

## Feeding state and age dependent changes in melanin-concentrating hormone expression in the hypothalamus of broiler chickens

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We aimed to quantify the gene expression changes of the potent orexigenic melanin-concentrating hormone (MCH) in chicken (*Gallus gallus*) hypothalamus with quantitative real-time polymerase chain reaction (qPCR), and for the first time determine peptide concentrations with a novel radioimmunoassay (RIA) under different feeding status. Three different experimental conditions, namely *ad libitum* feeding; fasting for 24 h; fasting for 24 h and then refeeding for 2 h, were applied to study changes of the aforementioned target and its receptor (*MCHR4*) gene expression under different nutritional status. The relative changes of MCH and *MCHR4* were also studied from 7 to 35 days of age. Expression of *PMCH* and *MCHR4* along the gastrointestinal tract (GIT) was also investigated. We found that expression of both targets was significant in the hypothalamus, while only weak expression was detected along the GIT. Different nutritional states did not affect the *PMCH* and *MCHR4* mRNA levels. However, 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. A decreasing trend with age was observable for both, the *PMCH* and *MCHR4* mRNA levels, and also for the MCH-like immunoreactivity. Correlation analysis did not result in a significant correlation between MCH peptide concentration and abdominal fat mass in *ad libitum* fed birds. In conclusion, MCH peptide concentration altered in response to 24 h fasting, which indicated that this peptide may take part in feed intake regulation of broiler chickens.

**Key words:** chicken, feeding states, hypothalamus, MCH, qPCR, RIA

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**Abbreviations:** Cq, quantification cycle; GIT, gastrointestinal tract; MCH, melanin-concentrating hormone; *MCHR4*, melanin-concentrating hormone receptor 4; *PMCH*, pro-melanin-concentrating hormone; qPCR, quantitative real-time polymerase chain reaction; RIA, radioimmunoassay; SEM, standard error of the mean

### INTRODUCTION

Feed intake regulating neural and endocrine systems are highly conserved among animals. The hypothala-

mus is the main site integrating peripheral and central signals regarding nutritional state in the mammalian, as well as in the avian species. The lipostatic theory of feed intake proposed that the hypothalamus also receives signals which represent the current state of fat depots and makes adjustments in feed intake to maintain the body's main energy storage (Baile *et al.*, 2000). The list of hypothalamic peptides influencing feed intake is growing; there are several peptides which are quite well studied in mammalian species, but still less studied in chickens at the protein level due to the lack of specific antibodies. The melanin-concentrating hormone (MCH) was first isolated from the chum salmon pituitary by Kawauchi and coworkers (1983), and recognized as a regulator of skin color change. Later, it was revealed that MCH also plays a significant role in the regulation of feeding behavior of mammals (Chagnon *et al.*, 2007). In rats, feed consumption increased after a central injection which suggests a role in the hypothalamic regulation of body weight (Qu *et al.*, 1996). Mice that lack MCH or melanin-concentrating hormone 1 receptor (*MCHR1*) are hypophagic, lean and hyperactive (Shimada *et al.*, 1998; Marsh *et al.*, 2002). Expression of *PMCH* and MCH receptor is mainly found in the central nervous system, with peak expression in the hypothalamic second-order neurons (Bittencourt *et al.*, 1992), but weak peripheral expression in mammals (Saito *et al.*, 1999; Pissios *et al.*, 2006) and chickens was also reported (Cui *et al.*, 2017). Presence of MCH in the circulation is also recognized (Naufahu *et al.*, 2017), but its role outside the central nervous system remained unclear.

The structure of the precursor mRNA and pro-MCH was comprehensively summarized by Presse & Nahon (2013). The mature MCH is a cyclic peptide with a disulfide bridge between two cysteines which are essential for MCH activity. A final form of MCH is created by proteolytic cleavage from its precursor called pre-MCH, along with two other peptides called neuropeptide glycine-glutamic acid (NGE) and neuropeptide glutamic acid-isoleucine (NEI). In mammals, one more neuropeptide, the MCH gene overprinted polypeptide (MGOP) can be potentially created by alternative splicing (Presse & Nahon, 2013). These peptides, beside MCH, have not been identified in chickens so far. The chicken pro-MCH consists of 163 amino acids with relatively low sequence similarity to mammalian counterparts (Sun *et al.*, 2013). However, the final form of MCH with 19 amino acids

shows high conservation between vertebrate species, as 100% sequence similarity exists between human, chicken and rat (Vaughan *et al.*, 1989; Breton *et al.*, 1993; Cui *et al.*, 2017).

