

Gamete biology: Perspectives for Bluefin Tuna Aquaculture.

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Abstract

Taking care of fish reproductive physiology and gamete biology allowed significant progresses in fry production control for many commercially interesting fish. Can it be a similar situation for bluefin tuna?

Fish gamete production results from long processes controlled mainly by climatic conditions, mediated by neuroendocrine and endocrine factors. The possibility to monitor the development of germinal cells by successive sampling in individual fish which easily breed in captivity, has allowed learning about the sequences of gametogenesis and their control, as well as important characteristics of fish gamete biology.

The female ovarian cycle involves a progressive accumulation of yolk in the oocytes (i.e., vitellogenesis), followed by cytoplasmic and nuclear events related to maturation and ovulation. The phases of vitellogenesis and maturation are of different duration in different species. In captive fish the endocrine regulation of reproduction may be disrupted by stress, thus preventing spontaneous spawning, while the application of exogenous hormones at the conclusion of gametogenesis allowed production of good quality gametes. In males, gamete production is not continuous as in mammals. Spermatozoa are produced after a cycle that includes a resting period, followed by continuous or discontinuous sequences of spermatogenesis (spermatogenesis sensu stricto, spermiogenesis and spermiation). The discontinuity of fish sperm production may induce a phenomenon of ageing along the period of spermiation, which decreases the quality of semen with time.

Wild bluefin tuna breed in specific areas during a short summer spawning season. When kept in cages in the area of Cartagena (Spain), they present developed gamete stages at the same time as wild fish, but, until now, neither mating behavior nor eggs have been observed as was the case in Japan. It has not been possible to monitor individual gonad recrudescence by successive sampling, due to inability to handle the fish without causing mortality, so that the reproduction failure remains unexplained. Hormonal stimulation may be an interesting alternative but its use requires an assessment of gonadal stage to be performed properly. The high post-handling mortality of bluefin tuna in captivity is a real bottleneck for reproduction control.

The improvement of handling and the use of less stress-susceptible individuals may be a major progress for bluefin tuna reproduction control. The occurrence of a second generation of juveniles in Japan, subjected to first domestication selection may provide very interesting experimental fish.

Introduction

Mediterranean Bluefin tuna (*Thunnus thunnus thynnus*) recruitment is very sensitive to overfishing. As the market demand increases and the fishing technics improve, it is now considered as an endangered species the fisheries of which is regulated an international commission (ICCAT) covering both West and East Atlantic and Mediterranean stocks. The development of captive broodstock with controlled reproduction may be a complementary source for recruitment particularly to provide fry for aquaculture.

The success of reproduction is not only in relation to parental factors like heredity, domestication, health and physiology but also to environmental factors and physiological status of gametes themselves when they are produced. In the present paper, the most important features of fish gamete biology for embryo success will be described and the current state of captive tuna gametes in Mediterranean will be analysed taking into account this basic knowledge.

Parental physiology

As is the case for most of the poikilotherms, fish reproduction is highly dependant on external factors and particularly temperature and photoperiod driving to a seasonal breeding period in temperate species. Schematically, these factors are received through sensitive receptors of the

central nervous system and particularly pineal, olfactory bulbs and eyes. They are integrated by the brain and transformed into neuroendocrine signals in hypothalamus. By the periodic release of a decapeptide called gonadoliberin or GnRH (Gonadotropin Releasing Hormone), this part of the brain controls the release of glycoprotein hormones by the pituitary into blood circulation. These proteins or gonadotropins stimulate the gonadal function.

In testis, gonadotropins induce the synthesis and release of 11 ketotestosterone (11KT), a steroid which controls the whole spermatogenic cycle up to spermiogenesis. Spermiation occurs for high 11KT blood levels but it may be controlled by complementary hormonal inputs and particularly maturation inducing steroid (see below). Generally gametogenesis can occur in captive fish although sperm collection may be difficult or impossible.

The seasonal female gametogenesis includes two steps, respectively vitellogenesis and oocyte maturation. During vitellogenesis, the ovary secretes increasing quantities of 17β estradiol (E2) under the control of a FSH-like gonadotropin. This steroid exerts by feed-back to pituitary a regulation of its own synthesis. Moreover, E2 induces the hepatic synthesis of a vitellus precursor called vitellogenin the proteic skeleton of which aggregates minerals, sugars and lipids and carry them from hepatic cells to the ovary via the blood stream. Vitellogenin accumulates into oocytes by an active uptake controlled also by FSH until the oocytes reach a determined volume varying among species. During spawning time, variations of GnRH stimulates LH-like GtH which peaks in blood stream. This peak provokes the ovarian synthesis of a new steroid called MIS (maturation inducing steroid). This progestagen induces meiosis resumption (migration of nucleus, germinal vesicle breakdown) and concomitantly the ovulation (oocyte hydration and follicle rupture). This rapid overview of reproduction process clearly shows the different levels of endocrine control along the neuroendocrine axis. Unfavorable external conditions like stress and pollution may disturb reproduction by endocrine disruption at these different levels and prevent either vitellogenesis or maturation or ovulation. The use of additional hormonal treatment proved to be efficient to complete reproductive process in some cases particularly when spontaneous vitellogenesis occurs. Most of reproduction stimulation is performed by the use of pituitary extracts and more recently by GnRH analogs.

