Theses of Doctoral (PhD) Dissertation

GENE EXPRESSION ANALYSIS OF CHICKEN UNDER AD LIBITUM AND RESTRICTED FEEDING

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1 INTRODUCTION AND GOALS OF THE DOCTORAL DISSERTATION

The avian feed intake regulation is a significant scientific topic in animal science, as the amount of consumed feed, its digestibility and the breed's genetic capabilities interact to affect the performance of farm animals. The European Union's chicken meat production reached 14.8 million tons in 2016, which is 12.6% of the global production. This means almost 6.5 billion birds. For meat production, improved fast growing broiler chickens (meat type chickens) are bred. These broilers reach their slaughter weight of 2.2-2.5 kg by 35-40 days. This final weight is twice bigger than the value 50 years before. The EU is the second largest egg producer in the world with 390 million layer type chickens and 7.2 million eggs produced in 2016 (internet 1). The fast growing of broilers has side effects like increased carcass fat, more frequent appearance of skeletal disorders, ascites and the sudden death syndrome. Feed intake is regulated by physical and biochemical factors, the center of the latter is in the central nervous system, namely in the hypothalamus. Polymorphisms in genes encoding feed intake regulating peptides are often positively associated with the performance traits of farm animals. In case of birds we know little about how fasting affects the expression of orexigenic or anorexigenic feed intake regulating peptides in the central nervous system or in the peripheral organs, despite feed restriction or skip-a-day feeding are used in the growth period. Feed restriction and fasting would provide a good opportunity to study the genes encoding potential feed intake regulating peptides. Neuropeptides are small protein type signalling molecules consist of 50 or less amino acids. Neuropeptides takes part in pain perception, rewarding, learning and in feed intake regulation (Boswell, 2005; Denbow, 1994). In case of mammals, 95 neuropeptide encoding gene are already know which can produce more than a hundred of different peptide. In case of chickens the number of known neuropeptide encoding gene are 65 (Delfino *et al*., 2010) and the information regarding feed intake regulation is less than in case of mammals. My goal was to study some of the less studies genes, of which products are involved in feed intake regulation to provide information about the molecular basis of feed intake in chickens.

I aimed to investigate the following:

1. How does the 40% feed restriction affects the expression of some genes (*GAL, NMU, NMUR1, NMUR2, PAC1, PACAP*) encoding feed intake related products in the hypothalamus, proventriculus and jejunum.

2. How do the 24 h fasting and 24 h fasting 2 h refeeding affect the MCH expression at peptide and mRNA level in case of broiler type chicken? How does the aforementioned experimental setting affect the expression stability of 10 reference gene candidates in hypothalamus? To investigate the tissue expression pattern of *PMCH* and *MCHR4* in nine different tissues.

3. How does growing affect the gene expression of *PMCH* and *MCHR4* ?. Are there any correlations between hypothalamic MCH concentration expression and abdominal fat pad weight and adiposity signals (insulin, glucose)?

4. What kind of proteomic changes lies behind the already observed jejunal morphometric changes caused by 24 h fasting?

2 MATERIALS AND METHODS

2.1.1 Effect of 40% feed restriction on genes encoding feed intake regulating peptides and their receptors

2.1.2 Animals, experiment, sample collection

Sixteen female, 22 weeks old layer type chickens (Tetra-SL line) were set in the experiment. Birds were individually housed to ground pens. During the one week long adaptation period, each ones were fed with *ad libitum* layer 1 diet (briefly: 17.5% crude protein with a metabolizable energy of 11.7 AME_n MJ/kg dry matter, 3.8% Ca, 0.4% P). Lighting regime and concentrate were implemented in accordance with the Tetra-SL management manual. After the adaptation period, half of the birds were maintained on 40% feed restricted diet (restricted group, 8 chickens) for 5 days, whereas the other group was fed *ad libitum* (control group, 8 chickens). Water was freely available in each pen during the whole experiment. After 5 days, each bird was slaughtered by concussion.

Whole hypothalamus was macrodissected according to the chicken brain atlas (Griffin *et al*., 2001). After opening the abdominal cavity, the proventriculus and jejunum samples were immediately removed, opened along the longitudinal axes and washed in ice-cold PBS solution. Five mL blood from each bird was collected in EDTA coated blood collection tubes and centrifuged. Aliquots of plasma samples were immediately frozen in liquid nitrogen and stored at -70°C for further analysis.

