

# Systemic infusion of exogenous ghrelin in male broiler chickens (*Gallus gallus domesticus*). The effect of pulse frequency, doses, and ghrelin forms on feed intake, average daily gain, corticosterone, and growth hormone concentrations

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**ABSTRACT** There is limited information on the effect of exogenous ghrelin infusion on feed intake (**FI**) in chickens. Therefore, male broilers were used in 3 factorial experiments to determine the relationships between doses (0, 1, or 4 nM; Dose), frequency (once every two h; 2 h), once every 4th h (4 h) or continuous infusion, and ghrelin forms including acylated-ghrelin (**AG**) and desacylated-ghrelin (**DAG**) on FI, ADG, and concentrations of corticosterone and Growth Hormone (GH). Treatments were delivered via a jugular cannula, using programmable pumps for 11 consecutive days. FI and ADG were recorded, and plasma was collected. Data were analyzed using a factorial design. In Experiment 1 the effect of AG pulse frequency and doses were evaluated. There was a linear decrease in FI ( $P = 0.002$ ) and a linear increase in corticosterone ( $P = 0.033$ ) and GH ( $P = 0.011$ ) concentrations when AG was infused. However, ADG decreased with doses ( $P = 0.011$ ) only when

AG was given at 2 h. In Experiment 2 the effect of ghrelin forms and doses given at 2 h was evaluated. There was a linear decrease in FI when AG was infused and a linear increase in FI when DAG was infused ( $P < 0.05$ ). Birds infused with DAG gained more weight than those infused with AG. There was a linear increase in corticosterone and GH concentrations only when AG was infused ( $P < 0.01$ ). In Experiment 3 the effect of continuous infusion of 2 doses (0 and 1 nM) of AG and DAG were evaluated. There was a linear decrease in FI and ADG when AG ( $P < 0.001$ ) was infused and a linear increase in FI and ADG when DAG was infused ( $P < 0.05$ ). There was an increase in corticosterone concentrations only when AG was infused ( $P = 0.022$ ). However, GH concentrations were not affected by treatments. We concluded that AG and DAG pulse frequency and doses had a differential effect on FI, ADG, corticosterone, and GH concentrations in broiler chickens.

**Key words:** ghrelin, broilers, feed intake, infusion

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## INTRODUCTION

Ghrelin is a growth hormone (**GH**)-releasing peptide initially purified from rat stomachs (Kojima et al., 1999). In chickens, ghrelin is 26 amino acids peptide highly expressed and secreted primarily from the proventriculus (Kaiya et al., 2002). Two forms of ghrelin circulate in blood. The active form of ghrelin (acylated ghrelin; **AG**) undergoes a unique acylation process and is the only form that binds to its cognate receptor, a seven-transmembrane G protein-coupled receptor (**GHSR-1a**). Ghrelin O-acyltransferase is the enzyme that transfers an acyl group from fatty acids to the

serine-3 residue of ghrelin, thus making it active (Gutierrez et al., 2008). Desacylated-ghrelin (**DAG**) does not undergo the acylation process and has a very weak binding ability for GHSR-1a (Gauna et al., 2007; Callaghan et al., 2014).

Systemic or central infusion of ghrelin in mammalian species stimulates feed intake (**FI**) and induces adiposity (Tschöp et al., 2000; Wren et al., 2001). In avian species, most researchers have shown that intracerebroventricular (**ICV**) infusion of AG decreases feed intake (Table 1). However, there are conflictive results on the effect of ghrelin on feed intake when injected peripherally. In layer chickens, i.v. injection of ghrelin did not affect feed intake (Kaiya et al., 2007). On the other hand, i.v. injections of ghrelin in broiler chickens inhibited feed intake 30 to 120 min after treatments were applied (Geelissen et al., 2006). Similarly, a short-time inhibitory effect (approximately 30 min) on feed intake was reported when ghrelin was administered peripherally in neonatal broiler chickens (Buyse et al., 2009). However, when a ghrelin receptor agonist (Capromorelin) was

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**Table 1.** Effect of ghrelin administration on feed intake in different avian species.

