MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

THE ANALYSIS OF SPERM CRYOPRESERVATION AND PROPAGATION OF „SUDÁR" CARP LANDRACE (*CYPRINUS CARPIO MORPHA ACCUMINATUS*) AND HÉVÍZ DWARF CARP (*CYPRINUS CARPIO MORPHA HUNGARICUS*) IN AN INTENSIVE SYSTEM AND THE ESTABLISHMENT OF THEIR *IN VITRO* SPERM BANKS

Theses

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List of markings and abbreviations

Table 1. List of abbreviations in the doctoral thesis.

Abbreviation in the text	Full name
°C	degree Celsius
CASA	Computer-assisted Sperm Analysis
CRF	Controlled-rate Freezer
FAO	Food and Agriculture Organization of the United Nations
IUCN	International Union for Conservation on Nature and Natural Resources
LIN	Linearity
MA-HAL	Hungarian Aquaculture and Fisheries Inter-branch Organisation
mg	milligram
mL	millilitre
mm	millimetre
sec	second
N. D.	No data
pMOT	Progressive motility
RAS	Recirculating Aquaculture Systems
kg body weight	body weight kilogram
VCL	Curvilinear velocity

1. INTRODUCTION

1.1. Background of the thesis

Carp (*Cyprinus carpio*) is one of the essential freshwater farmed fish species in the world and Hungary, as well. (ALIKHUNI 1966; FAO 1996, 2020). There are 89 countries in the world that breed and produce carp, and it is of outstanding importance in 39 countries. In Hungary, the volume is 17 thousand tons, representing 79.5% of our market fish production. Hungary, with Poland and the Czech Republic, accounts for almost 68% of the annual EU production (FAO 2020; MA-HAL 2019; URBÁNYI & STASZNY 2018). Contrary to economic fish production, a slow but steady decline in wild carp (*Cyprinus carpio carpio*) populations have emerged due to the gradual loss of habitats and hybridization with breeding carp species. These days, the phenomenon has become widespread as the IUCN Red List includes the Danube subpopulation as a 'critically endangered' stock (KOTTELAT 1996). Due to this conservation aspect, it is extremely important to explore and protect genetically pure stocks. The genetic background of "sudár" carp landrace and Hévíz dwarf carp in a river basin district is of outstanding importance in Hungary, both in economic and nature conservation terms (UDVARI 2017).

Lake Hévíz is the largest natural-bedded, medicinal peat lake in Europe (KÖRMENDI et al. 2008). In addition to the several introduced fish species, there is only one native in the lake. The fish is a specialized carp species adapted extremely well to the specific lake conditions (SPECIÁR 2004). The morphologically distinct variant occurs exclusively here and forms a self-sustaining breed. As a result of its adaptation, the population consists of dwarf individuals and has extremely specific and unique genetic traits. It is resistant to the persistently high temperature, which is not tolerated by our other domestic carp landraces (HORVÁTH & URBÁNYI 2004). Its origin can be traced back to the Danube wild carp, which is also supported by genetic studies but is not on the IUCN Red List. Consequently, it forms a vulnerable stock concerning nature conservation (KOTTELAT 1996; LEHOCZKY et al. 2005a, b; 2007).

The "sudár" carp landrace is of great economic value in Hungary. Balaton Fish Management Non-Profit Ltd is the owner and maintainer of the officially state-recognized landrace (UDVARI 2017). The organization aims to preserve and increase the fish population of Lake Balaton. The company's main goal is to maintain natural populations and propagate and restock them year by year. The "sudár" carp landrace also has an important fishing touristic value, as it is an immensely popular (number one) prey fish in the lake and its basin. Preservation of

its genetic resource is important both economically and ecologically (NÉBIH 2011).

Like our other commercial fish species, freezing the male gametes of carp is of increasing importance year by year. Several cryopreservation methods have been developed for economically and environmentally important fish species (BLAXTER 1953; CABRITA et al. 2010; MOCZARSKI 1977). In addition to its economic significance, cryopreserved sperm may play a role in gene conservation, as it is an essential part of conservation biological action plans (CABRITA et al. 2010). The development of a method for freezing carp sperm dates to several decades (MOCZARSKI 1977). Due to the process, the frozen samples are controlled quality and can be dose accurately during propagation, even to fertilize large amounts of eggs. However, the involvement of the processes in fish production practice has not been carried out (URBÁNYI 2011). Due to the agglutination of carp sperm after thawing, it would be important to fully develop a uniform and species-specific method that can be reliably applied under hatchery conditions (MARTÍNEZ-PASTOR et al. 2017).

1.2.Aims

During my doctoral work, I aimed to carry out the following experiments:

The "sudár" carp landrace

1. Concerning the "sudár" carp landrace of economic importance, making the sperm cryopreservation process developed for carp more beneficial. Development of a large-scale sperm cryopreservation process (5 mL straw and a 5-10 mL cryotube) using a Styrofoam box and programmable freezer.
2. Elimination of agglutination after thawing characteristic of the carp male gametes with a recently applied pike extender.
3. Optimize the thawing period of the 5 and 10 mL cryotubes in a water bath using grayling and pike extenders.
4. The propagation in a closed intensive recirculation system with native and cryopreserved sperm.
5. The artificial propagation and larvae rearing in a closed intensive recirculation system. The monitoring of the growth and survival rate, as well as the malformations of the individuals.
6. With the establishment of an *in vitro* sperm bank for the landrace of economic importance, I may contribute to the conservation of the stock's genetic resources and the conservation of the natural population of the lake.

Hévíz dwarf carp

1. Hormonal induction of spermiation in wild-caught (directly on the lakeshore) or a closed intensive recirculation system maintained

male individuals.

2. The sperm cryopreservation of the vulnerable carp type in a Styrofoam box and a programmable freezer using grayling and pike extenders (eliminating agglutination).
3. The artificial propagation in a closed intensive recirculation system with native sperm. The monitoring of the growth and survival rate as well as the malformations of the individuals.
4. Establishment of an *in vitro* sperm bank for Hévíz dwarf carp, contributing to preserving the conservation biological value of the species.

