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New observations about the fertilisation capacity and latency time of sperm inseminated into the ovary of African catfish (Clarias gariepinus), an oviparous modelfish

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ABSTRACT

In our former study, we proved that sperm injected into the ovary by catheter through the oviduct 10-12 h before hormonally induced ovulation, retains its fertilizing capacity for several hours. Using this method, we produced viable larvae via in vitro fertilisation. The aim of this study was to investigate the time-dependent fertilizing capacity of sperm which were introduced into ovary. Sperm samples (2 mL sperm / bodyweight kg) were incubated in gonad lobes 5, 10, 15, 20, 25, 36 and 48 h before the gamete stripping. Ovulation was induced by extracted carp pituitary (CPE) hormonal administration (5 mg CPE / BW kg). There were no statistical differences ($p < 0.05$) among the hatching rates in the 5-25 h treatment groups, but we observed large individual fluctuations in fertilisation and hatching rate within the groups. However, at 48 h the treated group showed low fertilisation and hatching rate indicating loss of fertilizing capacity. In our second experiment cycle, three different inseminated sperm dosages were tested on fertilisation from the same pooled sperm batch; 2 mL, 1 mL and 0.5 mL sperm volume / BW kg. The 0.5 mL sperm dosage showed statistically ($p < 0.05$) higher fertilisation and hatching rate indicating that small milt volume was enough to apply for propagation. According to scanning electron microscopic analysis, this is the first observation that there is a chance for 'internal gametic association' reproduction strategy in fish species, where there is no direct contact between the two sexes in the spawning.

1. Introduction

The *in vitro* fertilisation (IVF) method is widely used in aquaculture. In general, calculated doses of different (sex) hormones are injected intramuscularly or intraperitoneally, by using a syringe and needle, to induce ovulation and spermiation. Catheters (thin tube) are used in aquaculture for different internal manipulations, such as sperm collection and ovarian biopsy. In smaller proportions, hormones are usually injected through catheters directly into the ovaries, a procedure called ovarian lavage (as described and used by Németh et al., 2012; Watson et al., 2009a, b; Zaucker et al., 2014), but abdominal implantation can also be applied (Lin et al., 1991, 1998; Lokman et al., 2015; Sato et al., 1995, 1997; Sherwood et al., 1988). Catheters can be used for other purposes as well. Müller et al. (2018a) tested a novel approach to deliver sperm to eggs in common carp (*Cyprinus carpio*) and concluded that sperm injection into the ovary through the oviduct leads to successful fertilisation and developing embryos. This result indicates that (i) spermatozoa can be stored in the ovary and oviduct for up to 12 h without loss of biological activity; and (ii) the ovulated eggs could be fertilised by the surrounding sperm after being released from the ovarian cavity. Müller et al. (2018b), Ittzés et al. (2020) published the novel approach to deliver sperm and hormone together to eggs in African catfish (*Clarias gariepinus*) and Jundia (*Rhamdia quelen*). They concluded that sperm + CPE injection into the ovary through the

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oviduct leads to successful ovulation, fertilisation and developing embryos at every experimental condition applied. It was demonstrated that the ovarian lavage with sperm and hormone, using seminal fluid as a delivery vehicle, can be used as an alternative for traditional *in vitro* fertilisation, where stripped eggs are fertilised by stripped sperm outside of the body of the mother. In our experiments described below, we applied only one latency time of inseminated sperm (10 h just before the expected ovulation) and one dose of sperm (2 mL sperm/ bodyweight kg). The aim was to reveal the biologically active period of the spermatozoa in the ovary and to investigate the effect of different sperm volumes on fertilisation capability by using fish propagation of ovarian lavage / artificial sperm insemination. The experiments were carried out on African catfish, a good model fish for fish propagation (Müller et al., 2018b, 2019; Van Oordt and Goos, 1987).

On the other hand, artificial internal injection of sperm into ovarian lobes is similar to unique ways of fish reproduction, which occur in nature. Although African catfish belongs to ovuliparous fishes (where female emits the ova into the environment then they are fertilised externally by the male), other fishes use alternative reproduction strategies of external fertilisation. Zygoparity refers to the release of fertilised ova or gynogenetically activated ova that are retained within the female genital tract for short periods. Embryoparity is the pattern of oviparous reproduction in which a definitive embryo is formed and may develop to an advanced state prior to its release from the female genital tract and subsequent hatching from the egg envelope. Munehara et al. (1989) discovered a subcategory of ovuliparity called "internal gametic association," which refers to sperm introduced into the ovary lobes by copulation. Spermatozoa of elkhorn sculpin, (*Alcichthys alcicornis*) can be stored at ovarian conditions for several days and after oocyte ovulation enter the micropylar canal of eggs within the ovarian cavity. There is no internal fertilisation because the spermatozoa stop just before the ooplasma in ovarian cavity. Water activation is needed for spermatozoon-egg fusion so the fertilisation not occurs until the eggs have been released into sea water. This species is a multiple spawner, which spawns at few days intervals for one month, and the sperm introduced into the ovary at the beginning of the spawning season retain their fertility for the entire period. Although it has been described for several cottid species (Abe and Munehara, 2005; Koya et al., 1993; Munehara et al., 1989; Munehara et al., 1991; Ragland and Fischer, 1987), but true internal fertilisation has not been documented (Muñoz, 2010). The driftwood catfish (*Trachelyopterus galeatus*) is unique among the Siluriformes because it presents internal fertilisation and expressed sexual dimorphism. Males have an intromittent organ on the first ray of the anal fin that is used to deliver sperm during copulation. In the females, spermatozoa are then stored in a saclike structure of the oviduct, and fertilisation occurs only at the moment of spawning (Ferraris, 2003; Meisner et al., 2000; Santos et al., 2013).

The sperm injection/ovarian lavage combines the simplicity of induced spawning, with a more time flexible delivery of the sperm compared to conventional in vitro fertilisation. Hormonal administration parallel with sperm injection into ovary may be applied in breeding programs or in intra- and interspecific hybridisation and it is a feasible method for using manipulated (cryopreserved or genetically modified) sperm also (Müller et al., 2018a, b, 2019; Ittzés et al., 2020). Other events where sperm injection could be favourable include: 1) Synchronising the availability of sperm and ovulated eggs may be rate limiting or where induction of egg release and or stripping is risky or technically unfeasible (marine fish species such as *Dicentrarchus labrax, Anarhichas lupus*, etc.). 2) Genome diversity can be increased by using sperm samples from several males for sperm injection and allowing the pairs (i.e. *Umbra krameri*) or groups to spawn (i.e. *Anguilla sp.*) after injection. 3) Successful application of sperm injection together with hormonal administration of these fish species may allow for changing the sex ratio in the stock, as egg batches would be fertilised by catheter delivered sperm and there is less demand for presence of additional males (i.e. *D. labrax, Sparus aurata*, etc.). 4) Artificially inseminated sperm samples could be introduced with different species to create hybrids as well, where there are too many barriers between the parental species due to size, spawning ethology etc. (i.e. *Ictalurus punctatus* × *I. furcatus*).

