THESIS

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ANALYSIS OF THE EXPRESSION PATTERNS OF INSULIN-LIKE GROWTH FACTORS, GROWTH HORMONE AND GROWTH HORMONE RECEPTOR GENES IN DIFFERENT FEEDING GROUPS OF *CLARIAS GARIEPINUS (*AFRICAN CATFISH)

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1. INTRODUCTION

Aquaculture farming is growing around the world due to its socio-economic merits associated with food supply and rural livelihoods. It is well known that aquaculture was unpopular in the 1970s and produced only 7% of the fish used in human consumption. Interest in aquaculture sparked interest about two decades ago and established reports express that fish products provided the world with macronutrients and food safety (FAO, 2022). By 2030, it is expected that the growth rates of global fisheries production, consumption, and trade will have slowed (FAO, 2022). By 2030, it is expected that fisheries will contribute 202 million tons to global food production. It is estimated that by 2030, aquaculture will have produced 106 million tons, up from the 100 million tons produced in 2027. By 2030, experts predict that the total amount of fish caught will have increased by 6%, reaching 96 million tons. Overfishing has decreased, waste, loss, and discards have decreased, and resource management has become more efficient, so this is the result. (FAO, 2022)

The Food and Agriculture Organization statistically projects an average of about 18.7 aquaculture farms globally, an increase of 6.1 million since 2000, As a result of growing acceptance of fish farming by small as well as medium sized stakeholders (SMEs). Several species, including tilapia and catfishes are exclusively produced by intensive fish farming mostly in Europe (FAO, 2016).

A consistent supply of oxygen from the aquatic environment is essential for fish survival. (Nicholas and colleagues, 2016). Fish have varied branchial reactions to low (hypoxic) and high (hypertoxic) DO levels in water; as a result, a minimum amount of dissolved oxygen (DO) in water is required for the development, growth, and reproduction of various fish species. (Jie et al., 2022; Wu et al., 2016).

Catfish are popular because they require less care than other species to maintain a sustainable agricultural system. They can breathe using their secondary breathing organ, allowing farmers to maintain a high stock density. High stock density reduces the amount of diffused oxygen levels in water, as well as its quality. They have superior flesh with less fishbone than carps. They have a robust immune system. (Pangni et al, 2008).

Nevertheless, Catfishes are mostly classified as obligate respiratory fishes unlike most species that depend solely on gill breathing. Catfish are equipped with an air breathing organ enabling then effect control oxygen depletion and dehydration. Due to these adaptive qualities, they are known to survive for longer periods out of water and can exclusively survive in oxygen depleted

environments (Belão et al, 2011). For this reason, fish farmers are growing more interested in catfish farming globally.

Despite the adaptive characteristics of catfish, they are sensitive of water temperature. Below 15-17 °C their immune system slowly stops (Quiniou et al,1998).

Its intensive farming is not different from other fishes due to the general high cost of fish feed in the world mostly attributed to the high level of fish meal products (Kumar et al, 2020)

Fish feed alone accounts for 40–75 % of aquaculture's investment costs, and the pricing of the ingredients popularly used in fish feed increased by 20–92 percent from June 2007 to June 2008. (FAO, 2014). The main components of fish feed have seen price increases of 40–75 percent over the past few years due to rising global commodity and energy costs (Agugliaro et al, 2012).

According to Miles et al, 2006 fish feed is costly because of its high content of fishmeal (fish protein and oil). Due to the high cost of fish feed, quality and moderation of fish feed must be worked on in parallel with expanding production, feed quality, and its impact on aquaculture, especially on the growth of fish. Knowing the properties of feed regular substances is critical to determine the growth rate of fish and finishing the monetary examination (FAO, 2016). Fishmeal typically contains minerals, water, and 60 to 72 percent fish protein by weight, 10 to 20 percent ash, and 5 to 12 percent fish oil, which contains the health-promoting omega-3 polyunsaturated fatty acids, PA and DHA (Cho et al, 2010).

Molecular Ecology Department's researchers were aiming for creating an African catfish line which is adapted to lower cost fish feed. The experimental fish feed's fishmeal content was partly substituted with soy meal. They made three positive selected lines (PS1, PS2, PS3) and one control selected lane (Kontrol) in Kisbajcs's African catfish fish farm. The PS fishlines got the experimental feed and the control fish lane got the control feed (conventional feed-high amount of fishmeal). Studies have shown that different kind of fishes can be adapted to lowered amount of fishmeal containing feeds (put studies here). After the fourth generation, 4 months old 300 fish were brought to Szent István Campus of Hungarian University of Agriculture and Life Sciences Fish Department where they were put for six weeks in a demonstration experiment. To supply the energy necessary to maintain biological activities, feed intake management must incorporate both exogenous and endogenous factors. This form of regulation is controlled by the endocrine system,

which sends hormones from one type of organ to another and is responsible for the release of hormones and the regulation of cellular activity. (Bertucci et al, 2019).

Besides exogenous factors, the act of growth involves endogenous interactions. One part of these interactions involves the hormonal background of growth. If we look at the main hormonal centers, we should consider the investigations of the HPS axis and the liver. Studies proved before (put studies here) vast majority of growth-related hormones comes from these organs. Starting from this background we analyzed four growth related gene's expression rate. Namely, growth hormone gene (*gh*), growth hormone receptor gene (*ghr*), insulin-like growth factor I (*igf-I*) and insulin-like growth factor II (*igf-II*). The expression was evaluated in the brain (*gh*, *ghr*) and in the liver (*ghr*, *igf-I*, *igf-II*), using Real-Time Quantitative PCR. We extracted the RNA at the end of the experimental time frame and evaluated the expression pattern results with $2^{*}\Delta\Delta CT$ statistical analysis.

1. Literature Review

2.1. Global State of fisheries and aquaculture

Global fish aquaculture has progressed, and Africa has grown by 14.5% since 2019 (excluding Egypt and Nigeria), while Asia has produced 91.6% of global output due to its large population. With 178 metric tons of aquaponic animal production and 36 metric tons of algae cultivation, the global consumption of fish products has increased steadily by 3% since 1961 because of the growing need for aquaculture in contrast to the population growth of 1.6%. Additionally, aquaculture earnings reached a record high of about 214 million tons in 2020, of which 157 metric tons, or 89% of them, were used for direct human consumption, up from 67% about six decades ago. (FAO,2022).

As Table 1 shows, the top 10 fish species which are produced around the world mostly carps, but we can find some catfishes too. Clarias catfishes are presented at the tenth place. But their popularity has grown about 25 times in the last two decades. Others, like carp's production has grown also, but just a few times in this time frame. In 2020 the total percentage of finfish in inland aquaculture was mostly carps, catfishes hold about 7,5 percentage of all. According to this trend, in the future we can expect on a rise of a catfish's popularity worldwide.

The percentage of total animal protein supplied by fish grew from 13.7% in 1961 to 16.0% in 1996. From then, it went down to 15.3 percent in 2005, a little improvement. Comparable statistics show that the worldwide rate rose from 12.9% in 1961 to 15.4% in 1989 before dramatically declining to 14.7% in 2005 (excluding China). The percentage of animal protein provided by fish ranged from 7.6% in North and Central America to over 11% in Europe, while it hovered closer to 19% in Africa, over 21% in Asia, and close to 19% in the LIFDCs, which include China. (FAO 2009)

African catfish is a key species for fishery and is produced in many places throughout the globe. The top producing countries nations are Nigeria, next is the Netherlands followed by Brazil, Hungary, Kenya, the Syrian Arab Republic, South African, Cameroon, and last but not the least Mali (FAO 2016)

Table 1Major aquaculture species produced in the world **Source: FAO 2022**

	2000	2020	Percentage of total, 2020
	thousand tonnes, live weight		
Grass carp, C. idellus	2 976.5	5 791.5	11.8
Silver carp, H. molitrix	3 034.7	4 896.6	10
Nile tilapia, O. niloticus	1 001.5	4 407.2	9
Common carp, C. carpio	2 410.4	4 236.3	8,6
Catla, C. catla	602.3	3 540.3	7,2
Bighead carp, H. nobilis	1 438.9	3 187.2	6,5
Carassius spp.	1 198.5	2 748.6	5.6
Striped catfish, P. hypophthalmus	113.2	2 520.4	5.1
Roho labeo, L. rohita	733.9	2 484.8	5,1
Clarias catfishes, Clarias spp.	48.8	1 249.0	2,5

2.2 State of fisheries in Hungary

Hungary is a landlocked country in Eastern Europe with no direct sea fishing fleet; producing only freshwater aquaculture products, as a result government stopped inland water fishing in 2016 to protect its limited aquatic environment and promote pond or tank fish farming on January 1st, 2016. As a result, aquaculture provides most of the domestic fish supply. (Jensen, 2021)

Whereas the EU average per capita intake is 20–22 kilograms per person per year, Hungarians eat just 5 kilograms of fish per year, despite importing marine seafood. The typical Hungarian is expected to consume 6 kilograms of fish annually by the 2030s. (FAO, 2014).

Hungary has always played a prominent role in Europe's freshwater fish production due to its hydrographic characteristics, as its gross production reached a thousand tons of fish in 2018 Pond farms and intensive farms combined gross fish production in 2021 was 14.4% lower than in 2007, 29.9% lower than in 2010, and 13.7% lower than in 2015. In 2021, the total number of employees in the fish farming sector, both male and female, was approximately 6.5 percent higher than in 2007, 16.8 percent higher than in 2010, but 12.1% lower than in 2015. (Bojtárné et al. 2019, Kiss et al, 2022).