2. MATERIALS AND METHODS

2.1. The general aspects of reproductive biology and sperm cryopreservation experiments

The “sudár” carp individuals were provided from the Irmapuszta stock of the breed owner Balaton Fish Management Non-Profit Ltd. In contrast, Hévíz dwarf carps were caught from the Lake Hévíz maintained by the operator. The experimental broodstock was kept in a closed intensive recirculation aquaculture system (RAS) belonging to the infrastructure of the Department of Aquaculture. Experimental tanks of 3 m^3 and 1 m^3 were used to maintain them. In all the cases, the broodstock was treated in the same way as the carp hatchery practice. After hatching, sperm was stripped after 24 hours concerning the “sudár” carp landrace and 12 hours regarding the Hévíz dwarf carp after injection. In male individuals, spermiation was induced by 2 mg body weight kg^{-1} of powdered carp pituitary. The ovulation of the female was induced using 4 mg body weight kg^{-1} carp pituitary. The motility of fresh and thawed sperm samples was recorded with a Computer-assisted Sperm Analysis (CASA) system. To activate the cells, a solution previously developed for the carp was used. The pMOT (straight line distance> 5 μm, pixel/μm ratio: 151–100), VCL, and LIN values of the cells were recorded. During cryopreservation, the grayling [200 mM glucose, 40 mM KCl, 30 mM Tris, pH 8.0±0.2 (HORVÁTH et al. 2012; BERNÁTH et al. 2016a)] and pike [150 mM glucose, 75 mM NaCl, 30 mM KCl, 1 mM Na_2HPO_4 * $12H_2O$, 1 mM $MgCl_2$ * $6H_2O$, 1 mM $CaCl_2$ * $2H_2O$, 20 mM Tris, and 0.5% BSA, pH: 8.0±0.2 (MOLNÁR et al. 2020)] extenders were applied According to the experimental design, dilution ratios of 1:1, 1:4, and 1:9 were used. During freezing, 10 % of methanol was applied as a cryoprotectant. In my study, diluted sperm was loaded into 0.5 mL and 5 mL straws, or 5 mL, and 10 mL cryotubes (Table 2).

Table 2. The summary of dilution ratios used for the different cryopreservation methods.

Cryopreservation capacity	Dilution ratios (mL)		
	1:1	**1:4**	**1:9**
0.5 mL straw	N. D.	N. D.	methanol: 0.05 sperm: 0.05 extender: 0.4
5 mL straw	N. D.	N. D.	methanol: 0.4 sperm: 0.4 extender: 3.2
5 mL cryotube	N. D.	N. D.	methanol: 0.4 sperm: 0.4 extender: 3.2
10 mL cryotube	methanol: 0.8 sperm: 4 extender: 3.2	methanol: 0.8 sperm: 1.6 extender: 5.6	methanol: 0.8 sperm: 0.8 extender: 6.4

Freezing was performed in a Styrofoam box or a controlled rate freezer (CRF), depending on the experiments. The cooling program varied depending on the capacity of the straws or cryotubes (Table 3).

Table 3. The summary of the different cryopreservation methods.

Cryopreservation capacity	Cryopreservation method and description
0.5 mL straw	Styrofoam box, at 3 cm, for 3 minutes (HORVÁTH et al. 2012)
0.5 mL straw	CRF. initial temperature: 7.5 °C, endpoint: -160 °C, cooling rate: 56 °C min^{-1}, (BERNÁTH et al. 2015)
5 mL straw	Styrofoam box, at 3 cm, for 7 minutes (BOKOR et al. 2010)
5 mL straw	CRF, initial temperature: 4 °C, end point: -160 °C, cooling rate: 15 °C min^{-1} (BOKOR et al. 2019)
5 mL cryotube	Styrofoam box, at 3 cm, for 7 minutes (following BOKOR et al. 2010)
5 mL cryotube	CRF, initial temperature: 4 °C, end point: -160 °C, cooling rate: 15 °C min^{-1} (following BOKOR et al. 2019)
10 mL cryotube	CRF, initial temperature: 4 °C, end point: -160 °C, cooling rate: 15 °C min^{-1} (BOKOR et al. 2019)

The cryopreserved samples were stored in a transportable canister tank with a capacity of 10 L and a 48 L storage dewar. Each stored sample was thawed in a water bath at 40 ºC. The thawing period varied depending on the sample capacity (Table 4).

Table 4. The summary of the different thawing periods.

Thawing capacity	Thawing period
0.5 mL straw	At 40 ° C, 13 sec (BOZKURT 2017; HORVÁTH et al. 2012)
5 mL straw	At 40 °C, 35 sec (BOKOR et al. 2010; CABRITA 2001b)
5 mL cryotube	It was determined experimentally.
10 mL cryotube	It was determined experimentally.

A Bürker chamber was applied to determine the cell number. The dilution ratio of the cell suspension varied between 5-fold and 1000-fold in my experiment, depending on the density of the samples. The fertilization process was accomplished using system water (30 sec). The eggs were swelled with Woynárovich's solution on a flatbed shaking table. The final stickiness of the eggs was eliminated with three replicates (20 sec, 15 sec, 10 sec) of tannic acid treatment. The hatching rate was determined at the moment of hatching. The abovementioned value (hatched larvae infertile eggs^{-1}) was determined at the moment of larvae hatching.

2.2. The experiments carried out in the "sudár" carp landrace

Table 5. shows the experiments performed on the broodstock.

Table 5. The summary of the experiments performed on the "sudár" carp landrace.

Chapter No.	Cryopreservation capacity	Extender	Cryopreservation method	The subject of the test	Sample size in the experiment
3.5.1.	5 mL straw and 10 mL cryotube	grayling	Styrofoam box, CRF	cryopreservation, motility	*N*=6
3.5.1.	10 mL cryotube	grayling	CRF	dilution ratio, motility	*N*=3
3.5.2.	5 mL straw and 10 mL cryotube	grayling pike	Styrofoam box, CRF	motility	*N*=7
3.5.3.	5 mL straw and 10 mL cryotube	grayling pike	Styrofoam box, CRF	cell concentration	*N*=5
3.5.4.	10 mL cryotube	grayling pike	CRF	thawing period	*N*=5
3.5.5.	5 mL cryotube	grayling pike	Styrofoam box, CRF	thawing period	*N*=5
3.5.6.	10 mL cryotube	grayling pike	CRF	motility, fertilization	♀: *N*=2 ♂: *N*=5
3.5.7.	N. D.	N. D.	N. D.	larvae rearing	♀: *N*=1 ♂: *N*=5

2.2.1. The comparison of the cryopreservation of a 5 mL straw and the 10 mL cryotube, and the development and optimal dilution ratio for the 10 mL cryotube

In my experiment, sperm samples from six individuals (*N=6*) were cryopreserved in a 5 mL straw and a 10 mL cryotube using a Styrofoam box and a controlled rate freezer (see Chapter 2.1, Table 3). In the second phase of my work, different dilution ratios applied during cryopreservation (*N=3*) in 10 mL cryotube were tested (see Chapter 2.1, Table 2). CRF was used as the freezing method (see Chapter 2.1, Table 3).

