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Biology and Control of Reproduction of Sturgeons in Fish Farm

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Abstract: Over the last century the reproduction of sturgeon has been intensively studied par Russian scientists who established a technology for the ranching of alevins which included artificial fertilization of gametes taken from wild broodfish and larvae rearing. More recently, since 1970, major contributions originated from teams in the USA, the EU, Japan and Iran. A rather good knowledge of the reproductive physiology of sturgeon is now available : gametogenesis, reproductive cycle, ovulation, spermiation and fertilization. The complete life cycle was obtained in captive conditions allowing the production of juveniles and caviar.

Key words : Sturgeon, reproduction, puberty, aquaculture

Introduction

The reproduction of sturgeons has been intensively studied par Russian scientists over the last century (Dettlaff *et al.*, 1993) but more recently major contributions originated from teams in the USA, the EU, Japan and Iran and their works were delivered to several symposia held in France Bordeaux (Williot, 1991), in New-York (Berstein *et al.*, 1997), Russia (Ghershanolivich & Smith,1995), in Piacenza Italy (Rosenthal *et al.*, 1999) and in the USA in 2001. By the end of the eighties a large body of knowledge was available in the field of reproductive physiology and technologies for the control of reproduction were rapidly established.

I-Captive broodstock

Mature males and females are now available in farm and are reared in all kind of structures (circular tanks, raceways, ponds) of appropriate dimension as some sturgeons may reach large sizes. The standard procedure is the same as for growing sturgeons but the density of fish should not exceed 20 kg/m^3. The shape of the rearing structure and the flow rate must allow the elimination of suspended solids. The brood fish are usually given salmonids feeds. The feeding rate depends on the water temperature, 0.2 to 0.7% body weight at 16-22°C. Exposure of females to low seasonal temperature is required in the white sturgeon *Acipenser transmontanus* and the Siberian sturgeon *A. baerii* for the completion of the entire reproductive cycle (vernalisation). Gravid *A. transmontanus* females exposed in November to constant temperature 18 °C did not ovulate in spring after hormonal treatment and showed follicle atresia. Females submitted to constant (15°C) or to seasonal (10-15°C) temperature were normally reproduced (Web *et al.*, 1999). In practice *A. baerii* should be kept at 10-14°C during 3-6 months before ovulation; changes in temperature should not exceed 2 °C/day (Williot, 2002).

II- Gametogenesis and reproductive cycle

Sex diferentiation :

The gonad is first represented by a thin thread of connective tissue (1 mm in diameter) in which are included the primary germ cells (diameter 8-12 µm). The ovary can be first recognised from a longitudinal groove on the side of the gonad and the formation of ovigerous lamella. The sexual differentiation usually occurs late in sturgeons and depends on the temperature, feeding and on the species. Age and weight at first maturity of 3 sturgeons species from farm are shown table 1.

Table 1: Age and weight at puberty of *A. transmontanus*, *A. ruthenus* and *A. baerii* from farm

	A. transmontanus		***A. ruthenus***		***A. baerii***	
	Male	Female	Male	Female	Male	Female
Age of puberty (years)	4	10	3-5	7-9	4-5	7-8
Body weight (kg)	10-15	25-30	0.7	1.4	5.3	7.9

Several methods are used to recognise the sex :

1) Direct observation of the gonads after laparotomy : 18 months (1.1-2.3 kg at 58-72 cm) in *A. transmontanus* and 3-4 years in *A. baeri*,
2) The measurement of circulating 11 Ketotestosterone which is characteristic of the female up to 6 years of age,
3) A direct examination under binocular of pieces of ovary or testis taken by biopsy,
4) Echography, a non invasive technique in which the shape of ovary can be recognised ; this require skilled people.

Spermatogenesis:

The testis of sturgeons is of lobular type and spermatogenesis occurs in cysts as in teleosts. In *A. transmontanus*, active spermatogenesis starts in 3 year old males with meiosis in October-November and is completed in February. In June, unshed spermatozoa are resorbed by Sertoli cells; males are mature at 4 years and weigh 10-15 kg (Doroshov *et al.*, 1997). In bester spermatogenesis starts in September and is completed by December. Androgen levels increase during spermatogenesis and remain high during prespermiation (Mojazi Amiri *et al.*, 1996a). The number of spermatogonia generation is not known but the GSI is high (testis may reach up to 10 % of body weight). The spermatozoa of sturgeon possess an acrosome found only in chondrosteans and chondrichthyans but not in teleots.

Oogenesis:

Russian scientists usually refer to 5 main stages of oogenesis (table. 2). In *A. transmontanus* female, some mitosis of primordial oogonia, recognised at 2-3 years, are organised in clusters of 10-20 cells of 8-15 µm surrounded by somatic cells ; oogonial mitosis are completed at the age of 4-5 years and the oocytes surrounded by a basal lamina and pre-follicular cells are found in the ovigerous lamella where they slowly develop (Doroshov *et al.*, 1997).

