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ORIGINAL ARTICLE

Effects of Ghrelin on Macronutrient Selection and Food Intake Patterns in Neonatal Broiler Chicks

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Running Title

Ghrelin and Nutrient Selection in Broiler Chicks

Abstract

Ghrelin, a hormone recognized for its role in stimulating appetite in mammals, exhibits distinct effects in birds, particularly concerning fat storage and appetite suppression. This study aimed to investigate the impact of ghrelin on macronutrient selection in neonatal chicks, addressing a gap in knowledge regarding non-mammalian species. In the first trial, four nearly isocaloric diets—normal, high carbohydrate, high protein, and high fat—were formulated, and eight-day-old chicks received intracerebroventricular (ICV) injections of chicken ghrelin to evaluate its effect on macronutrient intake. In the second trial, birds were given unrestricted access to all macronutrients simultaneously. In the third trial, chicks were raised on one macronutrient diet and subsequently switched to another. The results indicated that ghrelin significantly reduced intake across all diets, with notable variations based on diet type. Specifically, it decreased protein consumption while increasing the carbohydrate-to-protein intake ratio. This suggests that ghrelin may play a crucial role in energy management, particularly during activities such as flying, where energy demands increase while food availability may be limited. Consequently, low protein intake may lead to a preference for carbohydrates due to the influence of ghrelin. These findings enhance our understanding of avian feeding behavior and energy regulation, underscoring the necessity for further research on the implications of ghrelin for nutritional strategies in non-mammalian species.

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Introduction

Several lines of evidence indicate that strong brain mechanisms lead animals to avoid consuming previously harmful foods (conditioned taste aversion). To a lesser extent, there is evidence revealing mechanisms for choosing necessary and beneficial foods. These latter mechanisms result in a selective, macronutrient-specific appetitive drive (motivation). Thus, animals choose a macronutrient before consuming larger amounts of other macronutrients (1 – 5). Interestingly, chicks restrict their food intake in response to deficiencies in lysine, methionine, and tryptophan 24 h after hatching, showing that these mechanisms act during the neonatal period (6). Studies on selection among multiple diets have revealed that a wide range of species, including insects, fish, rodents, and pigs, regulate their intake around a specific protein to carbohydrate target (7 – 16). In a recently published article, using various dietary combinations as well as both sequential and choice feeding approaches in broiler chickens, the dietary balance between protein and energy is essential for optimal feed conversion efficiency (17).

Ghrelin is a peptide found in the brain's appetite control centers or the alimentary tract of both mammals and domestic poultry (18 – 24). Previous studies have explained that ghrelin plays a role in food intake, lipid metabolism, and energy expenditure (25 – 34). Unlike in other animal species where ghrelin is an orexigenic peptide, it has a potent anorectic effect in birds, including chicks, Japanese quail, and wild garden warblers (25, 32, 33, 35, 37).

In contrast to rodents, where ghrelin acts as an energy-conserving and fat-mass-enhancing peptide, evidence suggests that ghrelin plays a role as a lipolytic peptide in birds (27, 28, 38). This finding implies that some physiological functions of this peptide in birds are exclusive and unique.

In mammals, there have been numerous investigations regarding the effect of ghrelin on macronutrient selection, food choice and preference, flavor preference, and conditioned avoidance (39, 40). However, little is known about its role in avian species.

Given that ghrelin has different nutritional and metabolic effects in birds, it is interesting to determine how this peptide acts in the brain to choose the necessary macronutrients among various food sources. Hence, this study was conducted to evaluate the effect of ghrelin on the intake of four nearly isocaloric dietary combinations (normal and high fat, protein, and carbohydrate diets) in neonatal chicks using both sequential and choice feeding designs.

