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Association study between relative expression levels of eight genes and growth rate in Hungarian common carp (*Cyprinus carpio*)

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ABSTRACT

One of the most important issues in improving the competitiveness of the fish production sector is to improve the growth rate of fish. The genetic background to this trait is at present poorly understood. In this study, we compared the relative gene expression levels of the Akt1s1, FGF, GH, IGF1, MSTN, TLR2, TLR4 and TLR5 genes in blood in groups of common carps (*Cyprinus carpio*), which belonged to different growth types and phenotypes. Fish were divided into groups based on growth rate (normal group: n = 6; slow group: n = 6) and phenotype (scaled group: n = 6; mirror group: n = 6). In the first 18 weeks, we measured significant differences ($p < 0.05$) between groups in terms of body weight and body length. Over the next 18 weeks, the fish in the slow group showed more intense development. In the same period, the slow group was characterized by lower expression levels for most genes, whereas GH and IGF1 mRNA levels were higher compared to the normal group. We found that phenotype was not a determining factor in differences of relative expression levels of the genes studied.

1. Introduction

Due to the increasing population of the world, food security is one of the biggest challenges humanity will face. We will strive to produce more and better quality food products in the simplest and most economical way that is possible, while we try to protect our environment (FAO, 2018). One of the options available in the agricultural sector is the economical and fast development of fish farming (Olimov et al., 2019). Fish are well suited for producing high quality protein. However, within the fish stocks of the hatcheries it is often observed that some individuals grow more slowly than their peers of the same age, regardless of the same housing and breeding conditions. This phenomenon causes considerable economic loss. Therefore, one way of development in producing as much fish meat as possible can be the identification of genes that affect the growth of the species. The practical significance of gene expression studies is that if the activity of the genes studied can be correlated with the quantitative or qualitative traits being examined (for example: stress tolerance, resistance to disease, growth, reproduction, etc.), then by selection or influencing environmental factors, genes with higher activity could be used to produce fish stocks that are more economical (FAO, 2007). This could open up new opportunities for cheaper market fish, better quality and higher returns. To increase our knowledge of the genetic background of this phenomenon we examined the activity of eight genes to determine their role in and their association with growth rates. Our aim was to follow the activity of genes over a longer period of time in groups with different growth rates and phenotypes.

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In this way, the genes most involved in growth can be identified, which can become targets for further studies and selection programs.

In our study, we investigated the genetic causes of size differences in common carps (*Cyprinus carpio*), kept under the same conditions, using the RT-PCR method. The type of experiment was a long-term study using the same animals, which blood was taken from the individuals and then an RT-PCR method was used to examine the expression of the genes. There are several examples in previous long term research that specific gene expression were analyzed from blood, e.g., Qin et al. (2010) determined gene expression from complete peripheral blood profile after GH gene therapy in adult male rats. Yano et al. (2015) examined myostatin in bloodplasma and Ekwemalor et al. (2017) isolated RNA from goat blood. This study will discuss the relative expression of the following growth genes: Akt1 Substrate 1 (Akt1s1), fibroblast growth factor (FGF), growth hormone (GH), insulin-like growth factor-1 (IGF1), myostatin (MSTN) and Toll-like receptors 2;4;5 (TLR 2;4;5). We chose these genes because, together with their interaction with each other, they can influence changes in growth parameters, and certain genes are already active in the embryogenesis stage, thus they are involved in shaping the body early on. If individuals are exposed to stress, it can have an effect on sexual maturity, which in turn has an effect on growth (Milla et al., 2009). The GH-releasing hormone of the hypothalamus induces the GH gene in the pituitary gland to produce GH, which stimulates the release of IGF1 in the liver, which can accelerate somatic development (Moriyama et al., 2000). The myostatin (MSTN) protein negatively regulates the proliferation and differentiation of skeletal muscle cells, thereby increasing their growth (Sun et al., 2012); chronic stress caused by individual density also affects the immune response, so TLR genes may also indirectly affect the rate of growth (Pujante et al., 2015). Akt1S1 is a proline-rich substrate, also known as PRAS40, which is one of the cascades and components of rapamycin complex 1 (mTORC) (Nascimento et al., 2010). It can even be a negative regulator of mTORC if it binds to YWHAZ. Levels of phosphorylated Akt1S1 are generally high in advanced tumors, but its role in tumor development is still unclear (Andersen et al., 2010). However, Akt1S1 has a key role in the regulation of the insulin sensitivity of skeletal muscle (Wiza et al., 2013; Wiza et al., 2014). Akt1s1 knockout impairs the phosphorylation of mTORC1 substrates in certain celltypes, suggesting that Akt1s1 may also be important for mTORC1 signaling (Fonseca et al., 2007; Hong-Brown et al., 2010). In 2014, Wiza and colleagues (2014) investigated whether rash of the Akt substrate impairs this insulin-signaling pathway in skeletal muscle. Insulin-mediated Akt phosphorylation was enhanced after overexpression of WT-PRAS40 in both human skeletal muscle cells and mouse skeletal muscle cells. Furthermore, overexpression of AAA-PRAS40 also had an insulin-sensitizing effect, but to a lesser extent than WT-PRAS40 (Wiza et al., 2014).

Genes that promote tumor transformation during mutation or high expression are called proto-oncogenes. Also included are fibroblast growth factors, which are characterized by overexpression that causes a change in cells that results in increased GF activity that in turn can lead to uncontrolled cell proliferation (Harvey et al., 2000). In terms of their function, FGFs generally function by binding to the tyrosinekinase receptor (FGFR) on the cell membrane surface (Harvey et al., 2000). Proto-oncogenes are normal cellular genes involved in the regulation of growth, but tumors can develop if they become unregulated and poorly activated.

