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Effects of fasting and re-feeding on the expression of CCK, PYY, hypothalamic neuropeptides,
and IGF-related genes in layer and broiler chicks

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Abstract

Cholecystokinin (CCK) and peptide YY (PYY) have been investigated as gut hormones that send satiation signals to the brain in mammals. There is evidence that chicken PYY mRNA expression was the highest in the pancreas compared to other tissues. We recently suggested that insulin-like growth factor (IGF)-1 and its binding proteins (IGFBPs) may be involved in the appetite regulation system in chicks. In the present study, in order to evaluate the possible roles of CCK, PYY, and IGF-related proteins in the appetite regulation system in chicks, we analyzed changes in the mRNA levels of these genes in response to fasting and re-feeding in layer and hyperphagic broiler chicks. In layer chicks, 12 h of fasting reduced the mRNA levels of intestinal CCK, PYY, Y2 receptor, and pancreatic PYY, and these changes were reversed by 12 h of re-feeding. On the other hand, in broiler chicks 12 h of fasting reduced the mRNA levels of intestinal PYY and Y2 receptor, but not intestinal CCK and pancreatic PYY, and these changes were reversed by 12 h of re-feeding. Hypothalamic NPY mRNA significantly increased by 12 h of fasting in both chicks, and these changes were reversed by re-feeding. Also, 12 h of fasting significantly increased the mRNA levels of hypothalamic agouti-related protein and reduced the mRNA levels of hepatic IGF-1 only in broiler chicks, and 12 h of re-feeding did not change these. IGFBP-1 and -2 mRNA levels were markedly increased by 12 h of fasting in both chicks, and these changes were reversed by re-feeding. IGFBP-3 mRNA levels were increased by 12 h of fasting only in layer chicks, while re-feeding reduced the mRNA levels of IGFBP-3 in both types of chicks. These results suggest that several peripheral hormones, such as pancreatic PYY and intestinal CCK, may not play important roles in the regulation of food intake in broiler chicks.

Keywords: Anorexigenic peptides, Avian, Chicks, Feed intake, Gut.

1. Introduction

The appetite regulation system in birds is a sophisticated signal system that involves both central and peripheral regulation (Richards and Proszkowiec-Weglarz, 2007; Boswell and Dunn, 2017) as in mammals (Woods, 2009; Williams and Elmquist, 2012). Cholecystokinin (CCK) and peptide YY (PYY) have been investigated as gut hormones that send satiation signals to the brain in mammals (Woods, 2009) and possibly in chickens. For example, peripheral administration of CCK and PYY reduced food intake in mammals (Scott et al., 2005a; Sayegh et al., 2014) and chicks (Tachibana et al., 2012; Aoki et al., 2017). Plasma concentrations of CCK and PYY were elevated postprandially in mammals (Woods, 2009). There is evidence that the serum CCK concentration was elevated by food intake in chicks (Melo-Duran et al., 2019). PYY expression in the jejunum of chicks was down-regulated under fasting conditions (Aoki et al., 2017). The production areas of CCK and PYY are different between species. CCK is secreted from the duodenum, the upper part of the small intestine, in mammals (Côté et al., 2014), whereas CCK is primarily expressed in the ileum, the lower part of the small intestine in chicks (Honda et al., 2017; Reid and Dunn, 2018). Intestinal PYY is secreted by L cells in the intestinal mucosa and mainly expressed in the colon and cecum of mammals (Zhou et al., 2006), whereas in chicks, the major gastrointestinal site for expression of PYY is the jejunum (Aoki et al., 2017; Reid et al., 2017). Recent findings clearly demonstrated that chicken PYY mRNA expression was the highest in the pancreas compared to other tissues (Gao et al., 2017; Reid et al., 2017). These facts suggest that CCK and PYY act as anorexigenic hormones in both mammals and chicks and raise the hypothesis that different production areas may be involved in the species-specific mechanism of appetite regulation.