2.1.3 RNA isolation, cDNA synthesis and qPCR

Hypothalamus, proventriculus and jejunum samples were grounded in liquid nitrogen with a mortar and pestle. Total RNA was extracted from 30±3 mg grinded tissue using RNeasy Mini Kit with an on-column DNase I digestion step. Quantity and purity of isolated RNA were quantified using a NanoDrop ND-1000 spectrophotometer. Two μg RNA was reverse transcribed using a Maxima First Strand cDNA Synthesis Kit, with reaction conditions as recommended in the kit's manual. After reverse transcription, aliquots were stored in -20°C. Prior to qPCR, cDNA samples were diluted tenfold. cDNA specific primers were designed using Primer Express v3.0.1. Real-time PCR reactions were run in triplicates on 96 reaction plates in an ABI 7300 real-time PCR machine. Thermal profile was: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Real-time PCR reactions (20 μl) contained 50 ng of cDNA template, 1X Power SYBR Green Master Mix, and 200 nM of each primer. For the relative quantification, raw Cq values were normalized using the stable reference gene using the ΔΔCt method (Pfaffl, 2001).

2.1.4 Protein isolation, PACAP-38 radioimmunoassay

Hypothalamus, proventriculus and jejunum samples (75±7.5 mg) were lysed in 750 μl ice-cold AccuGENE water using a rotor-stator homogeniser for 30 sec at 30000 rpm. Homogenates were then centrifuged for 10 min at 20000 × g, 4°C. Supernatants were stored at -80°C for further analysis. The PACAP-38 peptide was extracted from 2 ml plasma by addition of a double volume of absolute alcohol and 20 μl 96% acetic acid. After precipitation and centrifugation (1500 × g, 10 min, 4°C) the supernatants were decanted and dried under nitrogen flow. PACAP-38 immunoreactivity in chicken plasma and tissues was determined with a specific and sensitive RIA technique as previously described (Jakab *et al*., 2004).

2.2 Selection of stable reference genes in broiler chicken hypothalamus under different feeding states

2.2.1 Animals, experiment, sample collection

One day old broiler type chickens (Ross 308) were obtained from a commercial hatchery. Male chickens were selected and allocated into ground pens. The ground pens had wood shavings and were equipped with bowl feeders and Plasson drinkers. Temperature was maintained at 32°C at placement using electrical heaters and fans, then gradually decreased by 1.5°C/week to ensure bird's requirement. Lighting regime was implemented in accordance with the Ross 308 management manual. At day 28 chickens were divided into three treatment groups: *ad libitum* fed (AL), fasted for 24 h (F24), fasted for 24 h then refed for 2 h (F24R2). Control male chickens were fed *ad libitum* with broiler grower diet (briefly: 20% crude protein with metabolizable energy of 13 AME_n MJ/kg). Water was freely available for all groups during the whole experiment. After the fasting period 8 animals were slaughtered from both groups, while the fasted group was refed with grower diet for 2 h. After refeeding, 8 animals were also slaughtered from the F24R2 group. Termination of birds and sample collection was the same as described in chapter 2.1.2.

2.2.2 RNA isolation, cDNA synthesis and qPCR

Whole hypothalamus samples were grounded in liquid nitrogen using a mortar and pestle. 25±2.5 mg grinded tissue was lysed in TRI Reagent using a rotor-stator homogenizer for 30 sec at 30000 rpm. Total RNA was extracted using Direct-zol RNA MiniPrep using an on-column DNase I digestion step. Isolated RNA amounts and purity were quantified in eluent using a NanoDrop ND-1000 spectrophotometer. Reverse transcription was conducted using 800 ng total RNA with qPCRBIO cDNA Synthesis Kit. Prior to qPCR, cDNA samples were diluted tenfold, and then stored in -20°C. Real-time PCR reactions were run in triplicates, same targets under same run using 384-well plates on a LightCycler 480 Instrument II. Thermal cycling conditions were the followings: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 30 sec. Real-time PCR reactions (10 µl) contained 4 ng of cDNA template, 1X Xceed qPCR SG Hi-ROX mastermix, and 200

nM of each primer. Primer specificities were tested using melt profile analysis after each run by gradually increasing temperature from 60 to 95°C.

2.2.3 Gene expression stability of 10 reference gene candidates

Five different approaches were applied to search for stable reference genes among the 10 candidates. Analysis by two Excel Visual Basic Application, geNorm (v3.5) and NormFinder (v0.953), the BestKeeper (v1) and the comparative ΔCt method was conducted on Microsoft Excel 2010. The method described by Chervoneva *et al*. (2010) was performed on SAS Studio release 3.5. Reference genes were ranked according to stability values of five algorithms.

