HUNGARIAN UNIVERSITY OF
AGRICULTURE AND LIFE SCIENCES

Natural spawning behaviour and induced spawning by using the novel fish propagation method of African catfish (*Clarias gariepinus*)

The Thesis of the PhD dissertation

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Gödöllő

2022

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1. INTRODUCTION

Aquaculture has played a significant food source for humans due to a high growth rate in world aquatic production, accounting for 52 percent of human fish consumption in 2016-2018. The productive proportion will continue to grow rapidly. Likewise, the production of the African catfish (*Clarias gariepinus*) also quickly increased since the early 2000s and reached about 248.208 tonnes in 2015. Considering the relatively low production costs of this species, low environmental requirements for breeding as well as the high meat quality, further intensive production growth should be expected.

Even through its location in Middle Europe, which is not ideal for producing warm water fish, Hungary is one of Europe's largest the African catfish producers due to the thermal water resources of the Carpathian Basin. *C. gariepinus* is produced in the second largest volume in Hungary, following the carp. The total African catfish production in intensive systems was 4.051 tonnes in 2020. With this production, Hungary is marked leader in the European Union. After 30 years of introducing this fish species, it can be concluded that the African catfish is the most important fish in the country's intensive systems. The position as mentioned earlier can only be maintained through continuous innovation, for which research projects involving domestic producer organisations and foreign partners are essential. One such element is the technological development of species propagation.

Despite the economic importance of the African catfish, its reproductive biology/spawning characteristics have not been fully revealed. The initial breeding behaviour of the species was described under hatchery and wild observation. However, the highlights of the mating during the 'amplexus' have not been shown, even though previous literature confirmed that the male discharges sperm during the pair fish remains motionless. The maturation and ovulation of female *C. gariepinus* typically have to be managed with exogenous reproductive hormones in captivity conditions. A variety of hormonal approaches have therefore been successfully performed to induce the maturation and ovulation of *C. gariepinus*, and also in hybridization of *C. gariepinus* and *H. longifilis*.

IVF is commonly managed extensively to produce mass production in aquaculture. In addition, IVF is the most appropriate method in many cases, such as breeding programs and intraspecific and interspecific hybridisation. Evidence that it contains several limitations in fish reproductive management. Ideally, after the hormonal injection of brood fish, it is returned to the pond, tank, or hapa to spawn spontaneously, even if development has been

hormonally persuaded. It would be essential to find a method of simplicity that combines artificially induced ovulation and spontaneous spawning in the hybrid catfish reproductive industry.

Early studies from MATE researchers demonstrated ovarian hormonal injection as a non-invasive method, which was proven to decrease the drawbacks of traditional approaches and successfully induced maturation and ovulation in several species. The further developed method as ovarian sperm injection was successfully conducted. The used method promises to (a) improve the fish hybridization techniques; (b) sustain the genetic diversity of the propagated species; (c) less time-dependent delivery of the sperm; (d) implications for economically important fish species induced spawning in spontaneous condition. Noticeably, C. *garepinus* females could store the artificially injected spermatozoa in the ovarian lobe, but there is no further information about the spermatozoa in the ovary during ovulation.

2. OBJECTIVES

Several expected objectives will be carried out in this research:
1. To find out the oral uptake of sperm bundles by female *C. gariepinus* during spontaneous mating.
2. To compare the reproduction effectiveness for induced spawning of *C. gariepinus* by using invasive and non-invasive in connection to different vehicles.
3. To test the practical application of inseminated sperm method for production of interspecific hybrids (*C. gariepinus* × *H. longifilis*).
4. To investigate the potential latency period of sperm in the ovary of African catfish (*C. gariepinus)*.

3. MATERIALS AND METHODS

3.1. Chapter 1. The oral uptake of sperm bundles by females African catfish (*C. gariepinus*) during spontaneous mating

3.1.1. Video recording of spawning in tank

The spawning experiments were recorded in a giant Zuger jar (conical up-welling, 200 L). Two waterproof cameras were employed to record the spawning, one in the conus of the jar (GoPro Hero 3 plus Silver, GoPro Inc, USA) and the other slightly below the water surface (GoPro Hero 6 Black, GoPro Inc, USA). After the first observation of the spawn, the camera was installed. A transparent plastic screen was inserted at the end of the cone of the jar to provide adequate space

between the camera and the fish to record the spawning behaviour accurately. The flow rate was 390 L/hour to maintain water quality (T = 25-26 °C). Three lamps (2 × G13/18W/230 V IP65) were used for video lighting.

Videos were collected of six spawning pairs, although one female did not release eggs during mating. The GoPro 3 and GoPro 6 cameras could record videos for 1.5 to 2 hours. During this time, periods of spawning phases and were only observed after eggs were ejected and fertilized (n = 5 pairs, $\Sigma_{\text{successful egg releasing}} = 34$, the total spawning number is 41 times).