The IGF1 system plays a central role in the neuroendocrine regulation of growth in all vertebrates. It is mainly the liver that produces its encoded protein, which is transported to other tissues by the bloodstream (Moriyama et al., 2000). The GH and IGF systems are very important promoters of bony fishes. In these fishes GH induces muscle growth by up-regulating the expression of some genes, like the myostatin (MSTN), and thus influence the skeletal muscle of fish (Fuentes et al., 2013). Norbeck and Sheridan (2011) investigated the effects of sexual steroidson IGF1 expression and thus on growth in young rainbow trout (*Oncorhynchus mykiss*). In this comprehensive study it was found that the hormone E2 (estradiol) inhibited growth better in muscle and liver than the other studied (testosterone) hormone. Evidence from some studies in a variety of vertebrate species suggest that the IGF-growth factor complex, composed of ligands, receptors, and high-affinity binding proteins, evolved early during vertebrate evolution. Among non-mammalian vertebrates, IGF signaling has been studied most extensively in fish, and there is evidence from numerous molecular studies in fish indicating that the IGF1 gene is transcriptionally active at all stages of fish development, including embryogenesis (Wood et al., 2005).

The GH protein is produced in the pituitary gland through induction of the GH gene by the GH-releasing hormone that is produced in the hypothalamus. Le Roith et al. (2001) investigated the somatomedin hypothesis, according to which tissue secretions of insulin-like growth factors (IGF) mediate the growth-promoting effects of pituitary growth hormone (GH) by endocrine and/or autocrine mechanisms. Norbeck and Sheridan (2011) found that body growth is also associated with reproductive capacity, because estradiol and testosterone reduce peripheral reactivity with GH (by inhibiting mRNA and inhibiting the functional expression of GHR-s), but these effects on tissue (GHR expression) and muscleforms (IGF expression) are specific. MSTN (myostatin) is a member of the transforming growth factor-β superfamily (TGF-β superfamily) that negatively adjust skeletal muscle development and growth, by inhibiting cell cycle progression (Sun et al., 2012). It is also called GDF-8 (Sun et al., 2012). Possible changes in this gene (for example single nucleotide polymorphisms = SNPs) lead to an increase in skeletal muscle mass (Lozier et al., 2018). In their study, Sun et al. (2012) screened partial genome fragments of MSTN for single-point nucleotide polymorphisms in common carp individuals. These individuals were a wild population. It was found that some of the SNPs in MSTN may have a positive effect on growth properties, and it has been suggested that MSTN would be a good marker gene.

TLR (Toll-like Receptor) is a family of genes encoding a group of proteins that play a key role in the body's natural immune system. TLRs, the first line of defense that recognizes microbial pathogenic molecules in the body, are one of the best-studied immune receptors for detecting infection in mammals (Palti, 2018). The major feature of TLRs in the fish genome is the high degree of similarity to the mammalian TLR system (Palti, 2018), but the number of duplicated TLR genes is high due to several fish-specific duplication events (Ohno, 1999). Causey et al. (2019) studied immunostimulated silver salmon (*Oncorhynchus kisutch*) from three experimental groups that had the same genetic background but showed strongly divergent growth rates. Transcript levels of the AMPK subunit gene were determined in skeletal muscle after altering immune status with bacterial and viral mimetics. Skeletal muscle was chosen for the study because it plays an important role in regulating the distribution of resources between different physiological systems. This immunostimulation caused a decrease in the expression of several genes encoding the AMPK subunit in GH transgenic fish. AMPK has been found to be widely transcriptionally regulated by GH transgenesis, so that salmon treated with the GH transgene express GH up to 40-fold higher than untreated. This in turn leads to a large increase in endocrine production of insulin-like growth factor (IGF1), which is excreted from the liver. In response to GH, both feed intake and growth increase. The dynamic expression responses they observed suggest that the AMPK system plays a role in balancing energy expenditure on mus-

cle growth according to the immunological status of silver salmon. TLR2 recognizes many microbial components including lipoproteins, lipopeptides, peptidoglycan parasitic glycosylphosphatidylinositol anchors and fungal zymosan (Takeda et al., 2003). The TLR4 gene is a central protein of receptors responsive to bacterial lipopolysaccharides (LPSs) (particularly Gram-negative) in mammals. This gene is not found in most bony fish, only in zebrafish and carp species (Palti, 2018). Basu et al. (2015) used several heat stress experiments to investigate the response of TLR2, TLR4, and TLR5 genes in catla (*Catla catla*) fish. It was observed that each gene underfunctions below or above a certain temperature, the change being most noticeable in the blood. The lowest expression of TLR2, TLR4, and TLR5 was observed in the blood. Compared to blood, TLR2 gene expression was 5.5-fold in gill and kidney, and TLR4 and TLR5 in liver were 7-fold and 150-fold, respectively. The TLR5 gene is linked to the recognition of bacterial flagellin. Typically, unlike most TLRs, activation is via the MyD88-dependent pathway on the cell surface of dendritic cell macrophages and intestinal epithelium (Hwang et al., 2010). Several studies demonstrate that the expression of trout TLR5S mRNA is primarily restricted to the liver, whereas the expression of TLR5M is found in all tissues examined. Two types of TLR5, membrane-bound and soluble, have been identified in fish species other than trout (*Fugu rubripes*, *Paralichthys olivaceus*) (Oshiumi et al., 2003; Ye et al., 2012).

