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Spectrophotometric method for determination of *Colossoma macropomum* (tambaqui) sperm concentration

Determinação da concentração espermática de tambaqui (*Colossoma macropomum*) por espectrofotometria

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Abstract
To assist the reproductive management of tambaqui (*Colossoma macropomum*) males in laboratory and commercial fish farming, a linear regression model was obtained from concentration curves using the spectrophotometric method. Twenty-two tambaqui males with an average age of three years old were selected and divided into two groups containing 11 animals each. Both groups alternately received a single dose of carp pituitary extract (CPE; 2.0 mg/kg body weight, intracoelomic). Sperm was collected 14 h after hormonal treatment and diluted (1:4000; sperm:formaldehyde saline). The concentration was estimated by counting spermatozoa in a Neubauer chamber and by using a spectrophotometer (λ=540 nm). Individual sperm concentration ranged from 11.40 to 71.13 × 10^9 sperm/mL. The degree of transmittance ranged from 62.1% to 95.0%. There was a significant correlation (r^2 = 0.966; p < 0.0001) between sperm concentration analyzed in a Neubauer chamber and transmittance at 540 nm. Analysis by spectrophotometry and the prediction provided by the equation Y=100.293 – 0.509X proved to be an efficient and fast method for estimating sperm concentration in tambaqui and can be used in routine procedures in artificial fish reproduction laboratories.
Keywords: fish; reproduction; semen.

Resumo
Visando auxiliar o manejo reprodutivo de machos de tambaqui (*Colossoma macropomum*) em piscicultura de laboratório e comercial, obteve-se um modelo de regressão linear a partir de curvas de concentração por método espectrofotométrico. Foram selecionados 22 machos de tambaqui com idade média de três anos. Eles foram divididos em dois grupos contendo 11 animais cada. Ambos os grupos receberam alternadamente uma única dose de extrato de hipófise de carpa (EHC; 2,0 mg/kg de peso corporal, intracelomático). O esperma foi coletado 14 horas após o tratamento hormonal e diluído (1:4000; esperma: solução salina formaldeído). A concentração foi estimada por contagem de espermatozoides em câmara de Neubauer e por espectrofotômetro (λ=540 nm). A concentração espermática individual variou de 11,40 a 71,13 × 10^9 espermatozoides/mL. O grau de transmitância variou de 62,1 a 95,0%. Houve correlação significativa (r2 = 0,966; p < 0,0001) entre a concentração espermática analisada em câmara de Neubauer e a transmitância em 540 nm. A análise por espectrofotometria e a predição pela equação Y=100,293 – 0,509X mostrou ser um método eficiente e rápido para estimar a concentração espermática de tambaqui, podendo ser utilizado em procedimentos de rotina em laboratórios de reprodução artificial de peixes.
Palavras-chave: peixes; reprodução; sêmen.

1. Introduction

Sperm concentration is the major variable in fish sperm analysis [(1)]. Monitoring and controlling this variable is crucial to ensuring consistent results regarding sperm cryopreservation and artificial insemination [(2)]. Moreover, it is considered an important indicator for determining sperm quality in terms of motility and morphology [(3, 4, 5)]. Sperm concentration can be used to assess the volume of fresh or post-thawed sperm that will be directed to artificial fertilization procedures, helping to determine the most suitable insemination dose [(6, 7)]. It can be also employed to identify the range of sperm production during the breeding season [(8, 9)], the response to hormonal treatment [(10)], and differences between bred and wild males [(4)].

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Sperm concentration may be estimated by (a) sperm count using a hemocytometer, (b) spermatocrit reading, (c) flow cytometry, and (d) optical density by spectrophotometry. The first method (a) is accurate but requires a lot of practice and time to be executed, which may lead to a loss of sperm quality in cryopreservation and artificial fertilization procedures, as these biotechniques demand rapid handling between sperm collection and its manipulation [11, 12]. The second method (b) is a quick and practical approach that is easily implemented in the field; however, results sometimes do not show linear correlations with those obtained by the hemocytometer or the spectrophotometer [13, 14]. Method (c) consists of the indirect analysis of cell concentration using fluorescence comparisons, but this technique is subject to criticism, as it may overestimate sperm concentration due to different fluorescence patterns caused by artifacts also found in the sperm [15, 16]. The last method (d) assesses the linear relationship between particle concentration and light absorption [17]. It requires an initial calibration with reading by a hemocytometer and provides accurate correlation indices with sperm concentration [18].

