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

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PhD dissertation

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Abbreviations

OM	Oocyte maturation
17OHP	17α-Hydroxyprogesterone
DHP	17α,20β-Dihydroxy-4-pregnen-3-one
DOCA	Desoxycorticosteroid acetate
PG	Pituitary Gland
CP	Carp pituitary
PE	Pituitary extract
CPE	Carp pituitary extract
SPE	Salmon pituitary extract
GnRH	Gonadotropin releasing hormone
GnRHa	Gonadotropin releasing hormone analogues
LHRH	Luteinizing hormone-releasing hormone
LHRHa	Luteinizing hormone-releasing hormone analogs
hCG	Human Chorionic Gonadotropin
DRA	Dopamine receptor antagonist
MET	Metoclopramide
DOM	Domperidone
PIM	Pimozide
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
GtH	Gonadotropin
IM	Intramuscular injection
IP	Intraperitoneal injection
OI	Ovarian lavage injection
OHI	Ovarian hormone injection
OSI	Ovarian sperm injection
OHSI	Ovarian hormone and sperm injection
IVF	*In vitro* fertilisation
CASA	Computer-assisted sperm analysis
PGSI	Pseudo-gonadosomatic index
GSI	Gonadosomatic index
C×B	♀ *Ictalurus punctatus* × ♂ *Ictalurus furcatus*
C×H	♀ *Clarias gariepinus* × ♂ *Heterobranchus longifilis*

1. INTRODUCTION

One of the social needs of the 21st century is to ensure that the ever-growing population of the Earth receives an adequate food supply. Fish and other aquatic foods from both freshwater and saltwater environments are critical for attaining food and nutrition aims (Gephart et al., 2020) and are one primary source of protein (Kucharczyk et al., 2019a). Aquatic foods contribute significantly to food and nutrition security and the future sustainability of healthy diets (Thilsted et al., 2016). 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 (Garlock et al., 2020; FAO, 2020). Likewise, the production of the African catfish (*Clarias gariepinus*) also quickly increased since the early 2000s and reached about 248.208 tonnes in 2015 (FAO, 2021). 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, particularly since it is a warm water species, which can be successfully cultured on a mass scale in different continents, e.g., in Africa, Asia, and Europe (Kucharczyk et al., 2019a).

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 (Sipos et al., 2019). 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, followed by the Netherlands (Lukácsik et al., 2021). 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. Farmers initially worked with *C. gariepinus*, but in the last 10-15 years, a hybridization process with the Sampa (*Heterobranchus longifilis*) has begun. The offspring get bigger, grow faster, and most importantly, mature later than the pure African catfish. The value of these hybrid specimens is extremely high, so it is critical to treat them properly in order to obtain a crosses egg with high efficiency. As mentioned earlier, Hungarian African catfish production is significant in EU. This position 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. In wildlife, the fish can reproduce when responding to environmental changes (Goos and Richter, 1996). The initial breeding behaviour of the species

was described under hatchery and wild observation in Van der Waal (1974) and Bruton (1979a). 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. Due to the lack of environmental conditions under hatchery, *C. gariepinus* is unable to spawn naturally (Amoah et al., 2020). Therefore, artificial reproduction technologies have been developed for fish breeding since the 1970s (Graaf and Janssen, 1996). The maturation and ovulation of female *C. gariepinus* typically have to be managed with exogenous reproductive hormones in captivity conditions (Richter et al., 1987a). A variety of hormonal approaches have therefore been successfully performed to induce the maturation and ovulation of *C. gariepinus* (Richter et al., 1985; Szabó et al., 2007; El-Hawarry et al., 2016; Müller et al., 2020; FAO, 2021), and also in hybridization of *C. gariepinus* and *H. longifilis* (Legendre et al., 1992; Nwadukwe, 1995; Sahoo et al., 2003). Similarly, artificial spawning and insemination have been successfully developed to produce the hybrid progeny between ♀ *Ictalurus punctatus* and ♂ *Ictalurus furcatus* (Myers et al., 2020) to overcome the reproductive barriers between species, allowing commercial production of hybrid catfish embryos (Dunham and Elaswad, 2018).

Artificial reproduction and IVF are commonly managed extensively to produce mass production, as this is an important scheme in aquaculture (Mylonas et al., 2010). In addition, IVF is the most appropriate method in many cases, such as breeding programs and intraspecific and interspecific hybridisation (Müller et al., 2018b). Evidence that it contains several limitations in fish reproductive management is mentioned in Mylonas et al. (2010), Perera et al. (2017), Müller et al. (2018a), and Myers et al. (2020). 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. This enables fish to exercise their regular breeding characteristics and achieve high seasonal fecundity using multiple asynchronous ovarian development spawning (Mylonas et al., 2010). 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. Additionally, physiological saline solution and the inseminated sperm as the vehicles can combine CPE to stimulate fish spawning, but there is no information from other inducing hormones in connection to this propagation method. Regarding the vehicle routes, physiological saline solution or even the inseminated sperm can be the vehicle of the hormones. Besides, the various delivery vehicles are used successfully in other fish. According to the hypothesis originating from Prof. László Horváth, there is a possibility that albumen from avian eggs introduced into fish ovaries may optimize the physiological processes of ovulation-ready oocytes due to the protein composition of albumen may promote OM and the rate of ovulation.

As mentioned earlier, further research should be extended to solve these bottlenecks in *C. garepinus*. This study aimed to explore the spawning behaviour in detail and develop several scientific aspects using the OI method.

2. OBJECTIVES

Several expected objectives will be carried out in this research:

1. To describe the unexplored aspects of spawning ethology in *C. gariepinus*.

2. To compare the reproduction effectiveness for induced spawning of *C. gariepinus* by using invasive and non-invasive in connection to different hormone 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 fertile spermatozoa under ovarian conditions of *C. gariepinus*.

3. LITERATURE REVIEW

3.1. General introduction

3.1.1. Aquaculture production

Aquaculture has played an important food source for humans. The annual contribution of aquaculture is approximately 82.1 million tons of fresh fish (FAO, 2020). Among the varieties of aquaculture fish species, one of the most important aquaculture fish species is the African catfish, *C. gariepinus* (FAO, 2021). The African catfish has attracted global interest as a strong, fast-growing species that survives well in poor conditions, such as high-density reservoirs or wastewater ponds. It has been cultivated in at least 50 countries worldwide, inside and outside its native boundaries (Srimai et al., 2020). Its world aquaculture production has grown rapidly since the early 2000s. In official statistics, Nigeria is the largest producer of the African catfish, followed by the Netherlands, Hungary, Kenya, Syrian Arab Republic, Brazil, Cameroon, Mali, and South Africa; these countries also produce mass production (FAO, 2021).

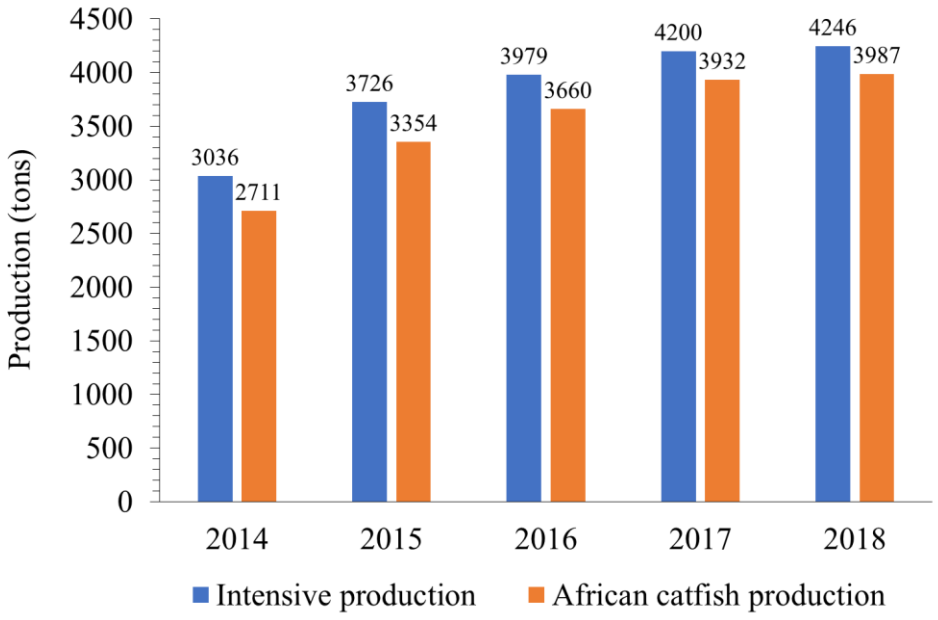

Figure 3.1. Intensive and African catfish production in Hungary between 2014–2018 (Kiss, 2019).

In Hungary, the fish mass production from intensive farms increased steadily from 2014 to 2018, in which the African catfish production accounted for 93.9 % (9.387 tons, Kiss, 2019) (Figure 3.1). The amount of fish produced in the systems was 5.277 tonnes in 2020, of which 4.051 tons was African catfish. The fish production of market size was 3.825 tons. With the production, Hungary was the first in the European Union, followed by the Netherlands with 2.700 tons. The fish size (500–700 grams) can be raised in about six months (Lukácsik et al., 2021).

3.1.2. Taxonomy and Biological features

Taxonomically the *C. gariepinus* belongs to Kingdom: *Animalia*; Phylum: *Chordata*; Class: *Actinopterygii*; Order: *Siluriformes*; Family: *Clariidae*; Genus: *Clarias* (Konings et al., 2019). The widely known name for *C. gariepinus* species is North African catfish (FAO, 2021) or African Catfish, African Sharptoothed Catfish, Barbel Catfish, Common Catfish, Mudfish, Sharptooth Catfish (Konings et al., 2019). The catfish genus is distinguished by an elongated cylindrical body, long dorsal and anal fins containing only soft fin rays. The outer pectoral ray is shaped like a spine, and the pelvic fin has six soft trays. The head is flattened and highly ossified, with skull bones forming a casque and a smooth, scaleless skin covering the body. The skin is generally darkly pigmented on the dorsal and lateral sides of the body. The color is marbled and uniform, ranging from greyish olive to blackish, depending on the substrate. The fish has four pairs of elongated barbels, one nasal, one maxillar on the vomer, and two mandibulars on the jaw. Tooth plates are found on both the jaws and the vomer. A supra-branchial or accessory respiratory organ is usually present. The air-chamber communicates with the pharynx and the gill-chamber. The accessory air breathing organ allows the fish to survive for many hours out of the water or for many weeks in muddy marshes. The African catfish males and females are easily distinguished because the male has a distinct sexual papilla located just behind the anus. Females lack this sexual papilla (Graaf and Janssen, 1996).

3.1.3. Geographical distribution

The African catfish have almost pan-African distribution (but are naturally absent from the Maghreb, Upper and Lower Guinea and Cape provinces). They are equally present in Jordan, Lebanon, Israel, and Turkey (FAO, 2021). The fish has also been introduced into most other countries as Argentina, Bangladesh, Brazil, Cambodia, China, Côte d'Ivoire, Czechia, Gabon, Greece, India, Indonesia, Iraq, Lao People's Democratic Republic, Lesotho, Mali, Myanmar, Netherlands, Philippines, Singapore, Thailand, Vietnam. It also confirmed to culture in Cyprus, France, Hungary, Mauritania, Poland, Russian Federation (Konings et al., 2019).

3.1.4. Natural habitat

The African catfish can be found in various habitats, including lakes, streams, rivers, marshes, and floodplains, all of which are prone to periodic drying. The most frequent habitats are floodplain wetlands and pools, where they may survive the dry season due to additional respiratory organs in the air. The fish undertakes lateral migrations from greater areas of water, where they feed and mature after about a year, to flood marginal regions and reproduce temporarily. These

reproductive migrations often occur shortly after the rainy season starts (Graaf and Janssen, 1996). In shallow marginal areas, larvae and small juvenile catfish live in flotsam and plant rootstocks. Larger juveniles live in densely vegetated marginal pools during the day and may venture out into open areas at night. Adult catfish inhabit offshore areas, with the highest density in the terrace and sheltered bay habitats and the lowest in slope and profundal habitats. Mature catfish migrate into shallow water at night and deeper water during the day. The phenomena is more pronounced when lake levels are low (Bruton, 1978).

3.2. Gonadal development

C. gariepinus shows seasonal maturity often associated with the rainy season and is therefore influenced by annual temperature changes and cyclic photoluminescence of water with recent spawning due to rising water levels (Richter et al., 1987b; Graaf and Janssen, 1996).

3.2.1. Males

Van den Hurk et al. (1985) described the testicular development and ovarian cycle of *C. gariepinus* at different stages. In detail, the four stages of testicular development can be morphologically identified during the annual cycle. Phase I: preparatory (spermatogonial multiplication); at this stage, the GSI is low (0.28±0.09). Phase II: pre-spawning (formation of spermatozoa, spermatids, and spermatozoa); the testicles have grown in size; the mean GSI was improved to 0.61±0.32. Phase III: fertility (functional maturity); the testicles are maximally developed; The average GSI is 1.01±0.38. Phase IV: post-spawning (regression); the mean GSI dropped strongly to 0.4±0.2, mainly due to sperm loss. Spermatozoa formation in male African catfish was divided into five histological stages. Stage I testes only had spermatogonia, whereas stage II testes had spermatogonia, primary spermatocytes, and meiotic germ cells. Spermatogonia, primary and secondary spermatocytes, and meiotic germ cells were present in stage III testes. Stage IV testes contained spermatids but no spermatozoa. All germ cell stages, including spermatozoa, were found in stage V testes (Çek and Yilmaz, 2007).

3.2.2. Females

The development of oocytes of wild *C. gariepinus* or adult fish has been elucidated by many researchers (Graaf and Janssen, 1996; Çek and Yilmaz, 2007; Tyor and Pahwa, (2017). Tyor and Pahwa (2017) divided the development cycles of wild catfish into the following phases: chromatin-nucleolus phase, peri-nucleolus phase, primary yolk stage, secondary yolk stage, tertiary yolk stage, migratory-nucleus stage, mature stage, and postovulatory follicle stage or spent phase. Besides, the development of six chronological stages of the oocyte can be seen (Graaf and Janssen,

1996). Phase 1, immature virgin: the oocyte is small (7-10 microns) and lacks yolk. The mitotic division increases the number of primary oocytes. Phase 2, developing virgin: the oocyte is small (7-10 micron) and lacking in the yolk. The number of primary oocytes grows through mitotic division, and by the end of this stage, the oocyte has grown to about 200 microns. Phase 3, ripening: The yolk of the oocyte is formed during this stage. The oocyte is the source of the yolk at this stage. Phase 4, maturing or ripe: the oocyte grows to a final size of 1000-1200 microns (1-1.2 mm). During this phase, oocyte yolk formation rises, and the proteins required for this process originate outside the oocyte. A large nucleus (0.2 mm) is clearly visible just outside the oocyte's center. At this stage, the oocytes are also referred to as "ripe eggs." They will remain in this stage until environmental factors cause them to ovulate. Phase 5, running or spawning: the eggs are translucent, flat, and translucent, with cytoplasm consolidated at the animal pole and visible as a reddish brown spherical cap. This is in contrast to the round eggs found in the ovaries prior to reproduction. Phase 6, spent: the ovary is flaccid, flabby, and bloodshot, with thick whitish tough walls. The female genital aperture appears inflamed. Some translucent and opaque eggs are visible to the naked eye.

3.3. Natural spawning behaviour

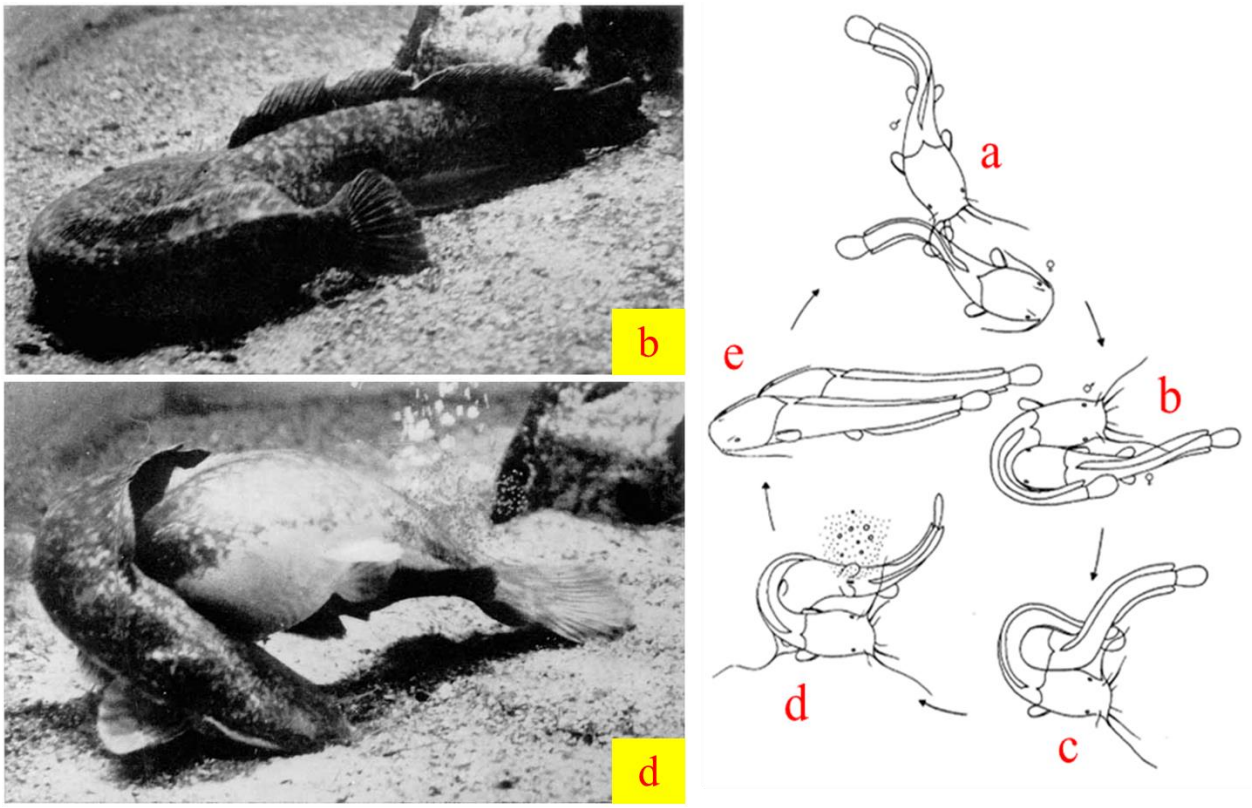

Figure 3.2. Courtship behaviour of *C. gariepinus*: Following (a), amplexus (b), sperm release (c), egg release (d), resting (e) (Van der Waal, 1974; Bruton, 1979a).

Normally, this catfish breeds in the summer after the rainy season, and a large number of them migrate to the "shallow submerged banks of rivers and lakes" (Richter et al., 1987b; Graaf and Janssen, 1996). The initial reproductive ethology cycle of *C. gariepinus* was described in the aquarium (Van der Waal, 1974) in the field (Bruton, 1979a). The process was divided into six stages: premigratory aggregation, migration, postmigratory aggregation, prenuptial aggression, courtship, and mating (Bruton, 1979a).

This writing focuses on the behaviour of the fish in the courtship and mating phase (Figure 3.2), which were considered lately in various literature. In general, males compete to establish courtship with females. After that, the winning fight approaches a female and touches the female's belly, head and tail. Towards the end, both fish start shaking and stop swimming; the female joins the male near the urogenital opening, while the male folds the body around the female's head, and then the pair attaches to a U-shaped position. After a few movements, the couple becomes immobile (Figure 3.2: a–b). During a loose amplexus form, males release invisible sperm (Figure 3.2: c). The male then slides behind the female body, always U-shaped, while in the female, the marked muscular tension is manifested behind the cephalic shield. The female then stiffens, sharply arches her back to one side and shovels her head under the male body and under the substrate (Figure 3.2: d). A large cloud of eggs and gas bubbles is released, and air also escapes from the buccal chamber through the gills. Gas bubbles can be used to transport eggs over a larger area. Within about 2 seconds of egg-laying, the female twists her tail from side to side, using her snout as an anchor to prevent forward movement and mixes and disperses the sperm and eggs. The couple usually rests after mating (Figure 3.2: e) and then resumes the next mating process.

3.5. Hormonal manipulations for maturation and ovulation

3.5.1. General regulation of fish maturation and ovulation

The ability to control the reproductive processes of fish in captivity and acquire high-quality gametes for the generation of marketable products is one of the prerequisites for the domestication and establishment of sustainable aquaculture. Hormonal manipulations can be used as management tools in most cultured fish species to improve egg production efficiency, increase spermiation, and facilitate hatchery operations. Hormonal therapies may also induce gamete maturation and enable artificial collection to carry out inter-specific hybridization, chromosome set manipulation, or artificial fertilisation for genetic selection programs (Mylonas et al., 2010). After the oocyte has completed its development phase, it becomes ready for the next phase of oogenesis, that is, the resumption of meiosis, which is accompanied by several maturational processes in the nucleus and cytoplasm the oocyte. This process, called OM, occurs prior to

ovulation and is a prerequisite for successful fertilisation; it consists of a breakdown of the germinal vesicle (GVBD), chromosome condensation, assembly of the meiotic spindle, and formation of the first polar body (Nagahama and Yamashita, 2008).

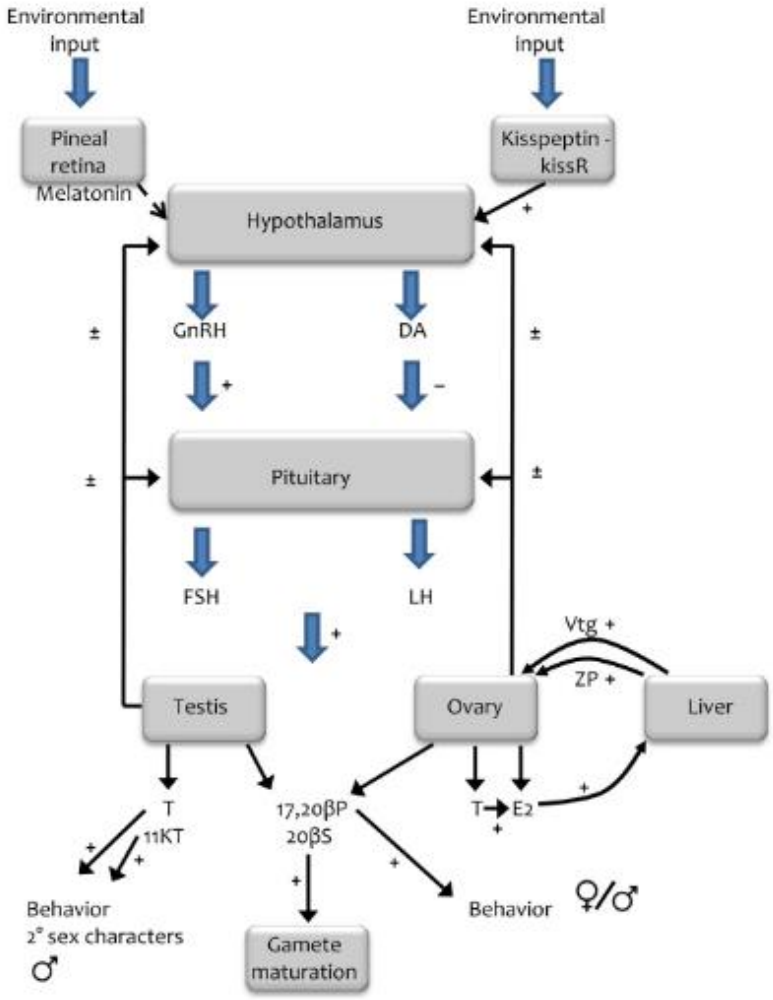

Figure 3.3. Schematic representative regulation of the hypothalamic-pituitary-gonadal axis in fish. Stimulatory effect (+); Inhibitory effect (-); dashed line indicates probable but unconfirmed regulatory input (Pankhurst, 2016).