2. Materials and methods

2.1. Experimental setup and rearing conditions

All 12 common carp individuals (*Cyprinus carpio*) were purchased from artificial propagation and belonged to the Böszörmény strain. Prior to the experiment fish were kept in a closed water recirculation system. At the beginning of the experiment, we decided on the duration experiment because the growth can be influenced by several complex parameters. With the same environmental parameters, growth can be influenced by individual density, amount and length of illumination (Karakatsouli et al., 2007), water temperature, pH (Abbink et al., 2012), and so on. As each individual may respond differently to these factors, we decided to take a blood sample, as no new individuals had to be included in the study, so individual differences could be ruled out. Common carp juveniles of 60 days post hatch (dph) were divided into four groups based on their growth rate and phenotype. The following groups were formed: scaled common carp with normal growth (average wet body weight: 34,10 ± 10,02 g, n = 3), mirror common carp with normal growth rate (average wet body weight: 30,44 ± 7,25 g, n = 3), scaled common carp with slow growth rate (average wet body weight: 5,05 ± 1,17 g, n = 3), mirror common carp with slow growth rate (average wet body weight: 7,08 ± 1,26 g, n = 3).

Prior to the experiment fish were distributed into 6 circular plastic tanks with a water volume of 350 L. In each tank 2 common carp juveniles were placed. Each tank contained 1 normal growth rate carp and 1 carp with slow growth rate. Scaled carps were placed in the first 3 tanks and mirror carp were placed in further 3 tanks. The treatments were set up in triplicate and completely randomized design.

Experimental groups were kept in the same water recirculation system provided with mechanical and aerated biofilter and UV lamp. Fish were fed three times daily (08:00, 12:00 and 16:00) with a commercially formulated dry feed; the feeding rates were 2.5% of the total biomass. Uneaten feed and faeces were removed daily. Oxygen saturation was maintained above 80% by aeration stones and temperature was controlled at 25.05 ± 0.5 °C. The photoperiod was 12 h light and 12 h dark. Water temperature and dissolved oxygen were checked daily with a HACH HQ30d portable meter (HACH CO., Loveland, CO, USA). The NO_{2-}, NO_{3-} and NH_{4+} concentrations were measured using the HACH Lange DR/3900 spectrophotometer (HACH CO., Loveland, CO, USA).

2.2. Sample collection

The first sampling was at 60 days post hatch. Further samplings were performed every 6 weeks. Standard body length and body weight of the fish were recorded each time. Standard body length was measured by tape measure and body weight was measured by digital scale.

Total blood were collected in order to use the same animals during the whole period of the experiment from the caudal vein with a blood collection syringe. Anesthetic used in this study was clove oil. Each fish was transferred from the stock tank to an anesthetic-containing aquarium (20 L). After the blood sampling anesthetized fish were transferred to a water tank (20 L with sufficiently aeration) for recovery. Blood samples were stored at −70 °C in RNAprotect Animal Blood Tubes (QIAGEN, Hilden, Germany).

The study was approved and carried out in accordance with the local ethics committee's guidelines of the University of Debrecen under the registration number DEMAB/15/2019.

2.3. Quantitative analysis of relativegene expression levels

Total RNA was isolated from blood of common carp using the RNeasy Protect Animal Blood Kit (QIAGEN, Hilden, Germany) by following the manufacturer's protocol. RNA quality and quantity were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (100 ng) was reverse transcribed in to cDNA with specific primers (Table 1) using the qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, United Kingdom). Quantitative real-time PCR (qRT-PCR) was performed using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The 10 μl reactions consisted of 5 μl PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.6 μleach of 10 μM forward and reverse primers, 1.3 μl dH2O (MilliporeSigma, Burlington, MA, USA) and 2.5 μl cDNA (2 ng/μl). For each sample, quantitative PCR was performed in triplicate. Thermal cycling was performed under the following conditions: 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Forward and reverse primers for common carp (Table 1) were designed by Primer Express v3.0.1 software (Applied Biosystems, Foster City, CA, USA) and checked for target identity using National Center for Biotechnology Information (NCBI) Primer Blast (Ye et al., 2012).

2.4. Statistical analysis

The same fish (n = 12) were used in all analyzes, but different groupings were formed. For example, in the growth-based grouping (n = 12), the group of normal growth rate fish (n = 6) consisted of 3 scaled and 3 mirror carp, just as the group of slow growth rate fish (n = 6) consisted of 3 scaled and 3 mirror carp. The same logic was used for phenotype-based analyzes, but the grouping factor was the phenotype (scaled and mirror) of the fish. Relative gene expression values were normalized to β-actin and analyzed with the Pfaffl method (Pfaffl, 2001). Primer efficiency was calculated with LinReg PCR version 2017.0 software (Ramakers et al., 2003). Statistical analysis was performed with SPSS 26.0 software (IBM Corp., Armonk, NY, USA). Independent samples *T*-test and Mann-Whitney *U* test were used to compare differences between two groups. The normality assumption was assessed using the Shapiro

Table 1
Data of applied primers.

Gene	Primer sequences (5′→3′)	Amplicon length (bp)
β-actin2	F: CCATCGGCAATGAGCGTTTC R: GCACAGCATAAGACTCACCCA	75
Akt1s1	F: AAGACCTCGCCTTAACACGG R: CGCGCAAACATACATACGCA	78
FGF	F: ACGGGGAAAACTGGTACGTC R: TTTGTCTGGTCCACCTGTCG	115
GH	F: TCTTCTCATTTGGGAGTGTCTCA R: AGACACCACTGTGAAATGTTCC	86
IGF1	F: CGCCTCGAGATGTATTGTGCAC R: CTGTATGCCGTTGCGCTCGT	73
MSTN	F: ATCTACTTGTCCGGTGCGTG R: ATGCATGTTCCAAAGCGTGC	91
TLR2	F: TGTGTGCGACACTCCATTCA R: GCACTAAGACAGCAGGGATCA	95
TLR4	F: GGCTATAGATCACCTGGACAGC R: CCAGACTTCATCATAGCTGGAGA	75
TLR5	F: TTGACGGCTCTGTTGTCCTC R: CACGTAAGTCACATGCGTAGG	77

Wilks test. Homoscedasticity was assessed using Levene's test. Independent samples *T*-test was used when the test of homogeneity of variances (Levene statistic) was not significant, and if significant, Welch's *T*-test was used. All statistical tests for two sample hypotheses were two-sided and considered significant if the p-value (p) was < 0.05.