Materials and Methods

Animals, Diets, and Drugs

One-day-old Ross broiler chicks (Ross 308-line, Mahan Chicken Meat Production Complex Co, Mahan, Iran) chicks were purchased. They were raised as a flock for 3 days under constant temperature (30 ± 1 ºC), humidity (40-50%), and continuous lighting. A normal diet (23% crude protein and 2850 kcal/kg ME) and water were provided *ad libitum*. After 3 days, the birds were transferred to individual cages and switched to feed multi-percent macronutrient diets (Table 1). The gradual diet switching was done. For this purpose, chicks were fed ad libitum with a normal diet and water from one day old until three days later. On the fourth day, the switching was started with a low-percent and then continued to a high-percent macronutrient diet at 24 or 36 h time intervals to adapt the chicks to a high-percent macronutrient diet. On day eight, central ghrelin injection was administered following a three-hour food deprivation period. Food intake was then measured at specified intervals (30, 60, 120, and 180 min) after the injection. Custom-synthesized octanoylated chicken ghrelin with 26-amino acids (GSS(n-octanoyl) FLSPTYKNIQQQKDTRKPTARLH, ASBIO Pharma Co. Ltd., Japan) was used for the study. Ghrelin was dissolved in a 0.1% Evans Blue plus 5% mannitol solution (32). Mannitol was also used as a control solution. ICV injection was done according to the Davis et al. method (41). Briefly, the head of the chick was inserted into a straining device, which positioned a hole in a plate overlying the skull immediately over the right lateral ventricle. The injection was performed using a micro syringe inserted into the right lateral ventricle through the hole with a total injection volume of 5µl. This method requires no anesthesia and the stress level of birds is insignificant (42). At the end of each experiment, the animals were anesthetized, then decapitated, and their brain was removed. Due to the color of Evans Blue in the injected solution, the data from birds that were not properly injected with the solution were not included in the analysis.

Trials and Experiments

In this study, three series of trials (16 experiments, twelve chicks for each experiment) were designed. In the first trial, four experiments were conducted to compare the effect of various doses of ghrelin on macronutrient intake. In experiment 1, the birds were fed with a normal diet, and ICV injection of ghrelin at the doses of 0, 5, 10, and 20 picomoles/individual was done in four groups. Experiments 2, 3, and 4 were identical to Experiment 1 except that the

birds were fed with high-protein, high-carbohydrate, and high-fat diet respectively, before ICV injection. In the second trial, three experiments were conducted. Chicks had free access to all high-protein, carbohydrate, and fat diets simultaneously before and after ICV administration of ghrelin at doses of 0 and 20 picomoles. In the first trial, 20 picomoles of ghrelin was the most effective dose. In the third trial, nine experiments were conducted, in each, chicks were raised on one of the above diets and then switched to another diet immediately after ICV injection. In these experiments, ghrelin at doses of 0 and 20 picomoles was administered.

Statistical Analysis

Food intake data were calculated as a percent of body weight and expressed as mean ± SEM. Data were analyzed using a two-way analysis of variance (ANOVA) with repeated measures. The model included ghrelin and diets as the between-subject factors and time as within within-subject factor for repeated measures. One-way ANOVA and post hoc Tukey test were used for multiple comparisons between treatment groups at each time point. In all experiments, significance was implied at $p < 0.05$.

Table 1. Composition of the experimental high-fat, high-carbohydrate, and high-protein diets.

Ingredients*	Fat (percent of diet)			Carbohydrate (percent of diet)		Protein (percent of diet)	
	4	7	11	70	80	32.04	41.97
Corn	53	48.1	42.8	70	80	42	19
Soybean meal	41.8	42.7	43.6	22.8	14.8	54.5	77.5
Soybean oil	0.8	4.7	9.1	3	1	-	-
Shell	-	-	-	1.4	1.4	1	1
Di calcium phosphate	1.4	1.4	1.4	1.6	1.6	1.9	1.9
Lysine	-	-	-	0.2	0.2	-	-
Methionine	0.2	0.2	0.2	0.3	0.3	-	-
Calcium carbonate	1.7	1.8	1.8	-	-	-	-
Sodium chloride	0.4	0.4	0.4	0.2	0.2	0.1	0.1
Vitamin and Mineral mix	2.5	2.5	2.5	0.5	0.5	0.5	0.5
AME (Kcal/Kg)	2930	3140	3380	3099	3105	3238	3238

*Diet was formulated to meet or exceed minimum recommended specifications for ROSS-308 broilers during the starter phase

Results

The results of the first trial indicated that the various doses of ghrelin decreased normal, high-fat, high carbohydrate and high protein intake (Figure 1). There was an interaction between the type of diet and the food intake-decreasing effect of ghrelin ($p<0.05$).

Among the four diets evaluated in the first trial, birds in the control group receiving a high-protein diet demonstrated the lowest feed intake. In the second trial (Figure 2), in a three-choice paradigm between high-fat, high-protein, and high-carbohydrate diets, ghrelin decreased the intake of all three diets, but specifically decreased protein intake (Figure 2B). This peptide increased the carbohydrate intake: protein intake ratio. Thus, it affects the preference for carbohydrate over protein intake (Table 2 and Figures. 2A, 2C,).

