



REPRODUCTIVE BIOLOGICAL INVESTIGATION OF ECONOMICALLY IMPORTANT GOLDFISH VARIETIES

Theses of the Doctoral (PhD) Dissertation

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1. BACKGROUND AND OBJECTIVES

The goldfish (*Carassius auratus*), belonging to the *Cyprinidae* family, is one of the best-known and most widely cultured ornamental fish species in the world. Through more than a thousand years of domestication and artificial selection, it has developed extraordinary phenotypic diversity, manifested in a wide range of modifications in body shape, coloration, scale patterns, and the morphology of fins, eyes, and the head (Smartt, 2001; Omori & Kon, 2019; Chen et al., 2022). Today, more than 180 goldfish varieties are known (Omori & Kon, 2019), and breeders are continuously creating new ones. Beyond its economic and cultural importance, the goldfish also serves as an important model organism for studying artificial selection and developmental biology (Smartt, 2001; Komiyama et al., 2009; Tsai et al., 2013; Ota & Abe, 2016; Chen et al., 2019).

In goldfish breeding, effective reproduction is essential for maintaining desired phenotypic traits (Glenn et al., 2011). Reproductive success is influenced by numerous factors, including broodstock condition, gamete quality, gonadal development, environmental conditions, and hormonal treatments applied during propagation (Mañanos et al., 2009). To increase breeding efficiency, it is crucial to evaluate both the quantitative and qualitative characteristics of gametes as well as the developmental status of reproductive organs (Fauvel et al., 2010). The gonadosomatic index, histological examination of gonads, and the assessment of gamete quality and quantity provide valuable information about sexual maturity and reproductive capacity (Kagawa et al., 1983; Kowalski & Cejko, 2019).

Egg and sperm quality fundamentally determine embryo and larval development. The examination of early developmental stages provides an opportunity to evaluate developmental abnormalities, survival, and hatching rate, thereby offering indirect information about the quality of the gametes used for fertilization (Bonnet et al., 2007; Bobe & Labbé, 2010). Although several studies have investigated certain goldfish varieties from morphological and genetic perspectives (Li et al., 2019; Ota, 2021), reproductive biological differences among varieties and the effects of artificial selection on reproductive processes have not yet been comprehensively studied.

In aquaculture, the short- and long-term storage of gametes has significant importance, as it contributes to the efficiency of breeding programs and the conservation of genetic resources (Contreras et al., 2019). However, microbial changes occurring during storage may significantly affect the quality of gametes intended for reproduction. Although substantial progress has been made in microbiome research in recent years (Foo et al., 2017), only a limited number of studies have investigated the microbial communities associated with fish gametes, particularly male reproductive products, and their changes during storage. In goldfish, the bacterial and fungal communities of sperm are currently unknown, despite their potential relevance from both animal welfare and reproductive biology perspectives.

I hypothesized that the phenotypic characteristics resulting from different selection objectives are reflected not only in external appearance but also in reproductive performance, early developmental processes, and certain microbiological and molecular traits. Therefore, the aim of my research was to conduct a comparative study of the reproductive, developmental, microbiological, and molecular characteristics of various goldfish varieties, and to investigate the impact of centuries of artificial selection on these traits.

Objectives

The aim of my research was to conduct a comprehensive study of the reproductive biological characteristics of goldfish varieties that differ markedly in phenotype, namely the common goldfish, shubunkin, black moor, and oranda.

1. To evaluate the fundamental reproductive parameters quantitatively and qualitatively, and to determine and compare fertilization and hatching rates among the different varieties.
2. To determine and compare growth performance, the incidence of developmental abnormalities, and survival rates during larval rearing.
3. To characterize the microbiota of male gametes (sperm) and investigate changes in its composition during short-term refrigerated storage and long-term cryopreservation.
4. To determine and compare the distribution and proportion of different stages of final gamete maturation among the varieties through histological examination of the gonads following the induction of spermiation and ovulation.
5. To investigate and compare the expression of the *rln3a* gene, which plays a key role in spermatogenesis, in brain and testicular tissues of the different varieties in order to identify molecular-level differences among them.

2. MATERIAL AND METHODS

The experiments were conducted at the facilities of the Institute of Aquaculture and Environmental Safety, Hungarian University of Agriculture and Life Sciences, Szent István Campus. The Institutional Animal Welfare Committee of the Hungarian University of Agriculture and Life Sciences, Szent István Campus, confirmed that all experiments were carried out in accordance with Directive 2010/63/EU and with its approval (approval numbers: MATE-SZIC/1742-1/2022 and MATE-SZIC/1745-1/2022).

2.1. Experimental broodstock maintenance and handling

The F2 generation goldfish broodstock used in this study originated from a Hungarian breeder. Sexually mature fish were separated by sex and maintained in a 3 m³ recirculating aquaculture system following a two-week quarantine period. The system was equipped with mechanical, biological, and UV filtration and operated under PLC control. Water quality parameters were continuously monitored and maintained as follows: temperature 18–19 °C, dissolved oxygen 7–8 mg L⁻¹, pH 7.8–8.1, with low ammonium and nitrite concentrations. Fish were fed once daily with a commercial dry feed at 2% of body weight.

Preparation of the broodstock for spawning followed the breeder's standard protocol. One week prior to the experiments, water temperature was gradually increased to 21–22 °C, and feeding was suspended for two days. Fish were anesthetized with 0.4 mL L⁻¹ 2-phenoxyethanol. Spermiation and ovulation were induced using common carp pituitary extract dissolved in physiological saline solution. Females received a total dose of 4 mg kg body weight⁻¹ administered in two injections: a priming dose (10%) 24 h and a resolving dose (90%) 12 h before the expected ovulation. Males received a single injection of 2 mg kg body weight⁻¹ 12 h before sperm collection. Gametes were obtained by gentle abdominal stripping according to conventional methods.

