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## **Chemerin impairs food intake and body weight in chicken :**

### **focus on hypothalamic neuropeptides gene expression and AMPK signaling pathway**

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**Running title:** Chicken chemerin reduces food intake

### **Footnote**

AgRP: Agouti-related peptide

BBB : blood-brain barrier

CART : Cocaine and amphetamine-regulated transcript

CCRL2 : C-C Motif Chemokine Receptor Like 2

CMKLR1 : Chemerin Chemokine-Like Receptor 1

CNS: central nervous system

CRH : Corticotrophin releasing hormone

CSF : cerebrospinal fluid

GPR1 : G Protein-Coupled Receptor 1

NPY : Neuropeptide Y

POMC : Pro-opiomelanocortin

RARRES2: Retinoic Acid Receptor Responder 2

TAS1R1 : Taste 1 Receptor Member 1

## **Abstract**

Unlike mammals, the role of adipokines and more particularly of chemerin in the regulation of food intake is totally unknown in avian species. Here we investigated the effect of chemerin on the food and water consumption and on the body weight in chicken. We studied the effects on the plasma glucose and insulin concentrations and the hypothalamic neuropeptides and AMPK signaling pathway. Female broiler chickens were intraperitoneally injected, daily for 13 days with either vehicle (saline; n=25) or chemerin (8µg/kg; n=25 and 16µg/kg; n=25). Food and water intakes were recorded 24h after each administration. Overnight fasted animals were sacrificed at day 13 (D13), 24h after the last injection and hypothalamus and left cerebral hemispheres were collected. Chemerin and its receptors protein levels were determined by western-blot. Gene expression of neuropeptide Y (*Npy*), agouti-related peptide (*Agrp*), corticotrophin releasing hormone (*Crh*), pro-opiomelanocortin (*Pomc*), cocaine and amphetamine-regulated transcript (*Cart*) and Taste 1 Receptor Member 1 (*Tas1r1*) were evaluated by RT-qPCR. In chicken, we found that the protein amount of chemerin, CCRL2 and GPR1 was similar in left cerebral hemisphere and hypothalamus whereas CMKLR1 was higher in hypothalamus. Chemerin administration (8 and 16µg/kg) decreased both food intake and body weight compared to vehicle without affecting water intake and the size or volume of different brain subdivisions as determined by magnetic resonance imaging. It also increased plasma insulin levels whereas glucose levels were decreased. These data were associated with an increase in *Npy* and *Agrp* expressions and a decrease in *Crh*, *Tas1r1* mRNA expression within the hypothalamus. Furthermore, chemerin decreased hypothalamic CMKLR1 protein expression and AMPK activation. Taken together, these results support that chemerin could be a peripheral appetite-regulating signal through modulation of hypothalamic peptides expression in chicken.

**Key words:** chemerin, food intake, hypothalamic gene, chicken

## 1. Introduction

In mammals, adipokines are mainly produced by white adipose tissue (WAT) (Luo and Liu 2016). They include proteins like leptin, resistin, adiponectin, visfatin and chemerin that play crucial role in the regulation of energy metabolism, insulin sensitivity but also in the appetite and satiety control (Spiegelman and Flier 2001). In rodents, recombinant leptin intravenous injection reduced food intake whereas central injection of recombinant visfatin or intravenous administration of recombinant adiponectin increased food intake (Brunetti, et al. 2012, Kubota, et al. 2007). Even if resistin deficient mice and rats treated peripherally with recombinant resistin did not change their food intake (Rajala, et al. 2003), central injection of recombinant resistin decreased food intake acutely in fasted rats (Tovar, et al. 2005). The actions of adipokines to regulate food intake fundamentally involve the hypothalamus (Henry and Clarke 2008). Indeed, several data showed that adipokines modulate feeding-related brain circuits that involve hypothalamic populations synthesizing anorexigens (pro-opiomelanocortin (POMC), cocaine,amphetamine-regulated transcript (CART) and corticotrophin-releasing hormone (CRH) and orexigens (Agouti-related protein (AgRP), neuropeptide Y (NPY)) (Klockars, et al. 2019). In mammals, chemerin is a small 16-kDa protein that is encoded by the RARRES2 gene (Mattern, et al. 2014). It is mainly expressed in WAT but also in liver (Mattern, et al. 2014). After secretion, chemerin is able to bind three G protein-coupled receptors (GPCRs) including CMKLR1, GPR1 and CCRL2 to initiate various actions depending on the cell type (Kennedy and Davenport 2018). Chemerin and its receptors are present in distinct brain regions including hypothalamus in rodents (Helfer, et al. 2016), baboons and chimpanzees (Gonzalez-Alvarez, et al. 2015) and pig (Smolinska, et al. 2019). In rat, chemerin could be involved in the structural remodelling of the hypothalamus (Helfer, et al. 2016). Furthermore, peripheral and central recombinant chemerin administration decreased food intake in rats suggesting a role of chemerin in the regulation of

satiety (Brunetti, et al. 2014, Helfer, et al. 2016). In peripheral tissues, chemerin regulates adipogenesis, glucose homeostasis (Helfer and Wu 2018), and could be a modulator of bone mass in mammals (Muruganandan, et al. 2010, Zhao, et al. 2019). In avian species, the structures and physiological functions of adipokines are largely less known than in mammals, particularly in humans and rodents (Mellouk, et al. 2018a). In chicken, the role of leptin in energy metabolism and the presence of the resistin gene are still a debate (Beauchair, et al. 2019, Dakovic, et al. 2014, Friedman-Einat and Seroussi 2019, Seroussi, et al. 2019). Concerning visfatin, central injection caused increased feed intake in chicks (Cline, et al. 2008, Li, et al. 2018). The role of chemerin in the feeding behavior, glucose metabolism and bone development is still unknown in chickens. Chicken chemerin and its receptors are expressed in peripheral tissues (Mellouk, et al. 2018c, Mellouk, et al. 2018d) and ovary (Mellouk, et al. 2018b). Furthermore, plasma chemerin levels are negatively correlated with the percentage of animal fat content (Mellouk, et al. 2018d) and with the percentage of hatchability of fertile eggs in broiler hens (Mellouk, et al. 2018b).

Considering the role played by adipokines in the control of appetite in mammals, in the present work we aimed to investigate the effects of daily administration of chicken recombinant chemerin (8–16 µg/kg) injected intraperitoneally, on food and water intake, body weight, and plasma glucose and insulin. Since several hypothalamic genes (*Npy* (Boswell, et al. 1999, Higgins, et al. 2010, Zhou, et al. 2005), *Agrp* (Song, et al. 2012), *Pomc* (Higgins, et al. 2010), *Cart* (Tachibana, et al. 2003), *Crh* (Denbow, et al. 1999, Honda, et al. 2007, Tachibana, et al. 2006) and *Tas1r1* (Byerly, et al. 2010) have previously been shown to be regulated in response to various feeding behaviors or nutritional states in birds, we investigated their expression following the chicken chemerin injections. We also studied protein levels of chemerin and its receptors and activation of the AMPK signaling pathway in chicken hypothalamus. Finally, we analyzed the impact of chicken chemerin on the volume

and surface of different brain subdivisions and on the development of the bones that could be potentially altered.

## **2. Materials and Methods**

### **2.1 Ethical issues**

All experimental procedures were performed in accordance with the French National Guidelines for the care and use of animals for research purposes (certificate of authorisation to experiment on living animals APAFIS number 10237-201706151202940v3). The Experimental Unit where birds were kept is registered by the ministry of Agriculture with license number C-37-175-1 for animal experimentation. Measurements performed in individual cages, blood sampling procedures, euthanasia procedures and scientific justification were approved by the ethics committee in Animal Experimentation of Val de Loire registered by the National Committee under the number C2EA-19.

### **2.2 Chicken recombinant chemerin and antibodies**

The chicken chemerin recombinant protein (full length, rRARRES2) was obtained from the *Gallus gallus* sequence (NM\_001277476.1), produced in *E coli* and purified by chromatography column-based on His-Tag under denaturation condition (Agro-Bio, La Ferté Saint Aubin, France). Mouse monoclonal antibody for vinculin was obtained from Sigma (Ref). Monoclonal chicken chemerin antibodies were produced by AgroBio (La Ferté Saint Aubin, France), and their specificity were tested as previously described (Mellouk, et al. 2018a). All antibodies were used at 1/1000 dilution in western blotting.

Specific antibodies against chicken CMKLR1 were produced by AgroBio (Orleans, France). Briefly, two peptides corresponding to 20 amino-terminal residues (DDSDTYDYLDYTYEPPGSV, CMKLR1-20) and 18 carboxy-terminal residues

(HRSFSKMSSMTEKETTTL, CMKLR1-18) of chicken CMKLR1 were conjugated to keyhole limpet hemocyanin using sulfhydryl chemistry (Sigma Genosys, Woodlands, TX). One hundred fifty micrograms of both conjugated CMKLR1-20 and CMKLR1-18 were emulsified with an equal volume of complete Freund's adjuvant and injected into two New Zealand White rabbits. Secondary immunizations were performed at day 14, 34, 57, 77, and 98 (relative to primary immunization) followed by bleeding on day 105. Blood samples were collected from an ear vein for titer testing in microtiter plates at day 42, 63, and 84. The specific humoral immune response to targeted antigens (CMKLR1-20 and CMKLR1-18) was evaluated by a direct ELISA using 96-well microtiter plates. The IgG from the antiserums were purified by protein G-affinity chromatography with the use of a HiTrap protein-G column (GE Healthcare), and the protein content was quantified with the BCA kit (Sigma-Aldrich). The specificity and activity of chicken CMKLR1 antibody were previously described (Estienne, et al. 2020). In the same manner, specific antibodies against chicken GPR1 were produced by AgroBio (Orleans, France). Two peptides corresponding to 18 amino-terminal residues (CYSYFYDLPEEEESPQST) and 18 carboxy-terminal residues (VELSAHHDENLHDLLQDC) of chicken GPR1 were conjugated to keyhole limpet hemocyanin using sulfhydryl chemistry (Sigma Genosys, Woodlands, TX).