Based on the results of the third trial (Figures 3), ghrelin specifically decreased protein intake in chicks that were raised with high fat diet (Figures. 3A, B and C) and high protein diets (Figures. 3D, E and F), and then switched to a high protein diet (Figures 3C and 3F). This means that chicks prefer carbohydrate and fat intake under the effect of ghrelin. However, in chicks that were raised with high carbohydrate diets (Figures 3G, H and I) and then switched to another diet, a significant decrease in carbohydrate intake by ghrelin was observed (Figure 3G). In chicks fed with high carbohydrate and then switched to high fat (Figure 3H) and high protein (Figure 3I) diets, the inhibitory effect of ghrelin was not observed showing that the birds prefer these diets.

Discussion

In this study, we found that ghrelin decreased the intake of all four diets but the pattern of decrease was different among diets (Figure 1). Additionally, among the different macronutrients, it seems that chicks consumed less protein than the others (Figure 1B). This is in agreement with

several pieces of evidence indicating that among foods rich in fat, protein, and carbohydrate, protein-rich foods have the highest satiating effect (1).

The results of the multiple-choice experiment (Figure 2) showed that under the influence of central ghrelin, chicks reduced their total food intake and showed a preference for carbohydrates over protein (Table 2). The reasons and mechanisms underlying this nutritional behavior are not clear. However, based on a model described by Kaiya et al. (43). The species-specific food-reducing effect of ghrelin for avian species may play a critical role in some situations, such as flying, where access to food for migratory birds is limited. In these conditions, increased levels of ghrelin in plasma and possibly in the brain can act as a satiety factor and also a lipolytic factor in response to negative energy balance. In broiler chickens, unlike rodents, peripheral injection of ghrelin decreases mRNA levels of fatty acid synthase in the liver (28). Ghrelin also reduced the respiratory quotient in broiler chicks (27), showing that ghrelin decreases the rebuilding of fat stores in birds. Interestingly, consistent with the above model, in a recently published paper, Goyman et al. (36) reported that plasma levels of acylated ghrelin in garden warblers, a long-distance migrant, with larger fat stores were higher than those of birds without fat stores. Furthermore, IP administrations of unacylated ghrelin decreased food intake and increased migratory restlessness (36). Based on our results and the results of other investigations mentioned above, it may be speculated that ghrelin plays a unique role in migratory behavior by (i) inducing a sense of satiety during long-distance flights to reduce the desire to land; (ii) increasing migratory restlessness and hence decreasing stopover duration (36); (iii) increasing the usage of lipid as an energy source during flights and finally, (iv) preferring carbohydrates intake (which is possibly more available than lipids) during mandatory stay at stopovers to recover their fuel reserves.

In the third trial, we investigated how ICV ghrelin influences macronutrient intake when chicks were previously exposed to a defined diet (Figure 3). Ghrelin caused a preferential suppression of protein intake in chicks that were raised with a high-fat diet before ghrelin injections (Figure 3c). This preference was also observed to a lesser extent in chicks that were raised with high protein (Figure 3f). However, these results again confirmed that ghrelin selectively decreased protein intake.

Table 2. Effect of intracerebroventricular administration of ghrelin at doses of 0 and 20 pmol on the percent of macronutrient intake in 3-h fasted neonatal chicks fed with a high carbohydrate, protein, and fat diet simultaneously.

Time(min)	Carbohydrate		Protein		Fat		Carbohydrate to protein ratio	
	Control	Ghrelin	Control	Ghrelin	Control	Ghrelin	Control	Ghrelin
30	46.23%	54.55%	20.60%	12.12%	33.17%	33.33%	2.24	4.50
60	44.65%	51.95%	25.46%	11.69%	29.89%	36.36%	1.70	4.40
120	37.80%	57.93%	32.44%	12.07%	29.76%	30.00%	1.60	4.70
180	38.62%	60.06%	32.23%	13.12%	29.15%	26.82%	1.20	4.57

Ghrelin has been structurally conserved during the evolution of birds and mammals. Specifically, the seven N-terminal amino acid sequence of both chicken and rat ghrelin is identical (20) with the third position (Ser) being acylated with octanoic or decanoic acid (44). This segment is necessary for the biological effect of ghrelin, including food intake, by binding to the receptor. Although it appears that this acylation is required to induce ghrelin's effect on food intake, some reports do not confirm this. For example, it has been shown that unacylated ghrelin decreased food intake in wild garden warblers (36).