3.1.2. Histological preparations

Three males were decapitated after spawning. Part of the testis, proximal and distal parts of the lobe of the seminal vesicle, and genitourinary papilla were fixed in Bouin's fluid. Samples were embedded in paraffin and five μm thick slices stained with hematoxylin-eosin for light microscopy. The number of spermatozoa in seminal versicles was estimated using cross-sectional thicknesses and scales, e.g., 100 μm × 100 μm × 5 μm, n = 3 sampling rectangle/image.

3.1.3. Collection of sperm samples

To assess sperm quality, four males were selected. Male fishes are killed by decapitation as a result of sperm collecting. The whole genital organ was carefully removed from the abdominal cavity without damage, then the testes and seminal vesicles were gently stripped with fingers to collect seminal vesicle secretion (SVS) and semen. All organs were removed from the blood with a surgical towel, and sperm samples were collected in PCR-labeled clean microcentrifuge tubes (0.5 ml). The first eight samples were collected, and the first three samples were eliminated from the study due to urine contamination. After collecting the sperm, the testicles were surgically separated. The testicles were then incised, and the spilled semen droplets were collected in a 2.5 ml Eppendorf tube. Control samples were used for testing within 5 minutes of collection (water activation 0 minutes). Two types of semen were removed from water wells using Micropipette after 1 second, 60 seconds, and 120 seconds from the water immersion. Then, the quality of sperm samples from the testis will be compared with that from mixed semen and SVS after activation.

3.2. Chapter 2. The reproduction effectiveness for induced spawning of *C. gariepinus* by using invasive and non-invasive in connection to different vehicles.

There were two experiments (EI, EII) carried out in this research to conduct various hormonal manipulations and a new vehicle of agent for inducing ovulation of fish. Thirteen males were used in EI, II. Each treatments contained 7 randomly selected females.

3.2.1. Experiment I. (EI):

- Group 1, 2. Ovopel were homogenised in NaCl solution (0.65 %) and given by IM and IP, respectively.
- Group 3. Suspended Ovopel in saline (NaCl 0.65 %) was administrated by ovarian lavage (OHI) method.
- Group 4. Ovopel pellets were homogenised in pooled albumen (ALB, albumen from chicken eggs, n = 3, Aranykorona Co., Székesfehérvár, Hungary) and managed by OHI method.
- Group 5. Powdered pellets of Ovopel were suspended in fish sperm and manipulated by OHSI method.

3.2.2. Experiment II. (EI):

- Group 1. The fishes were conducted as a description in Group 1, EI.
- Group 2. The spawners were conducted as an illustration in Group 1, EI. Parallel with hormone administration, pooled sperm was managed by OSI method.
- Group 3. The breeders were managed as described in Group 5, EI.

3.3. Chapter 3. Practical application of inseminated sperm method for production of interspecific hybrids (*C. gariepinus* × *H. longifilis*)

C. gariepinus brooders were induced to trigger ovulation by intramuscular injection with carp pituitary extract (CPE). Sperm from one specimen of *H. longifilis* was injected into the ovaries of four *C. gariepinus* ♀; females were then placed in pairs with four *C. gariepinus* ♂ in four separate spawning cages. The offspring from each spawning were reared in four replicates for up to 4 weeks to select and separate the genotypes from each other using morphological signs. To compare the ratio of genotypes obtained at the end of breeding to those obtained at the end of rearing, control spawning was also performed. Presumably, the hybridisation rate at the end of juvenile rearing and the initial stage (just after spawning) could be modified due to the interaction between the hybrids and pure *Clarias*; the control rearing groups were settled up. Individuals in the control groups originated from IVF. One female and one male *C. gariepinus* were treated with CPE via an IM, and the female was stripped after the latency period. The egg batch was divided into two

parts, one of which was fertilised with *Clarias* sperm and the other was the *Heterobranchus* sperm. *Heterobranchus* milt originated from the same samples, which were also used for insemination. In the control treatment, the pure and hybrid exogenous feeding larvae from the dry insemination method were randomly collected from each incubation tank and cultured together (50 + 50 larvae) in four replicate tanks. The exogenous feeding larvae of cage spawning pairs were selected from individual incubation tanks and randomly introduced (100 fish / tank, 4 replicates) in rearing tanks. All ontogeny fish were reared for up to day 28 for measurement of rearing parameters.

