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Evaluation of sperm quality in adult white fish (*Chirostoma estor*) Jordan 1879, Mexico

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Abstract
The sperm quality of the adult white fish *Chirostoma estor* was evaluated. The study was carried out over a period of 7 months (July 2014 to May 2015), at the facilities of the Regional Fisheries Research Center (CRIP-Pátzcuaro) Michoacán, Mexico. A total of 112 reproductive organisms of 24 months of age with a mean height and weight of 12.63±1.42 (cm) and 16.93±6.19 (g), respectively, were used. Mobility, viability and sperm concentration were determined. The results showed that the form of the spermatozoa of *C. estor* was cylindrical and uniflagellate. The highest recorded volume was 120 µL for the months of August and September and the lowest volume was recorded in the month of October with 41 µL. Additionally, the motility of the spermatozoa was a progressive forward movement with several movements from their flagellum for the months of April, May, October and November, but in July, 80-90% of the cells vibrated while others were immobile. With respect to viability, in all months it was 98-100% of live cells and the maximum sperm concentration was presented in the months of April and May with an average of 2.63 and 3.61 X 10^6 cells/µL, respectively.

Keywords: *Chirostoma estor*, motility, viability, sperm cells, aquaculture

1. Introduction
Chirostoma estor is a native and endemic species of Lake Pátzcuaro, Michoacán (Mexico). It has a great ecological, cultural and commercial value at local, regional, national and international levels [1, 2]. Nowadays, their situation in their natural habitat is vulnerable, so aquaculture has been the most viable alternative for the species since it has been able to close its life cycle in captivity, setting the optimal parameters for its development from egg hatching to breeding. However, it has been observed that under captive conditions their seminal quality and quantity decreased, as did their eggs. Low fertilization, low hatching percentage and deformed fry were also observed [3]. On the other hand, several studies have been carried out on the quality of gametes in aquatic organisms; however, these have been focused towards the females, leaving to the side to the males even though the poor quality of spermatozoa can affect the production of healthy larvae. Thus, evaluating the sperm quality in fish is very important since knowing if the sperm are viable we can improve the methodologies of artificial fertilization, as well as preserve the gametes to be used outside the breeding season. Short-term sperm preservation is a simple and practical technique that allows to prolong the availability of sperm in the management of broodstock by reducing the handling and stress originated in males to obtain sperm; It can also be a useful tool to increase genetic diversity in the stock of broodstock and complementary to cryopreservation, this conservation can be several hours, days or years depending on the purpose that is required to use, in turn the duration of the cryopreservation of gametes will depend on the final storage temperature and the choice of the method to be used. This biotechnology facilitates the crossing between improved organisms; the fertilization in organisms out of phase in maturation; the application of genetic improvement programs; the planning of spawning, avoiding the waste of sperm; besides optimizing the productive areas, by allowing the management of smaller number of males, with the decrease in production costs [4-7]. In recent years, research in Mexico has been carried out on the typical characteristics of gametes [6, 7], for freshwater and marine species to relate the quality of these with the ability to fertilize [8]. Some authors [9-14] who have performed studies on sperm quality state that this parameter is affected by several factors such as temperature, reproductive season, quantity and quality of food, stress conditions, reproductive management, photoperiod, salinity and frequency of sperm extraction, in addition to the

variability between the same genus and individuals, so generating a protocol to standardize sperm quality evaluation for any fish is very complex. Therefore, the objective of the present research was to evaluate the sperm quality of adult *C. estor* breeders by analyzing the mobility, viability, volume and concentration of their sperm cells in order to establish their reproductive potential, management protocols, and to control gametes that would allow improvement the productive processes of the species, thus reducing the pressure that this resource has on its natural habitat.

2. Materials and Methods

2.1 Obtaining of organisms

The present study was carried out over a period of 7 months (July 2014 to May 2015), at the facilities of the Regional Fisheries Research Center (CRIP in Spanish)-Pátzcuaro, Michoacán belonging to the National Institute of Fisheries and Aquaculture (INAPESCA in Spanish). 112 adult *Chirostoma estor* species of 24 months of age with a mean height and weight of 12.63±1.42 (cm) and 16.93±6.93 (g), respectively were used. Throughout the research, the *C. estor* were kept in tanks of 300 L capacity with 250 L of fresh water, and at a temperature of 25±1 °C with a natural photoperiod. The specimens were fed "Silver Cup" brand El Pedregal balanced food with 45% protein and a 2.5 mm size (mini pellets), providing 5% of their body mass, and were also provided live adult food of *Artemia franciscana* and *Daphnia magna*.