Table 2: Stages of ovarian development in sturgeon used in the Russian literature

Stage	Occurance	Cell category	Maxi ∅ of Oocytes	Adipose tissue*	Shape/color Of ovary
0		Oogonia		0	Filiforme
I	All year round	Oocytes	<100 µm	+	Filiforme
II	All year round	Oocytes,foll.cell	100-150	++	Yellowish
III	June-October	Vitellogenesis	500-1000	+++	Greyish
IV initial	Sept-April	Gv sub-central	1000-2000	++	Large size
Final	Feb-April	GV in migration	>2000	(+)	Large size
V	April-June	Atretic follicles			Regression

* Yellowish

In bester, Mojazi Amiri *et al.*, 1996b have identified several stages of oogenesis:

1) Nucleolus chromatin stage: the oocytes increase in size (from 10 to 60 µm) and show a high affinity for hematoxylin,
2) Perinucleolar stage: the cells reache 200 µm; a layer of nucleolus appears at the periphery of the nucleus. This is followed by the process of vitellogenesis itself occurring in the following stages,
3) Formation of oil droplets found in cells of 200-480 µm at the periphery of the nucleus,
4) Cortical alveoli stage 460-600 µm,
5) Primary yolk in the cytoplasm of 600-1000 µm oocytes,
6) Secondary yolk in cells reaching 1000-1600 µm, with deposit of large yolk granules of crystalline structure at the periphery,
7) Tertiary yolk stage ending with oocytes of 1600-3000 µm in diameter depending of the species,
8) Oocyte maturation *i.e.* the migration of the nucleus (or germinal vesicle GV) at the periphery (animal pole). During the reproductive cycle all size of oocytes can be found in the ovary but a few months before maturation only 2 size groups remain, large oocytes > 2mm and oocytes less than 0.5 mm (Williot, 2002).

Mojazi Amiri *et al.*, 1996b have shown in bester an increase of vitellogenin and various hormones (figure 1). The circulating viteliogenin reaches 3.5 mg/ml serum at the end of vitellogenesis (concentrations of 7 mg/ml have been reported in *A transmontanus*) and decreases sharply during oocyte maturation. Estradiol 17ß and 17,20ß-dihydroxy-4-pregnen-3-one increase during vitellogenesis and decrease immediately after; testosterone also increase but remain high during maturation.

Vitellogenesis is initiated at 4-8 years in *A. transmontanus* (15-30 kg) and is completed within 15-18 months. Yolk accumulation in the oocytes is more active during the summer months and stage IV initial is reached in the autumn and the ovary may remain in this stage for several months. But once the oocyte maturation has started ovulation will follow; if not the follicules will regress (Doroshov *et al.*, 1997). In adult Siberian sturgeon, in farm, the oogenetic cycle is 1 year (25 %), 2 years (54%) or 3 years (11%) (Williot & Brun, 1998).

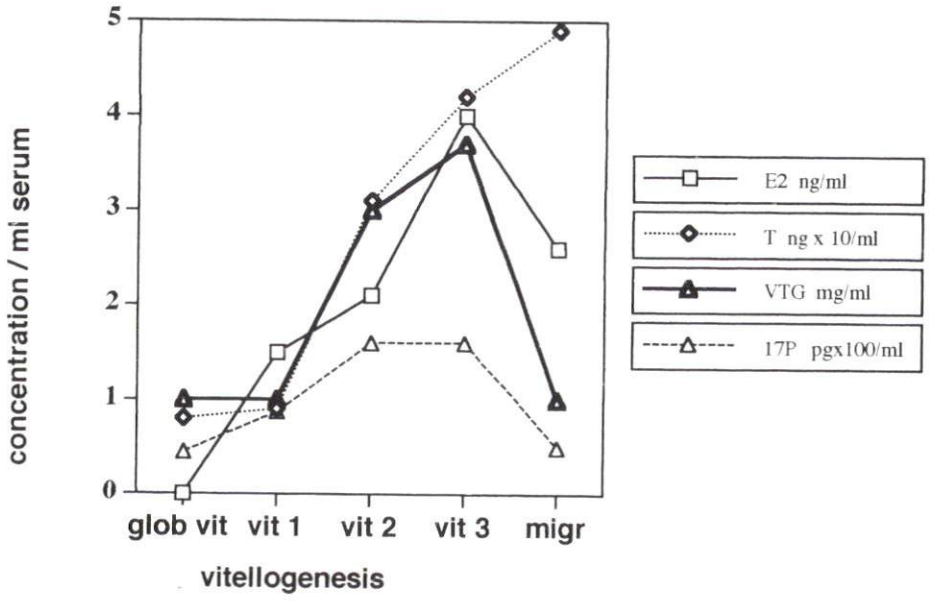

Figure 1 : Changes of circulating levels of vitellogenin (VTG) and some sex steroids (E2: estradiol, T: testosterone, 17P: 17,20ß-dihydroxy-4-pregnen-3-one) during vitellogenesis and oocyte maturation in bester ; stages of vitellogenesis oil droplet (glob vit), primary, secondary and tertiary yolk stage (vit 1-2-3), GV migration (migr) (redrawn from Mojazi Amiri *et al.*, 1996b).

The oocyte envelopes:

The two categories of envelopes surrounding the oocytes (acellular layers of the oocyte and two cell layer) are progressively organised during oogenesis (figure 2). 1) acellular envelopes around the oocyte (zona radiata interna and externa, secreted by the oocyte, and an external layer given different names (jelly coat, L3...) probably secreted by the follicular cells are part of the oocyte (pellucida). The zona radiata has two layers, 2) the layers of follicular cells (granulosa and thecal cells). The follicular cells are present early during oogenesis (maximum number is found in 0.6 mm oocytes). The 2 external cell layers are involved in the endocrine regulation of vitellogenesis and oocyte maturation.

