# Evaluation of milt quality of the yamú *Brycon amazonicus* under hormonal induction

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*Summary*

*Two-year old and first sexual maturation male Yamú Brycon amazonicus were selected, according to the presence of semen under gentle abdominal pressure (body weight BW 1.300 ± 3 g; Total length TL 41.4 ± 0.2 cm, mean ± SEM). Five treatments were carried out: three with mGnRH-a (10, 15 and 20 µg/kg in a single dose), one with carp pituitary extract CPE (4.4 mg/kg in two applications, 10 and 90 % with a 12 h interval) and a treatment control with application only of saline solution (0.9%). The volume, spermatic concentration, spermatocrit, fertility rate, percentage of live spermatozoa, motility and activation time were evaluated. The CPE caused an increase in volume, a decrease in sperm concentration and spermatocrit. A positive linear regression between the spermatocrit and the sperm concentration was found (p<0.05, r= 0.42).  The fertility rate was evaluated by the ration of spermatozoa/eggs, which oscillated between 3.06 ± 0.2 x 10^5 and 6.75 ± 0,3 x 10^5, without displaying an effect on fertility rate (p>0.05). For the other parameters evaluated, there were no differences, between the hormones utilized, or between them and the control. It was concluded that the CPE influences, the sperm fluidity, increasing the volume and decreasing the concentration, while the mGnRH-a does not cause any quantitative or qualitative changes in the yamú semen.*

**Key words:** *artificial reproduction, Bryconinae, CPE, mGnRH, semen*

## Introduction

The yamú *Brycon amazonicus* (syn = *B. siebenthalae*) a species of the Orinoco river basin, has a high potential for fish culture, because it is omnivorous and grows fast. Nevertheless, it has not been possible to offer this fish as a reliable alternative for cultivation, principally, because the reproductive techniques and the response of animals to the protocols used have not been satisfactory. Until now, hormonal induction studies have been conducted with carp pituitary extract (CPE), and favorable results have been obtained

in less than 80% of the females treated (8). Problems with males have been related to the variation in the quality and quantity of semen obtained after hormonal induction with CPE, observed through the fertility rates. In addition, the seminal characteristics after hormonal induction have not been described for the species.

This study evaluated some semen physical characteristics after hormonal induction, and compared the effect of two inductors, carp pituitary extract (CPE) and different doses of the mammalian gonadotropin-releasing hormone analogue (mGnRH-a).

**Materials and methods**

At the Laboratory of Tropical Fish Reproduction of the Instituto de Acuicultura of Universidad de los Llanos, Villavicencio, Colombia ($4°4'24''$ N, $73°34'57''$ W, 410 m elevation) male yamú raised in captivity since 1997, were kept in nurseries at a density of 300 $g/m^2$, fed with rations of 30% crude protein. When they reached sexual maturity at two years, with an average BW $1.300 \pm 3$ g and TL $41.4 \pm 0.2$ cm, individuals that exhibited presence of semen under the abdominal pressure were selected. They were weighed, measured, individually tagged and randomly distributed in the treatments, six fishes for each one. The mean temperature, relative humidity and precipitation conditions through on the experimental period were $26.4\,°C$, 84.5 % and 516.1 mm, respectively. Water physical-chemical parameters had small variations, with an average temperature of $27.6 \pm 0.1$ °C, dissolved oxygen $7.9 \pm 0.4$ mg/L and pH $6.5 \pm 0.1$.

The hormones employed were: carp pituitary extract CPE (Argent, USA) and mGnRH-a (des-Gly$^{10}$, [D-Ala$^6$]-LH-RH Ethylamide, Sigma Co., St Louis, Missouri). Intramuscular applications were made in the dorsal region. Five treatments were carried out, three with mGnRH-a (10, 15 and 20 µg/kg body weight BW in a single dose), one with CPE (4.4 mg/kg BW in two applications, 10% and 90% with a 12 h interval) and a treatment control of saline solution (0.9%).

Five hours after the last hormonal application, semen was obtained from each specimen, through gentle abdominal pressure. Contamination by feces, urine and blood was avoided. The semen was collected in mililiters-graded glass tubes, semen volume was immediately registered and stored under refrigeration at $5\,°C$ for later analysis. The males that did not release milt after the five hours were left for another five hours before considering the result as negative.

Twenty-five µL of semen were placed under a light microscope (X10) on a slide, and 50 µL of distilled water were added as a dilutor. The group movement of the cells was considered, and the percentage of the field in movement was established according to an arbitrary scale of 0 to 100%, expressing every 10% motile increment (0, <5, 10, 20, 30…100%), modified from Vuthiphandchai and Zohar (18). The motility duration was counted until at least 5% of the semen, displayed movement (15).