In mammals, the anorexigenic effects of CCK and PYY are mediated by the CCK-A receptor (CCKAR) (Beglinger et al., 2001; Dockray, 2012) and neuropeptide Y2 receptor

(Y2R), respectively, (Abbott et al., 2005; Scott et al., 2005b; Reidelberger et al., 2013). In chickens, high growth haplotype chickens showed decreased expression of CCKAR and resistance to the anorectic effect of exogenously administered CCK when compared to low growth haplotype chickens (Dunn et al., 2013). Chicken PYY showed high affinity binding to Y2R *in vitro* (Salaneck et al., 2000). However, the physiological changes in intestinal Y2R and CCKAR in response to food intake have not yet been examined in chickens.

Recently, we reported that intracerebroventricular administration of insulin-like growth factor (IGF)-1 significantly suppressed food intake in broiler and layer chicks (Fujita et al., 2017; 2019). Six hours of fasting significantly reduced the mRNA levels of IGF-1 in the liver, and this change was reversed by 6 h of re-feeding in broiler chicks (Fujita et al., 2017). In contrast, 6 h of fasting significantly increased the mRNA levels of Insulin-like growth factor-binding protein (IGFBP)-1 and -2 in the liver, and these changes were reversed by 6 h of re-feeding in broiler chicks (Fujita et al., 2018). IGFBPs were thought to be carrier proteins in the bloodstream, but the availability of blood IGF-1 to the receptor of target cells is limited by binding to IGFBPs (Allard and Duan, 2018). It is therefore likely that IGF-1, IGFBP-1, and -2 are involved in the regulation of food intake in chicks.

The hypothalamus is known as the central site for integrating satiety signals in mammals (Hussain and Bloom, 2013). Both the mammalian and avian hypothalamic arcuate nuclei contain anorexigenic pro-opiomelanocortin (POMC) and orexigenic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons that play important roles in the central regulation of food intake (Morton et al., 2006; Boswell and Dunn, 2017). Central administration of NPY and AgRP stimulates food intake, whereas central administration of α -melanocyte-stimulating hormone (α -MSH, a neuropeptide derived from POMC) suppresses it in mammals (Rossi et al., 1998; Edwards et al., 1999; Tung et al., 2006) and chicks (Tachibana et al., 2001; Saneyasu et al., 2011; Honda et al., 2012). Food deprivation induces NPY and

AgRP expression and suppresses POMC expression in the hypothalami of mammals (Bertile et al., 2003) and chicks (Fang et al., 2014). Therefore, the mRNA levels of hypothalamic NPY, AgRP, and POMC can be used as indicators for appetite.

In the present study, to clarify the physiological importance of CCK, PYY, and IGF-related proteins in the appetite regulatory system of avian species, we focused on the mRNA levels of intestinal CCK, CCKAR, PYY, and Y2R, pancreatic PYY, hepatic IGF-related proteins, and hypothalamic NPY, AgRP, and POMC in response to fasting and re-feeding in chicks. We also used two different types of chicks, layer and broiler chicks, because broiler chicks show hyperphagia that may be caused by weakened satiation signals.

2. Materials and Methods

2.1. Animals and diet

This study was approved by the Institutional Animal Care and Use Committee and was performed according to the Kobe University Animal Experimentation Regulations (25-08-01). One day old male layer (White leghorn) and broiler (Ross 308) chicks were purchased from local hatcheries (Japan Layer K. K., Gifu, Japan and Yamamoto Co., Ltd., Kyoto, Japan, respectively). The commercial chick starter diet met the nutritional requirements for both broiler and layer chicks. They were given free access to water and a commercial chick starter diet (NICHIIWA SANGYO Co., Ltd., Kobe, Japan) under a 23-h/1-h light - dark cycle, a light schedule commonly used in the poultry industry.

We previously showed that 12 h of fasting significantly decreased PYY mRNA levels in the ileum in chicks (Aoki et al., 2017). Reid et al. (2017) reported that 11 h of fasting significantly decreased PYY mRNA levels in the pancreas in broiler-layer hybrid chicks. However, 7.5 h of fasting did not influence CCK mRNA levels in the ileum in NOVOgen brown