C. garepinus females could store the artificially injected spermatozoa in ovarian lobe for 10 h and the seminal plasma was absorbed through ovarian lobe wall (Müller et al., 2018b, 2019; Van Oordt and Goos, 1987), but there is no information about what is happening with the spermatozoa in ovary at ovulation. The aim of this study was to investigate the potential latency period of African catfish sperm in the ovary and test the optimal or minimal sperm-egg ratio.

2. Material and methods

The present study consists of four experimental series. In the first two experiments (labeled as E1–&2), optimal latency period to maintain the biological activity/fertilisation capability of inseminated spermatozoa in ovarian cavity were investigated. In the whereas third one (E3), the optimal sperm-egg ratio was determined; in the fourth experiment (E4), the location of ovary injected spermatozoa on ovulated egg surface was investigated using scanning electron microscope.

2.1. Experimental conditions and animal welfare

All experiments were carried out at the Georgikon Fish Research Center of University of Pannonia. African catfish of 36 months of age were used as broodstock, which had been reared in recirculation system and fed on commercial pellets (Skretting Classic K 3P, Skretting, Stavanger, Norway). Males and females were sorted and kept separately during the trial (35 L/fish/tank). The water temperature when the fish were introduced to the tank was 27 °C which was maintained constantly throughout the study period. Natural photoperiod was applied and the light in the experimental room was dimmed. Before all manipulations, fish were anaesthetised in a solution of 100 mg L^{-1} benzocaine (ethyl 4-aminobenzoate, Norcaine) for subsequent handling. Key environmental conditions were maintained during the experiments. The water temperature was measured three times per day in all three experimental cycles (27 ± 0.5 °C). The experiments were reviewed and approved by the Scientific Ethics Council for Animal Experimentation; (GK-2675/2012) and the Food Chain Safety, Animal Health and Animal Welfare Directorate of the Government Office of Zala County (XX-I-100/2258–002/2012), with respect to scientific content and compliance with applicable research subject regulations.

2.2. Experimental designs

There were four experimental series:

E1: We investigated the effects of latency time on the fertilisation capacity of artificially inseminated sperm in ovary. Five treated groups were set up with five females in each group. Artificial sperm insemination were carried out with different times before the expected ovulation; 5 h, 10 h, 15 h, 20 h and 25 h (Fig. 1.)

E2: We analyzed the fertilisation capacity of artificially inseminated sperm in the ovary over one day. Two treatment groups were set up with five females per group. Artificial sperm insemination was carried out 36 h and 48 h before the gamete stripping (Fig. 1).

E3: The aim was to study the efficiency of different sperm volume ratios for artificial sperm insemination (Fig. 2). There were three experimental groups with five–six females each and groups were treated with different amount of sperm from the same pooled sperm samples (0.5 mL sperm/females, 1 mL sperm/female and 2 mL sperm/female). In order to check the fertilisation capacity of pooled sperm samples, in parallel with the sperm insemination they were also used for *in vitro* fertilisation (n♀ = 5).

E4: Here, we investigated location of ovary injected spermatozoa on ovulated egg surface just after gamete stripping without water

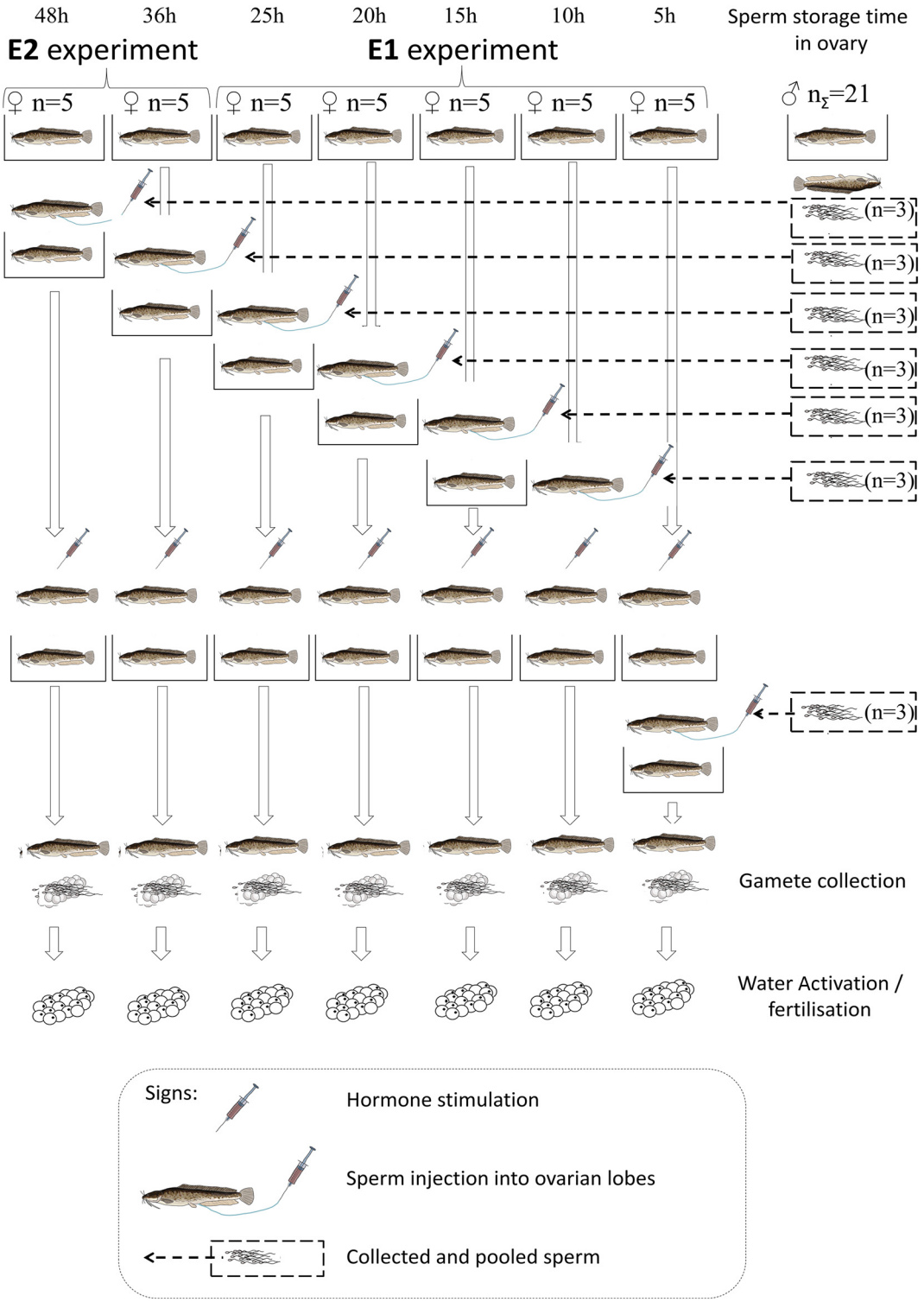

Fig. 1. Schematic representation of experimental design of the E1 and E2 experiments. Sperm samples were injected into the ovaries of African catfish specimens at different time points (5–48 h before stripping). Sperm samples were originated from surgically removed testis of decapitated males (21 individuals in total). Hormone stimulation occurred 10 h before stripping.

activation using scanning electron microscope.

