



Physiological and biochemical studies on the hypothalamic regulation of food intake in broiler and layer chickens

Saneyasu, Takaoki

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Doctoral Dissertation

**Physiological and biochemical studies on the hypothalamic
regulation of food intake in broiler and layer chickens**

(ブロイラーとレイヤーにおける視床下部摂食調節に関する生理生化学的研究)

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Graduate School of Agriculture Science,
Kobe University

Takaoki Saneyasu

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General Introduction

Broiler chickens have been genetically selected for their heavy body weight, high growth rate and high meat production, and genetic selection has introduced wide variations in food intake between broiler and layer chickens. There is the evidence showing a significant increase in food intake in broiler chickens compared to layer chickens from the neonatal period [48, 56]. Recently in the recent poultry industry, overeating in broiler has become a problem because of decreasing meat productivity due to overeating-induced excessive accumulation of fat tissue and a series of health-related complications (fatty liver, leg problems, reproductive efficiency, etc.). Therefore, it is important to understand the cause of excess food intake in broiler chickens to develop better management of food intake. However, little is known about the detailed mechanism involved in this increase in food intake in broiler chickens.

It is generally accepted that appetite is regulated by the balance between two orexigenic peptides, neuropeptide Y (NPY) and agouti-related protein (AgRP), and proopiomelanocortin (POMC)-derived anorexigenic melanocortin peptides, such as alpha-melanocyte stimulating hormone (alpha-MSH), in mammals [53].

In mammals, NPY and AgRP play an important role in the stimulation of food intake in the hypothalamus [53]. NPY and AgRP were highly expressed in the hypothalamic arcuate nucleus (ARC) [54, 55]. The mRNA levels of NPY and AgRP in the hypothalamus are increased by fasting [6] and reduced by peripheral anorexigenic hormone insulin [59, 65] and leptin [72, 74]. In fact, the hyperphagia of genetically obese animals such as leptin-deficient mice and leptin receptor-deficient rats is induced by an increase in hypothalamic NPY and AgRP mRNA levels [49, 72, 86]. The orexigenic effect of NPY is mediated by hypothalamic Y1 and Y5 receptors [22, 38]. Both the Y1 receptor antagonist and Y5 receptor antagonist significantly reversed NPY-induced food

intake [35, 39]. On the other hand, AgRP antagonizes anorexigenic neuropeptide alpha-MSH binding and signaling via the melanocortin 4 receptor (MC4R) [55].

In birds, AgRP increases food intake in layer chicks, but not in broiler chicks, suggesting that AgRP is not a cause of the increase in food intake in broiler chicks [76]. In contrast, central administration of NPY significantly increases food intake in broiler chicks [3, 15, 40, 42] and layer chicks [62, 78], as well as in mammals. NPY mRNA was highly expressed in the infundibular nucleus (IN), the avian equivalent of the mammalian ARC [36, 85], and the mRNA level of NPY in the hypothalamus is increased by fasting [36] and restriction feeding [10] and reduced by insulin [70] and leptin [19]. NPY-immunoreactive fibers were seen throughout the entire chick brain, but were more abundant in the hypothalamus [20]. The NPY content in the paraventricular nucleus (PVN) and IN significantly increased during fasting, and the NPY content of the PVN was restored to pre-fasting levels after 24-h refeeding [92]. NPY and its receptors (Y1, Y2, Y4, Y5, Y6, and Y7) have been cloned [8, 13, 29, 47, 63], and Y1 and Y5 receptors are expressed in the chicken hypothalamus [29]. Thus, NPY plays a critical role in the stimulation of food intake in chicks.

POMC neurons in the hypothalamus play an important role in transducing the afferent inputs, i.e., hormonal and metabolic signals, into a neuronal anorexigenic response in mammals [53, 66]. Hypothalamic POMC mRNA levels are increased by peripheral anorexigenic hormone insulin [4] and decreased by fasting [52] in mammals. In humans, POMC is processed into three melanocortin peptides: alpha-, beta- and gamma2-MSH in the hypothalamus [51]. In contrast, mice and rats produce alpha-MSH, but not beta- and gamma2-MSH, because Arg and Lys at the cleavage sites of prohormone convertase are replaced by Pro and Asp (mouse) or Pro and Ala (rat), respectively [11]. However, previous studies demonstrated that central administration of either alpha-, beta- or gamma2-MSH suppresses food intake in mice [83] and rats [1,

7, 50]. These findings suggest that, although mice and rats do not produce beta- and gamma2-MSH, alpha-, beta- and gamma2-MSH may act as appetite-suppressive peptides in the central regulation of food intake in mammals. The anorexigenic effect of alpha-MSH was suggested to be mediated by hypothalamic MC4R in mammals [2].

In chickens, the hypothalamic POMC mRNA level is increased by peripheral anorexigenic hormone insulin [30, 70] and reduced by feed restriction [27]. The predicted amino acid sequence of the chicken POMC showed conservation of all cleavage sites of prohormone convertase, suggesting that chicken POMC is processed into alpha-, beta- and gamma2-MSH, the same as in humans [23]. Central administration of alpha-, human beta- and human gamma2-MSH in chicks significantly decreases food intake [15, 37, 40, 73]. However, the amino acid sequences of beta- and gamma2-MSH are not conserved in humans and other vertebrates including chickens [23, 28, 44, 69]. In rats, central administration of human beta-MSH significantly decreased food intake, whereas a human beta-MSH mutant, which has only one amino acid substitution and binds to the MC4R with lower affinity than human beta-MSH, had no effect on food intake [7]. Thus, it is possible that the effects of chicken beta- and gamma2-MSH on food intake in chicks are different when compared with human peptides.

In the present study, I investigated the involvement of NPY and POMC-derived alpha-, beta-, gamma- MSH in the difference in food intake between broiler and layer chickens.

Chapter 1

Neuropeptide Y effect on food intake in broiler and layer chicks

1. Introduction

As mentioned in General Introduction, there is evidence that differences in food intake between broiler and layer chickens are observable in the neonatal period [48, 56]. However, the causes of the difference in food intake in neonatal period between broiler and layer chicks are not clear.

It is generally accepted that appetite is regulated by the balance between orexigenic peptide NPY and AgRP, and anorexigenic peptides alpha-MSH in mammals [53]. In birds, central administration of NPY significantly increases food intake in broiler chicks [3, 15, 40, 42] and layer chicks [62, 78], as well as in mammals. But AgRP increases food intake in layer chicks, but not in broiler chicks [76]. Thus, NPY plays a critical role in the stimulation of food intake in chicks.

In this chapter, to clarify the involvement of NPY in the difference in food intake between broiler and layer chickens, I compared the hypothalamic mRNA levels of NPY, the Y1 receptor, and Y5 receptor, and the orexigenic effect of NPY between broiler and layer chicks at 1, 2, 4, and 8 days of age.

2. Material and methods

2.1. Animals

Day-old male chicks of a broiler (chunky: Ishii Co., Ltd. Tokushima, Japan) and a layer (White Leghorn: Ghen Corporation, Gifu, Japan) were purchased from a

local hatchery. They were given free access to water and a commercial chick starter diet (Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan). Room temperature was maintained at $32^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with an automatically controlled 12-h light:dark cycle (6:00-18:00). All experimental procedures followed the guidelines for the care and use of experimental animals at the Rokkodai Campus of Kobe University in Japan.

2.2. Experiment 1: Daily food intake of broiler and layer chicks

In Experiment 1, at 1 day of age, eight chicks of each strain were housed in individual cages with free access to water and a commercial diet. Food intake was measured every day.

2.3. Experiment 2: Changes in the hypothalamic neuropeptide Y mRNA level in broiler and layer chicks

Broiler and layer chicks were sacrificed at the mid-point of the light phase (12:00) by decapitation at 1, 2, 4, and 8 days of age. Their brains were removed within 1 min of decapitation, weighed, frozen on powdered dry ice and stored at -80°C for further analysis. The hypothalamus was dissected from the frozen brain, by referring to a stereotaxic atlas [43], and was weighed. Total RNA was extracted from the hypothalamus using Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from 5 μg of DNase I (Ambion Inc., Austin, Texas, USA)-treated total RNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems Inc., Foster city, California, USA) with random primers. Complementary DNA of NPY (GenBank accession no. [NM_205473](#)) was amplified with the following primers: NPY sense, 5'-CTT GTC GCT GCT GAT CTG -3'; NPY

antisense, 5'-GCC TCA GAG CCG AGT AGT-3'. As an internal standard, chicken ribosomal protein S17 mRNA (GenBank accession no. NM_204217) was also amplified, using the following primers: sense, 5'-GCG GGT GAT CAT CGA GAA GT-3'; antisense, 5'-GCG CTT GTT GGT GTG GAA GT-3'. SYBR® Premix Ex Taq was purchased from Takara Bio Inc. (Shiga, Japan), and mRNA expression was quantified in triplicate using the Applied Biosystems 7300 Real-Time PCR system according to the supplier's recommendations. After the reactions, the specificity of amplifications in each sample was confirmed by dissociation analysis showing that each sample gave a single melting peak. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product (CT). CT was calculated for the NPY and for the chicken ribosomal protein S17. For each cDNA sample, the CT for chicken ribosomal protein S17 was subtracted from the CT for NPY to give the parameter ΔCT , thus normalizing the initial amount of RNA used. The amount of NPY mRNA was calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is the difference between the ΔCT of the two cDNA samples to be compared.

2.4. Experiment 3: Changes in hypothalamic mRNA levels of Y1 and Y5 receptors in broiler and layer chicks

Hypothalamic mRNA levels of Y1 and Y5 receptors were analyzed as described in Experiment 2. Complementary DNAs of Y1 receptor (GenBank accession no. NM_001031535) and Y5 receptor (GenBank accession no. NM_001031130) were amplified with the following primers: Y1 receptor sense, 5'-TAG CCA TGT CCA CCA TGC A-3'; Y1 receptor antisense, 5'-GGG CTT GCC TGC TTT AGA GA-3'; Y5 receptor sense, 5'-GGC TGG CTT TGT GGG AAA-3'; and Y5 receptor antisense,

5'-TTG TCT TCT GCT TGC GTT TTG T-3'.

2.5. Experiment 4: Effect of central administration of neuropeptide Y on cumulative food intake in broiler and layer chicks

Broiler and layer chicks were divided into three groups at 1, 2, 4, and 8 days of age. Chicken NPY (YPSKPDSPGEDAPAEDMARYYSALRHYINLITRQRY-NH₂) was purchased from Peptide Institute, Inc. (Osaka, Japan). NPY was dissolved in a 0.85% (w/v) saline solution containing 0.1% (w/v) Evans Blue. Either NPY (0.2, or 0.4 µg) or saline (as a control) was intracerebroventricularly administered according to the method of Davis et al. [18] at a volume of 10 µl after 3 h of fasting. Food intake was measured at 30, 60, and 120 min after administration. Each feeder filled with food was pre-weighed. A paper was put under the feeder. In each time point, any spilled food on the paper was collected and food consumption was weighed using an electric digital balance (readability 10 mg). Food intake was calculated as follows:

Food intake = (The amount of food decrease in the feeder) – (The amount of spilled food)

At the end of the experiment, the chicks were sacrificed by decapitation. Verification of injection was made by observation of the presence of Evans Blue dye in the lateral ventricle.

2.6. Data analysis

Data from Experiments 1, 2, and 3 were analyzed by Student's *t* test at each day of age. Data from Experiment 4 were analyzed by the Tukey-Kramer test at

each time point. All statistics was performed using a commercial software package (StatView version 5, SAS Institute, Cary, North Carolina, USA, 1998).

3. Results

The daily food intake of broiler chicks was significantly higher than that of layer chicks after 2 days of age (Fig. 1). The hypothalamic NPY mRNA level was significantly lower in broiler chicks than layer chicks at 1, 2, and 4 days of age (Fig. 2). The mRNA level of the Y1 receptor was significantly lower in broiler chicks than in layer chicks at 2 and 4 days of age (Fig. 3). The mRNA level of the Y5 receptor at 2 days of age was also significantly lower in broiler chicks than in layer chicks (Fig. 3). Central administration of NPY significantly increased food intake in both strains at 2, 4, and 8 days of age (Fig. 4).

4. Discussion

First, I compared the difference in food intake between broiler and layer chicks. The daily food intake of broiler chicks was significantly higher than that of layer chicks after 2 days of age (Fig. 1).

I next examined the hypothalamic NPY mRNA level in broiler and layer chicks. The hypothalamic NPY mRNA level was significantly lower in broiler chicks than layer chicks at 1, 2, and 4 days of age (Fig. 2), suggesting that the hyperphagia in broiler chicks is not caused by the increase in the hypothalamic NPY mRNA level. This view is supported by recent publications. Zhou et al. reported that the hypothalamic NPY content is lower in broiler chicks than layer chicks at 1 day of age [93]. Yuan et al. reported that broiler and layer chicks exhibit similar

hypothalamic expression of NPY at 7 days of age [91]. These findings and our results suggest that the difference in the expression of hypothalamic NPY is not a cause of the increase in food intake in broiler chicks.

I also examined the hypothalamic mRNA levels of Y1 and Y5 receptors in broiler and layer chicks. Unexpectedly, the hypothalami of broiler chicks did not express higher receptor levels than those of layer chicks. The mRNA level of the Y1 receptor was significantly lower in broiler chicks than in layer chicks at 2 and 4 days of age (Fig. 3). The mRNA level of the Y5 receptor at 2 days of age was also significantly lower in broiler chicks than in layer chicks (Fig. 3). These results suggest that the differences in the expressions of hypothalamic Y1 and Y5 receptors do not cause the increase in food intake in broiler chicks.

To compare the orexigenic effect of NPY between broiler and layer chicks, I finally examined the effects of central administration of NPY on food intake in these strains. Central administration of NPY significantly increased food intake in both strains at 2, 4, and 8 days of age (Fig. 4). These results suggest that the increase in food intake in boiler chicks can not be explained by the difference in the NPY responsiveness.

In the present study, I showed that mRNA levels of NPY and its receptors were approximately the same or lower in broiler chicks than layer chicks (Figs. 2 and 3). In addition, NPY responsiveness was similar between broiler and layer chicks at 4 and 8 days of age (Fig. 4). Tachibana et al. reported that AgRP does not lead to increased food intake in broiler chickens [76]. These findings suggest that other molecules than NPY and AgRP are involved in the increase in food intake in broiler chicks.

5. Conclusion

In summary, I examined the involvement of a potent orexigenic peptide NPY in the difference in food intake between broiler and layer chicks. I showed that daily food intake was significantly higher in broiler chicks than layer chicks after 2 days of age; however, the hypothalamic NPY mRNA level was significantly lower in broiler chicks than layer chicks except at 8 days of age. The mRNA levels of NPY receptors were also significantly lower in broiler chicks than layer chicks at 2 and 4 days of age (Y1 receptor) or 2 days of age (Y5 receptor). All our findings demonstrated that the increase in food intake in broiler chicks is not accompanied with the over-expression of NPY or its receptor.

Chapter 2 Section 1

Effect of chicken melanocortin peptides on food intake in chicks

1. Introduction

As mentioned in General Introduction, previous studies demonstrated that central administration of either alpha-, beta- or gamma2-MSH suppresses food intake in mice [83] and rats [1, 7, 50].

In chicks, central administration of alpha-, human beta- and human gamma2-MSH significantly decreases food intake [15, 37, 40, 73]. However, the amino acid sequences of beta- and gamma2-MSH are not conserved in human and other vertebrates including chickens (Fig. 5) [23, 28, 44, 69]. In rats, central administration of human beta-MSH significantly decreased food intake, whereas human beta-MSH mutant, which has only one amino acid substitution and binds to the MC4R with lower affinity than human beta-MSH, had no effect on food intake [7]. Thus, it is possible that the effects of chicken beta- and gamma2-MSH on food intake in chicks are different when compared with human peptides.

In this chapter, I have compared the effects of alpha-, chicken beta- and chicken gamma2-MSH on food intake in broiler chicks.

2. Materials and methods

2.1. Animals

Day-old male broiler chicks (chunky) were purchased from a local hatchery (Ishii Co., Ltd., Tokushima, Japan). They were given free access to water and a

commercial chick starter diet (23.5% crude protein and 3,050 kcal/kg, Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan). Room temperature was maintained at $32^{\circ}\text{C} \pm 2^{\circ}\text{C}$. All experimental procedures followed the guidelines for the care and use of experimental animals at the Rokkodai Campus of Kobe University in Japan.