Ghrelin <sup>1</sup>	Species <sup>2</sup>	Age <sup>3</sup>	Route	Dose	Time <sup>4</sup>	FI result <sup>5</sup>	Reference
DAG	<i>Ggd</i> (Layers)	5d	ICV	0–100 pM/bird	30–90 min	↔	Tachibana et al. (2011)
AG	<i>Ggd</i> (Layers)	7d	ICV	0–10 pM/bird	30–90 min	↓	Tachibana et al. (2011)
AG	<i>Ggd</i> (Broilers)	3d	ICV	0.38–1.5 nM/bird	30–120 min	↓	Saito et al. (2002)
mAG	<i>Ggd</i> (Broilers)	21d	ICV	0–1.2 nM/bird	15–180 min	↓	Taati et al. (2010)
mAG	<i>Ggd</i> (Broilers)	2d	ICV	0.38–1.5 nM/bird	120 min	↓	Furuse et al. (2001)
AG	<i>Ggd</i> (Broilers)	5d	ICV	0.6 nM/100 g	30–120 min	↓	Farrokhi et al. (2021)
AG	<i>Ggd</i> (Broilers)	4d	ICV	0–20 pM/bird	30–120 min	↓	Saito et al. (2005)
GHRP-6	<i>Ggd</i> (Layers)	5d	ICV	0.38–1.5 nM/bird	30–120 min	↓	Khan et al. (2006)
mAG	<i>Ccj</i>	Adult	ICV	0.05–1 nM/bird	2, 4, and 12 h	↓	Shousha et al. (2005)
AG	<i>Ggd</i> (Layers)	8d	i.v.	0.6 nM/100 g	30–120 min	↔	Kaiya et al. (2007)
AG	<i>Ggd</i> (Broilers)	7 d	i.v.	1 nM/100 g	0.5–2 h	↓	Geelissen et al. (2006)
AG	<i>Ggd</i> (Broilers)	1 d	i.v.	1 nM/bird	30 min	↓	Buyse et al. (2009)
AG	<i>Sylvia borin</i>	NR	i.p.	100–500 pM/bird	18 h	↔	Goymann et al. (2017)
DAG	<i>Sylvia borin</i>	NR	i.p.	500 pM/bird	18 h	↓	Goymann et al. (2017)
mAG	<i>Ccj</i>	Adult	i.p.	0.5–1 nM/bird	12 h	↑	Shousha et al. (2005)
mAG	<i>Ccj</i>	Adult	i.p.	3 nM/bird	12 h	↓	Shousha et al. (2005)
AG	<i>Ggd</i> (Broilers)	7 d	i.p.	0.5 or 2 nM/100 g	30–120 min	↓	Ochoń and Pietras (2011)
Capromorelin	<i>Ggd</i> (Broilers)	28 d	Oral	0–12 mg/Kg/d	0–5 d	↑	Ceron-Romero et al. (2021)

<sup>1</sup>Ghrelin form: DAG, chicken desacylated-ghrelin; AG, chicken acylated-ghrelin; mAG, mammalian AG.

<sup>2</sup>Species: *Ggd* = *Gallus gallus domesticus*; *Ccj* = *Coturnix coturnix japonica*.

<sup>3</sup>Age: NR = Age not reported by author.

<sup>4</sup>Time: Time at which feed intake measurement was obtained after treatments were applied.

<sup>5</sup>FI results: ↔ = No change in feed intake ↓ = decreased feed intake = ↑ increased feed intake.

administered orally to male broiler chickens, a significant dose-dependent increase in feed intake was observed (Ceron-Romero et al., 2021).

The inhibitory effect of AG on feed intake is thought to be associated with the activation of neurons expressing the anorexigenic corticotropin-releasing hormone (CRH). Suppression of feed intake in chickens was induced by ICV injections of ghrelin, but it was attenuated by co-injection of a CRH receptor antagonist (Saito et al., 2005). Similarly, in 1-day-old broiler chickens, a single intravenous injection of chicken ghrelin increased plasma corticosterone levels by almost 400% (Buyse et al., 2009), presumably by the direct effect on endogenous CRH, which in turn activates the hypothalamic–pituitary–adrenal (HPA) axis, leading to increased corticosterone release from the adrenal glands (Kaiya et al., 2002; Saito et al., 2005). Another line of research found that ICV injections of a CRH-like peptide (Urocortin-3; UCN-3) but not CRH increased plasma ghrelin concentration in chickens (Khan et al., 2014). Since UCN-3 has more affinity to the CRH type 2 receptor (Lewis et al., 2001), evidence suggests that the effect of ghrelin in feed intake is at least partly mediated by the CRH system and the two receptors present in the CRH family. Additionally, ghrelin can directly affect the histaminergic system (via H1 receptors) that in turn modulates CRH release (Taati et al., 2010). The GHSR-1a receptor exhibits a highly constitutive activity (Holst et al., 2003; Qin et al., 2022). Consequently, the ability of the ghrelin receptor to retain a certain degree of activity independently of the ligand may also play a role in the differential regulation of ghrelin in feed intake.