2.3. Gamete management

2.3.1. Hormonal induction of males

Hormonal induction of males (E1: $n = 15$, bodyweight (BW): 442.4 ± 100.1 g; E2: $n = 6$, BW: 364.6 ± 70.7 g, E3: $n = 10$, BW = 360.9 ± 78.3) was carried out by injection of carp pituitary extracts (5 mg CPE / 1 mL 0.9% NaCl solution / kg BW) intraperitoneally at the base of the ventral fin of the males, 24 h prior to the treatment of females.

2.3.2. Sperm collection and sperm quantification

Sperm was extracted from testes that were surgically removed from

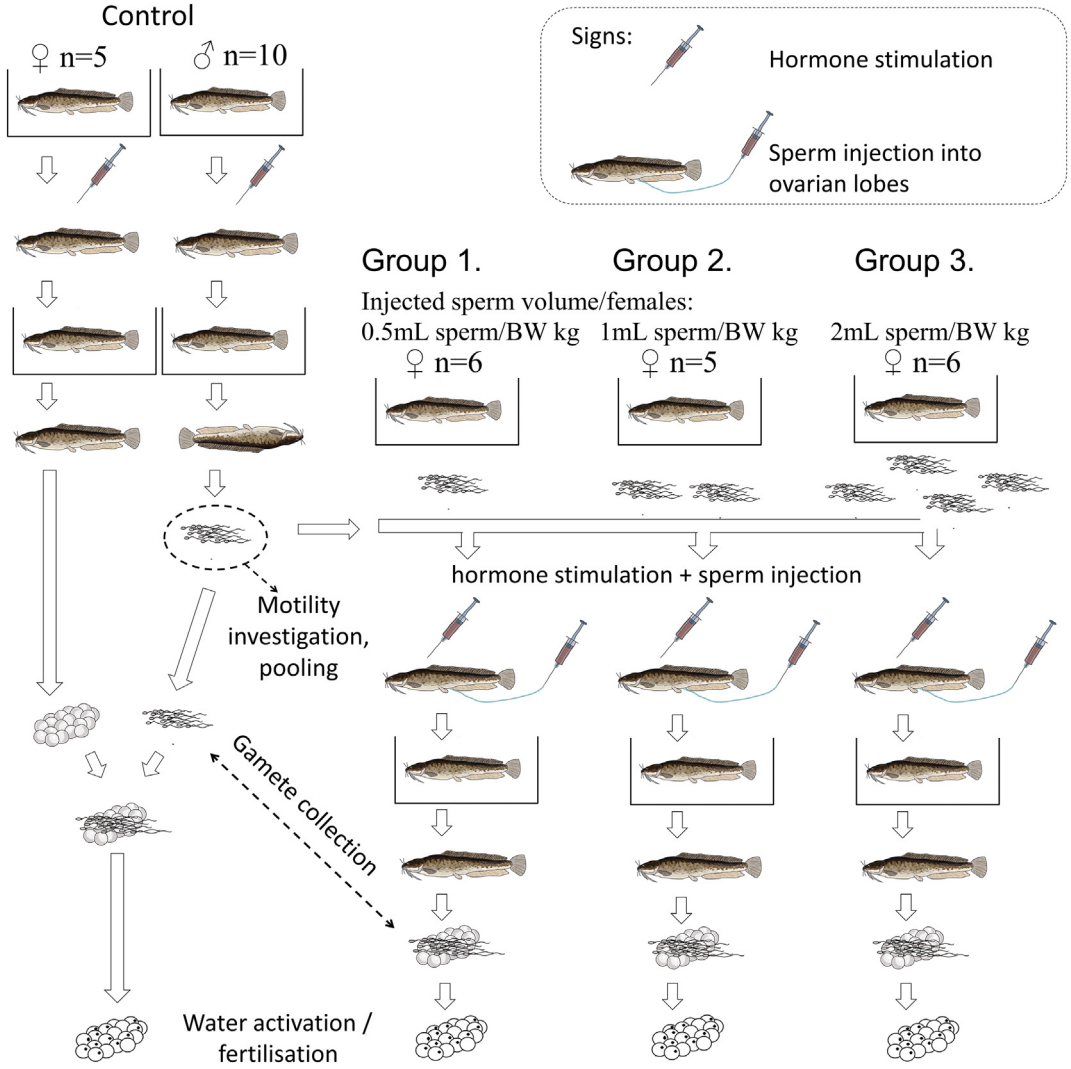

Fig. 2. Schematic representation of the experimental design of E3 experiment. Sperm samples were injected into the ovaries of African catfish specimens at different volumes (0.5 mL/BW kg, 1 mL/BW kg and 2 mL/BW kg). Sperm samples were originated from surgically removed testis of decapitated males and after sperm evaluation of sperm samples were pooled. In order to check the sperm fertilisation capability control females were fertilised by using part of native sperm as well just before sperm injection. Hormone stimulation occurred 10 h before stripping.

the body cavity of the fish (after decapitation following anaesthetic over dosage with Benzocaine). The sperm samples were subjected to motility analysis. Sperm motility was estimated after activation by adding 50 µL water to one drop milt on a glass slide under 100 × magnification (Olympus BX43, Olympus Corp., Tokyo, Japan). Sperm samples with estimated motility > 80% were selected for experimental purposes and were pooled for ovarian lavage (males number: Fig. 1., Fig. 2)."The sperm concentration of pooled milt samples was determined by using a Bürker chamber in E2, resulting in a 1.12×10^9 cell/mL value.

2.3.3. Sperm insemination

Sperm samples were introduced into the ovarian cavity of females through the genital papilla as shown in Müller et al. (2018b). We attached a silicone catheter (Feeding tube, 400 mm length, size: CH, outer diameter 1.3 mm, inner diameter 1 mm, Galmed, Poland) to a 2 mL syringe, and inserted the catheter about 10 cm deep into the oviduct up to end of ovary lobes via the genital opening. The calculated dosages were divided equally between the two lobes of ovary. Inseminations were conducted in anesthetised (with Benzocaine) status of fish.