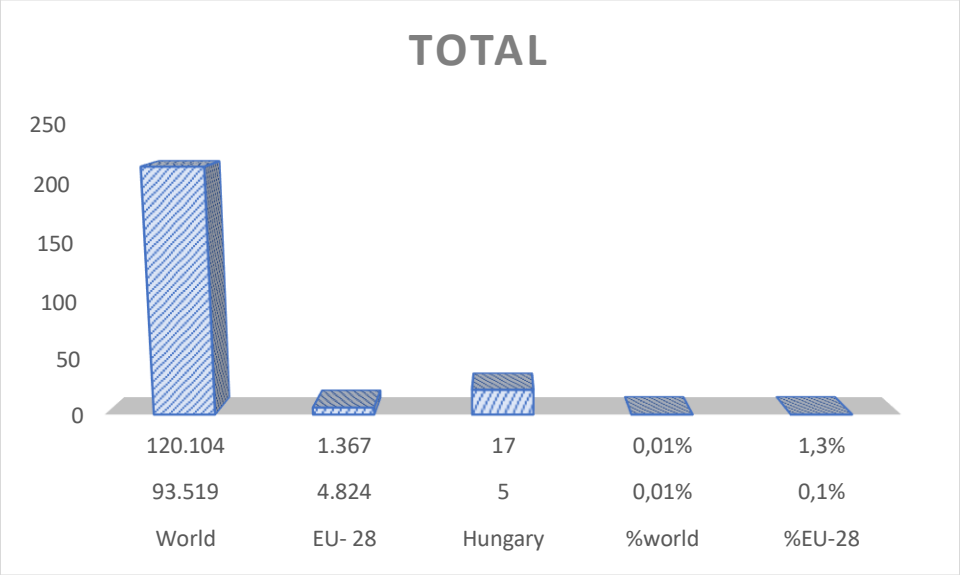


Figure 1illustration of fishery production in and fish intake of EU and Hungary against the world

Large-scale fish farming in ponds and intensive fish production utilizing geothermal water are two distinguishing characteristics of the Hungarian fish farming industry. Carp is the species of fish that is farmed the most often in ponds, although catfish and sturgeon are the most common species farmed using feedstuffs. Commercial opportunities may be available with tank systems that have a recirculation of the water. (Popp et al, 2018).

Preferential fishing for ecological purposes, such as nuisance fish eradication, accounts for a lower fraction of total harvest and may be offered for purchase with a catch permit. However, this total includes just 1% of the entire ecological harvest. Recreational fishers account for the vast bulk of catches in natural waterways. Statistics on fish consumption do account for the catch of traditional fishermen, who perform most of their fishing in natural waterways for mostly home use. There were almost 770 000 authorized fishermen in Hungary in 2020 alone, and their total catch was approximately 5081 tonnes. (Jensen, 2021).

The geographical, water and climatic conditions in Hungary are favourable for traditional pond fish husbandry and intensive fish production. Hungary's carp production is the third largest in Europe, its carp production technology is also used in other countries and its experts participate in aquaculture development programs. The major farmed species are common carp (Cyprinus carpio) and Chinese carps, and 7% of total aquaculture production comes from geothermal water heated intensive systems in which the main species is the North African catfish (*Clarias gariepinus*). Performance testing and the registration of carp varieties was begun in 1996 to increase the efficiency of fish production and to improve the reproductive capacity and genetic quality of carp stock. The Hungarian Fish Farmers' Association founded its Carp Breeding sector in 1997 and prepared a long-term production program detailing the general production goals, suggesting suitable production methods, describing the methods for performance testing and introducing a registering and origin certification system. (https://www.fao.org›fishery› naso_hungary). With current production statistics of 78% in ponds and 22% in tanks and raceways (Eurostat, 2019) there is a need to reduce production costs, preferably in fish feed because it accommodates the majority of fish production capital.

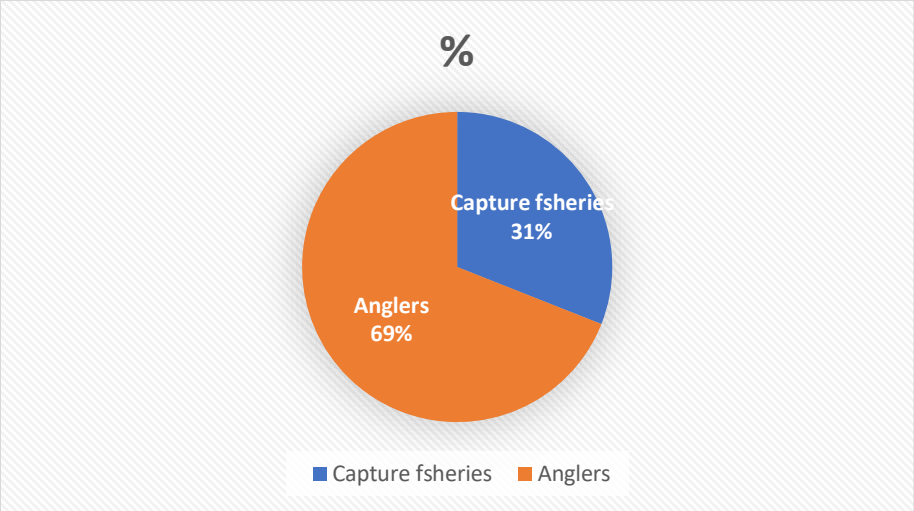


Figure 2: Proportion of harvest (tons) by anglers and capture fishers, 2010/2011

Source: Research Institute for Fisheries, Aquaculture and Irrigation

Figure 3Typical illustration of large scale extensive integrated open Aquaculture Systems in Hungary

Source: Popp et al, 2018

2.2.1 Aquatic species produced in Hungary.

Varying species and production technology are used in aquaculture, which has a diverse range of products. Fisheries technologies are currently used to raise about 100 different species all over the world. In the EU, shellfish account for more than 45% of aquaculture production, marine fish for more than 30%, and freshwater fish for more than 20%. (EUMOFA,2021).

Integrated pond fish fanning in Hungary increases natural yield and produces more food per unit. of area, facilitated by modern integrated multi-trophic aquaculture (IMTA) systems. The Strategic Research and Innovation Agenda (SRIA) of the European Aquaculture Technology and Innovation Platform identified research and development (R&D) work for the development of integrated pond aquaculture as an important part of SRIA (Popp et al, 2018).

Of the many non-native fish species that have been introduced to the European continent, the African catfish (*Clarias gariepinus)* has received relatively little attention and are farmed but in lower volumes. 9.000 tones mainly in Hungary and Netherlands. (EUMOFA,2021)

2.3 Catfish Species

Catfish are a diverse group of ray-finned fish, recognized by the enormous barbells on their heads (Laszlo et al, 2002). African catfish remain one of the most vital species in global aquaculture farming, mostly farmed in Brazil, Netherlands, Hungary, South Africa, Syrian Arab Republic, Mali, and Cameroon. (FAO, 2016). With more than 4000 species and over 12% of the population of teleost fishes, catfish belongs to the order Siluriform, which is one of the largest groups of freshwater fishes (Balasubramanian et al, 2021). There are four main catfish species; Amur catfish (*Silurus arsotus*), Channel catfish (*Ictalurus punctatus*), Stripped catfish (*Pangasianodon hypophthalmus*), and African catfish most commonly cultivated globally (Clarias gariepinus) and the female catfish is generally larger in size in these species (Dauda et al,2018)

Figure: 4Growth sexual dimorphism in Amur catfish. (A) Juvenile body weight at the specified time points following hatch (n=30). (B) The weight and height of adults who are two years old (n = 30). (C) Illustrations of a male and female Amur catfish at two years of age

Source: Shen et al, 2020

2.4 African catfish (Clarias gariepinus) biological properties

The anatomical structure of these species shows a unique organ in the gill cavity with a sizable surface area inverted by numerous blood vessels that allows the fish to take in oxygen from the surrounding air. African catfishes' floats to the surface level and fills the spaces between its gills with air. Enabling the fish to hold onto the air for a considerable amount of time after returning to the bottom. After that, the fish takes in oxygen from the air kept in this organ. The tough, undemanding species known as Clarias has done well to adapt to the unfavorable conditions that are frequently present in the transitory waters of Africa (Laszlo et al, 2002).

By isolating catfish in a cage-like enclosure and placing them in an experimental tank devoid of atmospheric air, (Balao et al., 2011) showed that C. gariepinus has high tolerance to oxygen deprivation. During the 30-hour observation period, the researchers found that the catfish persisted at the bottom, alternating extended periods of inactivity with sluggish movements for foraging, and displayed no significant behavioral differences between normoxic circumstances and

unfettered access to atmospheric air. In this situation, breathing was rather regular (ranging from 30 to 35 breaths per minute).

They also thrive well in salty, swampy environments polluted with poisonous fumes and organic matter such as ammonia than most other species. Clarias can eat a variety of diets including food waste and can withstand densely populated situations. All these attributes make this species well suitable for aquaculture, particularly in intensive recirculation systems as well as laboratory research. The fact that this fish needs warm temperatures makes it difficult to grow it in temperate settings. It becomes extremely susceptible to disease below 15°C and perishes. Together with the many characteristics, *Clarias* is very simple to grow, responds well to hormone therapy, and because of its ability to quickly create eggs, it can reproduce several times per year. Although this species well-dwells in Africa, it wasn't until the 1950s that aqua culturists began to recognize its exceptional features. The fish can be raised in open ponds during the summer. Now that sophisticated recirculating systems have been developed, Clarias are successfully raised in great numbers. Many people prefer boneless meat, and production expenses are inexpensive. Clarias reach sexual maturity quickly, making it possible to harvest 400–600 g fish from intensive aquaculture systems (8 months–1 year old). (Laszlo et al, 2002)

2.5 Fish feed

Catfish are omnivorous but primarily piscivorous, and they eat a variety of foods. This means that in the context of farming, they need a lot of dietary protein to function well, hence they must be fed feeds that are high in crude protein (35%–50%), Several nutrients are needed for catfish feed to optimize growth and boost profits for catfish growers. (Fregene et al,2020).

The increasing popularity of fish farming has resulted in an increase in the need for fish feed, and the protein component of fish meal is critical to worldwide fish farming. As a result, the amount of fish meal used in aquafeed is decreasing in favor of less expensive, more easily accessible protein sources. The price of fish feed is a significant barrier to fish farming on a worldwide scale as feed is a deciding component in the aquaculture value chain which ranges from 60–75% **(**Babalola 2010, Gatlin 2007**)** And 60-80% (Ragasa et al, 2022) of the total cost of fish production. Fish meal is an excellent protein source in fish feed due to its balanced amino acid profile and high digestibility. Fish meal has typically been the predominant source of protein in fish feed composition, especially for predatory fish species like catfish, salmon, and eel. and contains approximately 5 to 50% fish meal (Dersjant-Li, Y., 2021).

2.5.1 Types of fish feed

Nutrients such as bone meal, meat meal, and avian byproducts have been used to replace fish meal in fish feed. Animal proteins can largely replace fish meal because they are high-quality, low-cost protein sources (El-Sayed, 1998). Fishmeal can be substituted with other high-protein plant-based proteins, such as oil seeds. These ingredients are inexpensive and widely available (Tyapkova et al, 2016).