Gamete biology

Sperm assessment and physiological variations. Once produced by the testis spermatozoa remain in the testis until they are ejaculated during mating. As a particularity, fish sperm is unmotile in the testis and vasa deferens and becomes motile at the first contact with the external medium. In freshwater fish, the decrease of osmotic pressure and a lack of potassium are the triggering factor whereas in seawater, the hypertonicity of the medium activates sperm. The motility of sperm is maximum just at activation and then it decreases more or less rapidly among the species; For instance, all movement of sperm stops after 30 seconds in salmonids and seabass whereas they are still moving at 4 min in turbot and 20 min in tuna. The decreasing motility can be assessed by the number of motile cells, the frequency of flagellar beats or the velocity of the cells, those different parameters being correlated and dependent. Among the species, very variable amounts of semen can be collected from ripe males. In turbot only a droplet is obtained whereas in salmonids or seabass several milliliters can be release by a slight pressure of the abdomen. In some species as the sole the fish must be killed and the testis scarified to obtain sperm. The semen of fish may be also characterised by the concentration of spermatozoa. Several assessment techniques like use of counting cells, spermatocrit, spectrophotometry have been set up and have yielded convergent results. Among species it can vary from several billions to several tens of billions per milliliter.

An integrative parameter, the fertility of sperm can be evaluated by insemination trials with reduced sperm to egg ratio so as to avoid compensation of sperm quality by sperm quantity. In

seabass (*Dicentrarchus labrax*), secured ratio is 200,000 sp.egg⁻¹ in fry production purpose whereas experimental discriminating ratio is only 35,000 sp.egg⁻¹.

Finally, cryopreservation technics have been developed for fish sperm with variable success. For instance, the fertility of salmonid gametes is drastically decreased at thawing while the alteration of frozen sperm can be balanced by a slight increase of the sperm:egg ratio of in marine fish.

The characterisation of sperm status by the different techniques described above, allows monitoring physiological variations of sperm. The main concern is a decrease of quality along the reproductive season. In seabass for example, the volume of expressible milt decreases with time as increases the number of non spermiating males although the testis is still containing sperm. Moreover the assessment of sperm concentration of successive samples of a broodstock reveals a slight but significant decrease at the end of the season particularly in seabass. The motility of sperm is also altered at the end of reproductive season and shows a reduced duration of sperm movement. And at last, the recovery at thawing of frozen sperm is lower at the end of the reproductive season. The assay of ATP and its products reveals that the energetic charge of spermatozoa inside the testis decreases along the season. Such convergent studies may be explained by the discontinuity of sperm production in fish which result in an ageing of gametes stored in the testis for a long period.

Egg quality. Contrary to sperm, female gametes cannot be easily assessed for quality. Usually, the morphology of the egg and particularly the rotondity and the homogeneity of vitellus are observed to forecast development potential of the egg. However, if eggs with heterogenous vitellus and with deformities will surely fail, those showing perfect shape and vitellus aspect may be either well fertilized or not. So in the absence of specific indicators of quality, the fertilisation success is taken into account. In marine fish the fertilisation can easily be assessed just some hours after insemination due to the transparency of vitellus while in salmonids particularly, the recognition of fertilised eggs is delayed to eyed stage due to vitellus opacity (several days). Symetry of blastomere divisions, hatching rate, larvae deformities and ploidy of embryos were also explored to describe egg quality.

Among the causes of lack or loss of fertility of captive broodstock, we have particularly investigated the induction of spawn and the egg retention in genital tractus. As mentioned above, hormonal complementation can compensate stress effects which prevent spontaneous reproduction of captive broodstock or it can synchronise and program spawnings.

The success of spawn induction is depending on the stage of oocyte development at which hormones are applied. By the end of vitellogenesis, the oocytes stop growing by vitellogenin uptake and progressively present morphological modifications including peripheral vitellus differentiation, lipid coalescence and nucleus migration to the periphery. Our studies on seabass and red drum (*Sciaenops ocellatus*) determined 4 stages to characterise this post vitellogenic or prematurational period. The comparative application of LHRH analog at the different stage shows that the quality of egg depends on the physiological status of the oocyte. Precocious stimulation results in significantly lower fertilisation rate, smaller eggs, and uncontrolled response time. On the contrary the application of hormone at the beginning of nucleus migration results in precise ovulation time and higher fertilisation rate; in seabass at 13°C, ovulation occurs at 72± 2 h post stimulation.

