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Ovarian inseminated sperm impacts spawning success in zebrafish, *Danio rerio* (Hamilton, 1822) even in the absence of a male stimulus

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ABSTRACT

Reproductive obstacles have led scientists to develop novel techniques/technologies for artificial reproduction. We aimed to investigate the possibility of propagating zebrafish females using sperm ovarian lavage with and without presence of male stimulus. This experiment consisted of several treatments: traditional spawning approaches with females and wild-type males (AB♀ × AB♂); no males present with non-manipulated females (AB♀); no males present with females inseminated with NaCl into ovarian lobes [AB♀(inj.NaCl)]; no males present with females inseminated with sperm from transgenic males into ovarian lobes [AB♀(inj.Tg♂)]; non-manipulated females kept separately from wild-type males (AB♀|AB♂); females kept separately from wild-type males and inseminated with NaCl into ovarian lobes [AB♀(inj.NaCl)|AB♂]; and females kept separately from wild-type males and inseminated with sperm from transgenic males into ovarian lobes [AB♀(inj.Tg♂)|AB♂]. There were no released eggs in both negative control treatments (AB♀ and AB♀|AB♂). Egg production increased (ranged from 0 to 28.5 eggs/female) when females were injected in the presence [AB♀ (inj.NaCl) |AB♂] or absence of male stimulus [AB♀ (inj.NaCl) and (AB♀(inj.Tg♂)]. A further increase in egg production [relative to AB♀, AB♀ (inj.NaCl), and AB♀|AB♂] was detected when females were inseminated with pooled sperm from transgenic males in the presence of male stimulus [AB♀(inj.Tg♂)|AB♂; ranged from 2.5 to 55 eggs/female] or when using traditional spawning approaches (AB♀ × AB♂; ranged from 25 to 131 eggs/female). Females inseminated with sperm produced embryos, although no differences were detected when females were inseminated with pooled sperm from transgenic males in presence (11.8 ± 16.3%) or absence (average = 12.6 ± 9.2%) of male stimulus. Traditional spawning approaches produced the most eggs (81.2 ± 42.3 per female) and highest fertilization rate (81.3 ± 10.4).

1. Introduction

Fish are the largest group of vertebrates (~33,500 species; www.fishbase.org) and inhabit almost every aquatic environment on planet Earth. With the exception of hermaphrodite fish species, the majority of sperm and eggs develop separately within male and female gonads [1,2]. Fish ontogeny then begins with fertilization leading to and resulting in the fusion of the nuclei of the male and female gametes to form a diploid zygote [2,3]. Fertilization can occur either internally or externally of the female reproductive tract. External fertilization is by far the most common reproductive strategy in teleosts where eggs are ovulated from the ovarian follicles into the ovarian lumen or peritoneal cavity, usually following completion of the first meiotic division [4]. Once the mature egg has attained the metaphase of the second maturation (meiotic) division, it is released into the external aquatic environment and

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subsequently fertilized [1,2]. However, the external aquatic environment can be very hostile for gametes, and thus, the lifespan of activated sperm is usually short following contact with water, ranging from a few seconds to several minutes for the majority of fish species [5]. This limits sperm-egg interactions that control fertility and subjects gametes to strong selection or competition [6–9].

During in vitro fertilization, numerous steps can impact the final result of fertilization, from sperm collection to gamete mixing and incubation [10]. The success of fertilization in external aquatic environments can be aggravated by plugging of the micropyle [2], limited egg receptivity [11], and sperm longevity [12]. This scenario has been shown for zebrafish (*Danio rerio*, Hamilton, 1822), where if no sperm enter the egg during the first ~30 s post-activation, the fertilization process will be hampered [13]. Another barrier is non-synchronous spawning between sexes [14]. Together, these reproductive obstacles have led scientists to develop novel techniques and technologies for artificial reproduction, such as direct sperm injection into the fish's oviducts/ovary. For example, Müller et al. [15–18] developed a fish propagation method where sperm were delivered into the ovarian lobes of females by ovarian lavage in common carp (*Cyprinus carpio,* Linnaeus, 1758) and African catfish (*Clarias garepinus,* Burchell, 1822) parallel with hormonal induction. Together, their results showed that all sperm-injected females produced fertilized eggs that developed normally.