### **2.3 Animals and management**

Eighty-five broiler breeder female chicks from Hendrix Genetics (Saint Laurent de la Plaine, France) were studied from day (d) 1 to 28 d of age. Animals were distributed on the ground on the day of hatching until d 15, in homogeneous groups of 8-9 birds in 10 pens, each pen with an area of 3 m<sup>2</sup>. At 15 d of age, 75 chicks closest to the mean weight of 250g were raised in individual cages and randomly assigned to one of the three treatments until 28 d of age: treatment 1 (control, administration of saline, n=25), treatment 2 (administration of



chemerin 8 µg/kg, n=25) and treatment 3 (administration of chemerin 16 µg/kg, n=25). All the animals received *ad libitum* diet (cf **Table 1** for the composition) throughout the duration of the experiment.

#### **2.4 *In vivo* intraperitoneal treatment, food and water intake and body weight measurements**

Seventy-five female Cobb500 broiler chickens (15 days-old) with similar body weight were randomized into three groups of twenty five animals each, and injected intraperitoneally (i.p) daily, at 9 a.m for 13 days with either recombinant chicken chemerin (8 or 16 µg/kg of body weight) or vehicle (saline). The doses of recombinant chicken chemerin used were similar to those of rat chemerin injected in rat by Brunetti et al., 2014 (Brunetti, et al. 2014). Recombinant chicken chemerin for i.p administration was injected in a volume of about 0.2 ml. Briefly, the intraperitoneal injection was performed using a 26G3 / 8 (or 0.45 \* 10 mm) type needle suitable for the size of the animal and a 1 ml syringe. The needle was introduced at an angle of approximately 45°C in the lower right dial of the abdomen of the young animal then a slight aspiration was carried out, in order to ensure that there was no rising blood in the needle. The administration was then be continued by pushing the plunger steadily to deliver the full volume required. At the end of the administration, the needle was gently withdrawn from the abdomen of the animal. Twenty-four hours after each daily injection, food and water intake and body weight were recorded. Food eaten through the whole period was daily determined in each group by subtracting the remaining of food in the dispenser. Twenty-four hours after the last treatment animals were sacrificed and their hypothalamus, their left cerebral hemisphere and blood samples were collected and stored at -20°C before use.

#### **2.5 Plasma biochemical parameters**

Blood samples were collected from the occipital sinus into heparin tubes at 15 days-old (before the first chemerin or vehicle injection), 22 days-old and 28 days-old (just before slaughtering). Plasma was recovered after centrifugation at 5000 g for 10 min at 4°C and stored at -20°C until use. Plasma concentrations of glucose and insulin were determined by enzymatic assay using specific kits: glucose GOD-POD (MG981780, ThermoScientific, Asnières sur Seine, France), and ELISA (MBS741090, MyBioSource, San Diego, USA), respectively. The measurements were performed according to the manufacturer's protocol. For both assays, inter- and intra-experimental coefficients of variation were <12 and <8%, respectively. Samples were run in duplicate within the same assay.

## **2.6 RNA extraction**

Total RNA was extracted from the hypothalamus using TRI Reagent (Sigma–Aldrich, St. Louis, MO), according to the manufacturer's instructions. Briefly, each chicken hypothalamus was homogenized in 1 ml of TRI Reagent. The homogenate was centrifuged at 12,000 g for 10 min at 4 °C to remove insoluble material. The supernatant, containing RNA and proteins, was added to 0.2 ml chloroform, then shaken vigorously and incubated on ice for 15 min. The next centrifugation at 12,000 g for 15 min at 4 °C separated the mixture into three phases: a red organic phase, containing proteins, an interphase, containing DNA, and a colorless upper aqueous phase, containing RNA. The aqueous phase was removed and RNA was precipitated with 0.5 ml of isopropanol, stored for 30 min at -20 °C and pelleted by centrifugation at 12,000 g for 10 min at 4 °C. The RNA pellet was washed with 1 ml of 75% ethanol, air dried and finally suspended in RNase-free water. Contaminating DNA was removed using 2 units of RNase-free DNase-1 (DNA-free kit, Ambion, Austin, TX), according to the manufacturer's protocol. RNA purity and concentration were determined by spectrophotometry (Bio- Photometer Eppendorf, Hamburg, Germany). Concentration and

purity of isolated RNA were determined with a NanoDrop Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). The integrity of RNA was checked on 1.25 % agarose-formaldehyde gels.

## **2.7 Reverse transcription and real-time reverse transcription polymerase chain reaction (real-time RT PCR)**

The cDNAs were generated by reverse transcription (RT) of total RNA (1µg) in a mixture comprising: 0.5 mM each deoxyribonucleotide triphosphate (dATP, dGTP, dCTP, DTTP) 2 M of RT buffer, 15 µg / µL of oligodT, 0.125 U of ribonuclease inhibitor, and 0.05 U MMLV (Moloney murine leukemia virus reverse transcriptase, Promega, Charbonnières-les-Bains, France) for one hour at 37°C. Real-time PCR was performed using the MyiQ Cycle device (Bio-Rad, Marnes-la-Coquette, France), in a mixture of SYBR Green Supermix 1X reagent (Bio-Rad), 250 nM specific primers (Invitrogen™ by Life Technologies™, Villebon sur Yvette, France) (**Table 2**) and 5 µL of cDNA diluted to the fifth for a total volume of 20 µL. The samples were duplicated on the same plate. PCR amplification with water, instead of cDNA, was performed systematically as a negative control. After incubation for 2 minutes at 50°C and a denaturation step of 10 minutes at 95°C, samples were subjected to 40 cycles (30 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C), following by the acquisition of the melting curve. The primers' efficiency (E) was determined by using a five-points cDNA serial dilution. It was calculated with the following formula:  $E = 10^{-1/\text{slope value}}$  and was ranged from 1.85 to 2.00 (**Table 2**). PCRs were highly specific and reproducible ( $0.97 > R^2 > 1.05$ ). For each gene, expression was calculated according to primer efficiency (E) and quantification cycle (Cq), where expression =  $E^{-Cq}$ . Then, relative expression of the target gene to the reference gene was analyzed. We have tested several reference genes and selected a combination of three genes: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase

activation protein zeta polypeptide (*Ywhaz*), TATA box binding protein (*Tbp*), and Polymerase (RNA) II (DNA directed) polypeptide B (*Polr2b*) that were not affected by the variation of food intake and peripheral chemerin administration. Indeed, the geometric mean of multiple housekeeping genes has been reported as an accurate normalization factor (Vandesompele *et al.* 2002). Furthermore, *Tbp* and *Polr2b* genes are suggested to be used for normalization in chicken hypothalamus under different feeding status (Simon, et al. 2018).

## 2.8 Western-blot

Tissues (hypothalamus and left cerebral hemisphere) were lysed using an Ultraturax (Invitrogen™ by Life Technologies™, Villebon sur Yvette, France) in lysis buffer (Tris 1 M (pH 7.4), NaCl 0.15 M, EDTA 1.3 mM, EGTA 1 mM,  $\text{VO}_4^{3-}$  23 mM, NaF 0.1 M,  $\text{NH}_2\text{PO}_4$  1 %, Triton 0.5 %). The lysates were centrifuged for 20 minutes at 16,000 g at 4 °C and the supernatant containing proteins was collected and kept on ice. The protein concentration was measured using the bicinchoninic acid (BCA) protein assay (Interchim, Montluçon, France). Lysate protein (60 µg) was mixed with Laemmli buffer 5 X and proteins were denatured for 5 minutes by heat shock at 95 °C. Proteins were loaded in an electrophoresis sodium dodecyl sulfate-polyacrylamide gel (12 % for high protein weight (110 – 20 kDa) and 15% for low protein weight (< 20 kDa)). Then, proteins were transferred to a nitrocellulose membrane. Membranes were blocked with Tris-Buffered Saline Tween buffer containing 0.05% of Tween 20 and 5% of milk for 30 minutes at room temperature. Membranes were incubated overnight at 4 °C with the appropriate primary antibody (**Table 3**). The validation of all these antibodies was already reported in chicken (cf references in **Table 3**). Membranes were then incubated 90 minutes at room temperature with a HorseRadish Peroxidase-conjugated anti-rabbit or anti-mouse IgG (**Table 3**). Proteins of interest were detected by enhanced

chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer, Villebon-sur-Yvette, France) with a G-box SynGene (Ozyme, St Quentin en Yvelines, France) and GeneSnap software ((release 7.09.17), Ozyme). Then, proteins were quantified with GeneTools software (release 4.01.02).

## **2.9 Computerized Tomography Analysis**

Chickens bone parameters (volume of whole bone (mm<sup>3</sup>), volume of tibia cortical bone volume (mm<sup>3</sup>) and length of tibia (cm)) were investigated by CT scan (Siemens Somatom Definition AS, Siemens Corp., Germany) after the 13 days of daily chicken chemerin administration in 6 euthanased animals from each group. During the bone analysis, the chickens (n= 6 in each group) were placed on its back. The X-ray source was set at 100 kV and 120 mA/s. A total of 500 images were acquired every 0.6 mm, with a pitch 0.45. The images were reconstructed using a filter Safire I26. Analyses of all data were carried out using Acquisition Sinogram Image Processing IDL's virtual machine (ASIPro VM, Siemens Medical Solutions).