The mode of application and the quantity of hormone are also determinant for the success of stimulation. In seabass, the interval between spontaneous ovulations being long, the use of slow release implant induces a good quality first ovulation followed by low quality spawns. On the contrary, the application of implants to multiple short interval spawners like turbot and red drum, enhances the rhythm and the size of spawns. The comparison between LHRHa doses of 50 and 20µg kg⁻¹ of female in *S. ocellatus* showed significantly higher fertility for the

lower dose. In that case, we think that overdose stimulates unmaturing oocytes driving to non-competent eggs. In conclusion, hormonal supply can help obtaining spawns from captive stressed fish provided the dose and the time of application are adapted to the species.

The other important cause of egg quality deterioration is the loss of viability of gametes in the genital tract or overripening. In fact, egg release can be delayed from ovulation due to captivity (stress, lack of mating behavior, preventing spawns). The experimental retention of oocytes *in ovario* allows observing a rapid decrease of fertility with time. In trout, deleterious post-ovulation delay is about 20 days in old females whereas, it is about only some hours in turbot (10h), seabass (3h). The loss of viability is correlated to modifications of ovarian liquid where we observed a decrease of pH, an increase of potassium content and an increase of proteic content. In easy-to-handle captive species, overripening can be prevented by stripping the fish. Overripening has also intermediate effects like the increase of abnormality rate in the progeny and the increase of chromosomal aberration occurrence.

The case of Bluefin Tuna in the Mediterranean

Bluefin tuna caught in the spawning area of Balearic islands were towed to rearing cages in Puerto de Mazarrón, Spain, and have been maintained in captivity for 2 years in order to observe reproduction characteristics of the species in captivity. After unsuccessful trials of non-invasive sampling, we slaughter fish so as to get information about the genital status of the fish around the theoretical reproductive season.

In 2003, after one year of captivity, the males showed low gonadosomatic index (GSI) and they were not fluent. Only one fish had a small amount of testicular sperm presenting a very high viscosity. Nevertheless, the concentration assessed by both cell counting and spectrophotometry was only 1.92×10^{10} spz ml⁻¹. In 2004, i.e. after 2 years of captivity, some fish were fluent and all the sampled fish presented testicular sperm although the GSI was half that of wild-caught fish. As was the case at the first sampling, the viscosity of semen was very high but the concentration was 4.92×10^{10} spz ml⁻¹.

In terms of motility, an increase of motility duration was observed between the first (4 min) and the second year (7-20 min) of captivity. The motility of wild fish sperm observed previously lasted around 14 min.

Finally sperm of either wild or captive tuna shows a very good ability for short term (on ice) and long term (cryopreservation) storage.

Captivity seems to interfere on male genital activity on the first year probably due to high stress of the broodstock. An additional year of captivity clearly shows an increase in sperm quality and quantity demonstrating that long term captivity does not prevent male genital activity in Mediterranean conditions.

The large majority of the female sampled presented full vitellogenesis in 2004 after two years of captivity but 2 females presented undeveloped or regressed ovaries and one female underwent post-vitellogenesis. Tuna female may therefore breed in our European captivity conditions as is the case in Japan. However the regression observed shows the sensitivity of tuna to captivity. Moreover, although the vitellogenic process appears functional, neither mating behavior nor fertilized eggs were observed in the cages. First trials of hormonal stimulation drove to development of post-vitellogenic stages but did not allow hydration and spawning of eggs.

Perspectives and conclusion

Aquaculture seems to be an interesting alternative to fisheries and particularly for endangered species. However, the sustainability of this activity relies on a trustworthy supply of fish. In the case of bluefin tuna, fish is mostly brought by fisheries inducing unpredictable variations of supply and huge difficulties in management of both the fattening farms and the wild stocks.

Mastering the reproduction in captivity would be of considerable help to produce juveniles when required as is the case in other well established cultured species. Tuna seems to offer interesting possibilities since it already breeds in captivity in Japan and it shows good indications for reproductive success in European waters.

However, the process is not controlled to date and does not allow scheduling reliable productions of fry. The extreme difficulty to handle the tuna breeders and to perform non intrusive examination prevent the application of adapted treatments at the right stage to stimulate spawning. As a perspective, it can be recommended to develop research programs on stress control during handling of this large pelagic fish. On another point of view, the use of less stress-susceptible fish would allow solving the problem. The recent obtention by Kinki University, of progenies originating from tuna born in captivity will hopefully provide more domesticated individuals. These fish subjected to constant rearing constraint may be selected for their resistance and may present a better ability for handling. They are particularly interesting for studies on reproduction control and domestication process.

Aknowledgements

The present work was partly supported and funded by EU (Fair project QLRT-2001-01355, REPRODOTT).