Environmental cues are transduced into an endocrine signal through activation of the hypothalamic-pituitary-gonadal (HPG) axis (Mylonas et al., 2010; Yaron and Levavi-Sivan, 2011; Pankhurst, 2016) (Fig. 3.3), initially by synthesis and release of GnRH, a decapeptide, from the hypothalamus. The brain does not communicate directly with the gonads to control vertebrate reproduction (Wootton and Smith, 2014). According to current research, each species of vertebrate expresses two or three GnRH variants in various tissues, and these hormones have pleiotropic effects via a variety of receptor classes. Teleosts not only have the most GnRH variants discovered so far, with a total of eight, but recent data and whole genome analyses show that some species

have up to five functional GnRH receptors (Zohar et al., 2010). GnRH are expressed in different tissues, but the form produced in the hypothalamus appears to play an essential role in activating the HPG axis by synaptic stimulation of pituitary gonadotropin-producing cells. In some species, but not all species, pituitary gonadotrophs are also under the inhibitory action of DRA, and the balance between inhibitory DRA tone and GnRH stimulation determines the rate and level synthesis and release of gonadotropins (Chang and Jobin, 1994; Dufour et al., 2010; Zohar et al., 2010). The subsequent modulation of GnRH synthesis and release occurs through the newly discovered kisspeptin-kiss receptor system, which is increasingly believed to be the mechanism by which exogenous and endogenous environmental intake exerts its effect on the reproductive system (Nagahama and Yamashita, 2008; Zohar et al., 2010), although the mechanism of action remains undescribed. There is also a deduction of photoperiod modulation of the axis, synonymous with the hormones described in previous fish studies, such as gonadotropins (GtH-I, GtH-II). GtH-I and GtH-II in fish are LH- and FSH-like molecules in vertebrates, respectively. FSH and LH are released into the bloodstream to target on the gonad, where it stimulates the synthesis of sex steroid hormones, which are the final effectors of gonadal development (Mylonas et al., 2010). FSH is involved in stimulating the early stages of gametogenesis, while LH modulates the later stages of ovarian and testicular maturation (Levavi-Sivan et al., 2010). The receptor-ligand binding to both hormones activates adenylate cycles with a consistent increase in the cyclic intracellular concentrations of adenosine monophosphate (cAMP) and activation of a modulated cascade of synthesis of the protein kinase of steroid hormones and cleavage from the basal cholesterol molecule. The first stage of this cascade is the delivery of cholesterol to cleavage sites on the inner mitochondrial membrane under the acute steroid regulatory protein (StAR) regulatory activity, which seems to be one of the main steps limiting the rate in the neckline. The enzymatically regulated sequential cleavage of carbon atoms in the steroid nucleus and the addition of active groups result in the shorter 21C progestins and the shorter 19C androgens and 18C estrogens in the order of cleavage. A wide range of active steroid products are produced by gonadal tissues, including major active progestogens such as 17OHP, DHP, and 21-trihydroxy-4-pregnen 3-one (20βS) in both sexes, androgenic testosterone, 11-ketotestosterone and estrogen 17β-estradiol (Pankhurst, 2016; Yaron and Levavi-Sivan, 2011). Progestogens usually regulate the final maturation of gametes and are involved in some aspects of reproductive behaviour. Androgens and the precursors of estrogen production in females are responsible for the development of the testis in males and the development of secondary sexual characteristics. They are heavily involved in territorial and aggressive behaviour. Estrogen is primarily responsible for the estrogen receptor-mediated production of vitellogenin, the egg yolk precursor, by hepatic hepatocytes for subsequent absorption into growing oocytes during vitellogenesis and the production of proteins in the shell

egg or pellucid area. Interestingly, estrogen is also involved in regulating gene expression in the testis and is thought to play a role in stimulating spermatogonial proliferation. Steroids, in turn, can exert positive and negative feedback at higher levels in the HPG axis through classical action via intracellular receptors and short-term modulatory effects, although membrane-bound steroid receptors (Pankhurst, 2016).

3.5.2. Reproductive dysfunction in captivity

Reproductive problems are generally more severe in females and can be classified into three types. When kept in captivity, the first and most critical problem is not completely capable of vitellogenesis and spermatogenesis. The second type of reproductive dysfunction in female offspring is the absence of OM. The third type of reproductive failure in cultured females is a lack of spawning at the end of the reproductive cycle (Zohar and Mylonas, 2001; Mylonas and Zohar, 2007). Although there is a massive difference between fish, many cultivated species have some reproductive dysfunction. Hormonal therapies are essential to induce egg maturation, ovulation, and reproduction (Mylonas and Zohar, 2007).

Similarly, the reproduction of *C. gariepinus* under rearing conditions cannot reach the OM and ovulation in females (De Leeuw et al., 1985; Goos and Richter, 1996; Sharaf, 2012) due to the lack of natural reproductive stimulus (Mylonas et al., 2010) or stress (Goos and Richter, 1996). In combination with the inevitable stress, the absence of environmental parameters causes the blocking of the GnRH. Consequently, gonadotropin surge release fails to occur, which is enforced by an effective hypothalamic dopaminergic inhibition. The increase in LH induces the conversion of 17OHP to DHP, the final substance that induces maturation. Therefore, GnRH blockade in fish is effective by inhibiting the dopaminergic hypothalamus and has identified sex steroids that play a role in controlling the GtH release negative feedback. The sex steroids identified that have been shown to interact with the hypothalamic metabolism of dopamine to cause inhibition have been 11-KT and T (Goos and Richter, 1996). Therefore, the artificial propagation of *C. gariepinus* requires hormone-induced ovulation to overcome the barriers.

3.5.3. Applications of hormonal treatments

Hormonal reproduction has been used since the 1930s to induce ovulation, sperm, and reproduction (Mylonas and Zohar, 2000). Exogenous hormones are an effective manipulation to induce gonadal maturation and produce fertilized eggs. Therefore, hormonal manipulations can be used as management tools to increase gamete quality efficiency and facilitate reproductive performance in all farmed fish (Mylonas et al., 2010).

Table 3.1. Hormonal applications in the reproduction of the African catfish female. O rate-Ovulation rate; App-Hormonal delivery methods; CatPE-Catfish PE; Aquaspawn-20 µg GnRHa+100 mg DOM; Ovaprim-20 µg sGnRHa+10 mg DOM; Ovopel-18-20 µg mGnRHa+10 µg MET; IU-international unit; DMSO-Dimethyl sulfoxide; BSA-Bovine Serum Albumin; SMBS-Sodium metabisulphite; mGnRHa-mammalian GnRHa; sGnRHa-salmon GnRHa.

Hormone	App	Solvent	Dose/kg of BW	O rate (%)	References
LHRHa + DOM		-	25 µg + 5 mg	100	(Srimai et al., 2020)
sGnRHa +DOM (Ovaprim)	IM	-	10 µg + 5 mg	100	(Sharaf, 2012)
GnRHa	IM	Saline	40 µg	20	
	IM	Saline	20 µg	60	
GnRH + PIM	IM	saline + DMSO	20 µg +4 mg	90	
	IM	saline + DMSO	30 µg +8 mg	98	
	IM	saline + DMSO	40 µg +16 mg	100	
CPE	IM	0.9% saline	4 mg	88	(El-Hawarry et al., 2016)
CPE+DOM	IM	0.9% saline	4 mg + 10 mg	100	
LHRHa+DOM	IM	0.9% saline	50 µg + 10 mg	100	
GnRHa+DOM	IM	0.9% saline	40 µg + 10 mg	100	
CPE	OI	0.9% saline	3 mg	100	(Müller et al., 2018b)
CPE	IP	0.9% saline	3 mg	100	(Müller et al., 2019)
CPE	IP	Saline	4 mg	100	(Brzuska, 2011)
mGnRHa+MET (Ovopel)	IP	Saline	20 µg + 10 mg	83	
Preserved PG	IM	0.9 g/mL saline	0.9 g	100	(Okomoda et al., 2017b)
Fresh PG	IM	0.9 g/mL saline	0.9 g	100	
sGnRHa +DOM (Ovaprim)	IM	-	10 µg + 5 mg	100	
sGnRHa +DOM (Ovaprim)	IM	5 mL Saline	8 µg + 4 mg	100	(Marimuthu et al., 2015)
	IM	5 mL Saline	10 µg + 5 mg	100	
	IM	5 mL Saline	12 µg + 6 mg	100	
Toad PE	IM	2 mL of 0.9% saline	4 mg	100	(Salami et al., 1994)
	IM	2 mL of 0.9% saline	6 mg	100	
Frog PE	IM	2 mL of 0.9% saline	4 mg	100	
	IM	2 mL of 0.9% saline	6 mg	100	
hCG	IM	2 mL of 0.9% saline	2000 IU	100	
CatPE	IM	2 mL of 0.9% saline	4 mg	100	
	IM	2 mL of 0.9% saline	6 mg	100	
CatPE	IM	2 mL of 0.9% saline	1 gland	100	(Amoah et al., 2020)
LHRHa + PIM	IP	0.8% saline with 0.1% SMBS and 0.25% BSA	0.05 mg + 5 mg	100	(Richter et al., 1987a)
17α-OHP	IM	Dimethyl isosorbide	4 mg	100	
CPE	IP	Saline	4 mg	100	(Brzuska, 2004b)
GnRHa + DOM (Aquaspawn)	IP	Saline	20 µg + 50 mg	87.5	
LHRHa	IM	0.9% saline	10 µg	68	(Ude et al., 2005)
LHRHa	IM	0.9% saline	30 µg	73	
LHRHa	IM	0.9% saline	50 µg	82	
LHRHa	IM	0.9% saline	70 µg	85	
CPE	IP	0.9% saline	4 mg	90	(Brzuska, 2004a)
mGnRHa+MET (Ovopel)	IP	0.9% saline	20 µg + 10 mg	100	
hCG	IM	0.7% saline	6900 IU	93	(Inyang and Hettiarachchi, 1994)
hCG	IM	0.9% saline	500 IU	100	(Zidan et al., 2020)
	IM	0.9% saline	1500 IU	100	
	IM	0.9% saline	3000 IU	100	
	IM	0.9% saline	6000 IU	100	
sGnRHa +DOM (Ovaprim)	IM	0.6% saline	10 µg + 5 mg	100	(Ndimele and Owodeinde, 2012)
sGnRHa +DOM (Ovaprim)	IM	-	10 µg + 5 mg	100	(Kucharczyk et al., 2019a)

The OM and ovulation of the African catfish females cannot reach the hatchery due to the reproductive dysfunction; several hormones have been used appropriately to induce female reproduction (Table 3.1). Since the early 1970s, numerous methods for the artificial reproduction of *C. gariepinus* have been developed (Graaf and Janssen, 1996). When hormones are used, they are administered to females to stimulate ovulation and reproduction, and the hormones used during induction including DOCA, hCG, CPE, Catfish pituitary and Nile tilapia pituitary, 17OHP, hCG + CPE, LHRH or LHRHa and GnRH or GnRHa with or without DRA (Richter and Van den Hurk, 1982; Olaleye, 2005; El-Hawarry et al., 2016; Müller et al., 2018b).

The oldest and most widely used hormone for fish reproduction is the crude extract from PE of mature fish and has been used successfully in this species. However, unpredictable activity, difficulty in obtaining PE, and varying success rates in use (Dunham et al., 2000; Zohar and Mylonas, 2001) have promoted the wide use of hCG, which is characterized by wide availability on the market, along with its higher chemicals, purity that guaranteed a better efficacy. However, the high cost of hCG has led researchers to improve several farmed fish species in China with LHRHa and thus the evolution of the "Linpe method" (Peter et al., 1988). This approach induced fish reproduction from different forms of LHRH and LHRHa (Zohar and Mylonas, 2001). As a result, the use of LHRH has gained worldwide acceptance, overshadowing other hormone therapies that have been used for decades (El-Hawarry et al., 2016). Another widely practised hormone that has been conducted in the African catfish is synthetic hormones (Ovopel, Horváth et al., 1997; Dagin, Brzuska, 2011; Aquaspawn, Brzuska, 2003; Ovaprim, Kucharczyk et al., 2019 and Marimuthu et al., 2015). When GnRH alone does not work properly, it is usually used in conjunction with DRA (Hoga et al., 2018). The advantage of using DRA is that it blocks the inhibitory mechanism of dopamine, increasing the secretion of GnRH into the bloodstream while inducing maturation.

3.6. Hormonal delivery methods

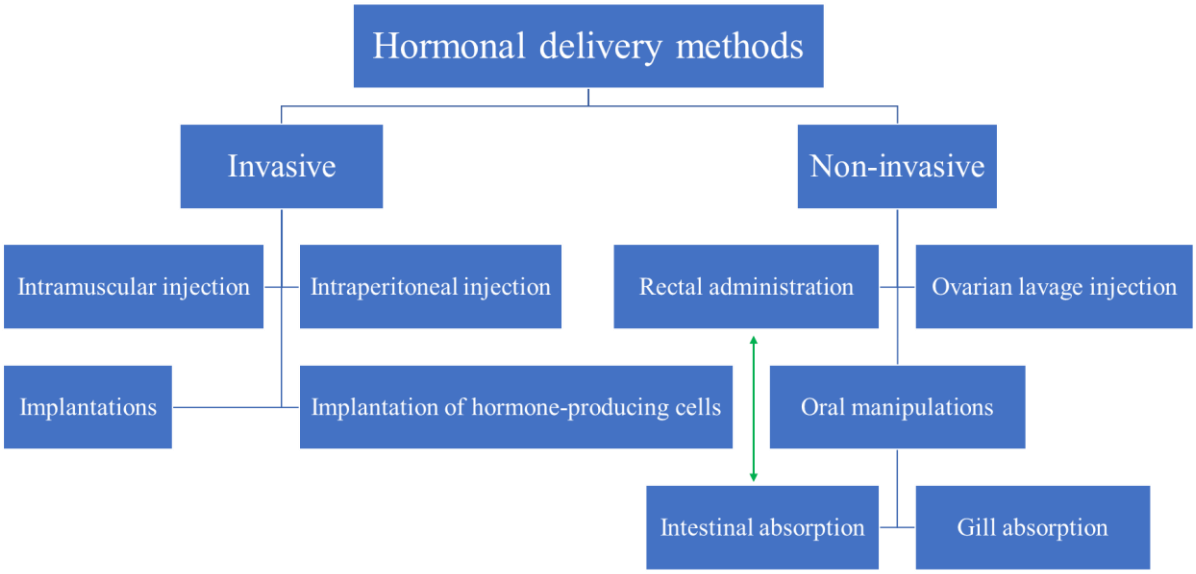

Figure 3.4. Hormonal delivery methods to induce reproduction in fish.

Introducing selected hormones and hormonal substances into fish species for reproduction is classified into two main methods: invasive and non-invasive. Invasive techniques contain IM, IP, implantations, and hormone-producing cells. Non-invasive methods include oral manipulation, rectal administration, and ovarian injection (Figure 3.4).

3.6.1. Hormonal injections

IM and IP are the common practices used for stimulated reproduction of captive fish (Zohar, 1989; Mylonas and Zohar, 2000; Watson et al., 2009a, 2009b). IP: the injection is managed through the peritoneum into the body cavity, most commonly at the base of the pelvic or pectoral fins. IM: The injection is introduced into muscle, most often into the tail shaft or back muscle at the level of the dorsal fins. Since part of the hormone preparation can return here due to the pressure of the solid muscle tissue, either the finger at the injection site and slowly pushes the injected solution into the injection site, or the needle with a long but small diameter a zigzag shape to prevent reflux. There are limitations to this method; injections can be stressful for small and delicate species or excessively laborious for low fertility species (Hill et al., 2005). The aim is to provide hormone preparations that induce ovulation using a hypodermic needle. In injection, the vehicle is often a liquid solution, most commonly saline (0.65 % NaCl or 0.9 % NaCl).

Generally, there was no difference in efficacy between the two routes of administration (Harvey and Carolsfeld, 1993). However, although there was no difference in the effectiveness of

the GnRHa+DOM treatment between the IM and IP, the IM achieved significantly better results than IP with the same amount of CPE and channel catfish PE in case of an ornamental cyprinid fish (*Balantiocheilos melanopterus*, Lipscomb et al., 2018). One of the advantages of IM is that the method of administration is much easier to standardize as an injection site and depth of administration. However, the treatment takes longer because the pressure of the muscle tissue is higher than the abdominal treatment, so it takes time to distribute the hormonal solution/suspension to prevent reflux. On the other hand, hormonal doses can be administered in significantly lower quantities than in the case of IP. In general, the amount to be issued is 0.5 ml/kg of body weight or less, and if it is required to inject a larger dose, it is advisable to treat the fish in several points. With IP, the treatment is much faster, and not sensitive to volume (2-3 ml/kg) than in the case of IM (Harvey and Carolsfeld, 1993). The disadvantage is that if the needle is inserted in the wrong place and/or the wrong depth, the calculated dose of the hormone can also enter the other organs. To reduce the risk, fish are usually treated at the scaly base of the chest or stomach. After dissolving in saline, the injected hormones enter the bloodstream within minutes, and then are metabolized, broken down, and excreted.

3.6.2. Implantations

The procedure consists of introducing hormone preparations into the fish body dissolved in a carrier from which the hormones are released in small doses over a long period. The advantage is that the stress on the fish is reduced as the number of manipulation decreases. Hence, with low doses of hormones entering the bloodstream evenly, it is possible to simulate the natural physiological function, thus obtaining a sexual product of better quality. Due to the carrier delay, treated fish and eggs are less sensitive to hormonal induction. In case of induced puberty, the weekly treatment must be continued for several months. Here, the implants' advantage is even more significant. The disadvantages are that their production at the factory level is unavailable, and the delivery has to be solved individually. When implanted in the muscle, the treated fish must reach a minimum size so that the implant does not cause physiological problems due to the size of the pellet. From now on, treatment with topical antibiotics is needed after the procedure to reduce the chances of infection. Some implants/pellets are inserted into the abdominal cavity or muscle tissue with an injection needle, hormone implant needle, or trocar. The other part is implanted anesthetically after surgery. The first sustained-release vehicle used was cholesterol (Weil and Crim, 1983) or a mixture of cholesterol and cellulose (Carolsfeld et al., 1988). The disadvantage of the method is that the hormone release rate varies from pellet to pellet, and cholesterol, an active biomolecule and precursor to steroid hormones, influences genital function (Mylonas and Zohar, 2000). Since then, the method has been perfected, and successful reproduction in different fish

species has been performed with different carriers; silicone or silastic rubber (Lee et al., 1986), water-in-oil-in-water emulsion - a mixture of lypophilized gelatin and cottonseed oil (Sato et al., 1997), carbopol (Szabó, 2008), ethylene-vinyl acetate copolymer (Mylonas and Zohar, 2000; Aizen et al., 2005), biodegradable microparticles (Mylonas and Zohar, 2000), osmotic pumps (Marte et al., 1987) and non-degradable implants such as methacrylate copolymer (Hirose et al., 1990). Depending on the type of vehicle and the water temperature, the duration of the hormone release ranges from 1–5 weeks (Mylonas and Zohar, 2000).

3.6.3. Implantation of hormone-producing cells

A project was started at the University of Leiden in the Netherlands to develop a hormone-induced sexual maturation method for European eels (*Anguila anguilla*) that does not require weekly hormone treatment for several months. The idea was to extract and transplant embryonic cell lines from zebrafish (*Danio rerio*) into eel eggs (*A. anguilla*), which are specifically responsible for producing of FSH-LH. Hence, elevated and continuous plasma levels of FSH ensure that vitellogenesis occurs without other treatments. The presence of transplanted cells was checked weekly for one month. During this period, morphological signs were used to demonstrate the initial sexual maturation of the eels and to prove that the genes enhance the expression of vitellogenin, qRTPCR concentration, were activated as a result of treatment (Schnabel et al., 2007). If the method can be improved, a breakthrough can be achieved in special areas of fish breeding (species that can be propagated by induced sexual maturation).

3.6.4. Oral manipulations

Oral hormone administrations are currently immature methods and can be divided into two subcategories; a) *gill absorption* and b) *intestinal absorption by using oral uptake*.

a) Gill absorption

Because gill treatment does not cause a mechanical problem on the gill plates, different compounds of small molecules can be absorbed into the bloodstream. Due to their physiological characteristics as external respiration and exchange of dissolved gases, the gill plates form the minimum distance between the external environment and the blood circulation (1–5 μm). The method is based on dissolving the hormone in solution, delivering it to the oral cavity, and then holding/closing the mouthpiece and gill caps for some time to absorb the dissolved hormone. With this method, Hill et al. (2005) proved that sGnRHa+DOM dissolved in dimethyl sulfoxide could reproduce *Epalzeorhynchos erythrurus*. This method has been developed for the reproduction of small fish, individual handling of the fish takes a relatively long time, due to their small size,

requires more careful preparation and handling. Using Ovaprim with the topical gill method, spermiation induction of silver rasbora was successfully conducted. An increase in semen volume, milt concentration, sperm motility, and sperm viability was detected (Al Adawiyah et al., 2019).

b) Intestinal absorption by using oral uptake

Table 3.2. Representative applications of hormonal administrations to induce fish ovulation by the intestinal absorption method. BW-body weight; Inj-injection; SB - Sodium bicarbonate; AgGnRHa-Antigen-grade GnRHa; L-lysophosphatidylcholine; Enhancing formulation-Tween 80 (4 %), oleic acid (0.6 %), ethylene diaminetetraacetic acid (EDTA; 0.25 %) and trypsin inhibitor (5 mg/ml), phosphate buffer (95 %).

Species	Hormone	Vehicles	Dose/BW kg	O-rate (%)	References
Oreochromis niloticus	GnRHa (Inj) + DOM (oral)	Saline + Sterile water	15 µg + 5 mg 30 µg + 5 mg	100 100	(Piamsomboon et al., 2019)
Scomber australasicus	AgGnRHa	Ethanol (50 %)	6.0 mg 1.2 mg 0.24 mg 0.048 mg	100	(Amezawa et al., 2018)
Euthynnus affinis	AgGnRHa	Guar gum	6.0 mg	100	
Puntius gonionotus	LHRH+DOM	NaCl 0.9 % + Citric acid	50 µg + 25 mg 50 µg + 50 mg 100 µg + 25 mg 100 µg + 50 mg	100 83 100 83	(Sukumasavin et al., 1992)
Anoplopoma fimbria	LHRHa	0.67 % saline + SB + L	1 mg	75	(Solar et al., 1990)
Cynoscion nebulosus	LHRHa	0.7 % saline	1─2.5 mg	100	(Thomas and Boyd, 1989)
Carassius auratus	PE	Phosphate buffer	2.62 mg	100	(Suzuki et al., 1988)
C. gariepinus	GnRHa	Enhancing formulation	5 mg	25-100	(Breton et al., 1995)
Cyprinus carpio	GnRHa	Enhancing formulation	5 mg	100	
C. gariepinus	GnRHa	Enhancing formulation	10-20-40 µg	50-90-100	(Breton et al., 1998)

The intestinal absorption process in fish and the enzymatic breakdown of organic macromolecules and hormone-containing synthetic short-chain amino acid compounds differ by species. These species lack a hydrochloric acid-pepsin digestive tract. At a pH of 6.7–7.7, the intestinal mucosa and pancreas break down proteins, lipids, and carbs. Solar et al. (1990) encountered difficulties due to the species' sensitivity to conventionally induced propagation procedures, which necessitated using a tiny tube to provide oral GnRHa therapy. In studies that employed mammalian and amphibian pituitaries, the potential for controlling fish reproduction by oral bioactive materials was indicated (McLean et al., 1999). Hence, dietary replacement or supplementation with PE has been observed to partially gravid and improve egg diameter, resulting in ovulation and a shortening in brood interval. Likewise, oral administration of SPE to goldfish *C. auratus*, induced ovulation and increased spermiation. The importance of the latter studies relates to the accompanying promotion in plasma GtH, testosterone and DHP (Suzuki et al., 1988), which provide a likely endocrine-based explanation for the observed effects of other pituitary preparations. Due to the problems inherent in using PE and partially purified hormones (Harvey and Carolsfeld, 1993), it would appear unlikely that such preparations will offer any significant

benefit concerning the control of reproduction in cultured fish using the oral route. A comparatively recent innovation in the control of maturation has been the application of GnRHa. The GnRHa provides perfect candidate molecules for the intestinal approach to control reproduction (see Breton et al., 1995, 1998; Amezawa et al., 2018). Indeed, sufficient experimental evidence has been collected (Table 3.2), such that oral delivery of GnRHa, with or without dopamine agonist (Sukumasavin et al., 1992), may be proposed as a viable method for controlling fish reproduction. This advance in reproductive biotechnology may be beneficial for species that are vulnerable to handling, are too small for safe injection due to offering the advantage of being stress-free (McLean et al., 1999).

