Analyses of regulators of fish sex with genomic tools

With over 34 thousand known species and over a dozen known sex determination (SD) and sexual differentiation (SDiff) strategies teleosts form the largest and most diverse vertebrate clade. The main goal of our project was to gain better understanding of the genetic and environmental regulators that form and maintain sex in teleosts. For the current project, the primary model was the zebrafish *(Danio rerio* Hamilton, 1822*)*, but we performed experiments on and analyzed data from several farmed fish species as well.

Although zebrafish is one of the most popular and best-characterized vertebrate model organisms (review: Choi et al., 2021), the regulation of its SD and SDiff is not fully understood. Wild-type zebrafish have a ZW/ZZ type Chromosomal SD (Wilson et al., 2014), but several domesticated zebrafish lines appear to have lost this trait resulting in lesser-known polygenic SD (see e.g., Liew et al., 2012; Liew and Orbán, 2014). Thus, a unique situation was formed: two different SD mechanisms exist within one species. Gonadal differentiation is also an unusual process in zebrafish: as many males start by generating a juvenile ovary, which later transforms into a testis (Takahashi, 1977; Wang et al., 2007). Although this process has been studied for over one and a half decades, several of its aspects are poorly understood (reviews: Orbán, Sreenivasan and Olsson, 2009; Liew and Orbán, 2014). The 'gonadal transition' process is a good model for natural sex reversal, thus we decided to deepen our understanding of its regulation.

A) Analysis of zebrafish sex with mutants and transcriptomics

Reverse genetics is an experimental approach that aims to reveal gene function by generating mutants and analyzing changes to phenotypes caused by the mutation (review: Hardy et al., 2010). In this project, we have worked on the characterization of

two genes that play an essential role in gonad formation: *heat shock factor 5 (hsf5)* and *bloom (blm;* also called *recql3* or *recQ protein-like 3)*.

The gene encoding the Hsf5 transcription factor was discovered earlier in our Singapore-based 'predecessor lab' as it has shown differential expression between male and female gonads of zebrafish during their differentiation (Sreenivasan et al., 2008). We have finalized the functional characterization of Hsf5 using loss-of-function mutants generated by CRISPR/Cas9. Our data have proven that Hsf5 is essential for efficient spermatogenesis in males, but its absence does not affect fertility in females **(*Saju et al, 2018)**. Males lacking functional Hsf5 showed a substantial decrease of their sperm count and severe malformations in many of their remaining sperm cells resulting in drastic loss of fertility. We showed that Hsf5 is required for progression through meiotic prophase 1 during spermatogenesis as suggested by the accumulation of cells in the leptotene and zygotene-pachytene stages and increased apoptosis in post-meiotic cells. RNAseq-based comparative transcriptome analysis of homozygous *hsf5*$^{-/-}$ mutants and wild types showed gonadal mis-regulation of a substantial number of genes with roles in cell cycle, apoptosis, protein modifications, and signal transductions, indicating an important role of Hsf5 in early stages of spermatogenesis. Hsf5 is conserved in most vertebrates whose genome has been sequenced and assembled so far. Its expression pattern in mammals is very similar to that seen in zebrafish, this transcription factor might play a similarly important role in the spermatogenetic processes across the whole clade. This story was published in Cell Reports **(*Saju et al., 2018)** and it was also described in detail on the Qubit blog (https://qubit.hu/2018/12/20/magyar-tudos-halspermavizsgalatai-segithetnek-feltarni-a-ferfi-sterilitas-genetikai-hatteret).

We have also participated in a collaborative project led by M. Varga and M. Kovács (both @ELTE Genetics) that aimed to characterize the *bloom* (*blm*; also called *recql3* or *recq protein-like 3*) gene and study its potential use in zebrafish as a model for Bloom syndrome (review: Ababou, 2021). RecQ homologs play important roles in the

maintenance of genome integrity (Croteau et al., 2014). The functions of Blm include repair of double-stranded DNA breaks, regulation of crossover patterning, and telomere maintenance, (review: Kaur, Agrawal and Sengupta, 2021). In order to gain a better understanding of the role of Blm protein in zebrafish we generated a mutant allele using CRISPR/Cas9 by targeting the fourth exon. Detailed analysis of the homozygous mutants indicated that Blm loss-of-function (LOF) does not impair early somatic development and DNA damage sensitivity of zebrafish. On the other hand, Blm LOF resulted in drastically shortened lifespan, severe male bias and loss of fertility in zebrafish males, but not in zebrafish females **(*Annus et al., 2022).** *Blm*$^{-/-}$ males displayed aberrant testis morphology and their histological analysis revealed an impairment in the formation of mature spermatozoa most likely due to the vastly reduced number of post-meiotic spermatid clusters **(*Annus et al., 2022).** Further examination by *in situ* hybridizations and electron microscopic imaging both pointed to large-scale cell death of spermatogonia and spermatocytes in *blm*$^{-/-}$ mutants resulting in defective meiosis.