Although the use of the spectrophotometer is limited due to its high cost, this method has been employed experimentally to assist analysis related to sperm cryopreservation of farmed or endangered species, as it is able to quickly determine sperm concentration [19, 20].

During artificial fertilization procedures, some commercial fish farms might not check the reproductive quality of males through sperm analysis. The sperm is usually simply collected and poured over the oocytes without previous assessment of its motility or concentration, or even the definition of the proper insemination dose. However, these variables are essential for selecting males that produce sperm of greater quality, in addition to allowing the optimized use of sperm volume from a single male to generate a greater number of doses, which can inseminate more oocytes. For tambaqui (*Colossoma macropomum*), despite its economic, social, and ecological importance [21], little information exists on sperm characteristics, especially on sperm concentration [5]. Such steps are important to ensure the constant supply of fish to this rising market, where tambaqui stands out for its appreciation by consumers.

Sperm concentration analyses such as the spectrophotometric method are a reliable alternative. Using raw data, a predictive equation can be obtained that allows a faster and more efficient manipulation of fresh or frozen sperm.

In addition, this technique may provide a rapid selection of males with suitable reproductive characteristics. Thus, the aim of this study was to evaluate the sperm concentration of hormonally induced tambaqui males by (1) determining transmittance values by spectrophotometry and correlating them with the results obtained by analysis in a Neubauer chamber (hemocytometer), (2) calculating calibration curves, and (3) generating a mathematical model able to predict sperm concentration.

2. Material and methods

Semen was collected monthly, over one year, at the Center for Research in Aquaculture (CPAq) at DNOCS, located in Pentecoste - CE, Brazil, (003°45'00" S; 39°10'24" W). Average ambient temperature in the region was 26.8 °C, with a maximum of 34.0 °C and a minimum of 20.6 °C. Average annual precipitation was 860 mm [22]. Sperm analyses were performed at the laboratory of the Integrated Biotechnology Center (NIB) at the State University of Ceará (UECE), in Fortaleza - CE, Brazil.

Twenty-two (n=22) tambaqui (*Colossoma macropomum*; Cuvier, 1818) males with an average age of three years (5,431.00 ± 29.70 g; 68.00 ± 4.25 cm), showing characteristics indicative of reproductive maturity, such as hyperemic urogenital papilla and easy sperm release by gentle abdominal pressure, were selected. The fish were identified by reading their electronic chips and were randomly assigned to two 350-m^2 earthen ponds, representing two experimental groups (Control and Treatment), with 11 males each. During the entire experiment, males were fed a commercial feed (finishing phase, containing 32% crude protein, at 5% of body weight) twice daily.

Animals from the treatment group (11 males) were induced with a single intracoelomic injection of carp pituitary extract (CPE; *Cyprinus carpio*, 2.0 mg/kg body weight) and their sperm were collected 14 h after induction. Control group males were not treated with any sort of hormonal induction. Sperm collections were performed monthly in both groups for 12 months.

Prior to sperm collections, the males were sedated with a eugenol solution at a 1:10:10000 ratio (eugenol: alcohol:tank water) to minimize stress. Then, each male was placed on a sponge with the eyes and caudal region wrapped in a damp cloth to facilitate their restraint and reduce visual stimuli. The genital region was cleaned with a paper towel to avoid sperm contamination with blood, feces, urine, or water. A gentle abdominal pressure was applied to release sperm [23], which was collected in 1.5-mL Eppendorf tubes. Subsequently, sperm from each male was fixed in physiological saline solution with 1% formaldehyde, at a 1:4000 ratio (sperm:fixer).

From this fixed sample, 10 µL were placed in a Neubauer chamber under a phase contrast microscope at 400x magnification. The sperm-cell count was performed in five quadrants, in both grids, 15 min after sedimentation. Three replicates were made per sample.