2.2.4 Effect of fasting and refeeding, growing and tissue expression of *MCHR4* and *PMCH* genes in the hypothalamus of broiler chickens

2.2.5 Animals, experiment, sample collection

Housing and feeding conditions were the same as described in chapter 2.2.1. In case of growth experiment 8 birds at day 7., 14., 21., 28. (same birds as in 2.2.1 chapter in *ad libitum* fed group) and 35 were slaughtered. Sample collection was the same as described in 2.1.1 and in 2.1.2 chapters. Live weight was recorded with a digital balance before slaughter, than the abdominal cavity was opened, and the abdominal fat pad was cut and its weight was measured. In case of four 28 day old *ad libitum* fed birds, tissue samples from the crop, proventriculus, ventriculus, duodenum, jejunum, ileum and rectum were also collected. Samples were collected to cryotubes and stored at -70 °C for further analysis.

2.2.6 RNA isolation, cDNA synthesis and qPCR

Steps were as the same as described in chapter 2.2.2.

2.2.7 Blood plasma glucose and insulin measurements

One ml (7 days old birds) and 5 ml (14-35 days old birds) blood was collected. Further steps were as the same as described in chapter 2.1.1.

2.2.8 Protein isolation, MCH radioimmunoassay

Steps for protein isolation were as the same as described in chapter 2.1.4. Measurements for MCH concentrations were described by Lelesz *et al*., (2016).

2.3 Effect of fasting on jejunal morphology and proteomics of broiler chicken

2.3.1 Jejunum morphometric measurements

Samples were acquired from 9 birds, from which 3 were *ad libitum* fed, 3 were fasted for 24 h and 3 were fasted for 24 h than refed for 2 h. Tissue sections were stained with hematoxylin-eosin, digitalized with h a camera equipped microscope. Morphometric measurements were conducted with Adobe Photoshop CC software.

2.3.2 Protein isolation, two-dimensional difference gel electrophoresis (2D-DIGE), image analysis, identification of proteins

Frozen jejunum samples (~100 mg) were crushed to fine powder with mortar and pestle in liquid nitrogen. Fifty mg of powdered samples were weighted with an analytical balance and 500 µl cold lysis buffer was added. Samples were placed on ice for 1 hour and mixed at every 10 min. After lysis, homogenates were centrifuged at 16,000 × g for 30 min at 4°C. Supernatants were collected to fresh tubes and the protein concentration was determined with RC DC Protein Assay kit using a microplate reader. Supernatants were stored at -70°C for further use. For each gel 50 µg of sample pool was labelled with 400 pmol of Cyanine2 NHS ester minimal dye and 50 µg from *ad libitum* and 50 µg from experimental group with each of 400 pmol of Cyanine3 or Cyanine5. Labelling reactions were carried out on ice for 30 min in dark, than 10 mM lysine stop solution was added to each reaction tube and incubated on ice for 10 min.

For the first dimension, 7 cm immobilized linear pH gradient strips (pH 5-8) were used. Strips were rehydrated by passive rehydration using samples (150 µg of protein) dissolved in 125 µl rehydration buffer for 16 h at room temperature. Isoelectric focusing was conducted in Protean IEF Cell. Focused IPG strips were equilibrated and proteins were separated by molecular mass (second dimension) on a vertical electrophoresis system. Preparative polyacrylamide gels for spot picking (containing 500 µg total protein) were stained with colloidal Coomassie G-250. Gels images were

recorded using a laser scanner, than the images were analysed with Delta2D 4.3 software. Spots were detected, quantified, and normalized according to the volume ratio of corresponding spots detected in the Cy2 image of the internal standard using the in-gel standard warping strategy.

Proteins were excised, digested from preparative gel (Shevchenko *et al*., 2006) and prepared to mass spectrometry according to Bhide *et al*. (2009). Proteins were identified using a LIFT™-MS/MS coupled matrix-assisted laser desorption ionization (MALDI) mass spectrometer.

3 RESULTS

3.1.1 Effect of 40% feed restriction on genes encoding feed intake regulating peptides and their receptors

The expression of the target genes with qPCR and the PACAP-38 concentrations with RIA method were measured in the 40% feed restricted and the *ad libitum* group. According to the Cq values all genes, expect one had mean Cq values above 15 in the examined tissues. *PACAP* showed the highest expression in the hypothalamus with mean Cq of 13.8 indicating an important role in the central nervous system, while in the proventriculus and jejunum *GAL* showed the highest expression (24.9 and 21.9). Feed restriction resulted a 1.63 fold increase of *NMUR1* and 1.49 increase in *PAC1* gene (Table 1.). In case of proventriculus *ADCYAP1* showed 1.88 fold increase. In jejunum samples *GAL, NMU, NMUR1* and *ADCYAP1* showed more than threefold increase, compared to the *ad libitum* group. PACAP-38 was present in a measurable concentration in all the examined samples. Feed restriction caused significant 21% increase in the proventriculus only (Figure 1.). This is the first study which investigated the concentration of PACAP-38 in chicken plasma. It was present in *ad libitum* group at 22.03 fmol/ml concentration, which is 13-times lower, than the concentration in case of goat, lamb or cattle plasma (Czeglédi *et al*., 2011). The PACAP-38 is present in similar concentration in the broiler chicken hypothalamus as well (Józsa *et al*., 2005).