Variations in the pro-melanin-concentrating hormone (PMCH) gene are often associated with performance traits of domestic animals. Single base substitution in the promoter region of *PMCH* is associated with average fat and grade fat (Helgeson & Schmutz, 2008), and further advantageous carcass characteristics in cattle (Walter *et al.*, 2014). There is also a variation in the non-coding site associated with shear force of breast and leg muscles of chicken (Sun *et al.*, 2013). This gene is also found to be associated with backfat thickness in pigs (Perez-Montarolo *et al.*, 2014). Despite the intense selection pressure, the DNA methylation pattern in the promoter region of *PMCH* remained highly conserved between wild and domestic chicken (Li *et al.*, 2015). MCH acts through two G protein-coupled receptors in humans, namely MCHR1 and MCHR2. Functional MCHR2 seems to be lost during evolution and only MCHR1 can be found in rodents (Chung *et al.*, 2011). A total of seven different MCHRs were predicted in vertebrates so far (Yun *et al.*, 2015). Recently, Cui and coworkers (Cui *et al.*, 2017) cloned and characterized two MCHRs in chickens. *MCHR2* is likely a pseudogene, as it does not encode a full-length receptor. Their functional study had shown that an MCHR1-like receptor (which is named *MCHR4* according to its evolutionary origin) is the only functional receptor for chicken MCH<sub>1-19</sub>.

The mRNA expression of the functional *MCHR* and *PMCH* has not been characterized in various tissues and the gene expression response to different feeding states of broilers has not been examined so far. A recently developed method (Lelesz *et al.*, 2016) measures not only *PMCH* expression, but the chicken MCH-like peptide levels (the developed assay is specific for the preprohormone) is assessed by RIA as well. Our study aimed to investigate the tissue distribution of *MCHR* and the *PMCH* with qPCR in the GIT of broilers because expression of feed intake regulating hormones is often restricted not only to the central nervous system (Ilnytska & Argyropoulos, 2008; Honda *et al.*, 2017). MCH is also involved in adiposity (Bradley *et al.*, 2000), therefore, we were interested to see if the hypothalamic level of MCH correlates with the adipose tissue weight. To the best of our knowledge, the response of MCH peptide and transcript levels to different feeding states in broiler chickens had not been studied. Here, changes in the peptide and mRNA levels of MCH were investigated under *ad libitum*, fasted for 24 h, fasted for 24 h and refed for 2 h conditions, and during growth (from 7 to 35 days of age), in the hypothalamus and some peripheral tissues. MCH peptide levels were investigated in the hypothalamus under different feeding status of chickens, and *MCHR4* and *PMCH* expression was assessed in various tissues under *ad libitum* fed conditions. We hypothesized that *PMCH* expression should increase after fasting, as it encodes an orexigenic peptide. Our results may provide information for better understanding of feed intake regulation for meat type chickens which may aid the selection of broilers with improved performance in the future.

## MATERIALS AND METHODS

**Ethical approval.** The experiment was approved by the local ethical boards of the University of Debrecen, Hungary (registration number: DEMAB/12-7/2015).

**Tissue distribution of *MCHR4* and *PMCH* transcripts, and effects of fasting and refeeding on the hypothalamic *MCHR4* and *PMCH* expression.** One day old broiler type chicks (Ross 308) were obtained from a commercial hatchery. Chickens were sexed using DNA extracted from wing feathers (Malagó *et al.*, 2002) and a PCR based method according to Li and coworkers (Li *et al.*, 2012). Male chickens were selected and allocated into floor pens. The floor pens had wood shavings as bedding and were equipped with bowl feeders and Plasson drinkers. The temperature was set at 32°C at the time of placement using electrical heaters, then gradually decreased by 1.5°C/week to meet the birds' requirement. A lighting regime was implemented in accordance with the Ross 308 management manual. At 28 days of age, chickens were divided into three treatment groups: *ad libitum* fed (*ad libitum* group), fasted for 24 h (F24h group) fasted for 24 h and then refed for 2 h (F24hRF2h group). Control male chickens were fed *ad libitum* with broiler grower diet (20% crude protein with a metabolizable energy of 13 MJ/kg). Water was freely available for all groups during the whole experiment. After the fasting period, 8 birds were sacrificed from both groups, while the fasted group was refed with grower diet for 2 h. After refeeding, 8 birds were also sacrificed from the F24hRF2h group. All chickens were terminated by concussion as recommended in 40/2013. (II. 14.) Hungarian act relating to animal experiments. Following decapitation, whole hypothalamus samples were macro-dissected within 10 minutes, as according to Griffin and coworkers (Griffin *et al.*, 2001). From the *ad libitum* fed broilers *ingluvies*, *proventriculus*, *ventriculus muscularis*, *duodenum*, *jejunum*, *ileum*, *cecum* and *colorectum* samples were also excised. All samples were snap chilled in liquid nitrogen and were stored in a freezer at -70°C.

**Age-dependent changes in *MCHR4* and *PMCH* expression and correlation analysis with phenotypic parameters.** Another set of one day old broiler type chickens (Ross 308) was housed, sexed, raised and sacrificed by the same manner as described in the previous section. Birds were given *ad libitum* access to water and feed during the whole experiment. Eight chickens were sacrificed at different time points, namely at 7, 14, 21, 28 and 35 days of age. Live weight was recorded before sacrifice, then the abdominal cavity was opened and the abdominal fat was excised, collected and weighed by a digital balance. The hypothalamus was excised and stored as described in the previous section. Plasma samples were also collected for glucose and insulin measurements. All samples were stored as described in the previous section.

