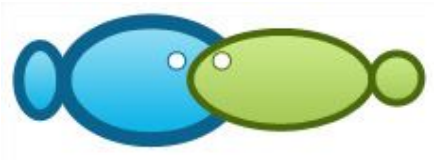

Ovarian lavage methods of fish propagation: a mini review on sperm artificial insemination and/or hormone delivery into the ovary

[1,2]Getachew W. Alebachew, [2]Nguyen N. Quyen, [3]Béla Urbányi, [3]László Horváth

[1] Sirinka Agricultural Research Center, P.O. Box 74, Woldia, Ethiopia; [2] Department of Freshwater Fish Ecology, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary; [3] Department of Aquaculture, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary. Corresponding authors: G. W. Alebachew, getaworkalebie@gmail.com; N. N. Quyen, quyenaqua@gmail.com

Abstract. Recently, the demand for fish source protein has been in increasing manner. The natural water fish seed sourcing is time overwhelming, inadequate, erratic, and an uneconomic way for the fish production system. Instead, *in vitro* (artificial) fertilization methods were used since long time ago. However, these methods have weaknesses including complications to the forecast of ovulation time, which affects on egg quality or cause stress on fish. Several fish do not lend themselves to hormonal injection due to their armored skin, small size, or inability to handle the stress involved. To overcome those problems new ovarian lavage sperm injection method has been tried. Similarly, this method is used for hormone delivery to induce ovulation, or delivery of sperm-hormone mix to induce ovulation, and at the same time to make fertilization happen in live-bearing fish species or after water activation in external fertilization fish species. A small-sized catheter tube coupled with a syringe is used to inject sperm and/or hormone into the ovary. Hence, this mini review aimed to address ovarian lavage sperm and/or hormones injection methods, application, importance, and impact on some reproductive traits on different fish species. Ovarian lavage hormonal induction is important to small fish species and removes the use of needles which produces injuries. Similarly, this application is important for artificial insemination to produce viable offspring either in the internal or external fertilization of fish species. Moreover, it is used to create hybrid fish that the two parental species do not ordinarily mate under natural conditions.

Key Words: artificial insemination, external fertilization, hormone, live-bearing, ovarian lavage, propagation, sperm.

Introduction. Population growth and searching for a healthy diet have increased the demand for fish meat (Hoga et al 2018). To attain the rising demand, the creation of sustainable aquaculture industries is one of the prerequisites. But the natural water fish seeds source is most erratic, inadequate, time overwhelming, and uneconomical; and creates the main limitation to the progress of intensive fish farming (Satia 1990; Fagbenro et al 1993; Adebayo & Fagbenro 2004). Controlling the reproductive processes of fish in captivity and collecting extraordinary top seeds for the grow-out of the marketable product is a solution (Ittzés et al 2020). Hence, technologies like artificial insemination and the use of hormones have significant importance. In an ovoviviparous fish (fertilization occurs externally), the use of very simple artificial insemination techniques results in original gamete physiological maturity after being diluted in the external medium (Bellard 1988).

Furthermore, the involvement of exogenous hormones can alter environmental factors and manage the reproduction time to outfit the production cycles (Woynarovich & Horvath 1980). Induction of ovulation of ready-to-spawned fish is properly set through the application of exogenous hormones (Lam 1982). The application of different hormones has been tried in different studies, for instance, ovaprim, ovopel (a mixture of dopamine receptor antagonist and mammalian GnRH analogue), and the two kinds of

gonadotropin (fish pituitary extract and human chorionic gonadotropin), and suprefact (Pontongkam & Miller 2006) to substitute the external environmental influences that cause reproduction in fish.

In numerous conditions (like a breeding program and intra-species and extra-species hybridization) external fertilization is an important method and is the only practicable approach used for the manipulation of sperm (genetically modified or cryopreserved). However, *in vitro* fertilization after stripping has numerous drawbacks such as (i) the ovulation time estimation after hormonal treatment is difficult, since eggs become overripe after ovulation and lose their fertilization potential (Cabrita et al 2009; Mylonas et al 2010, 2017); (ii) during long-term culture using inducing spawning can reduce the genetic diversity as some parents contribute more for the next generation (Cheng 1989); (iii) labor-intensive and inconsistent hybrid seed production (Perera et al 2017); (iv) in some species such as blue catfish (*Ictalurus furcatus*) males need to be sacrificed to collect the milt (Bart & Dunham 1996; Dunham & Argue 2000; Dunham et al 2000; Argue et al 2003; Hu et al 2011; Myers et al 2020a, b), because of its anatomy and reproductive physiology, it is difficult to strip sperm (Bart & Dunham 1996; Chereguini et al 1999), and (v) striping the egg from the ovary needs gentle work procedure, and is prone to contamination with urine and water that leads to premature activation of gamete (Dunham & Masser 2012), (vi) hormone injections can be stressful for small, delicate species (Watson et al 2009a, b).

