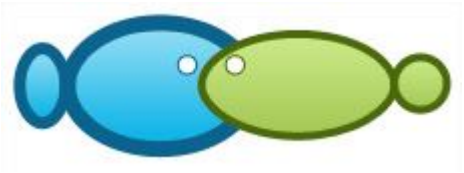

Techniques for *in vivo* extraction of gonads of male European catfish (*Silurus glanis*) for the artificial reproduction

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Abstract. Artificial reproduction of European catfish *Silurus glanis* was embarrassed by problems with stripping high-quality milt without contamination by urine. Analogical difficulties were noticed during milt stripping in male African catfish *Clarias gariepinus*. So fish-farmers sacrificed male catfishes for artificial propagation. Alternative method is non-lethal laparotomy for sperm collection. In this work we used surgical techniques for partial resection of testicular tissue in male catfish *in vivo* and a new method of urine descent before sperm selection. General anaesthesia was performed by immersion of fishes in tank with 25 L water with clove oil at the dose of 0.04 mL L^{-1}. Only a small incision (5-8 cm) was sufficient for ablation of part of testis. For the first time, separate sutures were applied to the peritoneum and skin for creating additional anastomoses which hold and fix internal organs. The regeneration of testis was noted. As alternative to surgical method we used catheterization of urine bladder before sperm collection. These methods are designed to keep alive male catfishes and to obtain high-quality milt without contamination by urine. The techniques of partial gonadectomy and urinary bladder catheterization could be used in farming and conservation aquaculture of European catfish and other silurids.

Key Words: European catfish, *Silurus glanis* L., artificial reproduction, bladder catheterization, laparotomy.

Introduction. The European catfish *Silurus glanis* is an important object of commercial and conservation aquaculture but as in all silurid fishes, males do not release semen under abdominal massage in captivity and sperm collection is practically impossible even after hormonal stimulation. The stripped volumes of milt are usually small and sperm is contaminated by urine causing spontaneous activation of spermatozoa motility (Linhart & Billard 1994; Viveiros et al 2002; Brzuska 2003; Viveiros 2003).

So farmers could not use stripped fluid for fertilizing mature eggs in European catfish (Szabó et al 2015). Analogical problems were noticed in African catfish *Clarias gariepinus*, Asian catfish *C. macrocephalus*, channel catfish *Ictalurus punctatus*, blue catfish *I. furcatus*, Indian catfish *Heteropneustes fossilis* because seminal vesicles actually block the sperm flow during hand-stripping. Some authors attempted stripping of milt from male African catfish after treatment by pituitary suspension or Ovaprim but milt was watery and bloody with no motile spermatozoa (Viveiros 2003; Diyaware et al 2010).

Fish farmers of Poland, Czech Republic, Hungary, Indonesia, Nigeria sacrificed male catfish to obtain sperm from the macerated testes for fertilizing female eggs. Though milt collection from male catfish after killing was effective for breeding purposes, it might bring shortage of males for further breeding. Sometimes farmers have to sacrifice 2-3 males before finding a specimen that has good milt despite the fact that the reddish genital papillae had been observed in all selected fishes. The further development of catfish farming needs in alternative technique of milt collection (Szabó et al 2015; Idahor et al 2018).

Some authors proposed non-lethal surgical methods for full (Sanap et al 2018) or partial (Diyaware et al 2010) resection of testicular tissue in male catfish using laparotomy. Gonadectomized fishes could be used for fattening and selling but not for

breeding. Partial removal of gonads with regeneration of testicular tissue could save males for re-utilization in next spawning period. The surgery could cause post-operational diseases, so constant control of testes regeneration is needed (Siwicki & Jeney 1985; Romanova et al 2017). The healing of wounds in silurids occurred sooner than in other fish orders (Guerra et al 2008).

To reduce stress during the surgical operation, fishes were dipped in the anaesthesia. The only anesthetic drug currently approved by the U.S. Food and Drug Administration (FDA) for use on food fish is tricaine methane sulfonate (MS-222). Other countries used different anesthetics: ethyl aminobenzoate (benzocaine), quinaldine, metomidate and etomidate, Aqui-S and other. At lower water temperatures, higher doses or longer exposure times are required with MS-222, benzocaine and 2-phenoxyethanol, because the absorption rate decreases at lower temperatures. The low pH of an anesthetic solution of some drugs, especially quinaldine can influence its efficacy (Coyle et al 2004). In Russia quinaldine, etomidate (Propiscin), benzocaine, lidocaine ($C_{14}H_{22}N_{2O}$), novocaine ($C_{13}H_{20}N_2O_2$) and clove oil are most popular anesthetics (Golovanova 2004; Zavyalova et al 2012; Chebanov & Galich 2013). At present three widely-used anesthetics worldwide are clove oil, MS-222 and 2-phenoxyethanol (Priborsky & Velisek 2018).