Studies showed that 20-100% of dietary protein from fish meal when replaced by up to 40% soy base protein concentrate (SPC) did not have any negative influence on growth performance. Whereas replacing fishmeal by soy base protein concentrate (SPC) or soy flour at high inclusion levels, in general, reduced growth rate in the Chinese sucker fish possibly due to the presence of antigenic proteins. (Yu et al, 2013).

The use of soybean meal (SBM) as a source of protein for fish meal was also studied by (Choi et al, 2020) by deactivating the antinutritive compounds present through mild heat and studies showed Soy meal could replace 40% of the fish meal in rainbow trout diets without influencing growth performance or feed conversion ratio. The study tracked the growth of rainbow trout fed with fermented soy product (FSM) for 8 weeks with the intention of eventually replacing conventional fishmeal (FM) with FSM. There were six different treatments used to substitute for 20%, 40%, or 60% of the FM. Up to 40% replacement, there were no significant differences in weight gain (WG), feed conversion ratio (FCR), and digestibility of crude protein and dry matter between the six treatment groups and the control groups. When all the FSMs were used in place of 60% FM, however, WG decreased and FCR increased dramatically. The eight-week feeding research concluded that both FSMs could effectively replace 40% of fish meal in rainbow trout without impairing the fish's growth or their capacity to utilize the FSM as food.

In the study by Shu et al. in 2013, the protein content and B. subtilis used for fermentation both significantly increased when compared to raw soybean meal. The protein content of the fermented soybean after 72 hours increased by 19%, and the total amount of hydrolyzed amino acids increased by 18.75%. Comparing fermented soybean meal to raw soybean meal, the free amino acid profile and quantity rose significantly by 374.9%. On a diet with a 37% protein and 7% fat content, the fermented soybean meal was also found to be an effective substitute for fish meal (FM). This agrees with Silvery-black porgy juveniles fed for a two-month period to test the amount of soy products (SP) that might replace fishmeal (FM) in formulated diets without affecting growth

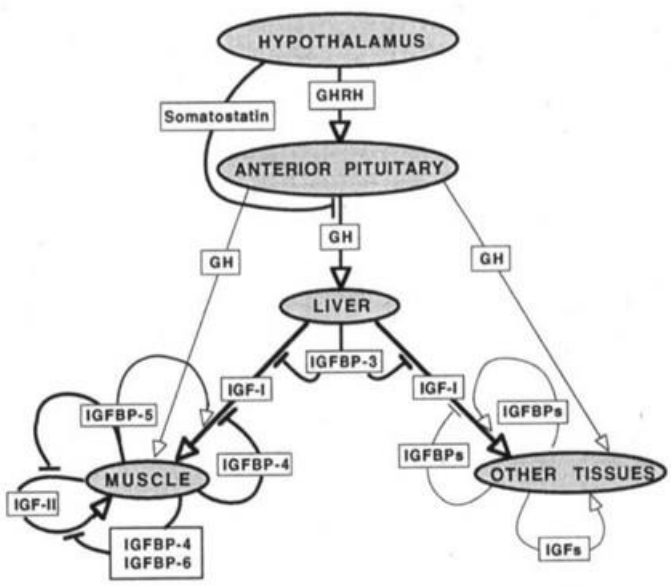
performance. FM was swapped out for SP, whereas FM served as the primary source of protein in the control diet (FM). By increasing dietary SP, feed intake, growth performance, feed utilization, and digestibility of dry matter, protein, and fat drastically improved. (Yagoubi et al, 2016)

2.6 Endocrinology of the Gh biosynthesis and release

Energy for biological functions is regulated through feed intake by combining exogenous and endogenous components. The endocrine system primarily produces and secretes growth related hormones and maintains cellular activity by relaying signals between the body's organs, responsible for this kind of homeostasis. **(**Bertucci **et al, 2019**).

The linear interaction of the hypothalamic-pituitary-somatotropic (HPS) axis, which includes the hormones growth hormone releasing hormone (GHRH), somatostatin (SST), insulin-like growth factors (IGFs), and other neuroendocrinology regulators like thyroid hormones (TH), glucocorticoids (GC), estrogens, and androgens, controls the growth of living cells. Its occurrence reflects the balance between the physiological processes of an organism and the consumption, utilization, and feed composition and quality. (**Volkoff et al, 2010; Triantaphyllopoulos et al 2020).**

Figure: 5 Simplified illustration of HPS axis, liver, and muscle hormonal connection



Source: Florini et al 1996

Growth hormone is a member of growth factors and contains about 191 amino acid residues. (Dai et al, 2015; Liu et al, 2018). Feedback mechanisms that function on positive and negative feedback systems, mediate the release of GH from the anterior pituitary gland. Somatostatin and growth hormone (GH) moderate their own release, which is controlled through ultrashort-loop feedback. Although ultrashort-loop feedback for GHRH has been hypothesized, there isn't enough proof to support it. Short-loop feedback between somatostatin, GHRH, and GH controls GH pulpability. GH stimulates somatostatin in the hypothalamus and may suppress GHRH release in the median eminence. Somatostatin, which limits GH release, goes to the pituitary gland after being released into the portal circulation, whereas GHRH increases GH release. Moreover, GHRH neurons are hypothesized to drive somatostatin neuronal activity, leading to somatostatin production, as somatostatin suppresses GHRH activity. A variety of peripheral factors influence how much GH is released in response to physiological demand such as long-loop feedback from insulin-like growth factor-1 (IGF-1) a potent mitogenic factor. Fish osmoregulation and the neuroendocrine regulation of development depend on the insulin-like growth factors IGF I and II. Since the plasma concentration of insulin-like growth factor can affect fish growth, it is widely employed as an indication in nutritional research studies to evaluate the effectiveness of specific nutrients. It is therefore essential for measuring growth, reproduction, and development in aquaculture. It is mainly produced in the liver in response to GH and results in the suppression of GH release by promoting the release of somatostatin and the inhibition of GH and GHRH (Fig 5.) (Steyn et al, 2016, Chandhini,2021)

2.6.1 Expression of genes in the organ's relation to somatic cell growth

Modifications in mRNA/protein levels of a particular hormone after fasting or feeding most likely reflect its physiological function in controlling appetite. Hence variations in gene expression and/or protein concentration levels of the hormones or receptors that control hunger are frequently linked to changes in eating habits and food nutritional quality in fish (Assan et al, 2021).

In these research, quantitative functional gene expression is crucial because it provides a clear understanding of the sophisticated molecular regulation process that functions under diverse physiological, developmental, and pathological states. The three methods for gene expression

investigations that are now most effective are real-time quantitative polymerase chain reaction (RT-qPCR), microarray, and northern blot. **(**Ouyang et al, 2019**).**

In 2013, Yang et al. used tilapias organs as a model. These organs were spiked with reference genes that included ubiquitin-conjugating enzyme (UBCE), beta-2-microglobulin (B2M), elongation factor 1 alpha (EF1A), tubulin alpha chain-like (TUBA), and beta actin (ACTB) in the brain, muscle, spleen, kidney, liver, heart, and intestine. All possible reference genes were shown to have transcriptional variations depending on tissue. EF1A was the most functional protein in the heart and muscle. GADPH was the most effective gene in the colon and brain. Under normal conditions, UBCE and 18S rRNA were the most ubiquitously expressed genes across tissues. These results suggested that examining gene expression in different tissues would benefit by using RT-qPCR with a combination of two or more reference genes.

Owing to its dynamic range, detection limit, precision, quantification accuracy, repeatability, low price, and greater ease of real-time expression monitoring, RT-qPCR is usually preferred and generally viewed as the superior standard for mRNA transcript quantification when compared to microarrays and RNA-seq **(**Derveaux et al, 2010**).** Furthermore, RNA quality, which can largely compromise the RNA purity and RNA integrity, sparked interest in gene expression analysis because it was demonstrated to have a significant impact on the performance and quantitative data of RT-qPCR. Other critical factors that affect RT-qPCR accuracy include cDNA quality, initial template quantity, primer specificity, and PCR amplification efficiency (Becker et al, 2010). Several researchers have embraced this wonderful strategy for analyzing gene expression.

3.0 METHODOLOGY

3.1. Experimental design

The research was carried out with the support of the iFishienci (Intelligent Fish feeding through Integration of Enabling technologies and Circular principles) program. This fish feeding research aims for higher quantity and quality of fish production while maintaining a sustainable economic environment. By this, in 2018 at the Kisbajcs's African catfish fish farm (Győri Előre Halászati Termelőszövetkezet) a breeding project was started. African catfishes' lines were made to test whether adaptation on lower cost (lower fishmeal containing) possible or not. There are three fish group lines which got the experimental feed (party substituted fish meal with soybean meal) and one control fish group (conventional feed). Each group was bred with their own group members using 4 multifactorial crossing (5 male x 5 female). When the 4th generation was made, they were feed the same way as their ancestors, but at the stage of 4 months about 60 individuals were brought from each group to Szent Istvan Campus of Hungarian University of Agriculture and Life Sciences. They were individually marked using PIT tags and mixed equally in 3 fish tanks (Table 2) for 6 weeks. Each tank had the same environmental properties (about 25 °C, drum filter cleaning, same light properties).

Table : 2 Demonstration trial design

	1. tank (Experimental feed)	**2. tank (Experimental feed)**	**3. tank (Control feed)**
PS1 fish group	**18 fish**	**18 fish**	**18 fish**
PS2 fish group	**18 fish**	**18 fish**	**18 fish**
PS3 fish group	**18 fish**	**18 fish**	**18 fish**
Control fish group	**18 fish**	**18 fish**	**18 fish**

Feeding was carried out once a day, around 10:00 o'clock. Fishes got 1 % of their weight in fish feed. Every week they were measured by weight and the fish feed amount was updated.

3.2. Sample collecting

After the end of the 6 weeks samples were taken. The fishes were put in sleep, complying with animal welfare regulations. Samples (the whole brain and one piece of the liver) were put in Tri-Reagent (MRC 118), then snapped frozen in liquid nitrogen. We put them in -80 °C refrigerator until RNA extraction.

3.3. RNA extraction

The RNA extraction was carried out using Trizol-Chloroform extraction method.