3. Results

3.1. Determination of morphological characteristics

Fish in all groups grew over time (60, 102, 144, 186, 228, 270, 312, 354, 396 days) in terms of body length and body weight (Fig. 1). In case of growth rating grouping, the body length and body weight of the normal group were significantly higher at the first three samplings ($p < 0.05$). From sampling IV on wards, there was no significant difference between the groups in terms of body length or body weight. However, the normal group maintained higher values for both parameters throughout the study. During the last three samplings, high standard deviations were observed in both groups.

In the case of phenotype-based grouping, with one exception (body weight at sampling IX, $p = 0.006$) no significant differences were found between groups in either body length or body weight. In the case of body weight, we also measured larger standard deviations within the groups for the last three samplings (Fig. 1). The scaled group showed a higher mean of body weight until sampling V, when the means of the two phenotypes were equal. Subsequently, the mirror phenotype was characterized by a slightly higher body weight mean. The scaled phenotype was characterized by a higher average body length in the first five samples. The mean body length values of the two phenotypes were equalized from sampling VI on.

3.2. Pcr primer design and optimization of amplification reactions

Fig. 2 shows the amplification of the Akt1s1, FGF, GH, IGF1, MSTN, TLR2, TLR4 and TLR5 genes on agarose gel. All designed primers showed successful amplification and proved to be applicable in subsequent examinations.

3.3. Quantitative analysis of relative gene expression levels in the blood of common carp in case of growth rate grouping

Relative mRNA expression levels relative to the normal group are shown in Fig. 3. The Akt1s1 relative mRNA expression level

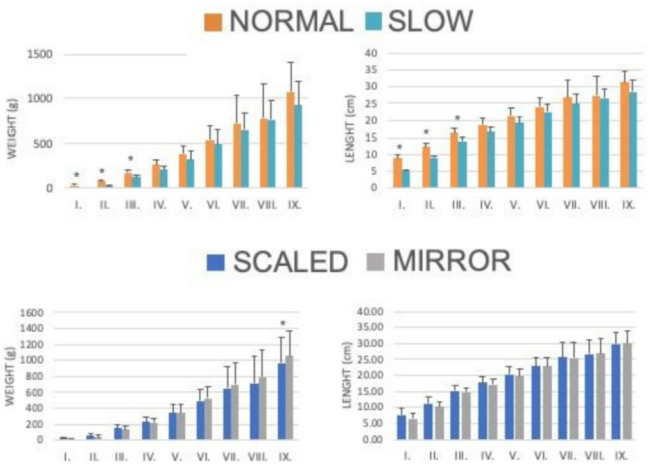

Fig. 1. Individual length and weight values based on phenotype.

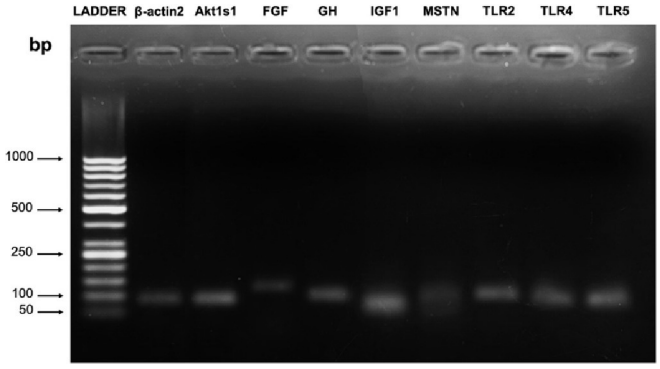

Fig. 2. Agarose gel electrophoresis results for the PCR products of designed primers.

of the slow growth group showed fluctuations compared to the normal group during the study (Fig. S1). Initially, the relative expression was almost the same in the two groups, then at sampling V relative expression of the slow group was significantly lower ($p = 0.018$) (Fig. S1). The trend was changed at sampling VII and the gene was expressed at higher levels in slow group. The difference was significant at sampling VIII ($p = 0.007$) (Fig. S1).

FGF mRNA relative expression level did not show notable differences between the two groups, except in the case of samplings IV and V, when the slow group showed lower mRNA levels. The difference was significant at sampling V ($p = 0.047$).

The GH gene has one of the most direct effects on fish growth. We measured growing trend of relative GH expression in the slow group at the first three samplings. The mRNA level was exceptionally high compared to the normal group at Sampling III. Sampling IV brought a trend reversal here as well. At this time, the normal group was characterized by a higher relative expression level. The higher relative mRNA expression level of the slow group stabilized at samplings V, VI and VII. During the last two samplings, the relative expression level of GH shows almost the same level in the two groups. For the GH gene, the difference in relative expression levels between the groups was not statistically significant in either case.

In the case of IGF1, samplings III, V and VII showed higher mRNA levels in the slow group than in the normal group. In the other samplings, IGF1 in the slow group was either underexpressed compared to the normal group or the means were equal. Statistically significant differences were found at samplings V ($p = 0.024$) and VI ($p = 0.047$).

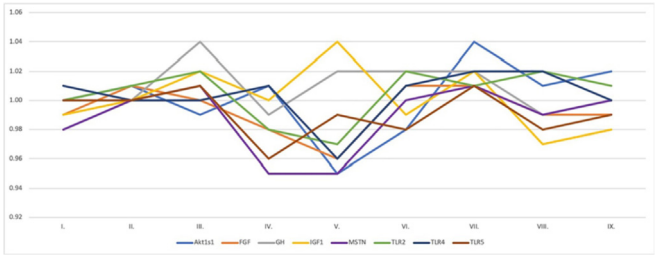

Fig. 3. mRNA relative expression levels of slow growth group relative to normal growth group (represented by value of 1.00).