## **2.10 Magnetic Resonance Imaging (MRI) method**

In this study we used a 3 Teslas (T) MRI scanner (Siemens Magnetom® Verio, Erlangen, Germany) to produce brain images of 8 anaesthetized chickens (n= 4 control and n= 4 chemerin). General anesthesia for a period of 2 hours was performed on overnight fasted animals. The anesthetic, a mixture of ketamine (75 mg / kg) and diazepam (2.5 mg / kg) was administered intramuscularly. Throughout the duration of the anesthesia, monitoring for bradycardia was performed. During the MRI examination, the animals were stored between two polystyrene cousins to prevent any movement of the animals. We used two separate 'loop' coils, with an inner diameter of 7 cm, so as to i) acquire the most normalized MRI

signal possible within the brain area and ii) and also to collect as much as MRI signal as the area is rather small compared to the size of the machine. Furthermore, the chicken heads were placed inside the two loop coils, which were positioned against, and parallel to each another. Two separate MRI image sequences (T1: spin-lattice or longitudinal relaxation time and T2: spin-spin or transverse relaxation time) were performed on the brains, in order to get two different image contrasts. The T1 3D and T2 3D were respectively the MPRAGE (Magnetization Prepared Rapid Acquisition Gradient Echo) and the SPACE (Sampling Perfection with Application-optimized Contrasts). The acquisition parameters for these T1 and T2 anatomical images were as follow:

- T1 3D: Repetition Time (TR) = 2120 ms, Echo Time (TE) = 3.72 ms, Inversion Time (TI) = 900 ms, flip angle = 9 degrees, Field-of-View (FOV): 81x81 mm<sup>2</sup>, matrix: 256x256<sup>2</sup> and a slice thickness of 0.32 mm resulting in a voxel size of 0.32x0.32x0.32 mm<sup>3</sup>. A bandwidth of 150 Hz/Px was used. An integrated Parallel Acquisition Technique (iPAT) of 2 was used with 4 Number of Excitations (NEX) producing an acquisition time of 19 min 40 sec.

- T2 3D: TR = 4000 ms, TE = 414 ms, TI = 380 ms, and bandwidth of 349 Hz/Px and a turbo factor of 309. The FOV was 180x180 mm<sup>2</sup>, matrix: 256x256<sup>2</sup> with interpolation making in plane images of 0.35x0.35, and slice thickness: 0.35 mm thus ending with a voxel size: 0.35x0.35x0.35 mm<sup>3</sup>. 4 NEX were used, generating an acquisition time of 12 min 02 sec.

## **2.11 MRI analysis method**

The analysis of structure's lengths was performed with the Syngo.Via software from Siemens. The analysis of the volumes on the MR images was done on the T1 images for the brainstem, cerebellum and telencephalon structures and on the T2 images for the lateral

ventricle as T2 contrast is clearly distinguishable for water content (**supplemental figure**). For the volume analyses, the Digital Imaging and Communication in Medicine (DICOM) images were converted to the Neuroimaging Informatics Technology Initiative (NIfTI) format. The NIfTI images were read using ITK Snap, a free post-processing software typically used to segment 3D medical image structures. The segmentation of ‘surface growth type was done automatically and then corrected manually. It was then possible to edit the volume of each segmented structure.

## **2.12 Statistical analyses**

The Statview® software (version 9.3) was used for all analyses. Data were tested for homogeneity of variance by Bartlett’s test and for normal distribution by Shapiro-Wilk test. One-way analysis of variance (ANOVA) with repeated measures was performed on data from food intake water intake, body weight, and glucose and plasma insulin. One-way analysis of variance (ANOVA) with non-repeated measures was performed on data from protein and mRNA expression levels and from brain structure volume and size. All experimental data are presented as means  $\pm$  SEM. The level of statistical significance was set at  $P < 0.05$ .

### 3. Results

#### 3.1 Effects of chicken recombinant chemerin intraperitoneal injections on food and water intake and body weight

We determined food (**Fig.1 A and B**) and water (**Fig.1 C**) intake and body weight (**Fig.2**) in female broiler breeder chicken daily injected with vehicle (0.2 ml saline) or chicken recombinant chemerin (8 or 16  $\mu\text{g}/\text{kg}$ ) at 9.00 am for 13 days. Compared to saline, chicken recombinant chemerin significantly decreased food intake from day 7 of treatment (repeated measures ANOVA,  $P < 0.05$ , **Fig.1 A** (daily) **and B** (cumulative)), while no significant effect on water intake was observed throughout the experiment ( $P=0.2$ , **Fig.1 C**). As showed in **Fig.2 A**, chicken recombinant chemerin significantly decreased body weight from day 6 (Repeated measures ANOVA,  $P < 0.05$ ). During all the experiment, at similar body weights, the food intake of animals injected with chemerin (8 or 16  $\mu\text{g}/\text{kg}$ ) was significantly lower compared to the control animals (repeated measures ANOVA,  $P < 0.05$ ) and this was observed consecutively from day 10 to day 13 of treatment (**Fig.2 B**).

#### 3.2 Effect of chicken recombinant chemerin intraperitoneal injection on the plasma glucose and insulin levels and bones development

As showed in **Table 4**, chicken recombinant chemerin administration (8 or 16  $\mu\text{g}/\text{kg}$ ) significantly decreased plasma glucose ( $P < 0.05$ ) and increased plasma insulin ( $P < 0.05$ ) in both treated groups as compared with vehicle in control group after 7 days and 13 days of treatment. Since a recent study showed a key role of chemerin and its CMKLR1 receptor in bone remodeling and metabolism (Zhao, et al. 2019), we evaluated bone development by CT scan. As showed in **Table 4**, the volume of the whole bone ( $P=0.5$ ), the tibia cortical bone volume ( $P=0.8$ ) and the length of the tibia ( $P=0.2$ ) were unchanged following 13 days of chicken recombinant chemerin treatment.



### **3.3 Effect of chicken recombinant chemerin intraperitoneal injection on the volume and the size of different brain subdivisions**

We studied the impact of the daily intraperitoneal injection of chicken recombinant chemerin (16µg/kg/day) for 13 days on the volume and the size of different brain subdivisions, such as the cerebellum, telencephalon, brainstem (**supplemental figure**) and lateral ventricle by using MRI. As shown in **Table 5**, intraperitoneal injection of chicken recombinant chemerin did not affect the volume and the size of these different cerebral structures when reported to the body weight of each animal.

### **3.4 Protein expression of chemerin and its receptors in chicken hypothalamus**

As determined by western-blot, we observed a significant higher protein level of the chemerin receptor CMKLR1 in chicken hypothalamus than in left cerebral hemisphere (cortex) ( $P < 0.05$ , **Fig. 3A**) whereas the amount of chemerin (**Fig. 3B**,  $P = 0.3$ ), GPR1 (**Fig. 3C**,  $P = 0.4$ ) and CCRL2 (**Fig. 3D**,  $P = 0.3$ ) two chemerin receptors, was similar between the two cerebral structures. The left cerebral hemisphere and hypothalamus exert different functions in chicken. Indeed, the left hemisphere is more involved in auditory habituation (Rogers and Anson 1979) whereas the hypothalamus plays a pivotal role in feed intake and energy balance regulation (Kuenzel 1994, Kuenzel, et al. 1999).

### **3.5 Effect of chicken recombinant chemerin intraperitoneal injection on the hypothalamic peptides expression**

We next investigated the effect of chicken recombinant chemerin on orexigenic and anorexigenic peptides mRNA expression in hypothalamus by RTqPCR. As observed in **Fig.4**, we showed a significant reduction in both orexigenic neuropeptides *Npy* (**Fig. 4A**,  $P < 0.05$ )

and *Agrp* (**Fig. 4B**,  $P<0.05$ ) gene expression in animals treated with chemerin (8 and 16  $\mu\text{g}/\text{kg}$ ). In contrast, the anorexigenic neuropeptides *Pomc* (**Fig. 4C**,  $P=0.6$ ) and *Cart* (**Fig. 4D**,  $P=0.4$ ) gene expressions were not modified with respect to vehicle-treated chickens. On the other hand, in animals treated with chemerin (8 and 16  $\mu\text{g}/\text{kg}$ ) we found a significant increase in both *Crh* (**Fig. 4E**,  $P<0.05$ ) and *Tas1r1* (**Fig. 4F**,  $P<0.05$ ) gene expression.

### **3.6 Effect of chicken recombinant chemerin intraperitoneal injection on chemerin system expression and AMPK activation in hypothalamus**

By western-blot we showed that the administration of chicken recombinant chemerin significantly reduced CMKLR1 protein content in hypothalamus (**Fig. 5A**,  $P<0.05$ ) with a dose effect whereas chemerin (**Fig. 5B**,  $P=0.3$ ), GPR1 (**Fig. 5C**,  $P=0.5$ ) and CCRL2 (**Fig. 5D**,  $P=0.4$ ) amounts were unchanged. In mammals, hypothalamic AMPK is involved in the regulation of energy balance and food intake (Minokoshi, et al. 2004). In chicken, activated AMPK is present in hypothalamic nuclei involved in regulation of food intake and energy balance suggesting similar effects to those observed in mammals (Proszkowiec-Weglarz, et al. 2006). As shown in **Fig. 6**, hypothalamic AMPK phosphorylation was significantly decreased in response to chemerin treatment (**Fig. 6**,  $P<0.05$ ), here again with a dose effect.