2.2. Statistical analysis

Data processing and statistical analyses were performed using Microsoft Excel, Microsoft Word, IBM SPSS 25, GraphPad Prism 8, Statistica, and R software. Data normality was assessed using the Shapiro–Wilk test and, when necessary, the Kolmogorov–Smirnov test. Non-normally distributed data were transformed using arcsine square root or logarithmic functions. Group comparisons were conducted using Analysis of Variance (ANOVA), Kruskal–Wallis tests, and appropriate post hoc analyses. Additionally, Odds Ratio, Linear Discriminant Analysis (LDA), Permutational Multivariate Analysis of Variance (PERMANOVA), and Pearson correlation analyses were applied where appropriate.

2.3. Research plan

The study was conducted in two consecutive experimental phases. During the first phase (2021), sperm quality, egg quality, propagation, and larval rearing experiments were carried out. During the second phase (2023), samples were collected for microbiological, histological, and gene expression analyses.

2.4. Gamete quality assessment and larval rearing

2.4.1. Sperm quality assessment

Sperm quality was evaluated by comparing eight males from each variety. Total sperm volume (mL) and relative sperm volume (mL kg body weight⁻¹) were determined. Sperm concentration (N , spermatozoa mL⁻¹) was calculated from the average number of cells counted using a Bürker chamber. Sperm motility (pMOT, %) and kinetic parameters (VCL, $\mu\text{m s}^{-1}$; VAP, $\mu\text{m s}^{-1}$; LIN, %; ALH, μm ; BCF, Hz) (Cabrita et al., 2008; Gallego & Asturiano, 2018) were recorded and analyzed using a digital camera connected to a CASA (Computer-Assisted Sperm Analysis) system.

2.4.2. Egg quality assessment and propagation trial

For females (common goldfish $N = 8$, shubunkin $N = 9$, black moor $N = 7$, oranda $N = 8$), the pseudo-gonadosomatic index (PGSI, %) and the number of eggs per gram were determined.

The efficiency of artificial reproduction was evaluated and compared among four goldfish varieties (common goldfish $N = 8$; 7, shubunkin $N = 9$; 9, black moor $N = 7$; 7, oranda $N = 8$; 7). Prior to fertilization, sperm motility and kinetic parameters were assessed using CASA. After quality evaluation, the five sperm samples with the highest pMOT (%) values were pooled and used for fertilization. Egg batches from individual females were pooled within each variety (40 g eggs/variety) and mixed thoroughly with pooled sperm (330 μL sperm/variety). Fertilization was activated using system water at an approximate water:egg:sperm ratio of 10:100:1 (Horváth et al., 1992). Fertilization was performed separately for each variety. Fertilization rate was determined 24 h after fertilization at the end of the segmentation stage. Hatching rate was assessed during synchronized incubation at the time of hatching.

2.4.3. Larval development assessment

Larvae obtained from the propagation trial were transferred to a PLC-controlled recirculating rack system. Rearing was conducted at 21–22 °C with a stocking density of 50 larvae per litre. Random sampling was performed on days 0, 3, and 10 post-hatch. At each sampling time, 60 larvae per variety were

evaluated. Before examination, larvae were anesthetized with 168 mg L⁻¹ MS-222 (tricaine methanesulfonate). For photography, larvae were immobilized in a 3% methylcellulose solution. The incidence of developmental abnormalities (somites, notochord, tail, fins, heart, cranial region, brain, jaw, and intestine), average total length (mm), and body weight (g) were determined and compared among varieties. Survival rate was assessed at the end of the rearing trial (10 days post-hatch, dph) (Bokor et al., 2015).

2.5. Investigation of sperm microbiota

For microbiological analyses, male broodstock (common goldfish $N = 3, 3$; shubunkin $N = 3, 3$; black moor $N = 3, 3$; oranda $N = 3, 3$) were hormonally induced and stripped as described in section 2.1. Prior to sampling, the urogenital pore was disinfected with 70% ethanol.

2.5.1. Short-Term storage

Sperm samples were stored at 4 °C for 96 h with sterile oxygen exchange every 24 h. Bacterial colony-forming units (CFU mL⁻¹) were determined on *Aeromonas* agar (AER) and tryptic soy agar (TSA). Selective cultivation on gentamicin-supplemented Mueller–Hinton (MH) agar was performed to detect potentially antibiotic-resistant bacteria. Fungi were cultured on streptomycin-supplemented potato dextrose agar (PDA). Representative bacterial and fungal colonies were identified by Sanger sequencing.

2.5.2. Long-Term storage

Sperm was cryopreserved in an extender previously published for chub (*Squalius cephalus*), containing: 205 mM glucose, 75 mM NaCl, 30 mM KCl, 1 mM Na₂HPO₄·12H₂O, 1 mM MgCl₂·6H₂O, 1 mM CaCl₂·2H₂O, 20 mM Tris, and 0.5% BSA (pH 8.0±0.02) (Bernáth et al., 2025). Samples were diluted 1:9 with extender, supplemented with 10% methanol as cryoprotectant, loaded into 0.25 mL plastic straws, and frozen. Cryopreserved samples were stored in liquid nitrogen (-196 °C) for five months. Thawing was performed in a circulating water bath at 40 °C (Bernáth, 2016). Sperm motility parameters and microbial communities were assessed before freezing and after thawing. Cultivation was performed on TSA and PDA supplemented with streptomycin. The extender and thawing water were also cultured to assess potential contamination.

2.5.3. Identification of isolated strains

Bacterial species identification was performed using partial 16S rRNA gene sequencing. Universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') were used, as they are among the most widely applied primer pairs for bacterial species identification (Frank et al.,

2008; Hou et al., 2018). Following DNA extraction, PCR amplification, and Sanger sequencing, taxonomic identification was performed using the EZBioCloud database based on sequences ranging from 700 to 1490 bp.

Fungal isolates were identified by sequencing the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA (nrDNA). The fungal-specific primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for species- or genus-level identification. Sequence analysis was performed using the NCBI BLAST system (BLASTn).