In the animal pole, a hole in the acellular envelopes of the oocyte, the micropyle allows a spermatozoa to enter into the oocyte. Several micropyles are found in sturgeons, less than 5 in *A stellatus*, between 5 and 10 in the beluga *Huso huso*, up to 30 in the Russian sturgeon *A. gueldenstaedtii*. The distance between two micropyles is 40-100 µm and they are all concentrated on an area of 120 to 300 µm. The opening is funnel shaped in *H. huso* and is cylindrical in *Acipenser* (Fig. 2). The lack of acrosome in teleosts is usually explained by the presence of micropyles which allows the spermatozoa to reach the cytoplasm and does not have to perforate the envelopes. In sturgeons which spawn in rapid current (1 m/sec) with fast dispersion of gametes the presence of several micropyles may be a way to increase the chance of spermatozoon to penetrate into the egg .

The process of penetration may be accelerated by the acrosomic reaction (Cheer & Clark, 1984) with a possible attachement of the 10 µm acrosomic filament to the oocyte membrane and a mechanical attraction of the spermaozoa toward the oocyte (the efficiency of the flagellum to bring the sperm head deep into the micropyle is probably limited). The same authors have suggested that the carbohydrate rich L3 layer (Fig. 3) triggers the acrosomic reaction as the sperm going through the micropyle reaches this L3 layer which is precisely at 10 µm from the surface of the oocyte surface.

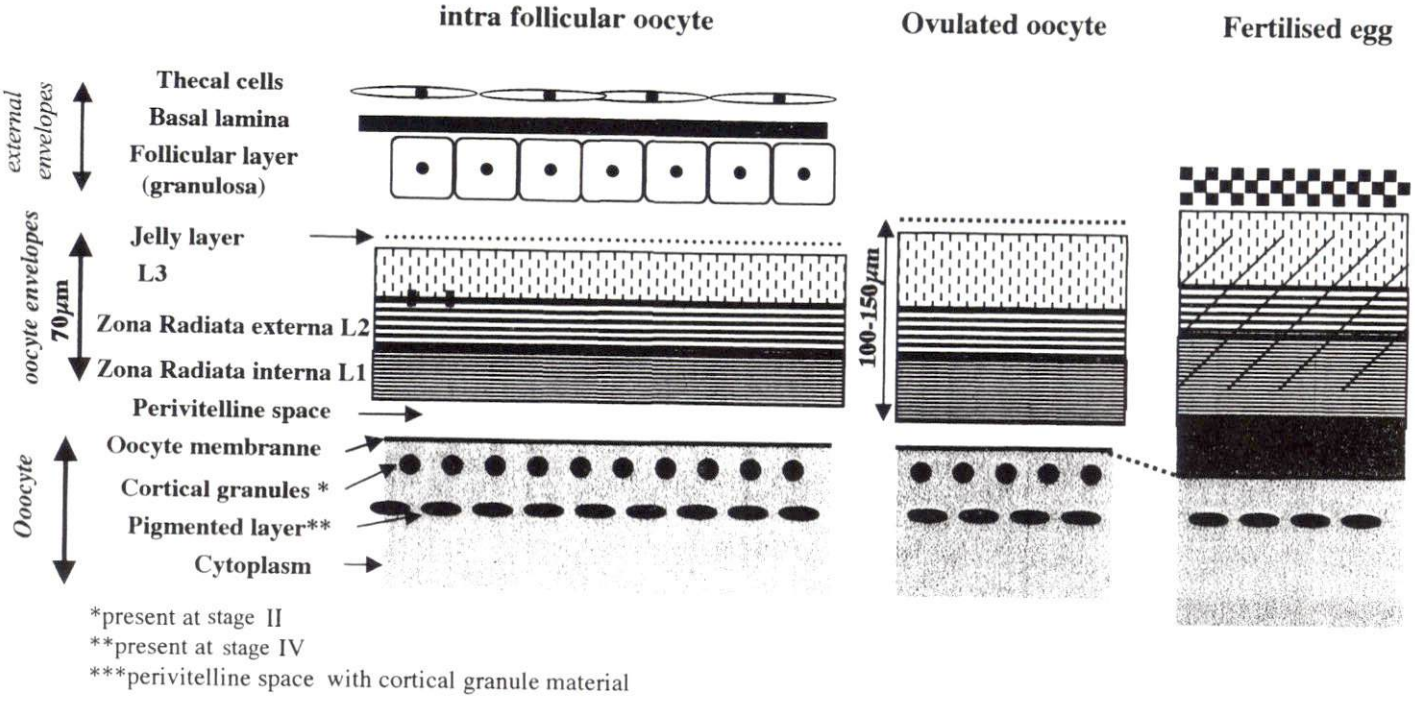

Figure 2 : Schematic representation of envelopes surrounding sturgeon oocytes inside the ovarian follicle (left) , after ovulation (middle) and after fertilization (right). The name of envelopes L1, L2, L3, Jelly coat are from Cheer & Clark 1984, 1985).