**RNA isolation and reverse transcription.** Whole hypothalamus samples were grinded in liquid nitrogen using a mortar and pestle. Powdered tissue (25±2.5 mg) was lysed in the TRI Reagent solution (Thermo Fisher Scientific, Waltham, MA, USA), with an Ultra-Turrax T10 at setting 5 for 1 min for efficient lysis and shredding of gDNA. Total RNA was extracted using Direct-zol™ RNA MiniPrep (Zymo Research, Orange, CA, USA) using an on-column DNase I digestion step with 30 U of the enzyme for 15 min. Elution was conducted once with 50 µl DNase/RNase-Free water pre-heated to 70°C. Isolated RNA amounts and purity (260/280 and 260/230 nm ratios) were quantified in the eluent using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was verified with 1% agarose gel electrophoresis. Reverse transcription was conducted using 800 ng total RNA with qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, UK) in 20 µl reaction volume with oligo(dT)s and random hexamers

Table 1. Details of primers and amplicons for reference and target sequences

| Gene symbol   | Sequence accession number | Chromosome | Description  | Encoded protein's function   | Primers (5'-3' sequence of forward and reverse)                                    | Reaction efficiency (E) <sup>†</sup> | Amplicon length (bp) | Amplicon length on gDNA (bp) | Amplicon Tm (°C) <sup>‡</sup> |
|---------------|---------------------------|------------|--|--|--|--------------------------------------|----------------------|------------------------------|-------------------------------|
| <i>ACTB</i>   | NM_205518.1               | 14         | Beta cytoskeletal actin  | Component of the cytoskeletal microfilaments.                            | <sup>1</sup> F: AGATCACAGCCCTGGCACCTAG<br><sup>1</sup> R: TTGCGCTCAGGTGGGGCAAT     | 1.920                                | 61                   | 416                          | 80.9                          |
| <i>B2M</i>    | XM_015279077.1            | 10         | Beta-2-microglobulin   | Component of the major histocompatibility complex class I.               | <sup>1</sup> F: ATCCCGAGTTCTGAGCTGTGC<br><sup>1</sup> R: CCGTCATACCCAGAAAGTGGCAT   | 1.888                                | 115                  | 1098                         | 83.2                          |
| <i>GAPDH</i>  | NM_204305.1               | 1          | Glyceraldehyde-3-phosphate dehydrogenase                                     | Takes part in glycolysis.  | <sup>1</sup> F: GCTGGCATTGCACTGAATGAC<br><sup>1</sup> R: CACTCTTGGATGCCATGT        | 1.853                                | 113                  | 470                          | 82.3                          |
| <i>HMB</i>    | XM_417846.5               | 24         | Hydroxymethylbilane synthase   | The third enzyme of the heme biosynthetic pathway.                       | <sup>1</sup> F: GCCTGAAGGAAACAATGCAGA<br><sup>1</sup> R: GCTGTGATGCCAACATGCT       | 1.886                                | 91                   | 543                          | 81.2                          |
| <i>LBR</i>    | NM_205342.1               | 3          | Lamin B receptor   | Localized in the nuclear envelope inner membrane.                        | <sup>1</sup> F: AAGGCACCTGAGGAAGACCTA<br><sup>1</sup> R: AAACCTGCCAATACGAGGGTTC    | 1.908                                | 98                   | 1032                         | 80.7                          |
| <i>POLR2B</i> | NM_001006448.1            | 4          | Polymerase (RNA) II (DNA directed) polypeptide B                             | Subunit of the DNA-dependent RNA polymerase II.                          | <sup>1</sup> F: ACACGTATGAATGCCGGGA<br><sup>1</sup> R: AGGTGCAATGCTCATAGACATCA     | 1.919                                | 110                  | 1262                         | 82.1                          |
| <i>RN18S</i>  | AF173612.1                | unmapped   | 18S ribosomal RNA gene   | Structural RNA of the small component of cytoplasmic ribosomes.          | <sup>1</sup> F: CTCTTCTCGATTCGCTGGGT<br><sup>1</sup> R: CATGCCAGAGTCTGCTTCGT       | 1.907                                | 96                   | 96 (single exon)             | 82.9                          |
| <i>RPS17</i>  | NM_204217.1               | 10         | Ribosomal protein S17  | Ribosomal protein, component of small subunit.                           | <sup>1</sup> F: TCCGGGGTATCTCCATTAAAGCTG<br><sup>1</sup> R: CATTTCCTGGTGTCGGGTC    | 1.901                                | 119                  | 1177                         | 85.7                          |
| <i>TBP</i>    | NM_205103.1               | 3          | TATA box binding protein   | Component of transcription factor IID.                                   | <sup>1</sup> F: ATCAAGCCCAAGAATTGTTCTGC<br><sup>1</sup> R: CTTCTGATTTCTGCTCGAACT   | 1.887                                | 85                   | 981                          | 78.2                          |
| <i>YWHAZ</i>  | NM_001031343.1            | 2          | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta | Adapter protein in different general and specialized signaling pathways. | <sup>1</sup> F: AGTCATACAAAGACAGCACGCTA<br><sup>1</sup> R: GCTTCATCTCTTGGGTATCCGA  | 1.886                                | 85                   | 416                          | 79.6                          |
| <i>MCHR4</i>  | KY196444.1                | 18         | Melanin-concentrating hormone receptor 4 (Also known as MCHR1)               | Only known functional receptor for MCH in chickens                       | <sup>2</sup> F: GCACAATTCTCCACGTCGG<br><sup>2</sup> R: CAACGCCACAGGAACAGATG        | 1.893                                | 113                  | 113                          | 84.4                          |
| <i>PMCH</i>   | NM_001195795.1            | 1          | Pre-melanin-concentrating hormone gene                                       | Pre-pro-MCH, precursor molecule of MCH                                   | <sup>3</sup> F: CAGTAAATTCCTCCATAGGAAGG<br><sup>3</sup> R: CAAAGAAAGTGCTGTAAGTCAGT | 1.877                                | 114                  | 399                          | 81.5                          |