2.6. Histological analysis

Broodstock used for histological examination (common goldfish $N = 6$ females, 5 males; shubunkin $N = 5$ females, 5 males; black moor $N = 5$ females, 5 males; oranda $N = 5$ females, 5 males) were maintained as described in section 2.1. Fish were euthanized by overdose of 2-phenoxyethanol (0.6 mL L^{-1}). Entire testes and ovaries were collected and weighed to calculate the gonadosomatic index (GSI). Gonads were stored in 8% buffered formaldehyde until processing. In testes, the proportions of tissues and cells at different spermatogenic stages (spermatogonia A, spermatogonia B, spermatocytes, spermatids, spermatozoa, empty lumen, connective tissue) were determined. In ovaries, the proportions of oocytes at different stages of oogenesis (perinucleolar, cortical alveolus, vitellogenic, mature, and atretic state) were quantified.

2.7. Gene Expression Analysis by RT-qPCR

To minimize the number of experimental animals, the same individuals used for histological analysis ($N = 5$ per variety) were used for gene expression studies. RNA was extracted from brain and testicular tissues, and its concentration and quality were assessed using a spectrophotometer. Complementary DNA (cDNA) was synthesized by reverse transcription. Primers for both the housekeeping gene and the target gene were designed specifically for this study. Quantitative real-time PCR (RT-qPCR) was performed using 5X Hot FIREPol EvaGreen qPCR Mix Plus and StepOne v2.3 software. Expression levels of the target gene *rln3a* were normalized to the expression of the housekeeping gene *β -actin* (Maruyama et al., 2006) and reported as normalized mRNA expression levels (Ivánovics, 2023).

3. RESULTS

3.1. Results of gamete quality and larval development assessments

3.1.1. Results of sperm quality assessment

Comparison of the basic spermatological parameters among the four goldfish varieties revealed no statistically significant differences in sperm volume, sperm volume per kilogram of body weight, or sperm concentration.

Regarding the motility parameter measured by the CASA system, namely pMOT, significantly higher values were observed in the shubunkin ($89\pm7\%$) and oranda ($91\pm2\%$) than in the black moor ($79\pm13\%$). However, no statistically significant difference was found between the black moor and common goldfish ($87\pm7\%$) for this quality parameter. No considerable differences were detected among the varieties in the sperm kinetic parameters VCL, VAP, LIN, ALH, and BCF.

Based on linear discriminant analysis (LDA), the common goldfish and black moor were clearly separated from the shubunkin and oranda varieties according to the examined sperm quality and quantity parameters. The differences among varieties were mainly explained by the pMOT, ALH, and SV BW⁻¹ parameters.

A statistically significant difference was observed among the varieties in the sperm motility parameter measured by CASA. In oranda ($78\pm11\%$), a significantly lower progressive motility value was recorded than in common goldfish ($92\pm3\%$) and black moor ($91\pm2\%$). The same parameter in shubunkin ($84\pm11\%$) did not differ significantly from the other varieties. No considerable differences were recorded among the kinetic parameters of sperm samples from the different varieties.

3.1.2. Results of egg quality assessment and propagation trial

Egg quality assessment

Significant differences were recorded among the varieties in the basic reproductive parameters of females. In oranda ($18\pm4\%$), significantly higher PGSI values were observed than in common goldfish ($9\pm5\%$) and shubunkin ($10\pm4\%$). In addition, this parameter was significantly higher in black moor ($16\pm3\%$) than in common goldfish. The number of eggs per gram was significantly lower in black moor (1091 ± 69) than in common goldfish (1733 ± 606).

Propagation trial

During the propagation trial, differences were recorded among the varieties in both fertilization and hatching rates. In oranda (93%), the fertilization rate was significantly higher than in common goldfish (71%), shubunkin (79%), and black moor (79%). In black moor (44%), the hatching rate was significantly lower than in the other three varieties [common goldfish (63%), shubunkin (69%), and oranda (71%)].

Results of larval development assessment

During the assessment of larval growth, no high degree of heterogeneity was observed in the individual development of the four varieties at the examined stages.

At hatching (0 dph), the total body length of common goldfish larvae (3.9 ± 0.2 mm) was significantly lower than that of shubunkin larvae (4.7 ± 0.2 mm). However, no significant differences were detected in black moor (4.2 ± 0.3 mm) and oranda (4.5 ± 1.0 mm) compared with the other varieties. At the same developmental stage, no difference was found in body weight among the varieties (common goldfish: 0.35 ± 0.06 mg; shubunkin: 0.47 ± 0.1 mg; black moor: 0.49 ± 0.07 mg; oranda: 0.5 ± 0.6 mg).

On the third day post-hatch (3 dph), the total body length of shubunkin larvae (5.3 ± 0.3 mm) was significantly lower than that of oranda larvae (5.6 ± 0.3 mm), while common goldfish (5.4 ± 0.4 mm) and black moor (5.5 ± 0.4 mm) did not differ significantly from the other varieties. No significant differences were observed in larval body weight (common goldfish: 1.1 ± 0.2 mg; shubunkin: 1.1 ± 0.2 mg; black moor: 1.4 ± 0.3 mg; oranda: 1.4 ± 0.3 mg).

On day 10 post-hatch (10 dph), similar total body length values were recorded in common goldfish (8.0 ± 0.8 mm), shubunkin (7.9 ± 0.7 mm), black moor (8.2 ± 0.8 mm), and oranda (8.2 ± 0.9 mm). However, the body weight of the elongated-bodied common goldfish (4.3 ± 1.35 mg) and shubunkin (3.9 ± 1.2 mg) varieties was significantly lower at this stage than that of the rounded-bodied black moor (5.4 ± 1.4 mg) and oranda goldfish (5.1 ± 1.6 mg).

During the morphological analysis of larvae, more than one abnormality per individual was observed in several cases. After hatching, morphological abnormalities were recorded at a significantly higher rate in shubunkin (70%) and black moor (75%) than in common goldfish (35%) and oranda (40%). On the third day post-hatch (3 dph), no significant difference was detected in the proportion of deformities among the varieties (common goldfish: 40%; shubunkin: 25%; black moor: 45%; oranda: 25%). On day 10, abnormalities occurred at a significantly lower rate in common goldfish (5%) than in the other varieties (shubunkin: 25%; black moor: 60%; oranda: 35%). A significantly higher proportion of deformed larvae was recorded in black moor than in common goldfish and shubunkin.