Earlier, we have analyzed the adult gonad transcriptomes of zebrafish by traditional Sanger sequencing and microarray analysis as well (Sreenivasan et al., 2008; Ribas et al., 2017). However, these approaches only covered the protein-coding transcriptome, entirely missing the differences in the expression profile of non-coding RNAs. We have set out to generate the complete transcriptome of both adult and developing gonads of both sexes. Samples were collected from adult testes and ovaries; total RNA were isolated and sent for RNA-sequencing of protein-coding and non-coding genes.

B) Individual analyses of the gonadal transformation process in zebrafish

Zebrafish is a 'gonochorist of late type', where the formation of male gonads involves complex processes. Takahashi (1977) was the first to note that gonad differentiation in zebrafish males start to from a 'juvenile ovary' which later transforms into a testis.

Hsiao and Tsai (2003) as well as Wang and colleagues (2007) followed this process with the help of transgenic reporter zebrafish lines and described the dynamics of the process based on the intensity of gonad-derived Egfp signals detected from a large group of fish. The exact timing of the transformation was analyzed by Takahashi (1977) and Maack and Segner (2003). Both of them reported, that a 'juvenile ovary-to-testis' transformation process occurs between 2-3 weeks post-fertilization (wpf). On the other hand, the initiation of gonad differentiation itself has been debated: estimates ranged from 10 dpf to 25 dpf (Liew and Orbán, 2014; King et al., 2020; Kossack et al., 2023). Another argument was on whether the 'juvenile ovary' phase is an essential feature of male development or not, in other words do all males or only some of them go through the gonad transformation process during their development. According to our knowledge, no one has analyzed these processes on individual live fishes.

For the purpose of monitoring gonad transformation on individual live fish, we selected two families (HFB.11; MHB.3) with male-biased sex ratio (71 and 75%, respectively) to increase the prevalence of neomale formation through bipotential ovary transformation. The sex ratio was determined from at least five F1 batches from both families.

Each experiment started with 48 zebrafish individuals, at the age of 2 weeks post-fertilization (wpf). They were kept in transparent mini-tanks to provide visually induced neural stimuli of each other. Two control groups were also established from the siblings of the individually kept juveniles, they were kept as groups (n=48 each) in two separate aquaria. All parameters, including feeding regime and water exchange were equalized.

We established a workstation to follow the gonad-derived fluorescent Egfp signal from live, tranquilized juveniles of the *tg{ddx4::egfp}* transgenic line (see section 'C' for more details). We used an Olympus SZ61 dissecting microscope equipped with digital camera (DP74) and MAA-03 Universal Light Source (BLS Ltd., Hungary). Serial

imaging of the same, separately kept individual started at the age of 3 wpf. Images were taken once a week until 4 wpf, then twice a week until 7 wpf, then once per week again. The last photos were taken at the age 9 wpf. Gonadal Egfp signal intensities were estimated on the scale of 0-5, where '0' was no signal from early gonad or differentiated testis, whereas '5' was a very strong signal originating from the developing ovary. Heat maps were built based on the fluorescent recordings. Subsequently, estimated signal intensities were quantified from the photographs with ImageJ FIJI Software.

Overall, based on the four successful experimental series we could verify that, the family-based sex ratios were not affected by the keeping conditions. The sex ratio of the individually reared population was similar to that of the two control groups. Based on the heat maps, data from the two male-biased families showed very different dynamics of gonad transformation. Most males of the MHB.3 family did not show any sign of early gonadal transformation: only seven individuals went through juvenile ovary-to-testis transformation at the expected early age (3 wpf). Later transformational events were seen in six individuals from 5-8 wpf with the similar maximum intensity as in the case 3 wpf transforming fish. Transition has never occurred from higher than level 3 intensity, yet all 19 females, except for one, reached 4.5 or level 5 values. Overall, we could summarize that the developing testis in MHB3 family showed three differentiation patterns: early transformation, late transformation and no transformation. In the case of the other male-biased family (HFB.11), more than 76% of the male offspring population formed their testis through transformation at least from the level 2.5; and in most cases level 3 was surpassed. All in all, the transformation events started from a substantially higher Egfp level, than in the case of the MHB.3 family.

These results strengthened the findings of Wang et al. (2007) who showed that zebrafish ovary development does not have a uniform timing within a given population. As these data are based on siblings as opposed to mixed offspring of

mass crosses, they clearly indicate that different transformation patterns can occur within a single family. We also showed that transformation of the juvenile ovary can occur at any level of Egfp signal, i.e. its developmental state (levels 1-5). We have identified individuals that became males extremely early (3 wpf) and others that changed extremely late (8 wpf). This is the first experimental system in which dedicated or transforming individuals can be observed in real time and sampled for subsequent RNAseq analysis.

