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Sperm features of captive Atlantic bluefin tuna (*Thunnus thynnus*)

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Abstract:

The present study aimed to establish some basic characteristics of Atlantic bluefin tuna sperm from captive mature males, treated or untreated by gonadotropin releasing hormones agonist (GnRHa). Intratesticular milt was collected from treated and untreated fish (mean weight ± SD: 122.9 ± 29.2 kg, n = 21). There was no significant effect of GnRHa treatment on GSI (1.33 ± 0.70%, n = 21) or on sperm concentration ($3.8 \pm 1.3 \times 10^{10}$ spermatozoa ml^{-1}, n = 21) estimated by optical density at 260 nm. Similarly, the percentage of motile spermatozoa measured at 30 s post activation: activating medium (AM seawater containing 10 mg ml^{-1} BSA) was not significantly different between control and GnRHa implanted males. On the other hand, a significantly higher Average Path Velocity (VAP) was assessed for sperm from GnRHa-treated fish, compared to controls. Regardless of hormonal treatment, the percentage of motile sperm decreased after a plateau of 5–6 min post activation, and any forwardly progressing movement ceased after 10–11 min. Linear trajectories of spermatozoa were observed in seawater while tighter circles were assessed when increasing the Ca^{2+}concentration in the AM. The storage capacity at 4°C was significantly lowered when NAM (Non Activating Medium: 50% seawater containing 10 mg ml^{-1} BSA) was added to sperm at a 1 : 1 dilution. These results demonstrated that treatment with GnRHa had little effects (except on VAP) on sperm characteristics in captive reared Atlantic bluefin tuna. Further investigations are required to improve the knowledge of tuna sperm biology and, in this respect, the comparison between intratesticular and released sperm features should be highly informative.

1. Introduction

Since the early 1990's, Atlantic bluefin tuna cage-culture of wild-caught individuals has developed in the Mediterranean Sea (Ottolenghi, 2008), reaching annually a production of about 20 000 tonnes (ICCAT, 2007). Because today fish are still captured in the wild and towed to offshore floating cages where they are farmed, the current "capture-based" aquaculture of Atlantic bluefin tuna is considered unsustainable and totally dependent on the heavily overfished stocks (Doumenge, 1999; Fromentin and Powers, 2005).
A proper aquaculture is necessary to alleviate the pressure from wild populations and enable a sustainable production of tuna in captivity. The first step towards controlled breeding of a species includes the study of its reproductive physiology to establish methods for controlled maturation, gamete release, egg incubation and juvenile rearing in hatcheries (Mylonas and Zohar, 2009).
The reproductive cycle of wild Atlantic bluefin tuna has been studied in both males and females (Medina et al., 2002; Corriero et al., 2003; Abascal et al., 2004; Medina et al., 2007). In addition, the reproductive function of captive fish has been initiated recently and methods have been developed to induce oocyte maturation and increase spermiation, using implants loaded with gonadotropin-releasing hormone agonist (GnRHa) (Corriero et al., 2007; Mylonas et al., 2007). However, little information is available on the biological characteristics and physiology of Atlantic bluefin tuna sperm. Because of their ultrastructure, tuna spermatozoa qualifie as aquasperm typical for fish with external fertilization (Abascal et al., 2002). High sperm concentrations and high percentages of motile spermatozoa were reported by Doi et al. (1982). Some features of tuna sperm movement were also recently described, including flagellar beat frequency and sperm swimming behaviour in relation to the time *post* activation (Cosson et al., 2008a, b).
This study aims to establish some basic features of Atlantic bluefin tuna sperm, such as sperm concentration and movement characteristics, after hormonal treatment of the broodstock.

2. Material and methods

Broodstock management

Rearing of the Atlantic bluefin tuna (mean weight ± SD: 122.9 ± 29.2 kg, n=21), sampling and hormonal treatment with GnRHa were as previously described (Mylonas et al., 2007). Briefly, tuna were caught around the Balearic Islands and were transported to the coast of Murcia (Spain). They were maintained in floating cages (diameter 25 or 50m, depth: 25m). Captive tuna were fed to satiation six times a week with frozen raw fish. Hormonal implants containing GnRHa were loaded onto a spear gun and were individually administrated by a diver (40–100 µg GnRHa kg^{-1}). Tuna were sacrificed 2 to 8 days after implantation. During sampling (July 2004 and 2005), water temperature ranged from 23 to 26°C. Immediately after excision from the fish, the whole testes were placed in a plastic bag, stored over ice and transported within one hour to the laboratory.