In this study, we used zebrafish as the model organism to further investigate this phenonemon. Zebrafish is an excellent model for studying aspects of reproductive biology, due to their growing popularity as a biomedical species, and the need to maintain numerous breeding lines [19,20]. Thus, advanced in vivo and in vitro reproductive studies will lead to more efficient propagation protocols, which can also be applied to other commercially viable species for hatchery production. More specifically, we aimed to investigate the possibility of propagating females using sperm ovarian lavage/sperm insemination without the presence of male stimulus.

2. Material and methods

2.1. Zebrafish lines and husbandry conditions

Zebrafish female broodstock were wild-type line [21] that have been incrossed for several years in the zebrafish laboratory of Szent István University, Hungary. Two male lines were used: i. wild-type line AB bred in the zebrafish laboratory [22], and ii. neutrophil-specific transgenic zebrafish line Tg(mpx:GFP)i114, that express green fluorescent protein (GFP) under myeloperoxidase (mpx) promoter [23]. AB females [n = 191, standard length (SL) = 10–28 mm] and males (n = 60, SL = 9–30 mm) were maintained under constant water quality parameters (25 ± 0.5 °C, pH 7.0 ± 0.2, conductivity 500 ± 50 μS, 0 mM CO_3^{2-}, 0.4 mM HCO_3^{2-}; degree German hardness < 0.5°; dissolved oxygen > 90%) in a Tecniplast ZebTec (Buguggiate, Italy) recirculating zebrafish housing system. The photoperiod was set at 14h light/10h dark. Zebrafish were kept in 3 L polycarbonate tanks and fed twice a day with Zebrafeed (Sparos, 400–600 μm) and twice a week with brine shrimp (Ocean Nutrition > 230.000 nauplii/g). Transgenic males used for experiments (Tg(mpx:GFP)i114, n = 15, SL = 19–25 mm) were kept together in a 3 L polycarbonate tank in the same system.

The protocols for fish propagation and experimentation (Scientific Ethics Council for Animal Experimentation; XIV-001-2306-4/2012 and PE/EA/742-7/2020) have been reviewed and approved by the National Food Chain Safety Office of Hungary and the Animal Health and Animal Welfare Directorate of Government Office of Pest County.

2.2. Experimental design

2.2.1. Sperm collection

Tg(mpx:GFP) homozygous males were removed from their breeding tank and anaesthetised with MS-222 (4.2 mL, 15.3 mM MS-222/100 mL water). After anesthetization the genital area was dried with a paper towel and sperm stripping was performed under a Leica M205 FA microscope. Sperm were obtained by applying gentle pressure to the sides of the transgenic males and collected using 0.2 to 2 μL pipette tips (Thermo Scientific Finnpipette™ F1) and G-1 glass capillary tubes (length = 90 mm, external diameter = 1 mm; Narishige Scientific Instrument Lab. Japan). After collection the sperm were pooled.

2.2.2. Sperm and isotonic solution insemination

Isotonic (physiological) NaCl solution 0.9% (Fresenius Kabi Deutchland GmbH) or a pooled sperm sample (0.4 μL) was artificially inseminated into one AB female (see Section 2.3 below). Females were anaesthetised, as above, ~1 h from the expected spawning time (i.e. when dark/light changes). Sperm was injected using specific pipette tips (Finnpipette™ F1) (Thermo Scientific Finntip™ 20 μL, CE marked), which were inserted ~2 mm deep into the oviduct through the genital papilla of anaesthetised females. Thereafter, females were placed back into their respective spawning tanks for recovery and spawning.

2.3. Experimental design

These experiment series consisted of 7 independent treatments which are highlighted below. For Treatment 1, traditional spawning approaches were used (absolute control, wild-type female × wild-type male: AB♀ × AB♂). Specifically, zebrafish propagation took place in ~1.7 L spawning tanks (Sloping Breeding Tank, ZebTec, Tecniplast S.p.a., Italy) which features a sloped interior, or “beach style”, that facilitates and promotes zebrafish spawning (tanks also used for all treatments). These tanks contain two interchangeable containers, where the bottom of the inner vessel is perforated to facilitate egg collection and movement of fish post-spawning. Propagating tanks were paired with one female and two wild-type males in the afternoon prior to spawning (n = 23♀, 46♂, Table 1, Fig. 1). Spawning took place the following morning, typically a few hours after the lights turned on, as zebrafish reproduction is strongly influenced by photoperiod [24].