The spermatozoa concentration was determined with a Neubauer chamber. Distilled water was used as a dilutor in a 1:800 semen-water ratio and mixed with a vortex. The Neubauer chamber was filled with the diluted semen; after one minute of rest two counts of $0.2$ mm$^2$ were conducted under a microscope (X 40).

Spermatocrit was measured in semen collected in capillary tubes 75 mm length and 1.1 mm of inside diameter, there were 70% filled and sealed, and then centrifuged by 10 min at 5000 rpm. The spermatocrit was defined as the percentage expressed ratio between the volume of sperm and the total volume of semen (11, 18).

A simple linear regression was calculated to determine if the spermatocrit could be utilised to estimate the sperm concentration (15). The data from the spermatocrit for statistical analysis had an angular transformation (11).

The percentage of live spermatozoa was determined through differential coloration. Milt samples were stained using an eosine solution, and for contrast stained, nigrosine (14), based on the principle that only the dead cells become permeable to the dye nigrosine/eosine. An analysis was conducted under a microscope (X40) by the arbitrary counting of 200 cells on the slide (18).

Fertility rate was tested with eggs pooled from six females, obtained by hormonal induction with CPE after Pardo-Carrasco *et al*. (8). Three 1.5 L incubators were utilized for each male with a ratio of eggs to semen of 5 g / 0.25 mL (9). One hundred eggs were counted from each incubator six hours after mixed with the semen. The fertilization rate was determined from the number of fertilized ova in relation to the total number of viable eggs. The eggs were defined as fertilized when they were spherical, translucent and contained a developing embryo in the blastopore closure stage. For each fertility test the proportion of spermatozoa per egg was determined.

Data for each parameter evaluated were compared between treatments using ANOVA to determinate statistical differences, followed by Tukey comparison test. Simple regression analyses were conducted between the evaluated parameters. Values of $p < 0.05$ were considered significant. For all statistical analysis Systac Versión 7.0 software (Spss Inc, Illinois, 1997) was used.

## Results

At the time of semen collection, two males of treatment 3 (15 µg/kg mGnRH-a) and three control males, did not release semen. In addition, in some repetitions of treatments 2 (10 µg/kg mGnRH-a), 3 (15 µg/kg mGnRH-a) and 4 (20 µg/kg mGnRH-a), the quantity of semen was not enough to proceed to all analyses; for this reason, the number of samples utilized in each analysis varied. The results for each parameter evaluated in the different treatments are shown in table 1.

In treatment 1 (CPE), the average volume of liberated semen was 6.2 ± 0.7 mL, which was equivalent to 4.8 ± 0.4 mL/kg BW, and was greater ($p < 0.05$) than observed in the other treatments. The treatments with mGnRH-a obtained similar results to each other and control.

The sperm concentration was lower in the treatment with CPE ($0.85 \pm 0.06 \times 10^{10}$ cell/mL), with a significant difference between the other treatments and control; no difference between the mGnRH-a treatments and control was observed. The total production of spermatozoa did not differ statistically between any of the treatments.

The spermatocrit varied between 0.6 and 43.3%. The treatment with CPE allows obtaining values inferior to that of spermatocrit ($p < 0.05$). A positive linear correlation was observed between spermatocrit and the spermatic concentration ($p < 0.05$, $r = 0.42$). The motility, cell production per kilogram, activation time, percentage of live cells and fertility rate were not different between the treatments.

## Discussion

In general, male fish do not need hormonal induction to produce sperm, although they can produce very viscous, scarce and difficult to disperse semen (6) and frequently of a lower quality (21). This study demonstrated that treatment with CPE produced an increase in the volume of semen, released in the Yamú, while the treatments with mGnRH-a where ineffective to increase the volume.

In the present study, the values of total sperm production did not exhibit any significant difference between the treatments; however, the values of sperm concentration did show a statistical difference, indicating that in the treatment with CPE the volume increased without increasing the spermatozoa quantity, causing a decrease in the sperm concentration, suggesting that the CPE had more influence on seminal fluid production that on spermatogenesis. Bedore (2) evaluated the semen of *Brycon orbignyanus* under induction with CPE and without induction, noting that males treated with CPE had a greater volume, although the concentration was similar between treated and control fish. The effect of CPE on increased seminal volume was observed for *Hypophthalmichthys molitrix* (19), and *Cyprinus carpio* (12). The reduction in the sperm concentration caused by CPE treatment, similar to this study results, was registered in *Cyprinus carpio* by Reenaselvi *et al.* (12).