Afterwards, the averages obtained from sperm-cell counts in the two grids were multiplied by the factor 200 × 10^6 (adapted from 24).

For the spectrophotometer reading, the equipment was initially calibrated with 1% formaldehyde saline solution at 100% transmittance. Then, 4 mL of each fixed sample were placed in a crystal cuvette into a digital Spectrophotometer 35-D (Coleman, São Paulo-SP, Brazil). A wavelength of λ=540 nm was used to mitigate a great part of interferences, and three transmittance readings were performed per sample.

The sperm concentration results obtained by the spectrophotometric method and by the Neubauer hemocytometer chamber were expressed as mean ± standard error and subjected to Pearson's linear correlation analysis ($p < 0.001$). Transmittance results were subjected to 2-log transformation (SAS 9.0, 2002). The prediction equation generated from the data was used to construct the table of sperm concentration relative to transmittance. The variables of transmittance percentage and sperm concentration were subjected to analysis of variance (ANOVA). In the case of significant differences, the Student-Newman-Keuls test was used for a pairwise comparison of means. The significance level adopted was 5%. "SigmaPlot 12.0" software was used in the statistical analysis of the results. The research was approved by the Ethics Committee on Animal Use at UECE (approval no. 10974342/2022).

3. Results

Fresh sperm samples collected after hormonal treatment had a mean volume of 5.05 ± 2.08 mL and a mean sperm concentration of 21.79 ± 4.02 × 10^9 sperm/mL. Males from the Control group produced sperm with mean volume and concentration of 0.55 ± 0.52 mL and 49.98 ± 18.63 × 10^9 sperm/mL, respectively. Thus, hormonally induced males produced a larger seminal volume and a lower sperm concentration ($p < 0.05$).

Table 1 shows the mean sperm concentration data of each animal (n=22), obtained by counting in a Neubauer chamber (10^9 sperm/mL), and their respective transmittances obtained by spectrophotometry (λ = 540nm). Using this data, a linear model was obtained (Y = 100.293 – 0.509X), in which Y = transmittance and X = sperm concentration (Fig. 1). Based on this equation, each unit increase in concentration (i.e. for every billion sperm/mL) was estimated to reduce transmittance by 0.51% ($p < 0.0001$).

Table 1. Transmittance percentages in a spectrophotometer (λ =540 nm) and sperm concentration in a Neubauer chamber (sperm × 10^9/mL) in two groups (Control and Treatment), shown individually per animal. Pentecoste - CE, Brazil.

Treatment			Control		
Male number	**Transmittance (%)**	**Sperm Concentration ×10^9 sperm/mL**	**Male number**	**Transmittance (%)**	**Sperm Concentration ×10^9 sperm/mL**
1	95.0	11.40	*12*	90.0	20.00
2	89.0	19.86	*13*	83.0	29.20
3	89.5	20.60	*14*	86.0	30.60
4	87.0	21.73	*15*	86.4	33.60
5	87.0	21.86	*16*	78.2	50.20
6	88.5	22.26	*17*	72.2	53.26
7	88.3	22.50	*18*	69.0	57.86
8	89.3	23.66	*19*	68.1	68.40
9	87.8	24.00	*20*	66.8	68.43
10	88.8	24.26	*21*	62.1	71.13
11	89.2	27.53	*22*	63.9	67.20
Mean*	89.04 ± 2.16^a	21.79 ± 4.02^B		75.06 ± 9.99^b	49.98 ± 18.63^A

*Lowercase letters represent the comparison between transmittances, while uppercase letters represent the sperm concentration comparison. Means with distinct lowercase letters are significantly different from each other by the Student-Newman-Keuls test ($p < 0.05$). Means with distinct uppercase letters are significantly different from each other by the Student-Newman-Keuls test ($p < 0.05$).

Table 2 shows the estimated values of tambaqui sperm concentration relative to the respective transmittance percentages calculated using the equation. An inversely proportional linear correlation was observed between sperm concentration in a Neubauer chamber and the transmittance at 540 nm (Fig. 1; Y = 100.293 – 0.509X), with a highly significant coefficient of determination ($R^2 = 0.966$, $p < 0.0001$), thus obeying the Lambert-Beer law.