In the next series of treatments (Treatments 2–4) the zebrafish spawning tanks did not contain males (n = 4 females/spawning tank, Table 1, Fig. 1). More specifically, Treatment 2 was the negative control, where females were not manipulated (AB♀). For Treatment 3 the females were artifically inseminated with only NaCl [positive control; AB♀ (inj.NaCl)], while in Treatment 4 the females were artifically inseminated with pooled sperm from the transgenic (TG) males [AB♀(inj.Tg♂)].

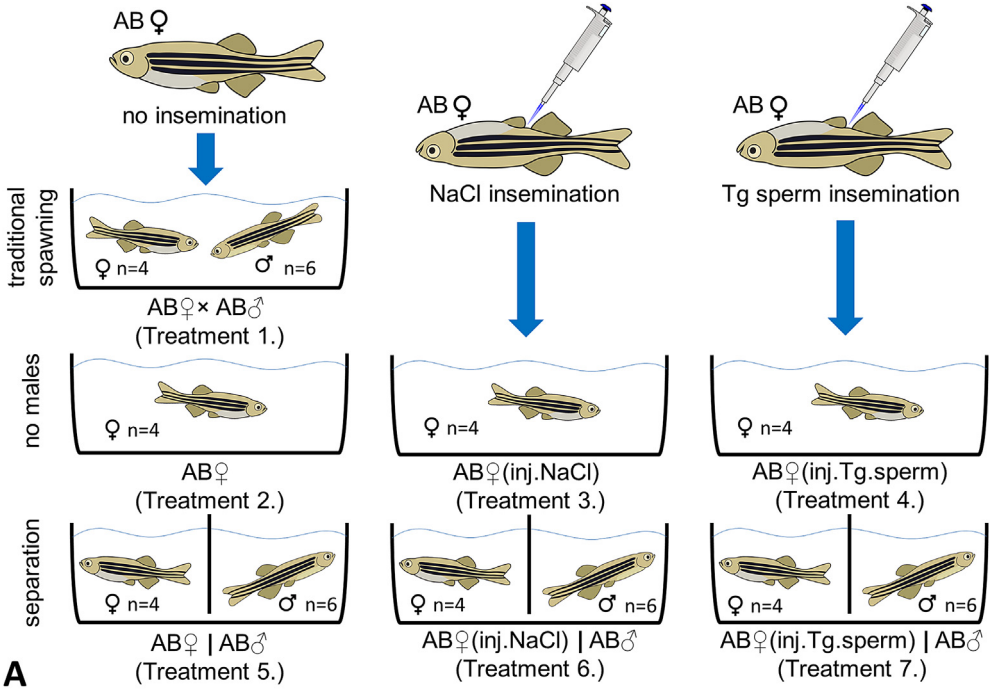
For the last series of treatments (Treatment 5–7) the females were kept separately from the wild-type males using a transparent divider in each spawning tank (n = 4 females and 6 males/spawning tank, Table 1, Fig. 1). Here, Treatment 5 was the negative control II (AB♀|AB♂) where females were not manipulated, while in Treatment 6 the females were inseminated with only NaCl [positive control II; AB♀ (inj.NaCl) |AB♂]. Finally, in Treatment 7 the females were inseminated with pooled sperm from transgenic males [AB♀(inj.Tg♂) |AB♂]. Males were selected randomly from the same broodstock (see 2.1. zebrafish lines and husbandry conditions) in every experimental series.

Table 1
Summary of treatments used to study how traditional spawning (Treatment 1) and ovarian inseminated sperm impacts spawning success in zebrafish (*Danio rerio*) without male stimulus (Treatments 2–4) and with male stimulus (Treatments 5–7).