3.6.5. Rectal administration

Rectal intubation, similar to oral administration, is an incomplete method. In this case, the hormone substance is dissolved in the solvent and introduced into the colonia through the anus of fish. For rectal manipulation, a flexible polyethylene tubing connected to an injection needle and tuberculin syringe is then introduced at 3 cm (Mikolajczyk et al., 2002), 4 cm (Roelants et al., 1992), or 6 cm (Mikolajczyk et al., 2001) up the descending intestine via the anus. Horseradish peroxydase plasma (HRP) level of *C. gariepinus* after 90 minutes administrated by rectal intubation method was higher than that done by IP. By contrast, the concentration of HRP was far lower than that in *Cyprinus caprio* after 390 minutes of manipulation (Roelants et al., 1992). The activities of sGnRHa + PIM were of the same order of magnitude after oral and rectal delivery since no significantly different LH release effect was observed at any sampling time. It suggested that intestinal absorption enhancement can introduce a low dosage of rectal and oral intubation of peptide drugs to the blood system of fish without substantial loss in bioactivity (Mikolajczyk et al., 2002). Even though the induced-ovulation was gained after rectal delivery in *C. carpio* and in *C. gariepinus* in the group with or without the enhancer (Breton et al., 1995), further studies are necessary to develop the rectal intubation method that is applicable in aquaculture media.

3.6.6. Ovarian lavage injection

This term describes hormone therapy delivery to the ovaries via a catheter. The goal is to deliver the hormone solution/suspension directly into the ovarian cavity through the genital opening and the fallopian tube using a catheter, biopsy sampler, or feeding tube. Some fish species are hypersensitive to invasive methods of administering hormones or, due to their small body size, find it technically more difficult to induce them by injection. Watson et al. (2009a, b) used a catheter to deliver dissolved hCG to the ovarian lobes of green-spotted pufferfish (*Tetraodon nigroviridis*), red eel (*Mastacembelus erythrotaenia*). hCG was absorbed through the ovarian wall,

entering the systemic circulation and inducing ovulation for some fish. Among domestic fish species, *Sander lucioperca* has been successfully propagated by this method connecting with CP suspension (Németh et al., 2012). The main finding was no statistically significant difference between the two hormone administrations (intramuscular and ovarian lavage groups) regarding reproductive parameters. These findings raise the possibility that, during hatchery breeding, the hormone is often not delivered into the "abdominal cavity" of farmed fish but into the ovarian lobes of the eggs before ovulation. This hypothesis led to the operative application of the catheter hormone delivery method, as it was hypothesized that the hormone is absorbed by the ovaries in the same way and exerts its effect as when it is injected into a muscle or an abdominal cavity.

The tolerance of the ovarian tissues to foreign substances proved significant (no rapid immune response after treatment, and even the mucopolysaccharide-type gonadotropic hormones, classified as macromolecules, were absorbed without degradation and loss of effect). Continuing this recognition, over the past three years, we have developed a new fish farming method, the physiological basis of which is that the spermatozoa delivered to the ovaries of externally fertilized eggs through a catheter remain viable for a long time (Müller et al., 2018a, b, 2019, 2020; Ittzés et al., 2020; Gazsi et al., 2021a, b). In these experiments, the Carp, the African catfish and South American silver catfish (*Rhamdia quelen*) were conducted by mixing powdered CP gland with sperm and injecting it into the ovarian cavity. The seminal sperm plasma used as a vehicle of the hormone was absorbed through the wall of the ovaries, and the ovulation-induced GtH was released from the crushed/powdered CP gland. Ovarian inactive sperm were activated in the aquatic ambient and were able to fertilize with ovulated eggs. The fertilisation results were not different from the data obtained with traditional breeding, including hormonal treatment and IVF. This new method is mainly possible for breeding fish species with physiological and/or technological barriers to the dry fertilisation process. Still, it can be easily replicated using artificial spawning for marine fish species.

3.7. Artificial insemination

The IVF, traditional fertilisation, or dry fertilisation, also known as artificial fertilisation or strip spawning, involves the physical removal of sexual gametes and artificial water activation for fertilisation. This method is standard in the mass production of the aquaculture sector. Concisely, the mature sperm and ovulated eggs should be collected in separate drying containers by stripping or surgical procedures, avoiding urine and water pollutants. The milt and eggs are then comprehensively mixed and fertilized before adding culture water, either fresh or seawater, depending on the species (Urbányi et al., 2009; Mylonas et al., 2010, 2017). The IVF process has the limitation that it is difficult to predict the time to ovulation after hormonal therapy (so called

latency time) of several fish species and egg can be overriped in female body cavity and loses fertility potential (Urbányi et al., 2009; Mylonas et al., 2017; Müller et al., 2018a, b; Ittzés et al., 2020). In the wet method, culture water was used immediately after mixing the milts and eggs, and insemination was performed with the culture water. In natural spawning, spermatozoa are generally immobile in the seminal plasma, and forward motility is initiated by dilution with the culture water (osmotic shock). Water and urine contamination during sperm stripping should be avoided in freshwater fish or most finfish species, as sperm motility lasts for a short time and may activate sperm before mixing with the egg (Mylonas et al., 2010). In OSI technique, the simplicity of induced spawning is associated with conventional IVF. Therefore, stripped fresh sperm is inoculated into the ovary through the oviduct via a catheter, and the delivered sperm maintain its fertilisation capacity for up to 10–40 hours, and for extended periods it remains inactive without loss of biological characteristic (Müller et al., 2018a,b; Müller et al., 2020). Then, the ovulated eggs and spermatozoa on their surface released from the ovarian cavity can be fertilised as a result of water activation.

3.8. Embryonic and larval development

Table 3.3. Embryonic developmental stages in *C. gariepinus* (Olaniyi and Omitogun, 2013). T-Time from fertilisation to the timing at 28.5 ± 0.5 °C.

Stages	Stage of development	T (minutes)	Major descriptions
1	Unfertilized oocyte	0	Oval-shaped, brownish in colour and opaque. Adhesive in nature with an absence of oil-like droplets
2	Fertilisation	0	Shrinkage of the yolk away from the perivitelline membrane
3	Blastodisc/Animal pole and Vegetal pole	24	Formation of blastodisc characterized with red spot
4	One-cell	36	Protoplasmic layer bulges at the animal pole or submicropylar area
5	Two-cell	38	First mitotic blastodisc cleavage
6	Four-cell	55	Second mitotic blastodisc cleavage
7	Eight-cell	67	Third mitotic division of two parallel rows
8	Sixteen-cell	80	Blastodisc meroblastic cleavage continues making a four × four blocking layer
9	Thirty-two-cell	83	Early morula staged cell
10	Morula	111	Further cell divisions led to numerous uncountable blastomeres
11	Blastula	129	Formation of a dome-shaped blastodisc
12	Gastrula	327	Blastoderm expanded; cell randomized transitional movement; formation of embryonic axis; Blastopore closure; polster and tail bud revealed
13	Somite	588	Early somite formation developed cephalocaudally
14	Hatching	1040	Break-off of the embryo out of the chorion or egg capsule

There are several ways for egg incubation in the case of *C. gariepinus*; troughs, vats, tanks, and trays made of different materials such as fibreglass, plastics, glass, concrete, and stainless steel are presently used. Optimum water temperature range is 28–32 ^{o}C (Graaf and Janssen, 1996). A chronological study of the embryonic stage of development in relation to the associated temperature of *C. gariepinus* is an essential factor in fish biology and subsequent manipulation (Bruton, 1979a; Baidya and Senoo, 2002; Olaniyi and Omitogun, 2013). A brief description of embryogenesis is shown in Table 3.3. The process was divided into 14 sub-steps, from 0 minutes of fertilisation to hatching through all stages. This study explains the fertilisation and hatching stages, often used as reproductive factors in assessing fish reproduction.

Unfertilized oocyte stage: The incompletely unfertilized eggs are oval, brown, and cloudy in shape. It has no oil droplets and adhesive in nature; and is about 1±0.1 mm in diameter possessing a thin perivitelline membrane whose space was filled with a layer of protoplasm (Olaniyi and Omitogun, 2013). Unfertilized eggs are indistinguishable from fertilized eggs because they initially swell in the same way and polarize in the same way. However, they are delayed in the first cleavage, and the poles of hillock-shaped animals are usually abnormally shaped, elongated, and pointed, as seen in treatment at temperatures of 24 °C and 32 °C (Anpe et al., 2017).

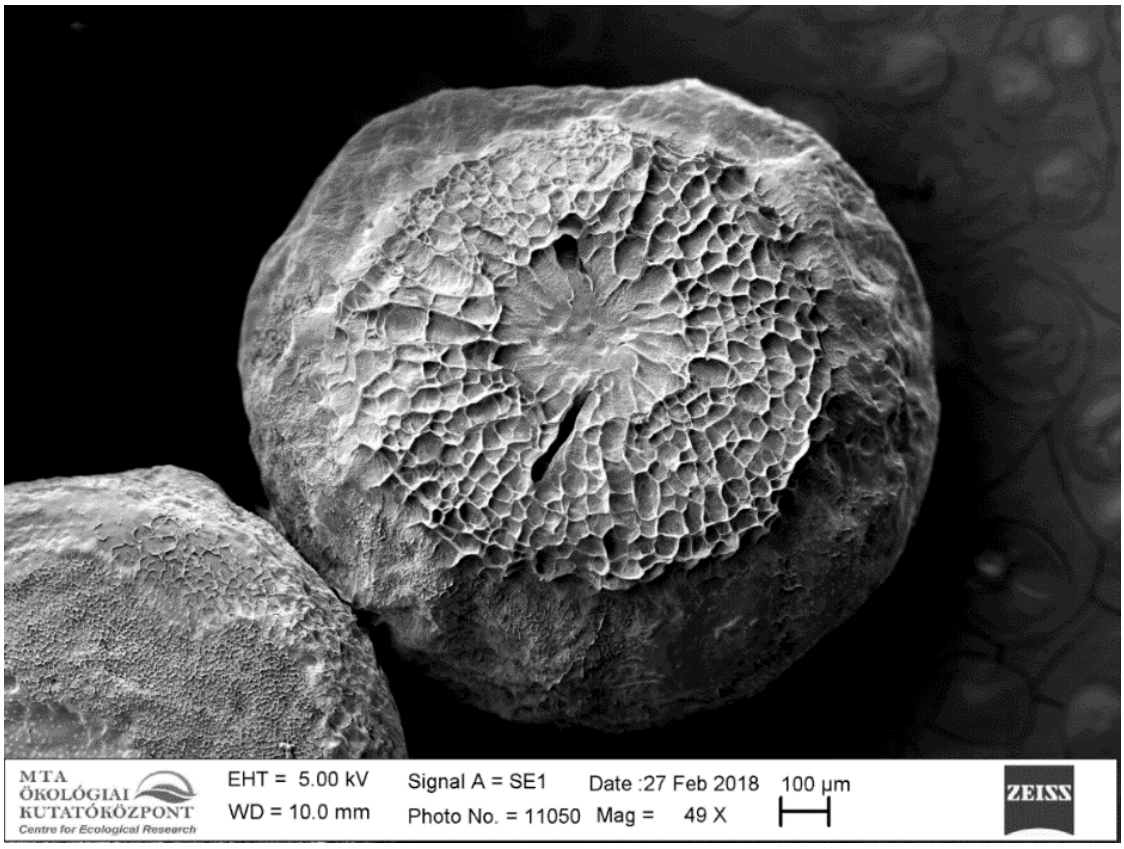

Figure 3.5. Electron microscopy study of the African catfish eggs (Photo: Prof. Éva Ács).

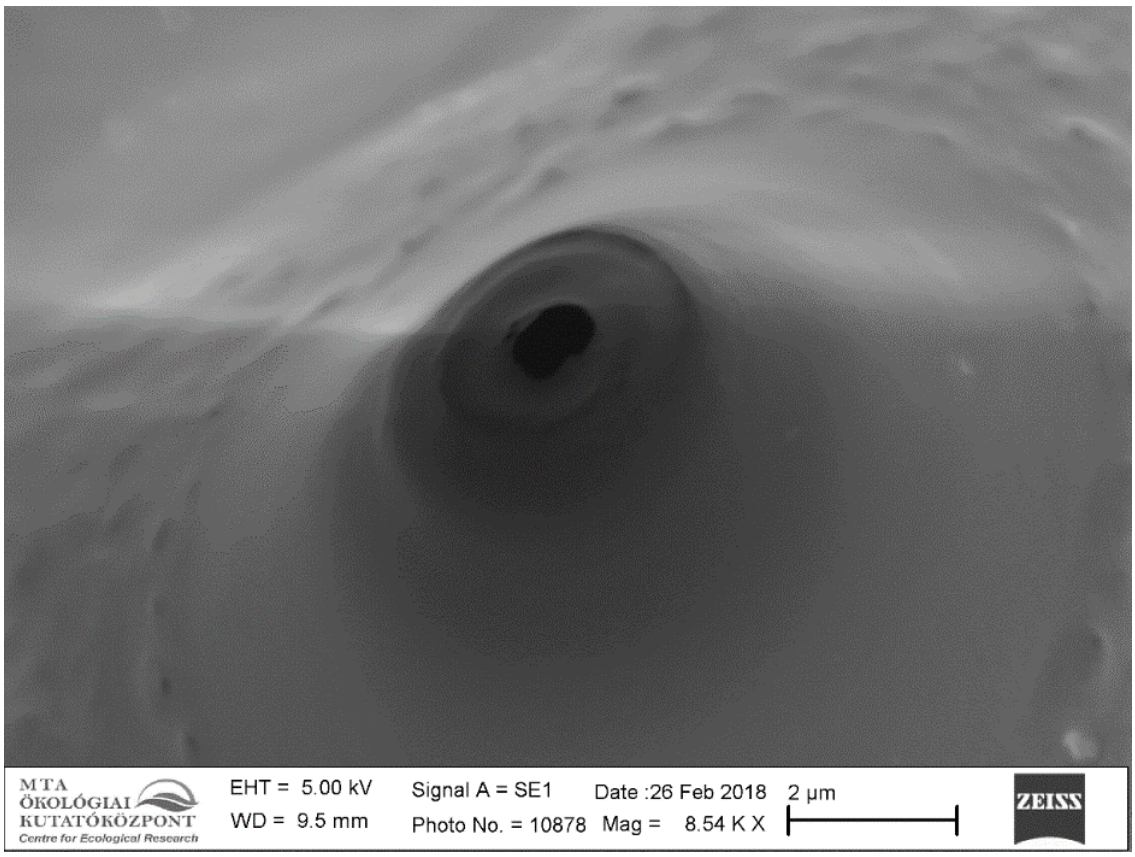

Figure 3.6. The micropyle region of the African catfish eggs (Photo: Prof. Éva Ács).

Fertilisation: Sperm enter the egg through a micropyle (Figures 3.5 and 3.6). Fusion of spermatogenesis and egg nuclei resulted in a slight contraction/reduction of yolk (Olaniyi and Omitogun, 2013). The fertilized egg has red spots on the opposite pole of the sticky disk (animal pole, Figure 3.5), and the yellow yolk becomes more orange then green-orange. *C. gariepinus* egg has no protective coating and is not resistant to drying (Bruton, 1979a). When a healthy egg reaches the closing stage of the original mouth, a clear distinction is made between good and bad eggs. Therefore, this is the stage where fertilisation can be determined (Anpe et al., 2017).

Hatching stage: Rupture of the embryo from the chorion or yolk sac through the tail is shown. Occasional embryonic peristalsis was noted before hatching. Therefore, the first respiration is recorded at a heart rate interval of 115–160/minute, and then the tailbud surrounding the yolk sac was recorded slightly off the central part. The anterior part of the head is elongated and bulbous, but the part of the brain/head is firmly attached to the yolk sac. Subsequent frequent contractions eventually destroyed it, and the tailbud broke the chorion or egg capsule covering (Olaniyi and Omitogun, 2013). Eggs hatched after 17.5 hours at 28.5 °C (Olaniyi and Omitogun, 2013), 17 hours at 26–28 °C (Baidya and Senoo, 2002), or 24–25 hours at 19–24 °C (Bruton, 1979a).

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

Observations on captive breeding/spawning of *C. gariepinus* have shown that females do not spawn individually but in groups with a significantly higher percentage of spawning females stocked at two and four pairs in each hapa (El Naggar et al., 2006). The mates show a complex breeding behaviour, which includes various preparatory actions culminating in a loose U-shaped amplexus (see Figure 3.7; II.1a, b), in which the male folds around the head of the female and the release of gametes (Van der Waal, 1974; Bruton, 1979a). Almost identical positions have also been described for other Clariidae, such as *C. batrachus* (Thakur, 1976), *C. fuscus* (Olshanskiy, 2019), *C. macrocephalus* (Olshanskiy et al., 2020). Similar positions have also been reported from other Siluriformes, such as *H. fossilis* (Thakur et al., 1977; Roy and Pal, 1986) and *H. longifilis* (Poncin et al., 2002). These authors emphasise the proximity of the male genital pore and the female's mouth during the amplexus. However, the release of sperm and their other path could not be traced.

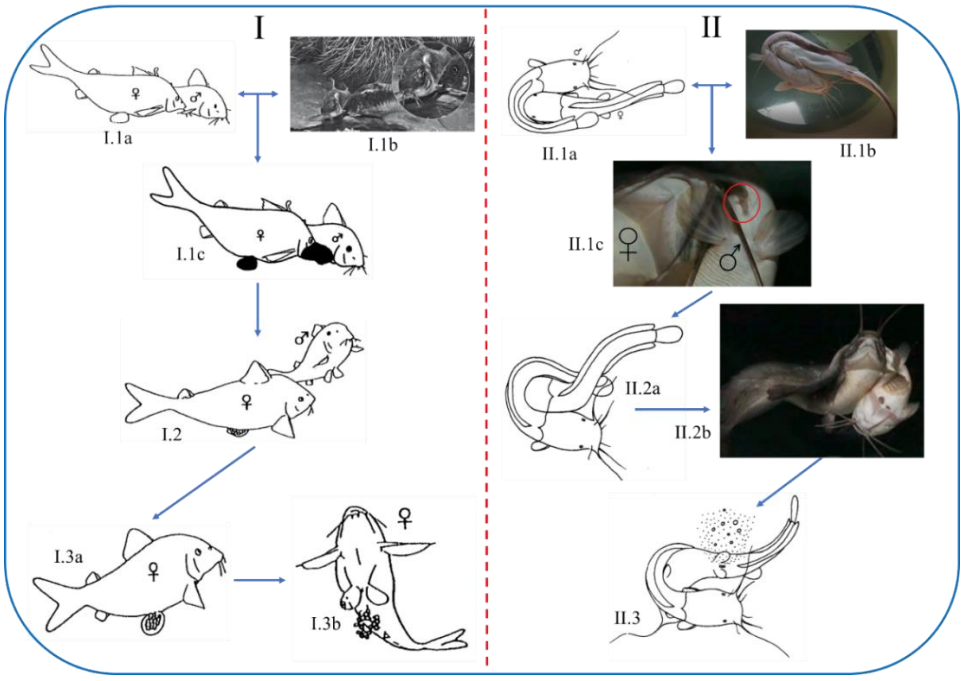

Figure 3.7. Mating behaviour of *Corydoras aeneus* (I) and Clariidae fish (II). Photos I.1a, I.1c, I.2, I.3a, and I.3b: Kohda et al. (1995); I.1b: Zarske and Greven (2015); II.1a, II.2a, and II.3: Bruton (1979a); II.1b: author; II.1c and II.2b: Olshanskiy et al. (2020). The female buts her mouth to the male genital or anal region in T-position (I.1a, b, and c) and U-shaped (II.1a, b, and c); The female drinks released sperm, then sperm pass via her intestine (I.1c-I.2), sperm droplet is discharged (II.1c, inside red circle). The female deposits eggs together with sperm into her pelvic pouch alone (I.2); The male stripped the abdomen of female after sperm releasing (II.2a-II.2b). Female finds the spawning place, then discharges eggs alone (I.3a-I.3b); Male helps the female for distributing eggs and ‘‘sperm cloud’’ to ambient water (II.3).

Regarding the release of sperm in *C. gariepinus*, Van der Waal (1974) stated that sperm "is not visible and ejected in very tiny quantities". Similarly, Bruton (1979a) also admitted that the released sperm "are invisible to the naked eye in the field" following the male "stiffens and arches his body". The author is completely uncertain about the frequency mating of the pairs, which is rarely up to five times, and the females can spawn with one or more males in a wild habitat, whereas the spawning activities can take over fifty times aquarium circumstance (Van der Waal, 1974). In *C. batrachus* (Thakur, 1976) and *H. fossilis* (Thakur et al., 1977; Roy and Pal, 1986), the discharged sperm was somewhat sibylline described as ''watery-textured".

The conspicuous proximity of the male genital opening and the mouth of the female during mating is also found in other Siluriformes, for example, in the genus *Corydoras* during the so-called T-position. In *Corydoras aeneus*, the females are proven to drink the sperm released in this position, which then passes quickly through her gut inseminating the eggs collected in the fin pocket formed by her pelvic fins (Kohda et al., 1995). However, both, especially the passage of sperm through the intestine, is controversially discussed (Figure 3.7, Zarske and Greven, 2015).

Olshanskiy et al. (2020) studied the mating behaviour of *C. macrocephalus* and mentioned that just before releasing the eggs, "a small drop of milt typically appeared near the male's genital opening'' (Figure 3.7; II.1c). The authors also discussed how this milt ultimately reaches the laid eggs, including (1) the minor water roughness could accelerate the extension of sperm; (2) During fifteen seconds after both fishes divide from the U-shaped, eggs adequately catch up the sperm cloud by supporting of noticeable turbulence; (3) The female reliably detects the moment of released sperm, then detect the sperm rapidly. However, that description seems to include some problems that need to be illuminated. Firstly, whether the sperm droplet can spread into ''sperm cloud'' by supporting water turbulence or an air bubble from females' gills help transport the milt over an expanding area as the equivalent mechanism with eggs in *C. gariepinus* (Bruton, 1979a). Besides, even though the male' reproduction system release sex pheromone to attract the female (Resink et al., 1987, 1989; Van den Hurk and Resink, 1992), it just accommodates a vital role for prenuptial spawning behaviour (Chowdhury and Joy, 2007) instead of insemination stage (Van den Hurk et al., 1987; Chowdhury and Joy, 2001). Lastly, the natural habitat of *C. microcephalus* as rivers, and canals (Tan-Fermin et al., 1997; Panprommin et al., 2008), where are conventionally unstable environmental factors can be affect sperm motility (Islam and Akhter, 2012). Those were challenges during insemination (Molony and Sheaves, 2001), and female fertility can be limited by sperm availability in external fertilisers (Wootton and Smith, 2015). The circumstance and the amplexus during mating of catfish have provoked us to revisit the hypothesis of sperm collection via the female's mouth.

3.10. Reproduction effectiveness for induced spawning of *C. gariepinus* by using invasive and non-invasive methods

Exogenous hormone conduction has been commonly used to induce artificial spawning in fish (e.g., Horváth et al., 1997; Zohar and Mylonas, 2001; Brzuska, 2004a,b; Kucharczyk et al., 2020). These hormonal treatments target the hypothalamic, pituitary, gonadal axis by increasing the gonadotropin level in the blood stream. The increase in gonadotropin is directly induced by an extract of fish PG or hCG or indirectly by administration of the GnRH or GnRHa. The combination of GnRHa and DRA further improves ovulation success by preventing dopaminergic inhibition of gonadotropin release from the pituitary gland. Optimal hormone preparations and their doses vary by species (Zohar and Mylonas, 2001; Yaron et al., 2009; Mylonas et al., 2010, 2017). In addition, many other factors influence successful ovulation, such as the optimal route of administration of spawning agents (Watson et al., 2009a,b; Lipscomb et al., 2018).

