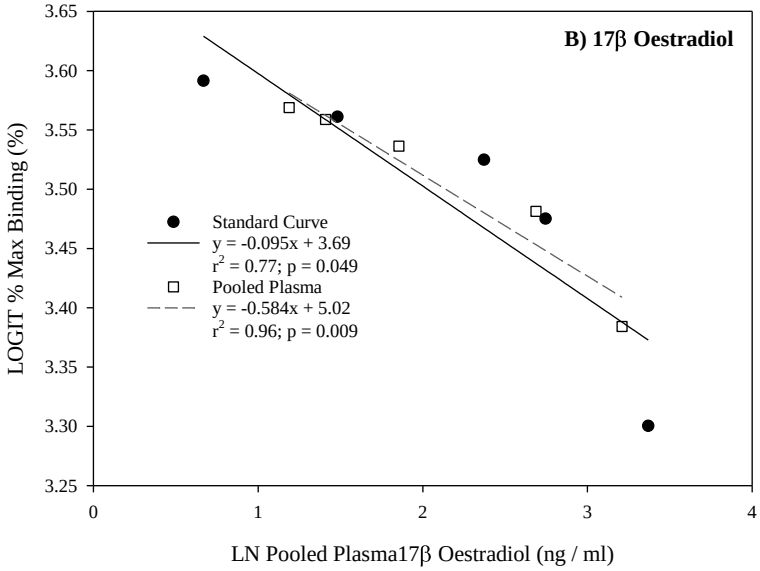

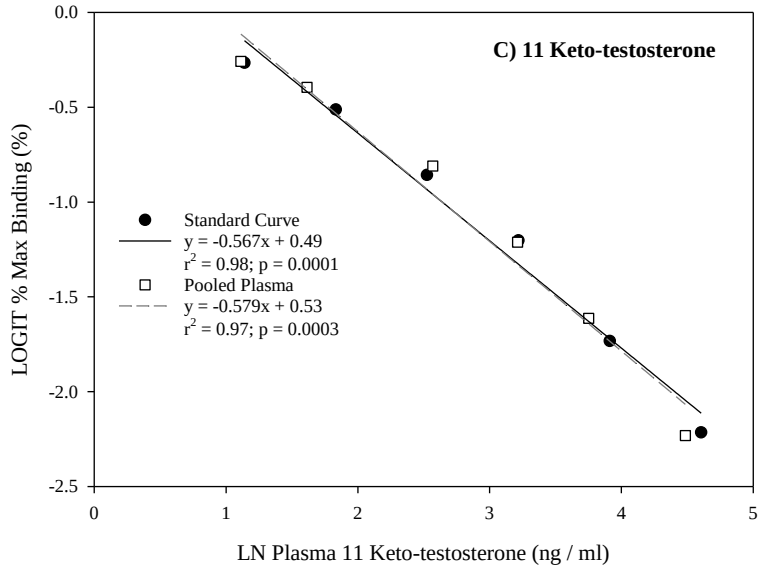

Supplementary Figure 1. Validation of radioimmunoassay (RIA) for (**A**) Testosterone (T) and (**B**) 17β-oestradiol (E_2); and enzyme-linked immunosorbent assay (ELISA) for (**C**) 11-ketotestosterone (11-KT) in blood plasma samples of *Arapaima gigas*.