In this study, mGnRH-a did not have an effect on any of the seminal parameters evaluated, although there are reports of the positive effect on other fish species. Zaniboni-Filho (20) found for the Pacu *Piaractus*

**Table 1.** Characteristics of Yamú *(Brycon amazonicus)* semen after hormonal treatments. The values correspond to mean ± SEM.

| Characteristics | CPE (n= 6) | GnRH-a 10* (n= 6) | GnRH-a 15* (n= 6) | GnRH-a 20* (n= 6) | Control (n= 6) |
|---|---|---|---|---|---|
| Volume (mL) | $6.2 \pm 0.7^{a}$ | $1.5 \pm 0.4^{b}$ | $1.4 \pm 0.8^{b}$ | $1.5 \pm 0.7^{b}$ | $0.6 \pm 0.3^{b}$ |
| Relative Volume (mL/kg) | $4.8 \pm 0.4^{a}$ | $1.1 \pm 0.3^{b}$ | $1.1 \pm 0.6^{b}$ | $1.1 \pm 0.5^{b}$ | $0.5 \pm 0.2^{b}$ |
| Motility (%) | $49 \pm 11^{a}$ | $72 \pm 5^{a}$ | $86 \pm 4^{a}$ | $69 \pm 13^{a}$ | $56 \pm 24^{a}$ |
| Concentration (cell/mL) | $85 \pm 6 \times 10^{8a}$ | $193 \pm 22 \times 10^{8b}$ | $191 \pm 26 \times 10^{8b}$ | $143 \pm 3 \times 10^{8b}$ | $192 \pm 9 \times 10^{8b}$ |
| Fertility rate (%) | $68 \pm 16^{a}$ | $57 \pm 20^{a}$ | $39 \pm 27^{a}$ | $50 \pm 17^{a}$ | $56 \pm 28^{a}$ |
| Sperm / egg ratio (cell) | $3.06 \pm 0.2 \times 10^{5a}$ | $7 \pm 0.9 \times 10^{5b}$ | $6.73 \pm 0.9 \times 10^{5b}$ | $5.03 \pm 0.4 \times 10^{5ab}$ | $6.75 \pm 0.3 \times 10^{5b}$ |
| Activation time (sec) | $34 \pm 2^{a}$ | $40 \pm 2^{a}$ | $38 \pm 4^{a}$ | $36 \pm 2^{a}$ | $33 \pm 4^{a}$ |
| Live cells (%) | $91 \pm 5^{a}$ | $97 \pm 1^{a}$ | $78 \pm 20^{a}$ | $95 \pm 2^{a}$ | $97 \pm 0^{a}$ |
| Spermatocrit (%) | $11 \pm 2^{a}$ | $28 \pm 4^{b}$ | $32 \pm 4^{b}$ | $32 \pm 4^{b}$ | $30 \pm 4^{b}$ |
| Production of spermatozoa / kg | $415 \pm 96 \times 10^{8a}$ | $203 \pm 50 \times 10^{8a}$ | $360 \pm 28 \times 10^{8a}$ | $214 \pm 92 \times 10^{8a}$ | $199 \pm 47 \times 10^{8a}$ |
| Spermatozoa / fish | $5.4 \pm 0.9 \times 10^{10a}$ | $2.6 \pm 0.6 \times 10^{10a}$ | $4.5 \pm 2.5 \times 10^{10a}$ | $2.8 \pm 1.2 \times 10^{10a}$ | $2.4 \pm 0.5 \times 10^{10a}$ |

\* Units: µg/kg BW
Control: saline solution (0.9%).
[ab] In the same line, means followed by different letters were significantly different ($p < 0.05$).

*mesopotamicus* that males treated with mGnRH-a (5-10 µg/kg) and CPE (2 mg/kg) had similar semen production of 13.1 mL/kg and 13.4 mL/kg, respectively. In addition, in the fertilization trials, the semen produced with mGnRH-a was similar to that produced with the utilization of CPE. The volume and the fluidity of the semen of *Pleuronectes platessa* and *Hippoglossus hippoglossus* was significantly increased in treatments with GnRH-a. (16,17). Tvedt *et al* (15) reported that for *Hippoglossus hippoglossus* the sperm concentration and spermatocrit decreased after treatment with GnRH-a, although motility was not affected. The mGnRH-a in this study was used without dopamine antagonist, which blocks the negative actions of dopamine on GnRH- induced LH release (10). It could be necessary in this species to utilize the LINPE method (10), which combines GnRH with a dopamine antagonist, a common practice for African catfish *Clarias gariepinus* and carp *Cyprinus carpio* (4). It is possible that the males of yamú did not respond positively to the hormonal treatment with mGnRH-a without the dopamine antagonist. Perhaps larger doses or longer response times are needed.