Invasive or non-invasive methods can be used as possible means of introducing hormones. Invasive treatment affects the tissues of the body to varying degrees. These are usually needle injections or minor surgeries. The non-invasive method does not damage the tissues of the body. This is especially important if the breeder is artificially propagated multiple times. Theoretically, oral hormone therapy can induce fish ovulation via introducing hormone through the gill plates (Hill et al., 2005) or intestines in the case of agastric species (Thomas and Boyd, 1989; Solar et al., 1990). However, these methods have not yet been implemented in hatchery practice due to their limitations.

Another novel method is sperm insemination. Sperm from several males alone or mixed with hormones are injected by catheter into the ovarian cavity of external fertilisation fish through the oviduct. Absorbed hormone induce ovulation, spermatozoa after several hour latency time in the ovaries, fertilise the ovulated eggs immediately with water activation after being released from the body cavity (Müller et al., 2018b). Both methods are less-invasive methods and do not cause damage to body tissues. This is especially important when the same spawners are bred artificially multiple times.

According to the hypothesis of Prof. László Horváth, albumen form chicken egg white introduced into the ovary of a fish can optimize the physiological process of oocytes ready for ovulation. The special protein composition of albumin can promote OM in the ovary follicles and increase the ovulation rate. However, information on the application of egg white as a hormonal implant for inducing ovulation in fish is not yet available. IM and IP are the most common way to penetrate muscle tissue or body cavity for induced spawning. These studies found no difference in efficiency between the two hormone delivery pathways (Harvey and Carolsfeld, 1993).

Exceptionally, there may be differences depending on the treatment. For small size fish (*Brycon melanopterus*), IM administration of carp and catfish PG resulted in higher ovulation rates than IP treatment. However, when treated with GnRHa+DOM, there were no significant differences between routes of administration (Lipscomb et al., 2018).

The experiment should be conducted using the African catfish as a model (Van Oordt and Goos, 1987) to compare the effectiveness of traditionally hormonal injections and modified sperm injection method. It should also studied albumen of the chicken egg as a novel vehicle for hormone delivery to induce fish ovulation by the ovarian injection method.

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

Distance hybridisation has long been carried out in a variety of fish to accelerate growth rate, improve flesh quality, create sterile animals, increase disease resistance and environmental tolerance, and improve other quality traits to make fish more beneficial (Bartley et al., 2000; Müller et al., 2004, 2011; Rahman et al., 2013). In addition to conventional breeding technologies, the hybridisation method is also widely applied to produce various types of hybrid offspring and is also frequently managed in the crossing of catfish, for example, C×H (Legendre et al., 1992; Olufeagba et al., 2016; Ndome et al., 2011), *C. gariepinus* × *C. macrocephalus* (Duong et al., 2017; Chaivichoo et al., 2020), *C. gariepinus* × *Pangasianodon hypophthalmus* (Okomoda et al., 2017a), and C×B (Bart and Dunham, 1996; Perera et al., 2017; Myers et al., 2020).

Conventionally, IVF is the most common technique used to produce hybrid embryos and thus the most widespread manipulation currently used in the catfish industry (Dunham and Elaswad, 2018). However, there are several limitations: (1) the ovulation time estimation after hormonal treatment can be problematic since eggs become overripe after ovulation and lose their fertilisation potential (Mylonas et al., 2010, 2017); (2) during long-term breeding, the application of induced spawning can reduce the genetic diversity in a certain stock where only a few parents contribute to the next generation (due to high fecundity) (NACA, 1989); and (3) striping the ovary is not only labour intensive but also requires a competent and gentle work procedure, which is prone to contamination that leads to early activation of the gamete (Dunham and Masser, 2012). Even if the maturation and ovulation of donor fish have been triggered hormonally, they are preferred to the pond, tank or hapa to spawn naturally in their raising fields (NACA, 1989). This permits the fish to exercise their natural breeding characteristics and discharge their gametes in synchrony; thus, high fertilisation success is achieved, and high seasonal fecundity is attained via multiple spawnings with asynchronous ovarian development (Mylonas et al., 2010). Hence, breeders are not disturbed by the stress from human intervention, and they spawn whenever they

are ready to reproduce (Żarski et al., 2015) and produce a number of high-quality eggs (Okamura et al., 2014). Spontaneous or induced mating/spawning methods have been used to propagate many species, for instance, European eel (*Anguilla anguilla,* Di Biase et al., 2017), Greater amberjack (*Seriola dumerili,* Corriero et al., 2021), Silver moony (*Monodactylus argenteus,* Thomas et al., 2020), South Pacific bonito (*Sarda chiliensis chiliensis*, Pepe-Victoriano et al., 2021), Chanel catfish (*I. punctatus,* Dunham and Elaswad, 2018), *C. macrocephalus* (Olshanskiy et al., 2009, 2020), and *C. gariepinus* (El Naggar et al., 2006; Priyadarshi et al., 2021). However, the application of this therapy to produce hybrid progeny has not been applied in most fish species for several reasons, such as different spawning ethology and different parent sizes. For instance, the C×B hybridisation is of economic importance (Dunham et al., 2000; Dunham and Masser, 2012). There are three spawning techniques: open-pond spawning, pen spawning and IVF. Open-pond spawning is rarely successful and cannot be recommended. "Pen spawning is a more consistent way of producing C×B hybrids than open-pond spawning. Average spawning success over 14 years of continuous research at Auburn University was approximately 15 percent. Therefore, pen spawning is not considered a dependable method of producing C×B hybrid fry" (Dunham and Masser, 2012).

The hypothesis is that the inseminated sperm / sperm ovarian lavage method (Müller et al., 2018a) combined with artificially inducing ovulation in pen / cage spawning / tank spawning or open-pond conditions may overcome the problems of the artificial fertilisation process and is also feasible for catfish hybridisation. This method has several theoretical and practical advantages: (1) the artificial administration of hormones corresponding to ovarian sperm injection may improve fish hybridisation techniques; (2) minimise the cost of larvae production; (3) sustain the genetic diversity of the propagated species by permits using a pooled sperm; (4) can combine the simplicity of induced spawning, with less time-dependent delivery of the sperm than in conventional IVF; (5) this has important practical implications for economically important fish species-induced spawning in tanks, ponds and where there is egg collection after spawning (Müller et al., 2018a, b, 2019, 2020; Ittzés et al., 2020; Gazsi et al., 2021a, b).

The aim of this study was to evaluate the OSI method combined with artificially induced ovulation as a feasible method for hybridisation of catfish in spontaneous spawning conditions.

3.12. The potential latency period of spermatozoa in the ovary of African catfish (*C. gariepinus*)

Müller et al., 2018a tested a new approach to distribute sperm to eggs in common carp (*Cyprinus carpio*) and concluded that injection of sperm into the ovary via the oviducts leads to successful fertilisation and embryo development. This result indicates that sperm can be stored in the ovary and fallopian tube for up to 12 hours without losing biological activity. The surrounding sperm can fertilize eggs after being released from the ovarian cavity. Müller et al. (2018b) published the novel approach to delivering sperm and hormone together to eggs in the African catfish and concluded that injection of sperm + CPE into the ovary via the catheter leads to successful ovulation, fertilisation, and embryonic development in any experiments state that is applied. It has been shown that ovarian lavage with sperm and hormone, using seminal fluid as a delivery aid, can be used as an alternative to traditional IVF, where eggs are fertilized by sperm outside the female's body. In the experiments, only one latency time of inseminated sperm (10 h before expected ovulation) and a dose of sperm (2 ml sperm/kg body weight) were investigated. It is needed to detect the biologically active period of the sperm in the ovary on the ability to fertilize using the proliferation of ovarian lavage/artificial insemination fish.

The aim of this study was to investigate the spermatozoa fertilisation capability under ovarian condition depending on time.

3.13. Possibilities of using the new fish propagation method

The practical advantages of using insemination method were described in Müller et al. (2020). The advantages of the new method are mainly found in the field of tank/pond spawning, ex situ and in situ conservation, biological activation, hybridisation, and reproduction for scientific research. Tank or pond spawning is less important in Hungary, but the total production of Asian carps - bighead carp, grass carp, silver carp and black carp - in freshwater aquaculture is very significant. In 2020, total production was 13 million tons (Yingchutrakul et al., 2022), and China produced >90 % of the world's carp fish (Cao et al., 2015). In China and other Asian countries, the traditional spawning method is still the most widely used method. For instance, in Asian seabass (*Lates calcarifer*) as a mass spawning species, methods under captive culture involve the aggregation of conditioned, sexually mature broodstock, typically at the ratio of 1 to 2 females to 3 to 5 males (Loughnan et al., 2013). By using the traditional spawning method, fish are allowed to reproduce naturally by hormonal induction in ponds, pools, cages, so-called hapas, and concrete pools with circular holes (Horváth et al., 2015). This method is also widely used in marine fish reproduction as well (e.g. Sea bass - *Dicentrarchus labrax,* gilt-head bream - *Sparus aurata,* Asian seabass - *Lates calcarifer* etc.). Their reproduction is based on induced spawning, where the

preparation of the broodstock is carried out solely by influencing environmental factors (water temperature, artificial control of the light programme). For example, hormone-induced spawning via LHRHa injections and environmental manipulation are generally necessary for final gonad maturation and to promote the release of gametes for artificial spawning in Asian seabass (*Lates calcarifer*) (Loughnan et al., 2013). Spawning occurs either spontaneously or is promoted by hormone treatment. The collection of the floating, fertilised spawn is carried out by various egg-collection devices installed in the effluent of the spawning pool. As the spawners in the spawning pool can spawn with several combinations, controlled cross-breeding (breeding in the strict sense) has been limited so far. However, with the method we have developed, controlled breeding can be carried out in these cases by reversing the sex ratio (Table 3.4.), allowing the collection of more fertile eggs from a single area in spawning pools, and mass fish reproduction can be replaced by the basis for planned breeding. Semen from top males with high genetic value can be used to fertilise multiple batches of eggs at the same time. For fish spawning in pairs, genetic diversity can also be increased by this method (by releasing 5-10 samples of males before induced spawning), which can be used for economically important fish species (e.g. catfish, perch, tilapia, climbing perch) or for species of conservation importance (e.g. European mudminnow – *Umbra krameri*). Manipulation of sperm (e.g. use of cryopreserved sperm) in induced spawning may be possible. The new method can also be integrated into hatchery breeding technology, producing a larger quantity of fertilised eggs per unit area at the same time, as there is no need to maintain tanks for males in the case of sperm injection. It is hoped that this novel breeding method will soon be put into practice and become widely applicable.

Table 3.4. Controlled breeding in cases of reversing the sex ratio.

	Traditional propagation		**Insemination method**	
	Sex ratio ♀:♂	Theoretical number of eggs that can be produced per unit area	Sex ratio ♀:♂	Theoretical number of eggs that can be produced per unit area
3 fish / unit	1:2	n	2:1	2n
5 fish / unit	2:3	2n	3:2 or 4:1	3n or 4n
Genetic diversity of offspring	limited, random combination		limited increase from female numbers, multiple male combination	
Breeding programme	limited control		can be controlled	

4. MATERIALS AND METHODS

Four main chapters were performed in this research, including:

Chapter 1. The oral uptake of sperm bundles by female the African catfish during spontaneous mating

Chapter 2. Reproduction effectiveness for induced spawning of *C. gariepinus* by using invasive and non-invasive methods

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

Chapter 4. The potential latency period of spermatozoa in the ovary of the African catfish.

The experimental trials, investigations, and analyses are carried out at the Fish laboratories of MATE, Hungary. Chapters 1 and 3 contained three experiments and one trial, which illustrated in Szent István Campus. Chapter 2 included two tests presented in Kaposvár Campus. Chapter 4 accommodated two investigations enforced in Geogrikon Campus. The detailed explanation of experimental designs for each chapter was documented in 4.3.

4.1. Broodstock management

For the experiments in chapters 1, 2, and 3, *C. gariepinus* broodstock was produced by artificial reproduction and grown in a recirculation system before the experiment in Kaposvár Campus. During rearing, fish were kept at 24–25 °C and fed daily with *Artemia* and a commercially formulated diet (Skretting Classic K 3P, Skretting, Stavanger, Norway) depending on age and size. A natural ambient photoperiod was applied, and the room in which the breeding unit was located was darkened to reduce stress. The used breeders in chapters 1 and 3 were transported to the Department of Applied Fish Biology, Szent István Campus, three days before the experiment. Subsequently, the fish were acclimatized in a 700 L fibreglass tank (T = 26 °C) connected to the external biofiltration system without feeding. The Sampa testes used in chapter 3 were collected from one mature male after dissection in anaesthetic in Research Institute for Fisheries and Aquaculture (HAKI), Szarvas, Hungary. Then, it was kept and transported on crushed ice to control the temperature for the subsequent handling in the experimental place.

Using 36-month-old African catfish as breeding stock in chapter 4, these fish were raised in the recirculation system in Geogrikon Campus and fed on commercial pellet feed (Skretting Classic K 3P, Skretting, Stavanger, Norway). During the experiment, males and females were selected and kept separately (35 L/fish/tank). When the fish was introduced into the tank, the water

temperature was 27 °C, kept constant throughout the study period. The natural light cycle was applied, and the light in the experimental room was dimmed. Main environmental factors were maintained during the experiments. The water temperature was measured three times per day during experimental cycles (27±0.5 °C).

The matured male breeders were identified by their pinkish-coloured papillae. The gravid females were recognised by free oozing out of eggs upon gentle pressure on their abdomen from the pinkish or reddish swollen vents (Olaniyi and Omitogun, 2013).

4.2. Administrative methods for hormones and sperm

4.2.1. The used hormones to induce spawning of *C. gariepinus*

The hormones for inducing propagation of *C. gariepinus* were Ovopel™ (Interfish Kft, Budapest, Hungary) and CPE. One pellet of Ovopel contains 18–20 μg of D-Ala6, Pro9 NEt-m GnRH and 8–10 mg of MET (Horváth et al., 1997). The used CPE in our experiments is the same type managed in Müller et al. (2018a, b) and Müller et al. (2019).

4.2.2. Hormonal induction and sperm administration methods

In various studies, the ovulation, spermination, and sperm manipulation of the fish were managed by traditional and novel methods.

1) IM: Reproductive hormone was homogenized in the vehicle and given about 3 cm posterior to the dorsal fin of fish.
2) IP: Reproductive stimulator was homogenised in the solvent and then injected at the dorsal fin base of the fish.

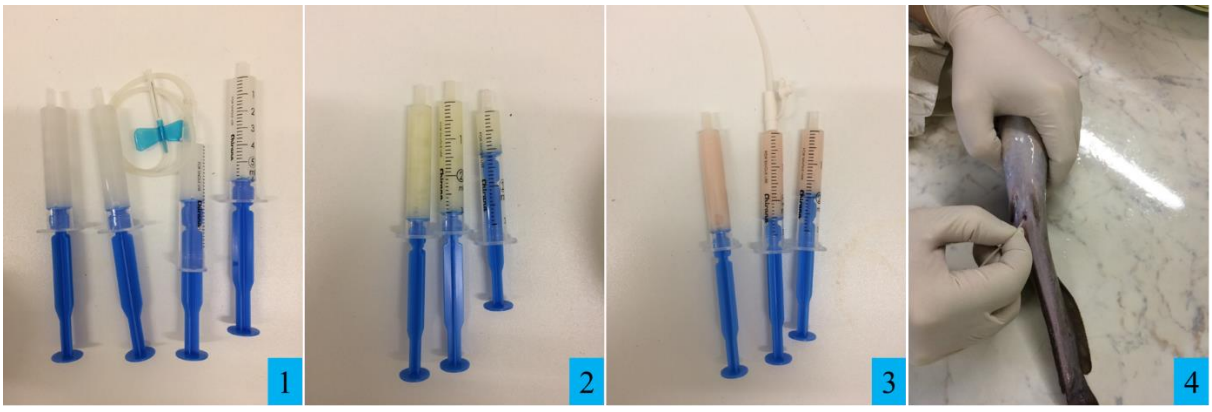

Figure 4.1. Photographs showing the ovarian injection of *C. gariepinus* (4) and the suspension of reproductive hormones in saline (1), avian albumen (2), sperm (3).

3) OHI: Suspended agent vehicle was introduced equally into the ovarian lobes of fish by using a silicone catheter (Feeding tube, 400 mm length, size: CH 4, outer diameter 1.3 mm, inner diameter 1 mm, Galmed, Poland) was attached 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 (see Figure 4.1).
4) OSI: Sperm samples were injected equally into the ovarian lobes by using a silicone catheter as described above.
5) OHSI: Hormone was homogenized in fish sperm and placed equally into the ovarian lobes by the method mentioned above.

4.3. Experimental designs

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

In order to reveal the spawning ethology of the *C. gariepinus* in as much detail as possible, a number of sub-experiments have been planned (Figure 4.2).

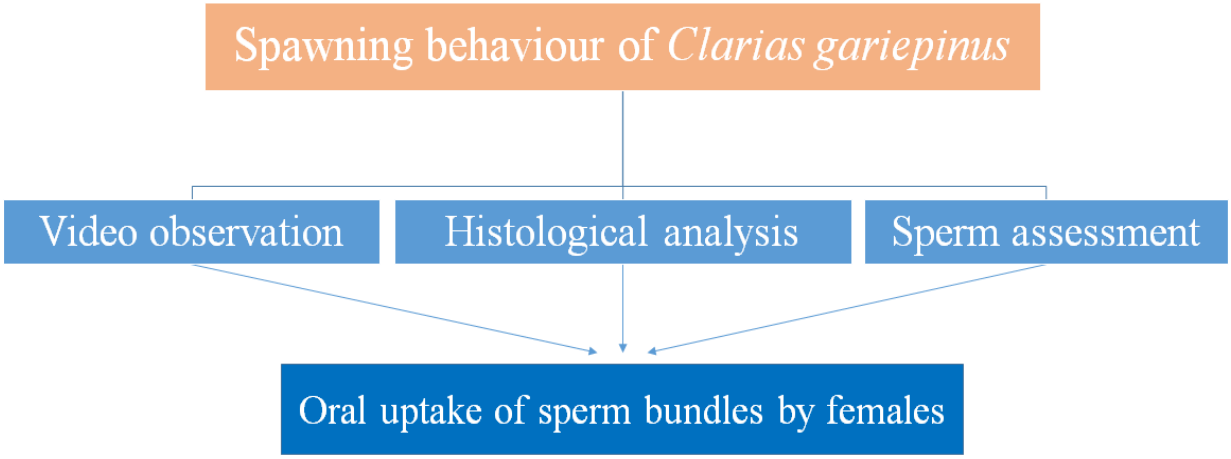

Figure 4.2. The experimental presentation of the African catfish spawning behaviour with focus on the mating stage. The detailed description of each sub-experiments was shown in text (Video observation: 4.3.1.1.; Histological analysis: 4.3.1.2.; Sperm assessment: 4.3.1.3.).

4.3.1.1. *Video observation*

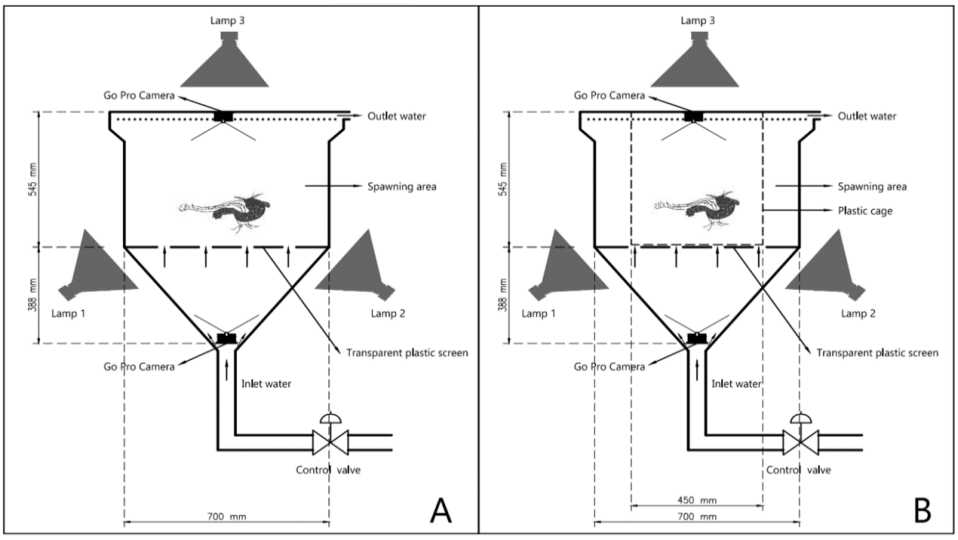

Figure 4.3. The system used for natural spawning and recording videos: None plastic cage (A), including plastic cage (B).

The spawning experiments were recorded in a giant Weiss jar (conical up-welling, 200 L) (Figure 4.3). 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 (diameter: 56 cm, diameter of holes: 2 cm, number of holes: 4) 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 (water temperature ranged between 25 and 26 °C). Three lamps (2 × G13/18W/230 V IP65) were used for video lighting.

Table 4.1. Summarised data of video recordings about the experiments. No of cameras 1 - GoPro 3 in conus and 2 - GoPro 6 above.

No of spawning	TL ♀ (mm)	TL ♂ (mm)	No of cameras	No of recorded mattings	Eggs in video
1.	373	399	1	4	✓
2.	321	371	1	7	Ø
3.	398	434	2	4	✓
4.	442	487	2	8	✓
5.	407	374	1	6	✓
6.	298	310	1	12	✓

Videos were collected of six spawning pairs, although one female did not release eggs during mating. Table 4.1 contains information on the pairs. The GoPro 3 and GoPro 6 cameras could record videos for 1.5 to 2 hours. During this time, periods of spawning phases and subphases were only observed after eggs were ejected and fertilized (n = 5 pairs, $\Sigma_{\text{successful egg releasing}} = 34$).

4.3.1.2. Histological analysis

For histology, three males were selected from broodstock (total length was 270, 303, 342 mm, respectively), and the application of hormones was the same used to observe spawning. 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.

4.3.1.3. Sperm assessment

Four males were selected for evaluation of sperm quality. Male fish were euthanised by decapitation due to sperm collection (Figure. 4.4a, b). 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, Figure 4.4.c). The first three batches from stripping were eliminated from the study due to urine contamination, and the next five from stripping were used for the analysis. After collecting the sperm, the testicles were surgically separated. The testicles were then incised, and the spilt semen droplets were collected in a 2.5 ml Eppendorf tube (Figure 4.4.d). Control samples were used for testing within 5 minutes of collection (water activation 0 minutes).

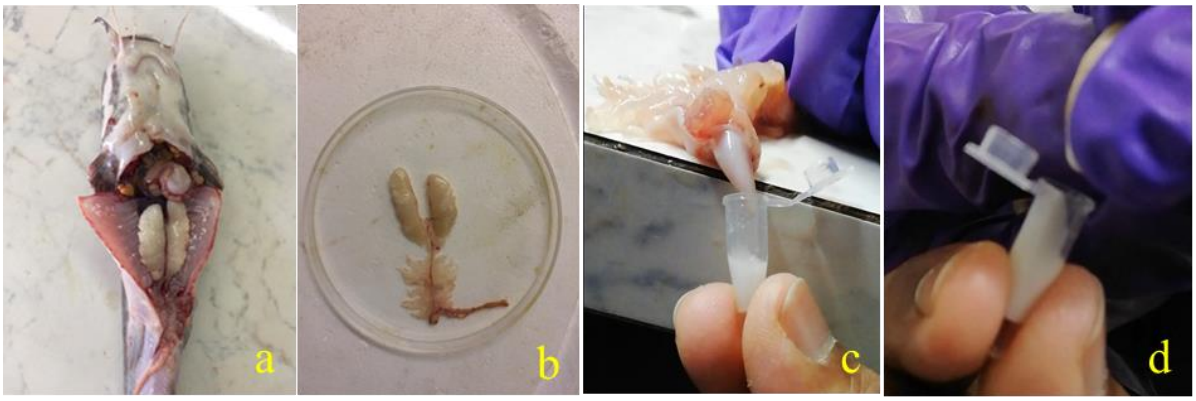

Figure. 4.4. Presentation of sperm collection. A - b) Genital organ, c) Stripping semen and SVS from testis via urogenital papilla, d) Collecting semen from the testis.