We used Electric Grinding Equipment for homogenization. The homogenization happened in the Tri-Reagent, then 100 µl was pipetted into a centrifuge tube. The tube was filled up to 1 ml with Trizol, then we centrifuged with 12.000g (rcf) for 15 minutes on 4 °C. The supernatant was taken out and put in a new tube. Chloroform was added and after 5 minutes of incubating on room temperature, we centrifuged again with 12.000g (rcf) for 15 minutes on 4 °C. The transparent phase was pipetted into a new tube, then it was filled up to 1 ml end volume with isopropanol. After 10 minutes of room temperature incubation the RNA was denatured and became visible. We spined again for 10 minutes on 7,500g (rcf) on 4 °C. The pellet stuck to the bottom. We poured down the unnecessary isopropanol and added 75 % EtOH to wash the pellet. We centrifuged on 7500 g, for 5 minute and on 4 °C.

The pellet was dried out and 20 µl nuclease free water (NFW) was added per sample, we incubated them on 55 °C for 10 minutes.

3.4. DNase treatment

For DNase treatment we used DNase I, RNase-free (Thermo Scientific) with the recommended protocol (Table 3). We incubated the samples for 30 minutes on 37 °C.

Table : 3 DNase treatment protocol

Component	Quantity (µl)1x sample
RNA	10
NFW (H_2O)	7,5
10x reaction buffer with $MgCl_2$	2
DNas enzym	0,5
∑	20

3.5. Concentration, purity, and RNA structure control

For spectrophotometric examination we used Nanodrop OneC. For blanking we used NFW.

We run the samples on 1,5 % gel for 25 minutes on 120 V.

3.6. RNA pools mix, cDNA writing and dilution.

The RNA pools are demonstrated in Table 16 in the results chapter. During the pool making we took out each sample 1000 ng of RNA and mixed in a centrifuge tube, then we took out 1000 ng of mixed RNA to write cDNA.

For cDNA writing we used Random hexamer (Thermo Scientific) and Revert Aid Reverse Transcriptase (200U/µl) (Thermo Scientific) with the recommended protocols (Table 4 and 5). For the hexamer primer annealing we incubated the reaction on 65 °C for 5 minutes. After the annealing phase we wrote the cDNA by adding the Table 11. mix to the annealed primer mix. PCR heat protocol for the cDNA writing: 25°C 10 minutes, 42°C 60 minutes, 70°C 10 minutes.

We diluted the cDNA with twofold dilution series (Table 6).

We used for endogenous control elongation factor 1 alpha (EF1A).

.

Table : 4 Random hexamer protocol

Component	Quantity 1x sample
Total RNA	0,1-5 µg
Random hexamer primer	2 µl
NFW (H_2O)	up to 13 µl

Table :5 Revert Aid Reverse Transcriptase protocol

Component	Quantity (µl)
5x HOT EvaGreen qPCR Super mix with ROX	4
dNTP Mix	2
Revert Aid Reverse Transcriptase	1

Table : 6 Dilution series of cDNA

Dilution (x)	1	2	4	8	16	32
cDNA (ng)	1000	500	250	125	62,5	31,25

3.7. Real-Time qPCR properties and protocol

For gene expression measuring we used Real-Time qPCR (StepOne™ Real-Time PCR System). The qPCR components protocol represented in the Table 7., the heat profile in Table 8., the qPCR map in Table 9. and the used primers in the Table 10.

Table : 7qPCR protocol

Components	Quantity (1x-µl)	Concentration (cc)
5x HOT FIREPOL qPCR Super mix	3	5x
NFW	7	-
F primer	1	6,6x
R primer	1	6,6x
Template	3	-
∑	15	-

Table : 8qPCR heat profile

qPCR steps	Heat (°C)	Time (min:sec)	Cycle (x)
Denaturation	95	10:00	1
Denaturation	95	00:30	45
Primer annealing*	57,5	00:10	45
Elongation	72	00:25	45
Melt curve	60-94 (↥0,5)	00:10	1

*Changing in the context of primers

Table 9qPCR primers used for gene expression measurement

Primer name	Primer sequence (5’ ↦ 3’)	Annealing temp (°C)	Source
Growth hormone forward	GAACCTGGGCAACCCTAA	56,7	Wang et al., 2017
Growth hormone reverse	AAGCAAGACAGCAGACGGA	54,7	Wang et al., 2017
Growth hormone receptor forward	ATTGTATTTCCAGACCCACCT	53.7	Molecular Ecology Department
Growth hormone receptor reverse	CCTCACCCTGACTTCATACTC	54.3	Molecular Ecology Department
Insulin growth factor I forward	TTTATTTCAGCAAGCCGACAG	53.6	Molecular Ecology Department

Insulin growth factor I reverse	TACATCCGATAGTTCCTCCC	53	Molecular Ecology Department
Insulin growth factor II forward	CTTCACAAGGATAGCACAAGG	53.4	Molecular Ecology Department
Insulin growth factor II reverse	TTAAACTTTCTGGAGCGGAG	52.3	Molecular Ecology Department

4.0 Results

4.1. Spectrophotometric examination results represented in Table 10.

Table : 10Spectrophotometric examination results

Sample name (PIT tag ID)	RNA pools (Fish group – feed type)	Concentration (ng/µl)	A260/280 (nm)	A260/230 (nm)
6754 Brain/Liver	PS1 – Exp. feed	2925/2830	2,07/2,10	2,08/1,5
6775 Brain/Liver	PS1 – Exp. feed	1138/5153	2,23/2,34	1,6/1,07
6720 Brain/Liver	PS1 – Exp. feed	2010/8885	2,18/2,37	2,09/1,39
1334 Brain/Liver	PS2 – Exp. feed	1257/2151	2,12/2,48	1,69/1,52
2476 Brain/Liver	PS2 – Exp. feed	3998/11745	2,3/1,96	1,99/1,49
1804 Brain/Liver	PS2 – Exp. feed	2409/1392	2,27/2,40	1,89/1,6
1991 Brain/Liver	PS3 – Exp. feed	1863/2964	2,25/2,34	1,90/1,09
1956 Brain/Liver	PS3 – Exp. feed	2884/5297	2,27/2,38	2,01/1,14
1998 Brain/Liver	PS3 – Exp. feed	3723/3868	2,17/2,29	2,07/1,39
1972 Brain/Liver	Control – Exp. feed	3477/3400	2,25/2,34	1,85/1,30
1961 Brain/Liver	Control – Exp. feed	1474/3806	2,29/2,44	1,86/1,17
1952 Brain/Liver	Control – Exp. feed	1049/2700	2,08/2,11	2,00/1,75
1330 Brain/Liver	PS1 – Control feed	1428/2939	2,22/2,25	1,77/1,33
1387 Brain/Liver	PS1 – Control feed	2205/9248	2,11/2,16	2,06/1,40
1368 Brain/Liver	PS1 – Control feed	1286/3217	2,08/2,47	2,01/1,20
2067 Brain/Liver	PS2 – Control feed	5111/3606	2,1/2,45	1,88/1,20
1765 Brain/Liver	PS2 – Control feed	1857/1770	2,24/2,40	1,89/1,53
1970 Brain/Liver	PS3 – Control feed	1278/4561	2,17/2,41	2,08/1,14
1973 Brain/Liver	PS3 – Control feed	3348/4270	2,25/2,31	1,79/1,46
1954 Brain/Liver	PS3 – Control feed	3410/8157	2,12/2,41	1,90/1,23
6760 Brain/Liver	Control – Control feed	1496/2328	2,12/2,28	1,93/0,99
6799 Brain/Liver	Control – Control feed	1951/3066	2,12/2,26	1,9/1,33
6728 Brain/Liver	Control – Control feed	1022/4261	2,14/2,32	1,92/0,98

4.2 qPCR results

4.2.1. Statistical analysis for growth hormone, growth hormone receptor, insulin-like growth I and insulin-like growth factor II genes

Table 11 represents the result of the growth hormone receptor gene (*gh*) expression pattern between the fish groups and the feed groups. There is no clear correlation between the feed and the fish (genetic background) groups because the PS3 groups have an opposite correlation against the other groups. If we do not consider the PS3 group, a direct correlation can be seen in the case of the other groups.

Table: 11represents growth hormone gene expression results. It contains beside the data of the run, the cycle threshold (CT) results which were used to calculate the 2*ΔΔCT values

Legend: PS1EB (Positive Selection group 1 – Experimental feed – Brain sample), PS1CB (Positive Selection group 1 – Control feed – Brain sample), GH (growth hormone gene primers), EF1A (elongation factor 1 alpha gene primers), 16X (16 times dilution), CT (cycle threshold)

	1	2	3	4	5	6	7	8
A	PS1EB GH 16X CT: 17,10	PS1EB GH 16X CT: 15,34	PS1EB GH 16X CT: 15,16	PS1EB EF1A 16X CT: 26,44	PS1EB EF1A 16X CT: 29,61	PS1EB EF1A 16X CT: 27,86	GH NEG CONT CT: 37,06	EF1A NEG CONT CT: 35,85
B	PS2EB GH 16X CT: 19,28	PS2EB GH 16X CT: 22,71	PS2EB GH 16X CT: 18,92	PS2EB EF1A 16X CT: 33,26	PS2EB EF1A 16X CT: 32,98	PS2EB EF1A 16X CT: 32,48		
C	PS3EB GH 16X CT: 18,76	PS3EB GH 16X CT: 21,46	PS3EB GH 16X CT: 22,46	PS3EB EF1A 16X CT: 32,76	PS3EB EF1A 16X CT: 31,22	PS3EB EF1A 16X CT: 28,51		
D	KEB GH 16X CT: 22,57	KEB GH 16X CT: 22,07	KEB GH 16X CT: 21,40	KEB EF1A 16X CT: 33,01	KEB EF1A 16X CT: 33,12	KEB EF1A 16X CT: 32,80		
E	PS1CB GH 16X CT: 23,80	PS1CB GH 16X CT: 22,54	PS1CB GH 16X CT: 24,53	PS1CB EF1A 16X CT: 32,66	PS1CB EF1A 16X CT: 33,13	PS1CB EF1A 16X CT: 33,19		
F	PS2CB	PS2CB	PS2CB	PS2CB	PS2CB	PS2CB		