<sup>†</sup>determined with LinRegPCR (range between 1 and 2, 2 is equals to 100% efficiency); <sup>‡</sup>determined with melt curve analysis. <sup>1</sup>(Simon *et al.*, 2018). <sup>2</sup>(Cui *et al.*, 2017). <sup>3</sup>designed in current study.

using Moloney Murine Leukemia Virus (MMLV) type reverse transcriptase. Reaction conditions were as follows: reverse transcription at 42°C for 30 min and RTase denaturation at 85°C for 10 min. Prior to qPCR, cDNA samples were diluted 10-fold, then stored in a freezer at -20°C until qPCR was performed.

**Quantitative real-time PCR (qPCR) assays.** Intron spanning cDNA specific primers were designed using Primer Express v3.0.1 software (Table 1) and *in silico* tested with NCBI primer BLAST (Ye *et al.*, 2012) to avoid false priming to pseudogenes or any other unexpected targets. Primers were also compared against the NCBI SNP database to avoid variations in priming sites. Amplicons were designed to be <120 bp for efficient reactions under fast cycling conditions and to make the assay independent from RNA integrity (Fleige & Pfaffl, 2006). Real-time PCR reactions were run in triplicates, with the same targets run in the same run using 384-well plates (4itude, Surrey, UK) on a LightCycler 480 Instrument II (Roche Life Science, Penzberg, Germany). Annealing temperatures were optimized using a PTC-200 thermal cycler (Bio-Rad, Hercules, CA, USA) between 58°C and 69°C. Reference gene selection was based on our previous publication (Simon *et al.*, 2018). Plates were centrifuged at 1500×g for 2 min before loading into the qPCR machine. Thermal cycling conditions were the following: initial denaturation at 95°C for 2 min, 50 cycles of denaturation at 95°C for 5 s, and annealing/extension at 60°C for 30 s. Reactions (10 µl) contained 4 ng of cDNA template, 1X Xceed qPCR SG Hi-ROX mastermix (Institute of Applied Biotechnologies, Prague, Czech Republic), and 200 nM of each primer. Primers were purified with standard desalting, stocks were diluted in molecular biology grade water and stored at -20°C (Integrated DNA Technologies, Leuven, Belgium). No template controls were included in each assay and reverse transcriptase negative controls were run in the case of *RN18S* and *MCHR4* to check for DNA contamination. Primer specificities were tested using the melt profile analysis after each run by gradually increasing temperature from 60°C to 95°C. Raw fluorescent data was collected with LightCycler software 1.5.0 (Roche Life Science) and converted using LightCycler 480 text file converter into input format for LinRegPCR (both available at <http://www.hartfaalcentrum.nl/index.php?main=files&sub=LinRegPCR>). Mean reaction efficiencies (E) for each primer pair were determined with linear regression analysis on individual amplification curves using LinRegPCR version 2017.0 (Ruijter & Ilgun, 2009), and also the quantification cycle values (Cq). Technical replicates with

Cq that deviated by more than one cycle from the other two were excluded from the averaging process. Five different approaches were applied to search for stable reference genes during growth. Analysis by geNorm (v3.5), NormFinder (v0.953), the BestKeeper (v1), the comparative  $\Delta C_t$  method and the method described by Chervoneva and coworkers (Chervoneva *et al.*, 2010) was performed as described previously (Simon *et al.*, 2018). Raw Cq values were normalized with stable reference gene Cq values (*TBP* for fasting-refeeding, and *ACTB* for age-related results), gene expression ratios were calculated, as described by Pfaffl (Pfaffl, 2001).