birds (Reid et al., 2018). Thus, In order to compare the physiological importance of CCK and PYY, we selected 12 h of fasting, which downregulates PYY expression in both ileum and pancreas. A total of 24 twenty-one-day-old male layer and broiler chicks were weighed and allocated to three cages based on body weight (eight birds in each group). Chicks were reared in electrically heated battery cages. The temperature was kept at $31 \pm 2^{\circ}\text{C}$ during the first 7 days, and then reduced gradually according to age until reaching $25 \pm 2^{\circ}\text{C}$ at 21 d. Chicks in the feeding group were euthanized by skilled person by decapitation after 0 h of fasting. Chicks in the fasting group were euthanized by decapitation after 12 h of fasting. After 12 h of fasting, chicks in the re-feeding group were refed for 12 h and euthanized by decapitation. Blood was collected from the carotid artery. Plasma was separated immediately by centrifugation at 1,910 g for 10 min at 4°C , and the plasma concentrations of glucose and triglycerides (TG) were measured using commercial kits (Lab Assay™ Glucose and Lab Assay™ Triglyceride, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, respectively). The liver, pancreas, and a central section of the jejunum and ileum were accurately excised, washed with saline, and frozen immediately in liquid nitrogen for real-time PCR analysis. The diencephalon was collected and preserved in RNAlater® tissue storage reagent (Sigma-Aldrich CO., St. Louis, Mo, USA), and the hypothalami were excised with reference to the stereotaxic atlas drawn by Kuenzel and Masson (1988). The septopallio-mesencephalic tract, third cranial nerves, 1.5 mm from the midline, and the dorsal section from the anterior commissure to 1.0 mm ventral to the posterior commissure were used as landmarks.

2.2. Real-time PCR

Total RNA was extracted from the tissues using Sepazol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from total RNA using Rever Tra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co.Ltd, Osaka, Japan). The

levels of mRNA were quantified for each primer using TB Green Premix Ex Taq II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan) according to the supplier's recommendations, in an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Complementary DNA of CCK (GenBank accession no. NM_001001741) and CCKAR (GenBank accession no. NM_001081501) was amplified using the following primers: CCK sense, 5' -GCG CTG CTG GCO AAG TA-3'; CCK antisense, 5' -GAC AGA GAA CCT CCC AGT GGA A-3'; CCKAR sense, 5' -TGG TTG CGT ATG GCC TCA TT-3'; CCKAR antisense, 5' -GGC GAT GCT GGT ACT TCC TT-3'. Complementary DNA of ribosomal protein S17 (internal standard) (Saneyasu et al., 2019), NPY, POMC and AgRP (Fujita et al., 2019), PYY, and Y2R (Aoki et al., 2017), and IGF-related proteins (Fujita et al., 2017; 2018) were amplified using primers as described previously.

2.3. Data analysis

Data were analyzed by one-way ANOVA. If a significant difference ($P < 0.05$) was detected, Fisher's PLSD test was performed for multiple comparisons. All statistical analyses were performed using a commercial software package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

3. Results

In layer chicks, the mRNA levels of intestinal CCK, PYY, Y2R, and pancreatic PYY were significantly reduced after 12 h of fasting, and these changes were significantly reversed by 12 h of re-feeding (Fig. 1A). Hypothalamic NPY mRNA levels were significantly increased after 12 h of fasting, and this change was reversed by 12 h of re-feeding (Fig. 1B). Plasma glucose and TG were significantly reduced after 12 h hours of fasting, and these changes were reversed by 12h of re-feeding (Fig. 1C).

In broiler chicks, the mRNA levels of intestinal PYY and Y2R were reduced after 12 h of fasting, and these changes were significantly reversed by 12 h of re-feeding (Fig. 2A). Hypothalamic NPY and AgRP mRNA levels increased after 12h of fasting, whereas 12 h of re-feeding significantly reversed the mRNA levels of hypothalamic NPY, but not AgRP (Fig. 2B). Plasma glucose and TG were reduced significantly after 12 h of fasting and these changes were reversed by 12 h of re-feeding (Fig. 2C).