2.2. Experiment 1: Effect of central administration of alpha-MSH on food intake in chicks

Initially, I carried out a screening experiment to examine the effect of central administration of alpha-MSH (H-SYSMEHFRWGKPV-OH, dose: 40-4,000 pmol) on food intake in broiler chicks. Alpha-MSH was purchased from Peptide Institute, Inc. (Osaka, Japan). I found that the central administration of alpha-MSH suppressed food intake, and higher doses (400 and 4,000 pmol) significantly suppressed food intake at similar levels (97.4% and 100%, respectively) at 60 min after administration. Based on these results, doses from 40 to 400 pmol were used in Experiment 1-3.

Four-day-old chicks were weighed and allocated to three groups based on body weight. Alpha-MSH was dissolved in a 0.85% (w/v) saline solution containing 0.1% (w/v) Evans Blue. The peptides were intracerebroventricularly administered according to the method of Davis *et al.* [18] at a volume of 10 μl after 3 h of fasting. Chicks were administered either the alpha-MSH (40 or 400 pmol) or saline (as a control). Food intake was measured at 30, 60, and 120 min after administration. Each feeder filled with food was pre-weighed. A paper was put under the feeder. In each time point, any spilled food on the paper was collected and food consumption was weighed using an electric digital balance (readability 10 mg). Food intake was

calculated as follows:

Food intake = (The amount of food decrease in the feeder) – (The amount of spilled food)

At the end of the experiment, the chicks were sacrificed by decapitation. Verification of peptide administration was made by observation of the presence of Evans Blue dye in the left lateral ventricle.

2.3. Experiment 2: Effect of central administration of beta-MSH on food intake in chicks

Four-day-old chicks were divided into three groups based on body weight. Chicken beta-MSH (H-DGGSYRLAHFRWHAPLKD-OH) [12] was purchased from Peptide Institute, Inc. (Osaka, Japan). Either the chicken beta-MSH (40 or 400 pmol) or saline (as a control) was intracerebroventricularly administered after 3h of fasting as described above. Food intake was measured, and verification of peptide administration was made as described above.

2.4. Experiment 3: Effect of central administration of gamma-MSH on food intake in chicks

Four-day-old chicks were divided into three groups based on body weight. Chicken gamma-MSH (H-YVMSHFRWNKFG-OH) [12] was purchased from Peptide Institute, Inc. (Osaka, Japan). Either the chicken gamma-MSH (40 or 400 pmol) or saline (as a control) was intracerebroventricularly administered after 3h of fasting as described in Experiment 1. Food intake was measured, and verification of peptide administration was made as described in Experiment 1.

2.5. Experiment 4: Effect of central administration of HS014 on the anorexigenic effect of alpha-MSH in chicks

First, I examined the effect of central administration of HS014 on food intake in chicks. Four-day-old chicks were weighed and allocated to four groups based on body weight. HS014 was purchased from Sigma-Aldrich (St. Louis, MO, USA). After 2.5 h of fasting, chicks were administered either saline or HS014 (50, 100, or 200 pmol) into the lateral ventricle as described in Experiment 1. Then after 30 min of fasting, food was given, and food intake and verification of peptide administration was made as described in Experiment 1.

Next, I examined the effect of central administration of HS014 on the anorexigenic effect of alpha-MSH in chicks. Four-day-old chicks were weighed and allocated to three groups based on body weight. After 2.5 h of fasting, chicks were administered either saline or HS014 (50 pmol) into the right lateral ventricle. Then after 30 min of fasting, chicks were administered either alpha-MSH (40 pmol) or saline into the left lateral ventricle as described in Experiment 1. Subsequently, food was given, and food intake was measured at 30, 60, and 120 min after administration. Verification of peptide administration was made by observation of the presence of Evans Blue dye in both right and left lateral ventricles.

2.6. Data analysis

Data were statistically analyzed with one-way analysis of variance and the Tukey-Kramer test as a post hoc test. Statistical significance was set at $p < 0.05$. The results are shown as means $\pm$ S.E.M. All statistical analyses were performed

using a commercial package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

3. Results

Central administration of alpha-MSH significantly suppressed food intake in broiler chicks ($p < 0.05$) (Fig. 6). At 120 min after administration, chicks treated with 400 pmol α -MSH consumed 71.7% less food than control chicks. Central administration of beta-MSH did not influence food intake in broiler chicks (Fig. 7). Also, gamma-MSH did not show any effect on food intake in broiler chicks (Fig. 8).

Central administration of HS014 did not influence food intake (Fig. 9). Based on this result, the dose 50 pmol was used in the following experiment. In the result, central administration of alpha-MSH significantly suppressed food intake ($p < 0.05$), while HS014 completely abolished the anorexigenic effect of alpha-MSH (Fig. 10).

4. Discussion

As shown in Figs 6-8, central administration of alpha-MSH significantly suppressed food intake in broiler chicks ($p < 0.05$) (Fig. 6), whereas beta- or gamma2-MSH did not influence food intake in chicks (Figs. 7 and 8). Similar results were obtained when these peptides were centrally administered in 8-day-old broiler chicks in our laboratory (data not shown). Alpha-, beta- and gamma2-MSH can be produced in equimolar amounts from the precursor peptide POMC in chickens [23] as well as in humans [5]. We can not exclude the possibility that beta- and gamma2-MSH inhibit feeding in chicks if much higher doses are injected. However, these findings suggest that alpha-MSH plays an important role in the regulation of

food intake by the central melanocortin system in chicks.

In mammals, alpha-MSH suppresses food intake via the MC4R [51]. There is evidence that the MC3R, MC4R, and MC5R were expressed in chicken brain [34]. Ling et al reported that HS014 displays clear selectivity for the chicken MC4R with about 90- and 300-fold higher affinity as compared with the MC3R and the MC5R, respectively [46]. Central melanocortin signaling through the MC4R is linked to the regulation of food intake in the ring dove [75]. In the present study, central administration of the MC4R antagonist HS014 completely abolished the anorexigenic action of alpha-MSH (Fig. 10). All these findings suggest the involvement of the MC4R in the inhibitory mechanisms of alpha-MSH for feeding behavior in birds.

In the present study, 400 pmol chicken beta-MSH did not influence food intake in chicks (Fig. 7). We previously showed that central administration of 300 pmol human beta-MSH in chicks significantly suppresses food intake [37]. The different responses between the present study and previous study are probably due to the differences between human and chicken amino acid sequences (Fig. 5). Biebermann et al. [7] reported that central administration of human beta-MSH significantly decreased food intake in rats, whereas human beta-MSH mutant, which has only one amino acid substitution, had no effect on food intake. These findings suggest that beta-MSH does not play an important role in the central regulation of food intake in chickens.

Smith et al. reported that intracerebroventricular administration of 3 nmol human gamma2-MSH in chicks significantly suppresses food intake, whereas 1.5 nmol human gamma2-MSH does not show any significant effect on food intake at 30, 60, and 120min after administration [73]. In the present study, 400 pmol chicken gamma2-MSH did not influence food intake in chicks (Fig. 8), although 40 pmol

alpha-MSH significantly suppressed food intake (Fig. 6). These findings suggest that gamma2-MSH does not play an important role in the central regulation of food intake in chickens.

Anorexigenic effects of alpha-, human beta-, and human gamma2-MSH have been confirmed in broiler chicks after 3h of fasting [37, 40, 73]. Therefore, in the present study, I have examined the effects of alpha-, chicken beta-, and chicken gamma2-MSH on food intake in chicks after 3h of fasting (Experiments 1-4) in order to compare the results in this study and the earlier studies. As a result, I found that only alpha-MSH significantly suppresses food intake in broiler chicks. However, I also showed that HS014 does not influence food intake in broiler chicks, suggesting that endogenous alpha-MSH does not function as an anorexigenic peptide under this experimental condition. Thus, further studies will be needed to examine the effects of alpha-, chicken beta-, and chicken gamma2-MSH under *ad libitum* feeding condition.

Amino acid sequences of beta- and gamma2-MSH in turtle, frog, and lungfish are also different from those of human peptides (Fig. 5). There is evidence that alpha-MSH acts as an anorexigenic peptide in amphibians and fishes [11, 14, 21, 60, 84]. However, the anorexigenic effects of beta- or gamma2-MSH have not yet been examined in reptiles, amphibians, and fishes. These findings and our results raise the hypothesis that beta- and gamma2-MSH might not act as anorexigenic peptides in birds, reptiles, amphibians, and fishes.

5. Conclusion

In summary, I examined the effects of alpha-, chicken beta- and chicken gamma2-MSH on food intake in broiler chicks. Central administration of

alpha-MSH significantly suppressed food intake. In contrast, beta- and gamma2-MSH did not influence food intake in broiler chicks. These results suggest that alpha-MSH may play an important role in the central regulation of food intake in broiler chicks.

Chapter 2 Section 2

A comparative study of the central effect of melanocortin peptides on food intake in broiler and layer chicks

1. Introduction

As mentioned in Chapter 2-1, central administration of alpha-MSH significantly reduced food intake in broiler chicks, while beta- and gamma2-MSH did not influence food intake. There is evidence that central administration of alpha-MSH also significantly reduces food intake in layer chicks [30, 80]. However, the effects of beta- and gamma2-MSH on food intake in layer chicks have not yet been examined. It is therefore possible that the absence of the anorexigenic effects of beta- and gamma2-MSH in broiler chicks is involved in the difference in food intake between broiler and layer chickens.

In this chapter, I examined the effects of central administration of alpha-, beta- and gamma2-MSH on food intake in layer and broiler chicks in order to clarify the involvement of POMC-derived melanocortin peptides in the difference in food intake between these strains.

2. Materials and methods

2.1. Animals

Day-old male layer chicks (White Leghorn) and broiler chicks (chunky) were purchased from a local hatchery (Ghen Corporation, Gifu, Japan and Ishii Co., Ltd., Tokushima, Japan, respectively). They were given free access to water and a

commercial chick starter diet (Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan). Room temperature was maintained at $32^{\circ}\text{C} \pm 2^{\circ}\text{C}$. All experimental procedures followed the guidelines for the care and use of experimental animals at the Rokkodai Campus of Kobe University in Japan.

2.2. Experiment 1: Comparison of hypothalamic POMC mRNA level in broiler and layer chicks

Broiler and layer chicks were sacrificed by decapitation at 8 days of age. Their brains were removed within 1 min of decapitation, weighed, frozen on powdered dry ice and stored at -80°C for further analysis. The hypothalamus was dissected from the frozen brain (by referring to a stereotaxic atlas drawn by Kuenzel and Masson [43]) and weighed. Total RNA was extracted from the hypothalamus using the Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from 5 μg of DNase I (Ambion Inc., Austin, Texas, USA)-treated total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems Inc., Foster city, California, USA) with random primers. Complementary DNA of POMC (GenBank accession no. [NM_001031098](#)) was amplified with the following primers: POMC sense, 5'-AGA TGG AGA AGG GTT GGAA-3'; POMC antisense, 5'-CGT TGG GGT ACA CCT TGA-3'. As an internal standard, chicken ribosomal protein S17 mRNA (GenBank accession no. [NM_204217](#)) was also amplified, using the following primers: sense, 5'-GCG GGT GAT CAT CGA GAA GT-3'; antisense, 5'-GCG CTT GTT GGT GTG GAA GT-3'. SYBR[®] Premix Ex Taq was purchased from Takara Bio Inc. (Shiga, Japan), and mRNA expression was quantified in triplicate using the Applied Biosystems 7300 Real-Time PCR system according to the supplier's recommendations.

2.3. Experiment 2: Effect of central administration of alpha-MSH on cumulative food intake in broiler and layer chicks

Eight-day-old chicks from each strain were divided into three groups. Alpha-MSH (H-SYSMEHFRWGKPV-OH) was purchased from Peptide Institute, Inc. (Osaka, Japan). Alpha-MSH was dissolved in a 0.85% (w/v) saline solution containing 0.1% (w/v) Evans Blue. The peptides were intracerebroventricularly administered according to the method of Davis *et al.* [18] at a volume of 10 µl after 3 h of fasting. Chicks were administered either the alpha-MSH (40 or 400 pmol) or saline (as a control). Food intake was measured at 30, 60 and 120 min after administration. Each feeder filled with food was pre-weighed. A paper was put under the feeder. In each time point, any spilled food on the paper was collected and food consumption was weighed using an electric digital balance (readability 10 mg). Food intake was calculated as follows:

$$\text{Food intake} = (\text{The amount of food decrease in the feeder}) - (\text{The amount of spilled food})$$

At the end of the experiment, the chicks were sacrificed by decapitation. Verification of peptide administration was made by observation of the presence of Evans Blue dye in the lateral ventricle.

2.4. Experiment 3: Effect of central administration of beta-MSH on cumulative food intake in broiler and layer chicks

Eight-day-old chicks from each strain were divided into three groups. Beta-MSH (H-DGGSYRMRHFRWHAPLKD-OH) [82] was purchased from Peptide Institute, Inc. (Osaka, Japan). Either the beta-MSH (40 or 400 pmol) or saline (as a control) was intracerebroventricularly administered as described in Experimenta 2.

Food intake was measured at 30, 60 and 120 min after administration and verification of peptide injection was made as described in Experiment 2.

2.5. Experiment 4: Effect of central administration of gamma2-MSH on cumulative food intake in broiler and layer chicks

Eight-day-old chicks from each strain were divided into three groups. Gamma2-MSH (H-YVMSHFRWNKFG-OH) [82] was purchased from Peptide Institute, Inc. (Osaka, Japan). Either the gamma2-MSH (40 or 400 pmol) or saline (as a control) was intracerebroventricularly administered as described in Experiment 2. Food intake was measured at 30, 60 and 120 min after administration and verification of peptide injection was made as described in Experiment 2.

2.6. Experiment 5: Comparison of hypothalamic melanocortin 4 receptor mRNA level in broiler and layer chicks

Hypothalamic mRNA level of melanocortin 4 receptor (MC4R) was analyzed as described in Experiment 1. Complementary DNA of MC4R (GenBank accession no. NM_001031514) was amplified with the following primers: sense, 5'-CCT CGG GAG GCT GCT ATG A-3'; antisense, 5'-GAT GCC CAG AGT CAC AAA CAC TT-3'.

2.7. Data analysis

Data from Experiment 1 and 5 was analyzed by Student's *t* test. Data from Experiment 2, 3, and 4 were analyzed by the Tukey-Kramer test at each time point.

All statistical analyses were performed using the commercial package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

3. Results

There was no significant difference in the hypothalamic POMC mRNA levels between these strains (Fig. 11). Central administration of alpha-MSH significantly suppressed food intake in both strains (Fig. 12). Central administration of beta-MSH significantly suppressed food intake in layer chicks, but not in broiler chicks (Fig. 13). Gamma2-MSH did not affect food intake in both strains (Fig. 14). There was no significant difference in hypothalamic MC4R mRNA levels between broiler and layer chicks (Fig. 15).

4. Discussion

I first examined the hypothalamic mRNA levels of POMC in broiler and layer chicks. There was no significant difference in the hypothalamic POMC mRNA levels between these strains (Fig. 11), suggesting that the hyperphagia in broiler chicks is not caused by the reduction in hypothalamic POMC mRNA levels. This view is supported by a recent publication; layer and broiler chicks exhibited similar hypothalamic expression of POMC at 7 days of age [91].

Next, I examined the effects of central administration of MSHs on food intake in these strains. Central administration of alpha-MSH significantly suppressed food intake in both strains (Fig. 12). These results are in good agreement with previous studies [30, 80].

Central administration of beta-MSH significantly suppressed food intake in

layer chicks (Fig. 13). On the other hand, central administration of beta-MSH did not affect food intake in broiler chicks (Fig. 13). It is therefore likely that the absence of the anorexigenic effect of beta-MSH might be related to the difference in food intake between broiler and layer chicks. We previously showed that central administration of human beta-MSH in broiler chicks potently suppresses food intake [37]. We confirmed that central administration of human beta-MSH in layer chicks potently suppressed food intake as well as alpha-MSH-induced anorexia (data not shown). There is evidence that central administration of human beta-MSH in rats significantly reduced food intake, whereas human beta-MSH mutant, which has only one amino acid substitution, had no effect on food intake [7]. It is therefore likely that only two amino acid substitutions in chicken beta-MSH have attenuated the anorexigenic action of beta-MSH in chickens.