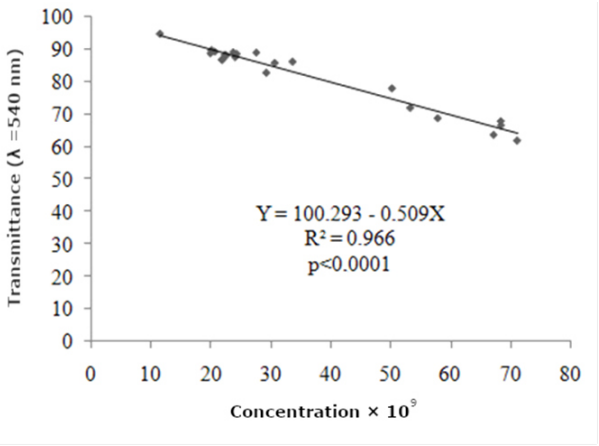

Figure 1. Sperm concentration curve of tambaqui (*C. macropomum*) obtained by the correlation between transmittance percentage using a spectrophotometer (λ = 540 nm) and sperm concentration obtained using a Neubauer chamber (sperm × 10^9/mL). Data collected in Pentecoste - CE, Brazil.

4. Discussion

Previous studies have reported that using hormonal treatment with CPE may result in the production of more dilute sperm [5, 25, 26, 27]. This occurs due to greater testicular hydration, which increases semen fluid volume, reducing sperm concentration [28, 29, 30]. Nevertheless, when this dilution occurs in species with higher sperm concentrations, such as tambaqui, it offers the possibility of better sperm use, since it might generate several inseminating doses.

Maria et al. [5] reported larger volumes of tambaqui sperm when two hormonal doses (0.25 and 2.5 mg/kg) were injected. In this case, 12.6 mL of sperm were collected per male. Other authors obtained results more alike to those of the present experiment, describing smaller volumes of tambaqui semen after using CPE (Leite et al. [7] - 4.5 mL; Oliveira et al. [31] - 4.3 mL; Martins et al. [32] - 3.3 mL).

Table 2. Simulation of transmittance percentage (% T) obtained using a spectrophotometer (λ = 540 nm) and tambaqui sperm concentration (sperm × 10^9/mL) estimated using the prediction equation Y=100.293 – 0.509X. Pentecoste - CE, Brazil

% T	Sperm × 10^9/mL	% T	Sperm × 10^9/mL	% T	Sperm × 10^9/mL	% T	Sperm × 10^9/mL	% T	Sperm × 10^9/mL
0	198.18	*20*	158.62	*40*	119.05	*60*	79.49	*80*	39.92
1	196.20	*21*	156.64	*41*	117.07	*61*	77.51	*81*	37.94
2	194.22	*22*	154.66	*42*	115.09	*62*	75.53	*82*	35.96
3	192.25	*23*	152.86	*43*	113.12	*63*	73.55	*83*	33.99
4	190.27	*24*	150.70	*44*	111.14	*64*	71.57	*84*	32.01
5	188.29	*25*	148.72	*45*	109.16	*65*	69.59	*85*	30.03
6	186.31	*26*	146.75	*46*	107.18	*66*	67.62	*86*	28.05
7	184.33	*27*	144.77	*47*	105.20	*67*	65.64	*87*	26.07
8	182.35	*28*	142.79	*48*	103.22	*68*	63.66	*88*	24.09
9	180.38	*29*	140.81	*49*	101.25	*69*	61.68	*89*	22.12
10	178.40	*30*	138.83	*50*	99.27	*70*	59.70	*90*	20.14
11	176.42	*31*	136.85	*51*	97.29	*71*	57.73	*91*	18.16
12	174.44	*32*	134.88	*52*	95.31	*72*	55.75	*92*	16.18
13	172.46	*33*	132.90	*53*	93.33	*73*	53.77	*93*	14.20
14	170.48	*34*	130.92	*54*	91.36	*74*	51.79	*94*	12.23
15	168.51	*35*	128.94	*55*	89.38	*75*	49.81	*95*	10.25
16	166.53	*36*	126.96	*56*	87.40	*76*	47.83	*96*	8.27
17	164.55	*37*	124.99	*57*	85.42	*77*	45.86	*97*	6.29
18	162.57	*38*	123.01	*58*	83.44	*78*	43.88	*98*	4.31
19	160.59	*39*	121.03	*59*	81.46	*79*	41.90	*99*	2.33