Effects of fasting and re-feeding on the mRNA levels of IGF-related proteins in the liver are shown in Fig. 3. Fasting and re-feeding did not influence hepatic IGF-1 mRNA levels in layer chicks (Fig. 3A). On the other hand, hepatic IGF-1 mRNA levels were significantly reduced after 12 h of fasting, and this change was not reversed by 12 h of re-feeding in broiler chicks (Fig. 3B). The mRNA levels of IGFBP-1 and -2 were markedly increased by fasting and reduced by re-feeding in both types of chicks (Fig. 3A and B). Hepatic IGFBP-3 mRNA levels were significantly increased by fasting only in layer chicks, and this change was reversed by re-feeding (Fig. 3A). In broiler chicks, re-feeding significantly reduced hepatic IGFBP-3 mRNA levels, although fasting did not influence them (Fig. 3B). Re-feeding significantly reduced hepatic IGFBP-4 mRNA levels only in layer chicks, although fasting did not influence them (Fig. 3A).

Discussion

Broiler chicks have undergone intensive selection for growth for more than 50 years. As a result, they do not adequately regulate voluntary food intake commensurate with their energy needs (Richards, 2003; Bornelöv et al., 2018). In the present study, we proposed that the gut hormones CCK and PYY, intestinal Y2R, and pancreatic PYY are involved in the post prandial changes in appetite in layer chicks. However, several changes were not observed in broiler chicks. All these findings suggest that the physiological roles of appetite regulation-

related genes may be different between broiler and layer chicks.

In the present study, we found that pancreatic PYY mRNA levels were significantly changed in response to feeding only in layer chicks. Gao et al. (2017) and Reid et al. (2017) clearly demonstrated that chicken PYY is highly expressed in the pancreas when compared to other tissues, including the intestines. Reid et al. (2017) also demonstrated that the mRNA levels of chicken PYY were significantly reduced by 11 h of fasting in the pancreas in two-week-old broiler-layer hybrid chicks. We previously reported that intravascular administration of chicken PYY significantly suppressed food intake in broiler chicks (Aoki et al., 2017). However, pancreatic PYY mRNA levels were not changed by fasting and re-feeding in broiler chicks in the present experimental condition. It is therefore possible that only jejunal PYY functions as a satiety signal in broiler chicks.

The mRNA levels of pancreatic PYY were reported in the frogs (Sundström et al., 2012) and fish (Cheung et al., 1991; Al-Mahrouki and Youson 1998; Chen et al., 2015), but the levels were not higher than intestinal PYY. In mammals, PYY functions as a neuropeptide in the central nervous system and gut hormone in the intestines (Ueno et al., 2008). However, pancreatic PYY may exhibit species-specific roles in chickens. Ding et al. (1997) reported that PYY-positive endocrine cells were scattered in the exocrine region in chickens and turtles when compared to eels, bullfrogs, mice, rats, guinea pigs, dogs, and humans. Tatemoto et al. (1992) reported that PYY suppresses pancreatic exocrine excretion in cats. In the present study, re-feeding significantly increased pancreatic PYY expression in layer chicks. It is therefore possible that pancreatic PYY suppresses the excretion of pancreatic juice in layer chicks in response to food intake.

The effects of gut hormones on food intake in chickens have been investigated in recent decades (Denbow, 1994; Honda et al., 2017). However, the interactions of endogenous gut hormones and their receptors in the appetite regulation of chickens have not been fully elucidated. In the present study, our findings clearly demonstrated that both fasting and re-

feeding affect the mRNA levels of not only PYY but also Y2R in the jejunum of layer and broiler chicks. This is the first report suggesting that expression of intestinal Y2R may be involved in the peripheral regulation of food intake in chicks. There is evidence that Y2R is expressed in the myenteric neurons of the intestines in mammals (Wang et al., 2010). The enteric nervous system is involved in the brain-gut axis in mammals (Bauer et al., 2016; Bliss and Whiteside, 2018). It is therefore possible that intestinal PYY functions as a satiety signal via the enteric nervous system in chicks. However, distribution of Y2R in the intestines in chicks has not been investigated. Further study is needed to identify the Y2R-expressing cells in chicken intestines.

In the present study, intestinal CCK mRNA levels were significantly reduced by 12 h of fasting, and restored after 12 h of re-feeding in layer chicks. However, in broiler chicks, no significant change was observed in the CCK mRNA levels. Reid and Dunn (2018) showed that neither short-term (6 h) fasting nor re-feeding influenced the mRNA levels of CCK in the ileum of 17-day-old brown chicks. Therefore, it is likely that CCK expression in the ileum is more responsive to long-term fasting or the re-feeding state or shows different responses in different chicken strains.