Based on LDA, common goldfish clearly separated from the other varieties in terms of larval deformities, showing an overall lower deformity rate. In shubunkin, jaw deformities were more characteristic, whereas in black moor, fin deformities and the overall occurrence of deformities were higher.

At the end of the 10-day larval rearing period, the survival rate was significantly higher in common goldfish (95%) than in shubunkin (91%) and oranda (92%). Black moor (93%) did not show a significant difference compared with the other varieties.

3.2. Results of sperm microbiota analysis

3.2.1. Results of short-term storage

Regarding colony-forming unit (CFU) on *Aeromonas* agar, the colony count in fresh samples ranged from 2.2×10^3 to 1.1×10^5 CFU mL⁻¹, while after 96 hours it ranged from 6.7×10^7 to 2.8×10^8 CFU mL⁻¹. Although there was no significant difference either between the variants at a given time point or among samples from a given variant at 0 and 96 hours, a clear increase in colony counts was observed by the end of the incubation period.

On TSA medium, fresh samples ranged from 1.7×10^4 to 3.4×10^6 CFU mL⁻¹, while after 96 hours a somewhat more uniform colony count of 1.1×10^8 to 2×10^8 CFU mL⁻¹ was recorded in the samples of the varieties. By the end of the 96-hour storage period, a clear increase was observed compared with the initial state (0 h); however, no significant differences were found either among varieties or between sampling times.

In fresh samples (0 h), 15 bacterial species were identified in common goldfish and 13 in oranda, while a slightly lower number, 10 species each, was identified from the samples of shubunkin and black moor. *Aeromonas veronii*, as well as several representatives of the genera *Microbacterium*, *Chryseobacterium*, and *Stenotrophomonas*, were present in all varieties' samples. Members of the genera *Brevundimonas* (1) and *Sphingomonas* (1) were isolated only from one individual each of common goldfish; *Acinetobacter* (1), *Micrococcus* (1), and *Rhodococcus* (1) species were isolated from one individual each of shubunkin, while *Glutamicibacter* (1) was isolated from two individuals. A *Serratia* (1) species was identified from black moor, while *Exiguobacterium* (1) and *Comamonas* (1) species were identified from one individual each of oranda.

At the end of storage (96 h), members of the genera *Pseudomonas* (6), *Aeromonas* (6), and *Stenotrophomonas* (2) were predominantly present in all varieties. A *Shewanella* (1) species was identified only from common goldfish, while a *Morganella* (1) species was identified from shubunkin samples. A *Serratia* (1) species was detected exclusively in the black moor variety. From both fresh and stored samples, species belonging to the genera *Rhodococcus*, *Chryseobacterium*, *Pseudomonas*, and *Morganella* showed phenotypic resistance to streptomycin, while gentamicin resistance was recorded in a representative of

Comamonas. During the study, resistance to both antibiotics was observed in several genera (*Sphingobacterium*, *Delftia*, and *Stenotrophomonas*).

During the examination of fungal colonies, at least one member of the genus *Penicillium* was isolated from every goldfish variety at the initial time point of short-term storage (0 h). In addition, *Cladosporium halotolerans* was identified in black moor and oranda, while *Aspergillus versicolor* was identified from common goldfish and shubunkin samples. At the end of storage, after 96 hours, only *Penicillium* species were identified in the samples of the varieties. *Penicillium chrysogenum* was isolated from all varieties, while two additional species were also identified from black moor and oranda samples.

3.2.2. Results of long-term storage

During long-term storage, a slight decrease was observed in sperm motility parameters after thawing; however, no considerable differences were recorded among the varieties.

Colony-forming units per millilitre ranged from 2.6×10^6 to 1.1×10^7 in fresh samples and from 4.1×10^6 to 2.1×10^7 in frozen samples. Based on the analysis, no significant difference was found either among the varieties or between fresh and thawed samples. Although no substantial increase in colony-forming units was observed after thawing, the species composition became considerably more diverse.

At the initial time point of long-term storage, 3–5 species were identified per variety from fresh sperm samples. *Aeromonas veronii* and members of the genus *Vibrio* were present in all varieties. *Pseudomonas putida* was present in the sperm samples of common goldfish and shubunkin. In addition, *Microbacterium tenebrionis* was identified from the common goldfish sample, *Micrococcus luteus* from shubunkin, *Paracoccus yeei* from black moor, and one representative each of the genera *Staphylococcus* and *Kocuria* from oranda.

After five months of storage at $-196\text{ }^{\circ}\text{C}$, representatives of 18 genera were identified in the samples of the four varieties. *Pseudomonas* species were isolated in the highest numbers, and certain *Agromyces* species and *Shewanella xiamenensis* were also present in all varieties. After thawing, *Aeromonas veronii* was again detected in all samples, as was *Micrococcus luteus*, which had been isolated from shubunkin samples. The identified species were not variety specific. Four species were isolated from the extender used during cryopreservation, two of which, *Pseudomonas juntendi* and *Stutzerimonas kunmingensis*, were also present in the thawed samples. Nevertheless, the change in diversity observed in the samples was still substantial. Species isolated from the water used for thawing were not recovered from the samples.

During the examination of fungal species composition, one *Penicillium* species was identified in fresh common goldfish samples, whereas no fungal species were isolated from shubunkin. From the sperm samples of black moor, *Penicillium* (1), *Aspergillus* (2), and *Trichosporon* (1) species were identified,

while from oranda, *Penicillium* (3), *Aspergillus* (1), and *Cladosporium* (1) species were identified. After thawing, one *Aspergillus* species was isolated from black moor samples and one *Penicillium* species from oranda samples.