It has also been revealed that growth hormone-releasing peptide-2 (GHRP-2) which is unacylated and has structural similarity with ghrelin, reduces food intake in chicks (33). Since the administration of all rat, bullfrog, and chicken ghrelin suppressed food intake in chicks (33), it can be concluded that the opposite effect of ghrelin on food intake between birds and other species is possibly related to downstream pathways of ghrelin signaling. In this regard, it has been reported that the suppressing effect of ghrelin on food intake is mediated by central corticotrophin-releasing factor (32), gamma-amino-butyric-acid (GABA) (45) and AMP kinase (46) systems in chicks while the orexigenic effect of ghrelin in rat is mostly contributed to hypothalamic neuropeptide Y and AgRP (47).

Conclusion

In summary, the results of this study indicate for the first time that ghrelin selectively decreases the consumption of foods that are rich in protein, carbohydrates, and fat. This peptide also selectively increases the carbohydrate-to-protein ratio, meaning that under the influence of ghrelin, birds prefer to eat more carbohydrates while their total food intake is reduced. Further studies are needed to clarify the molecular mechanism underlying this behavior in the brain. This feeding behavior might have some benefits for migratory birds during long-distance flights. However, in

precocial species, unlike mammals, different diets can be fed from hatching, providing a research opportunity for the nutritional neurosciences field during neonatal life.

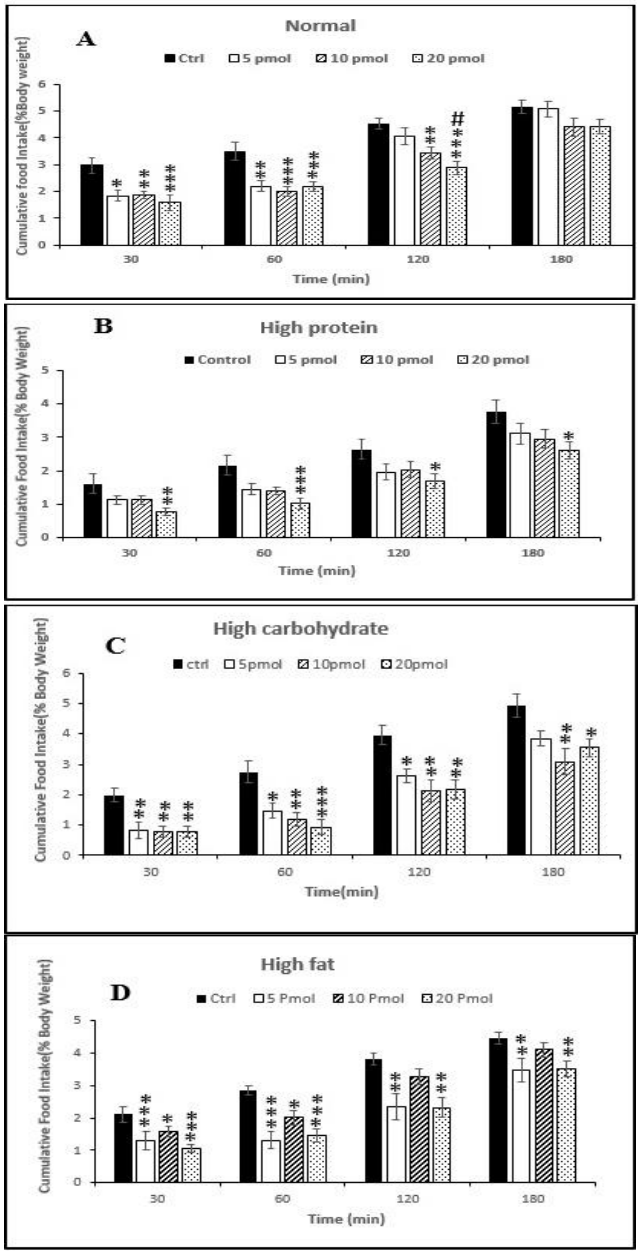

Figure 1. Effect of intracerebroventricular administration of various doses of ghrelin on macronutrient intake (g/100 g BW) in 3 h fasted neonatal chicks. Values represent the Mean ± SEM. A: normal diet intake; B: high protein diet intake; C: high carbohydrate diet intake; D: high fat diet intake.