**Plasma glucose and insulin measurements.** One mL (from 7 day old) or five mL (from 14-35 days old) of blood from each bird was collected in EDTA coated blood collection tubes (S-Monovette®, Sarstedt, Germany) and centrifuged at 2000×g for 10 minutes. Aliquots of plasma samples were immediately frozen in liquid nitrogen and stored at -70°C for further analysis. Blood glucose level was determined by the glucose oxidase method (Accu-Chek Active, Roche Diagnostics, Budapest, Hungary). The plasma insulin level was determined by immunoradiometric assay using a commercially available insulin kit (RK-400CT, Institute of Isotopes, Budapest, Hungary).

**MCH radioimmunoassay.** Powdered hypothalamus samples (150±1.5 mg) were homogenized in 1.5 mL ice cold molecular biology grade water (AccuGENE Water, Lonza) with a rotor-stator homogenizer (Ultra-Turrax® T10, IKA, Germany) for 30 sec at speed setting 6. Homogenates were then centrifuged for 10 min at 20000×g, at 4°C (Centrifuge 5810 R, Eppendorf). Supernatants were stored at -70°C for further analysis. In our RIA examination, the MCH specific antiserum (MCH1/5) was applied, which was raised against a conjugate of rat MCH and bovine serum albumin (BSA) coupled by glutaraldehyde in rabbits. The rat MCH peptide was used as a RIA standard. The range of concentration was between 0 and 200 fmol/mL. Mono-<sup>125</sup>I-labeled MCH was applied as a RIA tracer prepared in our isotope laboratory. Our assay buffer (0.05 mol/L, pH 7.4 phosphate buffer) contained 0.1 M NaCl, 0.05% NaN<sub>3</sub>, 0.25% BSA (Sigma). The 1 mL incubation mixture contained 100 µl MCH standards, 100 µl antiserum (working dilution 1:3500), 100 µl RIA tracer (3000 cpm/tube) and the assay buffer. After 48 hour incubation at 4°C the antibody-bound peptide was separated from the free peptide by addition of 100 µl separating suspension (10 g charcoal, 1 g dextran and 0.5 g commercial fat-free milk powder in 100 mL distilled water). After centrifugation

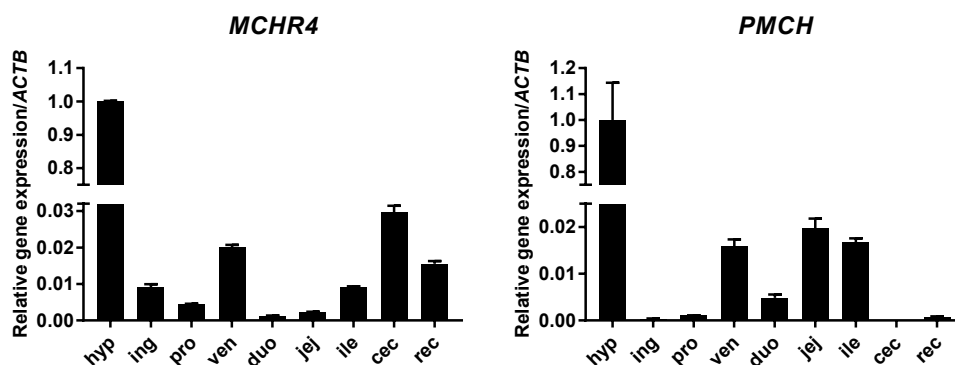
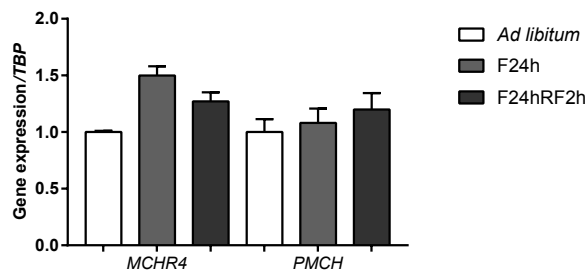


Figure 1. *MCHR4* and *PMCH* gene expression in nine different tissue types of broiler chickens as assessed by qPCR.

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 ± S.E.M. (n=4 in each group). Mean is presented as a fold difference compared to the value observed in the hypothalamus.





**Figure 2.** Effect of fasting and refeeding on the expression of *MCHR4* and *PMCH* genes in the hypothalamus of 28 day old broilers.

Data represent mean  $\pm$  S.E.M. ( $n=8$  in each group). *TBP* was used as a reference gene. No significant differences were found between groups.

(4000 rpm, 4°C, 20 min) the tubes were gently decanted. Radioactivity of the precipitates was measured in a NZ310 type gamma counter (Gamma, Budapest, Hungary). The MCH-like immunoreactivity (MCH-LI) of the unknown samples was read from the calibration curve.