Exp.	Treatment description		Abbreviation	# females and males/per spawning tank	Replicate
1	Traditional spawning		AB♀×AB♂	4♀×6♂	7
2	Females without males	Non-manipulated	AB♀	4♀	7
3		NaCl inseminated	AB♀ (inj.NaCl)	4♀	7
4		Sperm inseminated	AB♀(inj.TG♂)	4♀	7
5	Females separated from	Non-manipulated	AB♀|AB♂	4♀|6♂	7
6	males by transparent	NaCl inseminated	AB♀ (inj.NaCl)|AB♂	4♀|6♂	7
7	plastic screen	Sperm inseminated	AB♀(inj.TG♂)|AB♂	4♀|6♂	7

2.4. Egg collection and incubation

Water conditions for egg incubation were: Temperature = 25.5 °C; pH = 7.0 ± 0.2; and conductivity = 525 μS. Eggs were collected from all tanks and placed into Petri dishes (100 mm diameter) 4 h after initiation of light. Eggs were incubated in a thermostat with photoperiod set at 14 h light:10 h dark with daily water changes. After 72 h of incubation, genotypes (wild-type line or transgenic) of embryos were determined using a Leica M205 FA microscope with LAS X (Leica Application Suite X) 3.4.2.18368 software (Leica Microsystems CMS



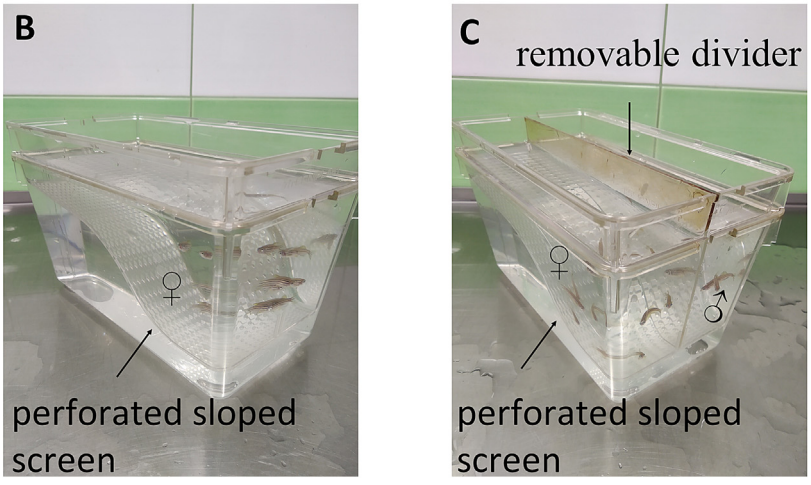


Fig. 1. A: Summarised schematic figure about the experimental design. **B-C:** Sloping Breeding Tanks for experiments, **B:** without divider screen **C:** with divider screen.

Gmbh). Offspring originating from injected transgenic sperm were counted upon detection of green fluorescence protein expression using an EGFP2 filter, where the maximal excitation and emission values were 489 nm and 508 nm, respectively.

2.5. Reproduction parameters

The number of eggs in each tank were quantified. Thereafter, egg production per female was calculated as: quantity of eggs counted per tank/number of females per tank. In addition, fertilization rate (%) was calculated as: (number of fertilized eggs at 72 h/number of eggs) × 100.

2.6. Statistical analysis

Data were analyzed using SPSS 22.0 for Windows. Residuals were tested for normality and homogeneity of variances. Data deviating from normality or homoscedasticity were transformed. Alpha was set at 0.05. Tukey's analysis was used to compare least-square means between treatments. Egg production and hatching rate data were subjected to one-way analysis of variance.

3. Results

Spawning treatment impacted both egg production ($P < 0.05$) and fertilization rate ($P < 0.05$). As evident in Table 2 there were no released eggs in both negative control treatments (Treatment 2 = AB♀ and Treatment 5 = AB♀|AB♂). Egg production increased when females were injected with NaCl in the presence [Treatment 6 = AB♀ (inj.NaCl) |AB♂] or absence of male stimulus [Treatment 3 = AB♀ (inj.NaCl) and Treatment 4 = (AB♀(inj.TG♂)]. There was also a high-degree of variation in egg production between spawns for these three treatment groups (Treatments 3, 4, and 6 ranged from 0 to 28.5 eggs per female), however as would be expected no eggs were fertilized from the positive controls (Treatments 3 and 6). Females from these treatments, in the presence (Treatment 6) or absence of male stimulus (Treatments 3 and 4), statistically released a similar amount of eggs.

A further increase in egg production (relative to Treatments 2, 3, and 5) was detected when females were inseminated with pooled sperm from transgenic males in the presence of male stimulus [Treatment 7 = AB♀(inj.Tg♂)|AB♂] or when using traditional spawning approaches (Treatment 1 = AB♀ × AB♂). A high-degree of within treatment spawning variability was also detected when using this sperm insemination treatment (Treatment 7 ranged from 2.5 to 55 per females) or the traditional spawning approach (Treatment 1 ranged from 25 to 131 eggs per female) (Table 2).