2.2.2. The comparison of two types of extender and different cryopreservation methods (motility test)

The cryopreservation of sperm obtained from seven individuals (*N=7*) using 5 mL straw in Styrofoam box and CRF, as well as 10 mL cryotube in CRF, was performed (see Chapter 2.1, Table 3). Two extenders with different compositions [grayling and pike (see Chapter 2.1)] were compared in my experiment.

2.2.3. The comparison of two types of extender and different cryopreservation methods (cell concentration test)

The effect of freshly stripped sperm and the cryopreservation in 5 mL straw and 10 mL cryotube using Styrofoam box and CRF on sperm density change (*N=5*) (see Chapter 2.1, Table 3) was investigated. The effect of the two extenders as mentioned above (grayling and pike) was also observed (see Chapter 2.1).

2.2.4. The assessment of a uniform thawing period for 10 mL cryotube using grayling and pike extenders in CRF

In my research, sperm from five individuals (*N=5*) was cryopreserved with a 10 mL cryotube. Pike and grayling extenders (see chapter 2.1) were applied for preservation. Freezing was performed in CRF equipment (see Chapter 2.1, Table 3). The effects of three different thawing periods (3 min 30 sec, 3 min 45 sec, and 4 min) were compared.

2.2.5. The assessment of an uniform thawing period for 5 mL cryotube using two extenders and two freezing methods

Sperm from five individuals (*N=5*) were frozen. After freezing, thawing period was standardized for the 5 mL cryotube. The experiment was carried out both in a Styrofoam box and in a CRF using two extenders presented earlier (see Chapter 2.1; Chapter 2.1, Table 3). The thawing period intervals of 2 min 45 sec, 3 min, 3 min 15 sec for grayling extender, 2 min 15 sec, 2 min 30 sec, and 2 min 45 sec for pike extender were compared.

2.2.6. The application of large-scale cryopreserved sperm (10 mL cryotube) during hatchery propagation

The most economical cryopreservation method (10 mL cryotube) was intended to develop intensive carp hatchery practice. In my research, grayling (see chapter 2.1) and modified pike extenders (205 mM glucose, 20 mM NaCl, 25 mM KCl, 1 mM Na_2HPO_4 * $12H_2O$, 1 mM $MgCl_2$ * $6H_2O$, 1 mM $CaCl_2$ * $2H_2O$, 20 mM Tris, and 0.5% BSA, pH 8.0 ± 0.2) was used. Freezing was performed with sperm stripped from five male individuals (*N*=5) in a CRF (see Chapter 2.1, Table 3). The study was implemented with fresh (control) and thawed (treated groups) sperm from the five males. In each group, 10 g of egg batch per unit was weighed, and fertilization was performed according to a

predetermined ratio (100 g of eggs; 10 mL of system water; 1 mL of sperm). During incubation of eggs, the batches were placed in a unique recirculation Rack system.

2.2.7. Intensive larvae rearing after propagation with fresh sperm

In my study, 332 g of eggs from one female (*N=1*) with a total sperm of 7.5 mL from five male individuals (*N=5*) was mixed. The incubation of the eggs and then the hatching process took place in a closed recirculation Zuger system. The hatched larvae were placed in a Rack system. Fifty individuals per litre in a rearing tank were placed. Feeding larvae were fed four times a day with freshly hatched brine shrimp (*Artemia salina*) larvae in *ad libitum*. During the research, the general body parameters (mean body length and body weight) and malformations of the selected individuals at the time of three developmental stages (hatching, absorption of the yolk-sack, one-week feeding larva) were recorded. During their development, morphological abnormalities (curved body, deformed tail development, yolk malformation, head deformity, edema, swim bladder distortion, haematoma) were recorded.

2.3. The experiments carried out in the Hévíz dwarf carp

Table 6. shows the tests performed on the experimental stock.

Table 6. The summary of the experiments performed on the Hévíz dwarf carp.

Chapter no.	Cryopreservation capacity	Extender	Cryopreservation method	The subject of the test	Sample size
3.8.1.	N. D.	N. D.	N. D.	spermiation, motility	*N*=8 *N*=8
3.8.2.	N. D.	N. D.	N. D.	spermiation, motility	*N*=7 *N*=7
3.8.3.	0.5 mL straw	grayling	Styrofoam box	motility	*N*=16
3.8.4.	0.5 mL straw	grayling pike	Styrofoam box, CRF	motility	*N*=5
3.5.5.	N. D.	N. D.	N. D.	larvae rearing	♀: *N*=4 ♂: *N*=5

2.3.1. The hormonal induction of the spermiation under field conditions

In my study, induction of spermiation was performed under field conditions in wild-caught individuals. Two groups from the stock were established. One group was hormonally injected (*N=8*), while the other was treated only with a fish saline solution of 0.65 % (control group) (*N=8*).

2.3.2. The hormonal induction of the spermiation in an intensive system with increasing hormone doses

Males were caught immediately before the experiment and placed in a closed intensive recirculation system. Two groups were established (*N=7-7*). In the first group, a series of increasing doses of the hormone for one week (1st treatment: 1 mg body weight kg^{-1}, 2nd treatment: 2 mg body weight kg^{-1}, 3rd treatment: 2 mg body weight kg^{-1}, 4th treatment: 4 mg body weight kg^{-1}) in 2-day intervals were used. According to the protocol mentioned above, the other group received only fish physiological saline solution (control group).