Gamma2-MSH did not show any significant anorexigenic effect in broiler or layer chicks (Fig. 14), suggesting that gamma2-MSH is not involved in the central regulation of food intake in chickens. In mammals, different results for the anorexigenic effect of gamma2-MSH were reported. For example, Millington *et al.* reported that central administration of gamma2-MSH strongly suppressed food intake in rats [50]. However, Tung *et al.* showed that equimolar amounts of gamma2-MSH were able to reduce food intake, but to a lesser degree than that seen with alpha-MSH [83]. In addition, Abott *et al.* reported that central administration of either alpha-MSH or beta-MSH significantly reduced food intake in rats when given in the early light phase, whereas gamma2-MSH did not influence food intake [1].

In mammals, the anorexigenic effect of alpha- and beta-MSH is mediated by hypothalamic MC4R [2, 45]. In chickens, MC3R, MC4R, and MC5R are expressed in the brain [34, 81]. Ling *et al.* reported that HS014, which is selective for the

mammalian MC4R [64], displays clear selectivity for the chicken MC4R with more than 90-fold higher affinity as compared with the chicken MC3R and MC5R [46]. In Chapter 2-1, I showed that HS014 completely reversed the anorexigenic effect of alpha-MSH in chicks, suggesting that alpha-MSH suppresses food intake via MC4R in chicks. Thus, I finally analyzed the mRNA levels of hypothalamic MC4R in both strains. However, there was no significant difference in hypothalamic MC4R mRNA levels between broiler and layer chicks (Fig. 15, $p = 0.159$), although the mean value of MC4R mRNA level in layer chicks was higher than that in broiler chicks. It is therefore possible that MC4R is not involved in the different effects of beta-MSH on food intake between broiler and layer chicks. However, MC4R protein levels on the target neurons of POMC neurons were not investigated in the present study. In addition, up to now, chicken beta-MSH binding affinity to the melanocortin receptors has not been examined. Sharma et al. reported polymorphisms in MC3R and MC4R and association with production traits in a commercial broiler line [67]. This finding and our results raise the hypothesis that the protein levels of melanocortin receptors and/or chicken beta-MSH binding affinity to the melanocortin receptors on the target neurons of POMC neurons in broiler chicks was lower than that in layer chicks. Further study will be needed to clarify this point and the mechanisms underlying the different effects of beta-MSH on food intake between broiler and layer chicks.

In the study of this chapter, I used 8-day-old chicks. The body weight of broiler chicks (about 150 g – 160 g) was heavier than that of layer chicks (about 80 g). However, brain weights of broiler and layer chicks are not much different between two strains at 8 days of age. Therefore, I did not decide the doses of MSHs based on their body weight. The results in the study of this chapter were quite similar to those in the study of Chapter 2 Section 1 using 4-day-old broiler chicks (average

body weight was about 75 g). Thus, the ineffectiveness of 400 pmol beta-MSH would not be due to the difference in their body weight or age between two strains.

5. Conclusion

In summary, I first compared the hypothalamic mRNA levels of POMC between broiler and layer chicks, and then examined the effects of central administration of melanocortin peptides on food intake in these strains. There were no significant differences in hypothalamic POMC mRNA levels between broiler and layer chicks. Alpha-MSH significantly suppressed food intake in both strains, whereas the anorexigenic effect of beta-MSH was found only in layer chicks. Central administration of gamma2-MSH did not influence food intake in either strain. It is therefore likely that the absence of the anorexigenic effect of beta-MSH might be related to the increased food intake in broiler chicks.

Chapter 3

Relationship between abdominal fat accumulation and appetite-related neuropeptides in broiler and layer chicks

1. Introduction

As mentioned in General Introduction, NPY and POMC play a critical role in the central regulation of food intake in both mammals and chicks. The gene expression of hypothalamic NPY and POMC is altered in response to circulating adiposity signals (insulin and leptin) which inform the brain of changes in body fat mass, in mammals and chicks. However, the relationship between adiposity and these neuropeptides remains unclear in chickens.

In the mammalian hypothalamus, POMC-producing neurons project from ARC to the PVN in the hypothalamus, and PVN contains corticotropin releasing factor (CRF)-producing neurons [66]. Thus, anorexigenic signals from POMC-producing neurons in the ARC are transduced to CRF neurons in the PVN.

Immunohistochemical studies have shown that hypothalamic POMC- or CRF-producing neurons in chickens localize in the same nuclei as those in mammals [23, 61]; POMC-immunoreactive perikarya are present in IN [23], and CRF-immunoreactive perikarya are present in the PVN [61]. The CRF receptor antagonist astressin significantly reversed the anorexigenic action of alpha-MSH in chicks [80]. These observations suggest that CRF is a downstream mediator of the POMC-induced anorexigenic pathway in chicks as well as in mammals. It is therefore possible that CRF is also regulated in response to body fat accumulation.

In this chapter, I investigated the relationships between abdominal adipose tissue weight and hypothalamic mRNA levels of NPY, POMC and CRF in broiler

and layer chicks.

2. Materials and methods

2.1. Animals

Day-old male chicks of a broiler (chunky: Ishii Co., Ltd. Tokushima, Japan) and a layer (White Leghorn: Ghen Corporation, Gifu, Japan) were purchased from a local hatchery. They were given free access to water and a commercial chick starter diet (Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan) under an automatically controlled 12-h light:dark cycle (6:00-18:00). This study was approved by the Institutional Animal Care and Use Committee (Permission number: 22-05-10) and carried out according to the Kobe University Animal Experimentation Regulation. Broiler and layer chicks were sacrificed at the mid-point of the light phase (12:00) by decapitation at 7, 14, 21, and 28 days of age. Body weight was measured before sacrifice. The abdominal adipose tissue was excised and weighed. The hypothalamus was dissected from the brain by referring to a stereotaxic atlas [43] and stored at -80°C for real-time PCR analysis.

2.2. Real-time PCR analysis

Total RNA was extracted from the hypothalamus using Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from 5 µg of DNase I (Ambion Inc., Austin, Texas, USA)-treated total RNA using a ReverTra Ace® qPCR RT Kit (TOYOBO CO. LTD., Osaka, Japan) with random primers. Complementary DNA of NPY (GenBank accession no. NM_205473), POMC (GenBank accession no.

NM_001031098), and CRF (GenBank accession no. AJ621492) were amplified with the following primers: NPY sense, 5'-CTT GTC GCT GCT GAT CTG -3'; NPY antisense, 5'-GCC TCA GAG CCG AGT AGT-3'; POMC sense, 5'-AGA TGG AGA AGG GTT GGAA-3'; POMC antisense, 5'-CGT TGG GGT ACA CCT TGA-3'; CRF sense, 5'-CTC CCT GGA CCT GAC TTT-3'; CRF antisense, 5'-CCT CAC TTC CCG ATG ATT-3'. As an internal standard, chicken 18S ribosomal RNA (GenBank accession no. AF173612) was also amplified, using the following primers: sense, 5'-AGT CCC TGC CCT TTG TAC ACA-3'; antisense, 5'-CCT CAC TAA ACC ATC CAA TCG-3'. THUNDERBIRD™ SYBR® qPCR Mix was purchased from TOYOBO CO. LTD. (Osaka, Japan), and mRNA expression was quantified in duplicate using the Applied Biosystems 7300 Real-Time PCR system according to the supplier's recommendations.

2.3. Data analysis

Hypothalamic mRNA levels of NPY, POMC, and CRF between 7 to 28 days of age were analyzed by two-way ANOVA. Comparisons between means of ages and chick types were made using the Tukey-Kramer test and Student *t* test, respectively when a significant interaction was not detected between chick types and ages. Comparisons between all means were made using the Tukey-Kramer test when a significant interaction was detected. Relative abdominal adipose tissue weights to body weight between 7 to 28 days of age in each type of chicks were also analyzed by the Tukey-Kramer test. Pearson's correlation coefficient analysis and simple regression were used to assess the relations between NPY or POMC mRNA level and abdominal adipose tissue weight (g/100 g body weight) and between the mRNA levels of POMC and CRF. Difference of correlation coefficients of the regression

lines obtained from broiler and layer was determined by testing the t -value. All statistics was performed using a commercial software package (StatView version 5, SAS Institute, Cary, North Carolina, USA, 1998).

3. Results

Two-way ANOVA did not show any significant interaction between chick types and ages with respect to the mRNA level of NPY, and hence, only the results of the main effects were presented in Fig. 16. Hypothalamic NPY mRNA levels significantly ($p < 0.05$) decreased at 14 and 28 days of age in broiler chicks when compared to 7 days of age (Fig. 16). In layer chicks, the hypothalamic NPY mRNA level was significantly ($p < 0.05$) decreased at 14 days of age when compared to 7 days of age, and the mRNA levels were not reversed at 21 and 28 days of age (Fig. 16). The weights of abdominal adipose tissue were significantly ($p < 0.05$) increased after 14 days of age in both strains (Fig17). The relationship between hypothalamic NPY mRNA levels and abdominal adipose tissue weights was significantly and negatively correlated in broiler chicks (Fig 18, $R^2 = 0.307$, $p = 0.001$). In layer chicks, the relationship between hypothalamic NPY mRNA levels and abdominal adipose tissue weights was negatively, but not significantly, correlated (Fig. 18, $R^2 = 0.109$, $p = 0.070$). There was no difference in the regression coefficient between broiler and layer chicks.

Two-way ANOVA showed significant interaction between chicken strain and age with respect to the mRNA level of POMC ($F = 4.76$, $p < 0.01$). Post hoc test showed significant difference ($p < 0.05$) in POMC mRNA levels between 7 days of age and 28 days of age in broiler chicks (Fig. 19). On the other hand, hypothalamic POMC mRNA levels were not affected throughout the experimental period in layer

chicks (Fig. 19). Also, there was no significant difference in hypothalamic POMC mRNA levels between broiler and layer chicks at each age tested. The relationship between hypothalamic POMC mRNA levels and abdominal adipose tissue weights in broiler chicks was negatively correlated (Fig. 20, $R^2 = 0.116$, $p = 0.056$). On the other hand, the relationship between hypothalamic POMC mRNA levels and abdominal adipose tissue weights was positively correlated in layer chicks (Fig. 20, $R^2 = 0.200$, $p = 0.012$).

Two-way ANOVA did not show any significant interaction between chick types and ages with respect to the mRNA level of CRF, and hence, only the results of the main effects were presented in Fig. 21. Hypothalamic CRF mRNA level was not affected throughout the experimental period in broiler chicks (Fig. 21). On the other hand, hypothalamic CRF mRNA level significantly increased at 28 days of age in layer chicks (Fig. 21). There was no significant difference in hypothalamic CRF mRNA levels between broiler and layer chicks at each age tested. The relationship between hypothalamic CRF mRNA levels and abdominal adipose tissue weights was negatively correlated in layer chicks ($R^2 = 0.169$, $p = 0.019$) but not in broiler chicks ($R^2 = 0.006$, $p = 0.672$) (Fig. 22). The relationship between hypothalamic POMC and CRF mRNA levels was significantly and positively correlated only in layer chicks (Fig. 23, $R^2 = 0.318$, $p = 0.010$).

4. Discussion

I first examined the relationships between abdominal adipose tissue weights and hypothalamic mRNA levels of NPY in broiler and layer chicks. Hypothalamic NPY mRNA levels significantly ($p < 0.05$) decreased at 14 and 28 days of age in broiler chicks and at 14 days of age in layer chicks, when compared to 7 days of age

(Fig. 16). These results suggest that the hypothalamic NPY mRNA level was higher during the first week post-hatching in both broiler and layer chicks than that after 14 days of age. Yuan *et al.* reported that broiler and layer chicks exhibit similar hypothalamic expression of NPY at 7 days of age [91]. As mentioned in Chapter 1, there was no significant difference in hypothalamic NPY mRNA levels between broiler and layer chicks at 8 days of age. In the result of this chapter, there was no significant difference in hypothalamic NPY mRNA levels between broiler and layer chicks at each age tested. These results suggest that the difference in the expression of hypothalamic NPY is not a cause of increased food intake in broiler chicks.

In mammals, in response to circulating signals which inform the brain of changes in body fat mass, hypothalamic gene expression of the NPY mRNA level is altered [65, 66, 74]. In chickens, the percentages of carcass fat are quite consistent with those from the abdominal adipose tissue weight [26]. Therefore, we analyzed the correlation between abdominal adipose tissue weight and hypothalamic NPY mRNA level. The relationship between hypothalamic NPY mRNA levels and abdominal adipose tissue weights was negatively correlated in broiler and layer chicks (Fig. 18, $R^2 = 0.307$, $p = 0.001$). These results suggest that hypothalamic gene expression of NPY is regulated in response to body fat accumulation in both strains.

I next examined the relationships between abdominal adipose tissue weight and hypothalamic mRNA levels of POMC in broiler and layer chicks. Hypothalamic POMC mRNA levels significantly ($p < 0.01$) decreased at 28 days of age in broiler chicks when compared to 7 days of age, and was not affected through the experimental period in layer chicks (Fig19). The relationship between hypothalamic POMC mRNA levels and abdominal adipose tissue weights was negatively correlated in broiler chicks (Fig. 20, $R^2 = 0.116$, $p = 0.056$). On the other hand, it was positively correlated in layer chicks (Fig. 20, $R^2 = 0.200$, $p = 0.012$). These results

suggest that hypothalamic gene expression of POMC is suppressed in response to the body fat accumulation in layer chicks but not in broiler chicks. There is evidence that dysfunction in the POMC-induced anorexigenic pathway can cause hyperphagia in mammals [31, 90]. Thus, it seems likely that the POMC-induced anorexigenic pathway does not function in response to fat accumulation, which in turn, results in hyperphagia in broiler chicks.

Finally, I examined the relationships between abdominal adipose tissue weight and hypothalamic mRNA levels of CRF in broiler and layer chicks. Hypothalamic CRF mRNA level was not affected throughout the experimental period in broiler chicks and increased at 28 days of age in layer chicks (Fig. 21). The relationship between hypothalamic CRF mRNA levels and abdominal adipose tissue weights was negatively correlated in layer chicks ($R^2 = 0.169$, $p = 0.019$) but not in broiler chicks ($R^2 = 0.006$, $p = 0.672$) (Fig. 22). I found a significant positive correlation between POMC and CRF mRNA levels only in layer chicks (Fig. 23, $R^2 = 0.318$, $p = 0.010$). Tachibana et al. reported that the anorexigenic effect of alpha-MSH was significantly attenuated by the coadministration of a CRF receptor antagonist in layer chicks [80]. These findings and our results suggest that the POMC-induced anorexigenic pathway, including CRF, function in response to body fat accumulation in layer chicks.

It is generally accepted that a catabolic circuit exists in which an increase of body fat is associated with increased insulin and leptin, increased MSH, and decreased NPY activity, and consequently increased activity of CRF and other catabolic signals; all of these lead in turn to reduced food intake and increased energy expenditure [87]. In fact, by measuring rectal body temperature, a physiological response that may reflect whole body energy expenditure, several studies reported that NPY can cause whole body hypothermia [9, 17, 57]. In

contrast, alpha-MSH and its receptor agonist were reported to increase heat production when administered centrally [32, 33, 89]. In chickens, central administration of NPY significantly increased food intake and significantly decreased rectal temperature [78], whereas CRF showed anorexigenic and hyperthermic effects [77]. Thus, the negative correlations between NPY and abdominal fat in broiler and layer chicks (Fig. 3) and the positive correlation between CRF and abdominal fat in layer chicks (Figs. 20 and 22) would not be the causes but rather the results of the abdominal fat accumulation. In chickens, Tachibana *et al.* reported that the injection of alpha-MSH did not affect oxygen consumption, carbon dioxide production and heat production during the 1 h post-injection period [79]. However, the negative correlation between POMC and abdominal fat in broiler chicks (Fig. 20) could be the cause of the abdominal fat accumulation. Further study is needed to clarify this possibility.