	GH 16X CT: 26,35	GH 16X CT: 22,57	GH 16X CT: 26,26	EF1A 16X CT: 33,93	EF1A 16X CT: 34,49	EF1A 16X CT: 33,15		
G	PS3CB GH 16X CT: 18,47	PS3CB GH 16X CT: 17,73	PS3CB GH 16X CT: 18,68	PS3CB EF1A 16X CT: 31,33	PS3CB EF1A 16X CT: 32,40	PS3CB EF1A 16X CT: 28,85		
H	KCB GH 16X CT: 25,69	KCB GH 16X CT: 22,99	KCB GH 16X CT: 18,35	KCB EF1A 16X CT: 31,68	KCB EF1A 16X CT: 32,92	KCB EF1A 16X CT: 32,01		

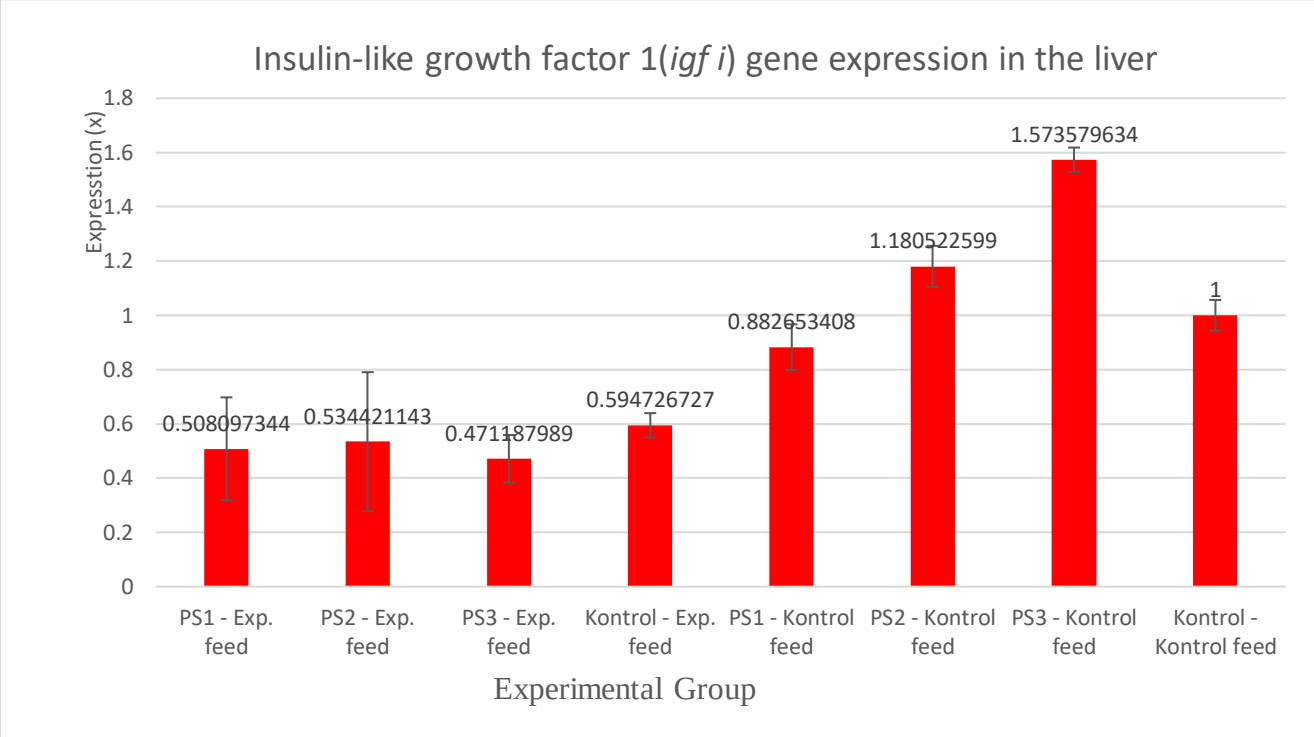


Figure: 6 The bars represent the graphical relationship between IGF-I gene expression performance of the treatments in the liver obtained by the 2*(Delta Delta Ct) average Ct values. The level of mRNA was generated using (qPCR machine Model) and IGF-I specific primer

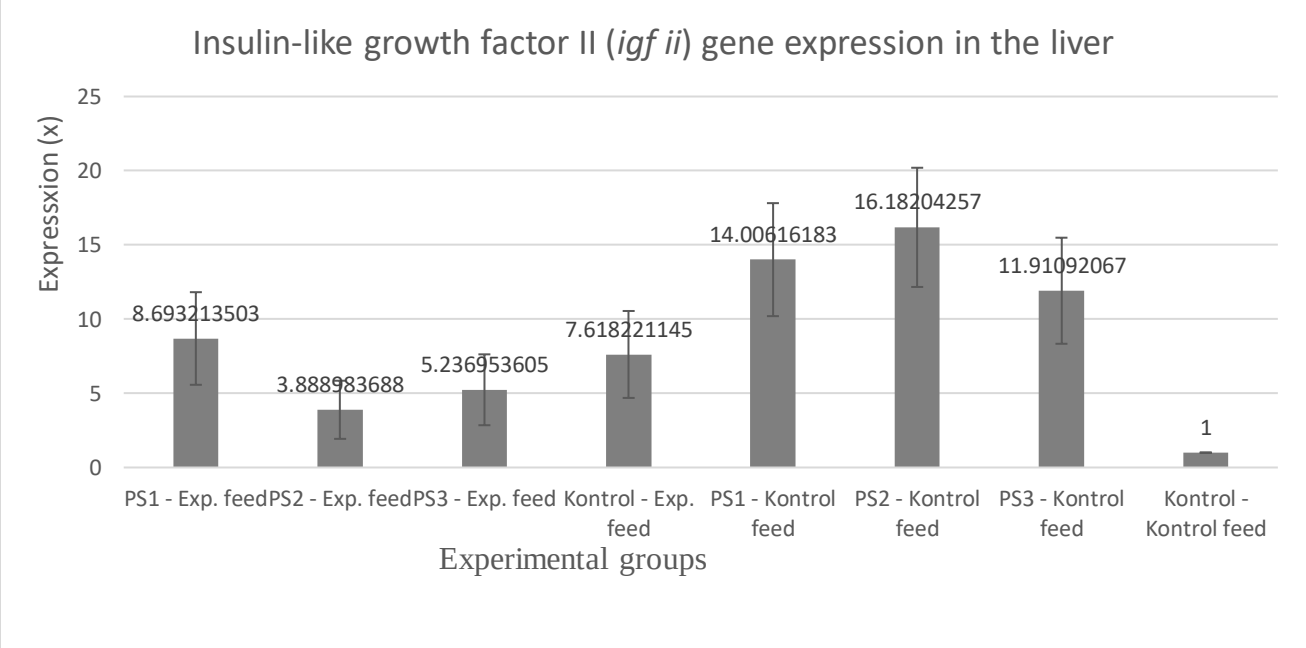


Figure : 7 The bars represent the graphical relationship between IGF-II gene expression performance of the treatments in the liver obtained by the 2*(Delta Delta Ct) average Ct values. The level of mRNA was generated using (qPCR machine Model) and IGF-II specific primers

.

Estimation of the levels of target gene IGF I and IGF II expression in the liver, as well as a comparison of the effects of the experimental feed and the control feed on the rate of growth as seen above. According to the data presented in the preceding histogram (fig 6, fig7), the right bars of fig 6 shows the full fishmeal feeds PS1 kon, PS2 kon, and PS3 kon, as well as the kon-kon feeds, all had higher levels of IGF I in the liver than the experimental feed.

When the interconnections between the various options are considered, the PS1 kon-kon feed was found to have expression values of 0.883 which was higher than the PS1 Exp feed, which had expression values of 0.508. This pattern was seen in every one of the PS Exp feeds as well as the control-control feeds that went along with them in IGFII as well (fig 6) where PS1 kon feed had an expression level of about 14.001 and PS1 Exp feed had expression value of 8.693, even though the changes were not the same, averagely there was about two times (2X) expression in each case indicating the feed conversion ratio of the fishmeal had better rate compared to the experimental feed. In the end, there was no correlation between the expression patterns that occurred within PS Exp feed and PS control feeds.

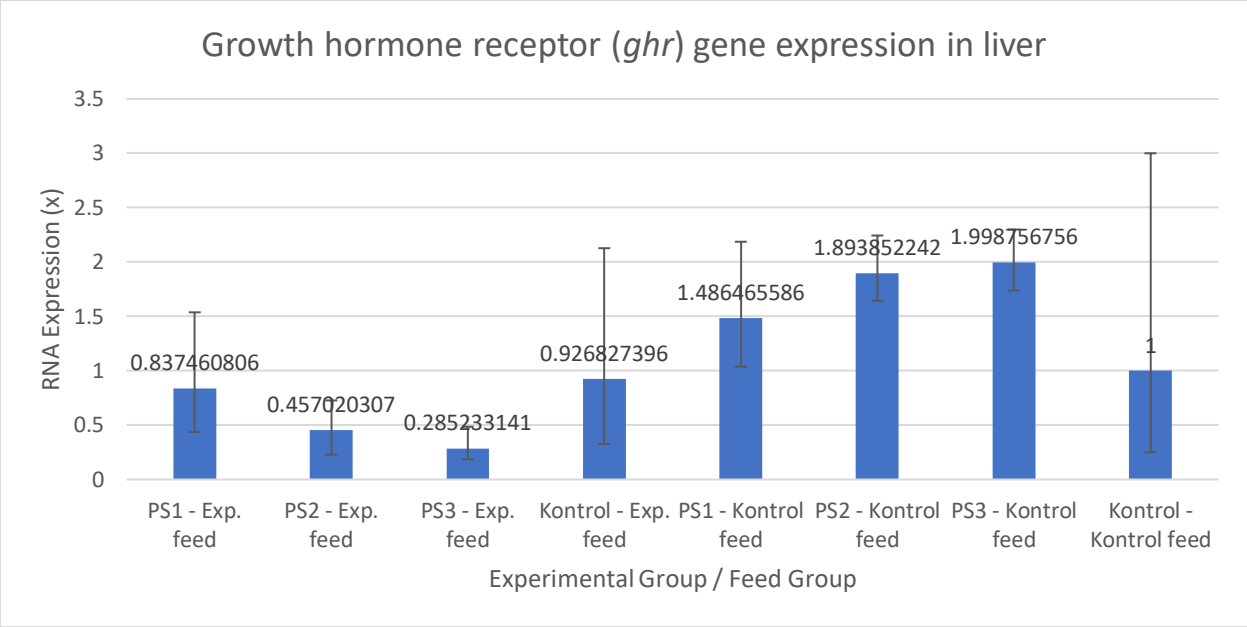


Figure: 8 The opened bars represent the graphical relationship between GhR gene expression performance of the treatments in the brain obtained by the 2*(Delta Delta Ct) average Ct values. The level of mRNA was generated using (qPCR machine Model) and GhR specific primers.