Females artificially inseminated with sperm also produced viable embryos, although no significant difference was detected when females were inseminated with pooled sperm from transgenic males in the presence or absence of male stimulus (Table 2). Specifically, fertilization rate was 12.6% (ranged from 0 to 24.7%) when no males were present [Treatment 4 = (AB♀(inj.Tg♂)] and was 11.8% (ranged from 0 to 39.3%) with male stimulus [Treatment 7 = AB♀(inj.Tg♂)|AB♂]. All embryos from the sperm inseminated group [AB♀(inj.Tg♂)|AB♂] were fertilized by transgenic sperm and it is indicated that all embryos were diploid (not haploid) and the AB males were not involved in fertilization (Fig. 2). Meanwhile, the traditional spawning approach (Treatment 1 = AB♀ × AB♂) had the highest fertilization rate (81.3%) with a high degree of variability (Table 2).

4. Discussion

Our previous studies indicated that artificial sperm insemination or sperm ovarian lavage is an effective tool for fish captive breeding [15–17,25,41]. In this study, spawning success of sperm inseminated zebrafish females was studied without males or without direct presence of males (i.e. males were separated from females with a divider) during spawning. Our results clearly showed no spontaneous spawning for the negative control treatments (Treatment 2 = AB♀ and Treatment 5 = AB♀|AB♂). In nature, zebrafish spawn in groups during the reproductive season [20,26]. This is also observed under lab conditions, where group spawning increases reproduction efficiency, including egg production, fertilization rate, hatching rate, larval viability, and genetic variability [20,24]. Here, group spawning was not enough to induce spontaneous ovulation for the untreated groups (AB♀ and AB♀|AB♂), despite the fact that females could observe males through transparent screens and receive visual stimulus and paternal pheromones.

Only a few infertile eggs (0–15.5 per female) were released from females that were injected with physiological saline solution into their ovarian lobes [Treatment 3 = AB♀ (inj.NaCl♂) and Treatment 6 = AB♀(inj.NaCl)|AB]. Generally, during in vitro fertilization procedures eggs can easily be released by gentle pressure on the females abdomen [27]. It is suggested that handling stress and saline injections or even the slope of the breeding screen can also induce female ovulation due to increases in internal pressure similar to the pneumatic egg collection method [28]. Our study along with others [29] have shown that females can spontaneously release eggs without the presence of males in captivity. For instance, percid females (i.e., pikeperch, *Sander lucioperca* Linnaeus, 1758; perch, *Perca fluviatilis* Linnaeus, 1758) can ovulate and spontaneously release their eggs/egg ribbons regardless of male presence [30,31]. Stagey et al. [29] investigated reproductive dynamics in goldfish, *Carassius auratus*, Linnaeus, 1758 and demonstrated ovulation without males under different spawning combinations, such as in the presence or absence of aquatic plants. After hormonal induction, several species can also release a proportion of their entire egg mass, including eel species, *Anguilla* sp. Garsault, 1764 [32–36], common carp [15,27], pikeperch, and perch [31]. In these cases

Table 2
Egg production and hatching rate from 7 treatments used to study how traditional spawning (Treatment 1) and ovarian inseminated sperm impacts spawning success in zebrafish (*Danio rerio*) without male stimulus (Treatments 2–4) and with male stimulus (Treatments 5–7). Treatments with different superscripts significantly differ ($P < 0.05$).

Treatment	Abbreviation	Egg production per female		Fertilization rate (%)	
		Mean ± SD	min-max	Mean ± SD	min-max
1	AB♀ × AB♂	81.2 ± 42.3^{c}	25–131.3	81.3 ± 10.4^{a}	61.6–93.2
2	AB♀	0	.	0	.
3	AB♀ (inj.NaCl)	3.4 ± 4.2^{a}	0–9.5	0	.
4	AB♀(inj.Tg♂)	16.1 ± 11.6^{ab}	0.5–28.5	12.6 ± 9.2^{b}	0–24.7
5	AB♀|AB♂	0	.	0	.
6	AB♀ (inj.NaCl) |AB♂	7.9 ± 4.8^{ab}	2.8–15.5	0	.
7	AB♀(inj.Tg♂) |AB♂	27.8 ± 20.6^{bc}	2.5–55	11.8 ± 16.3^{b}	0–39.3