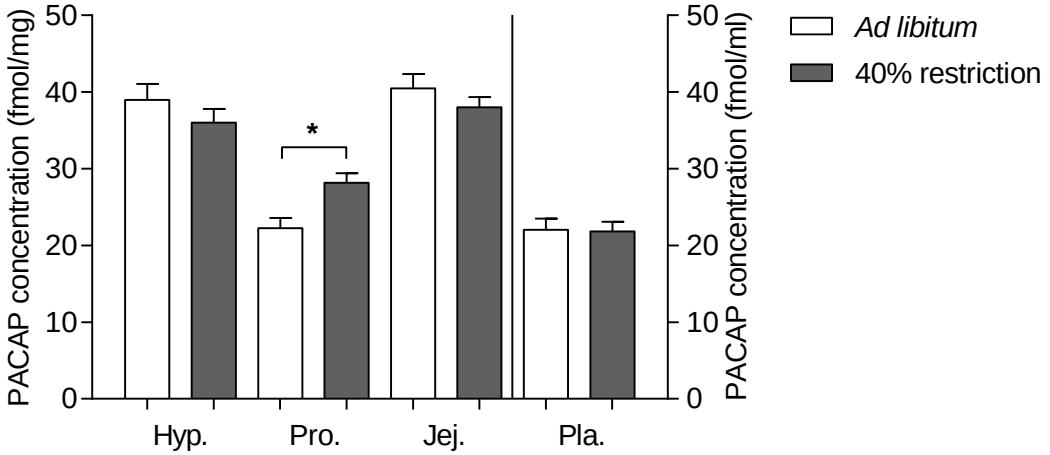

Figure 1. PACAP-38 concentrations in tissues of *ad libitum* fed and feed restricted hens measured with RIA. Hyp.: hypothalamus, Pro.: proventriculus, Jej.: jejunum, Pla.: plasma. Data are represented as group means ± SEM. **P*<0.05.

Table 1. Effect of feed restriction on the expression of the selected genes (fold change ± SEM), *P<0.05.

	hypothalamus		**proventriculus**		**jejunum**	
gene	*ad lib.*	40% restr.	*ad lib.*	40% restr.	*ad lib.*	40% restr.
ADCYAP1	1±0.06	1.26±0.10	1±0.05	1.88*±0.09	1±0.03	2.76*±0.08
GAL	1±0.05	1.3±0.14	1±0.11	1.52±0.32	1±0.02	3.08*±0.02
NMU	1±0.04	1.39±0.02	1±0.06	0.69±0.07	1±0.02	3.41*±0.19
NMUR1	1±0.02	1.63*±0.04	1±0.07	0.90±0.20	1±0.05	3.01*±0.09
NMUR2	1±0.02	1.5±0.10	1±0.01	1.04±0.06	1±0.02	0.52±0.01
PAC1	1±0.02	1.49*±0.03	1±0.03	1.07±0.12	1±0.13	1.01±0.02

In this experiment, the 5 day long 40% feed restriction did not affected the PACAP mRNA levels nor the PACAP-38 peptide concentrations in the hypothalamus, however PACAP receptor expression increased. Józsa *et al*. (2005) found that 36 fasting caused significant increase in PACAP concentration in broiler-type chicken's hypothalamus. Feed restriction has a great effect on gastrointestinal tract motility. It remained unanswered why *PACAP* expression remained unchanged while *PAC1* expression increased in the hypothalamus. At fasting it starts to slow down, than stop and reversed peristalsis can occur (Clench and Mathias, 1995). In addition to numerous feed intake regulating peptide, PACAP is present in the avian gastrointestinal tract where it takes part in motility regulation (Monir *et al*., 2014; Yoshida *et al*., 2000) as a smooth muscle relaxant (Ozawa *et al*., 1999). An increased *PACAP* expression was observed in the periphery by qPCR. I can be assumed that the PACAP increased concentration in the proventriculus is the result of decreased motility caused by lower feed intake. It is currently unknown how PACAP released to chicken's blood and from which tissue. Its plasma level remained unchanged in fed restricted group, to understand the role of PACAP-38 in chicken plasma requires further investigations. Intracerebroventricular injection of galanin stimulated feeding behaviour of layer and broiler chicks (Ando *et al*., 2000; Tachibana *et al*., 2008). Galanin in the jejunum has a motility affecting role (DeGolier *et al*., 1999; Salvi *et al*., 1999). It can be assumed that galanin increased expression in the jejunum is the result of decreased motility caused by lower feed intake.