In mammals, the ARC POMC neurones perform a primary sensory function in the central regulation of food intake by receiving metabolic information from circulating hormones [53, 66]. Insulin and leptin circulate at levels proportional to body fat content. Both hormones are sensed by the POMC neurons in the ARC; they act as important peripheral hormones in energy metabolism, and such hormones influence the regulation of food intake [53, 66]. However, several phylogenetic, bioinformatic and genomic arguments do not favor the presence of a leptin gene in the chicken genome [58, 68]. On the other hand, there is evidence that insulin suppresses food intake in chicks [30, 70], suggesting that it may function as an adiposity signal. Recently, Shiraishi *et al.* reported that central administration of insulin significantly reduced food intake in layer chicks, but not in broiler chicks, and hypothalamic insulin receptor mRNA expression levels were significantly lower in broiler chicks than in layer ones [71]. The anorexigenic effect of insulin was

completely reversed by coadministration of an MC4R antagonist in layer chicks [70], suggesting that insulin suppresses food intake via a POMC-induced anorexigenic pathway. These findings and our results raise the hypothesis that insulin resistance exists in the central nervous system of broiler chicks, which in turn, results in a down-regulation of the POMC-induced anorexigenic pathway. Further study is needed to clarify this point.

In this study, I have performed correlation analysis at each age in each chick type. However, any significant correlation was not detected, suggesting that more number of chicks might be needed to analyse the correlation. Further study is needed to analyse the correlation between hypothalamic gene expressions and abdominal fat weights at the same age in each chick type.

There is evidence that adipose tissue plays a critical role in maintenance of energy homeostasis through secretion of a large number of adipokines that interact with central as well as peripheral organs such as the brain, liver, pancreas, and skeletal muscle to control diverse processes, such as food intake, energy expenditure, carbohydrate and lipid metabolism, blood pressure, blood coagulation, and inflammation [25]. Several adipokines affect food intake in chicks. For example, central administration of visfatin significantly increased food intake in broiler chicks [16]. Recently, Yabuuchi *et al.* reported that agouti signaling protein (ASIP), an endogenous melanocortin-4 receptor antagonist, and POMC were expressed in chicken adipose tissue and their mRNA levels were changed in the negative-energy state [88]. Since MC4R agonist inhibits hypothalamic NPY expression [41], it is possible that chicken adipose tissue releases ASIP and alpha-MSH, which affect hypothalamic NPY mRNA levels in broiler and layer chicks. However, effects of adipokines on hypothalamic NPY and POMC levels in chicks have not been investigated yet. Further studies are needed to clarify the roles of adipokines in the

regulation of food intake in chickens.

It is generally accepted that adiposity signals leptin and insulin regulate hypothalamic neuropeptide at the transcriptional level [66]. On the other hand, the regulatory mechanism of the receptors of hypothalamic neuropeptide in response to the body fat accumulation was complicated. For example, Ghamari-Langroudi *et al.* identified three regulatory mechanisms important for the control of hypothalamic PVN-MC4 neurons: (i) direct postsynaptic modulation by leptin, (ii) regulation of MC4R mRNA expression and alpha-MSH responsiveness by leptin, and (iii) regulation by the constitutive activity of the MC4R signaling [24]. Therefore, we have examined the mRNA expression of hypothalamic neuropeptides as the first step to investigate the relationships between abdominal adipose tissue weight and hypothalamic regulation of food intake in broiler and layer chicks. Further study is needed to investigate the peptide content and release of neuropeptides in the hypothalamic nucleus and/or the expression and function of the neuropeptide receptors.

5. Conclusion

In summary, I investigated the effects of age and strain on hypothalamic mRNA levels of NPY, POMC, CRF in broiler and layer chicks and found that the relationship between hypothalamic NPY mRNA levels and abdominal adipose tissue weights was negatively correlated in both strains. In contrast, the relationship between hypothalamic POMC mRNA levels and abdominal adipose tissue weights was positively correlated in layer chicks, but not in broiler chicks. Therefore, it seems likely that POMC-induced anorexigenic pathway does not function in response to body fat accumulation, which in turn, results hyperphagia in

broiler chicks.

Summary

In this study, I investigated the involvement of NPY and POMC-derived alpha-, beta-, gamma- MSH in the difference in food intake between broiler and layer chickens.

In Introduction, I introduced recent information on the relation between appetite regulation and hypothalamic neuropeptides including NPY and POMC to explain the aim of this study.

In Chapter 1, I showed the involvement of a potent orexigenic peptide, NPY, in the difference in food intake between broiler and layer chicks. Daily food intake was significantly higher in broiler chicks than layer chicks at 2 days of age; however, the hypothalamic NPY mRNA level was significantly lower in broiler chicks than layer chicks except at 8 days of age. The mRNA levels of NPY receptors were also significantly lower in broiler chicks than layer chicks at 2 and 4 days of age (Y1 receptor) or 2 days of age (Y5 receptor). Central administration of NPY significantly increased food intake in both strains at 2, 4, and 8 days of age. All our findings demonstrated that the increase in food intake in broiler chicks is not accompanied by the over-expression of NPY or its receptor and the difference in the NPY responsiveness.

In Chapter 2 Section 1, I showed the effects of melanocortin peptides, alpha-, chicken beta- and chicken gamma2-MSH, on food intake in broiler chicks. Central administration of alpha-MSH significantly suppressed food intake, while the MC4R antagonist HS014 completely abolished the anorexigenic effect of alpha-MSH. In contrast, beta- and gamma2-MSH did not influence food intake in broiler chicks. These results suggest that alpha-MSH may play an important role in the central regulation of food intake in broiler chickens and that the inhibitory mechanisms of alpha-MSH in broiler chickens are exerted via MC4R.

In Chapter 2 Section 2, I showed the involvement of melanocortin peptides in the

difference in food intake between broiler and layer chicks. The hypothalamic mRNA levels of POMC between broiler and layer chicks were compared, and then the effects of central administration of melanocortin peptides on food intake in these strains were examined. There were no significant differences in hypothalamic POMC mRNA levels between broiler and layer chicks. α -MSH significantly suppressed food intake in both strains, whereas the anorexigenic effect of β -MSH was found only in layer chicks. Central administration of γ -MSH did not influence food intake in either strain. It is therefore likely that the absence of the anorexigenic effect of β -MSH is related to the increased food intake in broiler chicks.

In Chapter 3, I showed the effects of age and strain on hypothalamic mRNA levels of NPY, POMC, and CRF in broiler and layer chicks. The relationship between hypothalamic NPY mRNA levels and abdominal adipose tissue weights was negatively correlated in both strains. In contrast, the relationship between hypothalamic POMC or CRF mRNA levels and abdominal adipose tissue weights was positively correlated in layer chicks, but not in broiler chicks. Therefore, it seems likely that the POMC-induced anorexigenic pathway does not function in response to body fat accumulation, which in turn, results in hyperphagia in broiler chicks.

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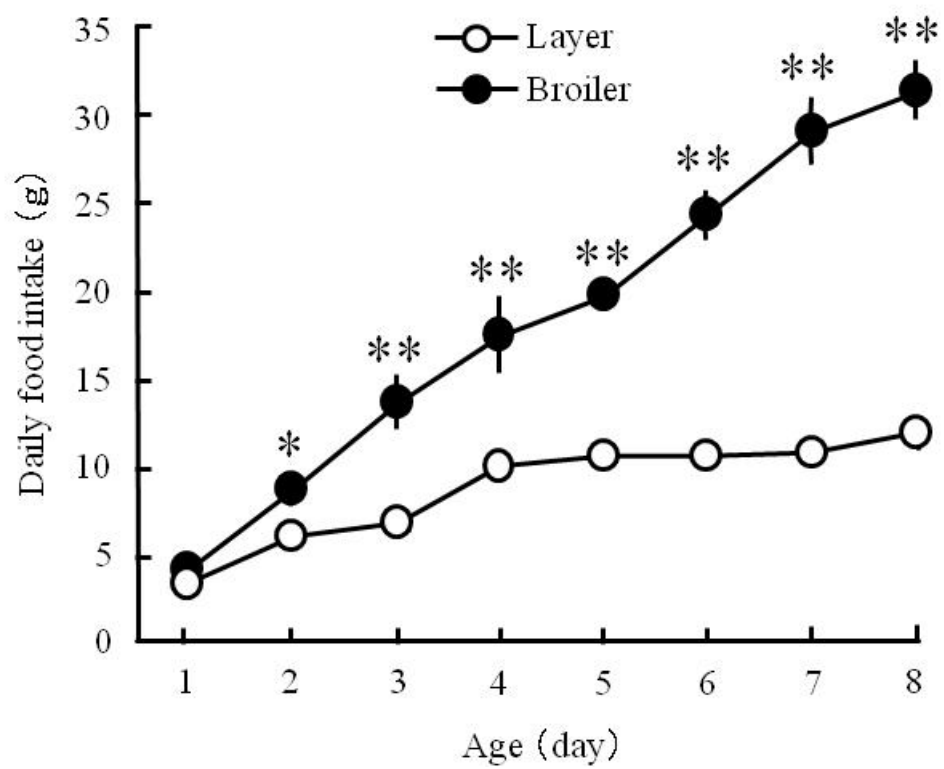


Fig. 1. Daily food intake of broiler and layer chicks. Data are means $\pm$ S.E.M. of eight chicks in each group. * and **, significant with respect to layer chicks ($p < 0.05$ and $p < 0.01$).

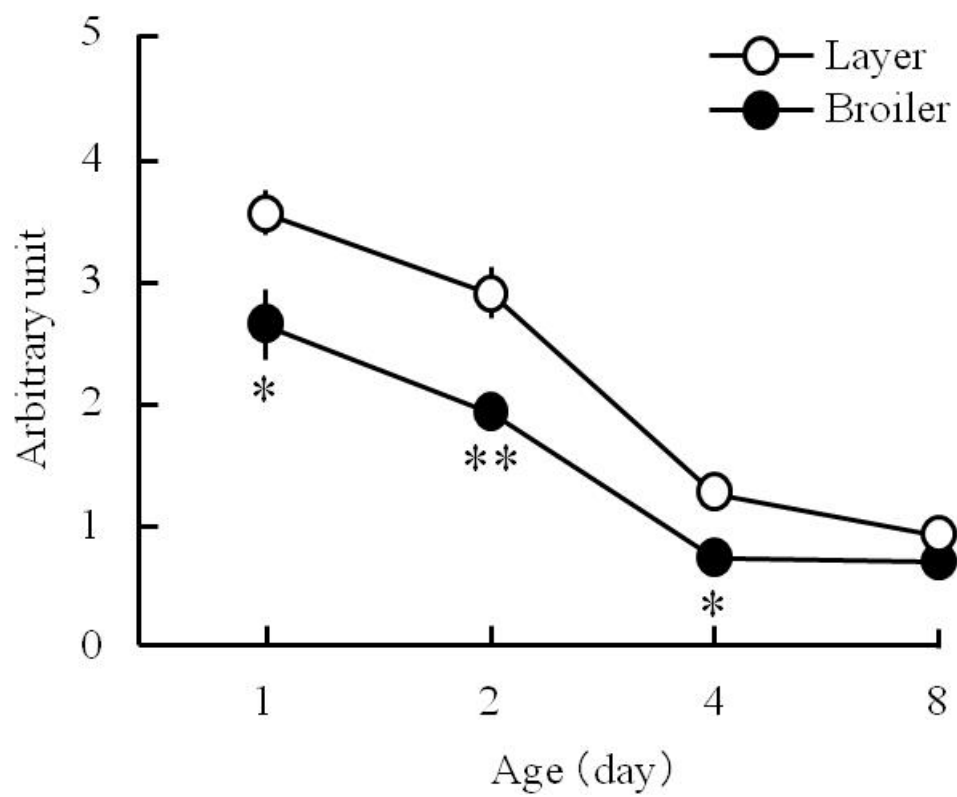


Fig. 2. Changes in the hypothalamic neuropeptide Y mRNA level in broiler and layer chicks. Data are means $\pm$ S.E.M. of six chicks in each group. * and **, significant with respect to layer chicks ($p < 0.05$ and $p < 0.01$).

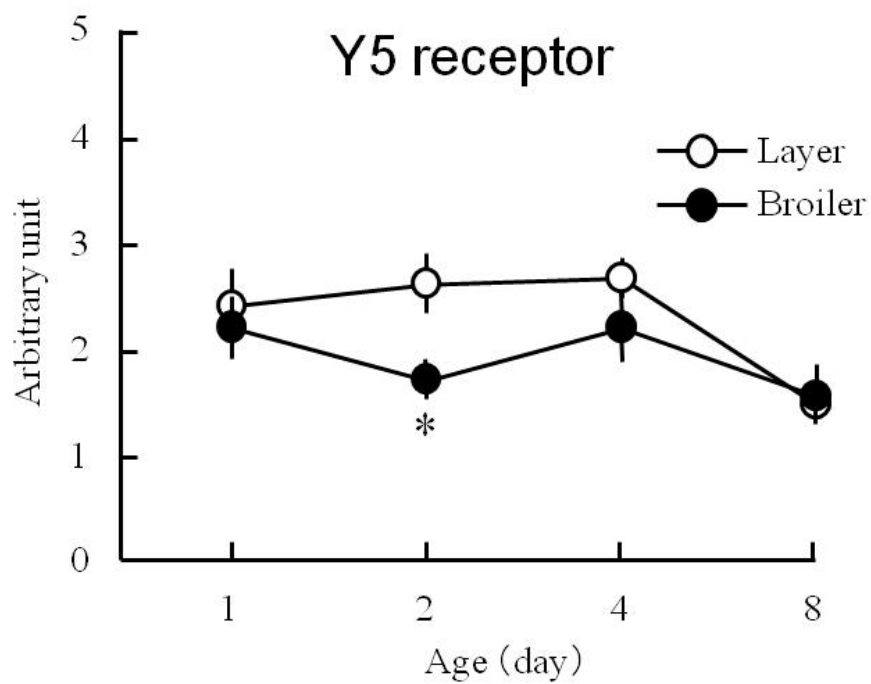
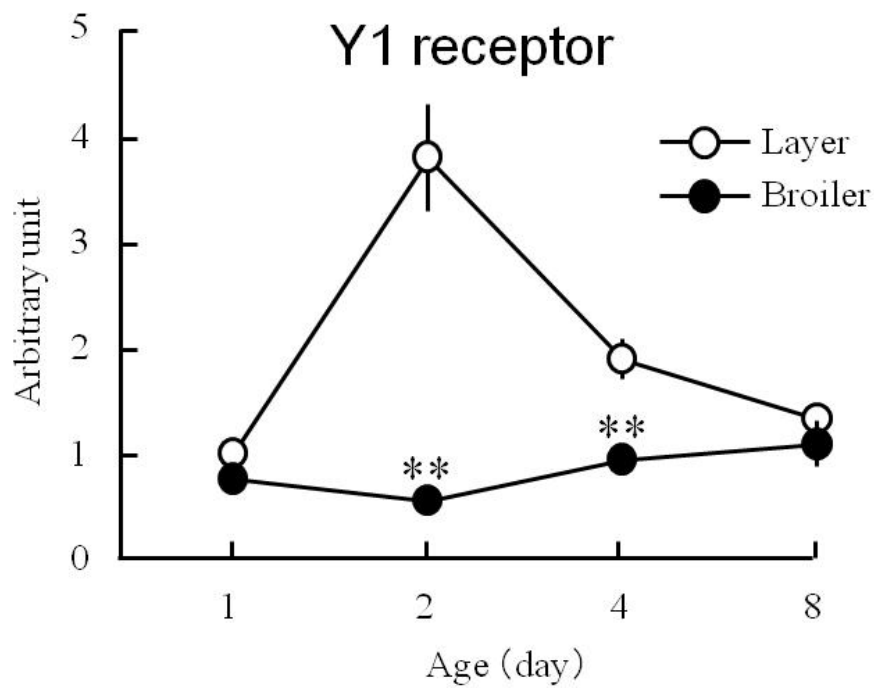


Fig. 3. Changes in hypothalamic mRNA levels of Y1 and Y5 receptors in broiler and layer chicks. Data are means $\pm$ S.E.M. of six chicks in each group. * and **, significant with respect to layer chicks ($p < 0.05$ and $p < 0.01$).