2.2 Spermatic quality

2.2.1. Gametes collection

Prior to the collection of sperm sample, the organisms were anesthetized by immersion for 4 to 5 minutes in a solution of benzocaine at a concentration of 7 mL/L, until the fish presented signs such as: poor response to changes in position, decrease in respiratory rate and total loss of balance (difficulty in straightening); once the organisms were anesthetized they were weighed and measured. For the extraction of semen, the urine bladder of the males was first emptied by abdominal pressure and the excess water was removed with absorbent paper to avoid contaminating or activating semen samples with feces, urine, blood or water.

The sperm was removed manually, by producing slight pressure in the abdominal area towards the genital pore. The samples were placed in 140 µL Eppendorf tubes, stored and transported in a cooler at 5 °C to the laboratory of the Regional Fisheries Research Center (CRIP).

2.2.2 Evaluation sperm motility

In a slide, 2 µL of semen were deposited, 5 µL of distilled water was added, and the microscope was visualized in a 40 X objective (VELAB Microscopes), the motility was quantified as established by (Betsy J and Sampath S) [15].

2.2.3 Sperm viability measurement

Viability was determined by smearing the slide with 1% Eosin-Nigrosine staining, placing 2 µL of semen and 1 µL of dye, observed under an optical microscope with a 40 X magnification. It was considered that live spermatozoa did not stain while dead spermatozoa took the coloration of Eosin-Nigrosine because the membrane becomes permeable by cellular death. 100 cells were counted in triplicate.

2.2.4 Spermatic concentration

The sperm concentration was quantified using a Neubauer chamber with a dilution of (1:99 µL) semen and diluent, respectively, for each of the samples. The total number of cells present in a (µL) was calculated as follows:

Spermatic concentration= ($\bar{X}_{cell.}$) * (25) * (D µL) / (0.1 µL)

($\bar{X}_{cell.}$).= Average number of spermatozoa observed.
25= Number of squares in the Neubauer chamber.
0.1= Neubauer chamber's Volume.
D= Dilution factor (µL of concentrated semen, diluted in µL of diluent).

2.3 Statistical Analysis

A two-way analysis of variance (ANDEVA) was performed to determine significant differences between the studied months. Finding significant differences (P<0.05), Tukey's method of comparing multiple means was applied. For the test of the analysis of variance, the technique of Tukey was used as well as the statistical program Systat 12 (Systat Software Inc., California, USA).

3. Results and Discussion

The spermatozoa of *C. estor* were determined as uniflagellated and with a cylindrical head.

Spermatic volume

The average value of the sperm volume of the white fish in shown in Fig. 1. It was observed that the highest volume was obtained in the months of August and September (120 µL), and the lowest volume obtained in the month of October with (41 µL), nevertheless the average values that laboratory cultivated fish of this same species present oscillate between the 11 to 101 µL [16]. This variation is related to the reproductive period in which the samples were taken, since this organism presents two reproductive peaks during the year, the most productive one from January to June [17, 18, 19, 20] and increasing significantly in the period from March and June and from September to November [19]. It should be mentioned that the extraction of the biological material was once a month because the continuous extraction reduces the quality of the sperm produced, and in addition alters the fertilization rate and sperm density during the reproductive period [21]. Regarding the statistical analysis (ANDEVA), this showed significant differences between the sperm volume and the study months.

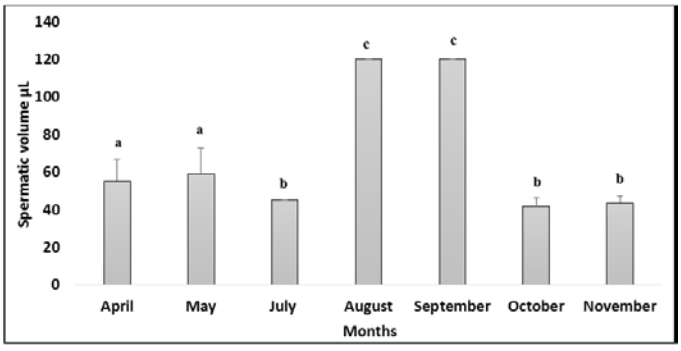

Fig 1: Sperm volume of mature males of *C. estor* obtained during the period from July 2014 to May 2015. Similar letters in columns do not present significant differences (P<0.05).

