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Novel method for induced propagation of fish: Sperm injection in oviducts and ovary/ovarian lavage with sperm

T. Müller[a,*], L. Horváth[a], T. Szabó[a], I. Ittzés[a], A. Bognár[b], P. Faidt[b], Á. Ittzés[a], B. Urbányi[a], B. Kucska[c]

[a] Department of Aquaculture, Faculty of Agricultural and Environmental Sciences, Szent István University, 2100 Gödöllő, Hungary
[b] BO-FA Fish Farm, 7252 Attala, Hungary
[c] Department of Aquaculture, Faculty of Agricultural and Environmental Sciences, Kaposvár University, 7400 Kaposvár, Hungary

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ABSTRACT

Motility of spermatozoa in most freshwater teleost species is suppressed by the osmolality of the seminal plasma in the testes, sperm ducts and initiated by a decrease of osmolality upon spawning in fresh water. We hypothesized that, similarly to internally fertilising fish species, spermatozoa of freshly stripped sperm, when injected directly into the ovary through the oviduct, will remain inactivated and maintain its fertilisation capacity for extended periods. Fertilisation is expected to occur after ovulated oocytes with spermatozoa on their surfaces are released into the water. To test this hypothesis, experiments were carried out on carp (*Cyprinus carpio*) as a model fish. Stripped and pooled sperm samples were injected by catheter into the ovarian cavity through the oviduct 2 h and 12 h before expected spawning. To prevent any loss of gametes the genital area of treated females were stitched by a standard protocol used in large-scale hatchery systems. At ovulation, eggs were stripped and fertilised with salt-carbamide solution. All sperm-injected females produced some fertilised eggs that developed normally while fertilisation rates varied considerably (24.2–81.2%). This proof of principle experiment demonstrates the potential for routine use as alternative to in vitro fertilisation for the propagation of fish species.

1. Introduction

One of the prerequisites for the domestication and establishment of sustainable aquaculture is the capacity to control reproductive processes of fish in captivity, and to acquire high quality gametes (i.e. eggs and sperm) for generation of the marketable product. In most cultured fish species, hormonal manipulations may be used as management tools to enhance the efficiency of egg production, increase spermiation and facilitate hatchery operations. In addition, hormonal therapies may be employed to induce gamete maturation and enable artificial collection in order to implement inter-specific hybridization, chromosome set manipulation or artificial fertilisation for genetic selection programmes (Mylonas et al., 2010).

In vitro fertilisation by induced breeding through hormone treatment followed by artificial fertilisation and incubation of fertilised eggs has several advantages (Woynárovich and Horváth, 1980) including better rates of fertilisation and hatching, protection against enemies and unfavourable environmental conditions, and better conditions for growth and survival. However, not all fish species ovulate in a predictable fashion. Several fish species including; striped bass *Morone saxatilis* (Kerby, 1986), white sturgeon *Acipenser transmontanus* (Conte et al., 1988), channel catfish *Ictalurus punctatus* (Phelps et al., 2007), pikeperch *Sander lucioperca* (Zarski et al., 2011), european eel *Anguilla anguilla* (Mordenti et al., 2014) respond best to final hormonal administration with high individual fluctuations due to different latency times of ovulation. Therefore, it is preferable to use induced spawning/captive spawning/natural-like method for such species. Egg fertilisation and hatching performance also exhibited better results than artificial insemination in Japanese and European eel (Tanaka, 2015; Di Biase et al., 2015) probably because the timing of spawning and fertilisation is optimized by the parent eels themselves (Okamura et al., 2013). The problem is that using induced spawning (for instance pikeperch) can reduce the diversity across the genome during long term culture as some parents contribute disproportionately to the next generation. This reduction of variation in the gene pool of stocks can in the culturing of the major Chinese carps; grass carp (*Ctenopharyngodon idella*), big head carp (*Hypophthalmichthys nobilis*), common carp (*Cyprinus carpio*), and silver carp (*Hypophthalmichthys molitrix*) in Asia. In these cases,

* Corresponding author.
E-mail address: Muller.Tamas@mkk.szie.hu (T. Müller).

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broodfish are returned to the pond, tank or hapa after hormonal injection and left to spawn naturally (NACA, 1989). The sex ratio of this spawning method is generally one female with two males, and again some parents produce large amount of juveniles.

Thus, it would better to develop a method, which can combine the simplicity of induced spawning with conventional in vitro fertilisation. To that end, we hypothesized that freshly stripped sperm samples might be injected into the ovary through the oviduct by using catheter and that the sperm thus delivered will maintain its fertilisation capacity and remain inactivated for extended periods. We also hypothesized that the release of ovulated oocytes (with spermatozoa attached to their surfaces) into water during natural or induced spawning and fertilisation would take place normally.

Our aim was to investigate if such internal delivery of sperm can lead to normally developing offspring and represent a technological alternative to other methods of induced propagation. The spermatozoa of all poecilids are produced in bundles, termed spermatozeugmata, and transferred to females using the gonopodium, a modified anal fin. Female poeciliids can store sperm in the folds of their ovaries and gonoducts (Constanz, 1989). In guppies, for example, stored sperm can continue to fertilise ova for up to 8 months (Winge, 1937), however recently inseminated sperm secures the highest rate of fertilisation (Constanz, 1984).