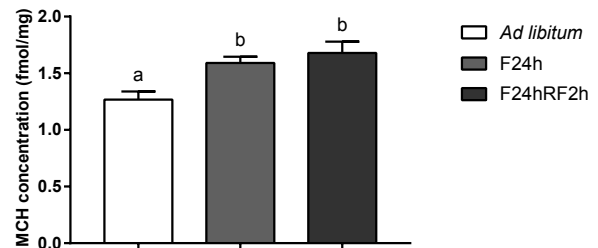
Antiserum “MCH1/5” used in the assay turned out to be C-terminal specific without affinity for the structurally similar peptides. The average ID50 value of the calibration curves was  $11.93 \pm 1.78$  fmol/mL, determined in ten consecutive assays. Detection limit of the assay for rat MCH was 0.2 fmol/mL. Intra-assay and inter-assay coefficients of variation were 6.84% and 9.32%, respectively (Lelesz *et al.*, 2016).

**Statistical analysis.** Statistical analysis was performed with GraphPad Prism® 7 (La Jolla, California, USA) software for Windows using One-Way ANOVA, followed by the Tukey-Kramer *post hoc* test for comparing every mean with every other mean. The ANOVA *post hoc* test for linear and non-linear trend analysis was also performed. Two-tailed Spearman correlation analysis was used to create a correlation matrix between the measured parameters. Results with  $P < 0.05$  were considered as statistically significant in all cases. All data was represented as mean  $\pm$  standard error of the mean (S.E.M.).

## RESULTS

### Tissue distribution of *MCHR4* and *PMCH* transcripts, and effects of fasting and refeeding on the hypothalamic *MCHR4* and *PMCH* expression

As the first step, we sought for stable control genes to be used in the subsequent RT-qPCR reactions. Searching for stable reference gene selection resulted in the expression of *ACTB* as the most stable among the selected targets during growth (Table S1 at [www.actabp.pl](http://www.actabp.pl)), and melt curves indicated specific reactions (Fig. S1 at [www.actabp.pl](http://www.actabp.pl)). Tissue distribution of *MCHR4* and *PMCH* investigated in the hypothalamus and along the GIT with qPCR are presented in Fig. 1. *PMCH* is expressed in



**Figure 3.** Effect of fasting and refeeding on the hypothalamic concentration of MCH in 28 day old broilers as assessed by RIA. Data represent mean  $\pm$  S.E.M. ( $n=8$  in each group). MCH contents are expressed as fmol/mg wet tissue weight. Group means with similar letters are not significantly different ( $P > 0.05$ ).

moderate levels in the hypothalamus with average Cqs of 28 and *MCHR4* in low levels with 37. Very low transcript levels of *MCHR4* and *PMCH* were measured in the GIT relative to hypothalamic expression ( $C_q > 34$ ), indicating that these transcripts are hardly expressed outside the central nervous system. *PMCH* expression was undetectable in the cecum. We measured the expression of *MCHR4* and *PMCH* with qPCR in the *ad libitum*, fasted for 24 h, and fasted for 24 h and refed for 2 h groups and MCH-like immunoreactivity with radioimmunoassay under the aforementioned conditions. There was no significant difference between the three groups ( $P=0.9730$ ) in regards to live weight (mean g  $\pm$  S.E.M.) before the experiment begun with the following values:  $1326 \pm 85.25$ ,  $1325 \pm 64.76$ , and  $1344 \pm 21.29$  in the *ad libitum*, F24h, and F24hRF2h groups, respectively. Mean live weight in the F24h group was significantly lower ( $P=0.0135$ ) when compared to the control group, but there was no significant difference between the *ad libitum* *vs.* F24hRF2h or in the F24h *vs.* F24hRF2h comparison ( $P > 0.05$ ).

Variance analysis of the parameters from different experimental settings had shown that fasting and refeeding did not affect the expression of these two genes significantly ( $P > 0.05$ ) when assessed by qPCR (Fig. 2). The effect of fasting for 24 h resulted in a significantly increased ( $P=0.0202$ ), i.e. 25.65% higher level of the hypothalamic MCH concentration measured by RIA. Birds fasting for 24 h and then refed for 2 h had even higher MCH level, with 32.51% increase ( $P=0.0033$ ), when compared to the *ad libitum* group (Fig. 3). Similar trends in changes of the *PMCH* expression and MCH concentrations were observed.