In E1, E2, and E4 every female was injected 2 mL/BW kg sperm, whereas in E3 females were injected with 0.5-02 mL sperm/BW kg as described above.

2.4. Hormonal induction of females

Females for both trials were injected intraperitoneally with 5 mg CPE in 1 mL 0.9 NaCl solution per kg bodyweight (Müller et al., 2018b, 2019). Injections were conducted on fish anesthetised with Benzocaine.

2.5. Fertilisation

Stripped and pooled gamete batches were fertilised through activation with aerated water. Five minutes after the water activation, three samples of eggs (average egg number per Petri dish: E1 = 76.3 ± 21.3, E2 = 188.9 ± 63.2, E3 = 154.4 ± 53.8) were collected from each batch and incubated in a Petri dish (105 mm diameter) at 25 °C. Water was changed continuously by using dripping system (flow rate 1700–1900 mL/h) in the Petri dishes until determination of the fertilisation rate at somitogenesis stage (12 h post fertilisation (hpf)), at organogenesis of the notochord (C-shaped embryo; 24 hpf, Osman et al., 2008) and hatching (36 hpf).

To evaluate induced egg production, the pseudogonadosomatic index [PGSI = (weight of stripped egg mass / BW before

stripping) × 100] was calculated.

2.6. Scanning electron microscopy (E4 experiment)

Two African catfish females (BW: 509-478 g) were artificially inseminated with sperm (2 mL/BW kg) 10 h before the expected ovulation parallel with intraperitoneal hormonal administration (5 mg CPE/ 1 mL 0.9 NaCl solution/BW kg). Two females were used as controls, they were injected with CPE, but not inseminated subsequently. For scanning electron microscopy, the eggs of controls and gametes (eggs with spermatozoa) from treated fish were fixed immediately without water activation in glutaraldehyde (5% in 0.1 M phosphate buffer) for 3–4 h at room temperature. Fixed samples were rinsed twice with phosphate buffer solution (pH 7) and shock frozen at 85 °C, then freeze-dried (until 2×10^{-2} mbar, at −60 °C for 6–8 h). After lyophilisation, the dried samples were mounted on metal stubs and sputter-coated with gold. The topographic features of the micropylar region of eggs (n = 20–20 eggs per treated females and 20 ovulated eggs from non-treated female) were visualised with an EVO MA 10 Zeiss scanning electron microscope (SEM) at an accelerating voltage of 5 kV.

2.7. Statistical analysis

Statistical analyses of fertilisation rates, bodyweights, egg number/ females and PGSI values were carried out by one way ANOVA (with Tukey post hoc test) in SPSS v22 for Windows. Treatment means were compared using $p < 0.05$ for significance.

3. Results

In the first two experiments (E1 and E2), we examined the biological activity or fertilisation capability of artificially inseminated spermatozoa injected into the ovaries at different times before gamete stripping (ovulation time). We determined the fertilisation rate of eggs as well as the ratio of developing embryos. As shown in Supplementary Table 1 and Fig. 3, all injected females produced egg batches from which developing embryos emerged at different stages (12 hpf, 24 hpf) at varying rates. Although there were great individual fluctuations in the survival within treatments there was no statistically significant difference among the average survival rates of injected batches during the 5 h–25 h sperm latency period ($p > .05$). When compared to the 36 h and 48 h groups that were analyzed in a different experiment (E2), fertilisation rate at 12 h and hatching rate at 36 h for the 48 h group showed a statistically significant decrease in the latter ($p < .05$; Fig. 3). It would be important to note that one female produced 68.3% fertilisation (24hpf) in the 36 h group. Based on these data, the estimated maximal latency period of biological active spermatozoa was at least 48–50 h.

In the third experiment (E3), we examined the effect of different injected sperm volume on the fertilisation capability. We determined the fertilisation rate of eggs as well as the ratio of developing embryos. As shown in Supplementary Table 2, Fig. 4. all injected females produced egg batches from which emerged embryos at different stages (12 hpf, 24 hpf) developed at varying rates. There was a statistically significant difference in the fertilisation rates among the treated groups (Fig. 3.), the survival rate was statistically higher value in groups of 0.5 mL sperm/BW kg compared to other treated and control groups.

In the fourth experimental series (E4), we investigated the location of spermatozoa on the surface of eggs by SEM. Our results clearly revealed that spermatozoa distributed close to micropyle region of African catfish oocytes that were obtained from sperm-injected ova (Fig. 5). The density of sperm cells could be very different, even between eggs originating from same ovum (Fig. 5A-B). In a sperm-injected female, two spermatozoa were found to have entered the micropyle and one farthest down the canal, just before the ooplasmic surface of the animal pole (Fig. 5C-D). There were no spermatozoa seen in the vicinity