Hence, varieties of hormones and sperm application methods and injection processes have been developed, from which, an ovarian lavage sperm insemination method is an alternative protocol of sperm and hormone application. Therefore, ovarian lavage with sperm or/and hormones injection methods are aimed to be addressed in this min-review.

History of artificial insemination in fish. The process by which sperm is collected from the male, processed, stored, and artificially hosted in the female reproductive tract for conception is known as artificial insemination (Webb 1992; McGovern-Hopkins et al 2003). In farm animals, tremendous artificial insemination success has been recorded, for which protocols are well established (Perry 1968). Methods for the external artificial fertilization of a variety of invertebrate eggs and those of several oviparous poikilothermic vertebrates have been widely employed by experimental embryologists, pisciculturists, etc. However, the artificial insemination of viviparous cold-blooded vertebrates (or poikilothermic vertebrates which lay fertilized eggs) has never been tried before the 50th (Clark 1950). Artificial insemination in ovoviviparous species with internal fertilization such as *Xiphophorus* (Samokvalova 1938; Clark 1950), and *Poecilia* (Billard 1966; Lodi 1979) has been practiced.

The first study about the ovarian lavage technique that used a catheter tube to inject a hormone into the ovarian lobe was reported by Watson et al (2009a, b). There are several publications to use this method in a variety of fish species with external fertilization such as spotted green pufferfish (*Tetraodon nigroviridis*) (Watson et al 2009a, 2009b), *Mastacembelus erythrotaenia* (Watson et al 2009b), pikeperch (*Sander lucioperca*) (Nemeth et al 2012), carp (*Cyprinus carpio*) (Müller et al 2018a), sperm and hormone mixture or hormone alone in *Clarias gariepinus* (Müller et al 2018b), cryopreserved sperm of *Clarias gariepinus* (Müller et al 2019), sperm and hormone mixture, or hormone alone in jundia *Rhamdia quelen* (Müller et al 2018b; Ittzés et al 2020), and in *Danio rerio* for ex-situ and in-situ conservation (Gazsi et al 2021).

Sperm collection in male fish and quality parameters. In the beginning, the male fish should be enhanced to spermiation using artificial hormones (Mañanos et al 2008; Mylonas et al 2017; Gallego & Asturiano 2018), such as gonadotropin-releasing hormone analog (GnRHa) (Zohar & Mylonas 2001; Mylonas et al 2010; Piamsomboon et al 2019), fish pituitary extracts, HCG hormone, gonadotropin hormone (GTH), luteinizing hormone-releasing hormone (LHRH) and LHRH agonists (LHRHa), gonadotropin-releasing hormone (GnRH), Ovatide, Dagin, Ovaryprim, Ovaprim, Ovopel, Ovupin-L, Ovulin and Aquaspawn (Olufeagba et al 2016; Zidan et al 2020). In some fish species such as African catfish (*C.*

gariepinus) only environmental manipulation (such as water temperature regulation) may be enough to induce maturation without the use of the artificial hormone (El Naggar et al 2006; Mylonas et al 2010).

After artificial hormone induction or natural maturation, the sperm is collected either by gentle hand-stripping using slight abdominal pressure (Haddy & Pankhurst 2000), or by surgical removal of the testis from the fish body and then collecting the milt by squeezing it (Bart & Dunham 1996; Dunham et al 2000; Viveiros 2002; Ataguba et al 2010) or crushing dissected testis (Yang et al 2007). Similarly, in catfish species, there are several possible methods of sperm collection such as hand-stripping, and surgical removal of the testis (Haddy & Pankhurst 2000; Bart & Dunham 1996; Dunham et al 2000; Viveiros 2002; Ataguba et al 2010, Yang et al 2007). To keep an aseptic condition, moisture from the exterior of the fish should be removed using absorbent paper (Watson et al 2009a). Before using the fresh sperm or cryopreserved sperm, it should be assessed for quality traits. Sperm quality traits such as sperm counts could be assessed by a numeration of the total quantity of sperm in the sample by spectrophotometer using a standard curve or a standard hemocytometer (Luer et al 2007; Mañanos et al 2008; Evans et al 2017). In addition, sperm viability can be measured using a fluorescence live/dead sperm assay (Invitrogen, Molecular Probes) (Evans et al 2017), or by induction of the acrosome reaction (Mañanos et al 2008).