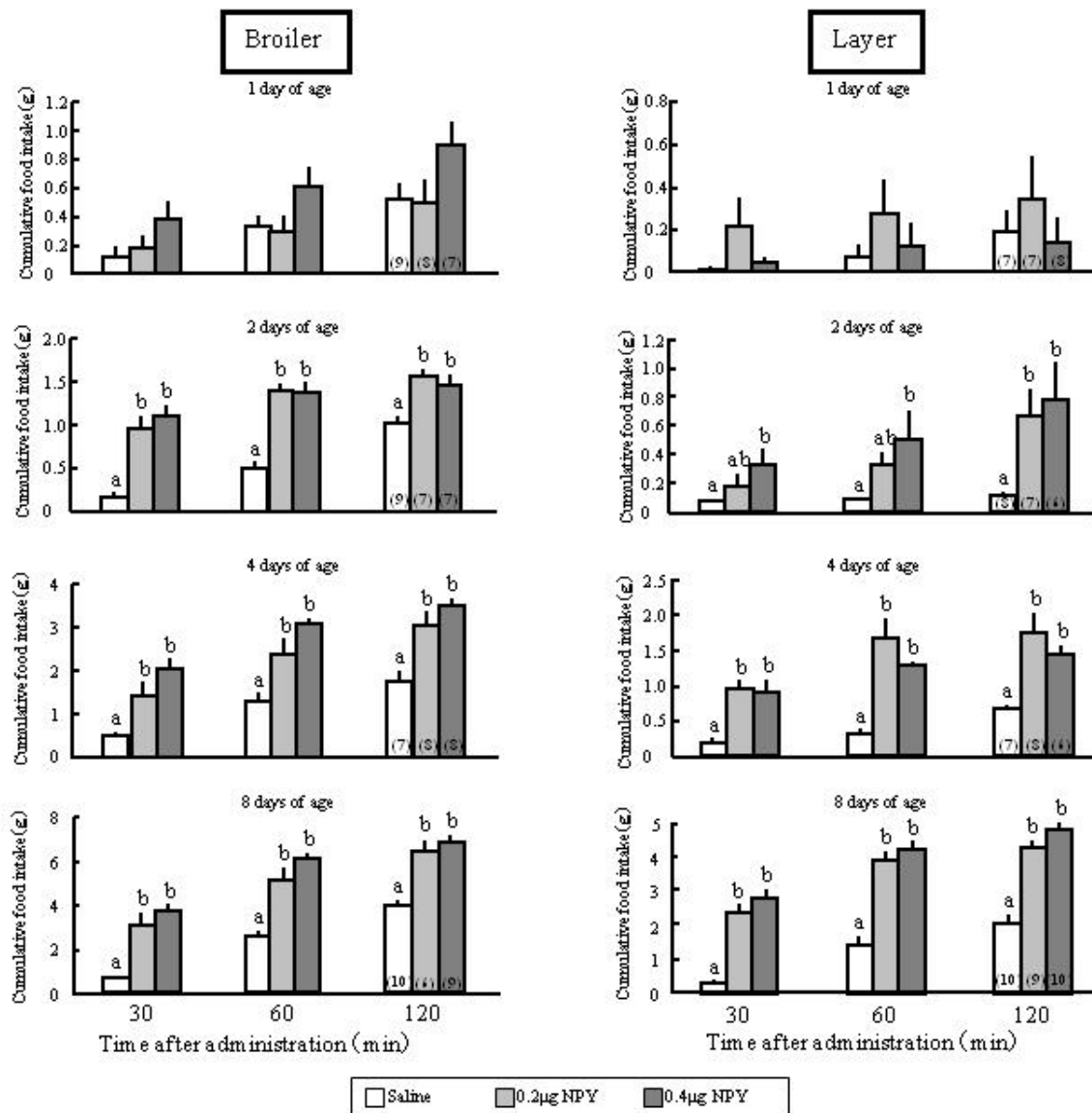


Fig. 4. Effect of central administration of neuropeptide Y on cumulative food intake in broiler and layer chicks. Data are means $\pm$ S.E.M. The number of chicks used is shown in parentheses. Groups with different letters are significantly different ($p < 0.05$) at each time point.

Alpha-MSH

Human : SYSMEHFRWGKPV
Chicken : SYSMEHFRWGKPV
Turtle : SYSMEHFRWGKPV
Frog : SYSMEHFRWGKPV
Lungfish: SYSMEHFRWGKPV

Beta-MSH

Human : DEGPYRMEHFRWGSPPKD
Chicken : DGGSYRIAHFRWHAPLKD
Turtle : DGNSYKMHHFRWNAPPKD
Frog : D-RKYKMHHFRWEGPPKD
Lungfish: D-RNYRMOHFRWNSPPKD

Gamma2-MSH

Human : YVMGHERWDREG
Chicken : YVMSHERWNKEFG
Turtle : YVMSHERWNKEFG
Frog : YVMSHERWNKEFG
Lungfish: YMMTHFRWDKEFG

Fig. 5. Alignment of melanocortin peptides from different species. Amino acid residues of melanocortin peptides nonidentical to human melanocortin peptides are marked by shading.

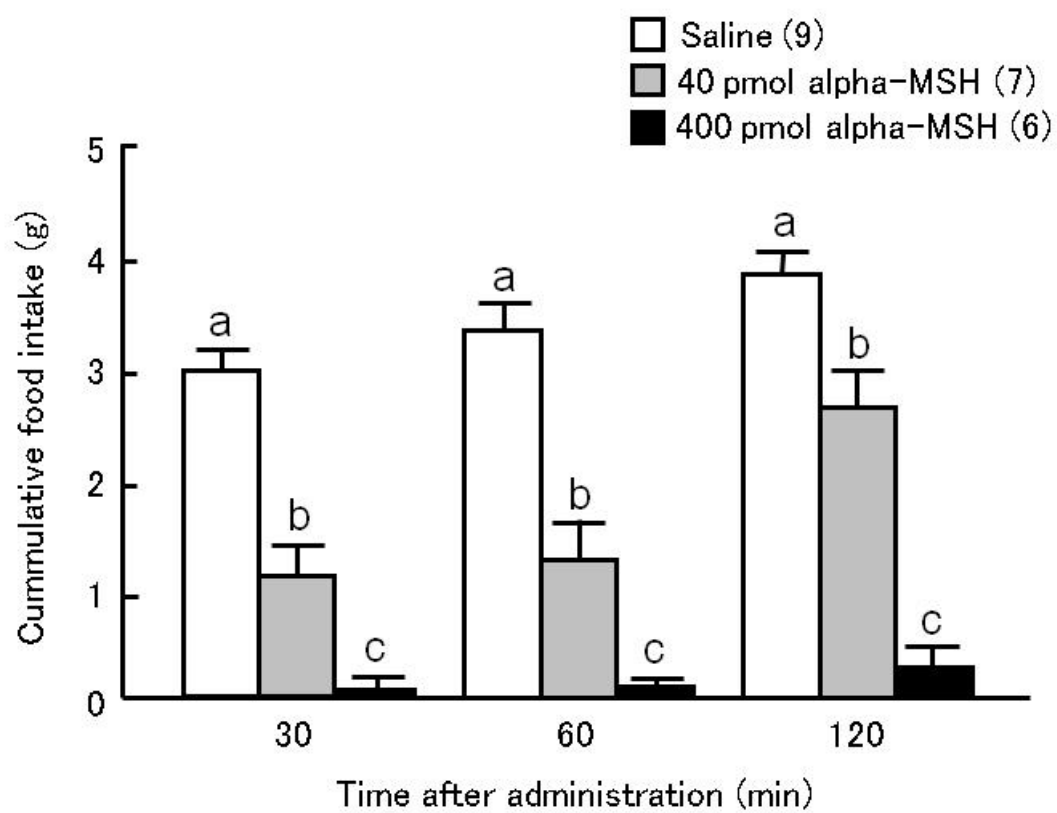


Fig. 6. Effect of central administration of alpha-MSH on food intake in chicks. Values are means $\pm$ S.E.M. The number of chicks used is shown in parentheses. Groups with different letters are significantly different ($p < 0.05$) at each time point.

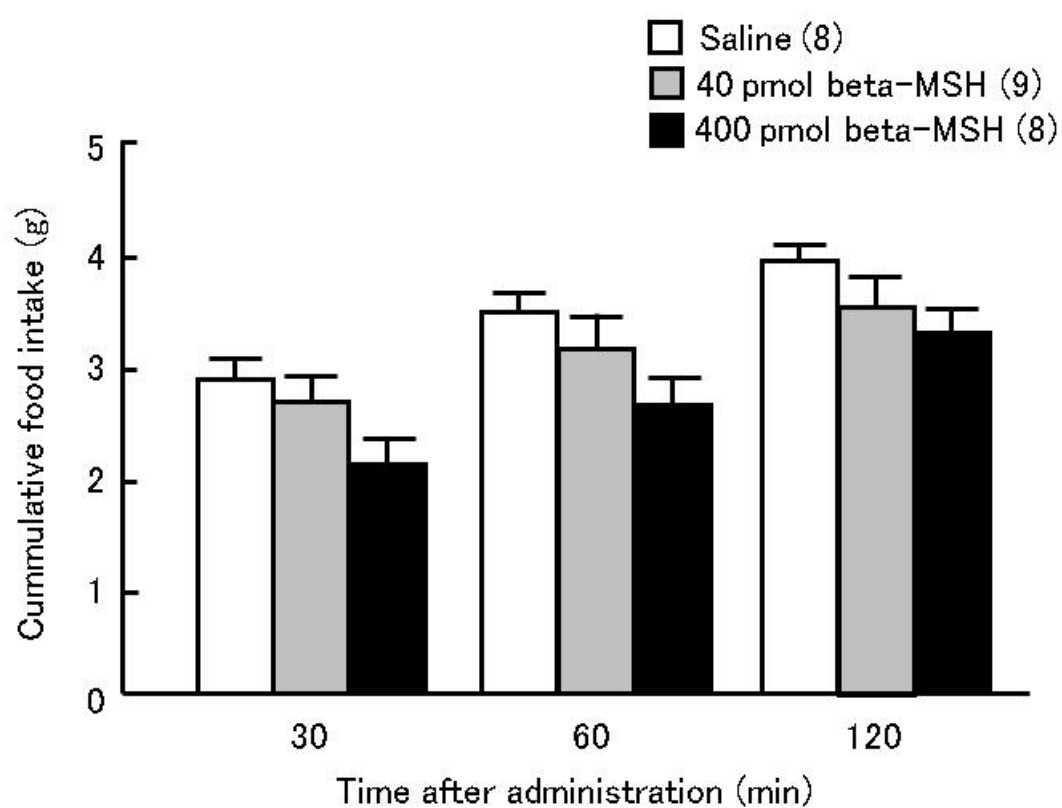


Fig. 7. Effect of central administration of beta-MSH on food intake in chicks. Values are means $\pm$ S.E.M. The number of chicks used is shown in parentheses.

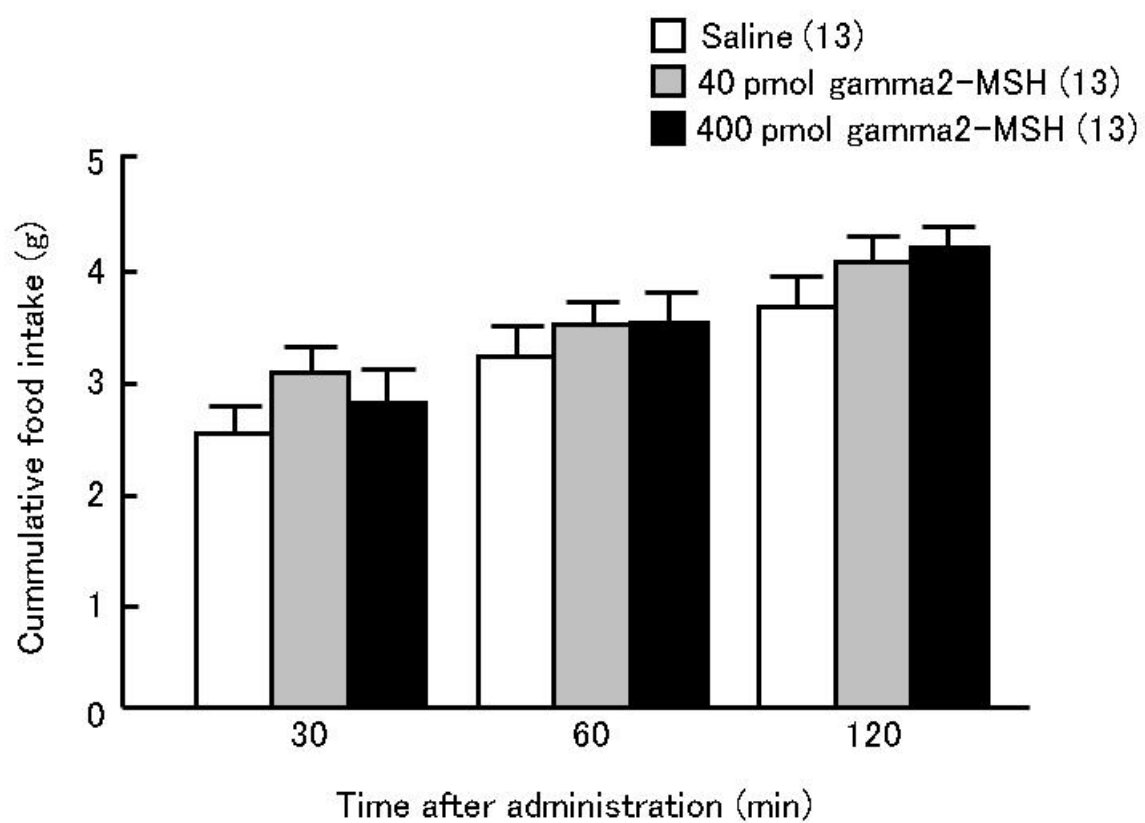


Fig. 8. Effect of central administration of gamma2-MSH on food intake in chicks.

Values are means $\pm$ S.E.M. The number of chicks used is shown in parentheses.

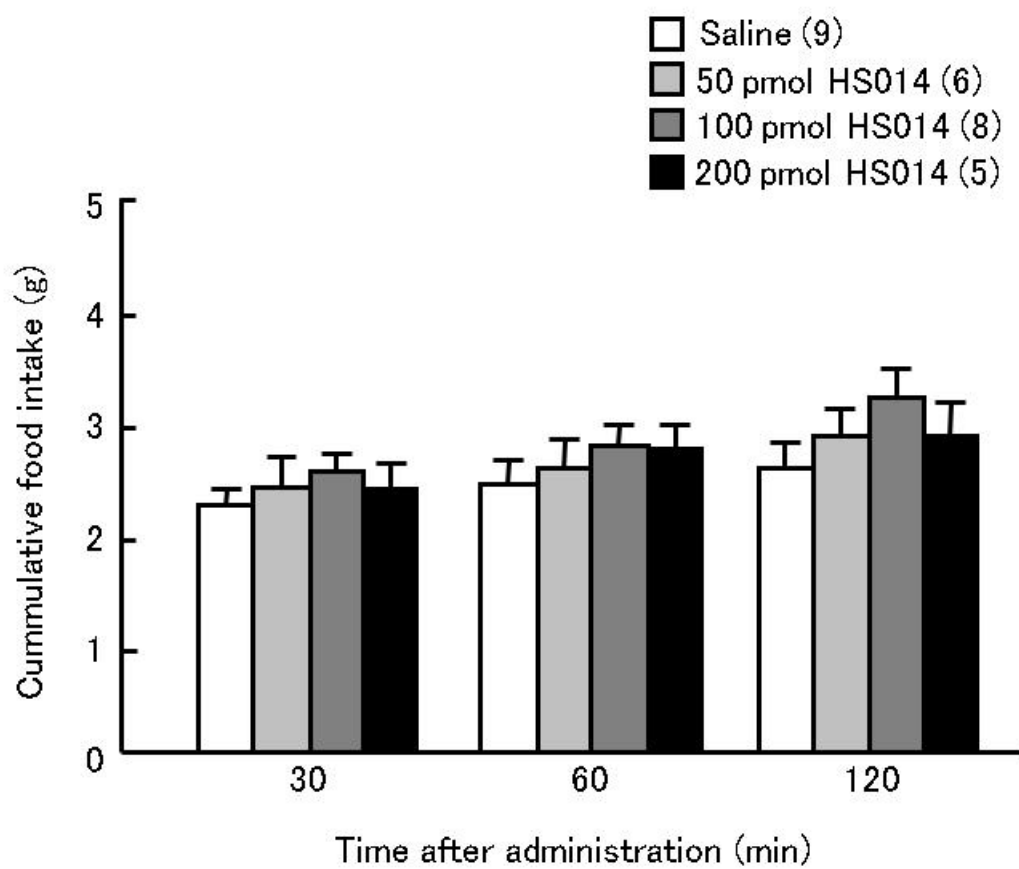


Fig. 9. Effect of central administration of HS014 on food intake in chicks. Values are means $\pm$ S.E.M. The number of chicks used is shown in parentheses.