In order to deliver sperm directly to the folliculi inside the ovarian cavity a method is required which allows direct access to the ovary. Catheters are generally used in aquaculture for different types of internal manipulations without harming the female. For instance catheters are used for ovarian biopsy, when oocyte samples are taken to check the reproduction phase of females or to time final hormonal injections to provoke ovulation (e.g., Ohta et al., 1997; Zarski et al., 2011). Ovarian lavage by the use of catheters has successfully been applied to species of fish that do not respond to conventional hormonal injection, such as *Tetraodon nigroviridis*, *Mastacembelus erythrotaenia* (Watson et al., 2009a,b) and was also successfully trialled for inducing ova in pikeperch (Németh et al., 2012).

We used carp as model fish for our experiments. Common carp is not only an important domesticated fish but is ideally suitable for induced spawning experiments. There are several methods for controlled propagation including induction of maturation by treating broodstock with hormonal administration gonadotrophic hormones, hCG and GnRH analogues. Consequently, a fish farmer skilled in the artificial propagation of common carp will have no problem in adopting this technology to the propagation of other valuable carps of the region such as other Cyprinids (Horváth et al., 2015, Targońska et al., 2010, 2011, 2012, etc.). Reproductive biology including sperm management of carp males has been well studied. Carp spermatozoa can be stored in atmospheric air conditions and after 4 days, about 40% of common carp sperm remain motile (Kowalski et al., 2014). Osmolality of seminal plasma of carp ranged 254–346 mOsmol kg^{-1} (summarised data from Alavi and Cosson, 2006) is practically no different from osmolality of ovarian fluid (290 mOsmol kg^{-1}) and the ovarian fluid of common carp does not activate carp spermatozoa motility (Horváth et al., 2010).

In summary, there are no reported biological and technical barrier factors that may hinder the introduction of fertile semen directly to ovaries those fish species where the ovarian fluid does not activate the spermatozoa motility.

2. Material and methods

2.1. Selection and maintenance of spawners

Experiment 1 was carried out at a private fish farm in Nagykarácsony, Hungary. Koi carps, aged three to four years, were used as broodstock (n = 18) reared in an outdoor pond system and fed by commercial pellet. Females were selected randomly and 3 groups each were maintained in 3000 L tanks. The water temperature when the fish were introduced to the tank was 17 °C. The photoperiod used was 16:8 h light:dark cycle maintained constantly throughout the study period. Males and females were sorted and kept separately after arrival at the facility. Before all manipulations, fish were anaesthetised in a solution of 100 mg L^{-1} benzocaine (ethyl 4-aminobenzoate, Norcaine) for subsequent handling.

Groups:

G1 (control). The steps of method of induced ovulation were applied according to described by Horváth et al., 2015 (n = 6).

G2. Sperm samples were injected into the ovary cavity about 2 h before stripping (n = 6).

G3. Sperm samples were injected into the ovary cavity about 12 h before stripping (n = 6).

Experiment 2 was carried out in a hatchery system of a carp producing fish farm (Attala Fish Farm Ltd., 7252 Attala, Hungary), 10–15 years old carp stock was used for the experiment (G12). The broodstock was reared in a pond and fed by natural food and commercial pellet. Females were selected randomly from broodstock and 5 individuals were kept in 10.000 L tank. The water temperature was 17 °C at the time when the fish were introduced to the tank. The photoperiod was constant throughout the study period with 16:8 h light:-dark cycle. Males and females were sorted and kept separately after arrival at the facility. Before all manipulations, the fish were anaesthetised in a solution of 20 drops 10 L^{-1} clove oil (*Syzygium aromaticum*) for subsequent handling.

2.2. Experimental conditions and animal welfare

All key environmental conditions were kept constant during the experiments (Table 1). Temperature, dissolved oxygen content, and conductivity were monitored by a dual–input portable multimeter INSA MDF 79 (INSA, s.r.o., Czech Republic). Other water quality parameters were determined by a compact photometer PF–12 NANOCOLOR® tube tests (Macherey–Nagel GmbH & Co. KG, Germany).

Table 1
The water quality parameters (mean ± SD) during the experiments.

	First experiment			Second experiment		
	9.00 h (At priming inj.)	22.20 h (At resolving inj.)	11:00 h (Fertilisation tests)	9.00 h (At priming inj.)	22.00 h (At resolving inj.)	11.45 h (Fertilisation tests)
Temperature [°C]	17.1–17.2	18.4–18.8	20.5–20.7	18.5	18.7	21.1
Dissolved oxygen [mg·L^{-1}]	–	7.03–8.45	7.00–7.91	–	7.3	–
Conductivity [μS·cm^{-1}]	–	740–770	–	–	559	–
pH	–	7.85–8.14	7.93–8.35	–	8.38	–
NO_3^- [mg·L^{-1}]	–	17.2–20.5	32.2–38.3	–	< 0.6	< 0.6
N-NH_4 [mg·L^{-1}]	–	< 0.02	< 0.02	–	< 0.02	< 0.02
N-NO_2 [mg·L^{-1}]	–	< 0.02	< 0.02	–	0.06	0.07
P-PO4 [mg·L^{-1}]	–	< 0.6	< 0.6	–	< 0.6	< 0.6
Total hardness [°d]	–	> 25	–	–	> 25	–