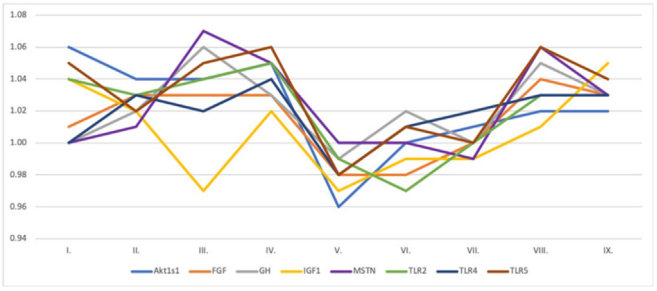

Fig. 4. mRNA expression levels of mirror phenotype relative to scaled phenotype (represented by 1.00 value).

MSTN relative expression levels increased steadily during the first three samplings in the slow group. MRNA levels showed a strong decline in samplings IV and V within the slow group. Subsequently, the values of the two groups were almost the same. No statistically significant difference in relative expression levels was observed between the groups for the MTSN gene.

TLR2 mRNA levels showed identical values at sampling I. The slow group was expressed at a higher than normal level at samplings II and III. Samplings IV and V also showed a trend reversal fort this gene. At this time, TLR2 was strongly under-expressed compared to the normal group. Starting from sampling VI, reversal occurred again and TLR2 showed higher relative expression in the slow group. Statistically significant differences were measured between groups at samplings VI ($p = 0.013$) and VIII ($p = 0.003$).

In the case of TLR4, the mRNA level of the slow group showed variance. At the first sampling, slightly higher mRNA levels were measured in this group, but the next two times the gene was expressed at the same level in both groups. In the slow group, mRNA levels were again slightly higher at sampling IV. The largest difference was observed at sampling V. At this time, TLR4 mRNA levels fell to nearly half that of the normal group. In the next three samplings, the relative expression level of the slow group was higher again. Finally, at sampling IX, the same values were measured again. Statistically significant differences between relative expression levels of the groups were found at samplings IV ($p = 0.042$), V ($p = 0.010$), VI ($p = 0.019$) and VIII ($p = 0.010$).

TLR5 mRNA levels showed the same value during the first two samplings. Even at sampling III the relative expression level of this gene was only slightly higher in the slow group. MRNA levels declined more markedly at sampling IV in the slow group and this trend was also characteristic of the next two occasions. Slightly higher relative expression values were measured in the slow group at sampling VII. Next, relative expression levels were higher in the normal group until the end of the experiment. No statistically significant difference was found between the values of the groups for TLR5.

3.4. Quantitative analysis of relative gene expression levels in the blood of common carp in the case of phenotype grouping

Data were also analyzed based on an alternative grouping. In this case, the phenotype of the fish was the grouping factor (Fig. 4, Fig. S2). As shown in Fig. 4, the change in mRNA levels of the genes shows a more uniform picture compared to the growth rate aspect analysis. There was a large-scale decrease in the relative expression levels of all studied genes of the mirror group between samplings V and VII. IGF1 shows a one-time deviation from this trend at sampling III. The mirror phenotype was characterized by higher mRNA levels during the first four samplings in most genes. An exception is TLR5, in the case of which no substantial differences were found between the relative expressions of the two study groups. There was a strong decline in mRNA levels in the mirror phenotype at sampling V. At this time, the group mean was below, or at least equal to, the mean of the scaled group. There was a gradual increase in the mirror phenotype in the last third of the study. At this time, in most cases, the mRNA level of the mirror group was higher than that of the scaled group. For the phenotype-based grouping, no statistically significant differences were found between mRNA levels. An exception is sampling VIII of the IGF1 gene ($p = 0.022$) (Fig. S2).

4. Discussion

Actual performance capacity may be limited in fish by environmental stress. Like fish kept in intensive system may often be subjected to several stressors of endogenous (e. g. social interactions) or exogenous (e.g. unfavourable water quality) (Karakatsouli et al., 2008) which elicits physiological and endocrinological changes and stress responses (Pickering, 1993). Therefore, in our study fish were kept under a recirculating water system which can help to minimize the occurrence of stressful conditions (Blancheton, 2000) and only the genetic effect could be observed. The stress response is an acute response in which fish use up their energy stores (Contreras-Sánchez et al., 1998). If environmental stress is constant, the stress response can have a detrimental effect on fish health by suppressing the growth rate (Pickering, 1993) and also disrupting reproductive processes (Milla et al., 2009; Bhatta et al., 2013). In a previously study that rearing of rainbow trout *Oncorhynchus mykiss* for 11 weeks under blue light (480 nm) had negatively affected growth and led to increased brain neurotransmitters indicating that blue light was perceived as stressful (Karakatsouli et al., 2007). The other influencing factor is the process of achieving sexual maturity. In fish, somatic growth is also accelerated in the early stages of sexual maturation (Le Gac et al., 1993), but in later stages physical growth decreases as resources are directed to gonadal development (Chen et al., 2018). As there were no stress factors or adverse environmental factors during the study, the genes we studied play an important role.