3.2 Selection of stable reference genes in broiler chicken hypothalamus under different feeding states

The stability of 10 reference gene candidates was investigated by with five different method under pooled conditions (samples from *ad libitum*, fasted for 24 h, fasted for 24 h refed for 2 h). Ranks obtained by ordering the stability values are presented in Table 2.

Table 2. Summary of gene stability rankings of the five different methods under pooled conditions

Gene	Overall rank[1]	Best-Keeper	geNorm	Chervoneva *et al*. (2010)	Norm-Finder	ΔCt method
ACTB	6	3	7 (0.332)[2]	8 (779.39)	6 (0.136)	6 (0.42)
B2M	9	8	9 (0.423)	9 (982.30)	10 (0.220)	9 (0.65)
GAPDH	5	7	5 (0.266)	6 (581.32)	2 (0.103)	5 (0.42)
HMBS	4	6	3 (0.231)	3 (189.67)	4 (0.116)	3 (0.40)
LBR	7	5	8 (0.362)	5 (542.01)	7 (0.138)	7 (0.47)
POLR2B	**2**	4	4 (0.239)	2 (36.32)	1 (0.096)	4 (0.42)
RN18S	10	10	10 (0.476)	10 (1023)	9 (0.189)	10 (0.69)
RPS17	**3**	2	1 (0.215)	4 (215.08)	5 (0.124)	2 (0.40)
TBP	**1**	1	1 (0.215)	1 (8.42)	3 (0.114)	1 (0.39)
YWHAZ	8	9	6 (0.301)	7 (725.94)	8 (0.164)	8 (0.49)

[1]obtained by ranking the geometric average of the five rankings, [2]stability values in parentheses.

TBP got the best rank under all experimental conditions, so it can be recommended as reference gene to be used for normalization for qPCR in chicken hypothalamus, because its expressional stability remains unaffected by feeding state. Despite their common appearance in gene expression studies (36.4, 33.9 and 7.4% overall), *ACTB*, *GAPDH* and *RN18S* are not the best choice for accurate normalization of qPCR results as their stability rank lagged behind TPB. The *TBP* gene encodes the TATA-box binding protein, which is a transcription factor, part of the RNA polymerase II pre-initiation complex and required for all the eukaryote cells basal transcription (Boeger *et al*., 2005).

3.2.1 Effect of fasting and refeeding, growing and tissue expression of *MCHR4* and *PMCH* genes in the hypothalamus of broiler chickens

The *MCHR4* and *PMCH* expressions were the highest in the hypothalamus, and along the GIT it was hardly measureable (Figure 2.).

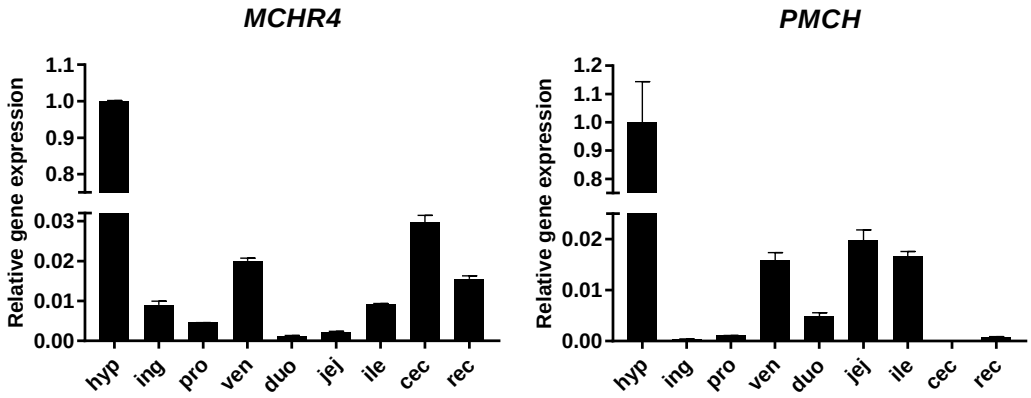

Figure 2. The *MCHR4* and *PMCH* gene expression in nine different tissue types of broiler chickens as assessed by qPCR. Samples listed in anteroposterior order were the following: *hypothalamus* (hyp), *ingluvies* (ing), *proventriculus* (pro), *ventriculus muscularis* (ven), *duodenum* (duo), *jejunum* (jej), *ileum* (ile), *cecum* (cec), *colorectum* (rec). Data represent mean ± SEM. (*n*=4 in each group). Mean is presented as a fold difference compared to the value observed in the hypothalamus.