3.4. Chapter 4. The potential latency period of sperm inseminated into the ovary of African catfish (*C. gariepinus*)

In this study, the optimum latency period to preserve the biological activity/fertilisation capability of inseminated sperm in the ovarian cavity was investigated in two experimental series, EI, and EII. In EI. Five treated groups were set up that consisted 5 females in each group. OSI was done at different times before the expected ovulation; 5-25 hours, 5-hour intervals. In EII. Two trial groups were conducted with five females per group. OSI was managed at 36 hours and 48 hours before the gamete stripping was investigated.

3.5. Statistical analysis

The reproductive factors and rearing parameters were calculated (Chapter 2., 3., and 4.):

1) Ovulated rate = (number of ovulated females / number injected) × 100
2) Fertilisation rate = (number of fertilized eggs / total eggs) × 100
3) Hatching rate = (number of hatched larvae / total eggs) × 100
4) Survival rate = (number of alive fish / total introduced fish) × 100
5) Hybridisation rate (Chapter 3.) = (number of alive hybrid fish / total alive fish) × 100

The obtained data was presented as mean ± standard deviation. The data were analysed by a one-way analysis of variance (ANOVA) and an independent sample t-test. Significance was accepted at $P < 0.05$. The statistical analysis was performed with Microsoft Excel and SPSS 22v or v26 for Windows.

4. RESULTS AND DISCUSSION

4.1. Chapter 1: The oral uptake of sperm bundles by females African catfish (*C. gariepinus*) during spontaneous mating

4.1.1. Spawning behaviour

Up to the amplexus, the mating behaviour of *C. gariepinus* is equivalent to that of the literature. However, the amplexus could be divided into four distinct stages.

Table 4.1. Summarised table about the spawning description in literature and the new findings reported in the presented study.

Phase (Bruton 1979)	**Literature description** (Van de Waal 1974, Bruton 1979)	**New observations** (Recent study)
Amplexus	The male gets ahead of the female, and when she settles down, he folds himself around her head and body. The mating posture, a form of loose amplexus, is usually held for 17–18 sec (42 timings by stopwatch, range 12–20 sec), after which the male suddenly stiffens and arches his body. This action probably accompanies the release of sperm (which are invisible to the naked eye in the field).	I. Position fixation by using fin hugging and barbels.
Sperm release		II. Sperm release and uptake of a sperm bundle by the female.
Egg release	The female now pushes her head forward into the substrate and flicks her tail vigorously for about 2 sec, distributing ova in all directions. Within about 2 sec of egg release, the female swishes its tail vigorously from side to side, using its burrowing snout as an anchor to prevent forward movement, and mixes and distributes the sperm and eggs.	III. Stripping the female by male; fertilisation of the eggs in the water column after the sperm has been discharged via the gill openings.

4.1.2. Histology

Testis: The testicles consist of twisted seminiferous tubules that run in the anterior-posterior direction to the gonad. According to macroscopy and histological characteristics, the males in spawning experiments were in stage 2 (developing, mid-spermatogenic phases). The lateral area of the testicles was white, and the testicular lobules were predominantly filled with developing spermatids and an increased amount of mature spermatozoa.

Seminal vesicle: The seminal vesicle consists of a system of finger-like lobes, each of which contains tubules. These tubules are filled with a fluid that sperm cells are stored. The fluid is secreted by epithelial cells.

Urogenital papillae: The estimated sperm density in the urogenital papillae was 1.2-1.4 $\times 10^6$ cell mL-1 (from histological preparations). Smooth muscle fibers surround the epithelium. No specific sperm bundles were seen on urogenital papillae. The sperm bundle formation can occur when the surface of SV sperm activates with the water.

4.1.3. Sperm quality analysis

The percentage of motile spermatozoa (progressive motility; spermatozoa combined with seminal plasma) has progressively decreased over time. In comparison, stripped sperm samples in which milt was combined with SVS within the first 60 seconds enhanced motilable spermatozoa. At 60 seconds after water activation, there was a statistically significant difference ($P < 0.05$) between the progressive motility measurements of stripped and testis sperm. Following that, the progressive motility estimates for SVS sperm declined in a manner comparable to that of testis sperm.

4.1.4. Discussion

The observations before the release of the gamete can be largely confirmed by our observations. A significant result of our observations is that the male's ejaculation cannot be described as "milt", nor does it have a "watery texture". Rather, it appears in the genital opening of the male as a compact structure, which also shows no signs of dissolution until it disappears, most likely in the female's mouth. These findings raise two consequent elements that need to be illumined, including (1) the responsible function of the male for this bundling and (2) the crucial roles of the female to this bundle altering after it has been picked up.

Likewise, when studying the spawning behaviour of other Siluriformes as *C. aeneus*, that females swallow the released sperm in T-position, which rapidly passes through her intestine, inseminating the eggs collected in the fin pocket created by her pelvic fins. Our observations and reasonable findings indicate that uptaken sperm flow out the buccal cavity of females together with air bubbles to fertilize the eggs in the water channel.

