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Inseminating dose for the artificial fertilization of *Brycon amazonicus* (Teleostei: Characidae)

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Summary

Knowledge of the sperm–oocyte ratio in fish fertilization serves as the basis for studies on artificial reproduction and gamete manipulation. The aim of this study was to determine the minimum insemination dose for *Brycon amazonicus* oocyte fertilization. Female and male gametes were used and tested with the following doses of spermatozoa oocyte^{-1} ml^{-1}: 10,000, 20,000, 40,000, 60,000 and 80,000 (in triplicate). Fertilization rates were calculated and estimated from the regression equation by applying the segmented regression model 'Linear Response Plateau' to determine the appropriate proportion of gametes. Based on the equation $\hat{Y} = 14.3415 + 0.0007836X$, the fertilization rate increased up to 63.34% as it reached a plateau with a proportion of 62,524 spermatozoa oocyte^{-1} ml^{-1}, which is the minimum insemination dose recommended for artificial insemination of the species.

Short Communication

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Introduction

The purpose of artificial insemination in fish is to successfully fertilize the largest number of fertile oocytes with the minimum number of males, bearing in mind the need to maintain a satisfactory gender balance (Billard *et al.*, 1995). Therefore, there is a need to establish an inseminating dose (or sperm ratio per oocyte) for each species that can be defined as the amount of sperm needed to fertilize an oocyte (Chereguini *et al.*, 1999). This dose can improve the use of breeders by increasing their reproductive efficiency through artificial reproduction, as it allows rational management of semen, reducing the number of animals and their maintenance cost.

Use of small or excessive sperm concentrations results in lower fertilization rates (Levanduski and Cloud, 1988; Ninhaus-Silveira *et al.*, 2006; Shimoda *et al.*, 2007). The insemination dose for fish, unlike terrestrial animals, is relatively higher and varies between species (Beirão *et al.*, 2019). In addition, variations in sperm to oocyte ratio may occur based on oocyte characteristics or semen quality (Suquet *et al.*, 1995), which directly interfere with fertilization potential (Rurangwa *et al.*, 2004).

Brycon amazonicus, know as matrinxã, is a neotropical fish whose reproductive biology is described, taking into account that many aspects are species specific. Reproduction in *B. amazonicus* does not always follow a standardized hormone induction protocol, with frequent failures in spawning, fertilization and hatching rates within and between reproductive periods (Romagosa *et al.*, 2001; Pardo-Carrasco *et al.*, 2002; Arias, 2006; Hainfellner *et al.*, 2012). Females have high relative fecundity (Arias, 2006) and the same female can spawn at least twice in the same reproductive cycle when given hormonal therapies (Honczaryk and Inoue, 2009). However, their large-scale production is limited by their intense cannibalistic behaviour in the early stages of development, with losses of up to 99% (Bernardino *et al.*, 1993) and a reproductive seasonality that restricts the availability of juveniles to a certain period of the year (Zaniboni-Filho *et al.*, 1988). Therefore, it is important to maximize the use of available gametes to produce a greater number of viable embryos, especially considering that *B. amazonicus* is an aggressive fish and highly sensitive to the entire process of artificial reproduction, and which justifies avoidance of gamete wastage and unnecessary use of breeders.

There have been studies for this species on its reproductive biology (Zaniboni-Filho *et al.*, 1988; Romagosa *et al.*, 1999), covering artificial reproduction (Bernardino *et al.*, 1993; Romagosa *et al.*, 2001), cryoprotectants, cryopreservation and conservation of gametes (Cruz-Casallas *et al.*, 2006; Ninhaus-Silveira *et al.*, 2006; Velasco-Santamaría *et al.*, 2006; Bashiyo-Silva *et al.*, 2015). However, the minimum insemination dose for the species has not yet been reported and unspecified volumes are being used for artificial fertilization, without any concern for better use of gametes. We highlight the importance of this work not only for artificial reproduction of the species, but also for tests in applied biotechnology, as manipulation of gametes, control of fertilization, polyploidy and androgenesis. The objective of this work was to determine the insemination dose for artificial fertilization of *B. amazonicus* oocytes, to attain a higher reproductive efficiency through the rational use of breeders.