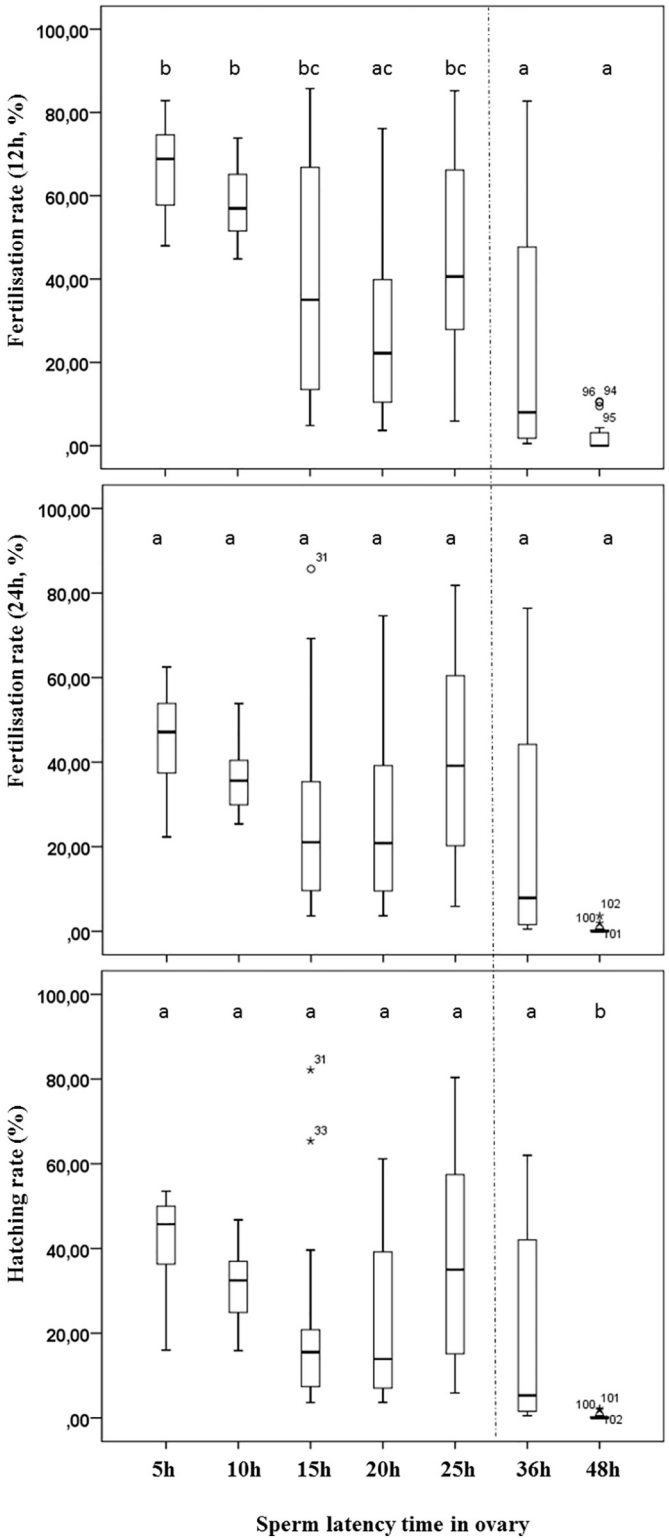

Fig. 3. Box plot graphs of fertilisation (12 and 24 hpf) and hatching rate (36 hpf) values of different treated groups in E1-E2. Different letters in the superscript indicate significant differences ($p \leq .05$, one way ANOVA, Tukey posthoc test) in the investigated parameters. The smallest and largest data values label the endpoints of the axis. The first quartile marks one end of the box and the third quartile marks the other end of the box. Approximately the middle 50% of the data fall inside the box. The "stars" extend from the ends of the box to the largest data values. The horizontal lines inside the boxes indicate the average values.

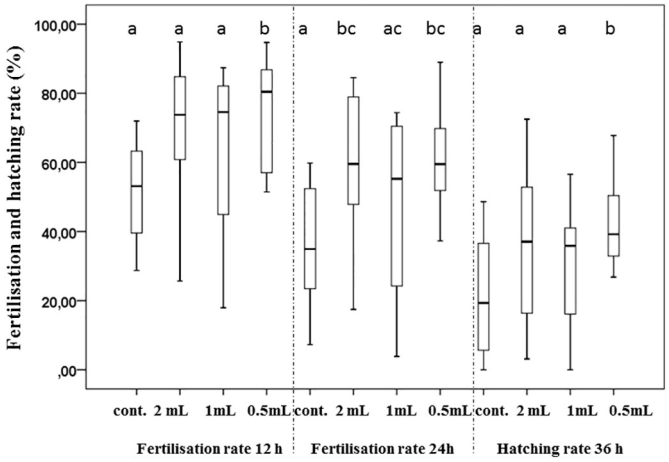

Fig. 4. Box plot graphs of fertilisation rate at 12 and 24 hpf and hatching rate values of different treated groups in E3. cont. = control, 2 mL = 2 mL sperm / BW kg, 1 mL = 1 mL sperm / BW kg, 0.5 mL = 0.5 mL sperm / BW kg. The smallest and largest data values label the endpoints of the axis. The first quartile marks one end of the box and the third quartile marks the other end of the box. Approximately the middle 50% of the data fall inside the box. The "stars" extend from the ends of the box to the largest data values. The horizontal line inside the boxes indicate the average values.

of micropyle region of control African catfish eggs (no lavage; Fig. 5E).

4. Discussion

Our previous experiments indicated that the artificial sperm insemination/sperm ovarian lavage method is effective tool for fish propagation (Müller et al., 2018a, b, 2019, Ittzés et al., 2020). Here we provide additional, repeated confirmations for these earlier observations. According to SEM investigations, we observed signs for 'internal gametic association' (IGA) in African catfish, which were first described in fertilisation pattern of cottids (Munehara et al., 1989) but it occurred another species within the Siluriformes as well, such as driftwood catfish, *T. galeatus* (Ferraris, 2003; Meisner et al., 2000; Santos et al., 2013). Similarly to the observation of Munehara et al. (1989), African catfish spermatozoa after oocyte ovulation entered the micropylar canal of eggs within the ovarian lobe. The spermatozoa stopped just before the ooplasma in ovarian cavity and water activation is needed for spermatozoon-egg fusion. Comparative analysis of externally fertilizing species, the mean number of sperm per stripped ejaculate increases with the mean number of ova released at spawning (Stockley et al. 1996). One of possible explanation is sperm limitation, meaning that sperm numbers was that large numbers of sperm necessary to ensure fertilisation. The other one is the mate-finding cost. It is a very interesting phenomena that altough the African catfish belongs in the group of external fertilised fish species, however artificial semen collection is difficult and the volume and concentration of sperm are very low. In large scale propagation of this species males are killed and isolated testes are macerated to obtain sperm (Viveiros et al., 2000). Considering the small amount of native sperm volume and our recent study where small amount of fish sperm could fertilise relatively large amount of eggs we can suppose that IGA can be artificially repeated and occured in other catfish species as well. It would be really important to monitor the condition of spermatozoa in the ovary *in vivo* to learn more about the process of spermatozoa activation in near future. One interesting aspect of this problem is the distribution of spermatozoa in the ovarian lobe. As previously observed, the seminal plasma with the hormonal agent is absorbed from ovarian lobe to the blood and circulates through ovarian wall (Müller et al., 2018b), thus spermatozoa keep their biological value in ovarian fluid until the last hours. It is supposed that at longer latency periods the relatively small amount of spermatozoa can distribute equally through the whole cavity of ovarian lobe. It taken relatively long time for spermatozoa to find the micropyle region of ovulated but not released egg in ovarian cavity. According to Szabó et al. (2010), African catfish eggs can be maintained in the ovarian fluid for at least 60 min without activation and loss of fertilizing ability, but this result was received at *in vitro* fertilisation under atmospheric conditions. In our experiment, there is a new, not known interaction between spermatozoa vs. ovarian fluid in contrast to sperm (including seminal plasma) vs. ovarian fluid. We showed this new relationship in order to learn more about the capacitation time and fertilisation capability of sperm within the ovarian cavity.

When sperm latency time in ovary was investigated no statistical difference was found of sperm latency period on PGSI, fertilisation rate at 24 h and hatching rate between 5 and 25 h in first experimental cycle. In the second experimental cycle, the fertilisation rate of group of 36 h latency time showed similar result to first one. As expected, the quality of eggs had a more pronounced effect on fertilisation capability than sperm latency period during the first 36 h, indicating that the ovarian lobes helped spermatozoa to maintain their vitality and biological activity. In practice, the optimal time for sperm injection into ovarian lobes is very similar to that of hormonal injection; 10 h before the expected ovulation at 25–27 °C. In this case, anaesthetised females can be handled at same time by using hormonal administration and sperm injection. In fact, one could consider mixing the sperm with maturation hormones as well (Müller et al., 2018b), but the potential conditions for such a combined injection must be tested experimentally.