Clove oil is an effective anaesthesia in carp (*Cyprinus carpio*) at 40 to 120 mg L^{-1}. In rainbow trout *Oncorhynchus mykiss* doses of 40 to 60 mg L^{-1} for 3 to 6 minutes gave effective surgical anaesthesia. Clove oil has a very high margin of safety; however, it also requires a relatively long recovery time compared to MS-222. The major advantages of clove oil are low cost and absence of unpleasant odor. Clove oil is not approved for use on food fish in the U. S. (Coyle et al 2004) but is wide-spread in Russia (Podushka & Chebanov 2007; Mikodina et al 2010; Zavyalova et al 2012) and other countries (Hamackova et al 2006; Park et al 2011). The purpose of this work was to develop methods of obtaining sperm from males of European catfish in vivo for artificial reproduction.

Material and Method. The experiments were carried out in 2015-2017 year in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, ETS №123, Strasbourg, 1986. The experimental protocol was approved by the Ethics Committee of the Federal State-Funded Scientific Institution All-Russian Research Institute of Irrigation Fish-Breeding.

Milt collection in male European catfish *in vivo* was carried out using surgical and non-surgical methods. Surgical method is laparotomy with partial resection of testicular tissue. A standard kit of sterilized surgical instruments and aseptic techniques were used for operation. Non-surgical method included urinary bladder catheterization through genital papilla before sperm collection. The fertility of semen was valued using sperm smears stained by Pappenheim method.

The physiological and immune state of fishes was characterized with methods of hematological and immunological analyses. Samples of blood were taken from the caudal vein *in vivo*. The leukocytic formula (WBC differential) was performed by differential counting on a digital microscope Optika DM-15. Non-enzymatic lysosomal cationic protein in neutrophils from fish blood was determined by cytochemical method with bromphenol blue (Pronina 2017).

The tested blood cells were divided into four groups (0-3 balls) according to their phagocytic activity: 0 – no cationic protein granules, 1 – individual granules, 2 – granules occupy approximately one-third (1/3) of cytoplasm, 3 – granules occupy half (1/2) of cytoplasm or more.

The mean cytochemical coefficient (MCC) was calculated using the formula:

$$MCC = \frac{0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3}{100}$$

Where N_0, N_1, N_2, N_3 are numbers of neutrophils with 0, 1, 2 and 3 ball activity;

$$N_0 + N_1 + N_2 + N_3 = 100$$

Serum biochemistry was carried out on the Chem Well Awareness Technology analyzer, using Vital reagents.

The mathematical treatment of data was performed by statistical methods according to Student's test with the application of such software as Excel from the Microsoft Office package. The differences were considered significant at $p < 0.05$.

Results and Discussion. We carried out artificial breeding European catfish in commercial farm in Chuvash Republic of Russian Federation in spring of 2015 and 2017. The average weight of selected males was 2.20±0.14 kg, the age - 3 years.

Our new approach with minimal operational access included separate stitches of peritoneum and skin for creating additional anastomosis between internal organs and for preventing infections. Total anaestesia by clove oil (0.04 mL L^{-1}) was stopped as fish reduced pain threshold. Skin incision was followed by: 1) separation of skin from peritoneum, 2) peritoneum incision, 3) removal testicular tissue (Figure 1A), 4) suturing the wound creating intermittent eight-shaped stitches on the peritoneum (Figure 1B) and skin (Figure 1C).

The total operation time was 12-15 minutes. While partial removal of testis the care was taken that other organs were not damaged. The dissected males were stitched with the absorbable surgical thread. After surgery, the fish were immersed in a bath with water without anesthetic and they immediately began to swim. After visual control of physiological state of operated catfishes on the next day all specimens were released into fishpond for rehabilitation and growth.