3.3. Results of histological examination

The gonadosomatic index (GSI) of the individuals used for histological and gene expression analyses showed significant differences among the examined goldfish varieties in both sexes. In females, shubunkin ($16.1 \pm 4.6\%$) and black moor ($14.1 \pm 4.5\%$) had significantly higher GSI values than oranda ($6.0 \pm 1.9\%$) and common goldfish ($1.8 \pm 0.9\%$). In males, shubunkin ($7.2 \pm 2.4\%$) and black moor ($6.0 \pm 1.9\%$) also showed the highest GSI values, which were significantly higher than those measured in common goldfish ($2.5 \pm 1.3\%$) and oranda ($2.9 \pm 1.2\%$). No statistically significant difference was observed between the latter two varieties.

During the examination of the ovaries after hormonal treatment, the vast majority of germ cells in all four goldfish varieties were present in primary growth stage I, the perinucleolar stage. However, the proportion of these cells showed considerable variability among varieties: $96.9 \pm 3.1\%$ in common goldfish, $82.4 \pm 10.1\%$ in oranda, and substantially lower values in shubunkin ($39.2 \pm 7.3\%$) and black moor ($59.9 \pm 7.4\%$). Significant differences were observed among all four varieties at this stage. The proportion of cells in primary growth stage II, the cortical alveolus stage, was significantly lower in common goldfish ($1.8 \pm 2.2\%$) than in shubunkin ($17.2 \pm 4\%$), black moor ($13.5 \pm 3.6\%$), and oranda ($8.3 \pm 2\%$). The proportion of cells in the secondary growth phase, the vitellogenic stage, was highest in shubunkin ($32.1 \pm 13.3\%$) and black moor ($25.2 \pm 10.4\%$). These values were significantly higher than those recorded in common goldfish ($0.6 \pm 1.4\%$) and oranda ($5.3 \pm 3.2\%$). In my study, mature and atretic oocytes were recorded in the lowest proportions among the maturation stages, and no differences were observed among the varieties in these values.

During the histological examination of testes, 12 hours after hormonal treatment, spermatozoa dominated the process of spermatogenesis in all four goldfish varieties and represented the most abundant cell stage. No significant difference was detected among the varieties (common goldfish: $74.5 \pm 0.8\%$; shubunkin: $74 \pm 5.1\%$; black moor: $60.3 \pm 7.2\%$; oranda: $58.4 \pm 20.7\%$). No significant differences were detected among the varieties in the early stages of spermatogenesis either (spermatogonia, spermatocytes, and spermatids).

3.4. Results of gene expression analysis

No significant difference was detected among the varieties in the expression of the *rln3a* gene measured in brain tissue.

In contrast to brain tissue, a statistically significant difference was observed in the expression level of the *rln3a* gene measured in testicular tissue. The normalized *rln3a* mRNA level in the testes was significantly lower in common

goldfish than in shubunkin. The values recorded in black moor and oranda did not differ significantly from those of the other varieties.

When individuals of the four varieties were analyzed together ($N = 20$), a significant, moderate positive correlation was detected between GSI values and *rln3a* mRNA levels measured in testicular tissue (Pearson correlation, $R = 0.5112$).

4. CONCLUSIONS AND PERSPECTIVES

4.1. Conclusions

Based on the available literature, it can be stated that differences observed in the reproductive biological parameters of goldfish may result from several factors. In addition to individual characteristics, such as size, age, and condition, reproductive performance is also strongly influenced by external factors. The physical and chemical parameters of the environment, the available space and feed, as well as the sex ratio, all affect the reproductive biology of fish. In my study, the examination of varieties of the same age, originating from the same population, and maintained and treated under uniform environmental conditions provided an opportunity to relate the observed reproductive biological differences to the phenotypic characteristics typical of the varieties and their presumed genetic background.

Based on my results, clear differences can be observed among the four varieties in basic reproductive parameters and in the early development of larvae. In females, significant differences were found in the qualitative and quantitative parameters of gametes, whereas in males, the separation among varieties was less pronounced. A certain tendency was observed regarding female body shape and reproductive parameters. In the rounded-bodied varieties (black moor and oranda), I recorded a higher pseudo-gonadosomatic index but a lower number of eggs per gram than in the elongated-bodied varieties (common goldfish and shubunkin). This suggests that body shape and the associated physiological characteristics may influence the relative gamete quantity and potential reproductive capacity of female broodstock. At the same time, females of the examined varieties did not show considerable differences from previously studied cyprinid species in terms of the ratio of gonad weight to body weight. Based on my results, the phenotypic characteristics typical of the varieties, and presumably their genetic background, may affect the reproductive parameters examined, influencing the reproductive biological traits of the two sexes in different ways.

During larval rearing, the proportion of individuals with morphological abnormalities decreased as growth and development progressed. While natural selection may have contributed to this trend, the results do not provide conclusive evidence that the observed reduction in deformity incidence can be attributed exclusively to this process. The type of developmental abnormalities showed a variety-specific pattern: in single-tailed, elongated-bodied varieties, deformities of the cranial region occurred more frequently, whereas in double-tailed, rounded-bodied individuals, fin deformities were more commonly observed. A body-shape-related pattern was also recorded in developmental rate, as by the end of the tenth day, the elongated-bodied varieties showed a considerable lag in body weight gain. The low hatching rate and higher proportion of developmental abnormalities observed in black moor were presumably attributable to lower-quality egg batches and to their specific phenotypic traits. Both the literature data

and my results clearly indicate that phenotypes formed through artificial selection cause differences among varieties from the embryonic stage onward. This affects hatching, larval development, and survival alike.