Difference was observed for the relative expression level of Akt1s1 (Fig. 4). Certain cellular functions of Akt1s1, such as regulation of the nuclear stress response, proteasome activity, and regulation of cell survival rate suggest that Akt1s1 may play a role in tumor progression. Furthermore, in Passtoors et al., 2012 examined whether transcriptional variation within the rapamycin complex 1 (mTORC) pathway was associated with human longevity in whole-blood samples from the Leiden Longevity Study. Gene analysis showed that seven genes in the 40 mTORC pathway had significant differential expression of at least 5%. Thus, the mTORC pathway plays a role not only in the regulation of disease and aging in animals, but also in terms of human health and longevity. Inhibition of the mTORC signaling pathway in yeast (Powers, 2006), worms (Vellai et al., 2003), flies (Kapahi et al., 2004), and mice (Harrison et al., 2009) results in prolongation of life. Inhibition of the mTORC

signaling pathway in mice provides cardiovascular benefits and improved metabolic function (Stanfel et al., 2009). In addition, mTORC2 regulates Akt phosphorylation in a portion of an upstream pathway that controls mTORC1 activation (Stanfel et al., 2009). Insulin-mediated activation of the insulin receptor substrate, the IRS1/Akt signaling pathway, regulates glucose uptake (Wiza et al., 2014). In contrast, overexpression of Akt1s1 inhibits proteasome activation and increases the stability of IRS1 (Wiza et al., 2014). This results in increased insulin sensitivity even with insulin resistance. Overexpression of Akt1s1 enhances insulin signaling through the IRS1/Akt axis in the heart and liver (Völkers et al., 2014). All of this requires phosphorus, which was certainly found in fish feed in the form of grains. The slow-growing group probably adapted more slowly to the nutritional values in the diet, and the large increase after the third sampling may also upset the equilibrium. The relative expression level of Akt1s1 mRNA in the slow growth group showed difference compared to the normal group, and at sampling V relative expression in the slow group was significantly lower and the main difference was significant at sampling VIII (Fig. 4). At sampling III, the significant difference between the two groups in both weight and length disappeared, and subsequently the mRNA level of the gene also decreased as compared to the normal group. The gene was less active during the period when slow fish began to catch up, which may also have been due to a better inhibition of the mTOR signaling pathway, thus improving metabolic function (Stanfel et al., 2009), so individuals were better adapted to the nutritional value of the feed. In the case of the slow group, samplings IV and V also showed lower levels of the relative expression of Akt1s1 and FGF. During this period the development required for sexual maturation was already underway, during which individuals utilized more energy (Moriyama et al., 2000), so the mTORC signaling system sensed the presence of less energy, and FGF also became an unregulated gene through a proto-oncogene and was improperly activated (Bhatta et al., 2013; Oppedal et al., 1997).

Growth and reproduction are closely related, as there is also an overlap with the endocrine system. Production of the GH protein is induced in the pituitary gland by the GH-releasing hormone of the hypothalamus it then stimulates the release of IGF1 mainly in the liver, thus accelerating somatic development (Moriyama et al., 2000). Transgenic carp treated with growth hormone (GH) show accelerated growth and delayed reproductive development, providing a viable model for understanding the overlap of growth and reproductive axes. Thus, Chen et al. (2018) studied the energy stability and reproductive development of GH-transgenic carp using multivariate RNA sequencing, real-time PCR, Western blot, ELISA, immunofluorescence, and in vitro incubation. It was found that although transgenic fish grow faster and more uniformly than wild-type fish, their reproductive abilities are significantly impaired due to overexpression of the GH gene. Changes in GH and myostatin levels are a regulated mechanism, acting through Stat5b; GH regulates the decrease in mature myostatin protein by increased expression and effect of IGF1 (Caicedo et al., 2018). The consequence is skeletal muscle growth. Although it has been found that the decrease in mature myostatin mRNA levels in the postnatal age begins in a phase of linear growth (McMahon et al., 2003; -Reisz-Porszasz et al., 2003) and has an effect on post-menopausal muscle mass development, it is the GH – IGF1 axis that is widely recognized as the major regulator of postnatal growth and is also essential for the development of sexual dimorphism (Le Roith et al., 2001). The structure of the growth hormone (GH) - insulin-like growth factor (IGF) axis consists of acentral neuroanatomical and regulatory-genetic systems, as well as a peripheral intracellular GH signaling pathway (Ranke and Wit, 2018). The effects of GH are mediated by Stat5a and 5b, thus regulating the function of IGF1 and the mature myostatin protein (Oldham et al., 2009). In contrast to myostatin activity, the activity of the growth hormone (GH – insulin growth factor-1 (IGF1)) axis results in muscle growth (Oldham et al., 2009; Brooks et al., 2011). Furthermore, in chronic stress, elevated cortisol levels may suppress the growth hormone/insulin-like growth factor-I (GH/IGF1) axis response (Pujante et al., 2015). In Cook et al., 2012 examined the organizational responses to a repetitive stress response in bluegill fish (*Lepomis macrochirus*). Blue-gill caught during the experiment were repeatedly subjected to standardized stressors and blood samples were taken (three times in 6 days). Sampling was performed after the cortisol concentration reached a peak. In this context, various indicators of fish were also examined, including the whole body, its size and physiology. Cortisol variability was correlated with the Fulton condition factor that smaller fish in poor condition had greater cortisol variability. The results have an impact on the correlations of GC concentrations, as they show that the consistency of stress responsiveness is also influenced by factors such as body size and health status. In an experiment on mice by Oldham et al. (2009), these genes were shown to be closely related because while myostatin was active, the weight of the animals did not increase and GH expression levels increased steadily, but due to puberty, GH function reduced myostatin activity. Slower developing mice also began to grow. Because the authors only examined myostatin activity, the anterior part of the pituitary gland was removed in the second half of the experiment to avoid an endogenous source of GH. Although exogenous GH may still activate Stat5b in young rats, some downstream target genes respond to Stat5b binding only after puberty (Choi and Waxman, 2000). Qin et al. (2010) examined the responses of rats undergoing GH gene therapy in 2010, in which the results showed that exogenous GH gene expression in normally developing subjects is likely to induce cellular changes in metabolism, signaling pathways, and immunity, which may result in body size changes. Long et al. (2019) investigated how the growth and immune response of the highly endangered Chinese sturgeon (*Acipenser sinensis*) is affected by keeping and breeding in a recirculation system at a given individual density. The fish were kept in three different density groups: low density (4.80 kg/m^2), medium density (8.99 kg/m^2) and high density (12.68 kg/m^2). The results showed that in the 12.68 kg/m^2 density group, the growth of the individuals decreased significantly, and the expression of several genes also decreased due to the stocking density, thus the immune response also showed a decreasing trend. Transcription of immune-related genes, including lysozyme, hepcidin antimicrobial peptide 1 (HAMP1), tumor necrosis factor α (TNF-α), interleukin 1β, and interleukin 8, was also significantly reduced to 12.68 kg/m^2 in groups ($P < 0.05$). On the somatotropic axis, serum levels and gene expression of both growth hormone (GH) and insulin-like growth factor-I (IGF1) also resulted in decreased regulation in the 12.68 kg/m^2 density group ($P < 0.05$) (Long et al., 2019) Previous study has also shown that cortisol reduced both plasma levels and transcription in the pituitary of GH and IGF, which led to growth suppression of fish under stress (Saera-Vila et al., 2009). Furthermore, Kajimura (2003) reported that both liver plasma levels and transcription of IGF1 were inhibited in tilapia (*Oreochromis mossambicus*) after intraperitoneal cortisol injection. These results highlighted that cortisol may interact with the GH/IGF1 axis and that inhibition of both GH and IGF1 secretion may be caused by high levels of cortisol in bone fish species when exposed to various stressful situations (Pujante et al., 2015). In 2009, Tymchuk et al. (2009) examined the growth of coho salmon (*Oncorhynchus kisutch*) and trout (*Oncorhynchus mykiss*) between wild populations and domesticated populations. During the experiment, GH, IGF1 and thyroid stimulating hormone (T3) were tested in several tissues and blood. The individuals had the same age and similar body size to successfully monitor the rate of growth. The results showed that in