C) Towards better understanding of zebrafish sex with improved tools

Early identification of the two sexes is an essential prerequisite of any research. However, gonad development and phenotypic sexual dimorphism is a relatively late-appearing trait in zebrafish (Conradsen and McGuigan, 2015). Previously, we have adapted a reporter line containing the *ddx4::egfp* transgene (earlier *vas::egfp*; Krøvel and Olsen, 2002; Krøvel and Olsen 2004) for this purpose (Wang et al., 2007). In this line, the accumulation of the Egfp reporter protein in the female germ cells results in a very strong signal that clearly labels the ovary in live females, whereas testes exhibit either a weak signal or an entire lack of signals not only in adults, but during gonadal development as well (Wang et al., 2007). Unfortunately, the pigmentation of the AB strain tends to weaken the gonad-derived Egfp signal, thereby interfering with the analysis of the gonadal transformation process, especially from the age of 4-5 wpf.

Recently, we have managed to improve this transgenic line by introgressing the reporter gene into a double-mutant *casper* background (White et al., 2008). The *casper* line was generated earlier by others through the crossing of two mutant lines: (i) *nacre* that lacks black melanophores (Lister et al., 1999); and (ii) *roy orbison* that does not produce reflective iridophores (D'Agati et al., 2007). The presence of both mutations in homozygous condition results in substantially reduced pigmentation in the abdominal area (White et al., 2008), consequently the *ddx4::egfp* transgene on the *casper* background offers potential advantages to the AB-based transgenic line.

Typically, the introgression of a transgene into a double mutant, recessive line would take at least three generations (Presslauer et al., 2016; Akhter et al., 2016). Therefore, we developed an efficient selection protocol that would allow us to identify homozygous transgenic *casper* individuals in somewhat more than two generations **(*Szabó et al., 2023a)**[1]. Homozygous mutant *(cas/cas)* zebrafish are crossed with homozygous transgenic *(ddx4::egfp/ddx4::egfp* or *tg/tg in short)* producing a uniform (cas/+; tg/+) F1 generation. In the F2 generation, both the mutant and the transgene segregate producing 64 genotypes. The selection procedure is performed on these F2 individuals and it is based on five steps: (i) very early visual selection of *nacre* and *casper* individuals based on lack of black pigmentation at two-three days post-fertilization (dpf); (ii) early microscopic selection of *casper* individuals based on the lack reflective pigmentation in their eyes at four dpf; (iii) identification of the transgenic *casper* females based on the strong ovarian Egfp signal at three months post-fertilization (mpf); iv) identification of transgenic male individuals through PCR-based detection of the *ddx4::egfp* construct from their fin-derived genomic DNA samples; and (v) identification of the homozygous transgenic *(tg/tg)* individuals using a duplex PCR reaction **(*Szabó et al., 2023b).** For the latter purpose, we determined the insertion site of the *tg{ddx4::egpf}* transgene by re-sequencing the genome of the transgenic line. Detailed analysis of the integration site revealed a three-kb deletion of chromosome 3 in the *tg{ddx4::egpf}* line **(*Szabó O. M, 2023)**. Primers designed to the deleted region allowed for a direct PCR-based selection of homozygous individuals without their propagation, thereby substantially reducing the duration of selection from 12 months to 4.5 months **(*Szabó O. M, 2023)**. The new homozygous transgenic line on *casper* background was named *gáspár* (pronounce: ghaashpar). We have now set up a stable line of *gáspár* by generating over 50 *gáspár* founders and by combining several hundred individuals from their mass crosses. We

[1] Papers, manuscripts and conference presentations published during the project are shown in bold and with a star.

are currently working on detailed characterization of the *gáspár* line that includes the analysis of the migration of primordial germ cells (PGCs) during early development and a thorough study of gonadal transformation. Our preliminary data clearly show that lack of black and reflective pigmentation in the trunk area allowed for far easier of the observation of processes and made counting of germ cells much easier **(*Szabó O. M, 2023)**. Our selection procedure might also be potentially useful for introgressing other transgenes into transparent mutant zebrafish lines.

Although the *tg{ddx4::egpf}* line is an excellent tool to study ovary development, but not for juvenile ovary to testis transformation. Gradual weakening of a very strong fluorescent signal is not easy to detect at the beginning, thus separation of the two sexes during this period is delayed. Therefore, we have decided to explore the possibility of generating a double-transgenic reporter line on *gáspár* background: in addition to the *ddx4::egpf* transgenic constructs that labels the females, we will introduce a second transgenic reporter construct. That construct will express a different reporter gene (e.g. tdTomato or mCherry) that can be detected in the presence of Egfp. The challenge is two-fold: (1) to find regulatory regions that are activated relatively early in the forming testis; and (2) to generate a strong fluorescent red signal that will can be recognized in the presence of Egfp. We have searched for conserved genes that are expressed primarily either in spermatogonia (see e.g., von Kopylow and Spiess, 2017) or early spermatocytes (see e.g., Qian et al., 2022; Sposato et al., 2023) and identified six potential candidates, including *deleted in azoospermia-like (dazl), zinc finger and BTB domain containing 16a (zbtb16a*), and *synaptonemal complex protein 2 and 3 (sycp2&3).* Their cloning and testing of their suitability for our purposes are in process.