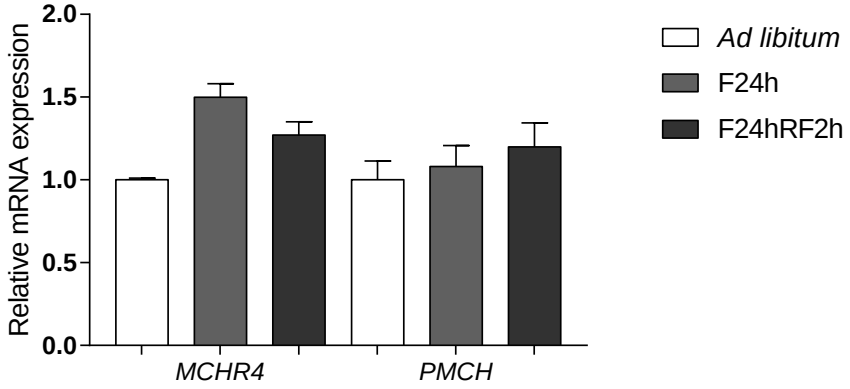

Figure 3. Effect of fasting and refeeding on the expression of *MCHR4* and *PMCH* genes in the hypothalamus of 28 day old broilers. Data represent mean ± SEM. (*n*=8 in each group). *TBP* was used as a reference gene. No significant differences were found between groups.

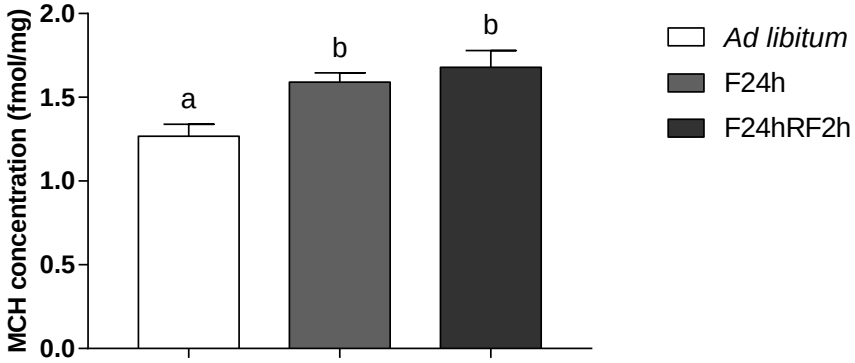

Figure 4. Effect of fasting and refeeding on the hypothalamic concentration of MCH in 28 day old broilers as assessed by RIA. Data represent mean ± SEM. (*n*=8 in each group). MCH contents are expressed as fmol/mg wet tissue weight. Group means with similar letters are not significantly different ($P>0.05$).

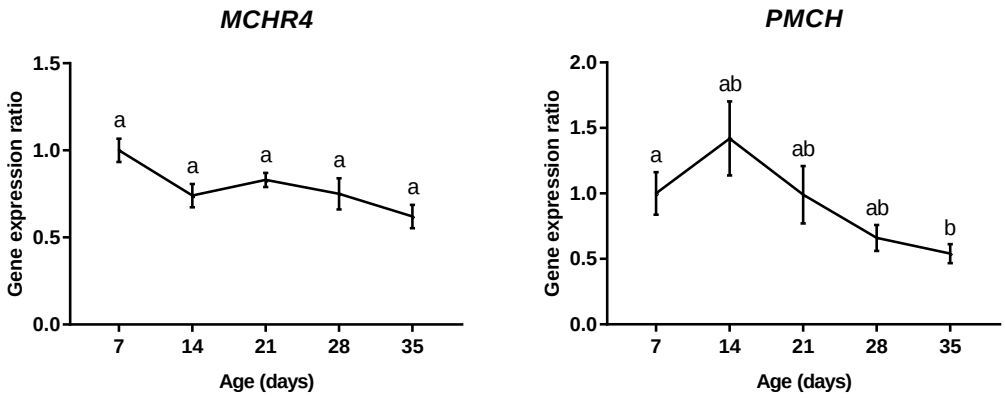

Figure 5. Growth dependent changes in *MCHR4* and *PMCH* gene expression in broiler chicken hypothalamus. Data represent normalized ΔCq mean ± SEM. (*n*=8 in each group), *ACTB* was used as a reference gene. Group means with same letters are not significantly different (*P*>0.05). Means are presented as a fold difference compared to the value observed at age of 7 days old.