4.2. Chapter 2: The reproduction effectiveness for induced spawning of *Clarias gariepinus* by using invasive and non-invasive in connection to different vehicles.

4.2.1. Reproductive performance

Throughout the first and second trials, all females in all treatment groups ovulated. In all trials, PGSI results were comparable across treatment groups. The hatching rate was significantly lower in treatments, where fish sperm was introduced to the ovary, whether it contained Ovopel (Group 5 in EI and Group 3 in EII) or not (Group 2 in EII) (Table 4.2).

Table 4.2. Data from EI and II on the bodyweight of females, the relative number of stripped eggs, and *hatching* rate in different treatment groups. For each experiment, means with the same superscript in each column are similar to each other ($P > 0.05$).

	Groups	Treatments	Statistics	Body weight (g)	PGSI (%)	Hatching rate (%)
Experiment I.	Group 1.	IM	Mean±SD	829.1±106.8	15.1±2.1	55.1±16.1[a]
			Min-max	700-1024	11.5-17.2	31.3 -70.8
	Group 2.	IP	Mean±SD	864.3±97.3	14.5±2.9	66.7±9.3[a]
			Min-max	764-1074	9.5-18.8	51- 79.2
	Group 3.	NaCl	Mean±SD	828.6±134.2	14.6±2.9	63.4±9.0[a]
			Min-max	692-1088	10.3-18.1	50-76
	Group 4.	ALB	Mean±SD	856.6±116.8	14.4±2 .7	65.0±13.1[a]
			Min-max	726-1054	8.9-17.1	50-83.3
	Group 5.	OHSI	Mean±SD	849.7±73.3	14±2.2	39.1±18.3[b]
			Min-max	748-942	10-16.9	4.2.55.2
Experiment II.	Group 1.	IM	Mean±SD	883.7±172	11.2±1.5	61.9±12.0[a]
			Min-max	668-1126	8.5-12.7	46.9-81.3
	Group 2.	IM+OSI	Mean±SD	765.1±152.6	12.6±3.5	46.0±9.2[b]
			Min-max	606-1078	9.2-17.6	29.3-59.3
	Group 3.	OHSI	Mean±SD	800.7±155.8	12±1.6	35.1±21.2[b]
			Min-max	678-1040	10.1-14.6	10.4.67.7

4.2.2. Discussion

During the EI and EII, PGSI data were also similar between treated groups in both experiments. In EI, the mean PGSI values ranging between 13.7% and 15.1% were similar among the independent treatments from the routes of administration.

The hatching rate was significantly lower for treatments where fish sperm combination Ovopel was introduced into the ovary. At the

same time, the parameter was similar when fish sperm was managed without Ovopel. The IM, IP, NaCl, and ALB treatments of EI produced the highest percentage of hatching. The OHSI treatment resulted in a significantly lower hatching rate than those from the other administration methods ($P < 0.05$). In EII, the IM+OSI and the repetition of OHSI treatment were performed with the lower hatching percentage, whereas the IM treatment resulted in a greater one, as shown in EI. The results of the inseminated groups in both experiments were lower than in the study, where CPE was suspended in sperm for ovarian lavage. The reason for this difference could be that hormone preparation Ovopel contains other components such as lactose, dextrose, calcium, and magnesia compounds, which may have negative effects on sperm viability. These temperatures could have negative effects on the viability of sperm in the ovarian lobe.

We hypothesized that native avian egg albumin injected noninvasively into the fish ovary by catheter would be play role in improving the physiological processes of preovulated oocytes, resulting in an increase in the number of ovulated oocytes. According to the findings of the experiments, albumin was a suitable hormone vehicle, however, there was no increase in relative egg production. It was not able to improve the hatching rate statistically compared to standard hormone treatments, but there was no harmful impact from the application of avian albumen.

4.3. Chapter 3: Practical application of inseminated sperm method for production of interspecific hybrids (*C. gariepinus* × *H. longifilis*)

4.3.1. Hybridisation performance

Three of the four spawning pairs were successful. Unsuccessful mating could be explained by several reasons: the unmaturation phase of the treated female, health status, improper pair (female or male) and consistent water level in the spawning tank.

A novel hybridisation technique was successfully established between the *C. gariepinus* and the *H. longifilis*. There were no statistical differences between the fertilisation capability of sperm samples, which were used in the control experiment ($P < 0.05$). As a result, in IVF trial, fertilisation rate and hatching rate were 81.35 ± 1.62% and 77.23 ± 0.87%, respectively, in *C. gariepinus* × *C. gariepinus*; 74.92 ± 6.6% and 71.1 ± 11.29%, respectively, for hybridisation. Therefore, the fertilisation ability of *H. longifilis* sperm was suitable for the ovarian insemination test in this study. The

fertilisation and hatching rates in the spawning cages were not investigated in this test.