2.3.3. The hormonal induction of the spermiation in male individuals kept in an intensive recirculation system and cryopreservation of sperm

In my research, hormonal induction of spermiation of the individuals (*N=16*) kept in a closed intensive recirculation system for a long time was performed. The injection protocol described in the previous chapter (Chapter 2.3.2) was used. A dilution ratio of 1:9 (see Chapter 2.1, Table 2) for 0.5 mL straw was applied during freezing. Freezing was performed in a Styrofoam box (see Chapter 2.1, Table 3).

2.3.4. The comparison of two types of extenders (grayling, pike) to eliminate agglutination during freezing

In my study, the post-freezing quality of the sperm obtained from Hévíz dwarf carp (*N=5*) after using the previously presented grayling and pike extenders (see Chapter 2.1) was compared. A dilution ratio 1:9 (see Chapter 2.1; Table 2) for 0.5 mL straw was used. Cryopreservation was performed in a Styrofoam box and a CRF (see Chapter 2.1; Table 3).

2.3.5. Propagation in an intensive system

The individuals (*N=24*; 12 females, 12 males) were hormonally injected on the lakeshore following catching them. Afterward, males and females were transported to a closed intensive recirculation system belonging to the infrastructure of the Department of Aquaculture. In my study, the eggs of 14.63 g from four females with a total sperm of 1.5 mL from five males were mixed. Fertilized eggs were placed in a Zuger jar of 7 litres. The hatching rate was determined on the 3rd day after fertilization.

3. RESULTS

3.1. The results obtained from the experiments performed in the "sudár" carp landrace

My results showed an agglutination of roughly 50 % after cryopreservation in using a grayling extender in all the cases. However, a uniform homologous cell suspension was observed using the pike extender. Furthermore, in most cases, compared to the fresh sperm, significantly lower pMOT and VCL parameters in the treated groups were recorded. In my studies, samples placed in the storage dewar and not thawed formed the basis for the sperm bank of the landrace.

3.1.1. The comparison of the cryopreservation of a 5 mL straw and the 10 mL cryotube, and the development and optimal dilution ratio for the 10 mL cryotube

In comparison with the fresh control, significantly lower motility values for both straw and cryotube were recorded. Furthermore, straw showed demonstrably lower values compared to the cryotube. However, after using the 10 mL cryotube, a significantly higher LIN was observed than the native sperm and the smaller volume method. In determining the dilution ratio, a verifiably lower pMOT as a result of the treatment compared to fresh sperm was measured. The fresh sperm showed a statistically higher VCL compared to the treated groups. The 1:1, as well as 1:9 dilution ratios, showed a demonstrably lower value than the fresh sample in the LIN parameter.

3.1.2. The comparison of two types of extender and different cryopreservation methods (motility test)

In my study of the 5 mL straw in a Styrofoam box, both grayling and pike extenders showed significantly lower values than fresh sperm. A similar trend was observed when measuring the VCL parameter.

After cryopreservation of the 5 mL straw in CRF, both extenders showed demonstrably lower value than the fresh sperm. Furthermore, a lower result for the pike extender compared to the grayling extender was measured. When examining VCL values, both cryopreserved groups showed significantly lower values compared to the fresh sample.

After cryopreservation in a controlled rate freezer of the 10 mL cryotube, a verifiable difference in pMOT and VCL parameters using the grayling and pike extenders compared to the fresh sperm was observed.

3.1.3. The comparison of two types of extender and different cryopreservation methods (cell concentration test)

The cell density after cryopreservation was lower for both extenders compared to the cell concentration of the fresh sperm.

A lower cell number for all three cryopreservation methods (5 mL straw Styrofoam box, 5 mL straw CRF, 10 mL cryotube controlled rate freezer) was recorded using a grayling the pike extender.

3.1.4. The assessment of a uniform thawing period for 10 mL cryotube using grayling and pike extenders in CRF

After the 10 mL cryotube cryopreservation, three different thawing periods (3 min 30 sec, 3 min 45 sec, 4 min) with both grayling and pike extenders were compared. After thawing, significantly lower values for grayling extender in progressive motility measurement compared to the fresh sperm were recorded at all three times examined. A similar result for the VCL parameter was obtained. A similar trend after using a pike extender was also observed. For the shortest thawing period, 30±12% pMOT, 48±8 μm s^{-1}, VCL, 86±3% LIN for the grayling; while for pike extender 26±14% pMOT, 41±7 μm s^{-1} VCL, 81±4% LIN values were recorded.

3.1.5. The assessment of an uniform thawing period for 5 mL cryotube using two extenders and two freezing methods

While assessing the thawing period of the 5 mL cryotube, the progressive motility of the samples was demonstrably reduced compared to the fresh sperm after cryopreservation in the Styrofoam box and the use of a grayling extender. A similar trend was observed when measuring VCL. For the fastest thawing period, a pMOT of 30±4%, VCL of 56±7 μm s^{-1}, and LIN values of 84±2% were recorded.

After cryopreservation in the CRF using the grayling extender 5 mL cryotube, the progressive motility of the samples reduced notably compared to the fresh sperm. A similar trend was observed in VCL. However, in the case of LIN parameters, verifiably higher values resulting from the treatments were measured. During the shortest thawing period, 37±6% pMOT, 58±8 μm s^{-1} VCL, and 87±1% LIN results were recorded.

While determining the thawing period of the 5 mL cryotube, the progressive motility of the samples was demonstrably reduced compared to the fresh sperm after cryopreservation in the Styrofoam box and the use of a pike extender. A similar trend was observed in VCL. For the fastest thawing period, a 19±5% pMOT, 55±3 μm s^{-1} VCL, and 84±5% LIN was measured.

In the CRF equipment, the progressive motility of the samples was demonstrably reduced after the use of a pike extender in the 5 mL

cryotube compared to the fresh sperm. A similar trend was described in VCL. During the shortest thawing period, 27±4% pMOT, 47±5 μm s^{-1} VCL, and 81±2% LIN results were recorded.