When the potential effect of different sperm volumes was tested (E3), no statistical differences of effectiveness were found among the control (traditional propagation method) and artificial sperm insemination method. It is important to note that despite our efforts we could not ensure the same environmental conditions among the control and sperm-injected groups. In order to check the sperm quality right after the collection and for pooling the sperm samples, we had to use up some of them for fertilisation tests. For that, maturation had to be induced in control females 10 h before sperm injection. The maturation process in control fish happened during daytime, while sperm-treated fish were maturing during the night. It seems likely that disturbing factors (noise, daily work rhythm, filtration cleaning, feeding, etc. in neighbouring rooms) influenced the success of final maturation of oocytes, which resulted in lower fertilisation rates in Experiment 3.

Naturally, there are lot of publications about artificial spawning of African catfish by using other types of hormones (Brzuska, 2003, 2011; El-Hawarry et al., 2016); Kovács et al., 2010; Richter et al., 1987). Our fertilisation rate (besides control) was mean ± $SD_{E1, E3}$: 62.1 ± 24%, min-max: 6–94.8% after 12 h of incubation. Fertilisation rate and hatching success of treated fish in our experiments showed lower results when compared to data published by others. All females originated from the stock of an intensive farm, and as such they were not in optimal condition for spawning (nutrition, keeping, etc.). This could explain the lower fertilisation and hatching rates. In this study, average PGSI values were similar across the different experiments with great individual fluctuations (mean E1, E2, E3: 10.8 ± 3.1%, min-max: 2.9–14.5%). This is similar which was reported in literature (9–15% (Brzuska, 2002, 2003; Rurangwa et al., 1998).

According to Rurangwa et al. (1998), the optimal ratio of spermatozoa/egg is 15,000: 1 for artificial insemination of African catfish *Clarias gariepinus*. Below a 3000:1 sperm/egg ratio, fertilisation success decreased significantly. Excessive sperm (> 15,000:1) partially inhibited fertilisation success. Our sperm:egg ratio could only be estimated, because we do not know anything about rest amount of the egg and spermatozoa which remained in ovarian lobes. In case of walleye (*Sander vitreus*) the optimal spermatozoa egg ratio was 25,000:1, higher amount of spermatozoa did not increased the fertilisation success (Rinchard et al., 2005). Our estimated sperm:egg ratio was: 30,377 ± 4,262:1 (2 mL/BW kg), 21,620 ± 14,969:1 (1 mL/BW kg), remarks two females from 6 released small amount of egg batches),

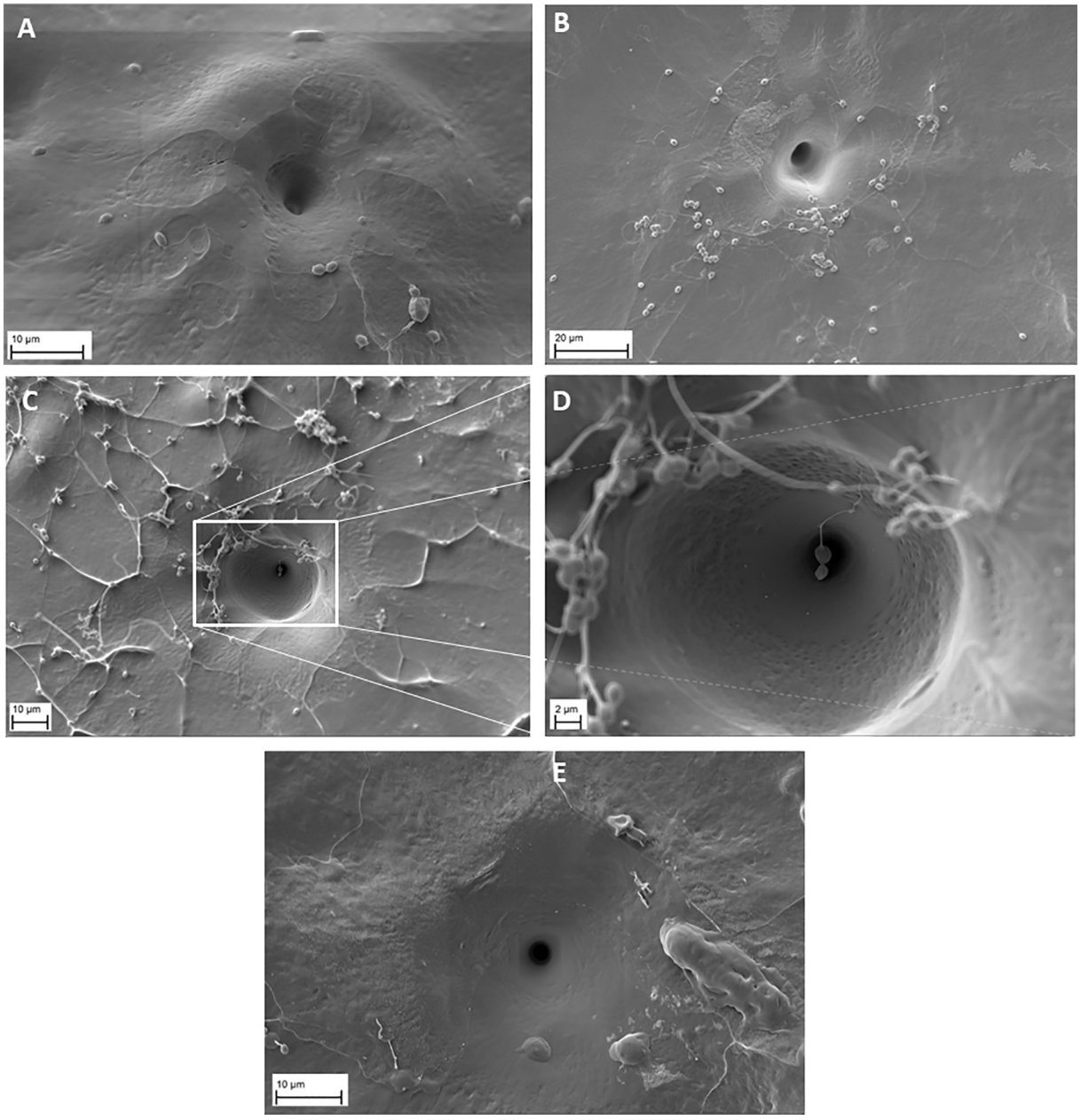

Fig. 5. Scanning electron micrographs reveal that sperm cells enter the mycropylar canal of eggs from ovarian lobules injected with sperm earlier. (A-B): Vastly different number of spermatozoa (arrowheads) are congregating around the external apertures of the micropyles of African catfish eggs originating from the same African catfish ovum. (C) Spermatozoa entering the micropylar canal of an African catfish egg derived from a sperm-injected ovarian lobule. (D) Enlarged image of the micropylar canal with the sperm cells shown on Panel C. (E) No spermatozoa are seen around the external apertures of the micropyle of a control African catfish egg (no lavage).