At the end of the raising period at day 28, considering the survival rate, the independent sample t-test revealed no significant difference between the hybrid and pure ratios of the control groups ($t = 0.385$, $P = 0.714$; Table 4.3).

Table 4.3. Summarised data of *C. gariepinus* and hybrid catfish of the control group. Survival rates (%) are presented as means and standard deviations (±S.D.).

Survival rate (%)	**Control group (n = 4)**		
	Total	***C. gariepinus***	**Hybrid catfish**
Mean ± S.D.	43 ± 12.19	45.79 ± 6.82	54.21 ± 6.82
(min - max)	(23 - 55)	(34.78 - 52)	(48 - 65.22)

Thus, fertilisation rates also showed post-spawning fertilisation hybridisation rates. Besides, according to the post hoc tests, all treated groups (T1–T3) showed significantly higher to total survival rates than that found in the control (Table 4.4).

The hybridisation rate on the day 28 was high in all inseminated groups (98.11 ± 1.59 %); in one case, this rate was 100%. Thus, the male *C. gariepinus* involved in mating had a negligible role in fertilisation (Table 4.4).

Table 4.4. Summarised data of *C. gariepinus* and hybrid catfish of treated groups. Survival rates (%) are presented as means and standard deviations (± S.D.).

Survival rate (%)		**Treated groups**		
		Total	***C. gariepinus***	**Hybrid catfish**
No 1 (n = 4)	Mean ± S.D.	65.25 ± 4.32	2.67 ± 0.6	97.33 ± 0.61
	(min - max)	(61 - 72)	(1.64 - 3.23)	(96.77 - 98.36)
No 2 (n = 4)	Mean ± S.D.	70.5 ± 4.72	0	100
	(min - max)	(64 - 75)		
No 3 (n = 4)	Mean ± S.D.	64.5 ± 5.41	3 ± 1.33	97 ± 1.33
	(min - max)	(58 - 72)	(1.64 - 4.48)	(95.52 - 98.36)
Summarised data	Mean ± S.D.	66.75 ± 5.52	1.89 ± 1.59	98.11 ± 1.59
	(min-max)	(58 - 75)	(1.64 - 4.48)	(95.52 - 100)

4.3.2. Discussion

Induced spawning hybridisation using the sperm insemination method was successfully managed, and the hybrid ratio was 95.5–100% from the investigated offspring. Because the hybridisation rate was similar at mating based on control experimental results, the role of the *C. gariepinus* male was only in the spawning ethology. These results were also supported in our previous works, in which *C. gariepinus* females had been inseminated with *C. gariepinus* sperm. After gamete stripping, the egg batches were divided into two parts. One was immediately activated with water, and in the other, freshly collected sperm was added. There were no statistical differences in fertilisation between the two egg batches; fresh sperm could not increase the fertilisation rate. In this test, sperm was injected equally up to the end of both ovarian lobes. The ovarian storage spermatozoa of *H. longifilis* could be as close as some μm to the micropyle or in it. Therefore, the released eggs and water transported to *C. gariepinus* spermatozoa had a significantly lower chance for fertilisation in the same eggs.

The presented results also confirm that by injecting sperm from different species into ovaries, hybrids can be produced through induction reproduction and gamete production. To our knowledge, this is the first report of the use of OSI as a novel method for distance hybridisation of fish in induced mating / tank spawning / pen spawning.

4.4. Chapter 4: The potential latency period of sperm inseminated in to the ovary of African catfish (*C. gariepinus*)

4.4.1. Reproductive performance

There were no statistically substantial changes between any parameter pairings within the treated groups in EI and EII. However, mean body weights were significantly different between the two treatments using one-way ANOVA with Dunn's post hoc analysis at $P < 0.05$ (Table 4.5).

As illustrated in Table 4.5, all injected females released egg batches from which developing embryos developed at different ratios 12 hpf. There were huge individual fluctuations in the survival within treatments, and there was statistically significant difference in the mean survival rates of injected batches over the 5–25 hour sperm latency period ($P < 0.05$). Similarly, there were statistically significant difference in the hatching rates within treatments in both EI and EII ($P < 0.05$). After 48 hours, fertilisation and hatching rate dropped. According to these results, the physiologically active spermatozoa had a maximum latency time of at least 48–50 hours.

Table 4.5. Summary table of the relationship between the time sperm spent in the ovary and the measured reproductive parameters of sperm-injected fish. Different letter signs indicate a significant difference between treatments for the same parameter (Experiment I: $P < 0.05$, ANOVA, Dunn's post hoc test. Experiment II: independent samples t test $P < 0.05$).