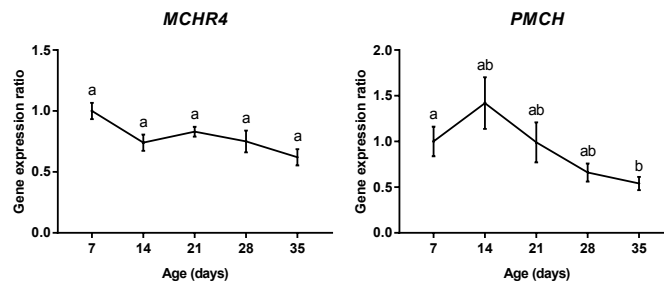
### Age-dependent changes in *MCHR4* and *PMCH* expression and correlation analysis with phenotypic parameters

Changes of abdominal fat and body weight and the percentage of abdominal fat depending on age are presented in Table 2. Analysis of variance revealed that

**Table 2.** Body and abdominal fat weight of chickens at different ages

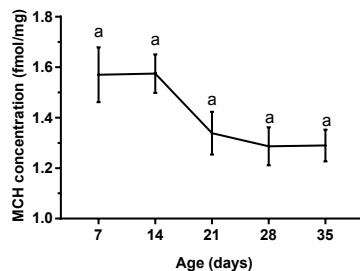
|                                 | Age (days)           |                     |                     |                       |                    |
|---------------------------------|----------------------|---------------------|---------------------|-----------------------|--------------------|
|                                 | 7                    | 14                  | 21                  | 28                    | 35                 |
| Abdominal fat (g)               | $1.05 \pm 0.102^a$   | $3.6 \pm 0.241^a$   | $9.24 \pm 0.623^b$  | $20.1 \pm 0.9^c$      | $24.8 \pm 1.28^d$  |
| Body weight (g)                 | $148.1 \pm 3.455^a$  | $311.6 \pm 12.15^a$ | $595.1 \pm 22.78^b$ | $1401 \pm 59.96^c$    | $1573 \pm 64.33^d$ |
| Percentage of abdominal fat (%) | $0.708 \pm 0.0645^a$ | $1.16 \pm 0.0658^b$ | $1.56 \pm 0.107^c$  | $1.44 \pm 0.056^{bc}$ | $1.58 \pm 0.076^c$ |

Data are expressed as mean  $\pm$  S.E.M.,  $n=8$  in each group. Group means with same letters are not significantly different ( $P > 0.05$ ).



**Figure 4. Age dependent changes in *MCHR4* and *PMCH* gene expression in broiler chicken hypothalamus.**

Data represent normalized  $\Delta Cq$  mean  $\pm$  S.E.M. ( $n=8$  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.



**Figure 5. Age dependent changes of MCH concentration in broiler chicken hypothalamus.**

Data represent mean  $\pm$  S.E.M. ( $n=8$  in each group). MCH contents are expressed as fmol/mg wet tissue weight. Group means with same letters are not significantly different ( $P>0.05$ ).

the abdominal fat and body weight had significantly increased after 21 days of age ( $P<0.05$ ). The percentage of abdominal fat also increased in an age dependent manner and reached 1.58% of the total body weight, on average at 35 days of age. Gene expression analysis of selected targets with qPCR revealed that in *MCHR4* expression no significant changes were found between the time points and much higher biological variations were observed when comparing to the *PMCH*'s expression. For *PMCH*, a significant ( $P=0.0184$ ) 0.54 fold decrease in mRNA level was observable between the first and the last time point (7th vs 35th days). Non-significant changes were detected in the other comparisons (Fig. 4). Linear trend analysis resulted in a slope of  $-0.075$  for *MCHR4* ( $P=0.0013$ ) and of  $-0.168$  for *PMCH* ( $P=0.0066$ ) indicating a significant linear decreasing trend for both genes upon growth. Measuring MCH-like immunoreactivity with RIA resulted in hypothalamic changes that were non-significant ( $P>0.05$ ) by age (Fig. 5), but linear trend analysis (with slope:  $-0.0848$ , and  $P=0.0027$ ) indicated a decreasing trend in MCH with age. Non-linear trend analysis did not show any significant ( $P>0.05$ ) results which indicates that the observed trends were linear in all of the investigated cases. MCH concentration ranged between 0.971–2.029 fmol/mg of wet tissue weights in the hypothalamus in the age related experiment. Insulin plasma concentrations had increased by age, with slope: 0.5336, and  $P=0.0119$  (Fig. S2 at [www.actabp.pl](http://www.actabp.pl)). Spearman correlation analysis showed that abdominal fat and body weights are significantly positively correlated, which means these two parameters tend to increase together, as expected. However, no significant correlation was found in the other cases (Supplementary Table 2 at [www.actabp.pl](http://www.actabp.pl)).

## DISCUSSION

The peptide sequence of MCH is highly conserved in all mammalian and avian species. This high conservation suggests that MCH has a similar role in feed intake regulation in chickens, as well as in higher vertebrates. Altering feeding state from *ad libitum* to fasting is commonly used to investigate a candidate feed intake regulating neuropeptide gene response (Wang *et al.*, 2012; Cui *et al.*, 2017). We hypothesized that the MCH level should increase in response to fasting and decrease with refeeding as MCH functions as an orexigenic peptide in mammals. We also aimed to investigate whether tissue expression patterns are similar between mammals and chickens.