Regarding sperm concentration, our results were similar to those of Leite et al. [7] and Oliveira et al. [33] (19.0 × 10^9 and 20.63 × 10^9 sperm/mL, respectively). In contrast, Maria et al. [5] and Martins et al. [32] observed lower concentrations (7.9 × 10^9 and 10.4 × 10^9 sperm/mL, in this order) than those presented here, also with hormonal induction to reproduce (21.79 × 10^9 sperm/mL).

In this study, the sperm concentration (49.98 × 10^9 sperm/mL) of the non-induced tambaqui was higher than those found by Martins et al. [32] (7.9 × 10^9 sperm/mL), who used saline solution (0.9% NaCl) only, without hormone addition. As expected, the sperm volume (0.55 mL) was lower than those reported in other studies, which applied 2.5 mg CPE/kg (4.31 mL) [31] or 1.5 mg CPE/kg (2.32 mL) [33], corroborating the statement that reproduction hormonal inducers act to increase seminal plasma production [29, 30].

These variations in sperm concentration in individuals within the same genus or species are linked to the season [8, 9], nutritional status [3, 34], water quality [35], animal weight [14], and the type and dose of the hormone used [10, 27]. For instance, in an experiment evaluating the reproductive performance of *Brycon orbygnianus* and *Prochilodus lineatus* treated with buserine extract, Paulino et al. [10] found that this hormone inducer provided a more homogenous response in sperm concentration of

P. lineatus compared with *B. orbygnianus*. This result suggests greater affinity between the species receptors and buserine extract.

The significant coefficient of determination (R^2 = 0.966) obtained by the linear correlation between sperm concentration analyzed in a Neubauer chamber and transmittance determined in a spectrophotometer is in accordance with the Lambert-Beer law, which suggests that the intensities of incident and emerging radiation can be related to the concentration of material present in the solution.

According to Ciereszko and Dabroswki [19], the linearity of the correlations between optical density and sperm density offers a good correlation (r = 1) at wavelengths between 400 and 700 nm, proving to be a quick method for estimating the number of sperm cells per milliliter. However, the accuracy of determinations is influenced by factors such as the type of hemocytometer used [36], observer experience [37], and sample dilution rate [19].

When using this spectrophotometric method, extra attention is needed to avoid sperm contamination with blood, feces, or urine, as these contaminants interfere with the spectrophotometric reading due to the dilution and change in sample color. According to Loir et al. [38] and Ciereszko and Dabrovski [19], the interference of proteins from seminal plasma itself appears to be insignificant, since the protein concentration in fish sperm is very low [39], and especially if associated with the choice of wavelengths above 400 nm.

5. Conclusion

Sperm concentration analysis by the spectrophotometric method at λ= 540 nm is efficient and can be adopted as a routine protocol for commercial and research purposes to determine sperm concentration. As such, it allows estimating sperm concentration in a precise and easy manner and provides a more accurate dilution of sperm to obtain a greater number of doses, which may be used to fertilize more oocytes.

Conflict of interests

The authors declare that there is no conflict of interest.

Author contributions

Conceptualization: C. S. B. Salmito-Vanderley and J. F. Nunes. *Data curation*: M. J. da A. F. Vieira and T. M. Torres. *Formal Analysis*: M. J. da A. F. Vieira and T. M. Torres. *Funding acquisition*: C. S. B. Salmito-Vanderley and J. F. Nunes. *Investigation*: M. L. da S. Apoliano and C. H. S. Melo. *Methodology*: M. J. da A. F. Vieira and C. C. de M. Salgueiro. *Project administration*: C. S. B. Salmito-Vanderley and C. C. de M. Salgueiro. *Writing (original draft):* Salmito-Vanderley and M. J. da A. F. Vieira. *Writing (review & editing):* M. L. da S. Apoliano, C. H. S. Melo and T. M. Torres.

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