Furthermore, the use of sperm viability kits, combining the SYBR Green and propidium iodide (PI) stains, has become popular in fish research, because it allows the classification of spermatozoa as dead when nuclei show red fluorescence over the spermatozoa head and as alive when they show green fluorescence (Mylonas et al 2017). Additionally, sperm velocity is another quality parameter that is estimated through the computer-assisted sperm analysis (CASA) method. Of the measures that could be recorded by CASA, *a priori* selection on the speed at which sperm swam along their recorded trajectory curvilinear velocity was determined (Evans et al 2017). Besides, the CASA systems are the progression of several photomicrography revelations and video-micrography methods for spermatozoa track, demonstrating an objective, sensitive and precise procedure for procurement of sperm kinetic features. Most of the parameters measured by using CASA systems have been positively correlated with spermatozoa fertilization potential; consequently, CASA is a very useful device for evaluating sperm quality in fish reproduction research (Gallego & Asturiano 2018).

Insemination procedures. Ovarian lavage insemination method using catheters (Silastic tube attached syringe) has successfully been applied to species of fish that had small body size, armored skin, or inability to handle the stress involved respond to conventional hormonal injection such as *Tetraodon nigroviridis* and *Mastacembelus erythrotaenia* (Watson et al 2009a, b). In case of clearnose skate (*Raja eglanteria*) the sperm mixture was introduced into the female ovary using a syringe and a 28-gauge needle extended with PE 10 polyethylene tubing. The sperm mixture was directed into the right and left uterine horns or the common cloacal receptacle. Eggs laid after this procedure can be monitored for fertility (Luer et al 2007).

In case of sperm, the insemination method pooled sperm samples (spermatozoa with seminal fluid) were injected into the ovarian cavity of females through the genital papilla. Injections were carried out using a silicone catheter tube (feeding tube, 400 mm length, size: CH, outer diameter 1.3 mm, inner diameter 1 mm, Galmed, Poland) coupled with a syringe, and introduced directly into both the left and right ovary by inserting 5-15 cm deep into the oviduct (depending on the size of the fish) via the genital opening (Müller et al 2018a, 2018b, 2019, 2020a, 2020b; Ittzés et al 2020). Gazsi et al (2021) used a glass capillary which was inserted approximately 2 mm deep into the oviduct of *Danio rerio* through the genital papilla of anesthetized females using an automated pipette, sperm samples were injected into the center of the genital papillae, i.e., sperm distribution was not directed into the two oviducts (random distribution), and then females were put back into the spawning tanks.

Sperm artificial insemination in external fertilization fish species. Most freshwater teleost fish species spermatozoa motility is repressed by the osmolality of the seminal plasma in the testes and sperm ducts, and initiated by a reduction of osmolality upon spawning in freshwater (Müller et al 2018a). In carp fish, delivering sperm to the egg through the oviduct into the ovary leads to the effective rescue of developing embryos. This suggests that sperm cells are deposited inside the ovary and oviduct for up to 12 hours with their biological capacity and it could fertilize the released egg from the ovary. At the same time, the fish were treated in two steps: abdominal injection for hormone induction; and providing sperm cells into the ovary by catheter tube (Müller et al 2018a, b).

The improved method (artificial insemination) can combine the simplicity of induced spawning, with a less time-dependent delivery of sperm than with conventional, *in vitro* fertilization. In the field of aquaculture management, the importance of ovarian lavage with sperm and hormone preparations could also be known where it is advantageous to increase genetic diversity. For example, in induced spawning of paired spawning fish species (such as *Sander lucioperca*, *Silurus glanis*, *Umbra krameri*, etc.) sperm samples from 5-10 different males can be used for fertilization (Müller et al 2018b). Regardless of the spermatozoa reaching the ovaries, water activation (external fertilization) is always required to fertilize the egg batches (Müller et al 2018b, 2019; Gazsi et al 2021). Table 1 indicates data about fertilization by using the sperm insemination method on varieties of fish species. Furthermore, a study by Müller et al (2019) states that cryopreserved sperm inseminated into the ovarian lavage results in 17.7±13.2 of the mean hatching rate ± standard deviation, which is significantly lower than the external fertilization method.