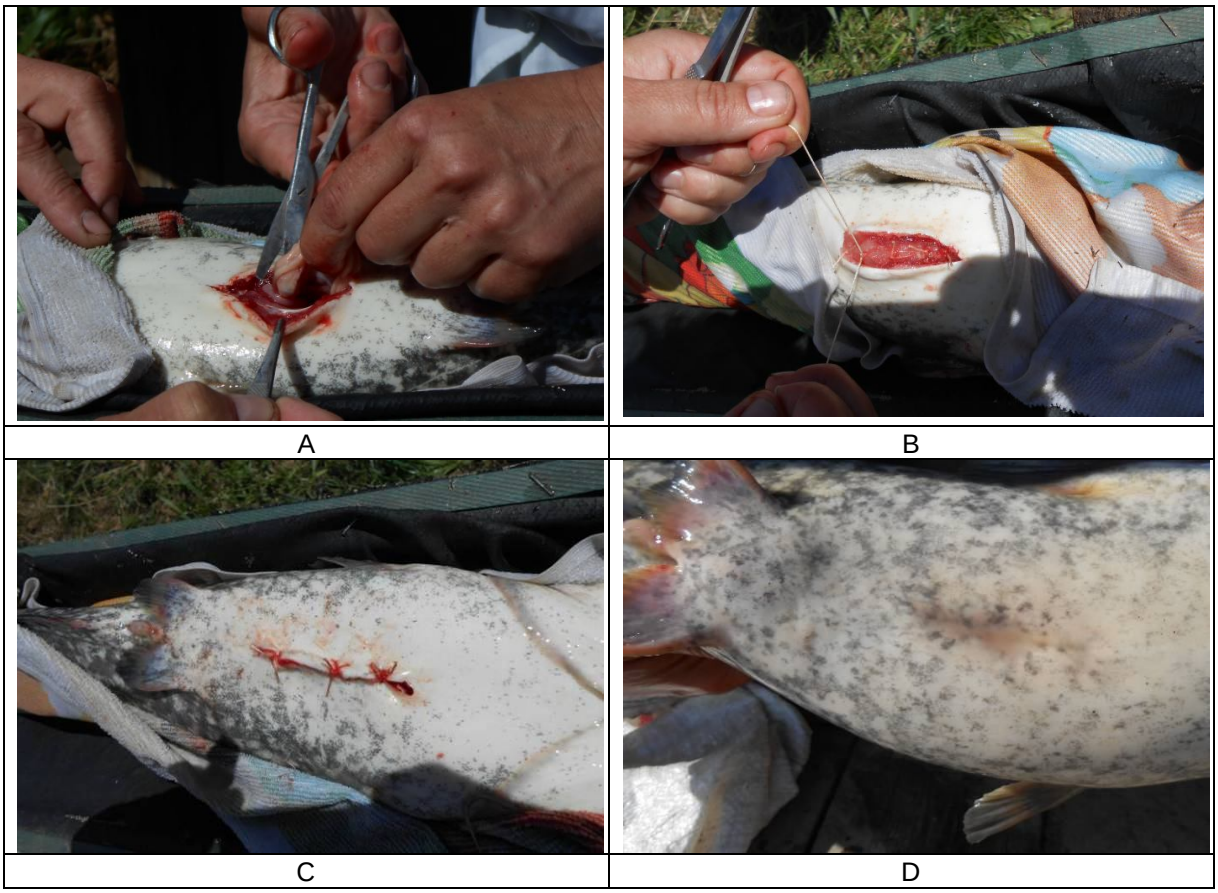

Figure 1. Elements of surgery: A - Getting the gonads of the catfish; B - Suturing of the peritoneum; C - Suturing of the skin; D - Hardly noticeable scar on the skin of operated fish (half year past operation).

Non-surgical method included bladder catheterization through urogenital papilla (Pronina et al 2017) before sperm collection (Figure 2). The sperm after catheterization of urinary bladder demonstrated high fertility in smears tests and could be used for fertilizing eggs.

Figure 2. Technique of urinary bladder catheterization through urogenital papilla before sperm collection in European catfish.

In six months after surgery (in autumn) monitoring of physiological state of operated specimens was carried out. All catfishes were caught and tested. The postsurgical survival was 100%, indicating the efficiency of the surgical procedure. The ventral skin on the place of dissection had a barely noticeable scar (Figure 1D). Operated fishes demonstrated fast growth. The average weight was 3.23±0.35 kg with gain more than 1 kg.

Physiological and immunological analyses (Table 1) showed that physiological parameters remained stable. These indices are in limit of physiological values for spring and fall seasons for 3-year European catfish (Pronina & Koryagina 2015).