Experimental design

At the laboratory, the excised gonads were weighed in order to calculate the gonadosomatic index as GSI (%). Intratesticular sperm was collected by syringe after an incision in the posterior part of excised testes and samples were stored in

a closed test tube (2ml) over ice until use (30 minutes to one hour). All sperm features were individually assessed.
After dilution in distilled water (1/2000), sperm concentration was assessed both using a haemocytometer and a spectrophotometer (wavelength: 260 nm). The paired data were used to produce a linear regression relating optical density to spermatozoa concentration.
Spermatozoa were activated using a two step dilution procedure: first, 1 µl of sperm was mixed with 499 µl of Non Activating Medium (NAM composed of 50 % seawater diluted by freshwater and 10mg ml^{-1} BSA, Bovine Serum Albumin) and second, 1 µl of this suspension was mixed with 50 µl of Activating Medium (AM composed of full seawater and 10mg ml^{-1} BSA). The spermatozoa were observed under a dark field microscope using a 20x objective. Changes in the percentage of motile spermatozoa (cells with progressive forward motility) in relation with time *post* activation were directly assessed in triplicate by the same observer in sperm from two GnRHa treated males. The effect of hormonal treatment on sperm Average Path Velocity (VAP in µm s^{-1}) was assessed using a CASA (Computed Assisted Sperm Analysis) system (Hobson Sperm Tracker, Hobson Tracking System Ltd, Sheffield, U.K.), according to Abascal et al. (2007). The effect of Ca^{2+} concentration was tested using AM (naturally containing 11 mM Ca^{2+}), or 0.5 M NaCl (same osmolarity as seawater) solution with 10 mg ml^{-1} BSA as an activating medium, without Ca^{2+} or containing 10 mM Ca^{2+}. The sperm trajectory diameters were measured.
Then, sperm samples were maintained at 4°C either diluted in NAM (1v./1v.) or undiluted, and the percentage of motile spermatozoa was assessed after 24, 48 and 72 hours.

3. Statistics

Data were calculated as means ± standard deviation. A linear regression was plotted between optical density and sperm concentration which was estimated by cell counting in a hemocytometer. Percentages (GSI and percentage of motile spermatozoa) were arcsin square-root transformed. After verifying variance homogeneity (Bartlett test), means obtained from various tests were compared by Student t-test or ANOVA. When results were significant, a Tukey *post-hoc* test was used for comparison of means.

4. Results

No significant effect of GnRHa treatment was observed on GSI, which averaged at 1.33 ± 0.70 %, or on sperm concentration that averaged $3.8 \pm 1.3 \times 10^{10}$ spermatozoa ml^{-1} (n = 25). The total sperm volume collected from each male (from both testes) ranged from 0.1 to 5 ml. A highly significant correlation ($P<0.001$; $R^2=0.841$) was observed between optical density at 260 nm and sperm concentration obtained with a haemocytometer (Fig. 1).
Spermatozoa were immotile in their seminal fluid, but were fully activated when diluted in AM. The percentage of motile spermatozoa observed at 30 s after activation was not significantly different between sperm of control (73 ± 10 %) and GnRHa implanted males (59 ± 32 %). Also, regardless of the hormonal treatment, the percentage of motile spermatozoa decreased as a function of time after a plateau of 5 - 6 min following activation. After 10 - 11 min, no forwardly progressive movement was observed (Fig. 2). A significantly higher ($P<0.01$) VAP was observed in sperm from GnRHa treated tuna

compared to untreated ones (Fig. 3). Sperm trajectory diameters were modified in relation with Ca^{2+} concentration of the activating medium : 45 to 50 µm for 0.5 M NaCl and 10 mM $Ca^{2+,}$ 80 to 140 µm for seawater and 800 to 1000 µm for 0.5 M NaCl without Ca^{2+}.
No significant difference of storage capacity at 4°C have been observed between hormonally treated males (n=3) and control ones (n=2). The results of the five males were plotted without any reference to hormonal treatment. A significant decrease ($P<0.001$) in the percentage of motile spermatozoa was observed after 24 and after 72 hours at 4°C. Furthermore, a significant decrease ($P<0.001$) of storage capacity at 4°C was observed when sperm was diluted with NAM (Fig. 4).