#### 4. Discussion

In the present study, we showed for the first time that chemerin and its receptors were expressed within the chicken hypothalamus. Furthermore, peripheral administration of chicken chemerin decreased food intake and body weight without altering water intake. At the hypothalamic level, these data were associated with a reduction in mRNA expression of orexigenic *Npy* and *Agrp* neuropeptides, a decrease of *Cmklr1* mRNA expression and a decrease in the AMPK phosphorylation. In contrast, we observed an increase of the anorexigenic *Crh* and *Tas1r1* mRNA expression. In addition, chicken chemerin administration did not affect the size or the volume of different brain subdivisions but it increased plasma insulin and reduced plasma glucose levels.

We showed that chemerin and its receptors were expressed at the mRNA and protein levels within the chicken hypothalamus. The proteins were detected by using specific antibodies targeting chicken chemerin, CMKLR1 and GPR1. Until now, chemerin and its receptors had only been identified only in peripheral and reproductive tissues of avian species (Diot, et al. 2015, Mellouk, et al. 2018b, Mellouk, et al. 2018c, Mellouk, et al. 2018d). However, the expression of the *Rarres2* gene was found in the hypothalamus of rodents (Helfer, et al. 2016, Miranda-Angulo, et al. 2014), baboon, chimpanzee (Gonzalez-Alvarez, et al. 2015) and pig (Smolinska, et al. 2019). In the brain, the hypothalamus is critical in sensing and integrating peripheral signals such as adipokines (Coll and Yeo 2013). In rat, *Rarres2*, *Cmklr1* and *Ccrl2* expression are localized in tanycytes and ependymal cells of ventricular zone and median eminence of the hypothalamus. These hypothalamic areas are involved in the regulation of the energy metabolism and are key sites of integration of the photoperiodic signal (Helfer, et al. 2016). However, in rat, *Gpr1* mRNA expression was not detected in the relevant hypothalamic regions associated with energy balance control (Helfer, et al. 2016). In

contrast, in mice, chemerin is predominantly expressed in ependymal cells but not in the tanycytes (Miranda-Angulo, et al. 2014) suggesting a species-specific expression pattern of this adipokine. In human, chemerin was found in the cerebrospinal fluid (CSF) (Zhao, et al. 2011) suggesting that in mammals, this adipokine could exert direct or indirect effects on the hypothalamus through its receptors. In birds, the hypothalamus is also the center of energy homeostasis and feeding behavior regulation as in mammals (Kuenzel, et al. 1999). In chicken, it will be interesting to determine specifically in which cell types are expressed chemerin and its receptors in the hypothalamus in order to better understand the molecular mechanism of chemerin in the regulation of the food intake in this species.

After seven day of daily chicken recombinant chemerin intraperitoneal injection, we showed a significant reduction of food intake and body weight. Our results are in a good agreement with data from literature reported in rodents. Indeed, Brunetti et al (2014) observed a decrease in food intake in rat after intraperitoneal injection of chemerin (8 or 16  $\mu\text{g}/\text{kg}$ ) (Brunetti, et al. 2014). In our experiment, we showed that the decrease of food intake was associated with a reduction in neuropeptides *Npy* and *Agrp* mRNA expressions and an increase of *Crh* expression. Like in mammals, NPY and AgRP are orexigenic neuropeptides while CRH is an anorexigenic neuropeptide in avian species (Kuenzel 1994, Richards 2003, Song, et al. 2013). CRH has been previously shown to decrease feed intake in chickens (Denbow, et al. 1999). The hypothalamus senses and integrates diverse signals such as the levels of glucose (Anand, et al. 1964, Oomura, et al. 1974) from blood vessels and through the third ventricle, and thereby regulates food intake. In our study, we also observed an increase in the expression of *Tas1r1* that is a hypothalamic sensor of glucose in mice (Ren, et al. 2009). In chicken, *Tas1r1* is expressed at higher levels in animals selected for increased body fat than in their counterparts selected for leanness (Byerly, et al. 2010). Since glucose is the energetic fuel in brain, the increase in hypothalamic *Tas1r1* mRNA may signal an

adaptation to the decrease in glucose levels observed after peripheral chicken chemerin injection. Intracerebroventricular administration of chemerin into F344 rats (one of the few rat strains that retained its photoperiod sensitivity and undergoes pronounced cycles of weight gain and weight loss under different photoperiodic conditions (Tavolaro, et al. 2015)) alters their food intake and body weight with associated changes in hypothalamic neuropeptides involved in feeding and growth. However, this response is transient and not sustained over long term (Helfer, et al. 2016). A study in Sprague–Dawley rats has reported that acute injection of chemerin into the arcuate nucleus of the hypothalamus has no effect on food intake and body weight nor the expression of appetite-regulating neuropeptides. This negative result may be explained by the dose of chemerin (8µg/kg) and the injection site (injection into the arcuate nucleus of the hypothalamus) used in this study (Brunetti, et al. 2011). Later, the same authors used peripheral chemerin injections and showed a reduction in food intake and body weight, but this seemed to have only a minor effect on hypothalamic neuropeptide expression (Brunetti, et al. 2014). While these studies showed seemingly contradictory responses due to different routes, mode of delivery and doses used, the results indicate that chemerin might not act directly through known neuroendocrine appetite-regulating pathways to exert its effects. This idea is supported by the finding that mice lacking CMKLR1 show reduced food intake and body weight but no changes in hypothalamic neuropeptides (Ernst, et al. 2012). In contrast with rodents, the reduction in food intake and body weight observed in chicken after peripheral chemerin injection is associated with variations of hypothalamic neuropeptides. These data suggest a role of chemerin in the regulation of avian hypothalamic circuits involving the orexigenic and anorexigenic neuropeptides. We do not know yet whether chemerin is able to cross the blood-brain barrier (BBB) in mammals and even less in birds. Even if it is well known that adipokines act on receptors located in CNS regions participating in the regulation of nutritional disorders, their interactions with the BBB are not

clear (Pan and Kastin 2007). In the present study, we observed that chronic peripheral chicken chemerin injections induce an increase in CMKLR1 protein level in the hypothalamus without alteration of GPR1 and CCRL2 protein amount suggesting that CMKLR1 could be the main chemerin receptors involved in the food intake regulation in chicken. In mammals, reduced food intake, body mass and adiposity, were found in mice with a disrupted *Cmklr1* gene as compared to the control animals (Ernst, et al. 2012). In contrast, *Gpr1*-knockout mice showed increased glucose intolerance on a high-fat diet compared to wild-type mice, but displayed no change in body weight, body composition and energy expenditure (Rourke, et al. 2014). CMKLR1 is a  $G_{i/o}$ -protein-coupled receptor that signals through mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK), phosphatidylinositol 3 kinase (PI3K)-AKT and adenosine monophosphate-activated protein kinase (AMPK) pathways to regulate biological functions such as angiogenesis, inflammation, and ovarian steroidogenesis (De Henau, et al. 2016, Goralski, et al. 2007, Reverchon, et al. 2014, Sell, et al. 2009, Wittamer, et al. 2003, Zhang, et al. 2019). Unlike CMKLR1 and GPR1, the binding of chemerin to CCRL2 does not induce downstream signaling pathways, calcium mobilization or ligand internalization, and it is therefore designated as an atypical, silent or non-signaling chemokine receptor. CCRL2 seems to have the ability to amplify local chemerin concentration for CMKLR1 interaction (De Henau, et al. 2016, Zabel, et al. 2008). In mammals, hypothalamic AMPK plays a pivotal role in the food intake regulation (Andersson, et al. 2004, Kim, et al. 2004, Minokoshi, et al. 2004). In avian species, there are several reports about the role of hypothalamic AMPK in the regulation of feed intake (for review (Song, et al. 2013)). Thus, the decrease in food intake after peripheral chemerin injection in broiler breeder chicken could be mediated by a reduction of hypothalamic CMKLR1 and /or a decrease of hypothalamic AMPK. Interestingly, we showed that peripheral chemerin injection did not affect the volume or the size of cerebellum, telencephalon, brainstem and lateral

ventricle as determined by MRI. MRI technique has largely been used in chicken embryo (Goodfellow, et al. 2016, Zhou, et al. 2015) but more rarely in post-natal chickens. These results indicate that the peripheral administration of chemerin at 8 or 16 $\mu$ g did not alter the post-natal development of the chicken brain.

In our study, we noted that chemerin significantly reduced plasma glucose and increased plasma insulin in chicken. In mammals, chemerin and its three receptors are expressed in pancreas and chemerin-deficient mice are glucose intolerant mainly due to increased hepatic glucose production and impaired insulin secretion (Takahashi, et al. 2011). Thus, we can hypothesize that chemerin injection in chicken increases insulin secretion and consequently decreases plasma glucose concentrations. Since it has been recently reported that CMKLR1 deficiency leads to lower bone mass in male mice (Zhao, et al. 2019), we investigated the bone development in chicken after chemerin injection. However, the volume of the whole and cortical bone and the length of the tibia were unchanged, suggesting that chemerin does not affect bone development in this species or the treatment duration was not sufficient or the animals were too old.

## **5. Conclusions**

Taken together, our results report for the first time the pattern of expression of chemerin and its receptor within the chicken hypothalamus. In addition, we found that peripheral chicken chemerin injections decrease food intake and body weight and this was associated to a modulation of hypothalamic neuropeptides expression and a reduction in AMPK phosphorylation. Furthermore, chemerin reduced plasma glucose and increased insulin secretion. Together, our results highlight a critical role for chemerin in the neuroendocrine control of energy metabolism. Further investigations are needed to evaluate the physiological relevance of chemerin in the regulation of food intake in chickens.