Experimental series	Sperm latency times (hours)	Bodyweight (g)	PGSI (%)	Fertilisation rate (%)	Hatching rate (%)
		Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Experiment I.	5	690,5 ± 259,0	10,8 ± 3,9	65,7 ± 11,3^{a}	39,3 ± 12,7^{a}
	10	784,1 ± 139,2	9,5 ± 2,3	57,9 ± 8,8^{ab}	31,6 ± 9,1^{ac}
	15	648,4 ± 248,7	8,6 ± 2,8	41,1 ± 29,0^{bc}	21,8 ± 23,1^{bc}
	20	617,2 ± 168,7	9,6 ± 3,3	29,8 ± 26,3^{c}	24,2 ± 20,9^{bcd}
	25	762,5 ± 145,4	8,8 ± 4,1	44,6 ± 25,5^{bc}	36,9 ± 24,3^{ad}
Experiment II.	36	458,6 ± 116,8	12,6 ± 3,0	26,5 ± 33,7	19,8 ± 24,1^{a}
	48	483,7 ± 183,7	11,7 ± 2,4	2,5 ± 4,4	0,4 ± 0,7^{b}

4.4.2. Discussion

Previously conducted research has shown that artificial sperm fertilization/sperm ovarian lavage is an efficient technique for fish reproduction. Both of the above experiments supported and confirmed these earlier results. As stated previously, seminal plasma carrying the hormonal substance is absorbed into the bloodstream by the ovarian lobes and circulates throughout the ovarian wall, enabling spermatozoa to remain biologically active in the ovarian fluid until fertilisation. It is assumed that over longer durations of latency, the comparatively tiny number of spermatozoa may distribute equally via the ovarian lobe cavity. Spermatozoa require a relatively long time to get the micropyle location of an ovulated but unreleased egg in the ovarian cavity. In this investigation, there is a novel, previously unknown interaction between sperm and ovarian fluid, as opposed to sperm vs ovarian fluid. This new relationship must be revealed to learn more about the capacity time and ability of sperm to fertilize in the ovarian cavity.

In this study, there was no significant difference in sperm latency period on PGSI. Besides, there was huge individual fluctuations in the fertilisation and hatching rate within treatments, and there were statistically significant difference in both parameters of injected batches. As predicted, egg quality had a greater effect on fertilisation potential than sperm latency during the first 36 hours, indicating that ovarian lobes assisted sperm in maintaining vitality and biological activity. In practice, the optimal time to inject sperm into the ovarian lobes is equivalent to a hormone injection: 10 hours

before the projected ovulation at 25–27 ºC. In this situation, anaesthetized females could be treated with hormonal therapy and sperm injection simultaneously. In reality, combining sperm with maturation hormones may be proposed, but the feasible circumstances for such a combination injection must be investigated experimentally.

As a result of the more accurate time prediction of ovulation, induced spawning is recommended and employed in practice for various fish species. In comparison to conventional IVF, our improved method combines the simplicity of induced spawning with a less time-dependent distribution of the sperm. Ovarian lavage with sperm and hormone preparations may also be advantageous in the sector of aquaculture management, where it is critical to maintain or enhance genetic diversity.

5. CONCLUSIONS AND SUGGESTIONS

The outcomes in Chapter 1 suggest that the reproductive strategy of *C. gariepinus* is probably equivalent in other *Clarias* species, in which one sperm bundle per spawning event is delivered and it's more or less directed onward transport to the eggs via the oral cavity, the gill openings and the respiratory stream, guarantees economical handling of the available sperm. The sperm and egg will meet in a small water channel, which may create a controlled fertilisation area in open water. At present, we can only speculate about the evolution of such a complex process as delivering sperm to the eggs via the mouth. It is conceivable that the need to force the partner into a certain position, which could have led to the male's mouth coming close to the female's gonopore. The short-term packaging of the sperm cells necessary for an effective oral uptake was already predetermined by the properties of the secretions of the seminal vesicles. Further researches should be extended to other *Claridae* species and the sperm competition in water column after coagulated semen discharge.

These findings in Chapter 2 indicated that sperm, egg albumen and saline were tailored vehicles of Ovopel to induce reproduction of this fish by OI method. Totally, the effects of artificial reproduction do not differ between traditionally invasive and non-invasive methods, except sperm insemination method. This gives an opportunity to improve the welfare of African catfish spawners during artificial spawning

In Chapter 3, the current results revealed novel feasibility of cross-breeding between *C. gariepinus* ♀ and *H. longifilis* ♂. Hormonal injected *C. gariepinus* female had received semen from *H. longifilis* by OSI method can propagate with hormonal stimulated *C.*

gariepinus male in spawning cage/tank circumstance to produce hybrid offspring. The overall average hybrid percentage was hight (98.11±1.59 %). Additional research is needed to extend this method in other highly commercial value species in the aquaculture industry and reveal the approach for both crosses in other artificially spontaneous conditions or natural waters.