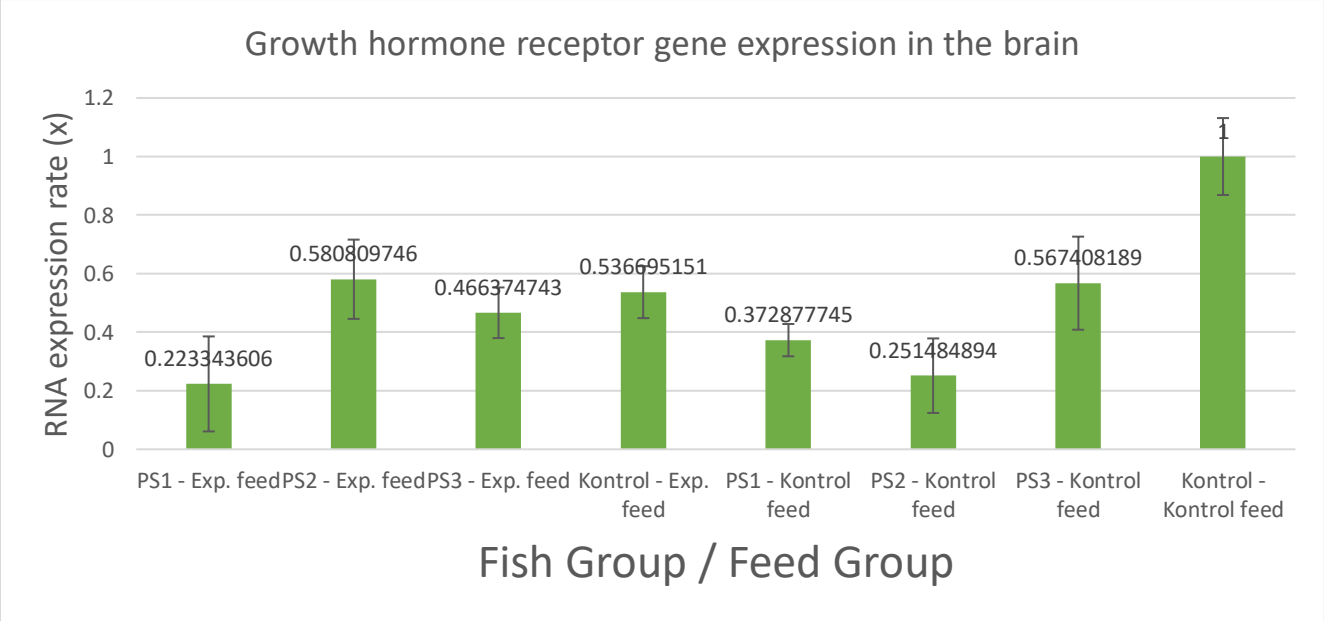


Figure 9: The opened bars represent the graphical relationship between GhR gene expression performance of the treatments in the brain obtained by the 2*(Delta Delta Ct) average Ct values. The level of mRNA was generated using (qPCR machine Model) and GhR specific primers.

Comparison of the secretion levels of GhR target gene in the brain and liver are presented above (fig 8 and 9), evaluating the effect of the two feed treatments on the release of GhR, the traditional fishmeal performed better than the experimental feed even though there was no significant difference between the Kon Exp. feed and Kon-Kon feed with values of 0.927 and 1 respectively in the liver with PS3 Kon feed being the highest expressed GhR gene in the liver. GhR gene expression in the liver seems to have similar pattern of expression to IGF I and IGF II in the liver.

However, this trend was not evident in the brain as Kon Exp. Feed and Kon-Kon feed gave expression values of 0.537 and 1.000 respectively, With Kon-Kon feed being the highest expressed GhR genes in the brain. From these findings, it is obvious that there was no correlation in GhR release in the liver and the brain and furthermore there was no connection among the various groups.

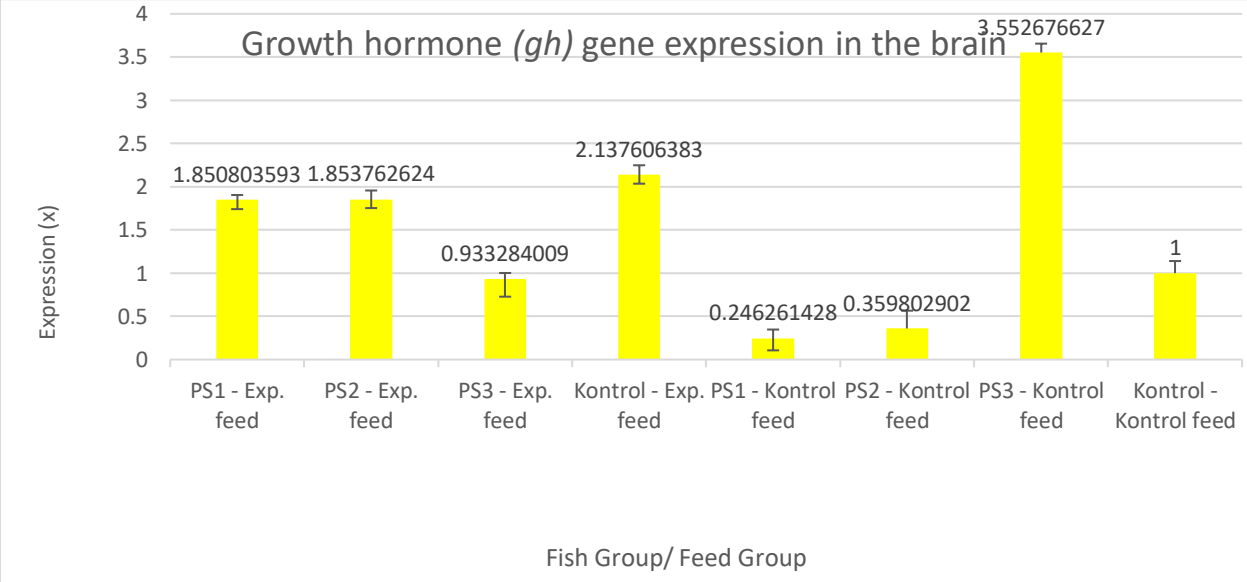


Figure: 10 The bars represent the graphical relationship between Gh gene expression performance of the treatments in the brain obtained by the 2*(Delta Delta Ct) average Ct values. The level of mRNA was generated using (qPCR machine Model) and Gh specific primers.

Growth hormone (GH) performance in the research work is described in the histogram above (Fig 10), PS3-Kon feed had the highest expression level followed by Kon. Exp feed with values of 3.553 and 2.138 respectively. PS1 Kon. Feed had the lowest expression rate with PSI-Exp feed and PS2 -Exp. Feed recording similar expression performance of 1.851 and 1.854 respectively. Surprisingly

PS1- Kon feed and PS2 Kon feed made of traditional fishmeal known to be effective protein source had the lowest expression values of 0.246 and 0.359 respectively.

5.0 DISCUSSION

The objective of this study was to find out the relationship between the growth performance of traditional fishmeal feed (FM) and Soybean Meal (SBM) incorporated fish feed. The role of the feed type is vital to the growth physiology and survival to fish breeds in depth knowledge of this would be useful in the development and management of aquaculture. (Wynne et al, 2005)

In the present study, we compared the growth-related mRNA gene expression levels in the brain and liver of the fourth generation of African catfish (*Clarias gariepinus)* put into two groups. Each group consisted of three positive selections and a control (PS1, PS2, PS3 and Kon). The groups were kept in the same environmental conditions and fed with different types of feed according to the research design. To assess the impact of the various feed types on the growth physiology of African catfish (*Clarias gariepinus*), quantitative real-time polymerase chain reaction (q RT-PCR) was used to measure the mRNA gene expressions of IGF I, IGF II, Gh, and GhR within the various groups.

For accuracy to be assured the extracted RNA was confirmed by running the sample through electrophoresis on a 1.5% agarose gel. This verified both the integrity of the RNA as well as the absence of primer dimers in the sample. After the data was collected, according to (Livak et al., 2001), the Delta-Delta Ct was found by measuring the Ct of mRNA synthesis at the point when the PCR curve hits the threshold of detection in the straight part of the curve. Our analyses revealed that there was no correlation in specific gene expression responses and the feed type among the various groups of African catfish. Furthermore, the fishmeal (FM) was generally seen to have better performance as compared to the soybean meal (SBM). This was not in agreement with various research works considered in the literature, according to (Yu et al, 2013) there was no significant differences between whole fishmeal and up to 40% soymeal replacement which agrees with (Choi et al 2020), Their research used soybean meal (SBM) as a source of protein replacement for fish meal by deactivating the antinutritive compounds through mild heat and could replace up to 40% of fishmeal.

6.0 CONCLUSION AND RECOMMENDATION

In conclusion this research did not agree with previous studies, since there was no clear positive correlation between the performance of the feeds among the groups.

As mentioned in the literature review, (Yang et al, 2013) validated the utilization of RT-qPCR to study gene expression using reference genes spiked into various organs of the Nile tilapia and concluded that all reference genes used showed transcriptional variations depending on tissue. This could be a reason for the variation of GhR gene expression in the brain and the liver as the IGF-I and IGF-II in the liver seems to have followed a particular pattern or correlation.

6.1 RECOMENDATIONS

- It is recommended that primers should be validated with a reference standard gene to check reliability.
- It is also recommended that younger African catfish should be used as growth related hormones are more active young animals.