* p<0.05: significant with the control group.
** p<0.01: significant with the control group.
*** p<0.001: significant with the control group.
# p<0.05: significant with 5 picomole ghrelin group

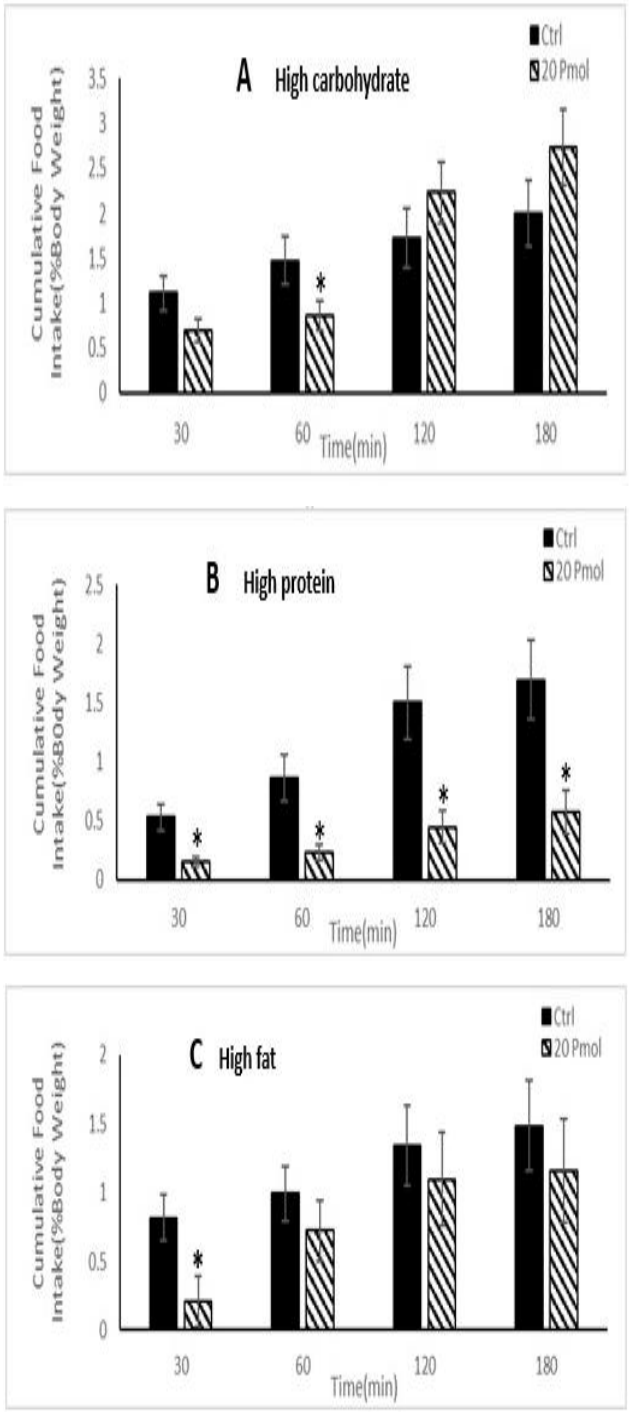

Figure 2. Effect of intracerebroventricular administration of ghrelin on macronutrient intake (g/100 g BW) in 3 h fasted neonatal chicks fed with three diets simultaneously 3 days before injections and 180 min after injections. Values represent the Mean ± SEM (n = 8-10/group). A: High carbohydrate intake, B: High protein intake, C: High fat intake.

* p<0.05: significant with the control group.