The examination of the sperm microbiome of goldfish revealed a diverse community of bacteria and fungi. No variety-specific composition was observed; however, several species overlapped among the samples of different individuals. This indicates that the microbiota of goldfish sperm is jointly influenced by the environment and the biological characteristics of the host organism. The culturable microbial community of sperm underwent substantial changes during storage processes, although the total bacterial count in the samples did not represent the changes in the composition of the bacterial flora. During short-term refrigerated storage, psychrophilic and psychrotolerant bacteria, often facultative or opportunistic pathogens, became dominant. In the case of fungi, somewhat higher diversity was observed in fresh samples (*Penicillium*, *Cladosporium*, *Aspergillus*), whereas after 96 hours, only *Penicillium* species could be isolated. This suggests that the applied storage conditions exerted selective pressure on the microbial community of sperm, promoting the proliferation of species that may potentially have a negative effect on sperm quality and, consequently, on fertilizing capacity and reproductive success. Species of the genus *Penicillium* had a competitive advantage under the examined conditions. Published studies, together with the increased bacterial species diversity recorded during cryopreservation, suggest that sample storage in liquid nitrogen using open straws may result in microbial contamination. This may not only lead to deterioration in sample quality but may also create the possibility of transmitting facultative and opportunistic pathogenic bacteria to the offspring stock. At the same time, the decrease in the number and diversity of fungal species suggests that sperm cryopreservation, storage under extremely cold conditions, and thawing exert strong selective pressure on the fungal community, and only certain stress-tolerant species can survive under these environmental conditions.

Despite identical conditions and treatment protocols, differences were observed among the varieties in the relative size of the gonads and in final gamete maturation. In females, the differences recorded in the proportions of the various maturation stages indicate that the varieties responded to the treatment with different efficiency. In addition, it can generally be concluded that 24 hours after injection was not sufficient to reach a high proportion of mature oocytes. However, the high proportion of sperm cells in all varieties indicates that males responded similarly to the hormonal treatment and that sexually mature individuals were in an advanced state of readiness for spawning. Overall, these findings suggest that the examined goldfish varieties may differ in their response to hormonal induction, resulting in differences in gonadal development and the progression of final gamete maturation. Such differences may be associated with the distinct genetic backgrounds of the varieties as well as physiological characteristics related to their phenotypic traits.

Based on the results of the molecular analysis, the similarity in *rln3a* expression levels recorded in the brain tissues of the varieties reflects the conservation of neuroendocrine functions, in accordance with the literature. In contrast, the differences measured in testicular tissue may indicate variety-dependent regulation of spermatogenesis.

Considering the GSI values, histological results, and *rln3a* expression together, it can be concluded that the individual varieties respond to hormonal induction to different extents. The mechanisms influencing the final maturation process of spermatogenesis show variety-dependent differences.

The effects of several thousand years of artificial selection have clearly left a detectable mark on the reproductive capacity and larval development of the examined goldfish varieties. My results contribute to a better understanding of the effects of artificial selection on reproductive biology.

4.2. Suggestions for further research

Based on the results of my doctoral research, I propose the following recommendations:

- a) Establishment of broodstock subjected to systematic gamete quality and quantity assessment for each variety:
 - I recommend that, prior to propagation, at least sperm motility should be evaluated individually for each male, although the recording of additional quality parameters is also advisable;
 - I recommend determining the optimal sperm-to-egg ratio for each variety.
- b) For females, I recommend extending and precisely determining, for each variety, the interval between hormonal induction by carp pituitary extract and stripping beyond the currently applied 24-hour period in order to achieve a higher proportion of mature oocytes.
- c) I recommend determining the optimal hormone dosage for each variety individually and synchronizing the timing of treatment between sexes in order to improve reproductive efficiency.
- d) Further refinement of sampling and storage protocols is recommended to minimize microbial contamination during sperm collection and storage. To prevent potential cross-contamination among samples, the use of sealable cryogenic vials and the maintenance of the microbiological purity of liquid nitrogen are recommended. Furthermore, the development of alternative approaches (e.g., probiotic-based methods) is proposed as a substitute for antibiotic treatment of stored samples.
- e) For economically important fish species, it is necessary to investigate the relationships between the fertilization capacity of gametes and their

associated microbiome. In addition, the effects of bioactive or toxic metabolites produced by isolated bacterial and fungal species on gamete quality and embryonic development should be examined.

- f) Investigation of *rln3a* gene expression during the different stages of the reproductive cycle is recommended.
- g) I recommend evaluating the applicability of the *rln3a* gene as a molecular marker of testicular function.

5. NEW SCIENTIFIC RESULTS

1. I demonstrated that significant differences exist in the qualitative and quantitative characteristics of gametes among individuals of goldfish varieties (common goldfish, shubunkin, black moor, and oranda) maintained under identical environmental conditions and of the same age. For the first time, I determined the number of eggs per gram in goldfish, including these four varieties. Furthermore, under standardized conditions, I was the first to compare fertilization rates (common goldfish: 71%; shubunkin: 79%; black moor: 79%; oranda: 93%) and hatching rates (common goldfish: 63%; shubunkin: 69%; black moor: 44%; oranda: 71%) among the four varieties and demonstrated significant differences between them. In female goldfish, I found that the final stage of gamete maturation differs among varieties following identical hormonal treatment, whereas in males, maturation proceeds at a similar rate across the examined varieties.

2. I demonstrated that the incidence of developmental abnormalities and survival rates differ significantly among the four examined goldfish varieties. The proportion of larvae exhibiting developmental abnormalities immediately after hatching was 35% in common goldfish, 70% in shubunkin, 75% in black moor, and 40% in oranda. By day 10 post-hatch, these values were 5%, 25%, 60%, and 35%, respectively. Survival rates following larval rearing were 95% in common goldfish, 91% in shubunkin, 93% in black moor, and 92% in oranda. Furthermore, I observed that larval growth rates and the body regions affected by deformities were associated with the characteristic body shape of each variety.

3. Using four goldfish varieties, I was the first to characterize the culturable microbial community of goldfish sperm in fresh samples, during short-term refrigerated storage, and during cryopreservation. My results demonstrate that both the diversity and the species- and genus-level composition of the goldfish sperm microbiota undergo substantial changes during short-term refrigerated and cryopreserved storage.

4. I was the first to compare the expression of the *rln3a* gene in brain and testicular tissues of goldfish varieties exhibiting distinct phenotypes. My investigations demonstrated that *rln3a* expression is conserved in brain tissue, whereas differences occur in testicular tissue among the varieties, indicating variety-specific differences in testicular function.