In the present study, hepatic IGFBP-3 mRNA levels were significantly increased by fasting only in layer chicks. IGFBPs binds IGF-1 in plasma and blocks the binding to the receptor (Allard and Duan, 2018). In chickens, only 6% of serum IGF-1 exists in free form, suggesting that the functions of plasma IGF-1 are significantly influenced by IGFBPs (McMurtry et al., 1997). It is therefore possible that the plasma concentration of free form IGF-1 is decreased by increasing IGFBP-3 under the fasting condition in layer chicks. Interestingly, a reduction in free IGF-1 after fasting has been reported in humans (Frystyk et al., 2002) and rats (Frystyk et al., 1999), but serum IGFBP-3 was reduced by fasting in rats (Frystyk et al., 1999). It seems likely that the physiological roles of IGFBP-3 are completely different between rats and layer chicks. Further studies will provide insights into the different roles of IGFBP-3

not only between in layer and broiler chicks, but also in mammals and birds.

In mammals, the hypothalamus plays an important role in directly sensing nutrients and hormones such as glucose, insulin, and leptin, and the area postrema in the caudal brainstem receives circulating metabolic signals including gut hormones (Clemmensen et al., 2017). Thus, the regulation of food intake is coordinated by a complex neurocircuitry involving multiple brain regions. Although, the role of CCK and PYY in the appetite regulatory pathway in chick brains has not yet been identified, we have reported that the hypothalamus may be involved in the IGF-1-induced anorexigenic pathway in chicks (Fujita et al., 2019). It is therefore possible that CCK, PYY, and IGF-1 affect the expression of hypothalamic neuropeptides via different pathways. Further studies are needed to clarify the target region of CCK and PYY in the brain and the interaction of the effects of gut hormones and IGF-1 in chicks.

Shurlock and Forbes (1981) reported that infusion of glucose into the hepatic portal vein depressed food intake in 28-week-old layer chickens. Lacy et al. (1986) reported that lipid infusion intrahepatically depressed food intake in 11-week-old layer chickens. In the present study, re-feeding significantly increased not only the mRNA levels of PYY but also plasma glucose and TG levels in layer and broiler chicks. These findings suggest that PYY and plasma metabolites may coordinately induce satiety in both types of chickens.

Hypothalamic NPY expression was upregulated by 12 h of fasting and these changes were reversed by 12 h of re-feeding in layer and broiler chicks, suggesting that changes of feeding conditions influence appetite in both types of chicks. On the other hand, significant upregulation of hypothalamic AgRP was found only in broiler chicks, and this change was not reversed by 12 h of re-feeding. It is therefore likely that 12 h of re-feeding is not enough to suppress appetite in broiler chicks. However, Tachibana et al. (2001) reported that central administration of AgRP significantly suppressed food intake in layer chicks but not in broiler chicks. Therefore, further study is needed to evaluate the physiological importance of AgRP in the central regulation of food intake in broiler chicks.

The effects of fasting on the hypothalamic POMC expression in chicks were different in previous reports. For example, 24 h of fasting in 14-day-old yellow-feathered broiler chicks (Fang et al., 2014) and 3 h of fasting in 5-day-old low or high body weight line chicks (Yi et al., 2015) significantly reduced hypothalamic POMC mRNA levels. On the other hand, Song et al. (2012) reported that 48 h of fasting did not influence the mRNA levels of hypothalamic POMC in 7-day-old broiler chicks. In the present study, 12 h of fasting did not influence the mRNA levels of hypothalamic POMC in either layer or broiler chicks. It is therefore likely that the regulatory mechanism underlying fasting-induced hypothalamic POMC expression is different depending on age, strain, or experimental conditions.