There is an increasing demand in the zebrafish community for early identification and separation of the two sexes in non-transgenic, wild-type lines. In order to develop potentially suitable tools for this purpose, we have searched for expressed sex

markers in the tail fin of zebrafish. Tail clipping is a traditional method for tissue collection from live fish, for the minimal invasiveness of the procedure and the rapid regeneration of the tail.

RNAseq analysis was performed from the tail fin of 15 adult male and 15 adult female individuals, whose sex was confirmed by the quantification of gonad-derived Egfp signals and visual observation following dissection. Illumina paired-end sequencing was done by Novogene, yielding ca. 12 Gb sequence reads per sample. Bioinformatic analysis of differentially expressed genes was performed using the PyDeseq2 package, a python implementation of the DESeq2 workflow for bulk RNA-seq data (Muzellec et al., 2023). Following basic QC, removal of low-expressing genes and individuals showing the signs of potential sample contamination we have compared the gene expression profiles of 10 male and 13 female individuals. Genes, whose expression level showed statistically significant differences ($p_{adj}<0.05$) between the two sexes were called differentially expressed genes (DEGs). Altogether, 387 DEGs were identified: 84 of them (21.7%) showed higher expression in the female fins, whereas the rest (303; 78.9%) exhibited higher levels in the male fins. The statistical significance for some of the DEGs was extremely high: e.g., 2E-123 for *O-acyltransferase-like* (or *oacyl* in short), 1.7E-90 for *sulfotransferase family 2, cytosolic sulfotransferase 3 (sult2st3)* and 2E-62 for *cytochrome P450, family 8, subfamily b2 (cyp8b2)*.

Next, we have selected those of the 387 DEGs that appeared suitable for a rapid sexing based on a normal PCR-based (i.e., not real time PCR-based) test based on the following three criteria: (i) fold change >5; (ii) on average >1,000 hits in the sex with higher expression; and (iii) no outlier individual with hit count within the range of values in the other sex. With these conditions 26 potential male markers and two potential female markers were identified. These 28 markers will be validated first on adult tail fins, then tested on tail fins isolated from individuals at earlier stages of their development. Should they work at earlier developmental stages than the appearance

of phenotypic sexual dimorphism, they could be potentially useful for the analysis of gonadal differentiation in the AB line, and possibly in other wild-type lines. Previously, other labs have searched for markers differentially expressed in the tailfin of adult male vs. female zebrafish (see e.g., Hosseini et al., 2019) and adult guppy males vs. females (Dick et al., 2018), but they have not tested their applicability for potential sex markers. When we searched our 28 selected tail-derived DEG markers against those that were described by Hosseini and colleagues (2019), we found 23 of them present in both of datasets. Nineteen showed DE into the same direction and only four showed the opposite result. On the other hand, comparison with the tail fin-based DEGs reported from guppy (Dick et al., 2018) resulted in two common DEGs only, both of them exhibiting higher expression level in the male tail. As no information related to sex-specific expression in fishes could be found for these two genes, we looked at their function in other vertebrates. The first, *diacylglycerol O-acyltransferase 2 (dgat2)* acts upstream of or within lipid homeostasis in the endoplasmic reticulum and it was reported to exhibit up-regulated expression in mice (see e.g., González-Granillo, 2019). The second, *xanthine oxidase/dehydrogenase (xdh)* is predicted to be involved in xanthine catabolic process and located in peroxisome. It was shown to have higher protein levels in the liver of male rats, compared to the females (Decker and Levinson, 1982).

D) Analyzing the effect of environmental factors onto the gonad differentiation process of zebrafish

Earlier, the former laboratory of the team leader has run a collaborative study with the team of Francesc Piferrer (CSIC, Barcelona) to study the effects of heat-induced masculinization on the sex ratio and gonadal transcriptomes of zebrafish. Their results showed that in heat-responsive families most females were masculinized to fully fertile neo-males, whose gonadal gene expression profile was near-identical to that of genetic males (Ribas et al., 2017). The masculinization process was found to be

stepwise with several intermediate steps between females and males (Ribas et al., 2017). Since these transcriptomic data were obtained with a microarray that covered only an estimated 40-50% of the whole zebrafish protein-coding gene set with no information on non-coding sequences, we have decided to revisit this topic in this project.

In order to study the process of heat-induced masculinization, we had to establish zebrafish families with known female to male sex ratios. Since the domesticated zebrafish strains have polygenic SD (Liew et al., 2012; Wilson et al., 2014), consequently there is a substantial variability among the sex ratio from different families, whereas multiple broods from the same family have similar male to female ratio (Liew et al., 2012). In total, we generated families with by crossing 51 pair of homozygous or hemizygous *tg{ddx4::egfp}* transgenic fish **(*Pethő, 2022; *Pethő, 2023; *Hirth et al., 2023a)**. We determined the sex ratio for each family using sexually dimorphic traits and differences in Egfp signals based on the strong transgene expression from ovary with fluorescence microscopic technique **(*Hirth et al., 2023b).** Altogether, we screened over 7,700 individuals from 51 families (ca. 150/family). Male-biased sex ratio was observed in 38 families with more than 50% of males, the remaining 13 families with a sex ratio of 25-50% males were intended originally for heat treatment. (The use of a female-biased population in heat-induced masculine experiments offers a better chance for identifying masculinized individuals or neo-males.) Unfortunately, some of the female-biased families failed to yield offspring on a consistent basis, therefore we had to extend the range to use those with 25-62% male offsprings for the heat treatment. We subjected to heat treatment multiple broods from 13 such families.