6. PUBLICATIONS RELATED TO THE TOPIC OF THE DISSERTATION

6.1. Publications in international journals

Bernáth, G., **Nagy, B.**, Bartucz, T., Csókás, E., Petényi, R., Bartos, I., Hegyi, Á., Csorbai, B., Ivánovics, B., Molnár, J., Urbányi, B., Bokor, Z. (2025). Large-scale cryopreservation affects sperm characteristics and hatching rate but not growth, malformation and survival rate in chub (*Squalius cephalus*) larvae. *Aquaculture*, 602, 742362.

DOI: [10.1016/j.aquaculture.2025.742362](https://doi.org/10.1016/j.aquaculture.2025.742362)

Nagy, B., Csorbai, B., Várkonyi, L., Staszny, Á., Molnár, J., Láng, Z. L., Bartucz, T., Ittész, I., Urbányi, B., Bokor, Z., Bernáth, G. (2024). Comparative study on the gamete quality, artificial propagation and larval development of common goldfish, shubunkin, black moor, and oranda variants of goldfish (*Carassius auratus*). *Aquaculture*, 582, 740502.

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Bokor, Z., Fodor, F., Várkonyi, L., **Nagy, B.**, Láng, Z. L., Ferincz, Á., Staszny, Á., Molnár, J., Lefler, K. K., Csorbai, B., Vancsura, Z., Szári, Zs., Urbányi, B., Bernáth, G. (2024). Biological parameters and spermatogenesis in razorfish (*Pelecus cultratus*) population inhabiting the largest shallow lake of Central Europe (Lake Balaton): studies for in vitro conservation purposes. *Aquaculture Research*, 2024(1), 6695280.

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DOI: [10.1007/s10695-023-01245-x](https://doi.org/10.1007/s10695-023-01245-x)

Bernáth, G., Csorbai, B., **Nagy, B.**, Csókás, E., Molnár, J., Bartucz, T., Láng, Z. L., Gyurcsák, M., Hegyi, Á., Kobolák, J., Griffiths, J. D., Ferincz, Á., Bokor, Z. (2023). The investigation of post-thaw chilled storage and the applicability of large-scale cryopreservation in chub (*Squalius cephalus*) sperm. *Cryobiology*, 113, 104588. DOI: [10.1016/j.cryobiol.2023.104588](https://doi.org/10.1016/j.cryobiol.2023.104588)

6.2. Publications in national journals

Bernáth, G., Csorbai, B., **Nagy, B.**, Kaszab, E., Suhajda, Á., Farkas, M., Urbányi, B., Bokor, Z. (2023). A mikrobiom kutatás szerepe az akvakultúrában. *Acta Agronomica Óváriensis*, 64(2), 164–178. 15 p.

- Nagy, B.**, Bernáth, G., Várkonyi, L., Molnár, J., Láng, L.Z., Izsák, T., Bartucz, T., Ittész, I., Ittész, Á., Urbányi, B., Bokor, Z. (2022). The comparison of sperm motility and density in four different goldfish (*Carassius auratus*) types. *Acta Agraria Debreceniensis*, (1), 135–140.
- Láng, L.Z., Bernáth, G., Bokor, Z., Csorbai, B., Fodor, F., Szári, Zs., Molnár, J., **Nagy, B.**, Csenki-Bakos, Zs., Urbányi, B., Várkonyi, L. (2022). A balatoni sudár ponty (*Cyprinus carpio morpha accuminatus*) indukált szaporítása és intenzív rendszerben történő lárvanevelésének vizsgálata. *Állattenyésztés és takarmányozás* 71: 1 pp. 36–43., 8 p.

6.3. Publications at international conferences

Oral presentations

- Várkonyi, D., Kaszab, E., **Nagy, B.**, Csorbai, B., Farkas, M., Bartucz, T., Kathi, L., Bartos, I., Ádám, R., Radóczy, J., Bokor, Z., Dzsudzsák, E., Harkai, P., Urbányi, B., Kobolák, J., Bernáth, G. (2025). Investigation on the relationship between reproductive biology and the microbial community in common carp (*Cyprinus carpio*). In: Book of Abstracts of Aquaculture for Everyone – Aquaculture Europe 2025, Valencia, Spain. 2025.09.22-25. pp. 1844–1845.
- Kaszab, E., Farkas, M., Várkonyi, D., Csorbai, B., **Nagy, B.**, Harkai, P., Radóczy, J., Ekici, A., Demircan Akyasan, M. D., Çanak, Ö., Kobolák, J., Bernáth, G. (2025). The analysis and identification of potential probiotic candidates from common carp (*Cyprinus carpio*) gut and gametes. In Abstracts of the 7th Central European Forum for Microbiology, Siófok, Hungary, 2025.10.6-8. pp. 32–33.
- Nagy, B.**, Bokor, Z., Kaszab, E., Suhajda, Á., Farkas, M., Bartucz, T., Csorbai, B., Urbányi, B., Bernáth, G. (2024). Detailed analysis of bacterial and fungal communities of sperm isolated from four different goldfish lines during chilled storage. In Book of Abstracts of 9th International Workshop on the Biology of Fish Gametes. p. 32. León, Spain. 2024.07.15–18.
- Nagy, B.**, Bokor, Z., Csorbai, B., Várkonyi, L., Molnár, J., Bartucz, T., Láng, L.Z., Ittész, Á., Ittész, I., Urbányi, B., Bernáth, G. (2022). The investigation of the differences in artificial propagation and larvae development by four different goldfish types (*Carassius auratus*). In: Andrzej, Ciereszko; Daniel, Źarski 8th International Workshop on the Biology of Fish Gametes, Book of Abstracts pp. 39–39., 1 p. Gdansk, Poland, September 22–23, 2022.