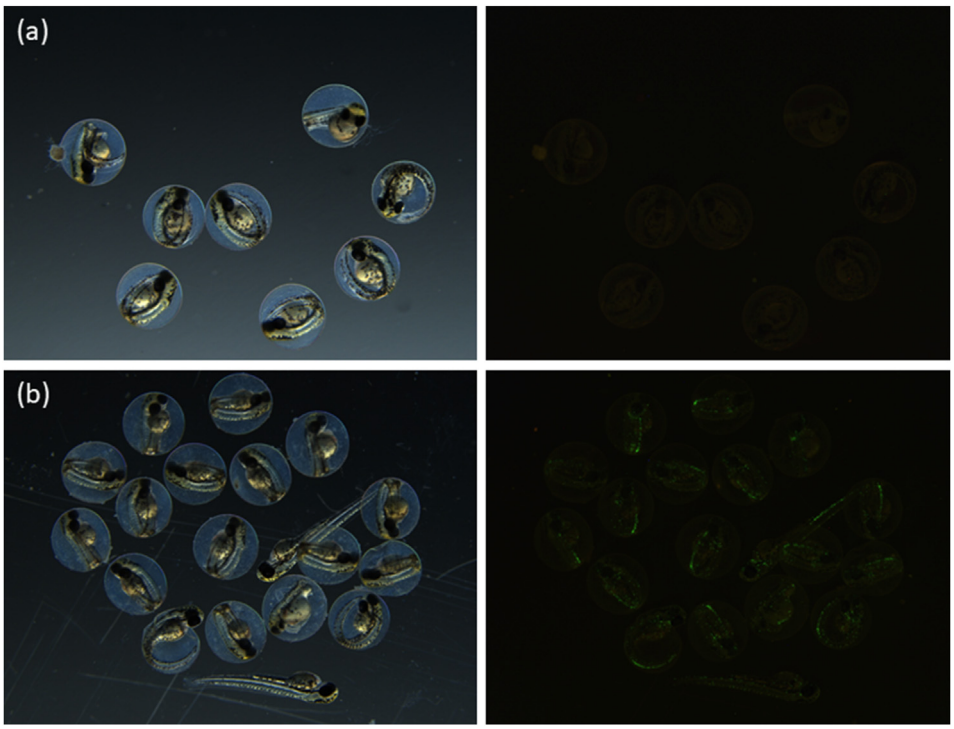


Fig. 2. Phenotypic appearance of offspring from different spawning treatments as a marker for transgenic (Tg) and wild type (AB) genotypes. Zebrafish embryos (72 h old) were imaged in the dark (left) and under fluorescence microscopy (right), resulting from crosses as follows: (a) AB × AB; (b) AB♀(inj.Tg♂) |AB♂.

(among others), hormonal stimulation is enough to induce ovulation without other stimulating factors.

Sperm inseminated zebrafish [Treatment 4 = AB♀(inj.Tg♂) and Treatment 7 = AB♀(inj.Tg♂)|AB♂] or those females injected with saline in the presence is male stimulus [Treatment 6 = AB♀(inj.NaCl) |AB♂] produced more eggs then those injected with just saline in adsense of males indicating that both males and injected milt provided an ovulation stimulus. Seminal plasma of sperm typically contains androgens, such as testosterone and 11-ketotestosterone, as well as the progesterone, 17α,20β,21-trihydroxy-4-pregnen-3-one [37–39]. These seminal plasma hormones are likely absorbed through the ovarian wall [16] and impact blood serum hormone levels that partly control ovulation processes and the general physiology of females. At present, information on this topic is limited. Thus, we speculate that if these absorbed seminal hormones manipulate the hypothalamic-pituitary-gonadal (HPG) axis they may infact improve artificial induction of ovulation for large scale propagation. Furthermore, if we can better understand the specific mechanisms of spontaneous egg release and group spawning behaviours these phenomena can be applied for improving induced spawning techniques for hatchery production of fishes.