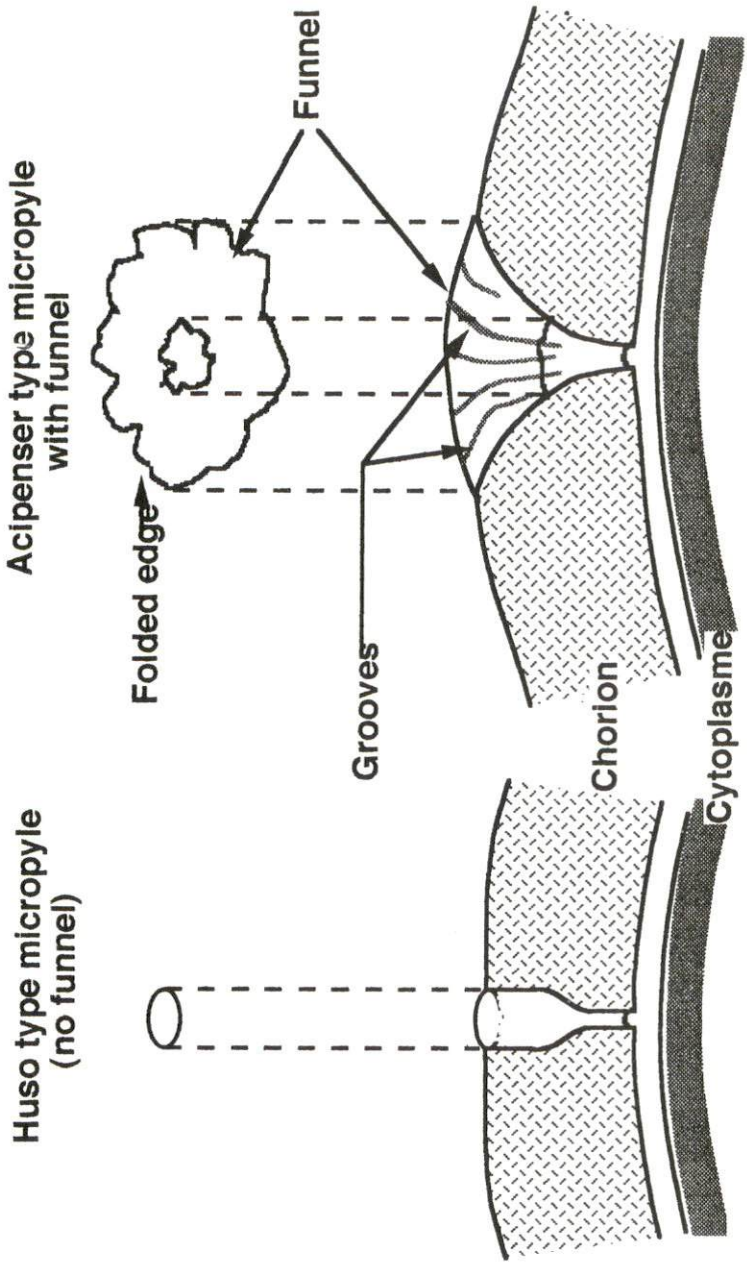

Figure 3 : Schematic representation of the morphology of the external aperture of micropyles in *Huso* and *Acipenser* (after L. Debus pers. comm. 2000).

III- Oocyte maturation and ovulation:

The oocyte maturation, immediately followed by ovulation, is the final step of oogenesis. It includes the migration of the germinal vesicle toward the animal pole of the oocyte, the rupture of the nuclear envelope (GVBD: germinal vesicle breakdown) and some changes in cytoplasm structures. In most fish species the final phase of oogenesis (oocyte maturation and ovulation) is induced by a sharp release of pituitary gonadotropin in response to some signals from the environment (in sturgeons, probably water temperature, flow rate and velocity, presence of spawning substrate,...). The importance of this surge of gonadotropin is not known in sturgeons. In captivity spontaneous oocyte maturation and ovulation do not occur likely due to a lack of precursors in the steroidogenesis pathway (Mojazi Amiri *et al.*, 1999a).

During ovulation the oocyte is freed from the follicle after rupture of the layer of follicular cells. Some parts of this layer may remain few hours at the surface of the oocyte covering sometimes the micropylar area and preventing fertilisation. For this reason it is advised to wait one hour after egg collection to carry out the insemination (more if there is evidence that ovulation has just occurred).

IV-The control of reproduction:

The main problem with sturgeon is that the oocyte maturation/ovulation and to a less extent spermiation do not occur spontaneously in captivity; as a result hormonal stimulation is needed. The collection (and sometimes storage) of sperm and eggs, artificial fertilization and incubation are also to be under control.

Identification of the oocyte maturation stage:

The stage of oocyte maturation when gonadotropin hormone is injected should be known precisely. The position of the migrating germinal vesicle in the oocyte is a classical criterion. Williot *et al.*, (1991) made a first selection in October-November (females with follicles > 2.8 mm and males which exhibit firm testes). A second selection is carried out in the spring at the time of reproduction to know the exact stage of maturation before hormonal injection.

Oocytes in follicles are taken by biopsy, placed 2-3 min in boiling water and cut in the axe of the 2 poles. The GV is seen and the distances A and B (Fig. 4) are

measured and the ratio A/B or polarization index (PI) established. The treatment is usually successful when the PI values are below 0.7 *i.e.* when the GV reach the periphery of the oocyte.

The receptivity of the follicles was measured by an *in vitro* test (Williot, 1997). Follicles are incubated in saline solution in Petri dish with 17,20ß-dihydroxy-4-pregnen-3-one added (1 µg/ml). When 90 % eggs are mature 24 h later, the female has good chances to ovulate. The method was refined by measuring the time when 50% of oocytes are mature (TE50). No female will ovulate when TE50>20 h, few ovulation when TE50>13.7 h and 100 % when TE50<13.7 h (Williot, 2002). The measure of circulating levels of testosterone and progesterone allowed Safi *et al.*, (1999) to discriminate fertile Persian sturgeon *A. persicus* females. .