Heat-induced masculinization was performed by increasing the water temperature from 28 to 36 °C as described by Ribas and colleagues (2017). However, due to the different setup, we had to optimize several conditions, including density, volume of water, and monitoring techniques of gonad differentiation. We also tested the

duration of the heat treatment: offspring were subjected to 3 days, one week and two weeks of heat exposure **(*Hirth et al., 2023).** Eventually, we used two-week duration for most treatments. Organ and tissue samples have been collected from Egfp-sorted and euthanized individuals originating from several families for RNAseq analyses. From adults we collected head, gonad, trunk, tail and tail fins samples, whereas from juveniles we archived the head, trunk and tail samples.

Earlier, microarray-based comparative transcriptomic analysis performed on testes could not identify substantial differences between untreated genetic males and heat-induced neo-males (Ribas et al. 2017). Here we checked for DEGs that would allow us to differentiate between genetic males *vs.* heat-induced neo-males or at least heat-treated *vs.* control samples. Tail fin samples were sequenced with Illumina paired-end RNAseq (protein-coding only) technology from nine heat-treated individuals and their transcriptome was compared to that of ten untreated, control male tail fins. Although 1,230 DEGs showed statistically significant differences ($p_{adj}<0.05$) in their expression levels between heat-treated and control samples, the data were very noisy. None of these genes showed consistent values in all individuals within the group; there were at least 1-2 clear outliers per group distorting the mean values, thereby affecting the fold changes. Gonadal samples are being prepared for whole RNA sequencing (protein-coding and non-coding) from adult individuals. Similarly, trunk samples from heat-treated and untreated juveniles will also be sequenced. Revisiting the analysis of heat-induced effects on gonadal transcriptomes in zebrafish is expected to deepen our understanding on how high heat masculinizes the gonads of teleosts.

In collaboration with the team of F. Piferrer, we also analyzed the inheritance of the effects of elevated temperature on sex differentiation in domesticated zebrafish. In five families, results showed a consistent increase in male percentages in the parental generation as a result of heat treatment. Sex ratios were then determined in the untreated F1 and F2 offspring of the above two groups, which were all raised at 28

°C. Whereas a male-biased sex ratio in the unexposed F1 generation was observed in three families, no transgenerational effects were observed in the F2 generation of any of these families **(*Valdivieso et al., 2020)**. When DNA methylation was analyzed in the testes of P, F1 and F2 males derived from exposed and non-exposed fathers and grandfathers, it was significantly decreased only in the testis of the 35°C-derived males in the F1 generation but not of the F2 generation and, surprisingly, neither in the 35°C-exposed males of the P generation **(*Valdivieso et al., 2020)**. In summary, our results indicate that not only the sex ratio response to elevated temperature, but its inheritance also varies among the families. The altered epigenome in the testis of F1 males reveals possible lasting effects of temperature in the unexposed offspring of heat-exposed parents in case of fluctuating increases of water temperatures.

E) Genomic studies on the sex and physiology of other teleosts

E1) Male-specific DNA marker from African catfish

African catfish or sharptooth catfish *(Clarias gariepinus)* has become an important food fish species for European aquaculture and especially Hungary. Our country is now the leader for the continent with an annual production exceeding 5,000 metric tons. At the beginning of the millennium, we described two Y-specific sex markers from the genome of African catfish (Kovács et al., 2001). In a collaborative study with the team of Balázs Kovács (MATE), we have successfully validated one of these male-specific genomic sex markers. The marker was tested on several offspring groups from targeted crosses in three subsequent generations and it was found to predict the sex 96.43% accuracy on over 600 individuals (***Balogh et al., 2023**). As an earlier study suggested that both XX/XY and ZZ/ZW SD systems might coexist in this species (Nguyen et al., 2021), but their putative 'moderately sex-linked loci' failed to produce sex-specific amplification in our samples. Similarly, the possibility for temperature-induced masculinization proposed by others (Santi et al., 2016) was also investigated, but no such effect was detected in our stocks (***Balogh et al., 2023**). In summary, our

results support an exclusive XX/XY CSD system in our African catfish stock and indicate a good potential for future use of this male-specific DNA marker in research and commercial production.