Central and peripheral tissue mRNA distributions for both, MCH and its receptor, were examined. We found very low levels of expression except for the hypothalamus, which is the main site of feed intake regulation. Outside the CNS, *PMCH* and *PMCH* mRNA were undetectable in nearly all tissues except for spleen and testes, respectively, as examined by Cui and coworkers (Cui *et al.*, 2017) in layer type chickens. Our results are in agreement with these findings as we found that both transcripts are in very low levels along the GIT. This indicates that the expression of MCH and its receptor occurs mainly in the central nervous system. This result is inconsistent with mammalian studies as some of them clearly demonstrate the presence of MCH mRNA and MCH immunoreactivity in the peripheral tissues, like testis, stomach, pancreas and intestine (Hervieu & Nahon, 1995; Pissios *et al.*, 2007). Therefore, MCH probably does not play as much of a significant role in the peripheral organs of chickens as in mammals.

Our experimental settings allowed us to investigate the impact of feeding states on the expression of the selected genes. No significant changes were observed in the expression of *MCHR4* or *PMCH* transcripts, however, the significant increase in MCH peptide level indicates that *PMCH*'s final gene product is feeding state dependent in the hypothalamus. Mean hypothalamic MCH peptide concentration measured by RIA in the current study was similar in broiler chickens (1.27 fmol/mg) as compared to rats (1.3 fmol/mg, Lelesz *et al.*, 2016). Intracerebroventricular injection of MCH into 3 day old broiler chicks did not result in increased feed intake (Ando *et al.*, 2000), however, this does not reflect how endogenous MCH peptide concentration responds to different feeding states. Previous studies conducted with layer type chickens showed that *PMCH* mRNA level was greater in low bodyweight selected than high bodyweight selected chicks, but *PMCH* expression did not differ between the fed and fasted states (Yi *et al.*, 2015). Fasting for 48 h significantly increased *PMCH* mRNA levels by

more than 1.5 fold in male broiler chickens when compared to control fed birds and returned to normal levels after 24 h refeeding (Song *et al.*, 2012). These results indicate that *PMCH* expression differently responds to different energy status in chickens. In the fasting-refeeding experiment, the short refeeding period chosen (2 h) did not significantly affect the *PMCH* expression when compared to 24 h fasted group, which is in agreement with a study conducted with rats, where *PMCH* expression did not change in the hypothalamus between fasted for 16 h and then refed for 2 h experimental states (Uchoa *et al.*, 2012). These observations may indicate that the MCH expression reflects the overall energy balance of the body similarly in both species.

In our age-related experiment, a decreasing trend was observable both, in the *MCHR4* and *PMCH* expression, and in the MCH concentrations in the hypothalamus. Similar results were found when Saneyasu and coworkers (Saneyasu *et al.*, 2013) investigated one of the most potent orexigenic peptide encoding gene expression, namely neuropeptide Y (NPY), and found that mRNA levels of these genes are age-dependently decreased in the broiler chicken hypothalamus. NPY expression was also found to be decreasing in layer type chickens with age (Honda *et al.*, 2015) and pro-opiomelanocortin which encodes anorexigenic peptides was found to be increased with age. An age-dependent increase in cocaine-amphetamine regulated transcript (an anorexigenic peptide) level was found in the chicken hypothalamus (Cai *et al.*, 2015). Our observation is in accordance with the literature, as the expression of genes encoding orexigenic peptides is decreasing, while expression of genes encoding anorexigenic peptides is increasing with developmental stages of chickens.

It was hypothesized that the MCH expression decreases as a negative feedback to the increasing fat deposits with age. The lipostatic theory suggests that the current state of fat depots can also take part in the regulation of feed intake to maintain the body's main energy storage (Baile *et al.*, 2000). The main mammalian signaling molecule produced in adipose tissue is leptin. Studies showed that leptin exists and functions in birds, including zebra finch, chickens and ducks. Leptin receptor is present in the chicken hypothalamus (Liu *et al.*, 2007). However, leptin may not be an adipocyte-derived endocrine hormone to control avian energy balance, as its expression is low in the fat tissue and does not correlate with the fat pad mass (Huang *et al.*, 2014; Seroussi *et al.*, 2016). Insulin is also a proposed adiposity reporter in chickens. Plasma insulin concentration increases with body weight (Tokushima *et al.*, 2003) and can alter anorexigenic neuropeptide expression (Zhang *et al.*, 2015). Insulin receptor is also present in the chicken hypothalamus (Shiraiishi *et al.*, 2011) and that could be an evidence for insulinergic control of feed intake. In our studies, plasma insulin concentration showed an increasing trend with age, however, no correlation was found with the MCH peptide concentration, which may indicate no connection between MCH expression and adiposity.

## CONCLUSIONS

We found that *MCHR4* and *PMCH* are mainly expressed in the hypothalamus of broiler chickens. Gene expression of the selected transcripts is independent of the feeding state, but MCH-like immunoreactivity was increased with fasting followed by refeeding which indicates a role in feed intake regulation of broiler chickens.

Both gene transcripts and MCH-like immunoreactivity displayed decreasing trends in the hypothalamus with age which raises further questions as to why these genes' expression is altered during ontogenesis.

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