Table 1

The impact of artificial insemination method on mean fertilization rate ± SD in external fertilization fish species

Species	*Experimental group*	*Control group*	*References*
Danio rerio	Sperm insemination spawning III (42.9±27.5)[a] Sperm insemination spawning IV (41.5±23.7)[a]	Traditional spawning I (21.7±30.7)[b] Traditional spawning II (43.3±29.4)[a]	Gazsi et al (2021)
Clarias gariepinus	Post-thaw of cryopreserved sperm (23.5±16.1)[b] Post-thaw of cryopreserved + additional fresh sperm (17.6±13.7)[b]	Fresh sperm dry fertilization (71±14.4)[a]	Müller et al (2019)
Cyprinus carpio	Sperm samples were injected into the ovary cavity about 2 h before stripping (46.7±15)[b] Sperm samples were injected into the ovary cavity about 12 h before stripping (41±15.7)[b]	Traditional dry method (85.8±4.2)[a]	Müller et al (2018a)
Rhamdia quelen	Sperm injection and hormone treatment separately (82.1±9.4)[ns] Sperm + CPE hormone mix ovarian lavage (76.5±4.4)[ns]	Traditional dry method (75.6±9.3)[ns]	Ittzés et al (2020)
Clarias gariepinus	CPE hormone + sperm into the ovary (74.7±18.4)[ns]	CPE hormone + sperm into the ovary plus fresh sperm for dry fertilization (75.1±11.5)[ns]	Müller et al (2018b)

Note: Different letters in the superscript on the same row indicate significant differences ($p < 0.05$) within the experimental groups; 'ns' indicates a non-significantly difference between the experimental and control groups; CPE - carp pituitary extract.

Sperm artificial insemination in live bearing (internal fertilization) fish species. Live-bearing (i.e., viviparous) fishes reproduce by internal fertilization (Yang et al 2007). Artificial insemination has been practiced in live-bearing fish species such as in *Raja eglanteria* (Luer et al 2007), a cross between *Xiphophorus* species (Yang et al 2007, 2012a, 2012b), *Poecilia reticulata* (Liu 2018), and molly (*Poecilia latipinna*) and *Xenotoca eiseni* (Huang et al 2009). Comparatively, viviparous fish species are smaller in body size than the oviparous fish species that use external fertilization (Yang et al 2007). Hence, the insemination procedure is held with a very small insemination silicon tube connected with a gel-loading pipette tip at one end (Yang et al 2007, 2012a). Table 2 indicates some artificial insemination experiment results in some important reproduction traits in live-bearing fish species.

Table 2

The impact of artificial insemination on some reproduction traits in live bearing (internal fertilization) fish species

Species	*Experiments and results gain*	*References*
Raja eglanteria	Sperm into the common cloacal receptacle of female A (8 pairs of fertile eggs resulted) Sperm into the right uterine horn of female B (17 pairs of fertile eggs resulted) Sperm into the left uterine horn of female C (no fertile eggs resulted)	Luer et al (2007)
Xiphophorus variatus	*Xiphophorus variatus* fresh sperm (1 female and 1 male offspring produced)	Yang et al (2012a)
Xiphophorus hellerii	*Xiphophorus variatus* cryopreserved sperm (4 female and 3 male offspring produced)	
Xiphophorus variatus	*Xiphophorus variatus* cryopreserved sperm (1 male offspring produced)	
Xiphophorus sp.	Cryopreserved sperm insemination (10% fertilization rate (fertilized/all-female))	Liu (2018)
Poecilia reticulata	Cryopreserved sperm insemination (50% fertilization rate (fertilized/all-female))	
Xenotoca eiseni	Cryopreserved sperm insemination (10% fertilization rate (fertilized/all-female))	
Poecilia reticulata	Cryopreserved sperm insemination (50% fertilization rate (fertilized/all-female))	Huang et al (2009)
Poecilia latipinna	Cryopreserved sperm insemination (20% fertilization rate (fertilized/all-female))	
Poecilia reticulata	In 1st brood females produced mean±SD of 5.93±2.79 babies (range 1-11)[a] In 2nd brood females produced mean±SD of 7.96±2.76 babies (range 3-15)[b]	Gasparini et al (2018)
Xiphophorus helleri	*X. maculatus* cryopreserved sperm inseminated group 1 (5 offspring) *X. maculatus* cryopreserved sperm inseminated group 2 (6 offspring) *X. maculatus* cryopreserved sperm inseminated group 3 (1 offspring) *X. maculatus* cryopreserved sperm inseminated group 4 (5 offspring)	Yang et al (2012b)
Xiphophorus maculatus	*X. helleri* fresh sperm inseminated (8 female offspring) *X. helleri* cryopreserved sperm inseminated (18 female offspring)	Yang et al (2007)

Note: Different letters in the superscript on the same row indicate significant differences ($p < 0.05$) within the experimental groups.