Two types of semen were removed from water wells using a Micropipette after 1 sec, 60 sec, and 120 sec from the water immersion. Then, the quality of sperm samples from the testis was compared with that from mixed semen and SVS after activation. Sperm motility parameters were determined using a CASA system (in details chapter 4.5.1.: Sperm measurement).

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

Two experiments were carried out in this research to conduct various hormonal manipulations and a new vehicle of agent for inducing ovulation of fish (Figure 4.5). For the experiment I, 8 males were used; 5 males (883.2 ± 96.1 g) for IVF and 3 males (856 ± 56.1 g) for insemination. For the experiment II, 5 males (906.4 ± 41.7 g) were used.

In experiment I, five treatments were set up. Each treatments contained 7 randomly selected females. According to the results of experiment I, experiment II was conducted to reveal the exact effects of ovarian injected sperm with and without Ovopel on the fertilisation. Two treatments were identical comparing to experiment I (IM and OHSI) and sperm samples were inseminated as positive controls (OSI).

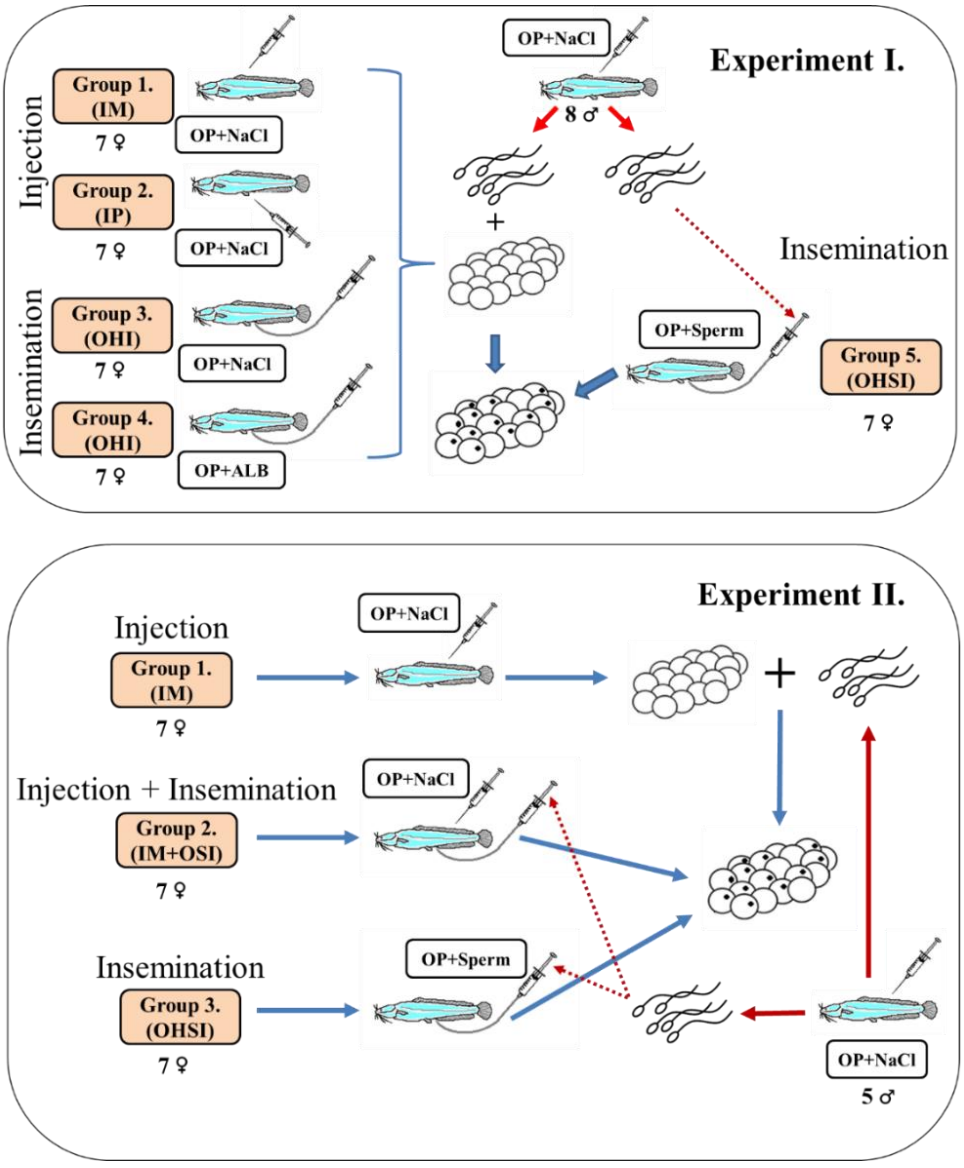

Figure. 4.5. Schematic representation of the design of the experiment I and II; Ovopel-OP, Sodium chloride solution (0.65 %)-NaCl.

4.3.2.1. Experiment I (EI)

- Group 1, 2. Ovopel pellets 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 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.

4.3.2.2. Experiment II (EII)

- 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.

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

To advance the timing of spawning, the African catfish brooders were induced to trigger ovulation by IM with CPE. Sperm from one specimen of the Sampa (Figure 4.6) was injected into the ovaries of four *C. gariepinus* ♀ (BW = 750.5 ± 51.18 g); females were then placed in pairs with four *C. gariepinus* ♂ in four separate spawning cages (Figure 4.7).

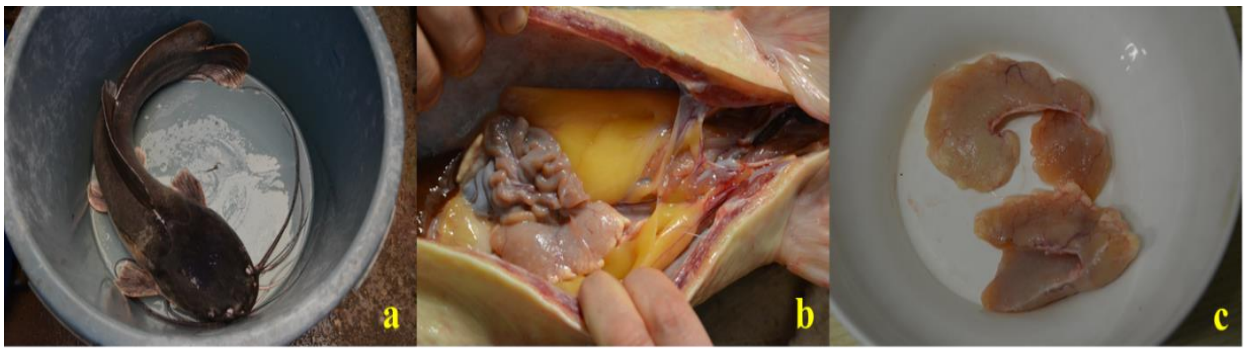

Figure 4.6. The representation of collecting the Sampa testis: male fish (a), fish surgery (b), and testes (c).

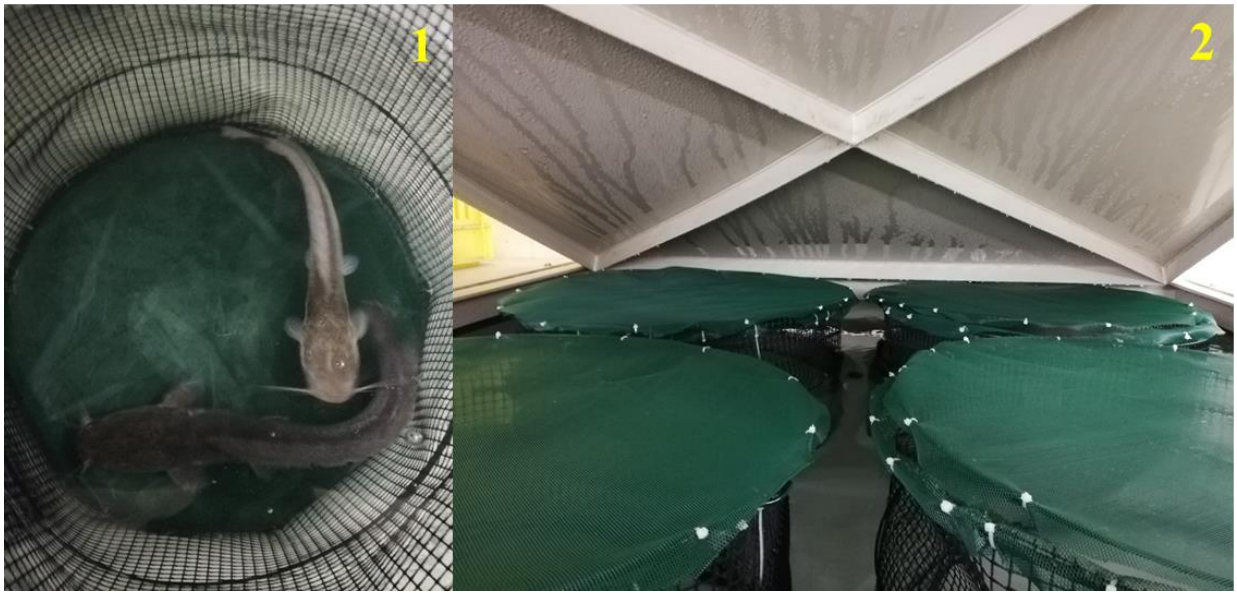

Figure 4.7. Schematic representation of cages/tank spawning for inter-specific crosses (C×H). After inducing spawning of four pairs *C. gariepinus* (1), in which the females were then injected the milt from *H. longifilis* by OSI method (Müller et al., 2020), the pairs were introduced separately into the spawning cages (2).

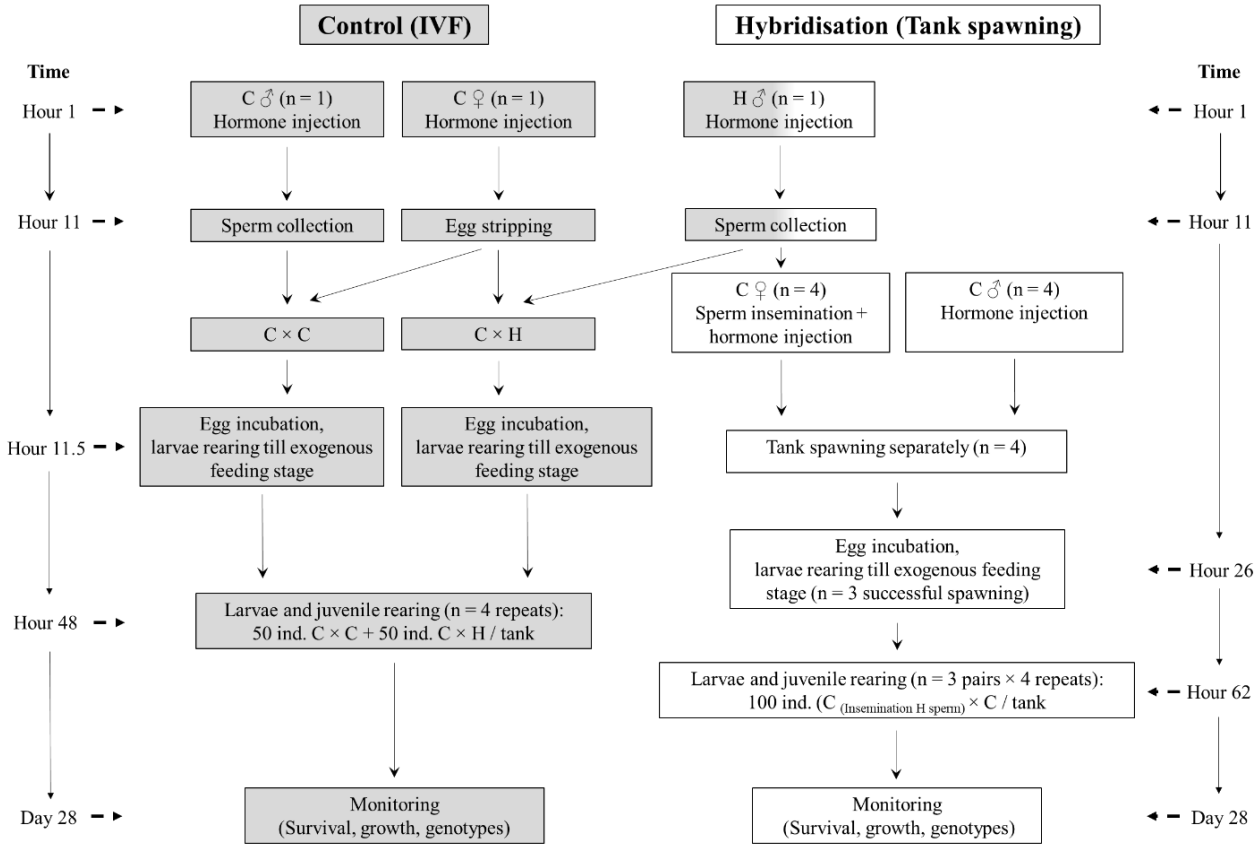

Figure 4.8. Schematic diagram of the experiment. C-*C. gariepinus*; H-*H. longifilis*; Ind.-individuals.

The offspring from each spawning were reared in four replicates for up to 4 weeks to select and separate the genotypes (hybrid and pure *Clarias* individuals) 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 using. 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 (body weight: 796 g) and one male *C. gariepinus* (bodyweight: 956 g) 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 (Figure 4.8).

4.3.4. 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, experiment I (EI), and experiment II (EII) (Figure 4.9).

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.

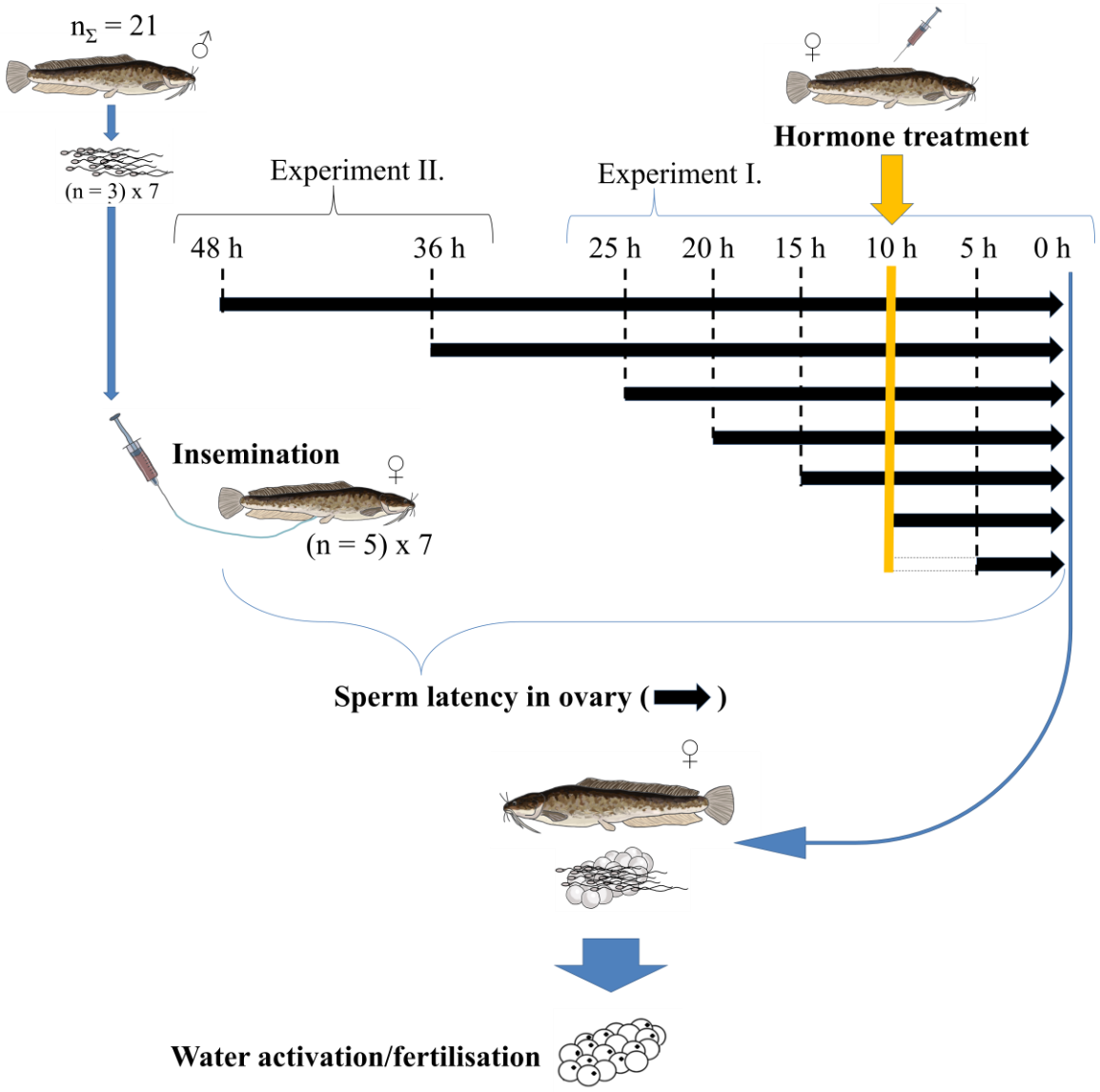

Figure 4.9. Schematic representation of the experimental design of the EI and EII. Pooled sperm samples were inseminated into the ovaries of the African catfish from 5 to 48 hours before stripping. Sperm samples were originated from surgically removed testis of 21 males. Five females were applied in each treatment. Hormonal administration was applied according to the description of Müller et al (2020).

4.4. Hormonal induction dosage of spawners

Hormonal induction of *C. gariepinus* in chapter 1 and 3 was carried out by IP therapy at a dosage of 3 mg CPE/1 mL 0.9 % NaCl solution/kg body weight for males, and 5 mg CPE/1mL 0.9 % NaCl solution/kg body weight in females following the description in Müller et al. (2018a, 2019, 2020). The *H. longifilis* male was conducted by injection of CPE (4 mg/kg BW, Brzuska and Adamek, 2008). In chapter 4, hormonal induction of males and females was administrated by the IP method of CPE (5 mg CPE/1 mL 0.9 % NaCl solution/kg BW).

All females in chapter 2 were treated with the Ovopel with the dosage of 1 pellet/kg of BW. In both experiments, the hormone was grinded, the appropriate vehicles were then added in the ratio of 1 pellet/2 mL for each treatment. In each experiment, male hormone therapy was given with Ovopel suspended in 0.65 % NaCl solution by IM method 10 hours prior to female treatment for OSI. In the case of IVF, males and females were injected concurrently.

After hormonal administration, the females and males were kept separately in suitable tanks/cages to avoid the aggressive invader behaviour.

4.5. Sperm measurement and injection dosage

The testes were collected from male fish by opening the abdomen, and surgical testes were then cut and gently stripped to collect the milt. The semen samples were recorded and subjected to motility analysis. Selected sperm samples, for which expected motility was over 80 %, were selected for experimental purposes and were pooled for ovarian lavage administrations.

4.5.1. Sperm quality analysis

In chapter 1, sperm motility parameters were determined using a CASA system (Sperm VisionTM v. 3.7.4, Minitube of America, Venture Court Verona, USA) coupled to an Olympus BX 41 microscope using a 20 × negative phase contrast objective). The main motility parameters examined in this study were progressive motility (%), average path velocity (VAP; μm/s), curvilinear velocity (VCL; μm/s), linearity (LIN; %) and path straightness (STR; %). All sperm quality assessments were performed using a regularly used activating solution in cyprinids (Saad et al., 1988) comprising 45 mM NaCl, 5 mM KCl, 30 mM Tris (pH = 8). In this case, the following technique of water activation was used: A 10 μl sperm sample was transferred into a 6 Well Multiwell Plates (cell culture multiwell plate, 6 well, PS, clear, cellstar®, TC, lid with condensation rings, Greiner Bio-One International) by using Micropipette with pipette tips (Tips LTS 10 uL, Metler Toledo). Each well contained 4 mL of system water of Zebrafish (water parameters: 25.5±0.5 °C, pH 7.0±0.2, conductivity 550±50 μS). After 60 and 120 seconds following water activation, sperm samples were removed from wells using a Micropipette for sperm analysis (description of sperm analysis was same to control studies) and analyzed using the CASA system. One sperm sample/well/treatment/male was analysed only once, but all treatments were replicated four times with the same methods (n = 4 males).

In chapter 2, the collected sperm samples were recorded and subjected to motility analysis. A drop of milt (about 5 mm^3) was placed on a slide, activated by 50 μL water, and covered by a cover glass. Motility of spermatozoa was observed and measured using a microscope (200× magnification; Müller et al., 2018a, b).

In chapter 4, a drop of sperm was placed on a slide activated by 50 µl water and covered by a cover glass. After that, the motility of spermatozoa was monitored and quantified using a microscope (100 × magnification, Olympus BX43, Olympus Corp., Tokyo, Japan). Likewise, the sperm concentration of pooled milt samples was evaluated using a Bürker chamber in EII, resulting in a 1.12×10^9 cell/mL value.

4.5.2. Sperm injection dosage

In chapter 3, milt dosage was inseminated at 1 mL sperm/fish, and the applied dose in chapter 2 and 4 was 2 mL/kg of BW. The calculated dosages were divided equally between the two ovaries.

4.6. Fertilisation and incubation

Artificial sperm fertilisations are conducted at different times and routines that depend on the ovulation of fish. The fish were checked for ovulation by a slight pressure of the abdomen (Brzuska, 2002).

Chapter 2. Gametes stripped from inseminated females (group 5 in EI and group 2 and 3 in EII) were activated with aerated water in petri dish (Ø = 150 mm). In group 1, 2, 3 (EI) and group 1 (EII), eggs were stripped from each female. From each egg batch, 5.0 g of eggs were removed and put into a petri dish (Ø = 150 mm) and fertilized with 0.1 ml sperm. Five minutes after water activation, randomly selected egg samples (n = 96) from each female were placed into a 96-well cell culture plate (PS; U–bottom, Clear, Cellstar® TC, sterile, Greiner Bio-One Hungary Ltd., Mosonmagyaróvár, Hungary) and kept at a temperature of 28 °C for 24 hour (photo number 1, 2 of Figure 4.10). No water exchange was applied.

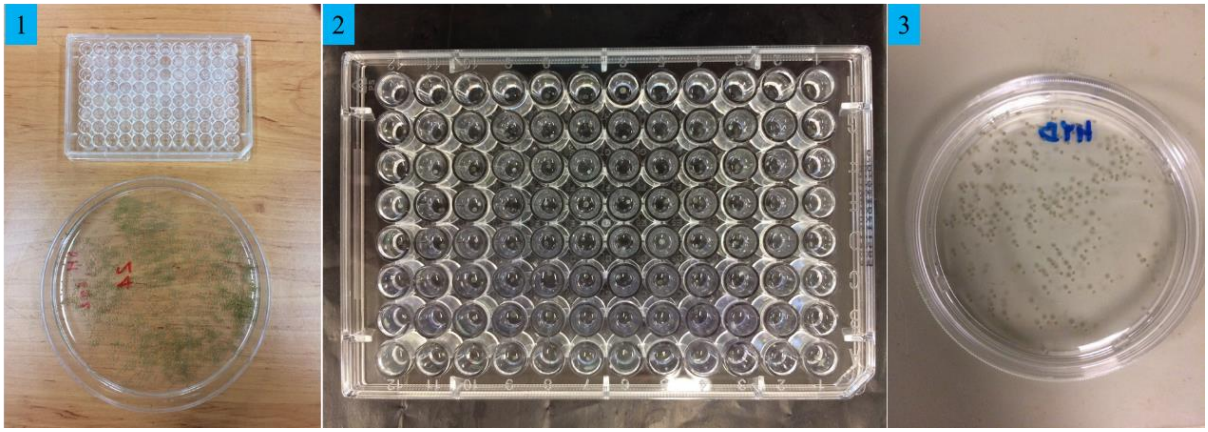

Figure 4.10. Fertilisation of eggs in a petri dish (1, Ø = 150 mm) and incubation of fertilised eggs in 96 - Well Plates (2) and Petri dish (3, Ø = 105 mm).