In the present study, plasma TG levels were significantly higher under the re-feeding condition in both layer and broiler chicks. The reason for significant increases in plasma TG is not clear. However, Leveille (1969) reported that refeeding for 3 days following a 3-day fast significantly increased liver fat upon refeeding chicks when compared to control chicks. Leveille et al. (1975) also demonstrated that hepatic synthesis of fatty acids was markedly depressed by fasting, but increased rapidly upon refeeding, “overshooting” the control rate of fatty acid synthesis in chicks. Thus, one possible explanation is that 12 h of re-feeding following 12 h of fasting produced an “overshoot” in lipogenesis to levels surpassing that of the *ad libitum* feeding group.

3. Conclusion

We examined the effects of fasting and re-feeding on the appetite regulation-related genes in broiler and layer chicks and found that several genes showed different responses in the two types of chicks. Our findings suggest that pancreatic PYY and ileum CCK may not play important roles in the regulation of food intake in broiler chicks.

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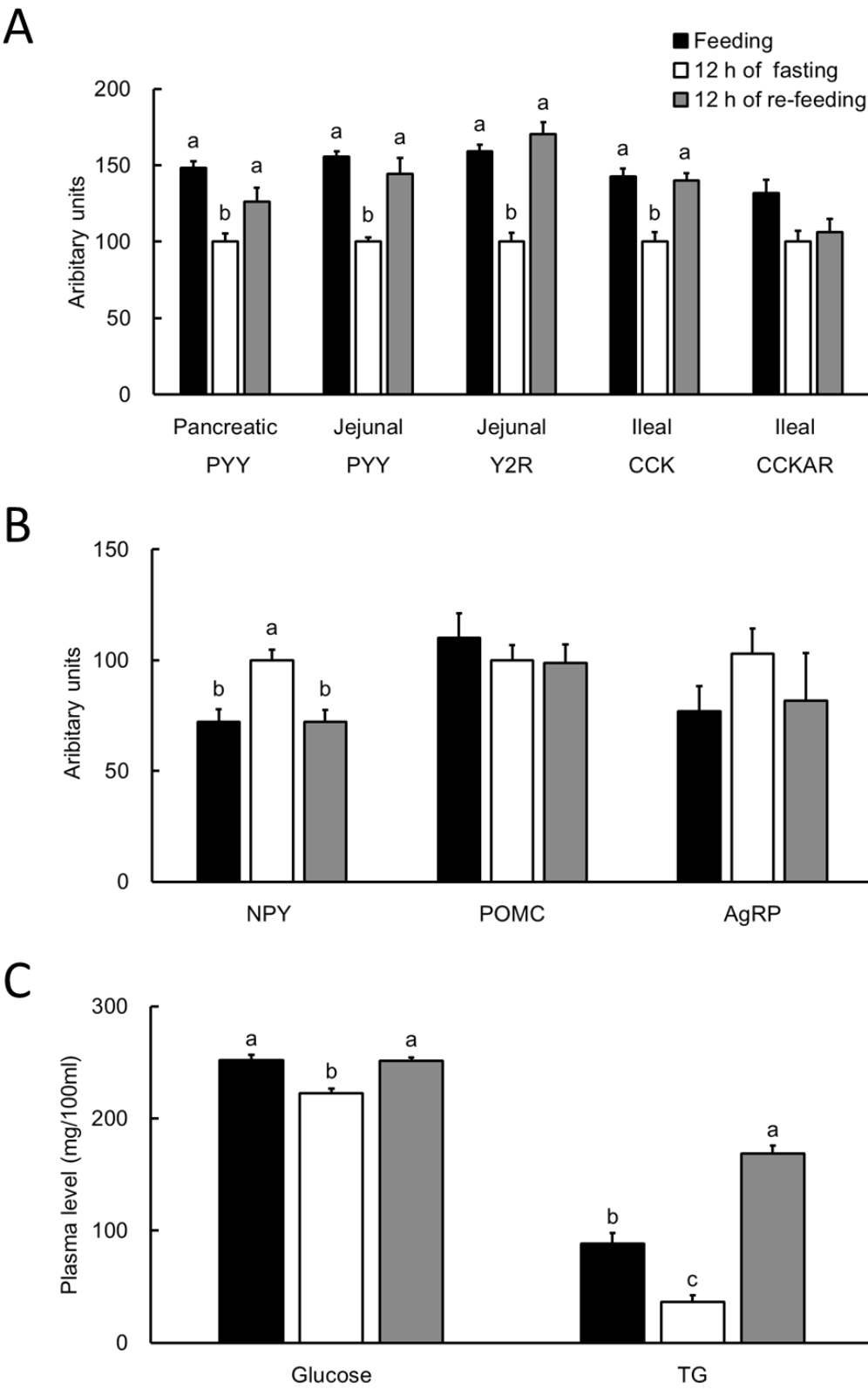
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Figure Captions