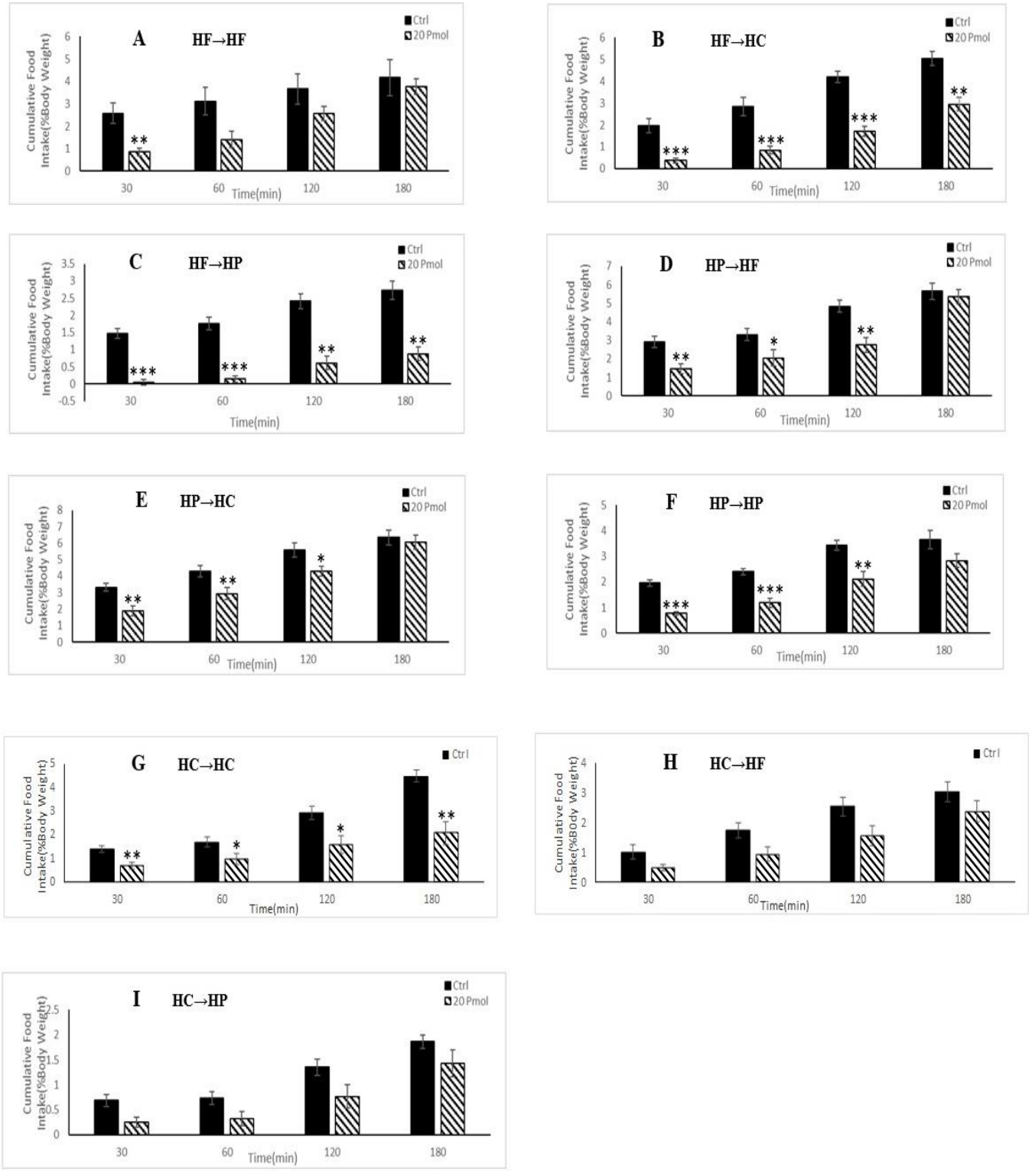

Figure 3. Effect of intracerebroventricular administration of ghrelin on macronutrient intake (g/100 g BW) in 3 h fasted neonatal chicks raised with: a high fat diet (A, B and C), a high protein diet (D, E, and F), and a high carbohydrate diet (G, H and I) 3 days prior injection and then switched to feed another diet. Values represent the Mean ± SEM (n = 8-10/group). HP: High protein diet, HF: High fat diet, HC: High carbohydrate diet

*p<0.05: significant with the control group.
**p<0.01: significant with the control group.
***p<0.001: significant with the control

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Authors' Contributions

Mehran Pourmashayekhi: methodology, data analysis, draft writing, **Hossein Jonaidi:** conceptualization, methodology, review and editing, **Iman Zadsar:** writing the original draft, **Hirouki Kaiya:** review & editing

Data Availability

All data are available if needed.

Ethical Approval

All experimental procedures involving animals were conducted in accordance with the ethical standards and guidelines for the care and use of laboratory animals.

Conflict of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Consent for Publication

Not applicable.

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