The expression of melanin-concentrating hormone gene (*PMCH*) and its receptor's (*MCHR4*) expression was measured by qPCR and also the MCH peptide concentration by RIA. Feeding states did not affected *PMCH* nor *MCHR4* mRNA expression (Figure 3.). The 24 h fasting caused 25.65% increase in MCH peptide concentration, furthermore the 2 h refeeding caused 32.51% further increasing compared to *ad libitum* state (Figure 4.). Similar trend was observable both in *PMCH* expression and MCH concentration changes. Decreasing trend was observable in *PMCH* and *MCHR4* mRNA levels with growth, this was also observable in MCH peptide concentrations (Figure 5.). According to correlation analysis the MCH peptide concentration and the abdominal fat did not correlated significantly, nor with adiposity signals like, plasma glucose or insulin levels in the *ad libitum* fed birds. The MCH peptide concentration measured in *ad libitum* fed chickens hypothalamus was (1.27 fmol/mg) which is similar to rat peptide concentrations (~1.3 fmol/mg) as measured by Lelesz *et al*. (2016).

The *MCHR4* expression did not changed significantly with growth. In case of *PMCH*, significant 0.54 fold decrease was observable between at age of day 7. and day 35. sampling times. There was no difference between the other comparisons (Figure 5.). Linear trend analysis showed that the mean average decreasing between the time points was -0.075 in case of *MCHR4* and -0.168 in case of *PMCH*. Decreasing trend was also observable in MCH peptide concentration with mean decreasing of -0.0848 with growth. The Spearman correlation analysis showed that abdominal fat and body weight

highly correlates. In the other comparisons (r<0.4) there was no significant correlation (Table 3.).

Table 3: Spearman correlation analysis of variables

	Abdominal fat	**Body weight**	**Glucose**	**Insulin**	**MCH conc.**
Abdominal fat	1	0.642 (0.009)[1]	0.088 (0.753)	0.223 (0.421)	0.027 (0.920)
Body weight		1	0.174 (0.533)	0.255 (0.356)	-0.106 (0.694)
Glucose			1	0.266 (0.334)	0.215 (0.437)
Insulin				1	-0.136 (0.334)
MCH conc.[2]					1

[1]*P* values are in parenthesis, [2]MCH peptide concentration in the hypothalamus

The *MCHR4* and *PMCH* expression showed decreasing trend in the hypothalamus. These results are in agreement with the literature. The orexigenic neuropeptid Y (NPY) show decreasing trend in broiler hypothalamus as investigated by Saneyasu *et al*., (2013). The NPY expression also showed decreasing trend in layer type chicken hypothalamus while, the anorexigenic POMC gene increased with growth (Honda *et al*., 2015). Similarly the CART peptid (anorexigenic) expression increased with growth (Cai *et al*., 2015). Fasting for 48 h significantly increased *PMCH* mRNA level more than 1.5 fold in male broiler chickens compared to control fed birds and returned to normal levels after 24 h refeeding (Song *et al*., 2012). In overall it can be concluded that MCH take part in feed intake regulation in broiler chickens.

3.3 Effect of fasting on jejunal morphology and proteomics of broiler chicken

Morphometric changes, induced by fasting, were measured in the chicken duodenum, jejunum and ileum in effect of fasting. In case of duodenum, the villi length increased with 17% in effect of refeeding, while there were no significant changes in other parameters. Fasting caused a 21.4% decrease in jejunum, than refeeding caused a significant increase, but the villus length was still 14.3% smaller than the control value. In case of ileum the 24 h fasting than 2 h refeeding increased the villus area with 33.3% and 10% the crypt depth. The width of muscularis externa remain unaffected in all case. Fasting for 32-48 h or more cause decreased performance of broilers, which can be

attributed to the delayed development of small intestine (Bhanja *et al*., 2010), decreased villus area, crypt depth, number of dividing cells and the decreased length of the three intestine section (Gonzales *et al*., 2003). Mass spectrometry successfully identified 11 proteins (Table 4.). According to UniProt protein database 4 of them are secreted to the extracellular space (APOA1, APOA5, MUC6, EXFABP), 4 are part of the cytoskeleton (ACTA2, ACTB, KRT14, TPM1), 1 is located in the cell membrane (MAGT1), 1 cytoplasmic protein (CHP1), 1 is located in the nucleus (HSP90AA1). With regard to their biological function these proteins are part of the cytoskeleton, takes part in fatty acid transport, in the general stress response, or in the ion- and vesicle transport. Morphometric measurements revealed that jejunal villi area decreased to 24 h fasting, than a significant increase happened after 2 h refeeding. It can be supposed that the change of expression in cytoskeletal proteins are related to this morphometric change, because there was no observable other morphological change. Decreasing mucin layer thickness after fasting was already observed (Smirnov *et al*., 2004). This can be partially explained by the decreased mucin 6 protein expression. The altered level of proteins which take part in lipid metabolism can be explained by the increased reverse lipid transport in effect of fasting (Peebles *et al*., 2004).