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## Figures legends

**Fig. 1. Time course of food (daily (A) or cumulative (B)) (g) and water (C) (ml) intakes in two-weeks old female chicken fed with a standard diet and treated with vehicle (0.2 ml saline) or chemerin (8–16 µg/kg).** Vehicle or chicken recombinant chemerin was administered by intraperitoneal injections, during the light phase, at 09:00 h, for 13 consecutive days, and individual food and water intakes were daily recorded until 24 h after the last injection. Data are expressed in grams (g) for food and milliliters (ml) for water as mean ± SEM for each group (n= 25) and analyzed by repeated measures analysis of variance (ANOVA), followed by Newman–Keuls *post hoc* test. Different letters indicate significant difference at  $P < 0.05$ .

**Fig. 2. Time course of daily body weight (g) (A) and ratio between daily food intake (g)/body weight (g) (B) in two-week old female chicken fed with a standard diet and treated with vehicle (0.2 ml saline) or chemerin (0.2 ml 8–16 µg/kg).** Vehicle or chemerin



was administered by intraperitoneal injections, during the light phase, at 09:00 h, for 13 consecutive days, and individual body weights and food intakes were recorded daily. Data are expressed in grams (g) as mean  $\pm$  SEM for each group (n= 25) and analyzed by repeated measures analysis of variance (ANOVA), followed by Newman–Keuls *post hoc* test. Different letters indicate significant difference at  $P < 0.05$ .

**Figure 3: Protein level of CMKLR1 (A), chemerin (B), GPR1 (C) and CCRL2 (D) in hypothalamus and left cerebral hemisphere determined by immunoblot in control 28 days-old female chicken.** Vinculin was used as a loading control. Blots were quantitated, and the ratios of chemerin and its receptors to vinculin were represented. Data are expressed as the mean  $\pm$  SEM; n = 8 animals for each tissue. Results were analyzed by one-way analysis of variance (ANOVA). Different letters indicate significant difference at  $P < 0.05$ .

**Figure 4: Gene expression of hypothalamic neuropeptides in response to peripheral chemerin treatment in female chicken**

Gene expression of hypothalamic orexigenic (*Npy* (A) and *AgRp* (B)) and anorexigenic (*Pomc* (C), *Cart* (D) and *Crh* (E)) neuropeptides and nutrient sensing (*Tas1r1*)(F), 24 h after the last treatment with vehicle or chemerin (8–16  $\mu$ g/kg), were determined by real-time RT qPCR. Data are expressed as mean  $\pm$  SEM for each group (n=8) and analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test. Different letters indicate significant difference at  $P < 0.05$ .

**Figure 5: Protein level of CMKLR1 (A), chemerin (B), GPR1 (C) and CCRL2 (D) within the hypothalamus determined by immunoblot in response to peripheral chemerin treatment in female chicken**

Proteins amount of hypothalamic chemerin and its receptors, 24 h after the last treatment with vehicle or chemerin (8–16 µg/kg) were evaluated by western-blot. Vinculin was used as a loading control. Blots were quantitated, and the ratios of chemerin and its receptors to vinculin were represented. Data are shown as the mean ± SEM; n = 8 animals for each group and analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test. Different letters indicate significant difference at  $P < 0.05$ .

**Figure 6: Phosphorylation and protein levels of hypothalamic AMPK determined by immunoblot in response to peripheral chemerin treatment in female chicken**

Hypothalamic phospho and total AMPK, 24 h after the last treatment with vehicle or chemerin (8–16 µg/kg) were evaluated by western-blot. Vinculin was used as a loading control. Blots were quantitated, and the ratios of phospho-AMPK/total AMPK, and total AMPK/vinculin are represented. Data are shown as the mean ± SEM; n = 8 animals for each group and analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test. Different letters indicate significant difference at  $P < 0.05$ .

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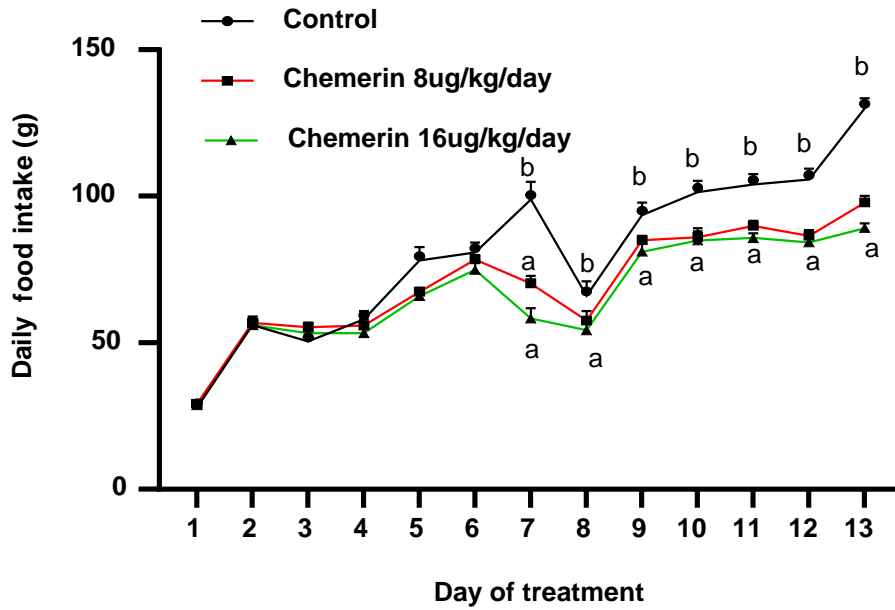
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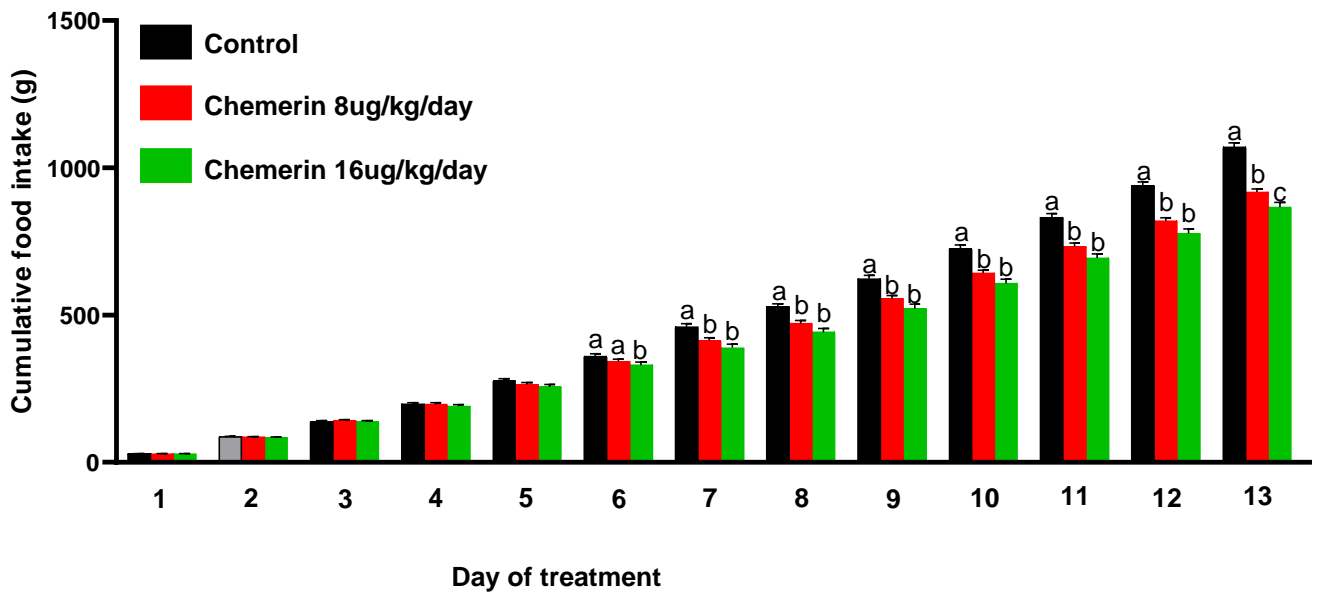
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A.



B.



C.