response to anthropogenic selection pressure in both salmon and trout, genetically substantiated differences in hormone expression and regulation developed, particularly for IGF1. Saera-Vila et al. (2009) also analyzed the function and time course of the GH/IGF axis of the golden plover (*Sparus aurata*) based on blood samples, and examined the selected stress markers. The fish were not fed, thus inducing stress responses, as a result of which growth hormone function was only indirectly reduced due to the IGF1 response. In the case of our study, by the time of the third sampling sexual development of the slow-growing fish had begun, therefore myostatin activity decreased due to GH, as a result of which the slow-growing group began to catch up, whereas the fast-growing group already devoted their energy to developing gonads, therefore their growth stagnated (Fig. 4).

In the case of our experiment, the levels of IGF1 at samplings V and VI were higher in the normally growing than in the slow-growing group, but overall, there was not much difference between the two groups. IGF1 is a 70 amino acid peptide with a molecular weight of 7649 Da (Dehkhoda et al., 2018). It is able to bind to insulin receptors but with low affinity. Most IGF1 is excreted by the liver and acts as an endocrine hormone, although many other tissues can excrete it (brain, muscles, kidneys), and hepatocytes generate the majority of circulating IGF1 in the blood (Dehkhoda et al., 2018; Moriyama et al., 2000). IGF1 has been shown to be an important growth-related hormone that has a GH-independent growth-promoting effect and in some cases acts synergistically with GH (Laron, 2001). It has been shown that in teleosts hepatic IGF1 expression is dependent on the presence of GH. The association of GH and GHR activates a post-receptor signaling system that stimulates transcription of target genes such as IGF1 (Xue et al., 2012). Exogenous IGF1 has been shown to stimulate growth when administered to hypophysectomized rats (Schoenle et al., 1982). IGF1 can then stimulate cell proliferation, differentiation, and ultimately body growth by acting on target tissues (Pierce et al., 2005). In the event of a gene failure, growth abnormalities develop due to the lack of insulin-like growth factor. On the other hand, IGF1 plays a role in skeletal muscle hypertrophy, in attenuating age-related skeletal muscle atrophy, and in restoring and improving muscle mass (Barton-Davis et al., 1998) They also affect the development of skeletal muscle in fish, as IGF1 expression is dependent on growth hormone. For example, IGF-binding proteins (IGFBPs) have a potential role in muscle growth (Moriyama et al., 2000).

In the first 3 samplings myostatin was strongly expressed, whereas in samplings IV and V we saw a strong decrease in myostatin expression. The data of samplings IV and V show a steady increase, i.e. the growth rate of the slow group was higher than that of the normal group during this period. The degree of myostatin expression may be associated with sexual maturity, as MSTN transcripts in the ovaries of many trout were shown to be prone to regulation by ovulation in several individuals (Roberts and Goetz, 2001). In the slow group, in samplings III and V-VII the rate of growth was higher. Myostatin expression was lower (compared to the other genes) in slow fish compared to normal. As expected, MSTN levels decreased in the slow group as they began to catch up and increased intensively. Myostatin (MSTN) is a member of the transforming growth factor β superfamily, and this protein negatively regulates the proliferation and differentiation of skeletal muscle cells, and thus their growth (Sun et al., 2012). Possible changes in this gene (e.g. point mutations/SNP) lead to an increase in skeletal muscle mass (McPherron et al., 1997). In sharp contrast to mammals, MSTN orthologs of various fish species are widely expressed with a tissue-specific expression pattern. In mammals, it is expressed primarily in skeletal muscle, at lower levels in adipose tissue, mammary gland, and heart muscle. In fish, MSTN mRNA has also been detected in other tissues/organs (brain, eye, intestine, gill fibers, gonad, kidney) (Maccatrozzo et al., 2001). Two distinct myostatin genes have also been found in some fish species (Roberts and Goetz, 2001; Østbye et al., 2007). Comparison of myostatin sequences revealed that myostatin was extremely well conserved during evolution, so much so that in bony fish, such as salmonoids, copies of additional MSTN genes could be retained by due to duplication of the ancient genome (Gabillard et al., 2013). Some of the potential SNPs in MSTN may have a positive effect on growth properties: for example, in mice, disruption of the myostatin gene causes a significant increase in muscle mass due to a combination of hypertrophy and hyperplasia, and in cattle, natural mutations are also followed by a significant increase in muscle mass. Myostatin is a gene that inhibits the activation, proliferation and differentiation of satellite cells. Fish MSTN, on the other hand, is responsible for cell proliferation and overall inhibition of cell growth, so although it regulates tissue mass, it does not specialize as a strong muscle regulator (Gabillard et al., 2013). In contrast to mammalian muscle growth, where postnatal muscle growth occurs almost exclusively with hypertrophy, in most fish species postnatal muscle growth is a combination of hyperplasia and hypertrophy (Galt et al., 2014).