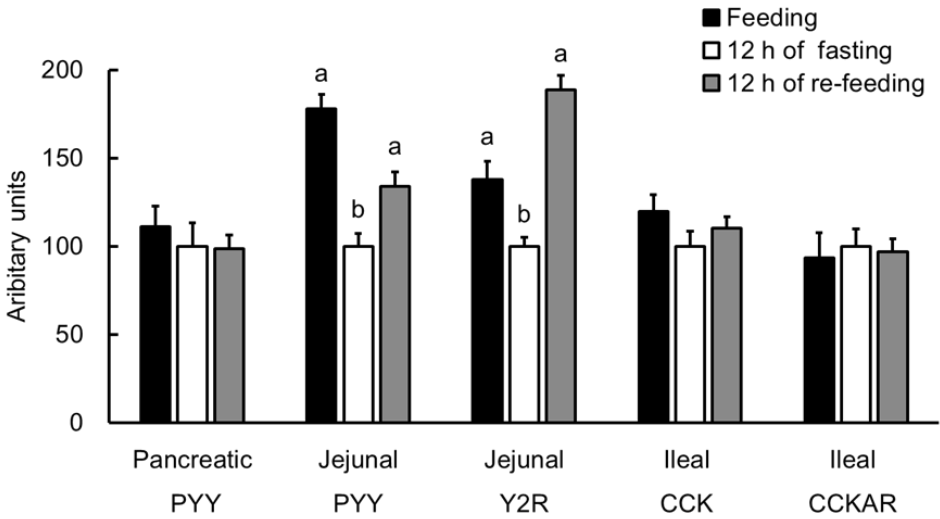
Fig. 1. Effects of fasting and re-feeding on the mRNA levels of (A) intestinal CCK, Jejunal PYY, and their receptors, and pancreatic PYY, (B) hypothalamic NPY, POMC, and AgRP, and (C) plasma metabolites in layer chicks. Data are the means \pm S.E.M. of eight chicks in each group and are expressed as a percentage of the mean in the 12 h fasting group. Groups with different letters for each gene are significantly different ($P < 0.05$).

Fig. 2. Effects of fasting and re-feeding on the mRNA levels of (A) intestinal CCK, Jejunal PYY, and their receptors, and pancreatic PYY, (B) hypothalamic NPY, POMC, and AgRP, and (C) plasma metabolites in broiler chicks. Data are the means \pm S.E.M. of eight chicks in each group and are expressed as a percentage of the mean in the 12 h fasting group. Groups with different letters for each gene are significantly different ($P < 0.05$).

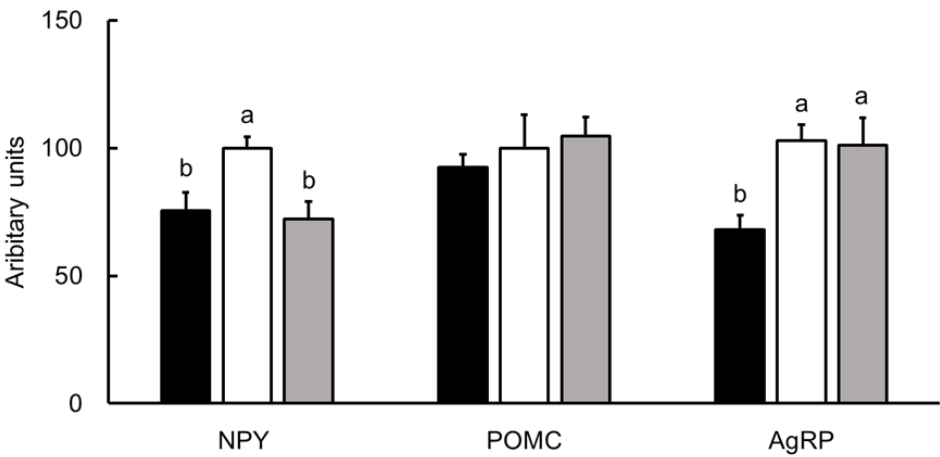
Fig. 3. Effects of fasting and re-feeding on the mRNA levels of hepatic IGF-1 and IGFBPs in layer (A) and broiler (B) chicks. Data are the means \pm S.E.M. of eight chicks in each group and are expressed as a percentage of the mean in the 12 h fasting group. Groups with different letters for each gene are significantly different ($P < 0.05$).