Motility

Sperm motility is considered the main factor in sperm quality, and in this study the sperm motility that the organisms presented during the different months were fluctuating; for April, May, October and November all sperm cells (95- 99%) had a progressive movement with several movements in their

flagellum, by August most spermatozoa (80-85%) had a strong forward vibration while others only vibrated *in loco*. In respect to the month of September, all spermatozoa (90-95%) exhibited a weak vibration *in loco* and for the month of July the cells (80-90%) vibrated while others were immotile. The motility time they had was 185±32.2 seconds for all months except for July, in which it was 95±2.4 seconds. These values are within the range reported by different authors, where they compare the motility of different freshwater and marine species [9, 22- 25 13, 14, 26]. In the case of this species, there are no records of mobility in time so it is necessary to continue with these types of evaluations. The percentage of motility and the duration of this depend on factors such as feeding, management, and stress, and it has been observed that the duration time is directly dependent on the type of solution with which the sperm cells are activated, since this should have a greater or lesser osmotic pressure than the seminal fluid. Another key factor to promote the movement of spermatozoa is salinity since in increasing this factor, the time of mobility is higher, and it has also been observed that the pH plays an important role in activating motility. For rainbow trout [27], it was determined that at a pH of 7.8, sperm motility is not induced. However, other studies have shown that spermatozoa are activated in extender solutions with pH of 5.5 to 10.5, with the optimum for fertilization being between 8.5 and 10 [28, 29]. In addition, it was determined that sperm contamination with urine significantly affects pH-dependent motility, activation can occur at a pH of 5.5, and if the sample is contaminated with urine, motility is not activated [28-30]. The best solutions that have had an effect greater than 50% are D532, NaCl, Erdhal and Graham 323 mOsmol/kg, DIA 512 and 532 [31-38]. In this study, pond water was used for reactivation, with activation percentages of 99% for April, May, October and November. However, further testing with activating solutions at pH greater than 8.5 is required.

Viability

The percentage of viability obtained was 98-100% for live spermatozoa for all months. These results are higher than those of other fish of the same genus (80.43% and 92.14%) for *C. humboldtianum* and *M. estor*, respectively [16]. However, it is important to note that when applying the viability test, extreme care must be taken in the time that elapses from the moment the semen is obtained and the drying time of the smear because if it is allowed to dry slowly, some spermatozoa cells die and are dyed before the drying process is completed, causing a false indication of the percentage of live spermatozoa [39]. Additionally it is important to analyze the integrity of the cell membrane as it gives spermatozoa the ability to regulate different cellular activities and signal pathways that can lead, among others, to the activation of sperm motility [40, 41].

Spermatozoa concentration

The number of sperm in the semen of the fish at the time of successful fertilization is of great importance, since many die on the journey due to different factors. The spermatic concentration expressed in number of spermatozoa present per unit seminal volume is one of the variables that is most considered to evaluate the reproductive potential of an organism [42, 43]. When evaluating sperm samples from *C. estor* reproductive organisms, the highest sperm concentration was determined for May (3.61 X 10^6 cells/µL) and the lowest was for the month of July (5.49 X 10^5 cells/µL). In respect to the statistical analysis (ANDEVA), this showed significant differences between April and May compared to July and other months of study (P=0.001) (Fig 2), this concentration differs from that reported by two researchers who studied the same species, where they observed that with larger organisms (both in size and weight), concentration is greater at 2.21-2.55 X 10^9 cells/mL [16, 44], which means there is a relationship between the volume of the sperm and the weight of the specimens, and the sperm concentration is usually related to the volume of sperm produced. In addition, both parameters are highly variable between populations and individuals and in relation to the stage of the reproductive period [43], this is corroborated in the present investigation since, when analyzing a relation between the weight and the number of cells, it was found that there is a positive correlation between these two variables adjusting to the model of the line with a coefficient of low correlation of 0.53 of way, meaning that the number of cells depends on the weight of the organisms (Fig. 3). On the other hand, with fish of the same family, *C. humboldtianum* and *O. bonariensis* their concentration is superior to that found in this study, 1.82 X 10^{10} cells/µL and 4.0 X 10^9 cell/mL, respectively [45, 46]. These variations are attributed to the fact that the specimens were spawned with human chorionic gonadotrophin (hCG), and in their reproductive age obtained a higher concentration of cells as well as volume. In addition, it is mentioned that variations in sperm concentration within species may be due to individual characteristics among organisms, as well as differences in age of fish, sampling season, interval between repeated stripping's, temperature, handling conditions, genetics, size, geographical conditions, quantity and quality of food [33, 47-51]. It has been observed that in wild organisms their sperm concentration is higher in comparison to those grown in captivity because they respond to a reproductive strategy where reproduction is optimized to the maximum and therefore more on this factor than growth or increase in another variable. Likewise, these organisms can select and better satisfy their eating habits compared to those in captivity since the requirements that each species needs are not proportionate [52, 37]. Based on the past research, we assume that by providing a balanced feed fulfilling the species' specific requirements, its gametes (sperm cells and oocytes), larvae, juveniles, and breeders will be of better quality since there is no commercial balanced food formulated specifically for this specimen. The present study was offered food of the species (*Oncorhynchus mykiss*) without accepting in its different presentations.