Chapter 3. In the control group, the IVF method was applied to produce the pure ontogeny *C. gariepinus* and the hybrid ontogeny of C×H. Twenty milligrams of the African catfish eggs were stripped into a dry plastic bowl and divided into two parts. Subsequently, part one was fertilised with the African catfish sperm (0.5 mL), and the other part was fertilised with the Sampa sperm (0.5 mL). Then, both gamete batches were activated with aerated water. Five minutes after activation, fertilised egg masses from each batch were separately incubated in plastic tanks (60 × 30 × 20 cm, the volume of 30 L water and 120 L / hour water flow) up to exogenous feeding larvae. Meanwhile, fertilisation and hatching rates were recorded in the fertilised eggs of both genotypes. Six egg samples (mean ± SD: *C. gariepinus* × *C. gariepinus* = 280. 67 ± 22.01, n = 3; hybrid eggs = 170.33 ± 25.11, n = 3) were collected from both batches and incubated in six Petri dishes (105 mm diameter; Photo number 3, Figure 4.10) at 26°C. Water was changed every 5 hours until the fertilisation and hatching rates were determined.

In the tank spawning group, four spawning cages (0.6 m diameter × 0.5 m height) were placed in one spawning tank (2 m length × 1.5 m width × 0.5 m height; water level adjusted at 0.45 m, total volume was 1 m^3), which was connected with the external biofiltration system. The spawning cages were shielded and covered by a plastic mosquito net (mesh size = 0.6 mm) to prevent the released eggs from escaping and leaving a 5 cm gap, allowing *C. gariepinus* broodstock to take gulps of air at the free surface. Water quality parameters were maintained at optimal levels (T = 25–26°C, pH = 6.8–7.8, NO_3 = 40–75 mg/l, NO_2 = 0.25–0.45 mg/L) during the spawning period. The tank spawning water naturally activated the gametes from sperm-injected females in this group. The pairs were checked 15 hours after tank introduction, and egg batches were collected from the bottom of the spawning tanks. Collected eggs were separately incubated in plastic tanks (60 × 30 × 20 cm, a volume of 30 L of water and 120 L / hour water flow) up to exogenous feeding larvae. The incubation tanks were butted to an operational biofilter system, and the water quality parameters (T = 25–26 ^{O}C, pH = 7.4–7.8, NO_3 = 25–30 mg/L, NO_2 0.2–0.4 mg/L) remained constant during the incubation period.

Chapter 4. Stripped and mixed gamete batches were fertilised by the IVF method mentioned above. Five minutes after the water activation, three samples of eggs (average egg number per Petri dish: EI = 76.3±21.3, EII = 188.9±63.2) were collected from each batch and incubated in a Petri dish (105 mm diameter) at 25 °C. Water was changed continuously by using a dripping system (1700–1900 mL/hour) in the Petri dishes.

PGSI (%) = (weight of stripped egg mass/BW before stripping) × 100 was measured for each female. The fertilisation and hatching rates for eggs of two fertilisations (hybrid and pure *Clarias*)

were determined by calculating the embryo survival rate at 12 h post-fertilisation (fertilisation rate) and 36 h post-fertilisation (hatching rate), as mentioned in Müller et al. (2020).

4.7. Larvae rearing

Exogenous feeding larvae from all genetic groups of chapter 3 were held at a density of 100 fry / plastic basket (28 × 15 × 12 cm, 5 L volume) containing 2.5 L water. Each container of the control group was assigned 50 pure *C. gariepinus* larvae and 50 hybrid larvae, while the chambers of the treated group were stocked with 100 larvae. All treatments were carried out in four replicates. Each basket was joined to the operational biofilter system described in 4.6., and the circulatory water flow was maintained at 6 L / tank / hour. The larvae were fed *Artemia nauplii* to satiation five times daily during the first five days. Then, larvae were alternately fed with *Artemia nauplii* (2 times a day) and zebra fish feed (3 times a day, crude protein 63 %, Sparos, Purtugal) up to day 28. The waste and residual feed were removed by siphoning prior to each feeding.

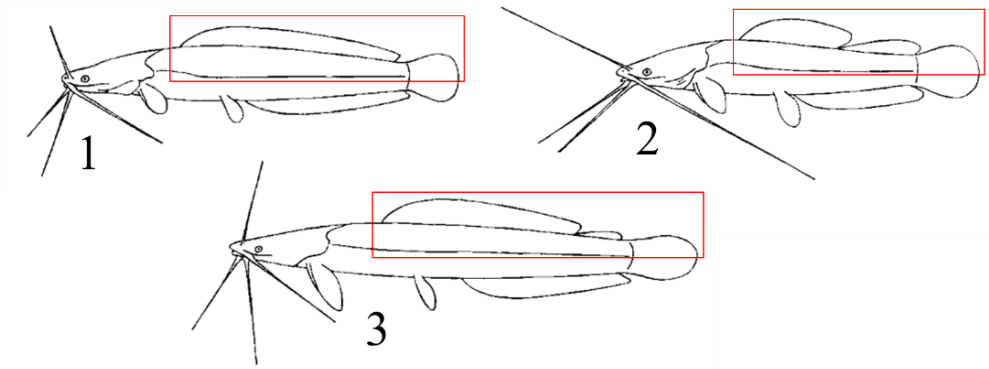

Figure 4.11. Schematic identification of (1) *C. gariepinus*, (2) *H. longifilis*, and (3) their crossing (Legendre et al., 1992).

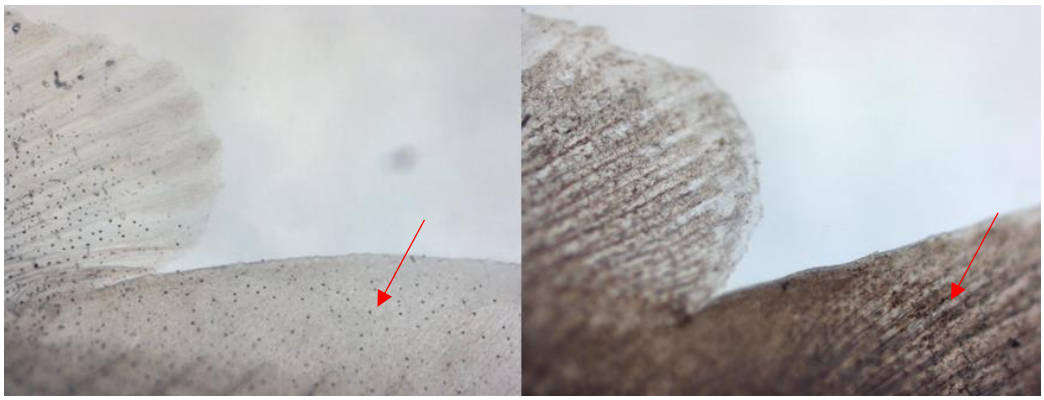

Figure 4.12. The difference between hybrid progeny (a small adipose fin, left image) and pure progeny (none adipose fin, right image) observe by microscope 4 ×.

Hybrid and purebred fry were distinguished based on the morphological differences between the two genotypes mentioned in Legendre et al. (1992) and Teugels et al. (1992) (Figure 4.11). In brief, a large adipose fin was revealed in pure *H. longifilis* but absent in *C. gariepinus*. The crossbreed specimens of C×H presented an intermediate exhibition with a small adipose fin. Genotype identification was observed at 4 × magnification using a microscope (Nikon Eclipse E600, Nikon Corporation, Tokyo, Japan) (Figure 4.12).

4.8. Statistical analysis

The obtained data were presented as mean±standard deviation. The statistical analysis was performed with SPSS 22v, v26 for Windows. Significance was accepted at $P < 0.05$.

The reproductive factors and rearing parameters were calculated:

1) Ovulation rate (%) = (number of ovulated females / numbers 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 / totals introduced fish) × 100
5) Hybridisation rate (%) = (number of alive hybrid fish / total alive fish) × 100

Chapter 1. Summary of CASA outputs of each measurement was used in statistical analysis. Progressive motility data within the organs were analysed by one-way analysis of variance (ANOVA), followed by Dunnett T3 post hoc test. Percentage data were subjected to Log10 transformation before statistical analysis. The progressive motility data between the original organs simultaneously were compared using an independent sample t-test.

Chapter 2. Data from body weight, PGSI values of treated groups, were analysed by one-way analysis of variance (ANOVA), followed by Duncan's post-hoc test. Data from hatching rate were analysed by Kruskal Wallis and Mann-Whitney tests. Percentage data on the hatching rates were subjected to log(x+1) transformation before they were analysed statistically where the means obtained from each replicate was used.

Chapter 3. Data from body length, body weight, and condition factor were log transformed before analysis. The data obtained were subjected to an independent t-test. Survival data were arcsine-square root transformed, and normality was tested using the Shapiro–Wilks W tests ($P > 0.05$ in each case). Fertilisation rate data for IVF were arcsine-square root transformed and analysed using an independent sample t-test.

Chapter 4. Statistical analyses of fertilisation rates, bodyweights, egg number/females, and PGSI values were carried out by one-way ANOVA (with Dunn's post hoc test).

4.9. Ethical issues

Before all procedures, experimental fishes were anaesthetised in a solution of 100 mg L^{-1} benzocaine (ethyl 4-aminobenzoate, Norcaine) for further intervention. The male fishes were over-anaesthetised with benzocaine solution and sacrificed by decapitation before collecting semen and histology samples.

Experiments were carried out following the license PEI/001/1719-2/2015 issued for the Department of Aquaculture of Szent István University by the Division of Food Chain Safety, Plant and Soil Protection of the Government Office of Pest County in Hungary (Chapter 1, 3). In Chapter 2**,** all procedures involving fish were conducted in line with the Hungarian legislation on experimental animals and were approved by the National Scientific Ethical Committee on Animal Experimentation (identification number of the license: KA - 3099). All effort was made to minimize the fish suffering. In Chapter 4, the experiment was 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), concerning scientific content and compliance with applicable research subject rules.

5. RESULTS AND DISCUSSION

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

5.1.1. Spawning behaviour

Up to the amplexus, the mating behaviour of *C. gariepinus* is equivalent to that of the literature (Van der Waal, 1974; Bruton, 1979a); for *C. macrocephalus* see Olshanskiy et al. (2020). However, the amplexus could be divided into four distinct stages, which we have defined.

I. Position fixation by using fin hugging

After being connected directly from the male, the female accepts the male to bend his body around her head and anterior body in a U-shape. Following this, the mates fix their positions at 3 points of contact (Figure 5.1):

1) The right (or left) pectoral fin of the female is stretched out perpendicularly to her body axis, and the male grabs and holds it with his left (or right) pectoral fin. This posture prevents the partners from slipping backwards if the female swims ahead and suddenly stop.

2a) The male's ipsilateral pelvic fin usually stiffens laterally under the female's head.

2b) The female barbels surround the male pelvic fin to consolidate the connection.

3) The body, including the anal fin of the male, is bent into a U-shape. The fin partially covers the female mouth. The time from position fixing to stripping (Figure 5.1) is 8–20 seconds. This sub-phase finishes when the partners stop and reach motionless.

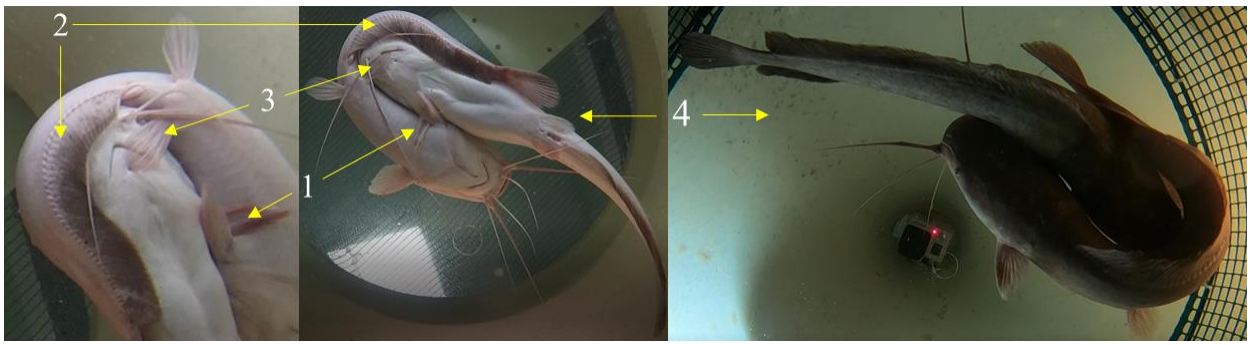

Figure 5. 1. 1. Pectoral fin (♀ - stretches, ♂ - locks), 2. ♂ Ventral fin (head support), 3. ♂ anal fin (turns towards ♀ mouth and trunk), 4. Two side of amplexus.

II. Sperm release – III. Uptake of sperm by female

From the videotapes, an evaluative volume of 1–3 µL of sperm is discharged from the urogenital papilla to form a sperm bundle (total papilla length: 200–500 µm, stage II maturation, see histological results). A rapid, pulsating (0.5–1 second) movement of the urogenital papilla is often observed. The urogenital papilla is always bent into a space created in front of the male anal fin, and sperm are released into the space. Then bundle disappears in the female's mouth while her operculum is closed (Figure. 5.2). The period between the release of sperm and the vanishing sperm is 1.2±0.3 seconds (n = 41).

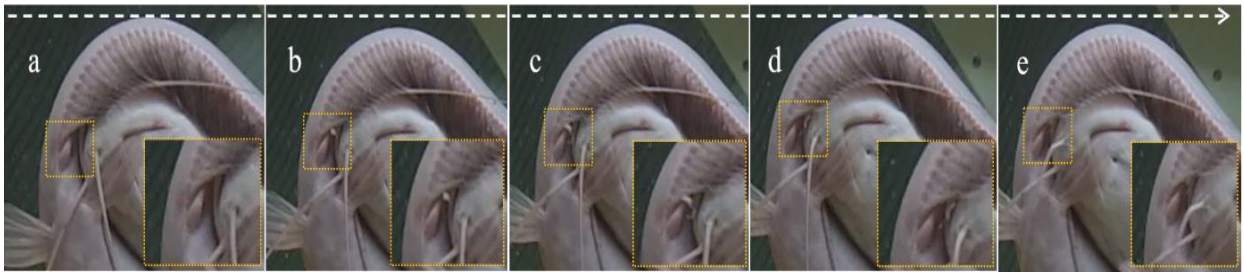

Figure 5.2. Sperm ejection (a–b), Uptake of sperm by female (c–e).

IV. End of amplexus

As soon as the sperm bundle has disappeared in the female's mouth, the female touches the male genital region with one or two of its longer barbels, previously fixed in the spawning position (Figure 5.3). Between sperm ejection and the end of the fixed position, there are 11.17±7.47 seconds (minimum 4–maximum 27 seconds).

Figure 5.3. Barbel signal, amplexus end (time: ~1 sec.).

V. Female stripping by male and fertilisation with help of an air bubble cloud

After the amplexus, the female barbels release the male's pelvic fin and the male release the female's head from his body embrace. The connection of two pectoral fins is continued to permit

the female to turn perpendicularly away from the male, which retains its U-shape. The male grabs the female from the abdomen with the head pointing to the ground. The female's posterior body is slightly lifted with the help of the male. In parallel, the male slides into the female's abdomen and quickly presses the female belly behind her pectoral fins, leading to the release of egg clouds. At the same time, the opening of the female's operculum allows an air bubble mixed with sperm to escape from her buccal chamber (observed in 34 of 41 successful matings; not seen in 7 of 41 successful spawnings). The "mixture" comes in contact with the released eggs, which are always located higher in the water column due to the increased turbulence caused by the air bubbles. It is essential to record that the air bubbles get out of the buccal chamber towards the area of urogenital papillae through the channel created by the turbulence and not from the genital pore as previously supposed (Figure 5.4). The subsequent steps correspond to previously described (Van der Waal, 1974; Bruton, 1979a).

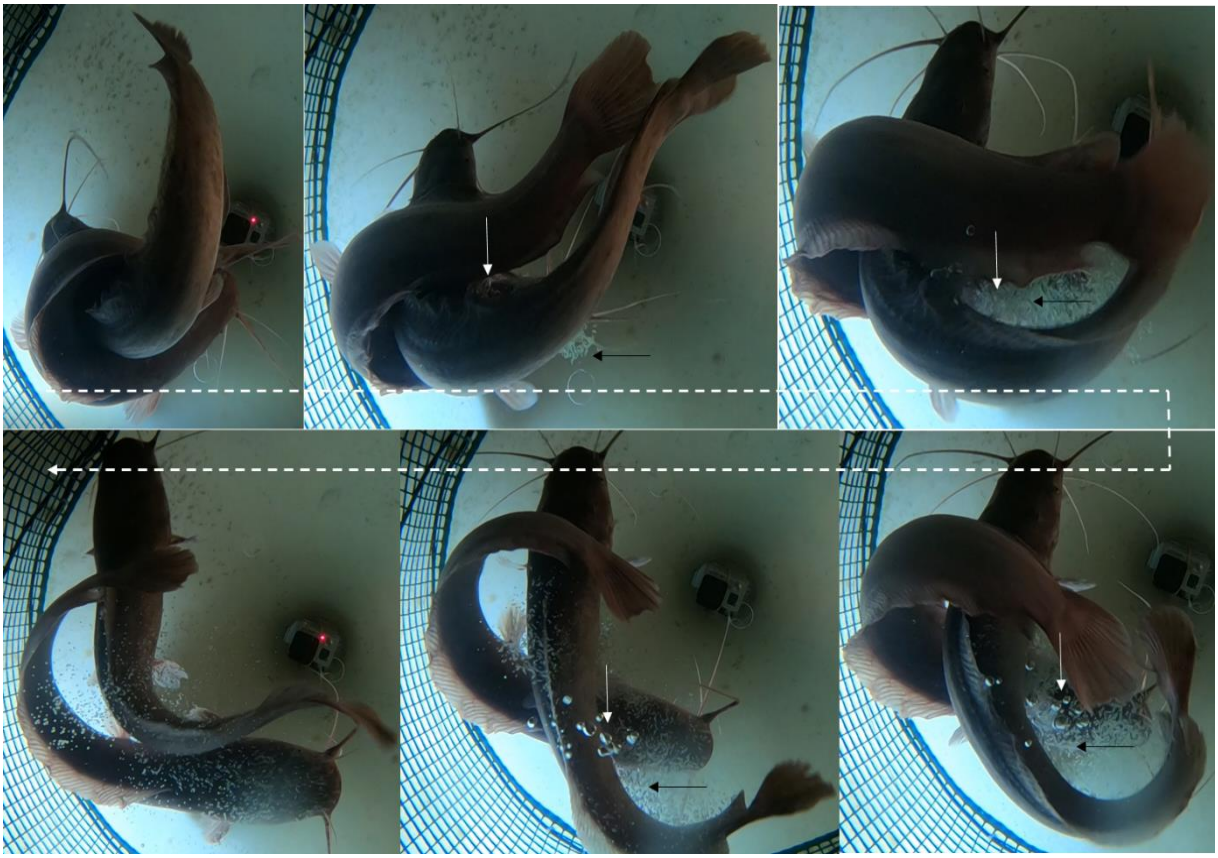

Figure 5.4. Female stripping by male and fertilisation with the help of air bubble cloud (white arrow – air bubbles, black arrow - eggs, time: 2.7±1.1 sec, n = 41).

5.1.2. 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 (Figure 5.5).

Seminal vesicle: The seminal vesicle consists of a system of finger-like lobes containing tubules. These tubules are filled with a fluid in that sperm cells are stored (Figure 5.5). 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 fibres surround the epithelium. No specific sperm bundles were seen on urogenital papillae (Figure 5.5). The sperm bundle formation can occur when the surface of SV sperm activates with the water (Figure 5.6).

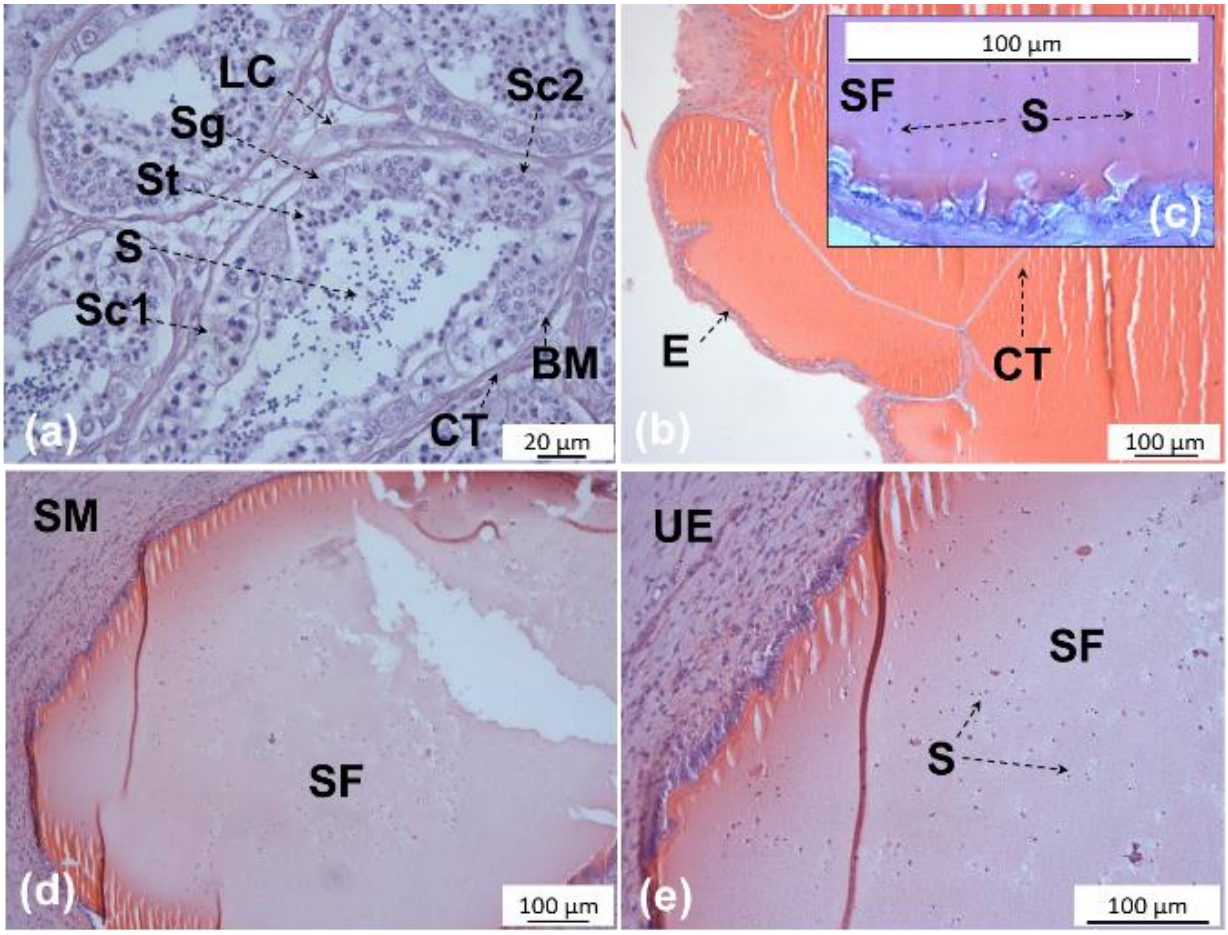

Figure 5.5. Cross section of (a) testis, (b)-(c) seminal vesicle lobe, (d)-(e) urogenital papille. Abbreviations and symbols: E = epithelium, BM = basale membrane, SM = striated skeletal muscle; UE = urogenital epithelium, S = spermatozoa, St = spermatid, Sg = spermatogonium, Sc1 = primary spermatocyte, Sc2 = secondary spermatocyte, CT = connective tissue, LC = Leydig cell, SF = secretion fluid.