3.1.6. The application of large-scale cryopreserved sperm (10 mL cryotube) during hatchery propagation

In my experiment, the quality of the native sperm after stripping and at the moment of fertilization was measured as well. Cryopreservation caused a decreased motility in the samples. However, the grayling extender showed significantly higher results than the modified pike extender. Compared to the value of the freshly stripped sperm and measured at the moment of fertilization, a verifiable decrease in the VCL parameter for both treatments was observed. For LIN values, a significantly higher result was recorded than for grayling extender compared to the fresh sperm. The LIN parameter of fresh sperm measured at fertilization was significantly lower than with the pike extender. After using a pike extender, a demonstrably lower value compared to a grayling extender was recorded. In assessing the hatching rate, a statistically significant reduction in the effect of grayling and pike extenders compared to native sperm was observed. Furthermore, the pike extender showed a demonstrably lower value compared to the grayling extender.

3.1.7. Intensive larvae rearing after propagation with fresh sperm

Based on my results, the body length of the freshly hatched “sudár” carp landrace larvae was 4.4±1 mm, and their body weight was 1.0±0.3 mg. After absorption of the yolk sac, the mean body length increased to 5.5±0.5 mm, and the mean body weight increased to 1.5±0.1 mg. The mean body length of one-week-old feeding larvae was 10.5±0.7 mm, and body weight was 12.1±1.7 mg. At the end of the study, the average survival rate of the larvae was 94±2%. During the larvae morphological studies, a significant change in the examined individuals was not observed.

3.2. The results obtained from the experiments performed in the Hévíz dwarf carp

In my studies, stored samples (not used for experiment) established the sperm bank of the vulnerable (from the aspect of conservation) fish species.

3.2.1. The hormonal induction of the spermiation under field conditions

After stripping treated and control groups, the injected individuals showed significantly higher progressive motility than the control group.

3.2.2. The hormonal induction of the spermiation in an intensive system with increasing hormone doses

In my experiment, individuals injected with fish saline solution (control) did not give a significant amount of sperm. Sperm from six individuals were collected successfully in the group injected with increasing hormone doses. During sperm quality assessment, high pMOT (72±8%), moderate VCL (93±12 μm s^{-1}), and high LIN values (88±2%) were recorded.

3.2.3. The hormonal induction of the spermiation in male individuals kept in an intensive recirculation system and cryopreservation of sperm

The pMOT and VCL values of the stripped sperm decreased significantly following cryopreservation. However, in the case of the LIN parameter, a significantly higher value was recorded compared to fresh sperm.

3.2.4. The comparison of two types of extenders (grayling, pike) to eliminate agglutination during freezing

A significantly higher result for the grayling extender than the pike extender was recorded using the controlled rate freezer. A significantly higher VCL was recorded using the grayling extender in the Styrofoam box compared to the values measured at the pike extender. A similar result was observed with CRF. A significantly higher LIN was also recorded in CRF using a grayling extender in comparison with the pike.

3.2.5. Propagation in an intensive system

In my study, the development of the fertilized egg batches incubated in the Zuger jars stopped. Only a single larva hatched.

3.3. Novel scientific results

1. I successfully cryopreserved the carp sperm using the previously developed extender for pike, which eliminated the agglutination after thawing, making it possible to determine a future exact fertilization unit during the hatchery use of the cryopreserved "sudár" carp landrace sperm.
2. I was the first to experimentally adapt an efficient cryopreservation method for the freezing of 5 mL and 10 mL cryotubes using both grayling and pike extenders in a CRF equipment (initial temperature: 4 °C, endpoint: -160 °C, cooling rate: 15 °C min^{-1}) and for the 5 mL cryotube in a Styrofoam box (3 cm, 7 min).
3. In the case of the 10 mL cryotube, I determined the thawing period of 3 min 30 sec at 40 °C after using the grayling and pike extenders. I determined the thawing period of 2 min 45 sec after using the 5 mL cryotube for the grayling extender and 2 min 15 sec after the use of the pike extender with a water bath at 40 °C.
4. In the case of the "sudár" carp landrace, I successfully examined the mean body length and body weight of the freshly hatched larvae after successful hatchery propagation in a closed intensive recirculation system. In the case of the landrace, I was the first to present an effective larvae rearing method in the case of individuals with a high survival rate and acceptable morphological characteristics in a closed intensive recirculation system.
5. I induced the spermiation of the extremely stress-sensitive Hévíz dwarf carp with high efficiency by injection in the field conditions directly on the lakeshore. In addition, after prolonged maintenance in a closed intensive recirculation system, with a prolonged hormone dose injection procedure (1^{st} treatment: 1 mg body weight kg^{-1}, 2^{nd} treatment: 2 mg body weight kg^{-1}, 3^{rd} treatment: 2 mg body weight kg^{-1}, 4^{th} treatment: 4 mg body weight kg^{-1}, in 2-day intervals), I successfully increased the sperm production of the Hévíz dwarf carp.
6. I successfully cryopreserved sperm of the Hévíz dwarf carp in a Styrofoam box and CRF equipment after using the grayling (previously used in cyprinids) and the newly applied pike extender in 0.5 mL straw at a dilution ratio of 1:9.
7. I successfully established an *in vitro* sperm bank using the stored sperm of the "sudár" carp landrace representing the economic value and the Hévíz dwarf carp, which is vulnerable from the aspect of nature conservation.