The time-dependent fertilizing capacity of sperm that were introduced into ovary was proven in Chapter 4. We indicated that sperm could store in gonad lobes 5, 10, 15, 20, 25, 36 and 48 hours; the optimal time to inject sperm into the ovarian lobes is 10 hours before the projected ovulation at 25–27 ºC. The estimated maximal latency period of active biological spermatozoa was at least 48-50 hours. 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 or in natural waters, as we described in such former studies.

6. NEW SCIENTIFIC FINDINGS

1. At present, we can only theorize about the evolution of such a complicated system as delivering sperm to eggs via the mouth. It is feasible that the need to force the mate into a specific position, which could have resulted in the male's mouth coming close to the female's gonopore, as seen in numerous Siluriformes, was essential to synchronize the pairing or to avoid sneaking. The properties of the seminal vesicles' secretions predetermined the short-term packaging of sperm cells required for effective oral uptake.

2. Albumen, sperm, and saline can be used as vehicles of Ovopel to induce reproduction of the African catfish using the OI method. The effects of artificial reproduction in *C. gariepinus* were not different from the use of traditionally invasive methods versus non-invasive methods, in which albumen and saline were used as the solvents.

3. The hormonally injected female of *C. gariepinus* received semen from *H. longifilis* by OSI manipulation can propagate with hormonal stimulated male of *C. gariepinus* in cage/tank spawning to produce hybrid offspring. Overall, the proportion of hybrids was high (98.11±1.59 %) and can obtain 100%.

4. The sperm can conserve 5, 10, 15, 20, 25, 36, and 48 hours in the ovaries. At least 48–50 hours were the maximum latency duration of biologically active spermatozoa. Nevertheless, in the 5–25-hour treatment groups, we observed a significant individual decrease in fertilisation and hatching ratios after 15-hour sperm stored in the ovarian lobes. Similarly, from 36–48 hours, the treated group demonstrated a deep decrease in fertilisation and hatching rate, indicating a loss of fertilising capacity

7. PUBLICATIONS

7.1. Publications in connection of the dissertation

Articles:

1. Quyến, N.N., Alebachew, G.W., Kucska, B., Kovács, G., Halasi-Kovács, B., Ferincz, Á., Staszny, Á., Horváth, L., Urbányic, B., Müller, T. (2022). Model experiment for practical application of inseminated sperm method for production of interspecific hybrids (*Clarias gariepinus* × *Heterobranchus longifilis*). Aquaculture Reports 27, 101418.
2. Kucska, B.*, Quyến, N.N.*, , Szabó, T., Gebremichael, A., Alabachew, G.W., Bógó, B., Horváth, L., Csorbai, B., Urbányi, B., Kucharczyk, D., Keszte, Sz., Müller, T. (2022). The effects of different hormone administration methods on propagation successes in African catfish (*Clarias gariepinus*). Aquaculture Reports 26, 101311 * These authors have contributed equally to the results presented in this paper.
3. Alebachew G. W., Quyến, N. N., Urbányi, B., Horváth, L. (2022). Ovarian lavage methods of fish propagation: a mini review on sperm artificial insemination and/or hormone delivery into the ovary. AACL Bioflux 15, 2181- 2190.
4. Müller, T., Ács, E., Beliczky, G., Makk, J., Földi, A., Kucska, B., Horváth, L., Ittzés, A., Hegyi, A., Szabó, T., Urbányi, B., Quyến, N.N., Orbán, L., Havasi, M. (2020). New observations about the fertilisation capacity and latency time of sperm inseminated into the ovary of African catfish (*Clarias gariepinus*), an oviparous modelfish. Aquaculture 522, 735109.

Proceedings:

1. Müller, T., Beliczky, G., Kucska, B., Horváth, L., Ittzés, Á., Hegyi, Á., Szabó, T., Quyến, N.N., Urbányi, B., Orbán, L., Havasi, M. (2019). New data about African catfish (*Clarias gariepinus*, Burchell) propagation by using ovarian lavage with sperm method, in: Innovation Challenges in the 21st Century : LXI. Georgikon Napok International Scientific Conference. Pannon Egyetem Georgikon Kar, Keszthely, Hungary, pp. 66–66.
2. Quyến, N.N, Alebachew, G.W., Kucska, B., Kovács, G., Halasi-Kovács, B., Ferincz, Á., Staszny, Á., Horváth, L., Urbányic, B., Müller, T. (2021). Hibridelőállítás indukált ivatásos-, és inszemináci módszerrel Clarias Gariepinus (inj. *Hertobranchus longifilis* sperma) *C. gariepinus,* in: Fisheries & Aquaculture

Development Vol 38. MATE AKI HAKI, Szarvas, Hungary, pp. 41–42.