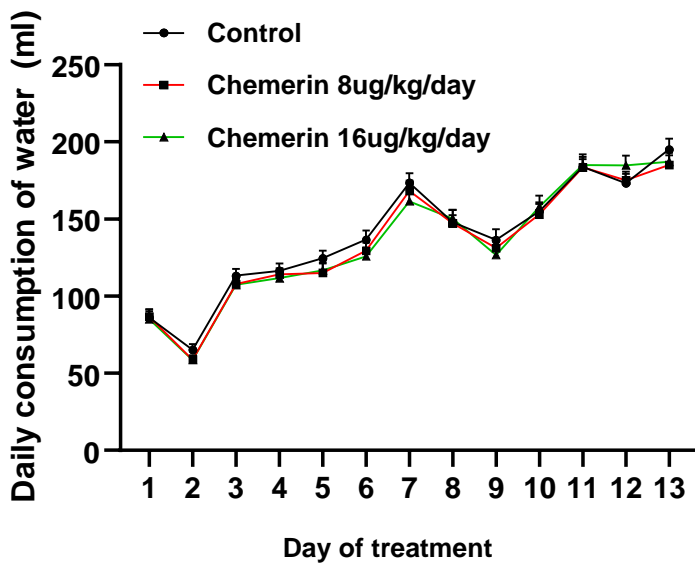
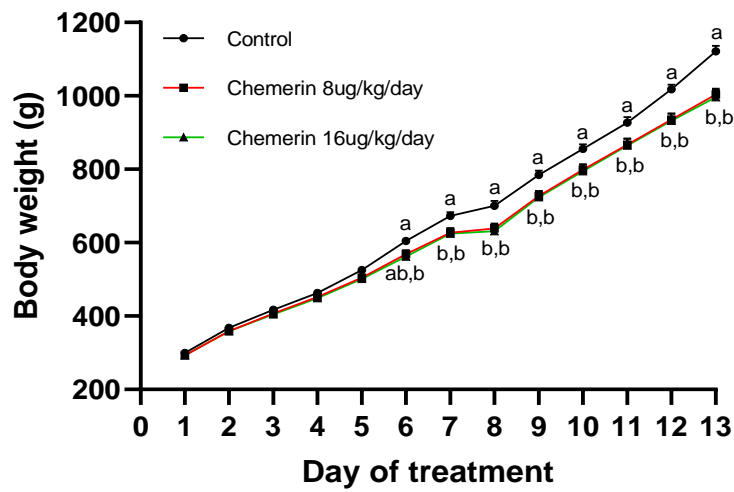


Figure 1

A.



B.

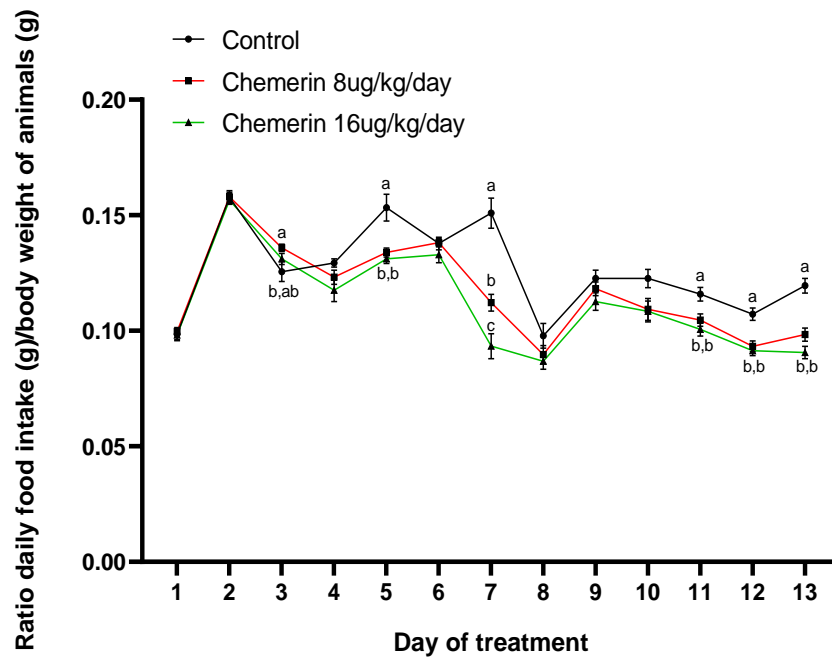


Figure 2



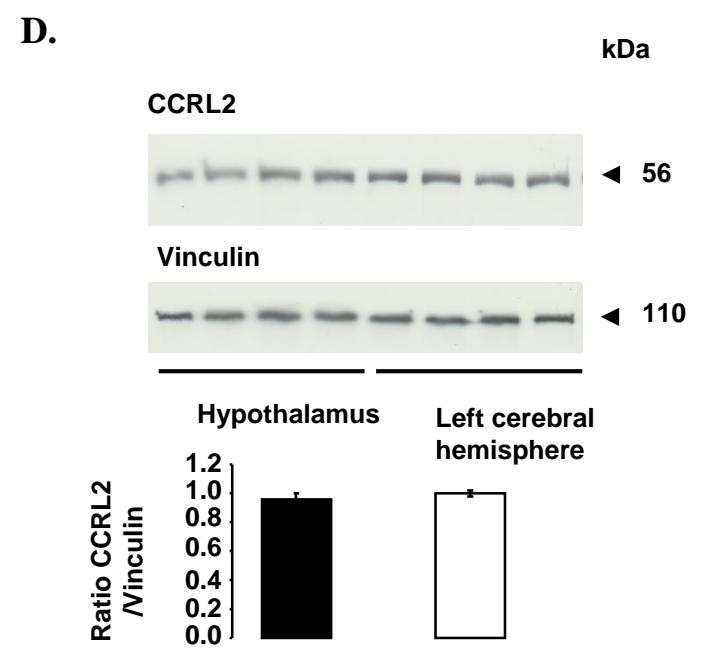
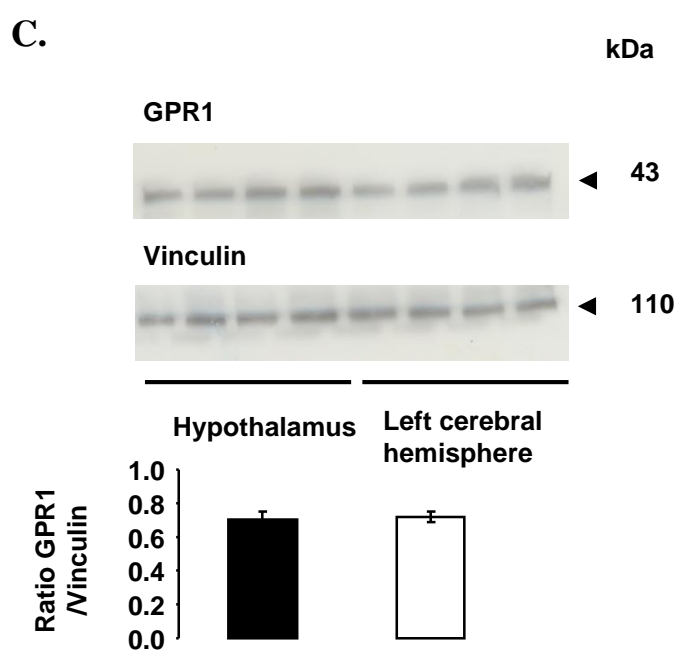
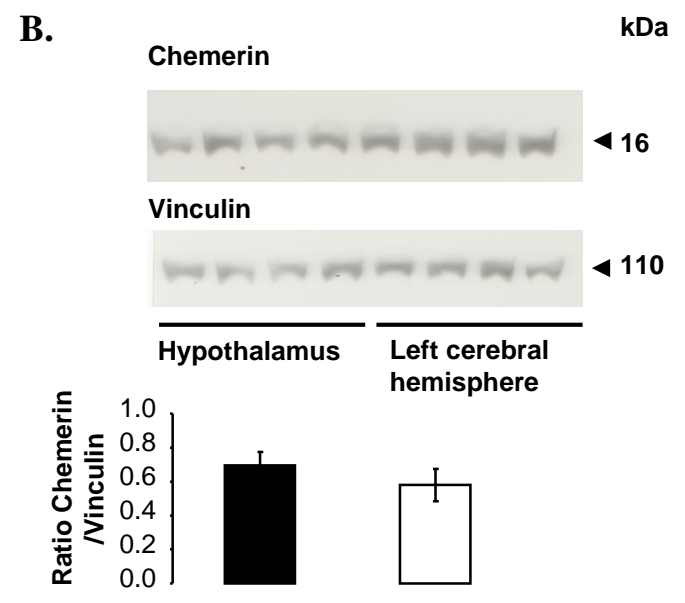
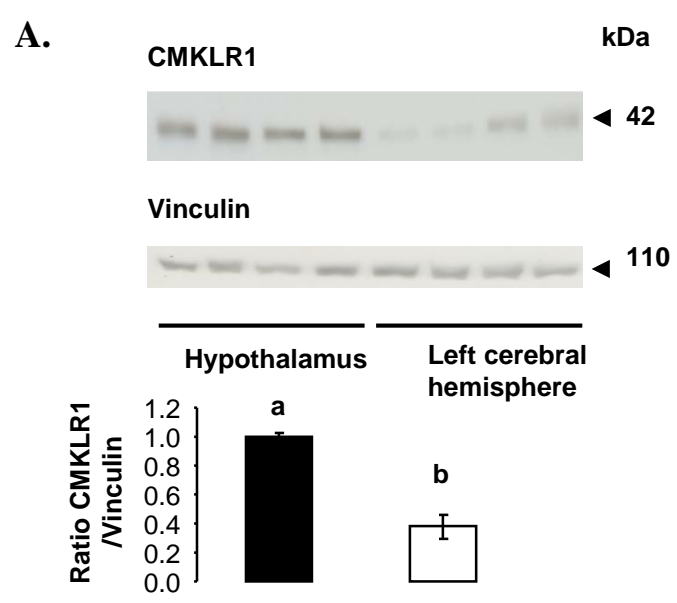
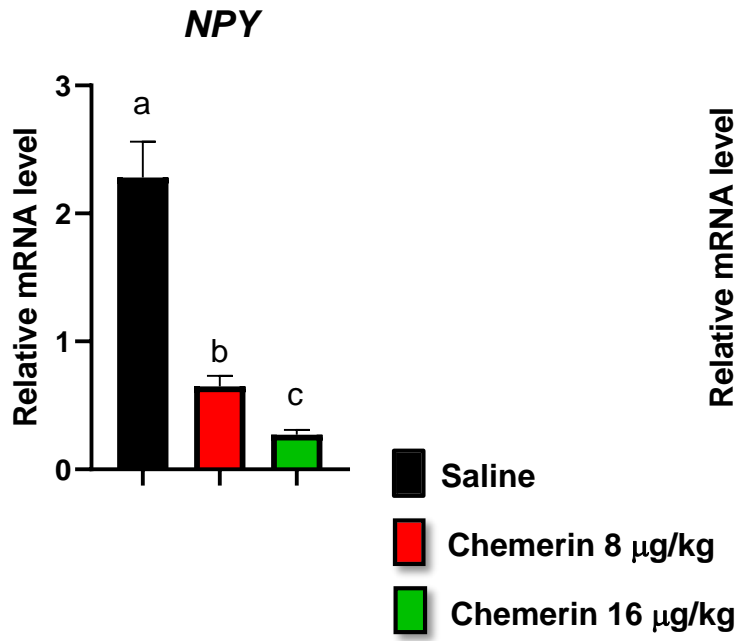
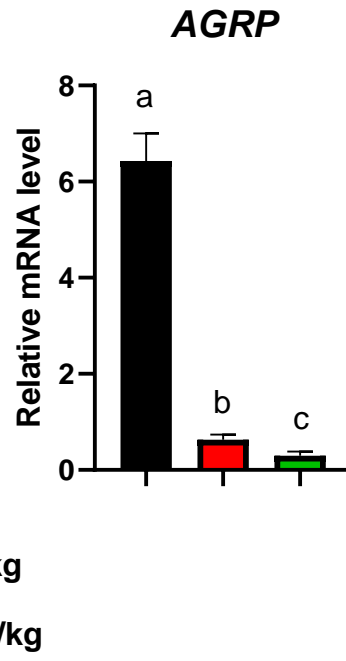