Summary

In recent decades, the need for high-quality and high-quantity fish products has been steadily increasing worldwide (FAO 2023). Unfortunately the fish stocks of the ocean can not ensure this amount of demand. This gave a big opportunity for inland fishfarming. Nowadays, many of our freshwater fish species, like sturgeons and catfish farmed intensively. In Hungary the second most farmed fish is the African catfish (*Clarias gariepinus*). As in the world, in Hungary also, the biggest expense on farms is the fish feed (50%) due to it's relatively high amount of fishmeal (fish oil and protein). Fishmeal is essential for optimal growth and development but lowering the animal content and complete it with alternative macronutrient sources could have a positive economic effect on fish farms' economic situation. Supplementation of fishmeal can reduce growth and overall fitness of fish, however studies suggest that through adaptation the deteriorating effect can be solved (Refstie et al 1997). The iFishIENCi (Intelligent Fish Feeding through Integration of Enabling technologies and Circular principles) international program aimed in 2019 to create an African catfish lane which is adapted to less costly fish feed (with soya meal supplementation) in Kisbajcs. Four lanes of catfish were made. Three of them (PS1, PS2, PS3) were fed with experimental feed (lowered amount of fishmeal) and 1 line with control feed (high amount of fishmeal). This thesis evaluates 4 genes in 2 types of tissue to investigate whether the adaptation has an impact on growth-related genes expression or not.

4 months old *Clarias gariepinus* were brought from Kisbajcs to Fish Department of Hungarian University of Agriculture and Life Sciences. After 6 weeks of demonstration experiment where the 4 lanes were equally mixed in experimental and control feed tanks, brain and liver samples were taken. Real-Time qPCR was used to establish the relative gene expression differences between the fish and feed groups at the case of growth hormone (*gh*), growth hormone receptor (*ghr*), insuline-like growth factor-I (*igf-I*), and insuline-like growth factor-II (*igf-II*) genes.

In the brain, the *gh* and the *ghr* genes relative expression rate between the fish and feed groups did not show coherent results. In the case of liver only two genes (*igf-I* and *ghr*) showed unidirectional results. Both of their expression patterns suggest the conclusion of the control feed makes the fishes to produce more igf-I peptide and gh receptor.

These results raise the possibility that there may be real differences between the fish and feed groups. However, the correlations found cannot be considered authoritative, as further research is needed.

To get consistent results more genes and fishes should be involved in the future. Every gene expression should be evaluated, which affects the growth-related key genes (*gh, igf-I, msnt, etc*) behavior. Expression pattern conclusion should be compared to the fish weight measurement result.

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APPENDICES

DECLARATION

on authenticity and public assess of final essay/thesis/master's thesis/portfolio[1]

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Student's Neptune ID:	TWUO7G
Title of the document:	Analysis of gene expression patterns of insulin-like growth factors, growth hormone and growth hormone receptor genes in different feeding groups of *Clarias gariepinus* (African Catfish)
Year of publication:	2023
Department:	Agriculture Biotechnology

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Table 12 represents igf1 gene expression results in the liver. It contains beside the data of the run, the cycle threshold (CT) results which were used to calculate the 2*ΔΔCT values.

Sample Name	Target Name	Cт	Average of Cts	Delta Ct	Delta - delta Ct	2^(–delta delta CT)
PS1 - Exp. feed	IGF1	28.2761269	28.25581423	-1.530680339	0.976823171	0.51
PS1 - Exp. feed	IGF1	28.1920872				
PS1 - Exp. feed	IGF1	28.2992287				
PS1 - Exp. feed	EF1A	29.6771145	29.78649457			
PS1 - Exp. feed	EF1A	29.5317307				
PS1 - Exp. feed	EF1A	30.1506386				
PS2 - Exp. feed	IGF1	28.4509277	28.62146759	-1.6035525	0.903951009	0.53
PS2 - Exp. feed	IGF1	28.5378361				
PS2 - Exp. feed	IGF1	28.875639				
PS2 - Exp. feed	EF1A	30.378273	30.22502009			
PS2 - Exp. feed	EF1A	29.7894878				
PS2 - Exp. feed	EF1A	30.5072994				
PS3 - Exp. feed	IGF1	29.1507607	29.25685438	-1.421878179	1.085625331	0.47
PS3 - Exp. feed	IGF1	29.3852406				
PS3 - Exp. feed	IGF1	29.2345619				
PS3 - Exp. feed	EF1A	30.6324654	30.67873255			
PS3 - Exp. feed	EF1A	30.7872543				
PS3 - Exp. feed	EF1A	30.616478				
Kontrol - Exp. feed	IGF1	27.9379253	28.00173696	-1.757802327	0.749701182	0.59
Kontrol - Exp. feed	IGF1	28.0316143				
Kontrol - Exp. feed	IGF1	28.0356712				
Kontrol - Exp. feed	EF1A	29.7451115	29.75953929			
Kontrol - Exp. feed	EF1A	29.8194923				
Kontrol - Exp. feed	EF1A	29.7140141				
PS1 - Kontrol feed	IGF1	28.4686718	28.47097333	-2.32742246	0.18008105	0.9
PS1 - Kontrol feed	IGF1	28.4045525				
PS1 - Kontrol feed	IGF1	28.5396957				
PS1 - Kontrol feed	EF1A	30.9151344	30.79839579			
PS1 - Kontrol feed	EF1A	30.6590939				
PS1 - Kontrol feed	EF1A	30.8209591				
PS2 - Kontrol feed	IGF1	27.6944199	27.79202334	-2.746929169	-0.239425659	1.18
PS2 - Kontrol feed	IGF1	27.8282604				
PS2 - Kontrol feed	IGF1	27.8533897				
PS2 - Kontrol feed	EF1A	30.426918	30.53895251			
PS2 - Kontrol feed	EF1A	30.6167068				
PS2 - Kontrol feed	EF1A	30.5732327				
PS3 - Kontrol feed	IGF1	27.709137	27.75181007	-3.161553701	-0.654050191	1.57
PS3 - Kontrol feed	IGF1	27.8271561				
PS3 - Kontrol feed	IGF1	27.7191372				
PS3 - Kontrol feed	EF1A	30.9420948	30.91336377			
PS3 - Kontrol feed	EF1A	30.8652248				
PS3 - Kontrol feed	EF1A	30.9327717				
Kontrol - Kontrol feed	IGF1	28.9655247	29.04212062	-2.50750351	0	1.00
Kontrol - Kontrol feed	IGF1	29.1312828				
Kontrol - Kontrol feed	IGF1	29.0295544				
Kontrol - Kontrol feed	EF1A	31.5909004	31.54962413			
Kontrol - Kontrol feed	EF1A	31.5659542				
Kontrol - Kontrol feed	EF1A	31.4920177				

Table: 13 represents igf1 gene expression results in the liver. It contains beside the data of the run, the cycle threshold (CT) results which were used to calculate the 2*ΔΔCT values.

Sample Name	Target Name	Cт	Average of Cts	Delta Ct	Delta - delta Ct	2^(–delta delta CT)
PS1 - Exp. feed	IGF2	23.8910408	23.77657954	-5.982009888	-3.119889577	8.7
PS1 - Exp. feed	IGF2	23.71863556				
PS1 - Exp. feed	IGF2	23.72006226				
PS1 - Exp. feed	EF1A	29.7327919	29.75858943			
PS1 - Exp. feed	EF1A	29.75845337				
PS1 - Exp. feed	EF1A	29.78452301				
PS2 - Exp. feed	IGF2	25.29738998	25.09469668	-4.821513494	-1.959393183	3.9
PS2 - Exp. feed	IGF2	24.99353409				
PS2 - Exp. feed	IGF2	24.99316597				
PS2 - Exp. feed	EF1A	29.98601913	29.91621017			
PS2 - Exp. feed	EF1A	29.94916916				
PS2 - Exp. feed	EF1A	29.81344223				
PS3 - Exp. feed	EF1A	24.93884659	24.88354111	-5.250848134	-2.388727824	5.2
PS3 - Exp. feed	EF1A	24.96078491				
PS3 - Exp. feed	EF1A	24.75099182				
PS3 - Exp. feed	IGF2	30.09779739	30.13438924			
PS3 - Exp. feed	IGF2	30.11256027				
PS3 - Exp. feed	IGF2	30.19281006				
Kontrol - Exp. feed	IGF2	23.88275337	23.7341404	-5.791574478	-2.929454168	7.6
Kontrol - Exp. feed	IGF2	23.61536407				
Kontrol - Exp. feed	IGF2	23.70430374				
Kontrol - Exp. feed	EF1A	29.55363274	29.52571487			
Kontrol - Exp. feed	EF1A	29.48646545				
Kontrol - Exp. feed	EF1A	29.53704643				
PS1 - Kontrol feed	IGF2	24.26107407	24.14203771	-6.670110067	-3.807989756	14.0
PS1 - Kontrol feed	IGF2	24.06302261				
PS1 - Kontrol feed	IGF2	24.10201645				
PS1 - Kontrol feed	EF1A	30.88857269	30.81214778			
PS1 - Kontrol feed	EF1A	30.76319695				
PS1 - Kontrol feed	EF1A	30.78467369				
PS2 - Kontrol feed	IGF2	23.50311661	23.42286237	-6.878442128	-4.016321818	16.2
PS2 - Kontrol feed	IGF2	23.45441246				
PS2 - Kontrol feed	IGF2	23.31105804				
PS2 - Kontrol feed	EF1A	30.42993927	30.3013045			
PS2 - Kontrol feed	EF1A	30.26554108				
PS2 - Kontrol feed	EF1A	30.20843315				
PS3 - Kontrol feed	IGF2	24.15450478	24.28663762	-6.436333338	-3.574213028	11.9
PS3 - Kontrol feed	IGF2	24.09351349				
PS3 - Kontrol feed	IGF2	24.61189461				
PS3 - Kontrol feed	EF1A	30.80675316	30.72297096			
PS3 - Kontrol feed	EF1A	30.67557335				
PS3 - Kontrol feed	EF1A	30.68658638				
Kontrol - Kontrol feed	IGF2	27.95172691	28.6739521	-2.86212031	0	1.0
Kontrol - Kontrol feed	IGF2	29.05700302				
Kontrol - Kontrol feed	IGF2	29.01312637				
Kontrol - Kontrol feed	EF1A	30.95843315	31.53607241			
Kontrol - Kontrol feed	EF1A	31.73118782				
Kontrol - Kontrol feed	EF1A	31.91859627				

Table: 14 represents igf1 gene expression results in the liver. It contains beside the data of the run, the cycle threshold (CT) results which were used to calculate the 2*ΔΔCT values.