2418 ± 635:1 (0.5 mL/BW kg). Informatively sperm/egg ratio was estimated in our study, which was 2.418:1 in lowest sperm injection but in contrast the Rurangwa et al. (1998) there was no statistically differences for other groups where the sperm/egg ratio was higher.

In vitro fertilisation is the most appropriate method for fish propagation in several situations, including breeding programs and intra- and interspecific hybridisation. It is also the only feasible method for using manipulated (cryopreserved or genetically modified) sperm for fertilisation. However, in many cases predicting the time of ovulation is very difficult, leading to large fluctuations in the quality of stripped eggs. For this reason, induced spawning, which is more accurately timed, is preferred and used in practice for several fish species (e.g. pikeperch, channel catfish, seabass and seabream) (Müller et al., 2018a). Our improved method can combine the simplicity of induced spawning, with a less time-dependent delivery of the sperm compared to conventional in vitro fertilisation. An advantage of ovarian lavage with sperm and hormone preparations could also be in the fields of aquaculture management where it is important to maintain or increase genetic diversity. For instance, in propagation of fish species that exhibit pairwise spawning under natural conditions (such as *S. lucioperca* or *Silurus glanis*) mixed sperm samples from multiple males could be used to fertilise eggs during induced spawning.

Further investigations are needed to adapt this method to other fish species, as well as to reveal the relationship between the fertilisation capacity of the sperm and the incubation time in the ovary. Could this time period be increased further without substantial loss of fertilisation capacity of the sperm maintained in the ovum, then the protocol of propagation in farm conditions can be beneficially extended. Our

approach may help to increase the success rate of artificial propagation when the timing and synchrony of egg production is critical for practical reasons. This may be the case for both induced spawning in ponds (of economically relevant farm fish species) or in natural waters (for management of fish populations or for endangered fish species) as we described in a former study (Müller et al., 2018a).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735109.

Declaration of Competing Interest

None of the authors have any conflict of interest to declare.

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References

Abe, T., Munehara, H., 2005. Spawning and maternal-care behaviours of a copulating sculpin, *Radulinopsis taranetzi*. J. Fish Biol. 67, 201–212. https://doi.org/10.1111/j.0022-1112.2005.00728.x.

Brzuska, E., 2002. Artificial spawning of African catfish, *Clarias gariepinus*: stimulation of ovulation using carp pituitary or Ovopel. J. Appl. Aquac. 12, 13–22. https://doi.org/10.1300/J028v12n04.

Brzuska, E., 2003. Artificial propagation of African catfish (*Clarias gariepinus*): differences between reproduction effects after stimulation of ovulation with carp pituitary homogenate or GnRH-a and dopaminergic inhibitor. Czech J. Anim. Sci. 49, 289–296.

Brzuska, E., 2011. Stimulation of ovulation in African catfish *Clarias gariepinus* (Burchell 1822) following treatment with carp pituitary homogenate, Ovopel or Dagin. Pol. J. Natur. Sci 26, 121–137.

El-Hawarry, W.N., Abd El-Rahman, S.H., Shourbela, R.M., 2016. Breeding response and larval quality of African catfish *(Clarias gariepinus*, Burchell 1822) using different hormones/hormonal analogues with dopamine antagonist. Egypt. J. Aquat. Res. 42, 231–239. https://doi.org/10.1016/j.ejar.2016.06.003.

Ferraris, C.J., 2003. Family Auchenipteridae (driftwood catfishes). In: Reis, R.E., Kullander, S.O., Ferraris, C.J. (Eds.), Check List of the Freshwater Fishes of South and Central America, pp. 470–482. https://doi.org/10.1643/ot-04-142. Edipucrs.

Ittzés, I., Kronbauer, E.C., Szabo, T., Horváth, L., Urbányi, B., Müller, T., 2020. Propagation of jundia *Rhamdia quelen* (Siluriformes:Heptapteridae) by applying the ovarian sperm injection method. Aqua. Rep. (in press). https://doi.org/10.1016/j.aqrep.2020.100275.

Kovács, E., Müller, T., Márián, T., Krasznai, Z., Urbányi, B., Horváth, A., 2010. Quality of cryopreserved African catfish sperm following post-thaw storage. J. Appl. Ichthyol. 26, 737–741. https://doi.org/10.1111/j.1439-0426.2010.01561.x.

Koya, Y., Munehara, H., Takano, K., Takahashi, H., 1993. Effects of extracellular environments on the motility of spermatozoa in several marine sculpins with internal gametic association. Comp. Biochem. Physiol. A Physiol. 106, 25–29. https://doi.org/10.1016/0300-9629(93)90033-Z.

Lin, H.R., Zhang, M.L., Zhang, S.M., Van Der Kraak, G., Peter, R.E., 1991. Stimulation of pituitary gonadotropin and ovarian development by chronic administration of testosterone in female Japanese silver eel, *Anguilla japonica*. Aquaculture 96, 87–95. https://doi.org/10.1016/0044-8486(91)90141-S.

Lin, H.R., Xie, G., Li-Hong, Zhang, Wang, X.D., Chen, L.X., 1998. Artificial induction of gonadal maturation and ovulation in the japanese eel (*Anguilla japonica* T. et S.). Bull. Fr. La Pech. La Piscic. 349, 163–176. https://doi.org/10.1051/kmae:1998041.

Lokman, P.M., Wylie, M.J., Downes, M., Di Biase, A., Damsteegt, E.L., 2015. Artificial induction of maturation in female silver eels, *Anguilla australis*: the benefits of androgen pre-treatment. Aquaculture 437, 111–119. https://doi.org/10.1016/j.aquaculture.2014.11.026.

Meisner, A.D., Burns, J.R., Weitzman, S.H., Malabarba, L.R., 2000. Morphology and histology of the male reproductive system in two species of internally inseminating south american catfishes, *Trachelyopterus lucenai* and *T. galeatus* (Teleostei: Auchenipteridae). J. Morphol. https://doi.org/10.1002/1097-4687(200011)246:2<131::AID-JMOR7>3.0.CO;2-K.

Müller, T., Horváth, L., Szabó, T., Ittzés, I., Bognár, A., Faidt, P., Ittzés, Urbányi, Kucska, B., 2018a. Novel method for induced propagation of fish: sperm injection in oviducts and ovary/ovarian lavage with sperm. Aquaculture 482, 124–129. https://doi.org/10.1016/j.aquaculture.2017.09.025.