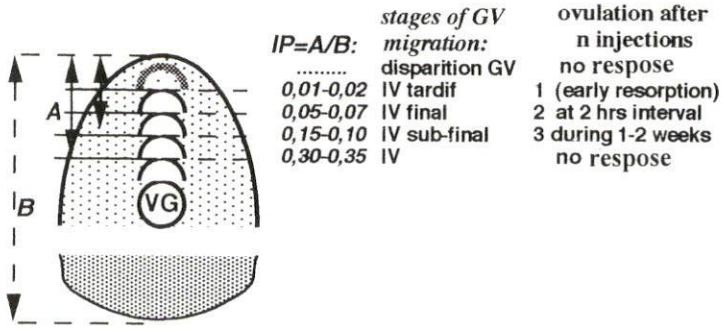

Figure 4: Measurement of the polarity index PI ; A : distance germinal vesicle (GV) to the animal pole, B :distance animal pole to vegetal pole. PI Values are given for different stages of GV migration and number of injections of pituitary extracts needed for ovulation (case of *A. stellatus* , N Patriche pers.comm. 1999)

Induction of oocyte maturation and ovulation

Use of pituitary preparations:

In Russia sturgeon pituitary extract were traditionally used to induce ovulation and spermiation. Preparations from carp are also potent and are used in France ad Italy. The biological activity of those preparations is not known and changes with time and hatchery managers tend to increase the doses to compensate for some loss of activity. Doses are usually 5 mg of acetone dried preparation per kg body weight suspended in saline solution grounded and injected.

Use of GnRHa:

Luteinising hormone releasing hormone LHRH is potent to induce ovulation in sturgeon at dose as low as 1.5-4.5 µg/kg (Barannikova *et al.*, 1989) (table 3) and analogues (GnRHa) are also active ; DAla^6, des-Gly^{10}-LHRH induced 80% ovulation at doses of less than 1 µg/kg in *A. stellatus, A. gueldenstaedtii* and *H. huso* ; eggs were of the same quality as the controls injected pituitary preparations, but the latency of response was slightly delayed in *H. huso* (Goncharov *et al.*, 1991). However poor results were obtained in the sterlet *A. ruthenus*. Strangely further works used much higher doses : >100 µg/kg in bester (Mojazi Amiri *et al.*, 1999b) or 50 µg/kg in paddlefish *Polyodon spathula* (Linhart *et al.*, 2000). Recently Williot (2002) used successfully ([D-Phe^6]- NH_2) in *A. baerii* at the dose of 10 µg/kg in females and 5 µg in males in a single injection. Now GnRHa starts to be used in hatcheries.

Table 3: Reproductive performances in *A. gueldenstaedtii* given LHRH and sturgeon pituitary extracts at 2 temperatures (Barannikova *et al.*, 1989)

Exp.	°C	Hormone	Dose Per kg	n female	% maturation	% ovulation	% devel[3]	% mort.[4]	Weight Gr
1	19-20	LHRH[1]	13-17µg	15	87	67	69	19	3.4
		EHE[2]	1.5-4.5µg	13	85	61	73	28	3.1
2	11-12	LHRH	7-13µg	18	94	90	79	45	2.7
		EHH	1.5-4.5µg	12	100	75	71	43	2.7

1 - Luliberin Calbiochem Behrin administration in 2 injections at 5 h interval

2 - EHE: sturgeon pituitary extracts, gonadotrope activity in frog units (µg)

3 - % normal embryos

4 - % mortality larvae rearing in ponds

5 - weight of the fry at 31-39 days

The latency of response is 20-40 hours and depends on several parameters : temperature (Fig. 5), stage of maturation, age of the fish, nature of hormone, number of injections. Ovulation occurs after 33 hours after one injection with carp pituitary extracts and 31.6 hours with sturgeon extracts ; at 11°C the latency is close to 50 hours (Williot, 2002). Practically in hatchery the injection may take place between 9 and 11 am so that ovulation will occur early the next morning. During the period injection-ovulation temperature should stay constant and oxygen concentration, near saturation

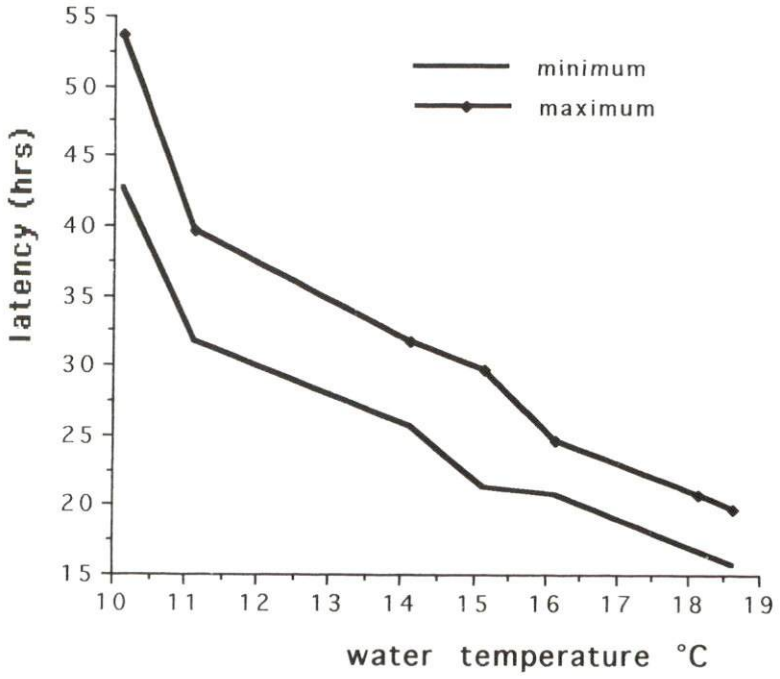

Figure 5 : Time between injection and ovulation in relation with water temperature in *A. stellatus* (N. Patriche pers. comm. 1999)