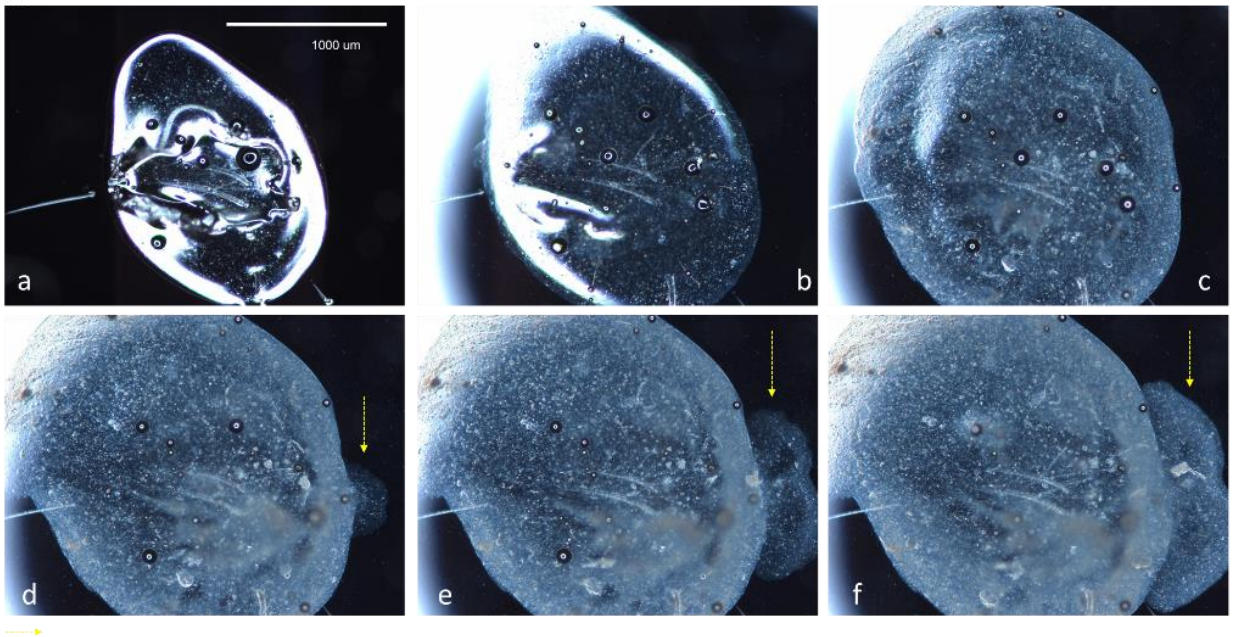

Figure 5.6. A - f. Sperm bundle formation in water activation, a) stripped sperm from seminal vesicle without water activation, b – f) water activation - protein coagulated forming fibrous or granular particles as “sperm bundle”. White arrow: sperm cloud (SVS with motile spermatozoa) from damaged sperm envelope and sub-sperm bundle formation by coagulation on the surface. SVS after water activation. Just before water activation, the time of water activation-yellow arrow indicates that formatting of sperm bundle by coagulated protein creates a layer around the SVS.

5.1.3. Sperm quality analysis

The percentage of motile spermatozoa (progressive motility; spermatozoa combined with seminal plasma) has decreased over time (Figure 5.7, 5.8). Stripped sperm samples in which milt was combined with SVS within the first 60 seconds enhanced motile 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 comparable to that of testis sperm.

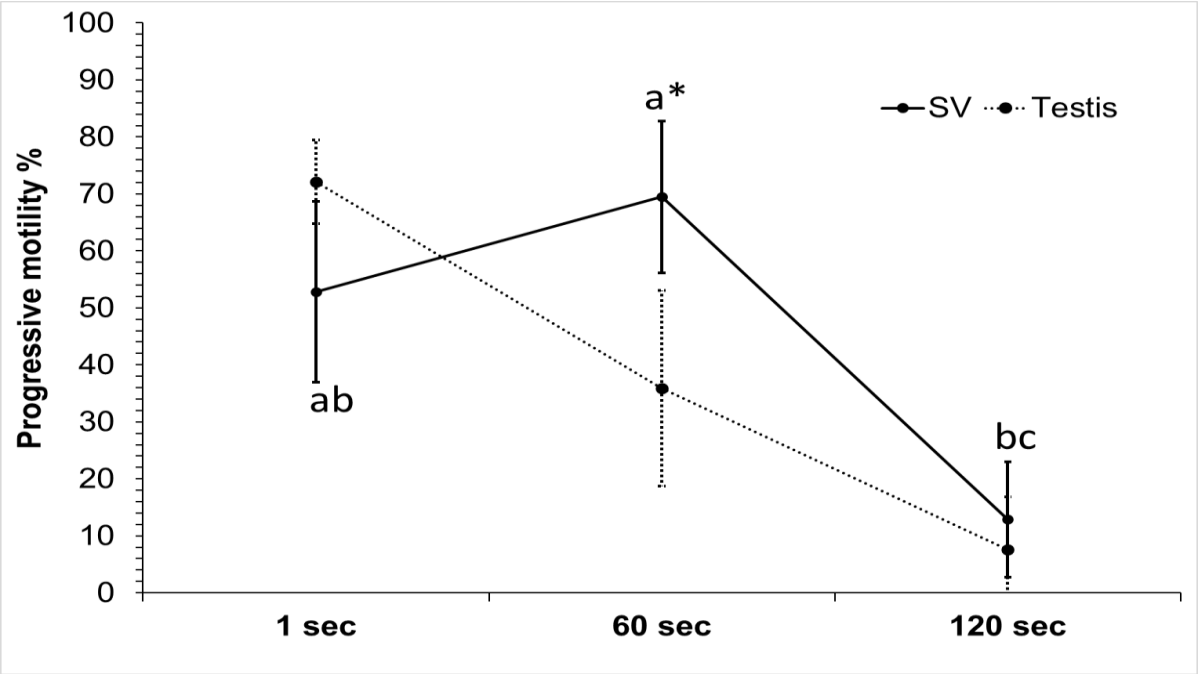

Figure 5.7. Progressive motility collected from the testis and stripped through the male genital organ from the testis to the urogenital papilla (SV). Different letters in the superscript indicate significant differences ($P \leqslant 0.05$, one way ANOVA) in SV. Stars in the superscript indicate significant differences ($P \leqslant 0.05$, independent t test) between the two types of semen.

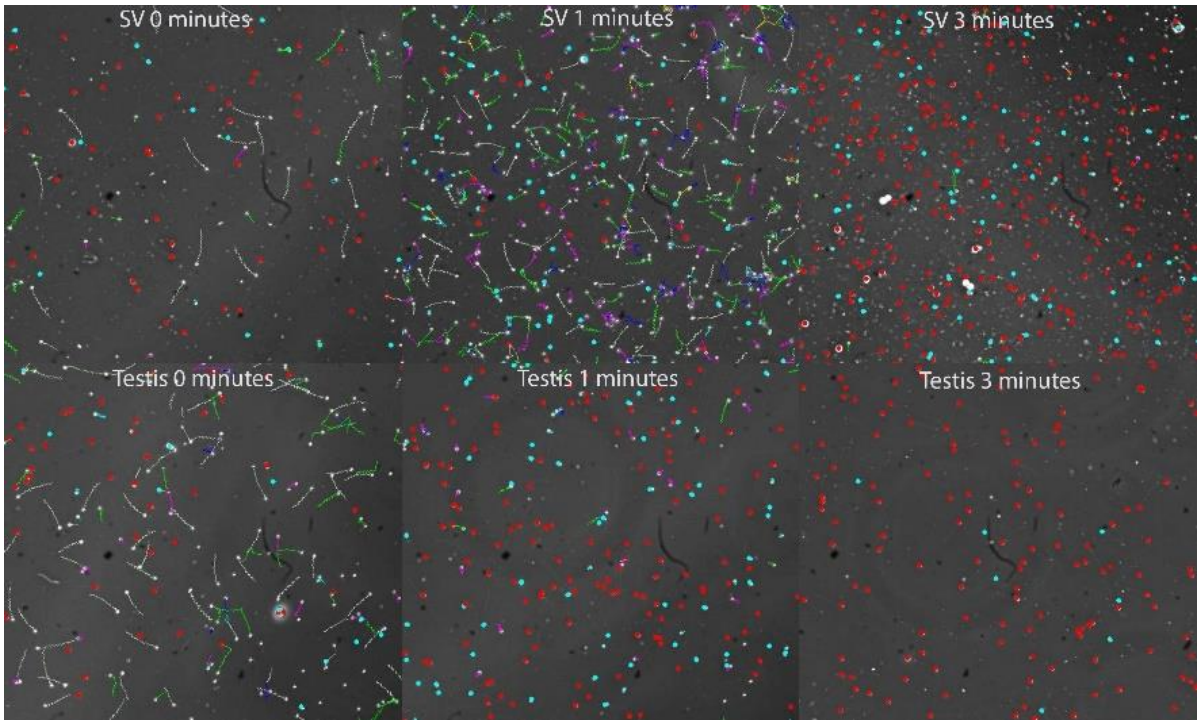

Figure 5.8. CASA photos of progressive motility from the males I, depending on water activation time. SV 1 second: 44.89 %, 60 seconds: 75.48 %, 180 seconds: 5.26 %, Testis 1 second: 64.44 %, 60 seconds: 21.12 %, 180 seconds: 0 %.

5.1.4. Discussion

The spawning behaviour of the African catfish was separated into various stages by Van der Waal (1974) and Bruton (1979a) based on aquarium and field observations. Their observations of gamete, including butting, nipping, mouth fighting, biting, spine tears, chasing, following, and amplexus can be primarily confirmed by our observations (Table 5.1). Male also accompanies specific behavioural features- and female-typical electric pulses, which are perceived via ampullary receptors in the skin and support egg release (*C. macrocephalus*, see Olshanskiy et al. (2020). In the following, we draw attention primarily to behavioural elements that have not been discovered so far (Table 5.1).

Table 5.1. Summarised table about the spawning description in literature and the new findings reported in the presented study.

Phase (Bruton 1979a)	**Literature description** (Van de Waal 1974, Bruton 1979a)	**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.

The unique U-shaped position during mating of various Clariidae has been reported repeatedly. It holds the female for a time in a posture that enables the mouth close to the male's genital opening, which is additionally supported by fin and barbel clinging. This fixation, in our

view, helps the exact receipt of the sperm, which the female, of course, takes orally. According to Olshanskiy et al. (2020), electrical pulses from the female cause "tetanus-like axial muscle contractions" in the male, which further intensify and strengthen the male's embrace.

A significant result of our observations is that the male's ejaculation cannot be described as "milt", nor does it have a "watery texture". Instead, 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 illuminated, 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.

Male could release the mixed semen and SVS like a clotted bundle during the mating phase in the amplexus. Males of Clariidae have a testicular duct system with seminal vesicles, i.e. fingerlike lobes organized in tubules that form a secretion (SVS) consisting of various proteins polysaccharides, glycoproteins proteins, and phospholipids (e.g., Van den Hurk et al., 1987; Mansour et al., 2002, 2004) and reaching the peak level during the spawning season (Van den Hurk et al., 1987; Fishelson et al., 1994). Various functions have been attributed to the SV secretions, many of which obviously do not apply (Van den Hurk et al., 1987; Mansour et al., 2002, 2004). Most interesting for the present discussion, however, is its viscosity and sticky nature, which appear to be primarily due to its glycoprotein content, and its changes when it comes into contact with water that can be observed in our findings and mentioned in Mansour et al. (2002, 2004). *In vitro* experiments show that the sperm were completely immobile in pure SVS, apparently due to the high viscosity of the secretion, and that after the addition of water, the SVS coagulated "forming irregularly spherical or fibrillous shaped protein remnants" (Mansour et al. 2004) and that the process could be reversed. We have made this process visible in Figure 5.6.

Further, Mansour et al. (2004) concluded that the SVS is "unstable in the environment relevant for natural spawning" and doubt whether the secretion is released together with the semen. In contrast, Fishelson et al. (1994) described that during reproduction, mature sperm accumulate in the proximal tubule of the vesicles and that sperm mixes with the secretion of the SV. Further, Mansour et al. (2004) stated that direct observations on the mode of semen release would be the key to elucidating the exact functions of SVS as it would be necessary to know how and when in the spawning process the secretion is released. However, this is complicated as spawning occurs at night, and in grass containing muddy water which turbidity still increases by the spawning activities (Van der Waal, 1974; Bruton, 1979a). We indicate that the secretions are released together with the sperm forming a sperm packet at least superficially stabilised by the coagulated SVS that can be picked up into the female mouth. There is an interesting parallel in mouthbrooding

tilapia fishes where females also ingest inactive sperm surrounded by a viscous, PAS-positive substance along with the eggs, and the sperm inseminate the eggs after activation in the oral cavity (Grier and Fishelson, 1995).

The events that occur after the intake of the sperm bundle by the female may only be determined indirectly. In the watery milieu of the oral cavity, the movement-inhibiting effect of the SVS must be canceled, and the sperm must be transported to the outside. It is done via the gill openings, and the activated sperm reach the released eggs with a directional flow of water and air bubbles. Sperm of *Clarias* species seem to be mobile in the water only for a short time (approx. 2 to 3 min, cf.. Mansour et al., 2002, 2004; Wagenaar et al., 2012; Olshanskiy et al., 2020). The movement of spermatozoa at a speed of about 0.1 mm/s is not enough to reach the eggs in a reasonable time. Olshanskiy et al. (2020) attributed the successful spawning mainly to the water roughness that occurs when the partners detach from the amplexus, supporting noticeable turbulence and assuming that the females olfactorily recognise the moment when the male releases the sperm. Likewise, when studying the spawning behaviour of other Siluriformes such as *Corydoras aeneus*, Kohda et al., (1995) indicated that females swallow the released sperm in T-position, rapidly passing through her intestine, inseminating the eggs collected in the fin pocket created by the 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.

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

5.2.1. Results

The effects of treatments from EI and EII on PGSI and hatching rate are shown in Table 5.3. All females in all treatment groups ovulated throughout the first and second trials. In all trials, PGSI results were similar among treatments in both experiments. 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).

5.2.2. Discussion

Many fish species need spawning agents, usually via injection, to produce mature gametes in hatcheries (Mylonas et al., 2010; Kucharczyk et al., 2020). Many farmed and wild species need hormonal drugs to induce final OM and ovulation (Szabó et al., 2002; Kucharczyk et al., 2019b, 2021). Hormonal agents also cause synchronization of reproduction, resulting in more and better gametes. For this reason, different hormonal drugs and their dosages are evaluated for reproductive

efficacy (Brzuska, 2004a; Kucharczyk et al., 2021). The non-invasive technique of delivering hormone substances to the African catfish spawners was successful and may be utilized in fish farms in the future.

Table 5.3. Data from experiment I, II on the body weight of females, the relative number of stripped eggs, and the hatching rate in different treatment groups. For each experiment, means with the same superscript in each column are similar to each other ($P > 0.05$). NaCl-Ovopel pellets were homogenized in fish physiological saline, then introduced equally into the ovarian lobes by catheter; ALB-Ovopel pellets were homogenized in chicken albumen, then placed equally into the ovarian lobes by catheter; OHSI-Ovopel pellets were homogenized in fish sperm, then placed equally into the ovarian lobes by catheter; IM+OSI-IM was conducted and pooled sperm was injected into the ovarian lobes by catheter.

	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 - 7 0.8
	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

Both in the first and second experiment, all females ovulated in all treatments. PGSI data were also similar between treatments in both experiments (Table 5.3). The reproduction success of the African catfish depends on many factors (Brzuska et al., 2004; Kucharczyk et al., 2019b; Müller et al., 2020). According to Brzuska (2002), mammalian GnRHa combined with DRA in the form of preparation Ovopel resulted in a higher ovulation percentage than CP treatment. In our experiments, all females ovulated independently of the vehicle used or the way the hormone was administered. In the experiment of Lipscomb et al. (2018), the IM of CP and channel catfish PE resulted in a higher ovulation rate than the IP in case of small size ornamental fish. However, in the same study, when fish were treated with Ovaprim (salmon GnRHa and DOM) there was no

difference in ovulation success between the administration routes. In EI, the mean PGSI values ranging between 13.7 % and 15.1 % were similar among the independent treatments from the routes of administration. These results are similar to those of Brzuska's (2002), where lighter weight the African catfish had 13.5 % and the heavier ones had 15.3% PGSI values. In EII, the mean PGSI values were lower, between 11.2 % and 12.6 %, but higher than in the experiment of Müller et al. (2018b), where the PGSI values were between 6.1 % and 13.0 % (mean value 9.4 % ± 2.3 %). The differences in PGSI values between the two experiments were significantly different independently from the treatments. The possible explanation could be that fish from EI and II were selected from the same broodstock. The selection of individuals from both experiments could have some negative effect on the ovulation success, expressed as PGSI values.

Hatching rates were significantly lower for treatments where fish sperm was introduced into the ovary by a catheter whether it contained Ovopel (treatment 5 in EI and treatment 3 in EII) or not (treatment 2 in EII). The percentage of hatching rates is one of the most common efficiency factors noted in fish artificial spawning (e.g., Kucharczyk et al., 2019a, 2020). In EI, the IM, IP, ALB, and NaCl, treatments resulted in the greatest hatching percentage. Mean values ranged between 55.1 % and 66.7 % without any significant differences (ANOVA, $P > 0.05$; see Table 5.3). The hatching rate of the African catfish in field studies done by Kucharczyk et al. (2019b) was much more varied between 11.2 % and 92.3 %. However, in the study by Samarin et al. (2018), grater hatching rates were found (81–88 %). The OHSI treatment resulted in significantly lower hatching rates ($P < 0.05$) than those from the other administration methods. EII was conducted to examine the possible reasons for the negative result of the OHSI treatment in hatching success. The IM+OSI treatment in EII, where females received the IM of Ovopel and sperm introduced into their ovary (OSI), resulted in a lower hatching rate than that in IM treatment ($P < 0.05$). The repetition of OHSI treatment resulted in the lowest fertilisation percentage, while the IM resulted in a higher fertilisation percentage like in EI. No significant difference was found between the OHSI and OSI Groups (ANOVA, $P < 0.05$; see Table 5.3). The results of the inseminated groups in both experiments were lower than in the study of Müller et al. (2018b) where CP 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. Moreover, the current experiment was carried out in higher water temperatures (28 °C), in contrast to Müller et al. (2020) that was 27 °C and Müller et al. (2018b) that was 25.8 °C and 26.9 °C. These temperatures could have negative effects on the viability of sperm in the ovarian lobe. It cannot be ruled out that the inseminated technique

described by Müller et al. (2020, 2018a, b) needs further refinements or should be applied only in special areas.

We hypothesized that native avian egg (chicken) albumin injected non-invasively 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. Because of the homologous composition of avian albumen, exogenous egg albumin promotes ovulation in the follicle. It may contribute to optimizing the quick protein synthesis of the granulosa cell layer rather than the slower processes of vitellogenesis or steroidogenesis, however this impact cannot be ruled out. According to the findings of the experiments, albumin was a suitable hormone vehicle (prolonged action hormone vehicle/implant), 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.

The production of seeds for further rearing in aquaculture is often based on the multiple use of the same spawners. For this reason, it is very important to develop reproductive protocols that cause the least distress to spawners. The result of ovarian lavage with hormone suspended in saline and albumen have shown that the effects this method do not differ from the effects of reproduction with the use of traditionally injection by needle. In the future, research should be undertaken to indicate whether the repeated use of a less-invasive method improves the reproductive efficiency of this species.

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

5.3.1. Results

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 African catfish and the Sampa. 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.

Table 5.4. Summarised data of *C. gariepinus* and hybrid catfish of the control group. Survival rates (%) are presented as means and standard deviations (±S.D.). There were no significant differences (Shapiro–Wilks W tests ($P > 0.05$)

Survival rate (%, 28 raising days)	**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)

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 5.4). 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 5.5).

Table 5.5. Summarised data of *C. gariepinus* and hybrid catfish of treated groups. Survival rates (%) are presented as means and standard deviations (± S.D.). No 1, 2, and 3 - identification of females.

Survival rate (%, 28 raising days)		**Treated groups**		
		Total	***C. gariepinus***	**Hybrid catfish**
No 1 (n = 4)	Mean ± S.D. (min - max)	65.25 ± 4.32 (61 - 72)	2.67 ± 0.6 (1.64 - 3.23)	97.33 ± 0.61 (96.77 - 98.36)
No 2 (n = 4)	Mean ± S.D. (min - max)	70.5 ± 4.72 (64 - 75)	0	100
No 3 (n = 4)	Mean ± S.D. (min - max)	64.5 ± 5.41 (58 - 72)	3 ± 1.33 (1.64 - 4.48)	97 ± 1.33 (95.52 - 98.36)
Summarised data	Mean ± S.D. (min-max)	66.75 ± 5.52 (58 - 75)	1.89 ± 1.59 (1.64 - 4.48)	98.11 ± 1.59 (95.52 - 100)

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 5.5).

There were no main aims, but the growth parameters were also investigated in the control and inseminated groups, considering the genotypes. There were no statistical differences in growth parameters (total body length and body weight, $P > 0.05$; Table 5.6) between the two genotypes in the control groups. However, the mean condition factor of *C. gariepinus* was significantly higher ($P < 0.05$) than that of hybrid offspring. In the inseminated group, the mean total body

length, body weight and condition factor were not different between the two genotypes ($P > 0.05$; Table 5.6).

Table 5.6. The summary of total lengths, body weights, and condition factor of the *C. gariepinus* and hybrid catfish in the control group and treated group at the end of experiment. Different letters in the same row within group indicate significant differences ($P < 0.05$) among both strains. Data are expressed as mean±S.D. Ind. - individuals; reps. - replications.

Parametres (28 raising days)		**Control group (n = 4 reps.)**		**Treated group (n = 4 reps. × 3 spawning ♀)**	
		***C. gariepinus* (n = 82 ind.)**	**Hybrid catfish (n = 90 ind.)**	***C. gariepinus* (n = 15 ind.)**	**Hybrid catfish (n = 786 ind.)**
Total length (mm)	Mean ± S.D. (min - max)	33.23 ± 5.11 (20.32 - 46.71)	33.61 ± 5.23 (20.44 - 55.57)	30.76 ± 2.85 (26.61 - 36.65)	30.84 ± 3.85 (17.8 - 43.77)
Body weight (mg)	Mean ± S.D. (min - max)	343.28 ± 143.02 (92 - 917)	337.22 ± 162.6 (76 - 1212)	276.67±67.73 (176 - 399)	264.86 ± 93.01 (54 - 769)
Condition factor	Mean±S.D. (min - max)	0.9 ± 0.14^{a} (0.6 - 1.24)	0.85 ± 0.16^{b} (0.51 - 1.68)	0.94 ± 0.13 (0.74 - 1.19)	0.88 ± 0.15 (0.42 - 1.63)

5.3.2. 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; the mating ritual facilitated the fertilisation of the eggs in a natural way. 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 (Müller et al., 2018b). Müller et al. (2020) investigated the localisation of injected spermatozoa on the surface of eggs just before water activation in *C. garepinus*, using electron microscopy observation. According to the study, the spermatozoa were distributed close to the micropyle of eggs and showed that ‘internal gametic association’ could occur just after ovulation. This means that spermatozoa could penetrate the channel of the micropyle, but in the absence of water activation, no fertilisation occurred. These observations were similar to a description of a subcategory within the ovulipar groups mentioned in Munehara et al. (1989). 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 fertilisation rate of this study using inseminated sperm was significantly higher than that of *Danio rerio* (Gazsi et al. 2021a). In that case, sperm insemination was injected into the centre of the genital opening due to the small body size of the fish (~2–3 cm). Therefore, the distribution of sperm injected into the ovarian lobes was random, and there were large fluctuations in the fertility rate (0–85 %), considering the fertilisation rate of mating males.

The presented results also confirm that by injecting sperm from different species into ovaries, hybrids can be produced through induction reproduction and gamete production (Müller et al., (2018a). The spontaneous hybridisation of fish can be observed in natural habitats or managed in captivity conditions in many species, as mentioned in Scribner et al. (2000), Albert et al. (2006), Müller et al. (2010), and Graham et al. (2020). However, the outcomes of spontaneous crossing fluctuated in C×B, as shown in Dunham et al. (2000) and Dunham and Masser (2012). 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.