3. Quyến, N.N., Alebachew, G.W., Kucska, B., Kovács, G., Halasi-Kovács, B., Ferincz, Á., Staszny, Á., Horváth, L., Urbányi, B., Müller, T. (2021). The novel method for induced hybridization in spontaneous spawning: *Clarias gariepinus* ♀ × *Heterobranchus longifilis* ♂, in: 7th Istanbul Scientific Research Congress. p. 1.
4. Quyến, N.N., Pataki, B., Kitanović, N., Ákos, H., Havasi, M., Keszte, Sz., Urbányi, B., Greven, H., Müller, T. (2020). Kísérletek az afrikai harcsa természetes ívási viselkedésének részletes feltárására. In: Nagyné Biró J. (szerk.) Halászatfejlesztés 37 – Fisheries & Aquaculture Development Vol. 37 NAIK HAKI Szarvas, Hungary, pp. 53–54.
5. Müller, T, Quyến, N.N., Getachew, W. A, Bógó, B., Horváth, L., Csorbai, B., Szabó, T., Gebretsadik, A G, Urbányi, B., Kucska, B. (2020). Különböző hormonbejuttatási módszerek hatása afrikai harcsa indukált szaporítása során. Megfigyelések vegyszermentes ikrakezeléssel kapcsolatban In: Nagyné Biró J. (szerk.) Halászatfejlesztés 37 – Fisheries & Aquaculture Development Vol. 37 NAIK HAKI Szarvas, Hungary, pp. 51–52.
6. Müller T, Quyến, N.N., Berta, I, Hoitsy, Gy., Hoitsy, M., Kiss, P., Havasi, M., Csenki, Zs., Urbányi, B., Kucska, B. (2020). Megfigyelések vegyszermentes ikrakezeléssel kapcsolatban In: Nagyné Biró J. (szerk.) Halászatfejlesztés 37 – Fisheries & Aquaculture Development Vol. 37 NAIK HAKI Szarvas, Hungary, pp. 49–50.

Publications not related to the topic of dissertation

Proceedings:

1. Kitanović, N., Marinović, Z., Quyến, N.N., Kovács, B., Müller, T., Urbányi, B., Bernáth, G., Horváth, Á., (2021). *In vitro* maturation and ovulation of African catfish (*Clarias gariepinus*) ovarian follicles, in: 56th Croatian and 16th International Symposium on Agriculture.
2. Kitanović, N., Marinović, Z., Quyến, N.N., Müller, T., Kovács, B., Urbányi, B., Bernáth, G., Horváth, Á. (2021). In vitro production of eggs from immature ovarian follicles of African catfish (*Clarias gariepinus*), in: Aquaculture Europe 21. pp. 634–635.
3. Kitanović, N., Marinović, Z., Quyến, N.N., Müller, T., Kovács, B., Urbányi, B., Bernáth, G., Horváth, Á. (2021). *In Vitro* System for

Maturation and Ovulation of African Catfish Ovarian Follicles. Vitr. Cell. Dev. Biol. 57, 757.

Advisable activities

Scientific Student Associations' Conference (TDK):

1. Bógó Bence (2021). Különböző hormonbejuttatási módszerek hatása az afrikai harcsa indukált szaporítása során. Supervisors: Dr. Tamás Müller, Nguyễn Ngọc Quyến,.Kucska Balázs,
2. Kiss Balázs (2021). Vegyszermentes ikra és lárvakezelés lehetőségei víziászka (*Asellus aquaticus*) felhasználásával. Supervisors: Dr. Kucska Balázs, Dr. Tamás Müller, Nguyễn Ngọc Quyến.

Thesis advice:

1. Alebachew, Getachew Worku (2021). Artificial hybridization in tank/cage spawning between *Clarias gariepinus* ♀ × *Heterobranchus longifilis* ♂ by using sperm. Supervisors: Dr. Tamás Müller, Dr. Kucska Balázs, Nguyễn Ngọc Quyến. MSc thesis.
2. Ehiorobo, Christopher Edosa (2021). Propagation of African catfish (*Clarias gariepinus*) using ovarian lavage method. Supervisors: Dr. Tamás Müller, Dr. Kucska Balázs, Nguyễn Ngọc Quyến. BSc thesis.
3. Bógó Bence (2021). Hévízi törpenövésű vadponty ex situ és in situ konzervációbiológiai kutatások (Ex situ and in situ conservation biological investigations of dwarf carp originating from Lake Hévíz). Co supervisors: Dr. Tamás Müller, Quyến Nguyễn Ngọc, Dr. Ádám Staszny. BSc thesis.