Creation of hybridization by artificial insemination. It is hoped that this method of artificial insemination may help facilitate genetic studies, particularly where the breeding of certain strains and hybrid combinations by natural methods has not been successful (Clark 1950). Hence, *Xiphophurus helleri* and *Platypoecilus maculatus* were successfully produced hybrid offspring (Samokvalova 1938; Clark 1950). Artificial insemination in fish to produce hybridization is usually conducted in situations where the male of the species cannot or should not be involved in the natural mating process. Researchers working with live-bearing fishes use the artificial insemination technique to crossbreed different species that normally would not mate under natural conditions (McGovern-Hopkins et al 2003).

A recent study result indicated that the artificial hybridization of external fertilization fish species between *Clarias gariepinus* ♀ × *Heterobranchus longifilis* ♂ by using sperm insemination followed by in tank/cage spawning with *Clarias gariepinus* ♂ method was successfully produced 98.8% of hybrid offspring; this result was far greater than the *in vitro* dry fertilization control group, which was 53.8% of hybridization (Alebachew 2021).

Application of hormone or sperm hormone mix into the ovary. Hormone administration for induction of ovulation is based on intramuscular or intraperitoneal injection in broodstocks. A syringe and needle were used. Injections using a needle, however, have a drawback that leads to the cause of injuries and stress for small refined fish species or is highly labor-intensive for species with low fecundity (Watson et al 2009a). In larger body size fish species, the seminal fluid was an appropriate delivery vehicle for the ovulation-inducing carp pituitary hormone via ovarian lavage (Müller et al 2018b). Additionally, the study on pikeperch shows that the non-invasive ovarian lavage method of hormonal injection is suitable for the induction of ovulation as the traditionally used intramuscular injection of hormonal products (Nemeth et al 2012).

In African catfish and in jundia, suspended sperm cells with carp pituitary hormone are deposited in the ovary lobes for up to 10 h without losing the sperm biological capability and it could fertilize the released egg from the ovary. Additionally, the seminal fluid was a suitable delivery vehicle for the ovulation-inducing carp pituitary hormone via ovarian lavage (Müller et al 2018b; Ittzés et al 2020). Table 3 indicates that an ovarian lavage hormone injection with or without additional sperm has recorded successful ovulation stories in some fish species.

Table 3

The effect of hormone applications into the ovary on PGSI and ovulation

Species	*Hormones*	*Mean PGSI±SD*	*Ovulation in % (no. successful ovulation/all observations)*	*Latency time (hrs)*	*References*
Tetraodon nigroviridis	hCG	17.73±7.9	100 (3/3)	36	Watson et al (2009a)
Clarias gariepinus	CPE + sperm	9.4±2.3	100 (7/7)	10-11	Müller et al (2018b)
Sander lucioperca	CPE	7.3±2.8	100 (10/10)	79.8	Nemeth et al (2012)
Siluriformes: Heptapteridae	CPE + sperm	10.2±5.4	71.4 (5/7)	10	Ittzés et al (2020)

Note: hCG - human chorionic gonadotropin; PGSI - pseudo-gonadal somatic index; CPE - carp pituitary extract.

Conclusions and prospectives. The ovarian lavage fish propagation is an important protocol in both live-bearing and external fertilization fish species. As an alternative to the *in-vitro* fertilization method, it is possible to use ovarian lavage for the sperm application to produce fertilized eggs after water activation in the external fertilization fish species. In live-bearing (internal fertilization) fish species, it is also an artificial insemination method to produce viable offspring. Furthermore, the ovarian lavage fish

propagation method allows hormone application directly into the female genitalia opening using a catheter tube, but without the use of injection which leads to injuries and stress conditions to the fish. Finally, the protocol is significant to produce hybrid offspring between different related fish species which are not natural mates. Nevertheless, the ovarian lavage method is still new, especially in the production of hybrid offspring from the external fertilization fish species, then it is important to experiment with different fish species such as in the hybridization between the American catfish, blue catfish (*Ictalurus furcatus*), and channel catfish (*Ictalurus punctatus*), and more other species.

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Authors:
Getachew Worku Alebachew, Sirinka Agricultural Research Center, P.O. Box 74, Woldia, Ethiopia, e-mail: getaworkalebie@gmail.com
Nguyen Ngoc Quyen, Department of Freshwater Fish Ecology, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary, e-mail: quyenaqua@gmail.com
Béla Urbányi, Department of Aquaculture, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary, e-mail: Urbanyi.Bela@uni-mate.hu
László Horváth, Department of Aquaculture, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary, e-mail: horvath.laszlo.matyas@gmail.com

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