E2) Analysis of a masculinizing mutation in common carp

An unusual gynogenetic common carp (/Cyprinus carpio/ L.) neo-male was identified earlier that has produced all-female offsprings when crossed with a normal female. According to our hypothesis, that individual contained a recessive masculinizing mutation (/mas/) in homozygous form at an XX sex chromosomal genetic background that has led to female-to-male sex reversal. A similar mutation was discovered and partially characterized earlier by others (Komen et al., 1992; Ruane et al., 2005), however the identity of the gene has not been determined. A gynogenetic family was generated from one of the F1 females, and grown at our collaborator's facility (P. Bársony, DE). Out of the over 250 gynogenetic F2 offspring, five confirmed males and three putative males were identified, whereas the rest turned out to be females. In addition to the appearance of males, phenotypic traits were also shown to be segregating in the F2 offspring. Genomic DNA was successfully isolated from the tail fin of six F2 males and six F2 females for re-sequencing using high throughput sequencing services. The genome of a male and a female individual was re-sequenced with Illumina paired-end technology to produce 46 & 47 Gb sequences, respectively, their bioinformatic analysis is in progress. Detailed analysis of the sequencing results might provide clues to the nature of the masculinizing mutation(s) present in this lineage.

E3) Understanding the adaptation of dwarf carp to a thermal environment

Lake Hévíz is a thermal pond with a peat bed located near Lake Balaton. The estimated formation of this lake is ca. 20,000 years ago. Its water is supplied by two hot springs, which provide ~24-28 °C and 33-35 °C water temperatures, during winter

and summer time, respectively. In that unique ecosystem, a special dwarf variety of wild common carp (*Cyprinus carpio carpio morpha hungaricus*) can be found, whose origin is unclear. Dwarf carp matures at one year of age, spawns in February and its maximal size is limited to 20-25 cm and 300-400 g. The sex ratio of dwarf carp population in the lake was shown to be heavily male-biased (75% males vs. 25% females; Tamás Müller, personal communication). The dwarf carp has been studied for over 15 years through a team effort spearheaded by T. Müller (MATE) and A. Specziár (BLKI), they collected samples and data from hundreds of individuals (see e.g., Varga et al., 2021; Müller et al., 2024). We have joined this collaborative effort to investigate how the dwarf carp has adapted to this unique environment through the analysis of its genome, transcriptome and physiology of this variety in comparison to a common carp landrace used in commercial aquaculture. We have performed a comparative lab-based growth trial of the dwarf carp and common carp individuals of the Attala landrace. The purpose of the trial was to find out whether the decreased growth potential of the dwarf carp in the lake is due simply to the decreased amount of food available and low oxygen environment in the lake or not. Our data seem to indicate the decreased growth rate of dwarf carp is genetically fixed and cannot be increased by adjusting the feeding regimes. When the two varieties were reared together, individuals of the commercial landrace dominated the dwarf carp: the growth rate and conditional factor of the former were both significantly higher. This is presumably due to the differences in the nutritional behavior: individuals of the Attala landrace were more aggressive, when searching for the feed, they chased away the dwarf carps even at similar size ranges **(*Hartl, 2023)**. At the same time, in response to an accidential bacterial infection individuals of the dwarp showed a substantially higher disease tolerance than those of the Attala landrace.

We have performed whole genome re-sequencing of the Hévíz dwarf carp and the Attala landrace using a MinION personal sequencer (Oxford Nanopore) to produce 25 Gb long-read sequences and by Illumina paired-end sequencing technology to

yield >40 Gb sequences as well. Preliminary results on the comparative analysis have not indicated large-scale differences (i.e., large deletions, insertions, inversions or duplications at the chromosome level) between the two re-sequenced genomes when mapped onto the common carp reference genome. Analyses of copy number variations, and selected gene families with potential role in thermal adaptation (e.g., hemoglobin genes, MHC genes and transposons) will be performed, together with whole genome methylation assays. The potential importance of dwarf carps is at least two-fold: (i) to provide new information on the mechanism of thermal adaptation in teleosts; and (ii) their possible incorporation into commercial selection programs might lead to increased tolerance against stress and diseases. The latter might be advantageous in the fight against koi herpes virus (KHV) and other infectious diseases that cause substantial losses in the global aquaculture production of the third most important freshwater food fish species (see e.g., Dixon et al., 2009; Hwang et al., 2020).

E4) Generating a reference genome for paradise fish

We have also participated in the sequencing and assembly of the genome of paradise fish (*Macropodus opercularis)* spearheaded by Máté Varga (ELTE) and Shawn M. Burgess (NIH, USA). Paradise fish is an "air-breathing" teleost native to Southeast Asia, Southern China, Northern Vietnam, and Laos. It is an ethological model that exhibits a set of complex behaviors but lacks genomic resources. We generated the *de novo* reference genome sequence using 150X coverage of PacBio SMRT HiFi long-read sequencing. The size of the assembled genome is 483 Mb, showing an average intron length of 566 bp, i.e., less than half to the average teleost intron length 1,214 bp, and shorter intergenic regions. The N90 for the assembly consists of 23 contigs even without any scaffolding, including "telomere-to-telomere" assemblies for six contigs (***Fodor et al., 2024**). Preliminary search of the unassembled contigs revealed potentially sex-specific sequences, future collaboration is expected with the Varga lab into that direction.