5. Discussion

The mean spermatozoa concentration assessed in testis of captive Atlantic bluefin tuna was lower than values observed by Doi et al. (1982) in wild animals (5.0 to 6.5 $x10^{10}$ spermatozoa ml^{-1}) but also lower than values recorded in wild individuals (4.8 to 6.6 $x10^{10}$ spermatozoa ml^{-1}) of the Pacific congener bluefin tuna, *Thunnus orientalis* (Gwo et al., 2005). The duration of sperm motility was high compared to values recorded in other marine fish species (1 – 13 min; Cosson et al., 2008b). As in European seabass (*Dicentrarchus labrax;* Sorbera et al.; 1996), the sustained administration of GnRHa by implants did not significantly modify tuna sperm concentration though it did increase the percentage of captive tuna in spermiating condition (Mylonas et al., 2007). The sperm volume contained in the testes of captive Atlantic bluefin tuna (0.1 – 5 ml) was rather limited, regardless of hormonal treatment and was possibly linked to the low GSI values which were significantly lower than in wild Atlantic bluefin tuna (Medina et al., 2007).
As in most fish species with external fertilization (Cosson et al., 2008b; Cosson, 2010), tuna spermatozoa are immotile in the seminal fluid, however motility is triggered by environmental changes, when the released sperm is diluted in seawater. Sperm movement duration observed in the present work was shorter than values reported by Doi et al. (1982). The lower GSI values observed in this study (GSI: 1.33 ± 0.70 %) suggest that fish were not fully mature. Higher GSI values (2.87 - 4.27 %) were recorded in wild Atlantic bluefin tuna (Medina et al., 2007). Furthermore, a sperm “maturation” process was suggested in salmonids, spermatozoa acquiring their movement capacity during their passage from the anterior to the posterior part of the sperm duct (Morisawa and Morisawa, 1986). In tuna, because sperm stripping was not possible, intratesticular sperm was collected which has probably not totally acquired its ability for potent movement. Finally, our activation procedure using a high dilution rate and a two step dilution is considered adequate to evaluate sperm parameters because of synchronical activation of the sperm cells (Billard and Cosson, 1992). Methods used by Doi et al. (1982) probably did not achieve synchronous sperm activation (dilution rate: 1/100; one step dilution method) and this would result in longer motility. The above three reasons may explain the lower percentage of motile cells (59 – 70 %) and shorter movement duration (10 - 11 min) observed, compared to data reported by Doi et al. (1982).
Sperm movement characteristics were studied using high quality samples, showing 100 % motile cells 30 s *post* activation. Similar to our observations in Atlantic bluefin tuna, GnRHa implants (25 µg kg^{-1}) significantly increased swimming speed of Atlantic halibut (*Hippoglossus hippoglossus*) sperm (Vermeirssen et al., 2004).
Dilution in NAM was initially designed to prevent sperm activity. However, the storage capacity of Atlantic bluefin tuna sperm decreased rapidly in such medium compared to seminal fluid. The study of seminal fluid composition would help to design a specific diluent for short term storage. Similarly, the addition of an artificial

seminal fluid protected sperm and prevented the alteration of spermatozoa functions during storage at 4°C in turbot (*Psetta maxima*) (Dreanno et al., 1998).
In conclusion, some basic features of Atlantic bluefin tuna sperm biology have been characterized in the present work, but it is clear that further investigations are needed in order to improve this knowledge, especially with regard to comparison of intratesticular and spontaneously released sperm characteristics.