Figure 3

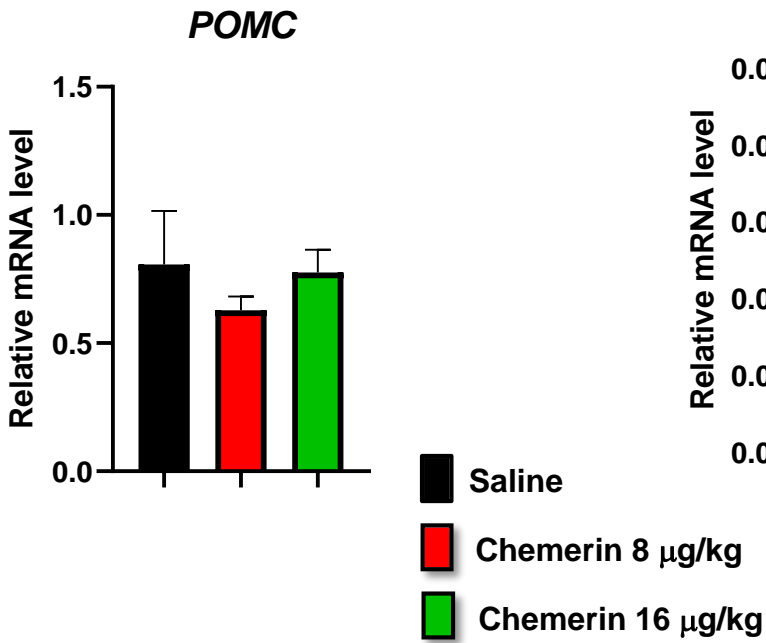
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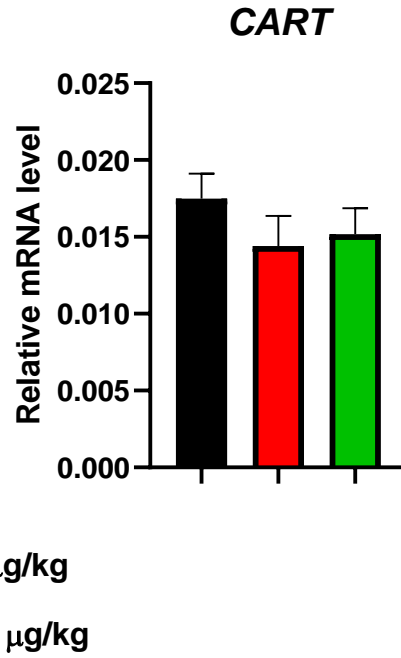
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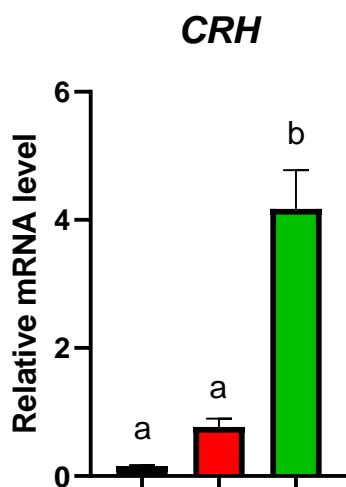
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D.



E.



F.