In our study, females which were inseminated with sperm produced viable embryos, but lower fertilization rates were detected. It is worth speculating that the relatively thick automatic pipette tip may have pressed against the urinary bladder of some females, which is very close to the urogenital papille. Thus, the inseminated sperm samples might have been contaminated with urine within the female reproduction organ decreasing sperm quality. In teleosts, contamination of sperm by urine leads to decreases in sperm quality [40]. Suboptimal inseminated sperm delivery into the oviduct may also lead to sperm leakage/release from the inseminated females, which would decrease fertility. Thus, further research is needed (e.g. sperm injection volumes, timing of injections) to optimize these insemination techniqiues and technologies for precise adaptation to other fish species [41]. Here, females were subjected to group spawning. Therefore, it was not possible to check egg production from individual females. Nevertheless, fertilisation rates for the natural spawning fish (AB♀ × AB♂) was four-fold higher than sperm injection groups with large individual differences between groups (61.6–93.2% fertilization rate).

Injected sperm does not cause internal fertilization. In ovulipar species, release of eggs from the female is followed by activation and fertilization within the external environment [42]. To the best of our knowledge, there are three studies where egg-laying fish species were internally fertilized and embryos developed within the ovarian lobes. For instance, Hayakawa and Munehara [43] reported that eyed embryos were found in the ovary of a marine sculpin species, *Hemilepidotus gilberti,* Jordan & Starks, 1904. The authors reported that all embryos died during early developmental phases or were deformed in the ovary. A single three-spined stickleback, *Gasterosteus aculeatus,* Linnaeus 1758 was also observed with embryos retained in the ovaries [44], but these embryos were not assessed for deformities or viability. Dean et al. [45] were the first scientists to detect living embryos in a non-copulatory, egg-laying teleost species, the three-spined stickleback. Here, larvae hatched and developed to maturity. During internal fertilization there needs to be sperm and egg contact. According to Müller et al. [18] simple sperm injection into the ovary before ovulation is not sufficient to stimulate internal fertilization, as water is also needed to activate the gametes. Munehara et al. [46] discovered a subcategory of ovuliparity, called “internal gametic association,” which refers to the association of male and female gametes in the female reproductive tract followed by release and fertilization in the external environment. Müller et al. [18] investigated this sperm ova interaction just after gamete stripping without water activation in African catfish using electron microscopy. They showed that sperm distributed near the micropylar

region and sperm were detected within the micropylar canal, similar to observations by Munehara et al. [46]. As a result, we suggest that sperm are inactive near the micropyle region (or in it) and just after gamete release the closest sperm to the micropyle will fertilize the egg after expulsion into the external environment.

During artificial propagation, hormonal injections typically help to induce spawning [27]. According to our previous observations on common carp [15] and the critically endangered *C. carpio* River Danube subpopulation [47] originating from Lake Hévíz [48] in spawning cages the females could release all their eggs after hormonal induction without the presence of males (unpublished data). Therefore, our insemination methods of reproduction can be improved by combining artificial induction of ovulation using different hormone treatments.

Further investigations are needed to develop this method in other fish species, as well as to reveal the biological background of fertilization capacity, including sperm competition, which may result in more viable embryos.

4.1. Conclusion

Spawning success of sperm inseminated zebrafish females (external fertilized fish species) was studied without males or without direct presence of males (i.e. males were separated from females with a divider) during spawning. The treated females partly ovulated and produced living embryos in different ratios without induced hormonal stimuli. Injected sperm does not cause internal fertilization. In ovulipar species, release of eggs from the female is followed by activation and fertilization within the external environment. This proof of concept experiment demonstrates successful spawning in case of external fertilized fish without direct presence of males by using sperm injection method.

CRediT authorship contribution statement

All authors contributed to this paper. **Gyöngyi Gazsi:** performed the experiments, Formal analysis, wrote the paper. **Ian A.E. Butts:** performed the experiments, Formal analysis, wrote the paper. **Vahid Zadmajid:** conceived and designed the experiments, Formal analysis, wrote the paper. **Bence Iványovics:** conceived and designed the experiments, Formal analysis, wrote the paper. **Luca Ruffilli:** performed the experiments, Formal analysis, wrote the paper. **Béla Urbányi:** performed the experiments, Formal analysis, wrote the paper. **Zsolt Csenki:** performed the experiments, Formal analysis, wrote the paper. **Tamás Müller:** performed the experiments, Formal analysis, wrote the paper.

Declaration of competing interest

The authors declare no conflicts of interest.

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