Milt and egg collection

Morphology of the genital tract in chondrostean :

The basic morphology of the genital tract is different in sturgeons by comparison with teleosts and the technology for gamete collection is sligthly different (Fig. 6). In chondrosteans males, sperm from the testis is transported to a

mesonephros via a rete testis and is mixed with urine in the Wolffian duct when it is released. For milt collection Tygon tubing 0.5-1 cm diameter; 5 cm long attached to a 10 ml syringe is introduced into the urogenital pore. With gentle suction milt is collected in the syringe. In sturgeon the spermatozoa are not activated by urine (cf. below). *A. baerii* semen can be collected over a period of few days after the gonadotrope treatment but for unknown reason, the best motility performances were recorded only after 2 days (Williot, 2002) (Fig. 7). A possible phenomenon of sperm maturation as shown in trout (Miura *et al.*, 1992) and carp (Barry *et al.*, 1989, 1990) exist in sturgeon and triggered by the hormone injection. After sampling milt can be stored in refrigerator in beaker over 2-3 days.

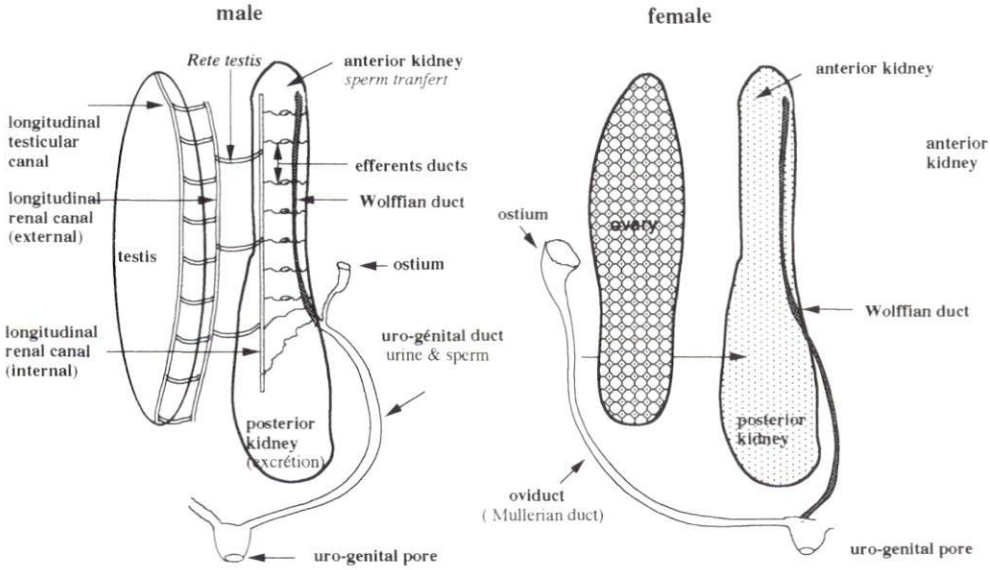

Figure 6: Schematic representation of the uro-genital organs in chondrostean

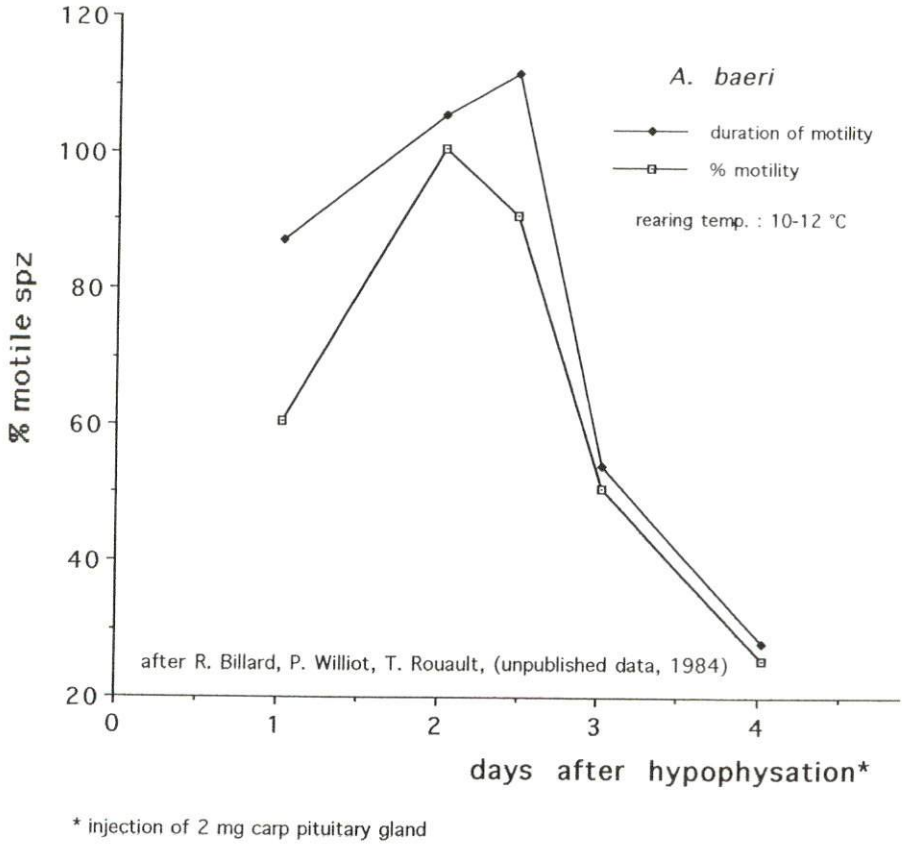

Figure 7: Changes in motility (% motile cells and duration of displacement) in *A. baerii*, rearing temperature 12 °C (R. Billard ; P. Williot and T. Rouault, 1984 unpublished).

In females the ovaries remain independent of kidneys and the Mullerian duct serve as an oviduct. In natural spawning the ova released in the body cavity are taken in this oviduct via an ostium and released at the level of the uro-genital papilla. In induced spawning mass ovulation occurs and all eggs cannot be stripped out via the ostium-oviduct system ; therefore surgical technologies are used (figure 8). Eggs free in the body cavity are collected by stripping via a micro caesarean section, 2-3 cm long, made just in front the anus (Conte *et al.