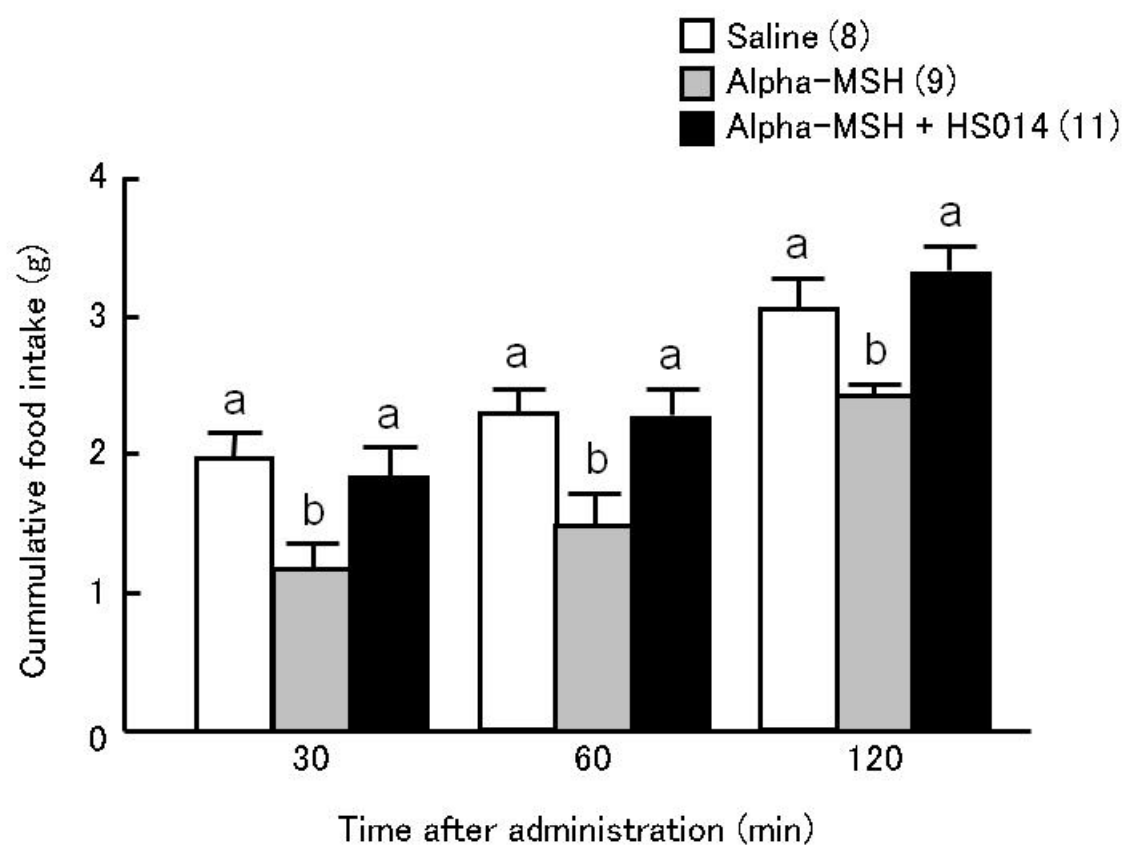


Fig. 10. Effect of central administration of HS014 on the anorexigenic action of alpha-MSH in chicks. Values are means $\pm$ S.E.M. The number of chicks used is shown in parentheses. Groups with different letters are significantly different ($p < 0.05$) at each time point.

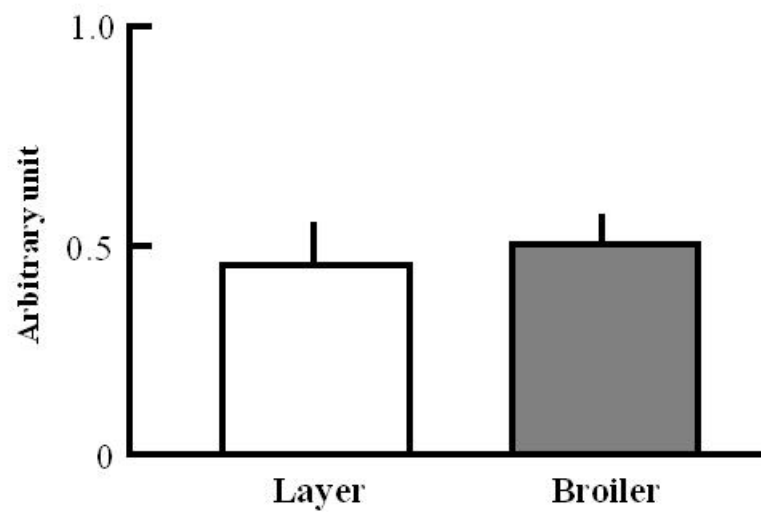


Fig. 11. Hypothalamic POMC mRNA levels in broiler and layer chicks. Values are means $\pm$ S.E.M. for eight chicks in each group.

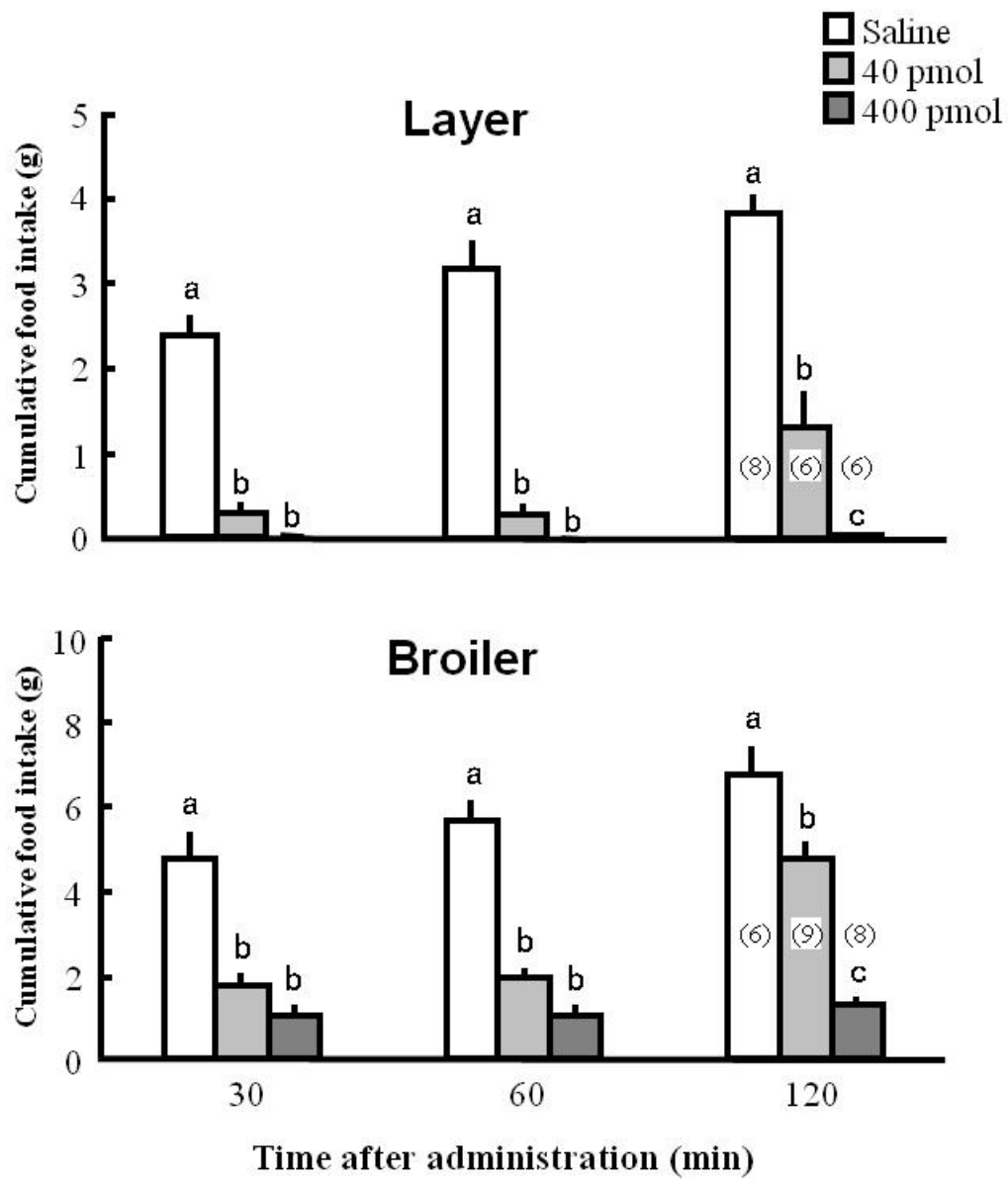


Fig. 12. Effect of central administration of alpha-MSH on food intake in broiler and layer chicks. Values are means $\pm$ S.E.M.. The number of chicks used is shown in parentheses. Groups with different letters are significantly different ($p < 0.05$) at each time point.

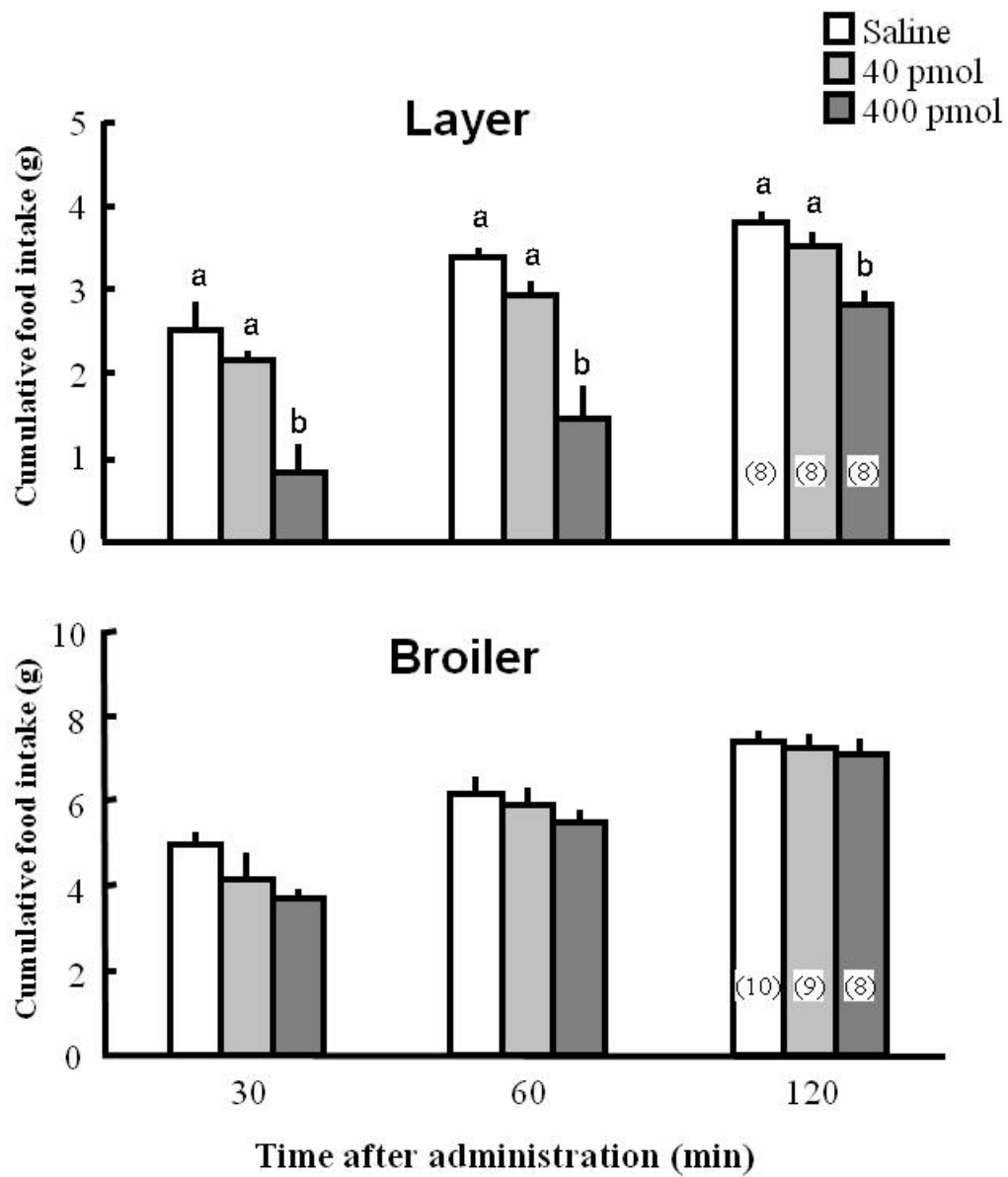


Fig. 13. Effect of central administration of beta-MSH on food intake in broiler and layer chicks. Values are means $\pm$ S.E.M.. The number of chicks used is shown in parentheses. Groups with different letters are significantly different ($p < 0.05$) at each time point.

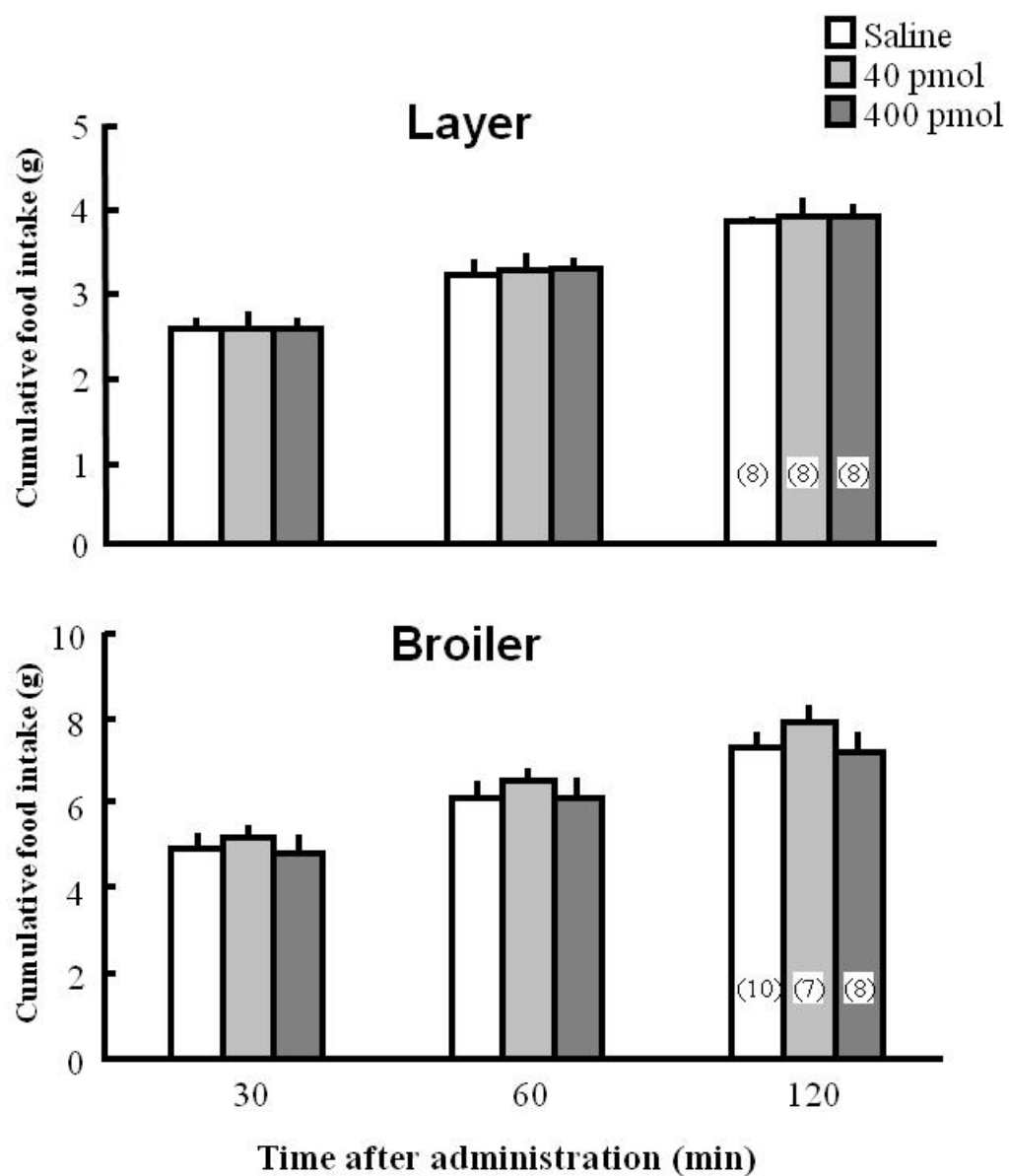


Fig. 14. Effect of central administration of gamma-MSH on food intake in broiler and layer chicks. Values are means $\pm$ S.E.M.. The number of chicks used is shown in parentheses.