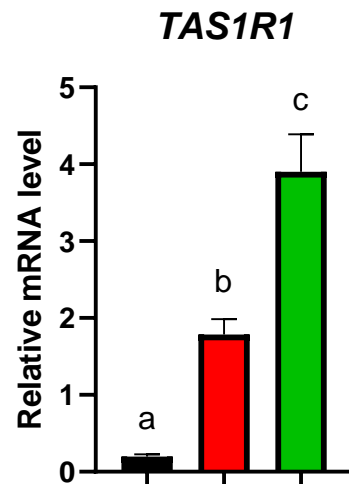


Figure 4

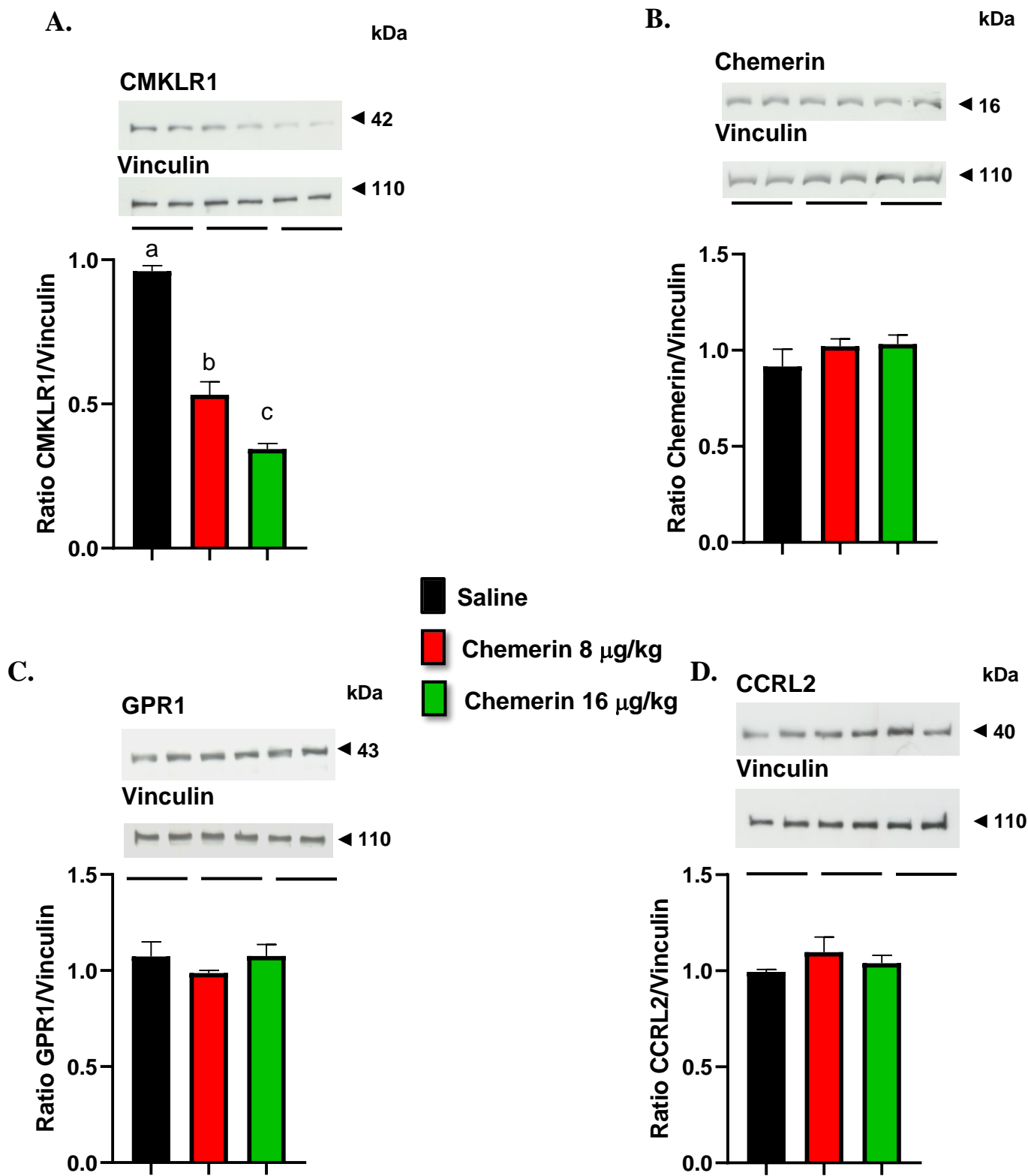


Figure 5

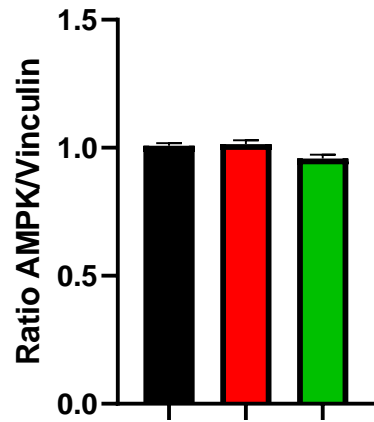
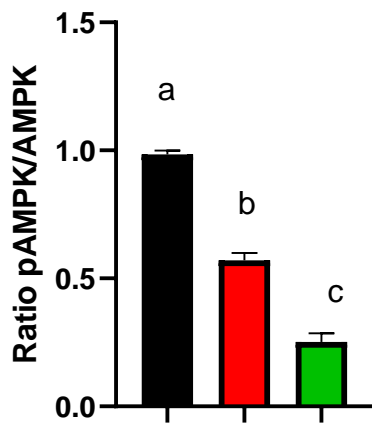
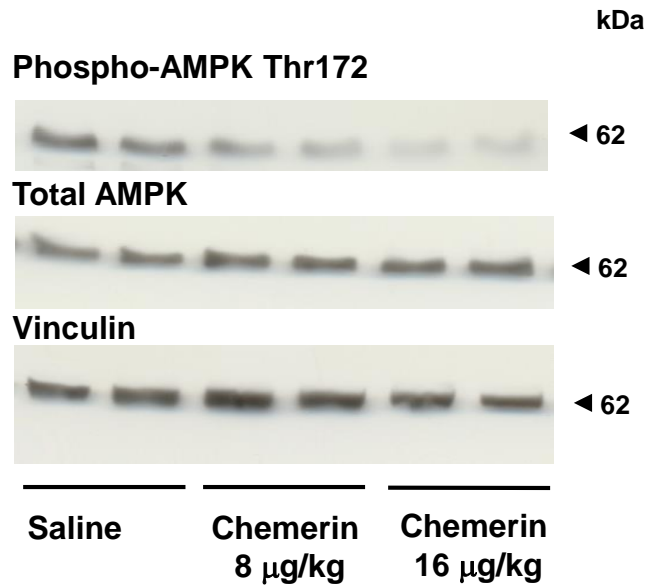
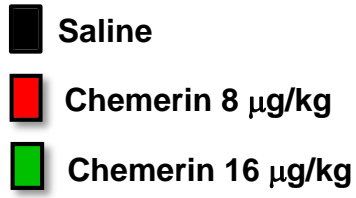


Figure 6

**Table 1: Composition (%) of the diet**

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Corn	39.64
Wheat	23.86
Soybean meal	29.3
Wheat bran	0
Soy oil	2.2
Sunflower oil	1
Sodium Bicarbonate	0.18
Calcium carbonate	0.86
Phosphate	2.06
Salt	0.3
Methionine DL	0.1
Lysine	0
Mineral premix	0.5
Soybean	0
Grape Seed Extract	0

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**Table 2: Oligonucleotide primer sequences**

Gene	Product size (bp)	Forward	Reverse	Number of accession	Efficiency
<i>Rarres2</i>	71	5'- CGCGTGGTGAAGGATGTG -3'	5'- CGACTGCTCCCTAAAGAGGAACT -3'	NM001277476.1	1.90
<i>Cmklr1</i>	64	5'- CGGTCAACGCCATTGGT -3'	5'- GGGTAGGAAGATGTTGAAGGAA -3'	NM001282407.2	1.95
<i>Gpr1</i>	249	5'- ACCTGCCTGAGGAAGAAGAA -3'	5'- AAAGGCCAGTGAAGCCCAT -3'	XM004942654.2	2.00
<i>Ccr12</i>	72	5'- CACGCAGTGTGCTTTAAAAGC -3'	5'- CAACAGCCACGTGACAATG -3'	NM001045835.1	1.92
<i>Ywhaz</i>	85	5'- AGTCATACAAAGACAGCAGCTA -3'	5'- GCTTCATCTCCTGGGTATCCGA -3'	NM001031343.1	2.00
<i>Polr2b</i>	110	5'- ACACGTATGAATGCCGGGA -3'	5'- AGGTGCAATGCTCATAGACATCA -3'	NM001006448.1	1.95
<i>Tbp</i>	85	5'- ATCAAGCCAAGAATTGTTCTGC -3'	5'- CTTCTAGATTTCTGCTCGAACT -3'	NM205103.1	2.00

**Table 3: List of primary and secondary antibodies used for protein detection by western blot.**

Primary antibodies	Dilution	Supplier	Host	Molecular weight (kDa)	Validation for avian species
VINCULIN	1/1000	Sigma (hVIN-1)	Mouse	110	Mellouk <i>et al.</i> , 2018
RARRES2	1/1000	Agrobio	Rabbit	16	Mellouk <i>et al.</i> , 2018
CMKLR1	1/1000	Agrobio	Rabbit	42	
GPR1	1/1000	Agrobio	Mouse	43	
CCRL2	1/1000	Sigma (SAB2100371)	Rabbit	40	Diot <i>et al.</i> , 2015
Phospho-AMPK Thr172	1/1000	New England Biolabs, Inc	Rabbit	62	Tosca <i>et al.</i> , 2006
Total AMPK	1/1000	Upstate Biotechnology, Inc	Rabbit	62	Tosca <i>et al.</i> , 2006
Secondary antibodies					
Rabbit	1/50000	Biorad	Goat		
Mouse	1/50000	Interchim	Goat		

**Table 4: Plasma glucose and insulin at day 0 (D0, no injection), 7 and 13 (A, n=25 animals in each group) and bone parameters at day 13 (B, n= 6 animals in each group) after intraperitoneal injection of vehicle and chicken recombinant chemerin 8 and 16 µg/kg,**

**A.**

Time (day, d)	D0			D7			D13			
	Treatment	C	Chem 8	Chem 16	C	Chem 8	Chem 16	C	Chem 8	Chem 16
Glucose (g/L)		2.11±0.06 <sup>a</sup>	2.10±0.05 <sup>a</sup>	2.11±0.05 <sup>a</sup>	2.14±0.05 <sup>a</sup>	1.92±0.04 <sup>b</sup>	1.84±0.05 <sup>b</sup>	2.09±0.04 <sup>a</sup>	1.90±0.03 <sup>b</sup>	1.87±0.04 <sup>b</sup>
Insulin (µg/L)		0.35±0.06 <sup>a</sup>	0.37±0.04 <sup>a</sup>	0.37±0.06 <sup>a</sup>	0.40±0.06 <sup>a</sup>	0.60±0.04 <sup>b</sup>	0.62±0.07 <sup>b</sup>	0.38±0.06 <sup>a</sup>	0.66±0.08 <sup>b</sup>	0.68±0.07 <sup>b</sup>

Different letter indicate significant differences at  $P < 0.05$ .

**B.**

Time (day, d)	d13			
	Treatment	C	Chem 8	Chem 16
Whole bone volume (mm <sup>3</sup> )		5.8x10 <sup>5</sup> ±1.7x10 <sup>6</sup>	5.9x10 <sup>5</sup> ±2.1x10 <sup>6</sup>	6.1x10 <sup>5</sup> ±1.5x10 <sup>6</sup>
Tibia cortical bone volume (mm <sup>3</sup> )		588.33±23.03	584.33±23.16	577.83±78.33
Length of tibia (cm)		7.13±0.09	6.91±0.06	7.13±0.10



**Table 5 : Effect of daily chicken recombinant chemerin intraperitoneal injection (16 µg/kg) for 13 days on the volume (A) and the size (B) of different cerebral structures of the brain (n=4 animals per group).**

**A.**

<b>Ratio volume (mm<sup>3</sup>)/ total body weight (g)</b>	<b>Control</b>	<b>Chemerin 16µg/kg/day</b>	<b><i>P value</i></b>
<b>Cerebellum</b>	0.45 ± 0.02	0.46 ± 0.008	0.76
<b>Telencephalon</b>	2.23 ± 0.07	1.98 ± 0.13	0.18
<b>Brainstem</b>	0.13 ± 0.005	0.12 ± 0.008	0.29
<b>Lateral ventricle</b>	0.02 ± 0.003	0.02 ± 0.0007	0.75
<b>Total volume</b>	2.84 ± 0.72	2.59 ± 0.12	0.17

**B.**

<b>Ratio size (mm)/ total body weight (g)</b>	<b>Control</b>	<b>Chemerin 16ug/kg/day</b>	<b><i>P value</i></b>
<b>Cerebellum ( Rostro-caudal axis)</b>	0.0009980 ± 1.877e-005	0.001055 ± 2.806e-005	0.17
<b>Cerebellum ( Dorso-ventral axis)</b>	0.0009800 ± 4.464e-005	0.0009921 ± 9.178e-006	0.80
<b>Telencephalon (Rostro-caudal axis)</b>	0.001582 ± 9.251e-005	0.001511 ± 5.024e-005	0.54
<b>Telencephalon (Dorso-ventral axis)</b>	0.0008223 ± 4.767e-005	0.0009013 ± 2.038e-005	0.20
<b>Brainstem (Rostro-caudal axis)</b>	0.001578 ± 6.577e-005	0.001576 ± 2.030e-005	0.98
<b>Brainstem (right ventro – lateral axis)</b>	0.0003640 ± 7.810e-006	0.0003825 ± 2.737e-006	0.09
<b>Brainstem (left ventro – lateral axis)</b>	0.0003450 ± 8.386e-006	0.0003499 ± 1.635e-005	0.80