4. DISCUSSION AND PROPOSALS

4.1. Discussion

4.1.1. Discussion of the results concerning the "sudár" carp landrace

In my experiments, an agglutination of approximately 50 % in each case after thawing the samples in the case of a grayling extender was observed. I was the first to apply large-scale cryopreservation in a Styrofoam box and CRF equipment for the landrace. In my preliminary experiment, "sudár" carp landrace sperm in a 5 mL straw with a Styrofoam box was successfully cryopreserved. A grayling extender, which had previously been effective for several carp species, was used (BERNÁTH et al. 2016a; BERNÁTH et al. 2017; BERNÁTH et al. 2018). Furthermore, the 10 mL cryotube for the "sudár" carp landrace using CRF equipment was efficiently applied in my experiment. Based on literature (HORVÁTH et al. 2003), a dilution ratio of 1:9 is the most appropriate for carp, which was also supported by the results of my studies. However, when using the extenders of different compositions, there was no significant difference between them with the 5 mL straw frozen in a Styrofoam box and the 10 mL cryotube preserved in the controlled rate freezer. However, the difference was confirmed for 5 mL straw after using the CRF equipment. Based on my results, it can be concluded that both grayling and pike extenders are suitable for performing large-scale sperm cryopreservation for the landrace. Based on my results, cryopreservation with pike extender resulted in a significantly higher cell concentration than grayling extender using any cryopreservation methods. In addition, the pike extender resulted in a uniform cell suspension in all cases. There was no difference between the different intervals using grayling and pike extenders for the three different thawing periods compared after using the 10 mL cryotube. Consequently, the fastest thawing is the most appropriate for the hatchery practice. There was also no verifiable difference between the different periods for the extenders mentioned above during the standardization of thawing after cryopreservation of the 5 mL cryotube in Styrofoam box and CRF. The application of the fastest thawing period can be perfectly integrated into the hatchery practice, thus allowing the frozen sperm to be easily applied during the propagation process. I was the first to apply cryopreservation of a 5 mL cryotube in a controlled rate freezer through my research. Furthermore, based on the publications in literature, I was also the first to apply the procedure in the case of a "sudár" carp landrace (HORVÁTH et al. 2007). I intended to prove the effectiveness of the cryopreservation methods during propagation. During cryopreservation of the 10 mL cryotube, the motility parameters were negatively affected by the freezing process. In my experiment, a

low hatching rate after the use of both fresh sperm, grayling, and modified pike extender was recorded. The possible reason for the low hatching rate may have been the incorrect distribution of the substrate of the fertilized eggs placed in the Rack system. It can also be assumed that poor egg quality may also have caused the low values. In the case of the grayling extender, a higher progressive motility and hatching result compared with the modified pike extender was recorded. According to my hypothesis, these random differences in each motility parameter can be explained by the individual variation of the samples and the strong selection effect on the sperm subpopulations during the freezing process (KRÓL et al. 2018). However, the newly tested pike extender allows the development of an accurate, applicable uniform sperm to egg ratio and a fertilization unit (HU et al. 2011). During my intensive breeding studies, I was the first to describe the mean body length and body weight parameters of the hatching larvae of the landrace. High growth values during larvae rearing were recorded. A survival rate of 94 ± 2% was measured in my study, which is much higher than the value found in the literature but described for pond stocking (45±21%) (HORVÁTH 2018). In my larvae morphology studies, the extent of the examined malformations was lower than the ratio described in other cyprinids (BERNÁTH et al. 2018; MARTINS et al. 2009) and corresponded to the ratio recorded for other wild carp types (ŁUGOWSKA & SARNOWSKI 2011; MARTINS et al. 2009).

4.1.2. Discussion of the results concerning Hévíz dwarf carp

Based on the results of my hormonal induction experiments in field conditions, the spermiation of the wild carp, which is vulnerable from the aspect of nature conservation and extremely stress-sensitive, was successfully injected. The individuals responded well to the injection, and I managed to strip the treated male individuals without mortality. After placing the individuals directly in a closed recirculation system, high pMOT, moderate VCL, and acceptable LIN values during the assessment of the stripped sperm using a series of increasing doses of the hormone were recorded. From the negative results obtained in the control group (I did not obtain a measurable sperm), it can be seen that high temperature similar to lake conditions itself is not sufficient for the induction of spermiation in the males. Prolonged hormone treatment is also necessary under hatchery conditions concerning Hévíz dwarf carp, which has vulnerable nature conservation value. The injection series presented above was applied after long-term maintenance in a closed recirculation system. After the efficient induction of sperm production, the injected individuals were stripped successfully, and high progressive motility values were recorded. During the cryopreservation, the extender was initially developed for the pike species, and the grayling

diluent previously used for cyprinids was successfully applied. Based on my results, similarly to the "sudár" carp landrace, agglutination in the thawed sperm during the application of the pike extender was not observed. Concerning Hévíz dwarf carp, I was the first to use a controlled rate freezer successfully. In my fertilization experiment, after the hormonal injection of the wild-caught broodstock, gametes were successfully stripped. However, the development of the embryos stopped during the incubation. The reason for this phenomenon may have included the asynchronous developmental stage of the oocytes. For several fish species, it has been noted that the asynchronous maturation of gametes generated problems during reproduction (ASTURIANO et al. 2004; CABRITA et al. 2006). Another possible problem of unsuccessful propagation may have included the non-natural propagation environment provided in a closed intensive recirculation system (WOYNÁROVICH & HORVÁTH 1980). Sperm quality assessment was not performed in my experiment (only a few volumes of sperm were available). As a result, it cannot be excluded that sperm quality influenced fertilization and egg incubation (KHOLODNYY et al. 2019).

4.2. Proposals

Based on my results, I intend to make the following suggestions in the case of the economically important "sudár" carp landrace and Hévíz dwarf carp representing nature conservation value:

<u>"sudár" carp landrace:</u>

1. When using the Styrofoam box, I recommend cryopreservation for 7 minutes at the height of 3 cm for the 5 mL straw and the 5 mL cryotube. In the case of a controlled rate freezer, I recommend the cryopreservation protocol for the 5 mL straw and the 5 mL and 10 mL cryotubes at an initial temperature of 4 ° C and an endpoint of -160 °C at a cooling rate of 15 °C min^{-1}. Both methods can freeze a large volume of carp sperm, and the methods are efficiently transferable to the hatchery propagation practice.
2. In the case of the "sudár" carp landrace, I recommend a dilution ratio of 1:9 when freezing a large volume of sperm.
3. For the thawing of the large volume (CRF equipment) 10 mL cryotube, I recommend thawing for 3 minutes 30 seconds after using the grayling and pike extenders. For thawing the 5 mL cryotube (Styrofoam box, CRF equipment), using the grayling extender it is 2 minutes 45 seconds, and for pike extender, use a thawing period of 2 minutes 15 seconds in a 40 °C water bath.
4. During the cryopreservation of sperm in the "sudár" carp landrace and the application in the propagation process, I recommend the use of the grayling extender previously used in cyprinids and the

extender of the newly applied pike extender. To determine a more accurate fertilization unit, I recommend the use of pike extender.

5. Based on my results, I recommend the long-term nursing (10 days) of the economically important carp landrace in a closed intensive recirculation system or under hatchery conditions.