Pathogen and nutrient sensing toll-like receptors (TLRs), also known as pattern recognition receptors, are evolutionarily conserved in most species. These transmembrane proteins play a key role in initiating innate immune responses in the detection and signaling of microbial pathogens or molecular samples expressed during cell-related stress, tumor, or viral infection (Gupta et al., 2017; Nadeem et al., 2019). Studies in recent years have also found several endogenous substances — i.e., the host's own substances — that are capable of binding to TLRs (Chang-Hong et al., 2017). Such may be an intracellular component released from a damaged cell, which may thus elicit an immune response in the absence of infection. Under stress, for example, heat shock proteins (HSPs) are overexpressed and may also act as alarm molecules. Among the heat shock proteins, HSP60, HSP70, and HSP90 have also been shown to be able to activate TLR2 and TLR4 receptors (Chang-Hong et al., 2017). Furthermore, Nadeem et al. reported in 2019 that the expression of TLR2 and TLR4 immunometabolic receptors in PBMC is significantly upregulated by H2O2-induced oxidative stress. Several studies support that dietary vitamin C can significantly regulate the expression levels of immune-related genes (HSP70, HSP90, BAFF, TNF-α, and IL-6) in the liver. These results indicate that dietary vitamin C can significantly affect fish growth performance, antioxidant status, and innate immunity. Furthermore, growth and immunity are two energy-intensive systems, both essential for survival and resilience. For this reason, they are closely interdependent in managing the balance of energy invested in growth and immunity, in which AMPK plays a role. In 2019, Causey et al. stimulated fish with two immunostimulants, peptidoglycan and polyinosinic acid, with the aim of eliciting an anti-inflammatory and antiviral response. This caused significant changes in the expression of several AMPK subunit genes. Salmon with the GH transgene express GH at levels up to 40 times higher than the wild type. This increases the relative expression of IGF1, so that the endocrine production of the anabolic hormone secreted by the liver in response to GH begins, resulting in a threefold increase in feed uptake. In our study, we examined the relative expression levels of TLR2, TLR4 and TLR5. In each case, the values changed during the sampling, but there is a statistically significant difference in the results of samplings IV, V, VI and VII for TLR4, when the relative expression level is halved (Fig. S1). Interestingly, during this period, the slow group showed higher mRNA levels, but at sampling V the ratio reversed drastically. However, this trend with minor differences can also be observed for the other genes examined, with the exception of GH and IGF1 (increased expression of these genes is associated with intense development). As

the growth rate of the slow group exceeded that of the normal group during this period, this indicates to us the relationship between individual development and TLR genes. It can be assumed that during periods of intense growth the function of genes associated with the immune system may be expressed at lower levels due to the increased energy requirement of the body. This should draw the attention of fish farmers to the increased consideration of animal health aspects during the intensive growth stages of stocks, as fish may be more vulnerable to adverse effects during this development phase.

Although in the phenotype-based analysis we found differences in mRNA levels for several genes, and higher relative expression was mostly observed in the case of the mirror phenotype, we could not detect a statistically significant difference with one exception (IGF1 at sampling VIII). Our results suggest that the activity of the genes we studied is related to the growth rate of fish, thus these may be potential targets for yield-enhancing treatments. Furthermore, we conclude that there is no relationship between the relative expression of the genes studied and the phenotype. In the future, we would like to conduct a diversity study to determine whether the mirror or scaled phenotype may be an influencing factor during growth. If this study does not show a significant difference either, it is not necessarily justified to maintain such phenotypic discrimination for strains.

The gene expression studies can serve as an important tool for the selection in fish populations. However, still little known about the genetic basis of adaptation in gene expression resulting from variation in the aquatic environment (e.g. temperature, salinity and oxygen) and the physiological effect and costs of such differences in gene expression (Larsen et al., 2011). Thus, in the case of fish, the transferability of the results to practice may not be as direct as in other domestic animals. In spite of that, gene expression an effective strategy to compare animals with extreme characteristics whitch differing in their breed, nutrition, growth rate, physiological state, management system or quality of their products (Cassar-Malek et al., 2008). Our studie have used this approach in order to provide results that can be transferred to practical fish management. We consider such a result that the phenotype (scaly, mirror) was not decisive for the growth rate and the expression of the genes showed essentially no significant difference between the two types. Although, as indicated above, further research is needed to confirm this, but this may be important information for breeders in developing selection strategies. Because breeders consider the character of scaling to be a very important selection aspect. One of the most important elements of the genetic potential of carp is related to growth and the resulting production of faster and heavier goods. This area could be a real breakthrough for the fish sector. In connection with growth, we found a significant relationship between the level of gene expression and the growth rate in several cases. These results may help draw attention to genes that merit further study in fish growth. We hope we have been able to contribute to the development of future genomic selection tools that will be available for practice and will be directly applicable to selection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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