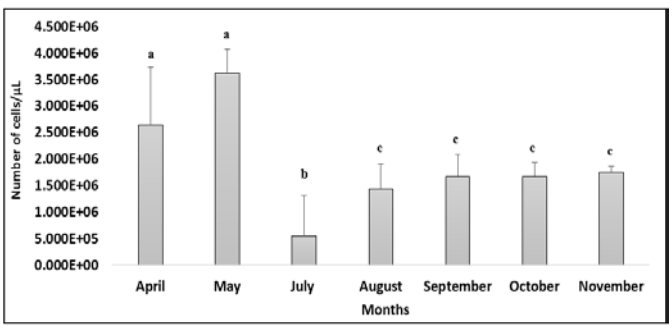

Fig 2: Sperm concentration of *C. estor* present in the different months of study. Similar letters in columns do not present significant differences ($P<0.05$).

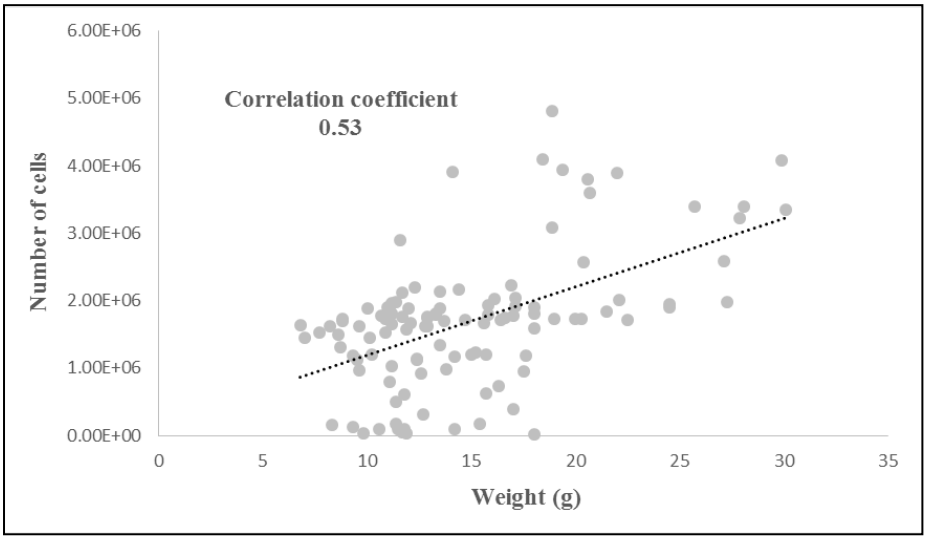

Fig 3: Correlation between the number of sperm cells and the weight of the *C. estor* reproducers.

4. Conclusion

In the present study, it is concluded that the reproductive peaks *C. estor* were present from April to May, increasing in the latter. The spermatozoid form was cylindrical and uniflagellated, presenting a sperm volume greater than 120 µL and a smaller one of 41 µL, with a viability of 98-100% of living cells, a motility of 99 to 80% with progressive movement forward and a spermatic concentration of 3.61 X 10^6 cells/µL in their reproductive age. These results are important in Mexico because it will allow us to establish the base for the management and control of male reproductive species in order to make the reproductive processes more efficient, optimize the fertilization in the females, and to increase the production of seed and the improvement of fry, thus reducing the pressure that this resource has on its natural habitat, giving way to increased production by aquaculture and allowing the recovery of natural populations in Lake Pátzcuaro.

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