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Materials and methods

Broodstock maintenance and hormonal induction

We used 4-year-old wild specimens that had been maintained since 6 months of age at the Experimental Farm Aquaculture Station of the Federal University of Amazonas (UFAM) Manaus, Amazonas, Brazil (2°38′56.1′′S, 60°03′14.7′′W). They were placed in earthen ponds (550 m^2, ca. 0.1 fish m^2) and fed twice daily to their apparent satiation with a commercial fish feed that contained 32% crude protein.

Four breeders were selected in December 2014. Two males that emitted semen under mild abdominal pressure and two females with hyperemic genital papilla, soft, bulging abdomens (Woynarovich and Horváth, 1983). They had >50% oocytes that presented eccentric germinal vesicles (Arias, 2006), which were observed after ovarian biopsy by insertion of a flexible catheter into the genital pore, followed by gentle aspiration and immersion of oocytes in Serra's fixative solution for a few minutes (Stoeckel, 2000). The animals were separated by sex in two circular fibreglass tanks with a capacity of 3000 L each. Water was constantly renewed and aerated, and an average temperature of 27.7 ± 1.0°C and pH 7.9 ± 0.2 was maintained.

Females (1.66 ± 0.16 kg) received 5.5 mg kg^{-1} carp pituitary extract divided into three doses and males (1.31 ± 0.69 kg) received a single dose of 2.0 mg kg^{-1} carp pituitary extract (Zaniboni-Filho and Barbosa, 1996), as intramuscular injections in the base of the dorsal fin. The animals had never been previously subjected to hormone therapy.

Gamete collection and evaluation

Each specimen was removed from the tank, and placed in a container containing 50 mg L^{-1} eugenol solution (Vidal *et al.*, 2007) to reduce the stress associated with this procedure and to collect the gametes.

Semen were collected 12 h after hormonal injection in ml-graduated tubes (16.5 ± 7.0 ml). Sperm motility was evaluated subjectively (94.16 ± 2.5% of mobile sperm and 33.66 ± 2.9 s of post-activation motility) for each sample in two steps as described by Billard and Cosson (1992). The first step consisted of diluting the semen in a 1% NaCl solution (1:40, semen:diluter) and, in the second step, the previously diluted sample was activated with distilled water (1:20, diluted sample:water), on a microscope slide coated with 0.05% bovine serum albumin to prevent sperm from adhering to the slide, under an optical microscope, ×40 magnification objective. Subsequently, a semen pool was constituted. A Neubauer haematimetric chamber was used to determine the pool sperm concentration (1.63×10^{10} sperm ml^{-1}), as recommended by the Brazilian College of Animal Reproduction (Colégio Brasileiro de Reprodução Animal, 2013).

Oocytes were released 5 h after the last hormone dose (ca. 140 accumulated thermal units) into plastic containers.

Insemination doses, artificial fertilization and fertilization rate

The insemination doses were prepared according to the methodology used for *Misgurnus anguillicaudatus* (Yasui *et al.*, 2009), with some dosage modifications for *B. amazonicus*, based on two previous experiments. Sperm were diluted in 1% NaCl solution to attain concentrations of 0.75×10^7, 1.51×10^7, 3.02×10^7, 4.53×10^7 and 6.04×10^7 spermatozoa ml^{-1}. This solution keeps the sperm still and viable until insemination. We pipetted 150 µl of the oocytes (755.2 ± 19.6 oocytes) into 18 glass beakers (50 ml) using a clipped pipette tip. Each oocyte mass was immediately inseminated with 50 µl of the previously diluted sperm solution. After insemination, gametes were activated with 800 µl distilled water, homogenized for 1 min and then hydrated with 20 ml of incubator water (28.9°C). These dilutions resulted in insemination doses of 10,000, 20,000, 40,000, 60,000 and 80,000 spermatozoa $oocyte^{-1}$ ml^{-1} (three replicates for each dose were made). A mass of oocytes mixed with only 50 µl of 1% NaCl solution was maintained as a control group.