Poster presentations

- Nagy B.**, Bernáth, G., Várkonyi, L., Molnár, J., Láng, L. Z., Izsák, T., Bartucz, T., Ittész, I., Ittész, Á., Urbányi, B., Bokor, Z. (2021). The comparison of sperm motility and density in four different goldfish (*Carassius carassius*) types. In: European, Aquaculture Society (eds.) Aquaculture Europe 21: Abstracts p. 859. Madeira, Portugal, October 4–7, 2021.
- Bernáth, G., Várkonyi, L., Csorbai, B., Láng, L. Z., Molnár, J., Bartucz, T., Nagy, B., Lehoczky, I., Szabó, G., Urbányi, B., Bokor, Z. (2021). The improvement of sperm cryopreservation methods in the endemic tench (*Tinca tinca*) and crucian carp (*Carassius carassius*) for conservation purposes. In: European, Aquaculture Society (eds.) Aquaculture Europe 21: Abstracts pp. 140–141.
- Láng, L.Z., Bokor, Z., Bernáth, G., Csorbai, B., Nagy, B., Bartucz, T., Izsák, T., Csenki-Bakos, Zs., Fodor, F., Szári, Z., Urbányi, B., Várkonyi, L. (2021). Artificial propagation and larvae rearing in recirculation aquaculture system (RAS) of the Hungarian carp landrace (*Cyprinus carpio morpha accuminatus*). In: European, Aquaculture Society (eds.) Aquaculture Europe 21: Abstracts Madeira, Portugal, October 4–7, 2021.

6.4. Publications at national conferences

Oral presentations

- Várkonyi D., Kaszab E., Csorbai B., Farkas M., **Nagy B.**, Bokor Z., Urbányi B., Harkai P., Bartucz T., Kirchner M., Kathi, L., Bartos, I., Radóczy J., Bernáth G., (2025). A szaporodásbiológiai mutatók és az azt befolyásoló mikrobiális közösség kapcsolatának feltárása ponty (*Cyprinus carpio*) fajban. *A XLIX. Halászati Tudományos Tanácskozás kiadványa*, pp. 75–80.
- Nagy B.**, Bokor Z., Solymosi E., Zsáky T., Koós Á., Pásztor V., Sztanó J., Ladányi J., Lódi Gy., Antal P., Szabó T., Bajári Zs., Urbányi B., Bernáth G. (2025). Algával dúsított takarmány hatása a ponty (*Cyprinus carpio*) hímivarsejt minőségére. *A XLIX. Halászati Tudományos Tanácskozás kiadványa*, pp. 40–47.
- Várkonyi, D., Farkas, M., **Nagy, B.**, Bartucz, T., Petényi, R., Csorbai, B., Kobolák, J., Urbányi, B., Bokor, Z., Bernáth, G., et al. (2024). Ponty (*Cyprinus carpio*) bél és ivartermék mintákból izolált mikrobaközösségek identifikációja. In Molnár, D., Molnár, D., Nagy, A.Sz. (Eds.), XXVII. Tavaszi Szél Konferencia 2024: Tanulmánykötet I. [Spring Wind Conference 2024] (pp. 33–43). Budapest: Doktoranduszok Országos Szövetsége (DOSZ).

- Nagy B.**, Bokor Z., Kaszab E., Suhajda Á., Bartucz T., Csókás E., Molnár J., Csorbai B., Urbányi B., Bernáth G. (2023). Különböző aranyhal (*Carassius auratus*) változatok hímivartermékének mikrobiom vizsgálata rövid távú tárolás során (előzetes eredmények). 28. Szaporodásbiológiai találkozó, Szarvas, 2023.11.10–11.
- Nagy B.**, Bernáth G., Csorbai B., Várkonyi L., Molnár J., Bartucz T., Láng L.Z., Ittész I., Ittész Á., Urbányi B., Bokor Z. (2022). Az aranyhal (*Carassius auratus*) különböző változatainak szaporítási és fejlődéstani vizsgálata. *A XLVI. Halászati Tudományos Tanácskozás kiadványa*, pp. 68–72.
- Nagy B.**, Bernáth G., Csorbai B., Várkonyi L., Bartucz T., Láng L.Z., Ittész I., Ittész Á. (2022). Különböző aranyhal (*Carassius auratus*) változatok spermatológiai vizsgálata. 25. Tavaszi Szél Konferencia, Pécsi Tudományegyetem. 2022. május 6–8.
- Nagy B.**, Bernáth G., Várkonyi L., Molnár J., Láng L.Z., Izsák T., Bartucz T., Ittész I., Ittész Á., Urbányi B., Bokor Z. (2021). A sperma motilitásának és -sűrűségének összehasonlítása négy különböző aranyhal (*Carassius auratus*) változatban. A jövő tudósai, a vidék jövője, Debreceni Egyetem. 2021. december 1.

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- Nagy, B.**, Bernáth, G., Lefler, K.K., Csorbai, B., Bartucz, T., Petényi, R., Bartos, I., Ittész, I., Urbányi, B., Bokor, Z. (2024). Gazdasági jelentőségű aranyhal változatok ivarsejt érésének vizsgálata. *A XLVIII. Halászati Tudományos Tanácskozás kiadványa* [Proceedings of the 48th Scientific Conference on Fisheries & Aquaculture] (pp. 197–202). Szarvas: Magyar Agrár- és

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Nagy B., Bokor Z., Csorbai B., Molnár J., Bartucz T., Gyurcsák M., Láng L. Z., Csókás E., Hegyi Á., Urbányi B., Bernáth G. (2023). A fejes domolykó (*Squalius cephalus*) indukált szaporítási technológiájának optimalizálása. *A XLVII. Halászati Tudományos Tanácskozás kiadványa*, pp. 14–24.

Bernáth G., **Nagy B.**, Hegyi Á., Csorbai B., Várkonyi L., Müller T., Urbányi B., Bokor Z. (2022). A mélyhűtött széles kárász (*Carassius carassius*) sperma felolvasztást követő tárolhatóságának és mozgási képességének vizsgálata. *A XLVI. Halászati Tudományos Tanácskozás kiadványa*, pp. 123–127.