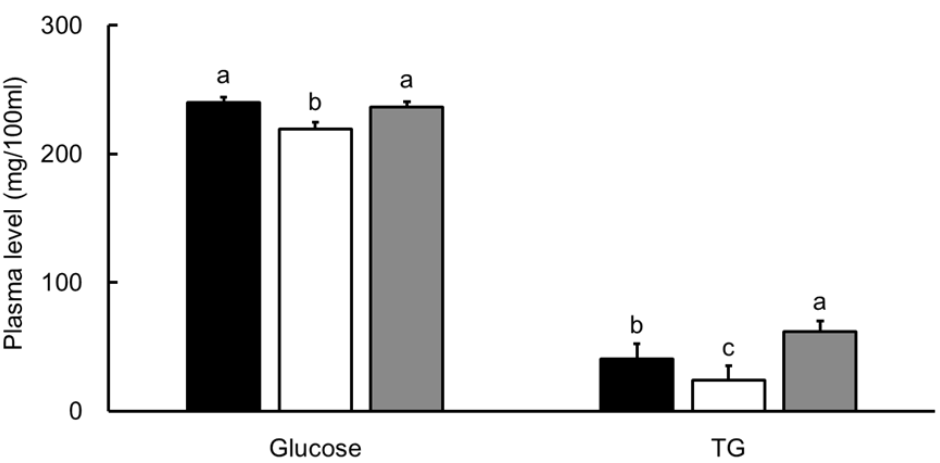
A



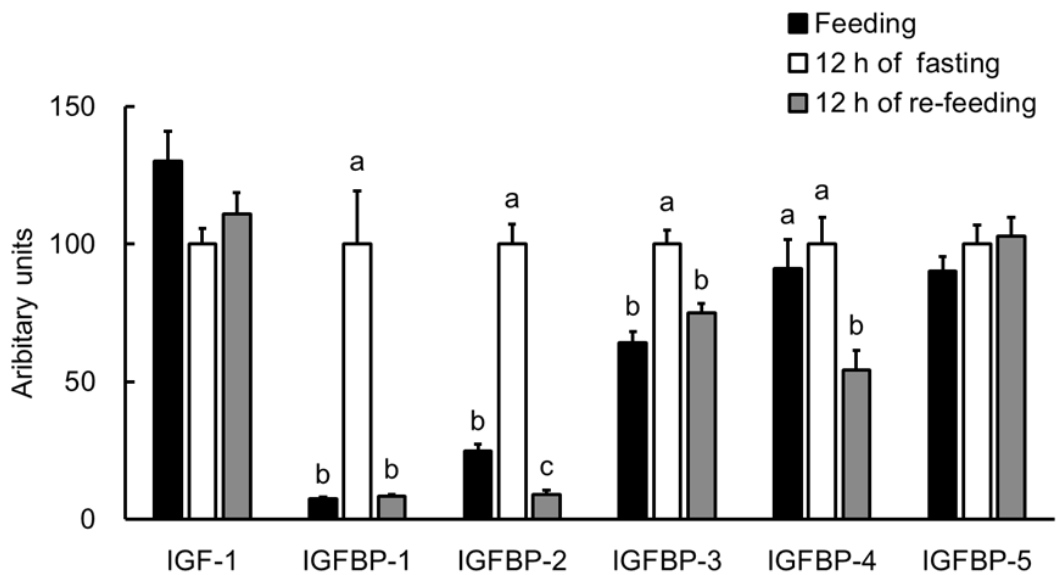
B



C



A: Layer chicks



B: Broiler chicks