*, 1988) or by a small incision (1-3 cm) in the posterior dorsal area of the oviduct (Stech *et al.*, 1999) (Fig. 8).

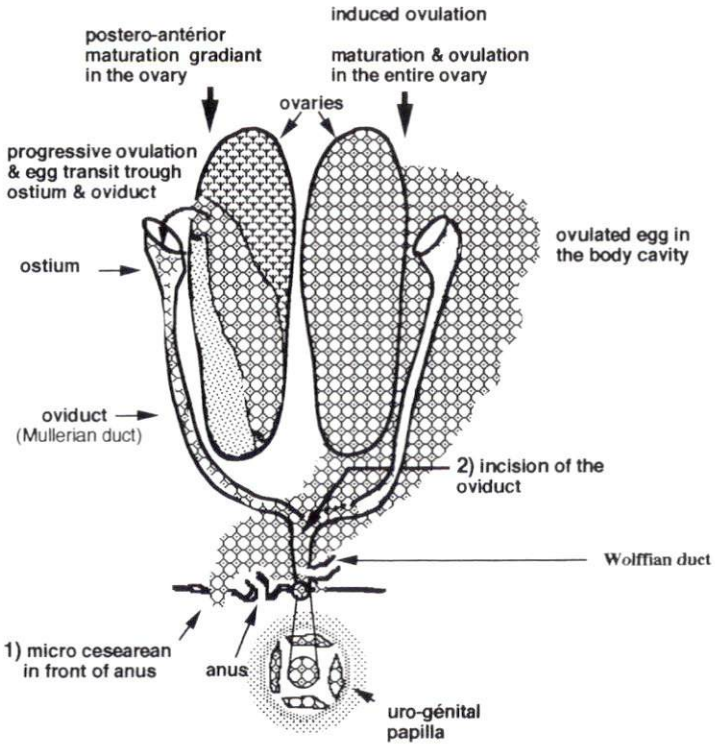

Figure 8 : Collection of ovulated eggs by microsurgery in sturgeons

Artificial insemination

Motility of spermatozoa:

Sturgeon spermatozoa are immotile in the genital tract due to the potassium content in the semen reaching 2.5 mM (and not, as in most teleosts, by the osmotic pressure of the seminal fluid which is low in sturgeon (38±3 milliosmoles /kg). The activation of motility occurs when sperm is diluted in water, which decreases K+ to a threshold level of 0.1 mM. The high K concentration (2.5 mM) is probably a mechanism to allow the mixing with urine without activation of motility as the dilution is not high enough. Motility should be checked before insemination. 1 µl of semen is mixed with 100 µl of water under the microscope stage and the % of motile cell and the duration of motility are measured (magnification X100). Semen with less than 90 % motility are discarded.

Fertilisation and egg treatment:

Ten ml of good quality semen (90 % motility) are mixed with 2 l of water and added to 1l of eggs in a dishpan and stirred with hands during 2 mn. This corresponds to the duration of motility (Fig. 9). If only poor quality sperm is available, it can be used provided that the volume is increased. The 1/200 dilution in water may look high ; the objective is to avoid a too high sperm concentration around the eggs and avoid phenomenon of polyspermy. To ensure a good mixing of gamete and restrict the losses in case of poor quality sperm or egg a good plan is shown in Fig. 10.