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Figures

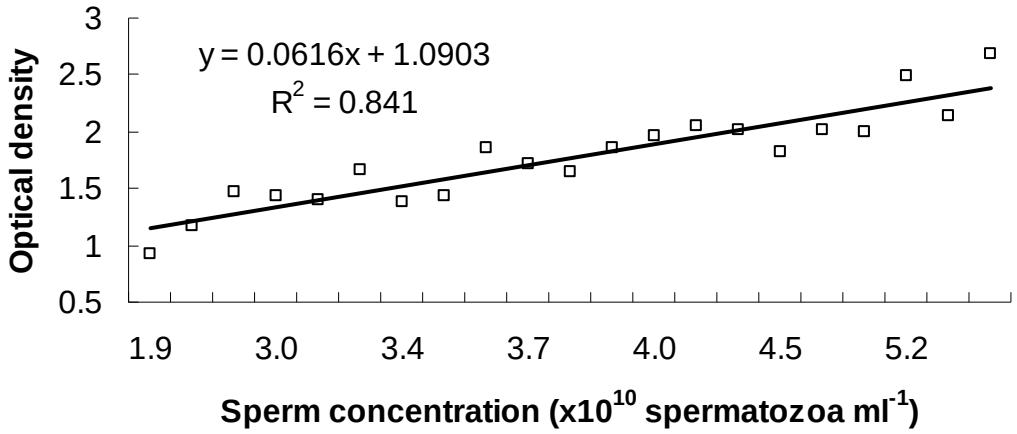
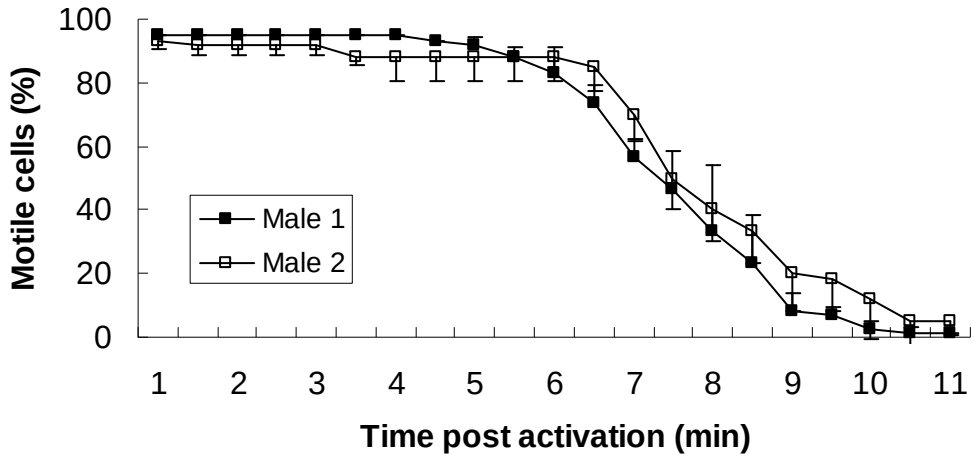


Fig.1. Linear regression of the optical densities of diluted Atlantic bluefin tuna sperm, recorded at a wavelength of 260 nm versus sperm concentration assessed using a haemocytometer (n=21 males).

Fig.2. Changes in the percentage of Atlantic bluefin tuna motile spermatozoa with time after activation in Activating Medium evaluated in triplicate (n=2 males; mean±SD; the samples presented in this graph were selected for their high initial percentage of motility).



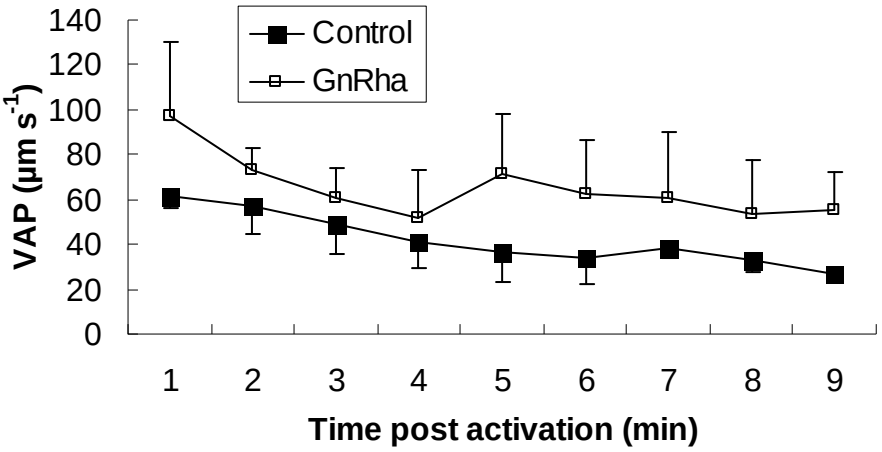


Fig.3. Effect of GnRHa treatment of Atlantic bluefin tuna on changes of sperm Average Path Velocity (VAP), evaluated with CASA system, with time elapsed after activation in Activating Medium (n=3 males; 20 to 30 spermatozoa for each analysis, mean±SD).

Fig.4. Effect of the Non Activating Medium (NAM) on Atlantic bluefin tuna sperm storage capacity at 4°C (n=5 males; mean±SD).