Table 4. Proteins identified by mass spectrometry

Identified protein	**Accession number**	**Encoding gene**	**N/C**[1]	**Fold-change**[2]	***P* value**	**MW (kDa)**[3]	**pI**[3]
Actin, cytoplasmic 1	P60706	*ACTB*	18/41	2.05	0.034	41.7	5.29
Actin, smooth muscle	P08023	*ACTA2*	17/52	9.97	0.013	33	5.46
Apolipoprotein A1	P08250	*APOA1*	16/31	1.57	0.048	30.7	5.58
Apolipoprotein A5	XP_417939	*APOA5*	21/34	-1.54	0.044	40.3	6.26
Extracellular fatty acid-binding protein	P21760	*EXFABP*	9/34	1.56	0.017	20.2	5.56
Heat shock protein 90 kDa alpha class A member 1	P11501	*HSP90AA1*	11/14	1.65	0.009	84	5.01
Calcineurin B homologous protein 1	Q5ZM44	*CHP1*	21/79	2.77	0.022	22.4	4.97
Keratin, type I cytoskeletal 14	Q6PVZ1	*KRT14*	19/20	4.32	0.014	51.0	5.02
Magnesium transporter protein 1	Q5ZJ06	*MAGT1*	10/19	11.07	0.013	36.7	9.66
Mucin-6	F1NBL0	*MUC6*	17/12	-1.63	<0.001	132	5.78
Tropomyosin alpha-1	P04268	*TPM1*	19/32	2.36	0.031	32.8	4.7

[1] Number of matched peptides/sequence coverage percentage (%)
[2] Fold-ratio of the protein expression of fasted group compared to the *ad libitum* group
[3]Theoretical molecular mass and isoelectric point according to SwissProt protein database

4 NEW AND NOVEL OBSERVATIONS OF THE THESIS

1. I measured the PACAP-38 concentration in chicken plasma for the first time (22.03 fmol/ml). The 40% feed restriction resulted a 1.88 fold increase in *PACAP* gene expression and 27% increase in PACAP peptide concentration in proventriculus.

2. An increased INSRα expression was observable in the epithelium and crypts of jejunum and in the epithelium of proventriculus after 40% feed restriction compared to ad libitum group.

3. The TBP gene expression stability is superior among the selected candidates in broiler-type hypothalamus under different feeding states so it can be used for similar gene expression studies as a reliable reference gene.

4. The *MCHR4* or *PMCH* mRNA levels were not affected by feeding states in the hypothalamus.

5. Decreasing trend with age was observable both for *MCHR4* and *PMCH* mRNA levels and also for MCH-like immunoreactivity, while plasma insulin concentration showed increasing trend. I found that *MCHR4* and *PMCH* expression are restricted to the hypothalamus.

6. Fasting for 24 h had significantly increased the MCH-like immunoreactivity by 25.65%. Fasting for 24 h and then refeeding for 2 h had further significantly increased the MCH peptide concentration by 32.51%, as compared to the ad libitum state.

7. At least 1.5-fold higher protein levels after 24 h fasting in case of actin (cytoplasmic type), actin (smooth muscle types), apolipoprotein A1, extracellular fatty acid-binding protein, heat shock protein 90 kDa alpha, calcineurin B homologous protein 1, keratin (cytoskeletal type 14), magnesium transporter protein 1 and tropomyosin alpha-1 chain.

8. While two protein showed more than 1.5-fold decreased expression namely: apolipoprotein A5 and mucin-6.

9. In regard of intracellular localization and function most of the identified protein are the part of cytoskeleton, or it’s secreted to the extracellular space, part of the general stress response, ion- and vesicle transport or part of lipid metabolism.

5 PRACTICAL UTILISATION OF THE RESULTS

The function of some gene's product was investigated, some of them proved to be involved in feed intake regulation according to the results. They can be useful in the future for marker associated selection for improved feed intake or they can be useful for animal health or animal welfare investigations of chicken.

It was found that the expression of *TBP* is stable under different feeding states. It can be useful for normalization for further experiments which will aim to investigate feed intake regulating gene expression.

The observed proteomic changes in the jejunum gives new information for further experiments to understand the effect of fasting on jejunal physiology.

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7 LIST OF PUBLICATIONS

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List of publications related to the dissertation

Hungarian scientific articles in Hungarian journals (1)

1. **Simon, Á.**, Jávor, A., Czeglédi, L.: A hipotalamikus neuropeptidek szerepe a gazdasági állatfajok takarmányfelvételének szabályozásában: irodalmi áttekintés.
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