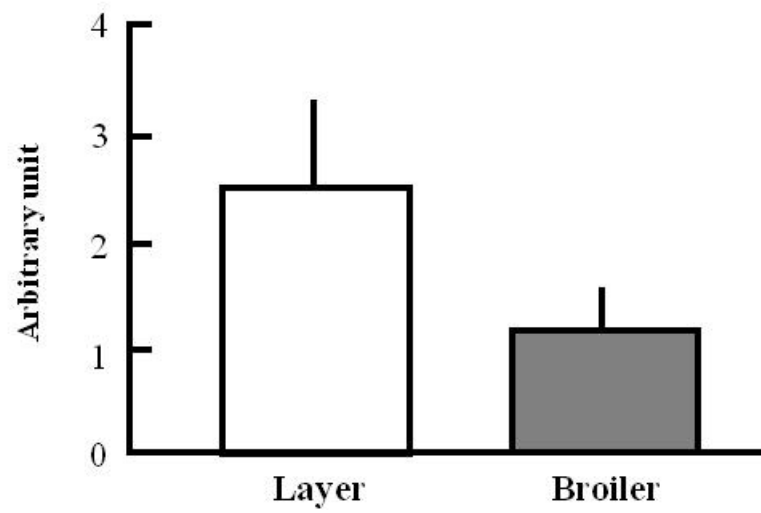


Fig. 15. Hypothalamic MC4R mRNA levels in broiler and layer chicks. Values are means $\pm$ S.E.M. for eight chicks in each group.

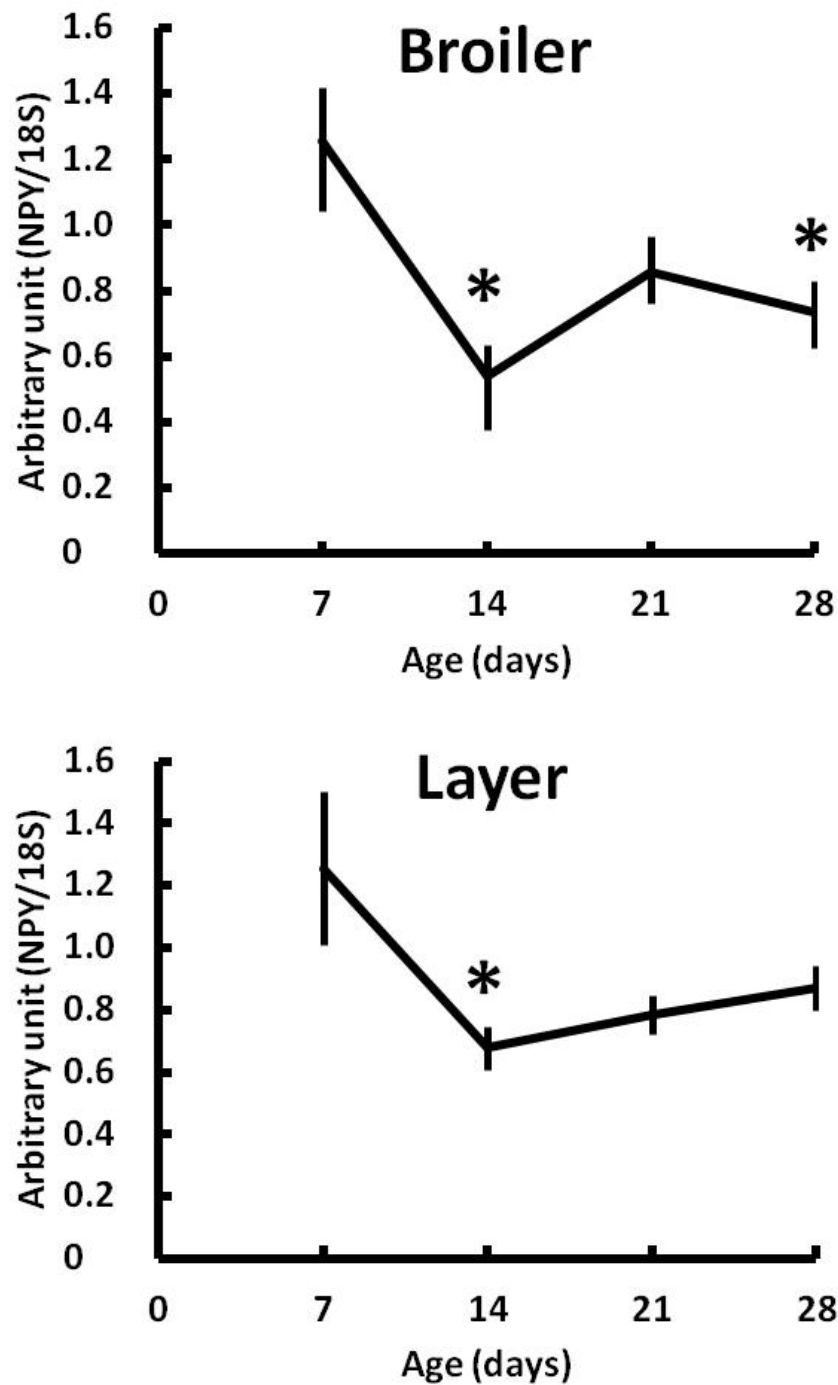


Fig. 16. Changes in the hypothalamic neuropeptide Y (NPY) mRNA level in broiler and layer chicks. Data are means $\pm$ S.E.M. of eight chicks in each group. *, significant with respect to 7 days of age ($p < 0.05$).

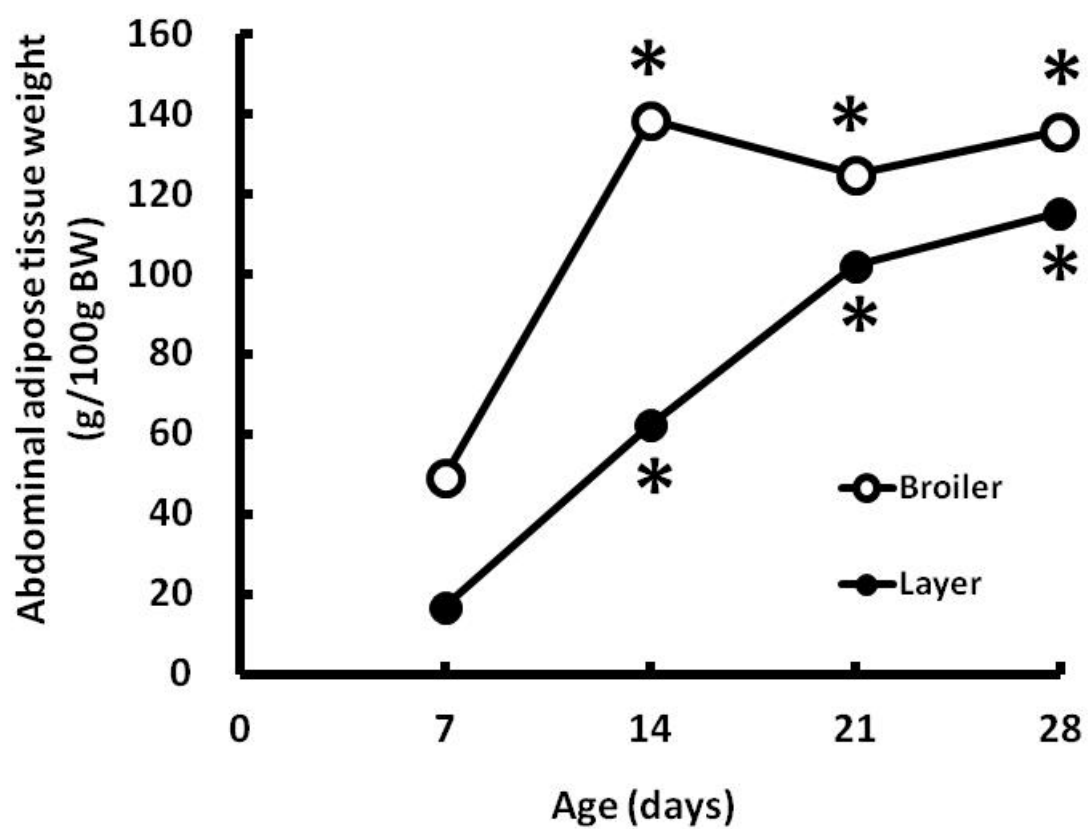


Fig. 17. Changes in the weight of abdominal adipose tissue in broiler and layer chicks. Data are means $\pm$ S.E.M. of eight chicks in each group. *, significant with respect to 7 days of age in each strain ($p < 0.05$).

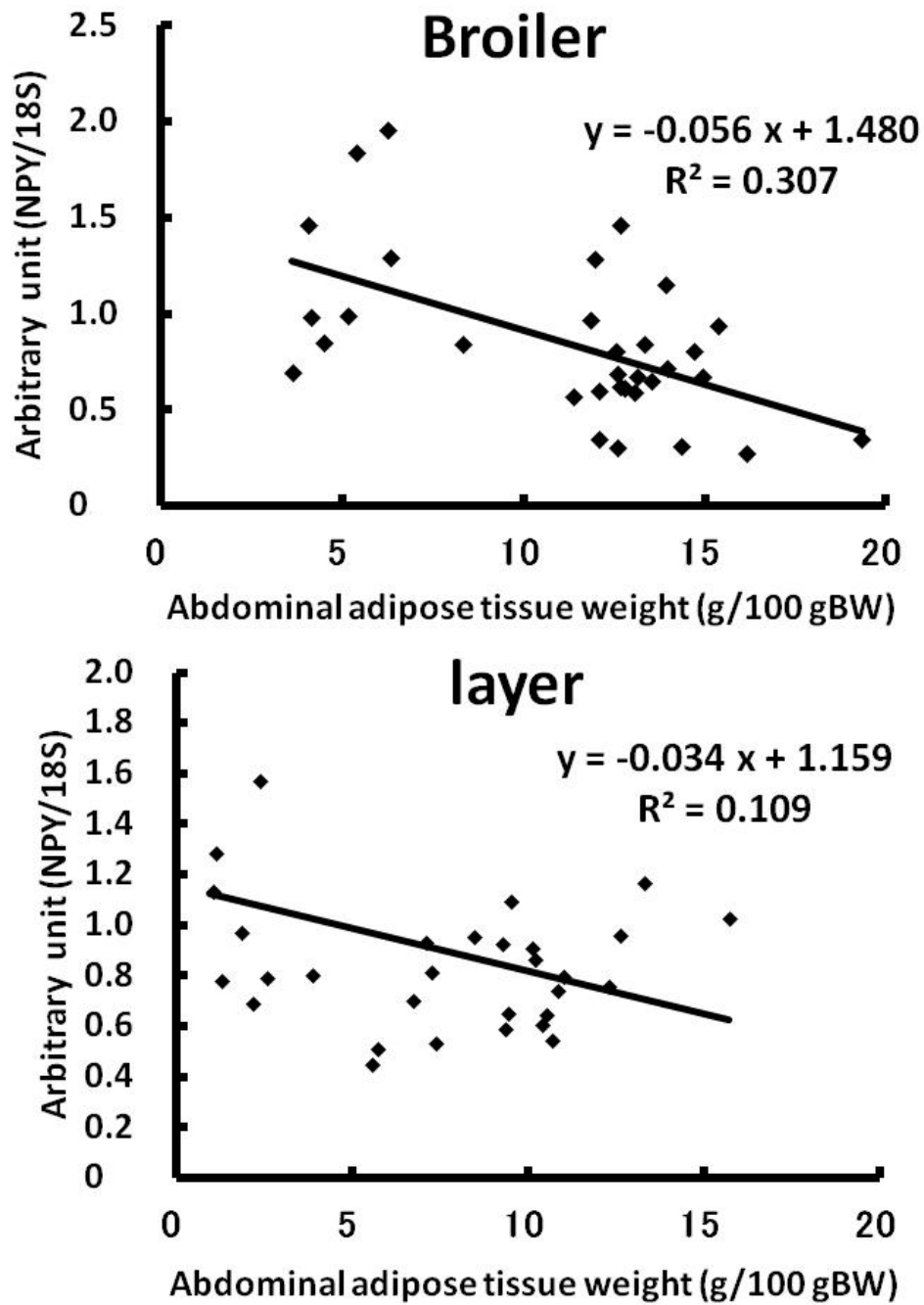


Fig. 18. Correlation between hypothalamic neuropeptide Y (NPY) levels and abdominal adipose tissue weights in broiler and layer chicks. Values include correlation coefficients with R^2 values.

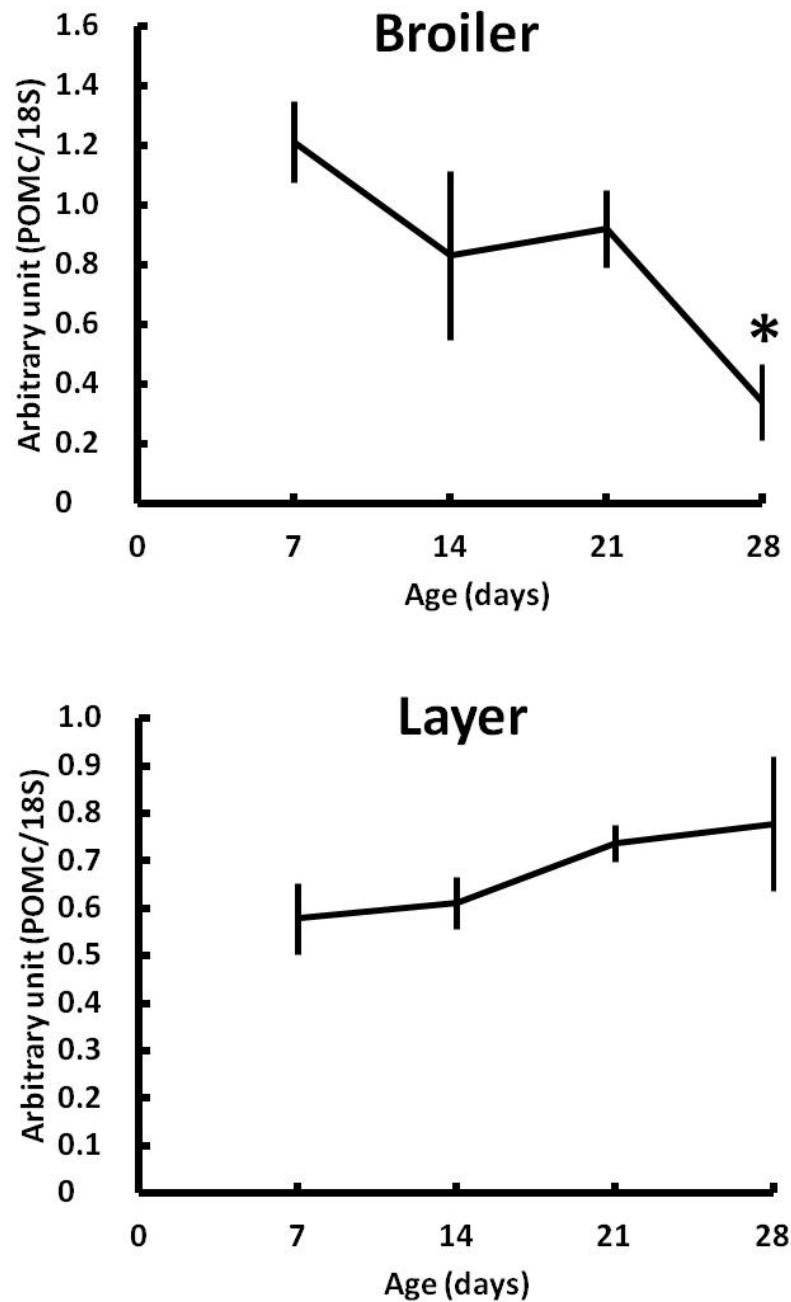


Fig. 19. Changes in the hypothalamic proopiomelanocortin (POMC) mRNA level in broiler and layer chicks. Data are means $\pm$ S.E.M. of eight chicks in each group. *, significant with respect to 7 days of age ($p < 0.05$).