Conventionally, incorporating spontaneous spawning and hormonal treatment in fish reproduction can utilise various advantages from both media, such as normal breeding behaviour, producing eggs of high fertilisation success, and undergoing multiple spawning in fish with asynchronous ovarian development, resulting in high seasonal fecundity (Mylonas et al., 2010), stress limitation reduction (Żarski et al., 2015), higher productivities of egg and fish yield (Okamura et al., 2014; Bosworth et al., 2020), least expertise, ease of visual inspection of spawning and egg masses, and fish ovulation determination (Dunham and Elaswad, 2018). Obviously, the incorporated protocol can also avoid limitations, as there is no possibility of controlled fertilisation and limited possibilities to predict the moment of spawning (Żarski et al., 2015) or the barrier of natural reproductive isolation between species (Dunham et al., 1998). In addition, the combination of their therapies might also reduce the drawbacks compared to using IVF, which is commonly administered in the distance hybridisation of fish, such as (1) the latency time estimation and losing their fertilisation potential (Urbányi et al., 2009; Mylonas et al., 2010, 2017; Müller et al., 2018a; Ittzés et al., 2020); (2) reducing genetic diversity (NACA, 1989; Müller et al., 2018b); (3) labour intensive and inconsistent hybrid seed production (Perera et al., 2017); and (4) prone to contamination (Dunham and Masser, 2012). The incorporation of ovarian sperm injection and artificial spawning induction (method) could frequently be applied to produce hybrid progeny in the aquacuture industry. For instance, the combined method is feasible to apply to create the C×B.

Noticeably, due to the lack of hybrid and inconsistent spawning success rates, open-pond and pen spawning cannot be used to cross the C×B (Dunham and Masser, 2012). Until recently, the open pond spawning technique had been used virtually unchanged for almost a century to propagate *I. punctatus* while requiring the least expertise. The hormones used to spawn this catfish comprised CPE, hCG and LHRHa. LHRHa implants are designated for consistency because of their effectiveness in producing C×B hybrids (Dunham and Elaswad, 2018). Combining the inseminated sperm and hormonal induction protocol solved the bottlenecks of these traditional therapies, also referred to as practically producing C×B hybrids. In other species, the method also potentially refers to other paired spawning fish species, such as European eel (*A. anguilla*), Greater amberjack (*S. dumerili*), Silver moony (*M. argenteus*), South Pacific bonito (*S. chiliensis chiliensis*), and Broadhead catfish (*C. macrocephalus*), or fish species spawning in small groups (Chinese carp; *C. idella, H. nobolis, H. molitrix, C. carpio*).

In IVF testing, the mean fertilisation rate and hatching proportion of hybrid crosses were 74.92±6.6 % and 71.1±11.29 %, respectively. These parameters were slightly lower than those in Nwadukwe (1995), Ataguba et al. (2009), and Olufeagba et al. (2016). Still, the hatching percentage was higher than that outcome obtained in 1989 (see Legendre et al., 1992), and (Nwadukwe, 1995). Differences in hatching rates arise from factors including breeding history (i.e., age, water quality); and the reason for the reduced hatching rate may be linked with gene compatibility of the different species; Hatchability is also a function of the quality of broodstock used (Ataguba et al., 2010).

Regarding the survival rates under investigation, the current findings were inconsistent between genotypes (Table 5.4). Our results are less than the range of 65.5 (53 days) to 93.6 % (15 days) reported for strains of crossbreeds by Legendre et al. (1992) and Ataguba et al. (2009) at 91.33 % after rearing for 15 days but similar to that (61.11 %) in Nwadukwe (1995) and Olufeagba et al. (2016) at 46 % after rearing for 15 or 28 days, respectively. Commonly, a considerable degree of problematic cannibalism during the rearing period has been observed in *C. gariepinus* (Graaf et al., 1995; Al-Hafedh and Ali, 2004; Mukai et al., 2013; FAO, 2019), or in *H. longifilis* (Baras et al., 1999; Coulibaly et al., 2007). Total survival rates in all treated groups were significantly higher than that in the control group. The difference survival rates between two treatments may be due to the different raising ration between hybrid and pure larvae (98.11±1.59:1.89±1.59 in treated group; 54.2 ±6.82:45.79±6.82 in control group). When pure and hybrid larvae are reared together, a higher hybrid percentage of larvae may lead to less problematic cannibalism.

Table 5.7. Some necessary IVF and hybridization experiments of *C. gariepinus* ♀ and *H. longifilis* ♂ with some essential early age performance traits. F-Fertilisation; S-Survival; Time-Number of days reared; BL-body length; BW-body weight.

F-rate (%)	BW (mg)	BL (mm)	S-rate (%)	Time (days)	References
-	-	-	66.5	22	(Aluko et al., 2001)
-	7.9	-	-	14	(Ataguba et al., 2010)
-	684	-	42.4	56	
-	86.7	-	93.6	15	(Legendre et al., 1992)
-	670	-	65.5	53	
90.11	27.39	24.89	46.03	28	(Olufeagba et al., 2016)
77.5	-	-	61.11	26	(Nwadukwe, 1995)
-	28	14.4	37.7	14	(Ojutiku, 2008)
-	-	146	-	78	(Oellermann, 1996)
-	-	-	81.1	4	
88.57	8.6	-	91.33	15	(Ataguba et al., 2009)
77.5	-	-	82	14	(Akinwande et al., 2012)
-	2.97	-	79.7	14	(Odedeyi, 2007)
-	32	-	73	70	

After rearing for 28 days, the body weights and total lengths of the purebred strains were equal to the hybrid crosses in both groups. Even though the condition factor of *C. gariepinus* was higher than that of the hybrid fry in the control group ($P < 0.05$), this parameter was not different in the treated group ($P > 0.05$). The body weights of the hybrid in the present study were far higher than the values reported for the same cross after a similar rearing period mentioned in Olufeagba et al. (2016) (Table 5.7). However, the values recorded for growth in this study were significantly lower than the weight gain at 1.520 mg after 26 days reported by Nwadukwe (1995). In contrast to the findings of this study, which showed no difference in the growth performance between each group, Olufeagba et al. (2016), and Ataguba et al. (2009, 2010) revealed that pure strain crosses of *C. gariepinus* showed better growth performance than the other crosses, but lower performance in pure *C. gariepinus* was revealed by Ojutiku (2008). This suggests that the growth of different crosses depends on the stage of development and the growth rates of fry, fingerlings, juveniles or adults may vary differently for each cross. Apart from this, the genetic effect of the parent individuals or due to the different rearing technologies could also cause these differences. A dissimilar result was found to the report by Olufeagba et al. (2016), as the mean final body length of pure *C. gariepinus* was found to be significantly higher than that of the hybrid. In contrast, Sahoo et al. (2003) reported that pure larvae had a significantly lower body length than the hybrid larvae of ♀ *C. gariepinus* × ♂ *C. batrachus* in IVF. However, Bruton (1979b) indicated that a reasonably similar condition factor was observed in pure *C. gariepinus*. The phenotypic variance

of a quantitative trait, such as growth and survival, is governed by genetic variance, environmental variance, and the interaction between genetic and environmental variance. Hence, a negative interaction between the genetic variance of the hybrid and the environment may have led to poor phenotypic expression of growth (Ataguba et al., 2010).

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

5.4.1. Results

In two studies, we assessed the biological activity or fertility of artificially inseminated spermatozoa injected into the ovaries at various periods prior to gamete stripping at ovulation time. 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 5.8).

Table 5.8. 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) Mean ± S.D.	PGSI (%) Mean ± S.D.	Fertilisation rate (12hpf) (%) Mean ± S.D.	Hatching rate (%) 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}

As illustrated in Table 5.8, all injected females released egg batches from which developing embryos developed at different ratios 12 hpf. There were large 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 differences 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.

5.4.2. Discussion

Previously conducted research has shown that artificial sperm fertilisation/sperm ovarian lavage is an efficient technique for fish reproduction (Müller et al., 2018a, 2018b; 2019). Both of the above experiments supported and confirmed these earlier results.

After comparative analysis, Stockley et al. (1996) determined that in externally fertilized species, the mean number of sperm per stripped ejaculate rises with the mean number of egg cells released during spawning. One reasonable suspicion is sperm limitation, which indicates that the required quantity of sperm is high enough to achieve fertilisation, and the other is the expense of finding a mate. Instead of the African catfish, which belongs to the species of externally fertilised fish, there is a fascinating phenomenon in which, since sperm extraction is practically impossible, the quantity and concentration of sperm is extremely low. Males are slaughtered, and the testes are macerated in large-scale reproduction of this species to collect milt (Viveiros et al., 2000). In order to understand more about the process of spermatozoa activation, it would be essential to measure the status of spermatozoa in the ovary *in vivo*. The placement of spermatozoa in the ovarian lobes is an exciting part of this issue. As stated previously, seminal plasma carrying the hormonal substance is absorbed into the bloodstream by the ovarian lobes and circulates throughout the ovarian wall (Müller et al., 2018a), 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. According to Szabó et al. (2010), the African catfish eggs could be stored in ovarian fluid for at least 60 minutes without activation or loss of fertilisation ability. However, this was achieved by IVF under atmospheric circumstances. In this investigation, there is a novel, previously unknown interaction between sperm and ovarian fluid, as opposed to sperm (including seminal plasma) 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 large 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 (Müller et al., 2018a), but the feasible circumstances for such a combination injection must be investigated experimentally.

Generally, several studies have been published on artificial insemination of the African catfish using various hormones (Brzuska, 2003, 2011; El-Hawarry et al., 2016); Kovács et al., 2010; Richter et al., 1987a). Our fertilisation rate (out of control) was from 2.5±4.4 % (48 hours group) to 65.7±11.3 percent (5 hours group) after 12 hours of incubation; from 0.4±0.7 % (48 hours group) to 39.3±12.7 % (5 hours group) posterior to 24 hours of incubation. The fertilisation rate and hatching success of fish treated in our studies were lower than previously reported results. All females derived from the stock of an intensive farm, and therefore were not in an optimal egg condition (nutrition, keeping, etc.). This may explain the lower fertilisation and hatching rates. PGSI mean values were comparable across tests in a recent study, with considerable individual variation (EI, II: 8.6±2.8-12.6±3 %). This result is consistent and has been observed in the literature (9–15 %) (Brzuska, 2002, 2003; Rurangwa et al., 1998).

IVF is the most appropriate method for reproducing fish in various situations, including reproduction and crossing programs. Additionally, it is the only approach suitable for fertilisation using administrated sperm. Nevertheless, predicting ovulation may be very difficult in many cases, leading to large fluctuations in the quality of stripped eggs. As a result of the more accurate time prediction of ovulation, induced spawning is recommended and employed in practice for various fish species (e.g., pikeperch, channel catfish, and seabream) (Müller et al., 2018a). Compared 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 aquaculture management, where it is critical to maintain or enhance genetic diversity. For example, in the propagation of fish species that naturally spawn in pairs (such as *Sander lucioperca* or *Sander glanis*), mixed sperm samples from multiple males might be used to fertilise eggs during stimulated spawning.

6. CONCLUSIONS AND SUGGESTIONS

The reproductive behaviour of *C. gariepinus* in the spontaneous condition is relatively well described but lacks some details for a more accurate assessment of the mating stage. The presented 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. We do not know precisely how many sperm are presented per sperm bundle, but our estimates of sperm quantity and the fact that catfish, including *C. gariepinus* are generally considered oligospermia. After the gas bubbles and sperm are released from the gill openings, the mates slowly dissociate. Then, while the female's body is kept at a balanced position, her tail is vigorously switched so that the mixture of sperm and eggs will be distributed in all directions. 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 specific position, which could have led to the male's mouth coming close to the female's gonopore, as seen in several Siluriformes, was crucial to synchronise the pairing. 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 research should be extended to other Claridae species and the sperm competition in the water column after coagulated semen discharge.

The production of seeds for further rearing in aquaculture is often based on the multiple uses of the same spawners. For this reason, it is essential to develop reproductive protocols that cause the most minor distress to spawners in chapter 2. These findings indicated that the egg albumen with Ovopel resulted in one of the greatest hatching percentage, and albumen can be a perspective ovarian hormone vehicle. Saline and sperm were appropriate vehicle of Ovopel to induce reproduction of this fish by OI method. The effects of artificial reproduction do not differ between traditionally invasive and non-invasive techniques, in which suspended hormone (without sperm) were inserted in to the ovary. This allows improving the welfare of the African catfish spawners during artificial spawning. In the future, research should be undertaken to indicate whether the repeated use of a non-invasive method improves the reproductive efficiency of this species. At the same time, the non-invasive method of applying hormonal agents may be used in the future for wild fish, which return to the environment after gametes are obtained.

In chapter 3, the current results revealed novel feasibility of cross-breeding (C×H). Hormonal injected *C. gariepinus* female had received semen from *H. longifilis* by ovarian injection manipulation can propagate with hormonal stimulated *C. gariepinus* male in spawning cage/tank circumstance to produce hybrid offspring. The overall average hybrid percentage was high (98.11±1.59 %). Despite variance in growth, the total survival rate was no significant difference between genotypes after 28 raising days. This approach will enable the hybrid generation of gametes, embryos, and larvae for early life stage manipulations, as well as a consistent supply of juveniles and adults for genetic selection programs and the aquaculture sector. Sperm can remain in the ovary without significantly reducing their fertilisation ability; this approach has the potential to be used for inducing crossover in a wide variety of farmed fish species, particularly those with unpredictable egg production timing and synchronization. Additional research is needed to extend this method to 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 introduced into the ovary was proven in chapter 4. We indicated that sperm could store in gonad lobes for 5, 10, 15, 20, 25, 36 and 48 hours. The estimated maximal latency period of active biological spermatozoa was at least 48–50 hours. However, we observed large individual fluctuations in fertilisation and hatching rate of treatment groups; the reproductive outcomes were better in 5 and 10-hour groups. Similarly, at 48 hours, the treated group showed low fertilisation and hatching rate, indicating loss of fertilizing capacity. Further investigations are needed to develop this method in other fish species, as well as to reveal the relationship between the fertilisation capacity of the sperm and the incubation time in the ovary. Should this time period be possible to increase 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 or in natural waters, as we described in such former studies.

7. 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. The egg albumen contains Ovopel resulting from one of the highest hatching rates. 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 %. Despite variance in quantitative parameters like growth, the survival rate was insignificant between genotypes after 28 raising days.
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.

8. SUMMARY

World aquaculture production has grown rapidly, and this growth is expected to continue. The African catfish (*C. gariepinus*) is economically one of the most important freshwater cultured fish species due to its high technical tolerance and rapid growth rate. These characteristics also enable it to produce in environments unfavourable to other fish species; it is also successfully reared in reservoirs or wastewater ponds as well. African catfish are produced in 50 countries around the world.

The large-scale hatchery propagation of this species has long been developed: similar to other catfish species, requires killing the male fish in order to excise the testes and sperm collect directly from the excised testicle, as males are difficult to be stripped. The natural spawning behaviour of *C. gariepinus* was described more than 40 years ago, but has not been studied in detail since then, although many of its mechanisms are not known. Until now, the moment of fertilization was described on the basis of assumptions, because the spermiation was not observed. Our aim was to reveal all the peculiarities of the spawning ethology of the African catfish that have not been explored and/or described in detail. Using a combination of underwater video recordings, histological studies and computer assisted semen analysis (CASA), we were able to clarify in detail the spawning behaviour of African catfish. The spawning strategy is the closest to the known behaviour in *Corydoras* (a genus of Armored catfish).

The breeding methodology of most produced fish species has been already developed, however continuous improvement and efficiency of these procedures is crucial. In the second chapter our aim was to investigate the effect of a particular hormone preparation with different invasive or non-invasive methods on reproduction parameters. Physiological saline (0.9% NaCl), semen and avian albumen were used as hormone vehicle. There was no significant effect on fertilisation between physiological saline and albumin carrier treatments regardless of the procedure used (intramuscular and intraperitoneal injection, ovarian lavage), while sperm carrier and sperm inseminated fish were statistically significantly lower than the other treated groups ($p<0,05$).

Interspecific hybridisation by tank spawning is impossible for several fish species. As a novel method, the inseminated sperm method combined with hormonal induction was investigated to produce hybrid catfish by cage spawning in this chapter. *Heterobranchus longifilis* sperm was injected into the ovarian lobes of *C. gariepinus* parallel her hormonal induction. Inseminated Clarias females with Clarias males were used for cage spawning in pairs. After the spawning, fertilised eggs were collected and larvae reared for 28 days for identifying the genotypes by using

morphological analysis. Cage-spawned hybrid rates were 95.5–100 %. This approach could be an alternative method for producing hybrid catfish or other fish species without in vitro fertilisation.

The objective of the fourth chapter was to investigate the fertilisation rate after different sperm ovarian storage from ovulation. Sperm samples (2 ml sperm/kg body weight) were inseminated into ovarian lobes 5, 10, 15, 20, 25, 36 and 48 hours before programmed ovulation. There were no statistical differences ($p < 0.05$) among the hatching rates between 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.

Insemination as a new method combines the simplicity of induced/tank/pen spawning with advantages of in vitro fertilisation (dry fertilisation method) in external fertilised fish species. Hormonal administration parallel with insemination into ovary may be employed to breeding programs and intra- and interspecific hybridisation and it is the feasible method for using manipulated (cryopreserved or genetically modified) sperm.

9. ÖSSZEFOGLALÁS

A világ akvakultúra termelése gyorsuló ütemben növekszik és ez a növekedés várhatóan folytatódni fog. Az afrikai harcsa (*C. gariepinus*) gazdaságilag egyike a legfontosabb édesvízi tenyésztett halfajoknak, köszönhetően a nagy technológiai tűrőképességének, gyors növekedési erélyének. Ezek a tulajdonságaik képessé teszik más halfajok számára előnytelen környezeti tényezők közötti termelésre is; víztározókban vagy ülepítő tavakban is sikeresen nevelik. Közel 50 országában termelik.

A faj keltetőházi szaporítása régóta kidolgozottnak nevezhető: hasonlóan a többi harcsaféléhez, a tejeseket elölik és a termékenyítésre felhasznált spermát közvetlenül a kioperált heréből gyűjtik, mivel nehezen fejhetőek. A *C. gariepinus* természetes ívási viselkedését több, mint 40 éve leírták, azóta - habár több mozzanata nem ismert - nem kutatták behatóbban. Ez idáig feltételezések alapján írták le a termékenyítés pillanatát, mert nem volt megfigyelhető a tejesek spermaürítése. Célul tűztük ki, hogy feltárjuk az afrikai harcsa ívási etológiájának mindazon sajátosságait, amit eddig nem kutattak behatóbban és/vagy nem írtak le kellő alapossággal. Víz alatti videófelvételek, hisztológiai vizsgálatok és CASA spermaminőségvizsgálatok együttes alkalmazásával sikerült részleteiben pontosítani az afrikai harcsa ívási viselkedését. Az ívási stratégia a *Corydoras* (páncélosharcsa-félék neme) fajoknál ismert viselkedéshez áll a legközelebb.

A tervezhető termelés egyik alapkritériuma a biztonságos ivadék utánpótlás. A termelésbe vett legtöbb halfaj szaporítási módszertana már kidolgozásra került, de ezen eljárások folyamatos fejlesztése és hatékonyabbá tétele kulcsfontosságú. A második fejezetben célul tűztük ki, hogy megvizsgáljuk, hogy egy adott hormonkészítmény milyen hatást gyakorol a termékenyülési értékekre, ha különböző invazív, vagy nem-invazív módszerekkel kezeljük az ikrásokat. Vivőanyagként fiziológiás sóoldatot (0,9% NaCl), halspermát, és tojásfehérjét használtunk. Fiziológiás sóoldatú és tojásfehérje vivőanyagú kezelések között függetlenül az alkalmazott eljárástól (injektálás - intramuszkuláris, intraperitoneális -, petefészekmosás) nem volt szignifikáns hatása a termékenyülésre, míg a sperma vívőanyag és sperma inszeminált halak értékei statisztikailag igazolható módon elmaradtak a többi csoporttól.

Számos halfaj esetében a fajok közötti hibridizáció indukált ívatás (ívató medencés) szaporítási módszer esetében nem lehetséges. Ebben a fejezetben a hibrid harcsák előállításának lehetőségeit vizsgáltuk az új módszer alkalmazásával (ívató medence, hormonális indukcióval kombinált inszeminációval). A *Heterobranchus longifilis* spermát *C. gariepinus* petefészeklebenyébe

injektáltuk a hormonkezelésükkel egyidőben. Az inszeminált Clarias ikrásokat párban Clarias tejesekkel ívató ketrecbe telepítettük. Az ívás után a megtermékenyített ikrákat begyűjtöttük, a lárvákat 28 napig neveltük az utódok genotípusának meghatározására, amit alaktani bélyegek alapján értékeltünk. Az ívató ketreces szaporítás során a hibridek aránya 95,5-100 % volt. Kísérleti eredményeink alapján az inszeminácó egy alternatív módszer lehet hibrid harcsák vagy más interspecifikus halhibrid előállítására.

A negyedik fejezet célkitűzése volt, hogy megvizsgáljuk a spermiumok miként hatnak a termékenyülésre különböző petefészki tárolási időt követően. A sperma mintákat (2 ml sperma / testtömeg kg) petefészek lebenyekben injektáltuk 5, 10, 15, 20, 25, 36 és 48 órával a programozottan kiváltott ovuláció előtt. A 5-25 órás kezelési csoportok között nem volt statisztikalag igazolható különbség ($p < 0,05$) a kelési arányokban, de a csoportokon belül nagy egyéni különbségeket figyeltünk meg. A 48 órás kezelt csoportban azonban alacsony volt a megtermékenyítési és kelési arány, ami a termékenyítő képesség elvesztésére utal.

Az inszemináció mint új halszaporítási módszer egyesíti az indukált/medencés/ketreces ívás egyszerűségét az *in vitro* megtermékenyítés (száraz megtermékenyítési módszer) előnyeivel a külső megtermékenyítésű halfajok esetében. A petefészek inszeminációval párhuzamos hormonkezelés alkalmazható tenyésztési programokban, valamint fajon belüli és fajok közötti hibridizációban, valamint a módszer alkalmas manipulált; mélyhűtött vagy genetikailag módosított sperma felhasználására.

10. APPENDICES

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10.3. Supplemental figures

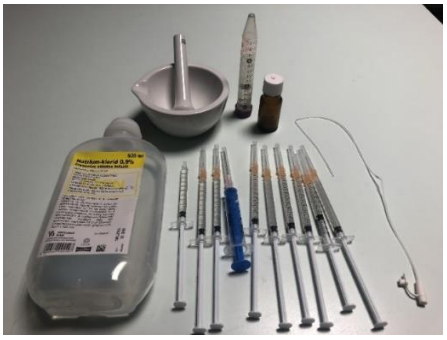

Figure 9.1. Hormonal preparation

Figure 9.2. Spawner management

Figure 9.3. Mixed Ovopel and sperm

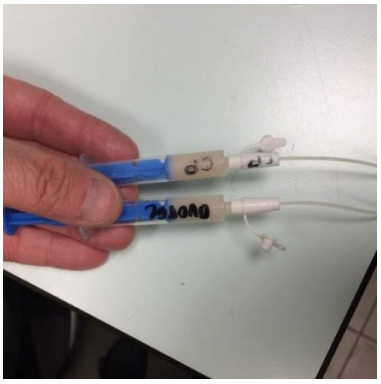

Figure 9.4. Homogenized hormone and vehicles

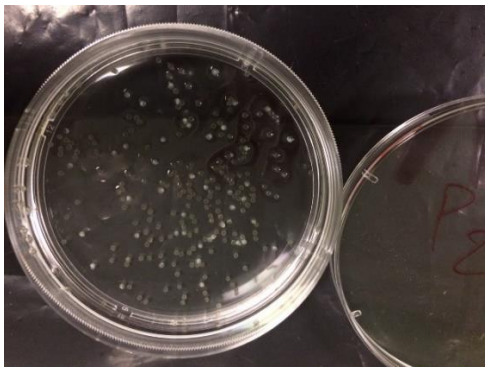

Figure 9.5. Egg incubation

Figure 9.6. Larvae rearing

Figure 9.7. Data collecting

Figure 9.8. Pure and hybrid fingerlings

10. PUBLICATIONS

10.1. 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.

10.2. 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.

10.3. 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.

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