According to Billard *et al* (3), the activation time is very short in fish, from 20 to 25 sec for trout and a little more for carp, between 80 and 90 sec. Yamú had motility values between 26 and 50 sec, within the values observed in other the teleost fish species.

Aas *et al* (1) recommended the use of spermatocrit as a fast and practical method to determine sperm density, and they found for *Salmo salar* a higher correlation between the spermatocrit and sperm concentration counted by the Burkers chamber (r= 0.92). Rakitin *et al*. (11) suggested that spermatocrit can be used to determine sperm density, although specific studies would be required to prove this relationship. This study found a positive linear correlation (p<0.05, r= 0.42), indicating that spermatocrit can be used as a practical substitute to counting method by Neubauer chamber. Tvedt *et al* (15) suggest that use of longer 40 min centrifugal times, to stable readings to allow raise the coefficient of correlation.

In the fertility tests, the sperm/egg ratio used varied between $3.06 \pm 0.2 \times 10^5$ and $6.75 \pm 0.3 \times 10^5$, without showing a relationship with fertility (p>0.05, r=0.119), indicating the possibility of using as even smaller ratio. Suquet *et al* (13) presented a review of the sperm/egg ratio optimal for several fish species, finding a great variability that ranges from $1.3 \times 10^4$ to $1.6 \times 10^6$. These authors also concluded that the rate of fertility is affected by the time of contact with the eggs and the percentage of their viability. These data indicate that for each species this relationship should be evaluated; unfortunately, this was not taken into account in our research.

In this study sperm motility varied between <5 and 90% and did not affect fertility (p>0.05, r= 0.145). According to Levanduski and Cloud (7), immotile spermatozoa interfered in the interaction of the motile ones with the eggs, causing a decrease in fertility, but the fertilization ability was reduced when the proportion of motile sperm was less than 10%. Billard *et al* (3) indicated that motility is highly variable between males and between successive samples of the same male, and is not a practical parameter to be used. Aas *et al* (1) found that semen motility between 35 and 95% did not affect the fertilization of *Salmo salar* eggs.

The volume and percentage of live cells in the semen of yamú was not related to the fertility rate, similar to that observed for *Salmo salar* by Krise *et al* (5). This lack of correlation indicates that the parameters used to evaluate the semen are not good indicators of quality.

An evaluation of the effectiveness of the LINPE method and identification of the forms of GnRH found in the brain and pituitary of the yamú is recommended to allow better understanding of the problem. Other parameters should be evaluated, such as the composition of the seminal plasma, and the use of biochemical tests that allow determining the fertilization capacity of the semen.

In conclusion, this study showed that CPE was effective in increasing the volume and decreasing the spermatic concentration and spermatocrit of *B. amazonicus* semen. The use of mGnRH-a did not affect the production of *B. amazonicus* semen.

*Resumen*

*Evaluación de la calidad seminal del yamú (Brycon amazonicus) bajo inducción hormonal*

*Machos de dos años de edad y primera maduración sexual de yamú Brycon amazonicus fueron seleccionados de acuerdo con la presencia de semen bajo una ligera presión abdominal (peso corporal PC 1300 ± 3 g, longitud total LT 41.4 ± 0.2 cm, media ± SEM). Fueron realizados cinco tratamientos: tres con mGnRH-a (10, 15 y 20 µg/kg en una única aplicación), uno con EPC (4.4 mg/kg en dos aplicaciones, 10 y 90 % con un intervalo de 12 h) y un tratamiento control con solamente aplicación de solución salina (0.9 %). El volumen, la concentración espermática, el espermatocrito, la tasa de fertilidad, el porcentaje de espermatozoides vivos, la motilidad y el tiempo de activación fueron evaluados. El EPC causó un incremento en el volumen de líquido espermático y consiguiente disminución en la concentración espermática y en el espermatocrito. Se halló encontrada una regresión lineal positiva entre el espermatocrito y la concentración espermática ($p<0.05$, $r= 0.42$).  La tasa de fertilidad se evaluó bajo una proporción de espermatozoides/huevos que osciló entre $3.06 ± 0.2 \times 10^5$ y $6.75 ± 0.3 \times 10^5$, sin presentar ningún efecto sobre la tasa de fertilidad ($p>0.05$). Para los otros parámetros evaluados, no hubo diferencia entre los grupos suplementados con hormonas ni entre el grupo control y estos. Se concluyó que el EPC tiene influencia sobre la fluidez del semen, incrementando el volumen de líquido espermático y disminuyendo consecuentemente la concentración espermática, mientras que el mGnRH-a no causa ningún cambio cuantitativo o cualitativo en el semen del Yamú.*

**Palabras clave:** *Bryconinae, CPE, mGnRH, reproducción artificial, semen*

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