Sample Name	Target Name	Cт	Average of Cts	Delta Ct	Delta - delta Ct	2^(–delta delta CT)
PS1 - Exp. feed	GH	19.04541779	18.94909414	-8.203684489	-0.888151805	1.9
PS1 - Exp. feed	GH	18.87910652				
PS1 - Exp. feed	GH	18.9227581				
PS1 - Exp. feed	EF1A	27.1502552	27.15277863			
PS1 - Exp. feed	EF1A	27.1854248				
PS1 - Exp. feed	EF1A	27.12265587				
PS2 - Exp. feed	GH	20.47807884	20.42233658	-8.205989202	-0.890456518	1.9
PS2 - Exp. feed	GH	20.3838501				
PS2 - Exp. feed	GH	20.4050808				
PS2 - Exp. feed	EF1A	28.68787003	28.62832578			
PS2 - Exp. feed	EF1A	28.76095581				
PS2 - Exp. feed	EF1A	28.4361515				
PS3 - Exp. feed	GH	19.05266762	19.02417183	-7.215920766	0.099611918	0.9
PS3 - Exp. feed	GH	18.93498421				
PS3 - Exp. feed	GH	19.08486366				
PS3 - Exp. feed	EF1A	26.29835892	26.2400926			
PS3 - Exp. feed	EF1A	26.28433418				
PS3 - Exp. feed	EF1A	26.13758469				
Kontrol - Exp. feed	GH	17.54408455	17.54005432	-8.411528905	-1.095996221	2.1
Kontrol - Exp. feed	GH	17.72598648				
Kontrol - Exp. feed	GH	17.35009193				
Kontrol - Exp. feed	EF1A	25.92118835	25.95158323			
Kontrol - Exp. feed	EF1A	25.94310379				
Kontrol - Exp. feed	EF1A	25.99045753				
PS1 - Kontrol feed	GH	21.54673386	21.39395142	-5.293795268	2.021737417	0.2
PS1 - Kontrol feed	GH	21.22581673				
PS1 - Kontrol feed	GH	21.40930367				
PS1 - Kontrol feed	EF1A	26.74860954	26.68774668			
PS1 - Kontrol feed	EF1A	26.61161613				
PS1 - Kontrol feed	EF1A	26.70301437				
PS2 - Kontrol feed	GH	21.58354187	21.58506139	-5.840811412	1.474721273	0.4
PS2 - Kontrol feed	GH	21.64145851				
PS2 - Kontrol feed	GH	21.53018379				
PS2 - Kontrol feed	EF1A	27.82678604	27.4258728			
PS2 - Kontrol feed	EF1A	27.16904831				
PS2 - Kontrol feed	EF1A	27.28178406				
PS3 - Kontrol feed	GH	18.31389046	18.14833832	-9.144439061	-1.828906377	3.6
PS3 - Kontrol feed	GH	18.1476841				
PS3 - Kontrol feed	GH	17.9834404				
PS3 - Kontrol feed	EF1A	27.27963829	27.29277738			
PS3 - Kontrol feed	EF1A	27.23434639				
PS3 - Kontrol feed	EF1A	27.36434746				
Kontrol - Kontrol fee	GH	19.93959999	20.07898521	-7.315532684	0	1.0
Kontrol - Kontrol fee	GH	19.9855957				
Kontrol - Kontrol fee	GH	20.31175995				
Kontrol - Kontrol fee	EF1A	27.51904297	27.3945179			
Kontrol - Kontrol fee	EF1A	27.40833282				
Kontrol - Kontrol fee	EF1A	27.2561779				

Table: 15 represents growth hormone receptor (GhR) gene expression results in the brain. It contains beside the data of the run, the cycle threshold (CT) results which were used to calculate the 2*ΔΔCT values.

Sample Name	Target Name	Cт	Average of Cts	Delta Ct	Delta - delta Ct	2^(–delta delta CT)
PS1 - Exp. feed	GHR	28.98900414	28.71162478	2.668031057	2.162663142	0.2
PS1 - Exp. feed	GHR	28.71422005				
PS1 - Exp. feed	GHR	28.43165016				
PS1 - Exp. feed	EF1A	26.08189964	26.04359372			
PS1 - Exp. feed	EF1A	26.01196098				
PS1 - Exp. feed	EF1A	26.03692055				
PS2 - Exp. feed	GHR	28.97772026	28.73775164	1.289230347	0.783862432	0.6
PS2 - Exp. feed	GHR	28.52757645				
PS2 - Exp. feed	GHR	28.70795822				
PS2 - Exp. feed	EF1A	27.482481	27.4485213			
PS2 - Exp. feed	EF1A	27.48404312				
PS2 - Exp. feed	EF1A	27.37903976				
PS3 - Exp. feed	GHR	28.74057961	28.66347885	1.605806351	1.100438436	0.5
PS3 - Exp. feed	GHR	28.63842583				
PS3 - Exp. feed	GHR	28.61143112				
PS3 - Exp. feed	EF1A	27.01523018	27.0576725			
PS3 - Exp. feed	EF1A	27.20710564				
PS3 - Exp. feed	EF1A	26.95068169				
Kontrol - Exp. feed	GHR	29.07893753	28.9221077	1.403193156	0.897825241	0.5
Kontrol - Exp. feed	GHR	28.80121422				
Kontrol - Exp. feed	GHR	28.88617134				
Kontrol - Exp. feed	EF1A	27.57684326	27.51891454			
Kontrol - Exp. feed	EF1A	27.51878738				
Kontrol - Exp. feed	EF1A	27.46111298				
PS1 - Kontrol feed	GHR	28.99633217	28.90927633	1.928593318	1.423225403	0.4
PS1 - Kontrol feed	GHR	28.80865669				
PS1 - Kontrol feed	GHR	28.92284012				
PS1 - Kontrol feed	EF1A	26.99119377	26.98068301			
PS1 - Kontrol feed	EF1A	26.96572685				
PS1 - Kontrol feed	EF1A	26.9851284				
PS2 - Kontrol feed	GHR	30.30504799	30.12487539	2.496824265	1.99145635	0.3
PS2 - Kontrol feed	GHR	30.11997604				
PS2 - Kontrol feed	GHR	29.94960213				
PS2 - Kontrol feed	EF1A	27.75836372	27.62805112			
PS2 - Kontrol feed	EF1A	27.62818336				
PS2 - Kontrol feed	EF1A	27.49760628				
PS3 - Kontrol feed	GHR	28.57963181	28.42381605	1.322909037	0.817541122	0.6
PS3 - Kontrol feed	GHR	28.21560097				
PS3 - Kontrol feed	GHR	28.47621536				
PS3 - Kontrol feed	EF1A	27.33338928	27.10090701			
PS3 - Kontrol feed	EF1A	26.97578239				
PS3 - Kontrol feed	EF1A	26.99354935				
Kontrol - Kontrol feed	GHR	27.96020889	27.71528244	0.505367915	0	1.0
Kontrol - Kontrol feed	GHR	27.54081726				
Kontrol - Kontrol feed	GHR	27.64482117				
Kontrol - Kontrol feed	EF1A	27.2834034	27.20991453			
Kontrol - Kontrol feed	EF1A	27.17845345				
Kontrol - Kontrol feed	EF1A	27.16788673				

Table: 16 represents growth hormone receptor (GhR) gene expression results in the brain. It contains beside the data of the run, the cycle threshold (CT) results which were used to calculate the 2*ΔΔCT values.

Sample Name	Cт	Average of Cts	Delta Ct	Delta - delta Ct	2^(–delta delta CT)
PS1 - Exp. feed	21.9361191	22.53990364	-6.572219849	-1.69798851	3.2
PS1 - Exp. feed	22.836256				
PS1 - Exp. feed	22.8473358				
PS1 - Exp. feed	29.3165112	29.11212349			
PS1 - Exp. feed	29.0614223				
PS1 - Exp. feed	28.958437				
PS2 - Exp. feed	23.951086	23.51814969	-5.698456446	-0.824225108	1.8
PS2 - Exp. feed	23.2814865				
PS2 - Exp. feed	23.3218765				
PS2 - Exp. feed	29.4651699	29.21660614			
PS2 - Exp. feed	28.9174328				
PS2 - Exp. feed	29.2672157				
PS3 - Exp. feed	24.9153404	24.22062365	-5.018339793	-0.144108454	1.1
PS3 - Exp. feed	23.8805523				
PS3 - Exp. feed	23.8659782				
PS3 - Exp. feed	29.4319077	29.23896345			
PS3 - Exp. feed	29.1625843				
PS3 - Exp. feed	29.1223984				
Kontrol - Exp. feed	21.8440762	22.18538411	-6.718498866	-1.844267527	3.6
Kontrol - Exp. feed	22.3712311				
Kontrol - Exp. feed	22.3408451				
Kontrol - Exp. feed	28.1765633	28.90388298			
Kontrol - Exp. feed	29.4289246				
Kontrol - Exp. feed	29.1061611				
PS1 - Kontrol feed	22.6986294	22.80753962	-7.400012334	-2.525780996	5.8
PS1 - Kontrol feed	22.8205948				
PS1 - Kontrol feed	22.9033947				
PS1 - Kontrol feed	29.9619122	30.20755196			
PS1 - Kontrol feed	30.1135139				
PS1 - Kontrol feed	30.5472298				
PS2 - Kontrol feed	21.8906288	21.96806844	-7.749450048	-2.875218709	7.3
PS2 - Kontrol feed	22.0228062				
PS2 - Kontrol feed	21.9907703				
PS2 - Kontrol feed	29.8531761	29.71751849			
PS2 - Kontrol feed	29.6170387				
PS2 - Kontrol feed	29.6823406				
PS3 - Kontrol feed	22.118288	22.1662178	-7.827229182	-2.952997843	7.7
PS3 - Kontrol feed	22.2190475				
PS3 - Kontrol feed	22.1613178				
PS3 - Kontrol feed	30.1367264	29.99344699			
PS3 - Kontrol feed	29.9156036				
PS3 - Kontrol feed	29.9280109				
Kontrol - Kontrol feed	24.0005932	16.07727051	-4.874231339	0	1.0
Kontrol - Kontrol feed	0				
Kontrol - Kontrol feed	24.2312183				
Kontrol - Kontrol feed	31.756094	20.95150185			
Kontrol - Kontrol feed	31.0984116				
Kontrol - Kontrol feed	0				