F) Novel, chemical-free approaches for improving the robustness of teleosts against infectious diseases

Parasitic infections pose a continuous threat during the production of farmed fish species (review: Shinn et al., 2015). Short-term treatments with high temperature have been shown repeatedly to activate both innate and acquired immunity and provide immunological memory on the long term (review: Scharsack et al., 2022). Early heat treatment of Asian seabass larvae and juvenile suffering from developing viral or bacterial infection has been shown earlier to help stocks to survive otherwise detrimental effects caused by the pathogens (Alain Michel, personal communication). In collaboration with K. Molnár, Cs. Székely and B. Sellyei (VMRI) and A. Michel Aquaculture R&D, France; now deceased) we decided to test the potential applicability of this approach to parasite-infected commercial food fish. For the model, we have used juveniles of European catfish heavily infected with *Thaparocleidus vistulensis* - formerly known as *Ancylodiscoides vistulensis* - a viviparous monogenean exoparasite, that exclusively attacks the gill. The parasite disrupts the tissue architecture, interferes with breathing thereby limits vital functions of the fish often resulting in its death.

T. vistulensis is especially harmful for stocks kept in intensive systems due to high density of potential hosts and lack of removal of intermediate forms by water exchange. Current options for treatment either involve potentially poisonous chemicals or drugs incompatible with EU regulations or are largely ineffective. Accordingly, this gill monogenean is among the biggest threats to the expansion of catfish aquaculture sector in Europe.

As the first step, we have developed a minimally invasive gill biopsy procedure for estimating the infection level of individuals to be tested. We investigated the microhabitat preference of *T. vistulensis* on the gills of infected European catfish. We

observed a balanced distribution on the two lateral gill sets and the decreasing trend in parasite numbers from anterior gill arches towards the posterior ones. Using these results, we developed a minimally invasive sampling protocol to estimate the parasite load of individuals (***Bognár et al., 2023, *Bognár et al., 2024**). The biopsy was taken from one of the hemibranches originating from the first gill arch on the left side. Biopsy-based estimates of parasite loads were validated by comparing them to full parasite counts of the same individuals and showed statistically significant correlations. Our biopsy-based method is designed to identify experimental animals with similar parasite load and create groups of hosts with comparable burden. We have also compared the suitability of two different tanks systems for short- to mid-term maintenance of gill monogenean-infected catfish individuals: one with static conditions including in-tank filtration and another with a continuous flow through system. Our data showed that both systems are suitable for keeping 20-40 infected catfish individuals for 2- months period of time (***Bognár et al., 2024**).

European catfish juveniles infected with *T. vistulensis* were collected from the facility following an accidental infection event. Conditions of the heat treatment were optimized through a series of trials: the final protocol included two hours of treatment at increased temperature on three consecutive days. Temperature increase was slow (0.1 °C/min), whereas tanks were allowed to return to room temperature with passive cooling. Once the conditions were set, experimental pairs of groups of European catfish were formed based on the result of gill biopsies collected on the previous day. These normalized pairs were then divided between the heat-treated and untreated control groups. Six independent experiments were conducted: three in our laboratory and another three in the facility of our collaborator. In five of the six experiments parasite counts showed a significant reduction in comparison to untreated controls ($p<0.05$). In one of the five successful treatments gill and spleen samples were collected from six treated and six control individuals. Samples were sent for RNA isolation and Illumina sequencing to Novogene (Oxford, UK). PolyA-based mRNA

isolation was followed by library construction (250-300 bp insert size) and libraries were subjected to at 75 bp single read sequencing with Illumina technology to yield 1.5-2.5 Gb raw data per sample.

Bioinformatic analysis of differentially expressed genes was performed using the PyDeseq2 package, a python implementation of the DESeq2 workflow for bulk RNA-seq data (Muzellec et al., 2023). Following basic QC, removal of low-expressing genes and individuals showing the signs of potential contamination we have compared the gene expression profiles of six heat-treated *vs.* six untreated gill samples as well as six heat-treated *vs.* five untreated spleen samples, respectively. Comparative transcriptomic analysis of gill samples revealed 29 DEGs whose expression showed a statistically significant difference (p_{adj}<0.05) between the heat-treated and untreated samples. Twelve of these genes showed up-regulation in the heat-treated samples, the fold-change for all, but one *(phosphatase and tensin homolog, pten)*, was over five-fold. The remaining 17 genes showed down-regulation as a result of the heat-treatment, the lowest fold-change was six-fold. Three of these seventeen genes *(slc52a3, clecl3a and doc3)* showed an over 100-fold decrease in their expression levels, whereas the expression of five of genes (*maf, sk6a18, ddr2, tnc1d2* and a gene with unknown function) appeared to be completely silenced by the heat treatment. Comparative transcriptomic analysis of spleen samples identified 613 DEGs with a statistically significant difference (p_{adj}<0.05) between the expression level in heat-treated *vs.* untreated samples. A total of 337 showed an up-regulation in the heat-treated samples, whereas the remaining 276 were down-regulated. Interestingly, the response to the heat treatment to the two immune-organs was entirely different. Very few genes showed significant changes in their expression level in the gills, some of them with very high fold changes. In contrast, over 600 DEGs were detected in the spleen, but the highest fold change was >8 only.