Hévíz dwarf carp:

1. Under field conditions, I recommend the hormonal injection of wild-caught broodstock on the shore to obtain the optimal sperm quality. However, in the case of the individuals maintained in a closed intensive recirculating system, the one-week injection protocol with prolonged increasing doses of hormone (1st treatment: 1 bodyweight kg^{-1}, 2nd treatment: 2 bodyweight kg^{-1}, 3rd treatment: 2 bodyweight kg^{-1}, 4th treatment: 4 bodyweight kg^{-1}, at two-day intervals) is recommended.
2. For gene preservation, I recommend using grayling and pike extenders for sperm cryopreservation using 0.5 mL straw in both Styrofoam box and CRF.

5.PUBLICATIONS RELATED TO THE TOPIC OF THE DISSERTATION

5.1. Publications in peer-reviewed journals

1. **VÁRKONYI, L.**, BOKOR, Z., MOLNÁR, J., FODOR, F., SZÁRI, ZS., FERINCZ, Á., STASZNY, Á., LÁNG, L. Z., CSORBAI, B., URBÁNYI, B., BERNÁTH, G. (2019): The comparison of two different extenders for the improvement of large-scale sperm cryopreservation in common carp (*Cyprinus carpio*). *Reproduction in Domestic Animals* 54 (3):639–645. p.
2. MOLNÁR, J., CSORBAI, B., BERNÁTH, G., **VÁRKONYI**, **L**., URBÁNYI, B., BOKOR, Z. (2019): Optimizing fish structure in angling ponds focusing on white fish. *Acta Agraria Debreceniensis Journal of Agricultural Sciences* 2019 (1) 33-36. p.
3. **VÁRKONYI**, **L.**, BOKOR, Z., FERINCZ, Á., STASZNY, Á., FODOR, F., SZÁRI, ZS., URBÁNYI, B., MOLNÁR, J., BERNÁTH G. (2018): The applicability of 10 mL cryotubes for sperm cryopreservation in a Hungarian carp landrace (*Cyprinus carpio carpio morpha accuminatus*), *Acta Agraria Debrecensiensis Journal of Agricultural Sciences* 2018 (75) 93-97. p.
4. BERNÁTH, G., CSENKI-BAKOS, ZS. I., BOKOR, Z., **VÁRKONYI L.**, MOLNÁR, J., SZABÓ, T., STASZNY, Á., FERINCZ, Á., SZABÓ, K., URBÁNYI, B., PAP, L. O., CSORBAI, B. (2018): The effects of different preservation methods on ide (*Leuciscus idus*) sperm and the longevity of sperm movement. *Cryobiology* 81, 125–131. p.
5. BERNÁTH, G., ITTZÉS, I., SZABÓ, Z., HORVÁTH, Á., KREJSZEFF, S., LUJIĆ, J., **VÁRKONYI, L.**, URBÁNYI, B., BOKOR, Z. (2017): Chilled and post-thaw storage of sperm in different goldfish types. *Reproduction in Domestic Animals*. 52 (4), 680-686. p.
6. BERNÁTH, G., ZARSKI, D., KÁSA, E., STASZNY, Á., **VÁRKONYI**, **L.**, KOLLÁR, T., HEGYI, Á., BOKOR, Z., URBÁNYI, B., HORVÁTH Á. (2016): Improvement of Common Carp (*Cyprinus Carpio*) Sperm Cryopreservation using a Programable Freezer. *General and Comparative Endocrinology* 237. 78-88. p.

5.2. Publications in peer-reviewed Hungarian journals

1. CSORBAI, B., URBÁNYI, B., BERNÁTH, G., SZABÓ, T., **VÁRKONYI, L.**, MOLNÁR, J., CSENKI-BAKOS, ZS. I., BOKOR, Z. (2020) Kisméretű recirkulációs keltetők felhasználásának lehetősége Magyarországon *Halászat Tudomány* 6 /1 8-13. p.
2. SZABÓ, T., BOKOR, Z., BERNÁTH, G., **VÁRKONYI, L.**, CSENKI-BAKOS, ZS. I., MÜLLER, T., MOLNÁR J., SZABÓ, K., URBÁNYI, B., CSORBAI B. (2019): A népesítési sűrűség növekedésre és megmaradásra gyakorolt hatásának vizsgálata a jászkeszeg (*Leuciscus idus*) intenzív rendszerben történő előnevelése során. *Halászat-Tudomány* 5/1. (2019) 3-6. p.
3. **VÁRKONYI, L.**, MÜLLER T., SPECIÁR A., URBÁNYI B., BERNÁTH G. (2018): A Hévízi-tó környezeti sajátosságai és az ott élő „törpenövésű" vadponty bemutatása. *Halászat* 111. (1) 29-30. p.
4. **VÁRKONYI, L.** (2017): Farok nélküli vadponty a Hévízi-tóból. *Halászat* 110 (1) 15. p.

5. BERNÁTH, G., ŻARSKI, D., KÁSA, E., STASZNY, Á., **VÁRKONYI, L.**, KOLLÁR, T., HEGYI, Á., BOKOR, Z., URBÁNYI, B., HORVÁTH, Á. (2016): A pontysperma mélyhűtés módszertani fejlesztése. *Halászat Tudomány* 2 (2) 18–26. p.

5.3. Proceedings in Hungarian

1. BARTUCZ, T., BOKOR, Z., IZSÁK, T., LÁNG, L. Z., MOLNÁR, J., NAGY, B., BERNÁTH, G., **VÁRKONYI, L.**, CSENKI-BAKOS, ZS. I., FERINCZ, Á., STASZNY, Á., CSORBAI, B. (2020): A domolykó (*Squalius cephalus*) ivadéknevelése recirkulációs rendszerben. In. GYÖRE P. (szerk): Szakkollégiumi és a Tudományos Diákköri Hallgatók Kutatásai. Debreceni Egyetem. 10-16. p. ISBN: 978-963-490-218-8. p.