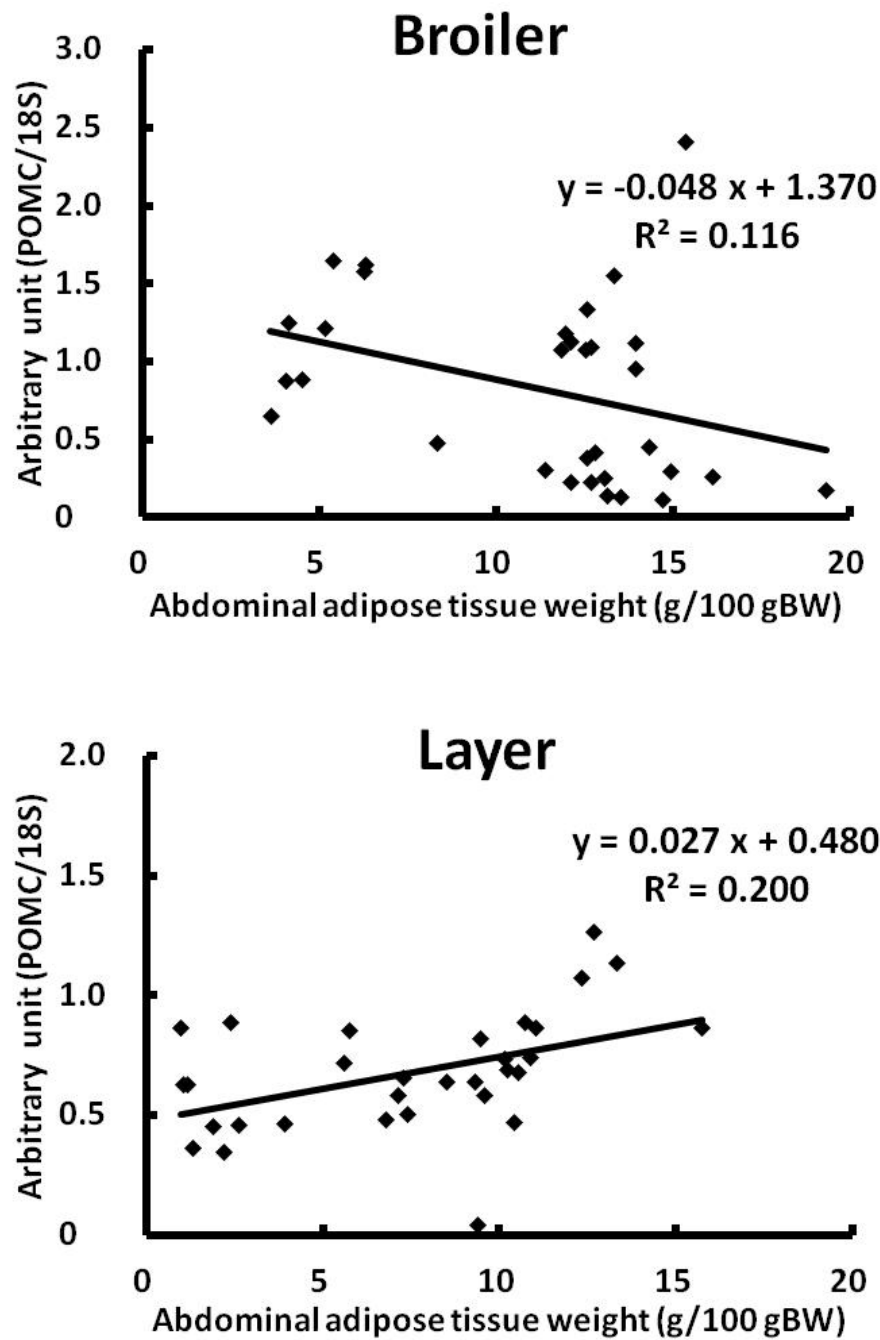


Fig. 20. Correlation between hypothalamic proopiomelanocortin (POMC) levels and abdominal adipose tissue weights in broiler and layer chicks. Values include correlation coefficients with R^2 values.

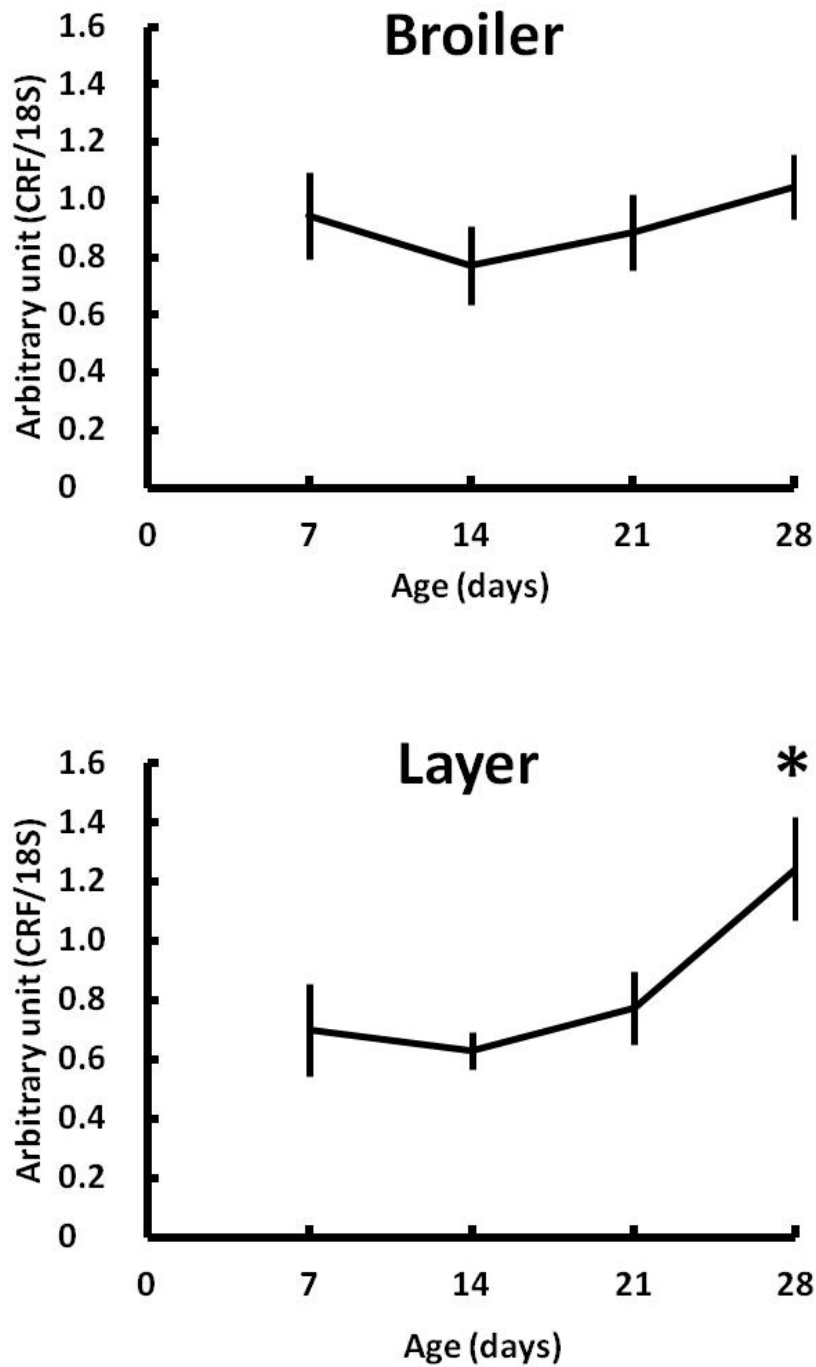


Fig.21. Changes in the hypothalamic corticotropin releasing factor (CRF) mRNA level in broiler and layer chicks. Data are means $\pm$ S.E.M. of eight chicks in each group. *, significant with respect to 7 days of age ($p < 0.05$).

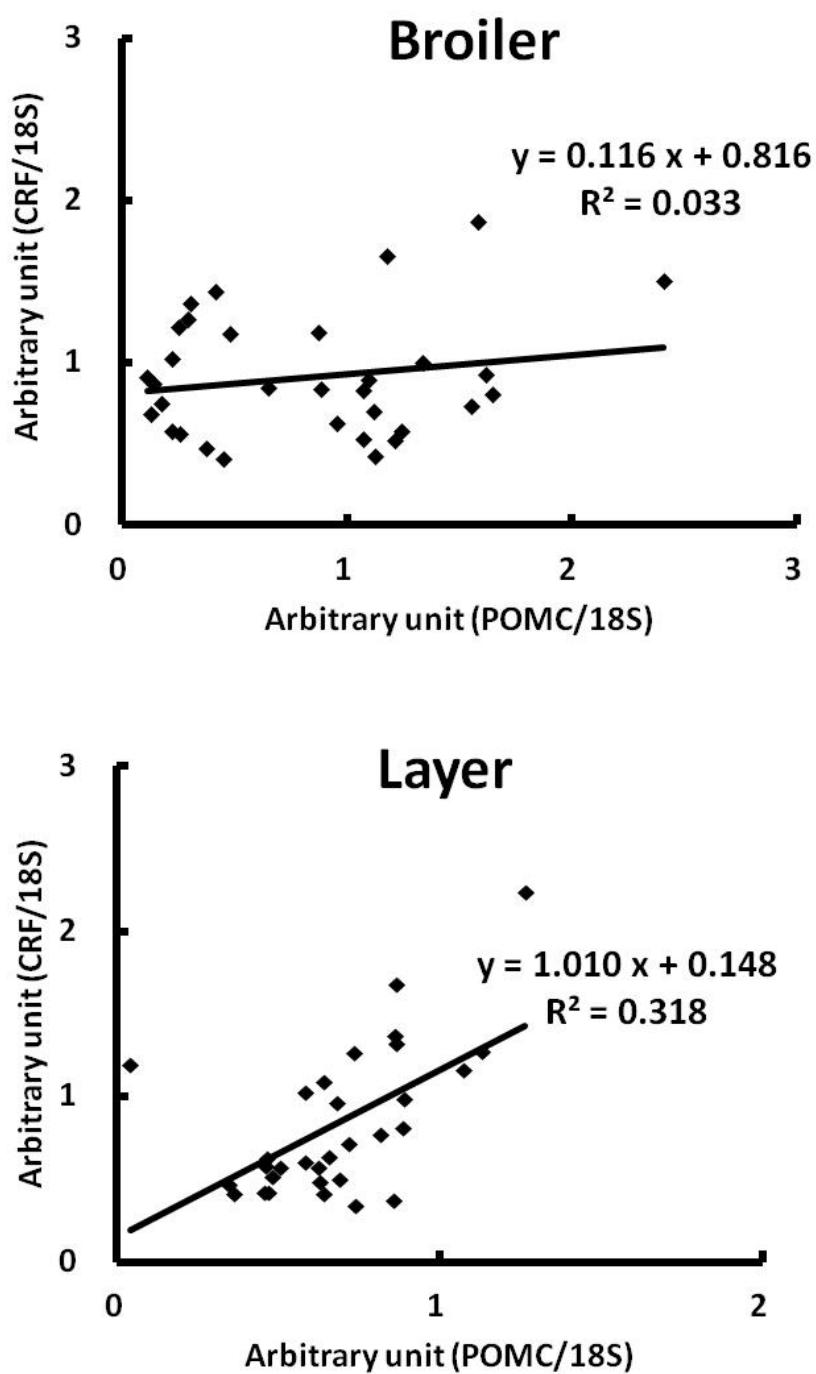


Fig. 22. Correlation between hypothalamic corticotropin releasing factor (CRF) levels and abdominal adipose tissue weights in broiler and layer chicks. Values include correlation coefficients with R^2 values.

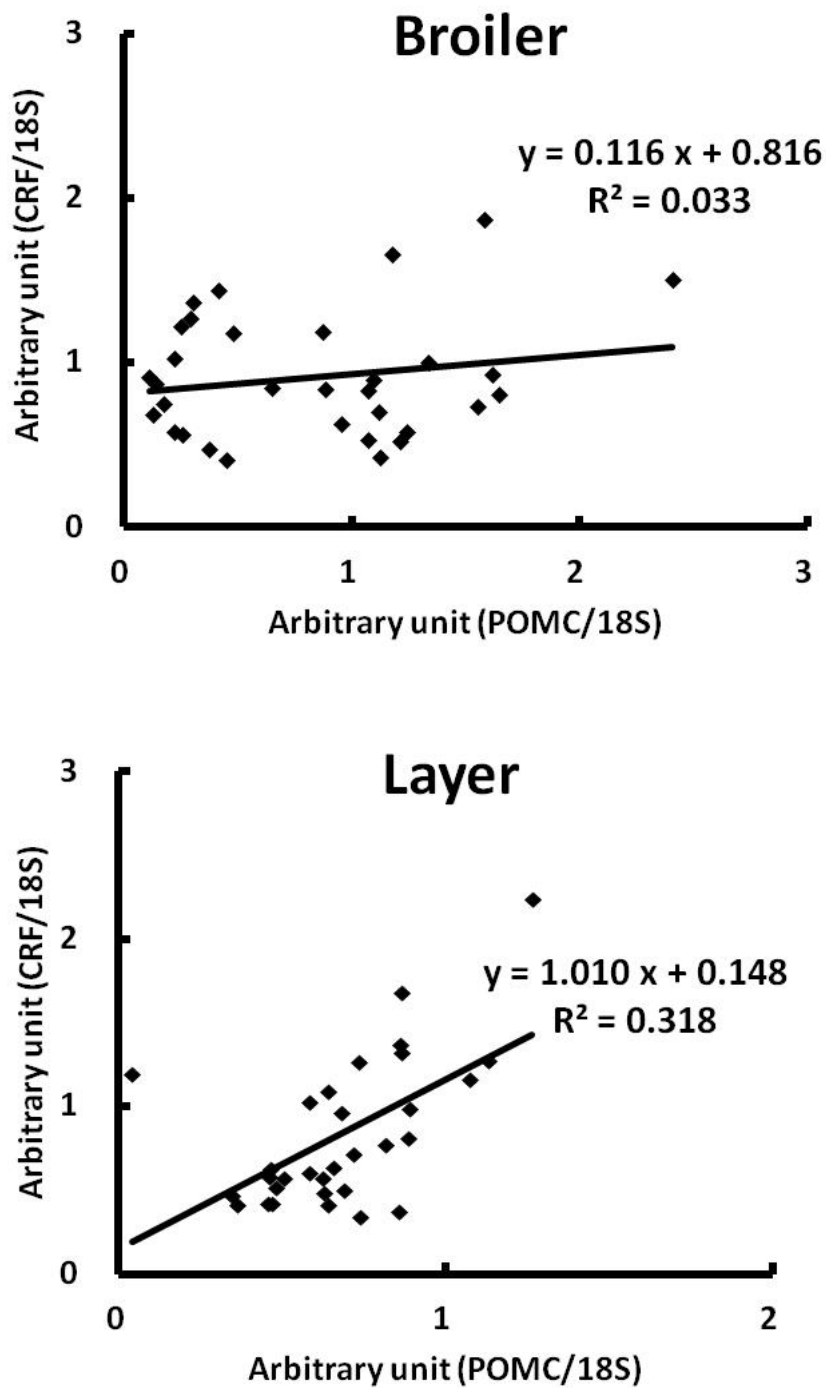


Fig. 23. Correlation between proopiomelanocortin (POMC) mRNA levels and corticotropin releasing factor (CRF) mRNA levels in the hypothalamus in broiler and layer chicks. Values include correlation coefficients with R^2 values.