Both of these DEG sets contained genes with unknown functions, indicating potential issues with the transcriptome and/or genome assembly or annotation. Although two

independent draft genome assemblies have been generated for the European catfish, the transcriptome data was relatively shallow for one (Ozerov et al., 2021), and completely missing from the other (Jensen et al., 2021). Moreover, all the published datasets were generated from healthy individuals, thus no transcriptomic data are available from diseased catfish.

We have induced systemic infection of a 1.5 kg European catfish individual by intravenous, subcutaneous and intraperitoneal injection of a bacteria cocktail composed of *Pseudomonas aeruginosa, Aeromonas hydrophila, Edwardsiella tarda, Staphilococcus aeruginosa* based on (Caruso et al., 2002; Zmysłowska et al., 2004). Once the signs of systemic infection became visible, the infected catfish individual was euthanized and samples were collected from the following five immune organs: gill, head kidney, skin, spleen and thymus. The same organs were also harvested from an euthanized, healthy control individual of the same size. mRNA samples were isolated from the organs and an RNAseq was performed with paired end Illumina technology (250-300 bp insert size) yielding ca. 50 million reads per sample. To further enrich the data, gill- and spleen-derived RNAseq samples (10M reads per sample) were added from twelve additional individuals. In total, these sequencing exercises yielded >600 M reads. These were combined with the 330 M transcriptome reads produced by (Ozerov et al., 2021) and assembled. Currently, the resulting contigs are being mapped onto the existing draft genome and annotated to yield an improved transcriptome. The improved transcriptome will be used as a reference for the re-analysis of our gill- and spleen-derived RNAseq data in the hope that a deeper and more informative results can be obtained.

Earlier, the previous laboratory of the project leader conducted farm-based challenges by exposing Asian seabass (*Lates calcarifer,* Bloch 1790) juveniles to complex natural environmental conditions. In one of these challenges, they collected

a total of 1,250 fish classified as either 'sensitive' or 'robust' individuals during the 28-day observation period. Then they constructed a high-resolution linkage map with 3,089 SNPs for Asian seabass using the double digest Restriction-site Associated DNA (ddRAD) technology and performed a search for Quantitative Trait Loci (QTL) associated with robustness. The results of these experiments were analyzed as part of this Frontline Research Excellence program. The search detected a major genome-wide significant QTL for increased robustness in pathogen-infected marine environment on linkage group 11 (ASB_LG11; 88.9 cM to 93.6 cM) with phenotypic variation explained of 81.0% **(*Shen et al., 2023)**. The QTL was positioned within a >800 kb genomic region located at the tip of chromosome ASB_LG11 with two Single Nucleotide Polymorphism markers, R1-38468 and R1-61252, located near to the two ends of the QTL. When the R1-61252 marker was validated experimentally in a different mass cross population, it showed a statistically significant association with increased robustness. Thirty-six potential candidate genes were located within the QTL, the majority of them have known functions related to innate immunity, stress response or disease (Table 2 of ***Shen et al., 2023**). When the list of these 36 genes potentially associated with robustness in the Asian seabass was compared to the DEGs identified in heat-treated European catfish samples, seven genes were found to be present in both sets.

Summary:

The most important findings of the project are as follows:

- We built and set up a fully functional genomic laboratory and a zebrafish facility from scratch.
- Our laboratory was the first to apply individual observation of the gonad development and large-scale live sexing of zebrafish juveniles. This allowed us to monitor the gonadal transformation process in real time. Our data clearly showed that the two male-biased families followed a very different path: while most males

of the MHB.3 family did not show any sign of gonadal transformation, 76% of the males of the other family formed their testis through that process. This indicates that not only the sex ratios, but the dynamics and timing of the transformation process are family-dependent.

- We have developed a new transgenic zebrafish line where the reporter construct is on a double-mutant background resulting in substantially reduced pigmentation. Identification of the integration site of ransgene made the development this line in 1.5 generations. This line allows observation of the gonad development in zebrafish without the potential masking caused by pigmentation in wild-types.
- We have identified 387 differentially expressed genes that show sexually dimorphic expression pattern in the tail fins of adult zebrafish. These markers can be used to verify the sex of those adults that do not show obvious phenotypic signs and could be potentially useful for sexing juveniles as well.
- We have adapted and optimized the protocol for the heat-induced masculinization of zebrafish. We determined the sex ratio of the offsprings for 51 families and tested for heat-sensitivity multiple broods from 16 of them. Comparative transcriptomic analyses of heat-treated vs. control groups of siblings are in progress.
- In another collaborative study, we took part in the successful validation of a Y-specific genomic sex marker in African catfish. We confirmed that neither signs of ZW/ZZ-type chromosomal SD, nor those of heat-induced masculinization can be found in the Hungarian stocks of this species
- We have successfully used a heat-based treatment to rescue European catfish individuals infected by a gill monogenean exoparasite. The treatment has substantially reduced the parasite loads on the gills in comparison to untreated, infected controls. Comparative analysis of the heat-treated vs. untreated gills and

spleens identified over 600 genes whose expression level changed significantly as a result of the treatment.

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