The fertilized oocytes were transferred to 18 cylindrical incubators (10 × 12 cm), with screened bottoms, properly identified and packed in a 3000 L fibreglass water tank with constant water and aeration renewal, at a temperature of 28.9 ± 1.0°C and a pH of 7.8 ± 0.1. Fertilization rates were calculated relative to the total amount of eggs ca. 6 h after artificial insemination, under a stereomicroscope, when the eggs were in the blastopore closure phase (Nakaghi *et al.*, 2014). In this phase of development, eggs that were whitish or broken were considered unfertilized or dead, and eggs that were translucent with perfect embryos inside the chorion were considered fertilized.

Statistical analysis

The regression equation that fitted the data for the variation in fertilization rates was estimated as a function of spermatozoa count per oocyte by applying the segmented regression model 'Linear Response Plateau' in the R statistical program. The appropriate ratio of spermatozoa to oocyte was calculated using this equation. Data were first tested for normality (Shapiro–Wilk test). Data are presented as the mean ± standard error. A P-value < 0.05 was considered to be significant.

Results

The estimated regression equation was $\hat{Y} = 14.3415 + 0.0007836X$ ($P < 0.05$), where $\hat{Y}$ = fertilization rate and X = spermatozoa per oocyte ($P < 0.05$; $R^2 = 0.827$). The fertilization rate increased linearly until it reached the maximum rate of 63.34%, remaining constant and starting to plateau at a ratio of 62,524 spermatozoa $oocyte^{-1}$ ml^{-1} (Fig. 1). This starting point of the plateau represents the minimum ratio required to achieve maximum fertility. Although the insemination dose increased after this point, the fertilization rate remained constant on a plateau of 63.34%.

Discussion

Knowledge of the appropriate inseminating dose is important for the development of semen and/or oocyte cryopreservation programmes, intended either for the conservation of genetic biodiversity of the species or even for breeding programmes in farms, in addition to the possibility of gamete optimization in aquaculture stations (Denniston *et al.*, 2000).

The quality of gametes observed in this study corroborates the results of other authors (Cruz-Casallas *et al.*, 2006, Ninhaus-Silveira *et al.*, 2006) and fertilization rates were similar to those reported by other authors (Romagosa *et al.*, 2001; Arias 2006). Therefore, the animals used were fit and the gamete quality parameters can be considered satisfactory for the artificial reproduction process.

According to Rurangwa *et al.* (2004), fertilization rates are related to sperm motility and may be influenced by the inseminating dose used, but it is poorly reported for fish species and presents

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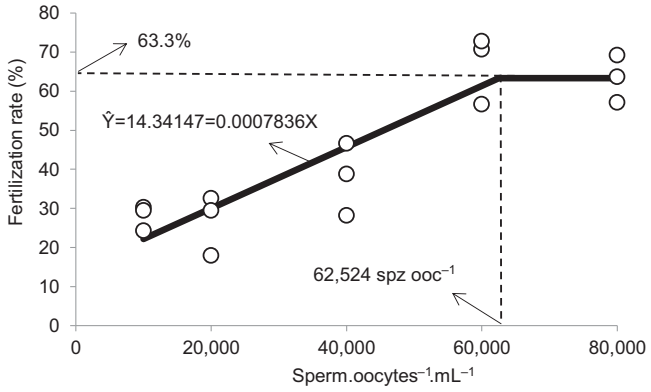

Figure 1. Values for oocyte fertilization rates of *Brycon amazonicus* as a function of spermatozoa oocyte^{-1} ml^{-1}.

great variation between species, which can probably be related to the characteristics of gametes, such as oocyte diameter, speed of spermatozoa, uptime and the distance travelled by the spermatozoa to reach the micropyle of the oocyte (Suquet *et al.*, 1995; Lahnsteiner 2000; Gage *et al.*, 2004; Bombardelli *et al.*, 2006).