Table 1

Hematological, cytochemical and biochemical parameters of investigated catfish

Parameters	*Before surgery (spring)*	*6 months later*
	WBC differential (%)	
Promyelocytes	-	0.3±0.2
Myelocytes	0.5±0.4	-
Metamyelocytes	3.1±0.9	1.8±0.4
Band neutrophils	0.7±1.1	2.5±0.5
Segmented neutrophils	4.3±0.8	6.4±0.7
Eosinophils	-	-
Basophils	0.3±0.4	0.2±0.2
Monocytes	3.3±1.8	4.1±1.2
Lymphocytes	87.8±2.2	84.7±0.9
	Cytochemical parameters	
MCC, units	1.28±0.14	1.82±0.03*
	Biochemical parameters	
ALT, U L^{-1}	44.8±4.4	35.5±4.5
AST, U L^{-1}	314±12.1	188.7±14.3*
Glucose, mmol L^{-1}	4.4±0.7	6.2±0.8
Total protein, g L^{-1}	38.7±4.2	27.6±1.3
Albumin, g dL^{-1}	16.6±0.8	17.1±0.8
ALP, U L^{-1}	29.0±17.9	10.9±2.5
Triglycerides, mg dL^{-1}	48.7±6.0	74.6±2.9*
Cholesterol, mg dL^{-1}	174.8±5.8	172.3±7.6

Note: * $p < 0.05$.

Significant differences were noted only in the lysosomal cation test (MCC), decreased activity of AST and accumulation of triglycerides in tissues but these changes we connected with fall adaptation to wintering.

One year after surgery (at next spawning season) the right and left testes practically did not differ in weight. The operated testis consisted of two parts with intermittent section at place of the ablation.

The comparison of sperm smears from control and operated testes demonstrated the similar state. The diameter of dead cells exceeded normal one in 2-3 times. The live germinal cells were dark but dead ones were pale pink. These features allowed easily differentiate live and dead spermatozoa. The percentage of live cells in operated testis was more than 75% (Figure 3) and did not differ from this index in control testis.

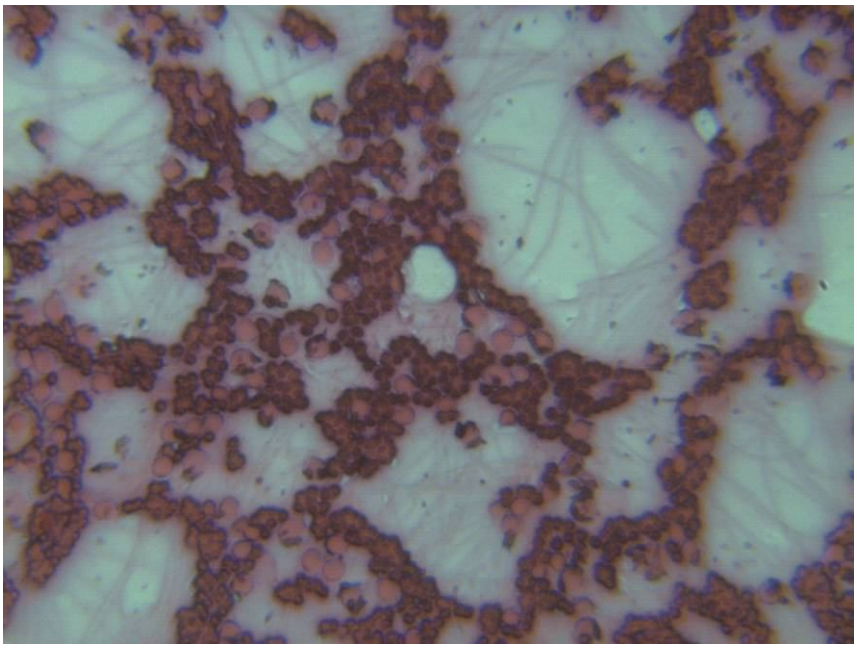

Figure 3. Microscopic picture of eosin-stained catfish sperm smear from operated testis at next spawning season. Magnification 400x.

These results suggested that partial gonadectomy could not alter the quality of sperm production in European catfish confirming data on African catfish (Guerra et al 2008; Diyaware et al 2010; Adebayo et al 2012). The sperm derived from regenerated testes performed effectively for fertilization of eggs. Hence the removal of part of testis could be recommended for semen collection in farming different species of Siluridae including European catfish.

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