Müller, T., Kucska, B., László, H., Ittzés, Á., Urbányi, B., Blake, C., Guti, C., Csorbai, B., Kovács, B., Szabó, T., 2018b. Successful, induced propagation of African catfish (*Clarias gariepinus*) by ovarian lavage with sperm and hormone mixture. Aquaculture 485, 197–200. https://doi.org/10.1016/j.aquaculture.2017.11.051.

Müller, T., Szabó, T., Kollár, T., Csorbai, B., Marinović, Z., Horváth, L., Kucska, B., Bodnár, Á., Urbányi, B., Horváth, Á., 2019. Artificial insemination of African catfish (*Clarias gariepinus*) using cryopreserved sperm. Theriogenology 123, 145–150. https://doi.org/10.1016/j.theriogenology.2018.09.034.

Munehara, H., Takano, K., Koya, Y., 1989. Internal gametic association and external fertilization in the Elkhorn sculpin, *Alcichthys alcicornis*. Copeia 1989, 673. https://doi.org/10.2307/1445494.

Munehara, H., Takano, K., Koya, Y., 1991. The little dragon sculpin, *Blepsias cirrhosus*: another case of internal gametic association and external fertilization. Japan. J. Ichthyol. 37, 391–394.

Muñoz, M., 2010. Reproduction in Scorpaeniformes. In: Cole, K.S. (Ed.), Reproduction and Sexuality in Marine Fishes: Patterns and Processes. Univ. Calif. Press, USA, pp. 65–87.

Németh, Á., Orbán, K., Faidt, P., Horváth, Á., Müller, T., Szathmári, L., Urbányi, B., Horváth, L., 2012. Induction of ovulation in the pikeperch (*Sander lucioperca* L.) by ovarian lavage. J. Appl. Ichthyol. 28, 914–915. https://doi.org/10.1111/jai.12067.

Osman, A.G.M., Wuertz, S., Mekkawy, I.A., Verreth, J., Kirschbaum, F., 2008. Early development of the African catfish Clarias gariepinus (Burchell, 1822), focusing on the ontogeny of selected organs. J. Appl. Ichthyol. 24, 187–195.

Ragland, H.C., Fischer, E.A., 1987. Internal fertilization and male parental care in the scalyhead sculpin, *Artedius harringtoni*. Copeia 4, 1059–1062.

Richter, C.J.J., Viveen, W.J.A.R., Eding, E.H., Sukkel, M., Rothuis, A.J., Van Hoof, M.F.P.M., van den Berg, F.G.J., Van Oordt, P.G.W.J., 1987. The significance of photoperiodicity, water temperature and an inherent endogenous rhythm for the production of viable eggs by the African catfish, *Clarias gariepinus*, kept in subtropical ponds in Israel and under Israeli and Dutch hatchery conditions. Aquaculture 63, 169–185. https://doi.org/10.1016/0044-8486(87)90069-X.

Rinchard, J., Dabrowski, K., Van Tassell, J.J., Stein, R.A., 2005. Optimization of fertilization success in *Sander vitreus* is influenced by the sperm:egg ratio and ova storage. J. Fish Biol. 67, 1157–1161. https://doi.org/10.1111/j.0022-1112.2005.00800.x.

Rurangwa, E., Roelants, I., Huyskens, G., Ebrahimi, M., Kime, D.E., Ollevier, F., 1998. The minimum effective spermatozoa: egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilization ability in *Clarias gariepinus*. J. Fish Biol. 53, 402–413. https://doi.org/10.1006/jfbi.1998.0711.

Santos, H.B., Arantes, F.P., Sampaio, E.V., Sato, Y., 2013. Artificial reproduction and reproductive parameters of the internally inseminated driftwood catfish *Trachelyopterus galeatus* (Siluriformes: Auchenipteridae). Ichthyol. Res. 60, 142–148. https://doi.org/10.1007/s10228-012-0324-9.

Sato, N., Kawazoe, I., Shiina, Y., Furukawa, K., Suzuki, Y., Aida, K., 1995. A novel method of hormone administration for inducing gonadal maturation in fish. Aquaculture 135, 51–58. https://doi.org/10.1016/0044-8486(95)01007-6.

Sato, N., Kawazoe, I., Suzuki, Y., Aida, K., 1997. Development of an emulsion prepared with lipophilized gelatin and its application for hormone administration in the Japanese eel *Anguilla japonica*. Fish Physiol. Biochem. 17, 171–178.

Sherwood, N.M., Crim, L.W., Carolsfeld, J., Walters, S.M., 1988. Sustained hormone release. I. Characteristics of in vitro release of gonadotropin-releasing hormone analogue (GnRH-A) from pellets. Aquaculture 74, 75–86. https://doi.org/10.1016/0044-8486(88)90088-9.

Stockley, P., Gage, M.J.G., Parker, G.A., Moller, A.P., 1996. Female reproductive biology and the coevolution of ejaculate characteristics in fish. Proc. R. Soc. B Biol. Sci. 263, 451–458. https://doi.org/10.1098/rspb.1996.0068.

Szabó, T., Erdei, P., Urbányi, B., 2010. Delayed in vitro fertilization of African catfish (*Clarias gariepinus*) and common carp (*Cyprinus carpio*) eggs in ovarian fluid of African catfish and rainbow trout (*Onchorhynchus mykiss*). J. Appl. Ichthyol. 26, 823–825. https://doi.org/10.1111/j.1439-0426.2010.01559.x.

Van Oordt, P.G.W.J., Goos, H.J.T., 1987. The African catfish, *Clarias gariepinus*, a model for the study of reproductive endocrinology in teleosts. Aquaculture 63, 15–26. https://doi.org/10.1016/0044-8486(87)90058-5.

Viveiros, A.T.M., So, N., Komen, J., 2000. Sperm cryopreservation of African catfish, *Clarias gariepinus*: cryoprotectants, freezing rates and sperm: egg dilution ratio. Theriogenology 54, 1395–1408.

Watson, C., Wood, A., Graves, J.S., 2009a. New technique for administration of human Chorionic Gonadotropin – ovarian lavage. 2009. Aquaculture America, Seattle, pp. 394 15-18.02.2009. Book of abstracts.

Watson, C.A., Hill, J.E., Graves, J.S., Wood, A.L., Kilgore, K.H., 2009b. Use of a novel induced spawning technique for the first reported captive spawning of *Tetraodon nigroviridis*. Mar. Genomics 2, 143–146. https://doi.org/10.1016/j.margen.2009.04.004.

Zaucker, A., Bodur, T., Roest Crollius, H., Hadzhiev, Y., Gehrig, J., Loosli, F., Watson, C., Müller, F., 2014. Description of embryonic development of spotted green pufferfish (*Tetraodon nigroviridis*). Zebrafish 11, 509–517. https://doi.org/10.1089/zeb.2014.0984.