Suquet *et al.* (1995) found that the insemination dose for *Scophthalmus maximus* was 6000 spermatozoa oocyte^{-1} with 87.3% fertilization. These authors concluded that this species needed a small amount of semen because of the size of its oocytes (0.91–1.2 mm) and also the semen had a long activation time. *B. amazonicus* oocytes are small (1.11–1.30 mm in diameter; Nakaghi *et al.*, 2014), as reported for another species of the same genus, *B. insignis* (1.3 mm; Shimoda *et al.*, 2007). However, the insemination dose for fertilization of *B. insignis* oocytes (314,481 spermatozoa oocyte^{-1}) was much higher than that found in our study, which suggested that other factors may influence interspecifically, such as contact time between gametes and the fertilization protocol used (Rurangwa *et al.*, 2004).

According to Cruz-Casallas and Velasco-Santamaría (2004) a proportion of 50,000 fresh sperm or 75,000 thawed sperm per oocyte is sufficient to obtain maximum fertilization percentages in artificial insemination of *Brycon siebenthalae*. In this study, the fertilization results showed effect pertinent to the treatments, presenting positive linear behaviour up to the proportion of 62,524 spermatozoa oocyte^{-1}. The fertilization rates reached a plateau at this ratio, and had no effect at the higher doses, remaining constant thereafter.

For *Brycon insignis* (Shimoda *et al.*, 2007), the fertilization rate increased to 87.8% and remained on this plateau at a ratio of 314,481 spermatozoa oocyte^{-1}. Other authors have also found this plateau from a given ratio of spermatozoa to oocyte on the fertilization rate of some species, such as *Astyanax altiparanae* (75.4%, 2390 spermatozoa oocyte^{-1}, Pereira-Santos *et al.*, 2017), *Colossoma macropomum* (84%, 102,486 spermatozoa oocyte^{-1}; Leite *et al.*, 2013), *Misgurnus anguillicaudatus* (62.84%, 684.42 spermatozoa oocyte^{-1}; Yasui *et al.*, 2009) *Salminus brasiliensis* (57.1%, 30,722 spermatozoa oocyte^{-1}; Sanches *et al.*, 2009) *Rhamdia quelen* (86.68%, 89,472 spermatozoa oocyte^{-1}; Bombardelli *et al.*, 2006).

Determination of insemination dose is important for evaluation of semen cryopreservation procedures and better economical use of large-scale artificial reproduction procedures, as lower insemination doses may trigger lower fertilization rates, and excess sperm reflects the inefficient use breeders. Therefore, fertilization tests, if performed with excessive amounts of semen, may not provide suitable conditions to identify the best treatment to be used, as eventual differences between methodologies may be attenuated by increase in the ratio of sperm to oocyte (Shimoda *et al.*, 2007). In addition, sperm economy will multiply the reproductive potential of higher performing or genetic breeding stock and, as a result, will benefit semen banks for species conservation and will also enable higher profitability for breeding companies in aquaculture.

Considering that the sperm concentration of *B. amazonicus* is ca. 1.4×10^9 ml^{-1} (Cruz-Casallas *et al.*, 2006, Ninhaus-Silveira *et al.*, 2006) and the number of oocytes per gram is estimated at 1500 cells and, based on the results found, it can be recommended that 7 ml of semen per 100 g of oocytes would be sufficient for good fertilization rates in this species. However, the use of a higher proportion of sperm per oocyte should be considered, if the semen has been cryopreserved, and to evaluate sperm concentration and quality under favourable incubation conditions. The sperm to oocyte ratio should only be considered as a reference value and not as an absolute or unalterable value for fertilization trials (Beirão *et al.*, 2019).

The insemination dose for fertilization of *B. amazonicus* oocytes will be useful for optimizing the use of breeding stock in the artificial propagation process, even more considering that this species is an extremely aggressive fish during reproductive management. This study will be the basis for conducting future studies of induced reproduction and gamete manipulation of fish.

In conclusion, the highest oocyte fertilization rates for *B. amazonicus* may be obtained with the use of a proportion of 62,524 spermatozoa oocyte^{-1} ml^{-1}.

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Data availability statement. Research data are not shared.

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Conflict of interest. The authors report no conflict of interest

Ethical approval. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee on Animal Use 'CEUA' of Federal University of Amazonas, protocol no. 019/2014). The Brazilian College of Animal Experimentation (COBEA) guidelines for the care and use of laboratory animals were followed.

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