5.4. Publications in conference abstract books

5.4.1. Presentations in English

1. **VÁRKONYI, L**., BOKOR, Z., MOLNÁR, J., FODOR, F., SZÁRI, ZS., FERINCZ, Á., STASZNY, Á., BIRKO-SULYOK, Z., LÁNG, L. Z., CSORBAI, B., URBÁNYI, B., BERNÁTH, G. (2018): The comparison of two different extenders and the improvement of large-scale sperm cryopreservation in common carp (*Cyprinus carpio*). Seventh International Young Researchers' Conference of NACEE, Gorki, Belarus, December 11-14, 2018. Abstract book. 24-25. p.
2. BERNÁTH, G., CSENKI-BAKOS, ZS. I., BOKOR, Z., **VÁRKONYI, L.**, MOLNÁR, J., KAJTÁR, A., SZABÓ, T., STASZNY, Á, FERINCZ, Á., SZABÓ, K., URBÁNYI, B., CSORBAI, B. (2017): The effects of different preservation methods on Ide (*Leuciscus idus*) sperm and the longevity of sperm movement. 6th International Workshop on the Biology of Fish Gametes, Czech Republic, České Budějovice, 2017. September 4-7. Abstract book 81. p.

5.4.2. Presentations in Hungarian

1. **VÁRKONYI, L.**, BOKOR, Z., CSORBAI, B., MOLNÁR, J., NAGY B., LÁNG, L. Z., IZSÁK, T., BARTUCZ, T., FEKETE, Á., FODOR, F., SZÁRI, ZS., URBÁNYI, B., BERNÁTH, G. (2020): A balatoni sudár ponty (*Cyprinus carpio carpio morpha accuminatus*) keltetőházi szaporítása, valamint intenzív recirkulációs rendszerben történő lárvanevelése. *Halászatfejlesztés* 37., 116-118. p.
2. MOLNÁR, J., BÉKÉSI, R., **VÁRKONYI, L.**, CSORBAI, B., CSENKI-BAKOS, ZS. I., MÜLLER, T., URBÁNYI, B., SZABÓ, T. (2020): Különböző népesítési sűrűségben történő márna (*Barbus barbus*) előnevelés növekedésre és megmaradásra gyakorolt hatásának vizsgálata intenzív körülmények között. *Halászatfejlesztés* 37., 21-23. p.
3. **VÁRKONYI, L.**, BOKOR, Z., FERINCZ, Á., STASZNY, Á., MOLNÁR, J., BIRKÓ-SULYOK, Z., LÁNG, L. Z., JUHÁSZ, V., FODOR, F., SZÁRI, ZS., URBÁNYI, B., BERNÁTH, G. (2018): A hévízi törpenövésű magyar vadponty (*Cyprinus carpio morpha hungaricus*), valamint a balatoni sudár ponty (*Cyprinus carpio morpha accuminatus*) szaporodásbiológiájának vizsgálata és *in vitro* spermabankjának létrehozása. XXXVII. Óvári Tudományos Nap, Mosonmagyaróvár, 2018. november 9-10. Kivonat 154. p.

4. **VÁRKONYI, L.** (2017): A hévízi törpenövésű magyar vadponty (*Cyprinus carpio morpha hungaricus*), valamint a balatoni sudár ponty (*Cyprinus carpio morpha accuminatus*) szaporodásbiológiájának vizsgálata és in vitro spermabankjának létrehozása. Jövő tudósai, a vidék jövője doktoranduszok konferenciája. Debrecen, 2017. november 24. Kivonat 6. p.

5.4.3. Poster presentation in English

1. **VÁRKONYI, L.**, BOKOR, Z., FERINCZ, Á., STASZNY, Á., MOLNÁR, J., BIRKÓ-SULYOK, Z., LÁNG, L. Z., IZSÁK, T., NÉMETH, F., URBÁNYI, B., BERNÁTH, G. (2019): The investigation of the reproductive biology of Hévíz dwarf carp (*Cyprinus carpio morpha hungaricus*) Aquaculture Europe 2019. Berlin-Germany, October 7-10, 2019, Abstract book 1569-1570. p.
2. **VÁRKONYI, L.**, MOLNÁR, J., LÁNG, L. Z., FERINCZ, Á., STASZNY, Á., FODOR, F., SZÁRI, ZS., URBÁNYI, B., BOKOR, Z., BERNÁTH, G. (2018): Methodical improvement of sperm cryopreservation in a Hungarian common carp landrace (*Cyprinus carpio carpio morpha accuminatus*). Aqua 2018, Montpellier, France August 25 - 29, 2018. Abstract book 774. p.
3. **VÁRKONYI, L.**, JÓZSEF, M., STASZNY, Á., FERINCZ, Á., BOKOR, Z., URBÁNYI, B., NÉMETH, G., BERNÁTH, G. (2018): The comparison of two hormonal induction method in the Heviz carp (*Cyprinus carpio carpio morpha hungaricus*). 8th International Water and Fish Conference, Belgrade, Serbia, June 13-15, 2018. Abstract book 272-275. p.
4. **VÁRKONYI, L.**, BERNÁTH, G., FERINCZ, Á., STASZNY, Á., FODOR, F., SZÁRI, ZS., URBÁNYI, B., BOKOR, Z. (2017): The establishment of spermbanks in Sichel (*Pelecus cultratus*), Volga pikeperch (*Sander volgensis*) and the wild Common carp (*Cyprinus carpio morha hungaricus*) focused on the population management at Lake Balaton. Aquaculture Europe ‘17 Dubrovnik, Croatia, October 16-20, 2017. Abstract book 1228. p.

5.4.4. Poster presentation in Hungarian

1. **VÁRKONYI, L.**, BOKOR, Z., CSENKI-BAKOS ZS. I., CSORBAI, B., MOLNÁR, J., NAGY, B., LÁNG, L. Z., IZSÁK, T., BARTUCZ, T., FEKETE, Á., FODOR, F., SZÁRI, ZS., URBÁNYI, B., BERNÁTH, G. (2020): A balatoni sudár ponty (*Cyprinus carpio morpha accuminatus*) keltetőházi szaporítása, valamint intenzív recirkulációs rendszerben történő lárvanevelése. XLIV. Halászati Tudományos Tanácskozás, Szarvas 2020. szeptember 23-24. Kivonat 116-118. p.
2. **VÁRKONYI, L.**, SPECZIÁR, A., HORVÁTH, L., URBÁNYI, B., MÜLLERNÉ TRENOVSZKI, M., MÜLLER, T. (2016): Hévízi törpenövésű vadponty indukált szaporítása az élőhelyén. LVIII. Georgikon Napok, Keszthely, 2016. szeptember 29-30. Kivonat 157. p.