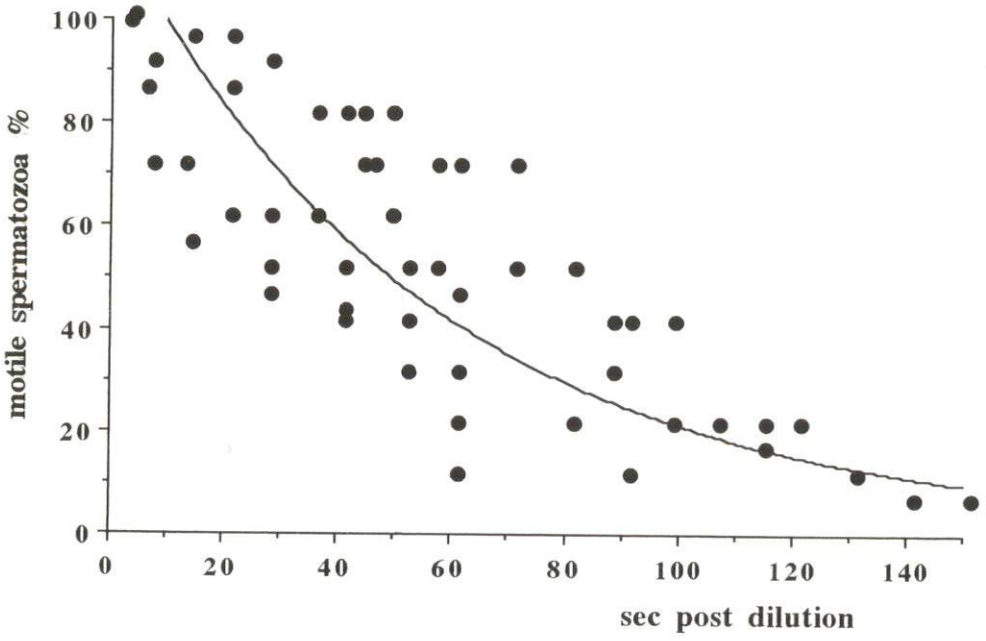

Figure 9 : Motility of *A. baerii* spermatozoa : changes with time of the % motile cells

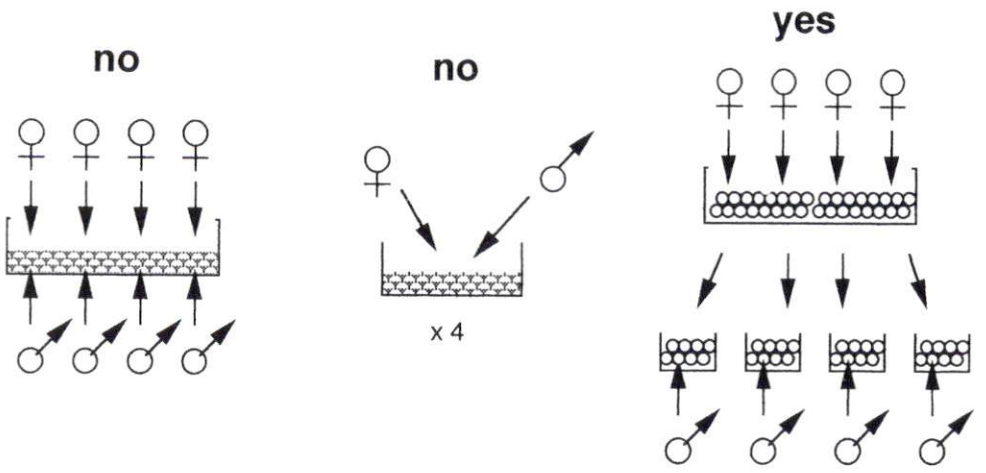

Figure 10: How to mix egg and sperm in the insemination procedure to keep a maximum of genetic variability and restrict the negative impact of poor quality gametes on the % of fertilization

To avoid the formation of the sticky layer the inseminated eggs are washed with water and treated during 1h with clay (benthonite) buffered at pH 7-8 (1l of egg in 5-7 l of water and 500g of clay). The eggs are rinsed several times and loaded in incubation jars (Zug, Mc Donald...) at temperature of 12 to 18°C, preferably 14°C (less development of fungus). The duration of incubation depends on the temperature and the species (Fig. 11), hatching takes place after 6-7 days of incubation at 16°C.

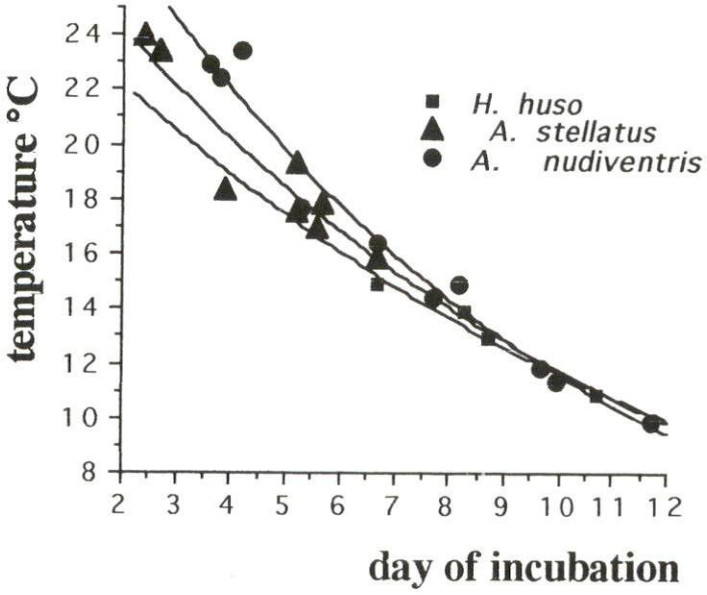

Figure 11: Changes in the time of incubation with temerature in 3 sturgeon species

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