Limited information is available on the effect of the two forms of ghrelin in avian species. In migratory garden warblers' birds, injection (i.p.) of DAG decreased feed intake, whereas injections of AG did not affect feed intake (Goymann et al., 2017). However, in other

migratory species (blackbirds) researchers were not able to find correlations between ghrelin and fat levels at departure time (Eikenaar et al., 2018). In layer chickens, ICV injections of DAG did not affect feed intake after 30 min of treatments, whereas AG decreased feed intake (Tachibana et al., 2011).

The physiological pattern of ghrelin secretion is pulsatile in humans with approximately 1 pulse every 1 to 4 h during fasting (Koutkia et al., 2004; Natalucci et al., 2005; Nass et al., 2008). However, in fasting rats, the interpulse interval was found to be more frequent with approximately 1 pulse every 23 min (Bagnasco et al., 2002). The amplitude and width of pulsatile ghrelin secretion appear to increase during regular meal patterns in humans, suggesting that ghrelin rises during meal anticipation (Frecka and Mattes, 2008). The spontaneous pattern of ghrelin secretion indicates that the production could be attributed to neuronal in addition to gastrointestinal signals (Koutkia et al., 2004; Natalucci et al., 2005).

Ghrelin increases the secretion of GH in rats (Date et al., 2000; Hosoda et al., 2000; Wren et al., 2000), humans (Takaya et al., 2000), and in chickens (Ahmed and Harvey, 2002). Acylated-ghrelin pulses are correlated with GH pulses (Nass et al., 2008). However, when total ghrelin was measured, ghrelin pulses did not correlate with GH pulsatility (Koutkia et al., 2004). Acylated-ghrelin stimulates the release of GH after binding GHSR-1a and causing an increase of intracellular calcium concentration via the inositol 1,4,5-trisphosphate signal transduction pathway (Howard et al., 1996; Kohno et al., 2003). When ghrelin was administered in vitro to chicken pituitaries, a downregulation of cGHSR-1a was observed within 15 min (Geelissen et al., 2003). These data suggest that GH and ghrelin provide a feedback mechanism that regulates GHSR-1a.

There is a lack of long-term experiments that evaluates the effect of ghrelin (AG or DAG) on feed intake,

ADG, corticosterone and GH concentrations in chickens. Most of the literature on exogenous ghrelin administration in avian species only provides information of measurements taken after a relatively short time post-ghrelin administration (Table 1). Moreover, the effect of different forms of ghrelin, pulse frequency, and doses has not been evaluated. Therefore, the objectives of the experiments presented here were to evaluate the effects of systemic infusion of AG and DAG for 11 consecutive days at different frequencies and doses on FI, ADG, corticosterone and GH concentrations in male broiler chickens.

## MATERIALS AND METHODS

### Animal Rearing and Treatments

One-hundred-fifty 1-day-old male broiler chickens (*Gallus gallus domesticus*; Ross 708) were obtained from a commercial hatchery and used in 3 replicated experiments during 3 consecutive years. Birds were randomly placed in 2 battery brooders and reared as recommended by industry standards with ad libitum feed and water consumption. Birds received a standard corn-soybean-based starter diet from 1 to 14 d of age (22 % CP, 1.0 % Lys, 1.2 % Ca, and 0.6 % P) followed by a grower/finisher diet until the end of the experiment (20 % CP, 1.2 % Lys, 1.1 % Ca, and 0.6 % P; Purina, St. Louis, MO). At 3 wk of age, birds (BW  $1.5 \pm 0.1$  Kg) were randomly transferred from the brooders to individual cages (0.6 × 0.5 × 0.5 m) and were fitted with a harness and collar before jugular cannulation. Chickens were housed at the Poultry Building (Alabama A&M University) and exposed to a 23L:1D photoperiod and  $22 \pm 1.5^\circ\text{C}$  temperature. The care, treatment, and experimental protocols were approved by the Institutional Animal Care and Use Committee of Alabama A&M University.

### Experiment 1

Birds ( $n = 5/\text{trt}$ ; yr 1) were randomly assigned to a  $2 \times 3$  factorial arrangement of treatments (**FAT**). The first factor in the FAT included the frequency of exogenous acylated ghrelin pulses (**Frequency**) infused (i.v.) once every 2 h (**2 h**) or once every 4 h (**4 h**). The second factor included different nano molar (**nM**) doses (**Dose**) of acylated-ghrelin: 0 nM/100 g BW/d (**0 nM**), 1 nM/100 g BW/d (**1 nM**), or 4 nM/100 g BW/d (**4 nM**). Birds in the 0 nM group were considered control birds and received a saline-citrate-gentamycin solution (**Saline**) via programmable pumps (see below). The experiment was conducted for 11 consecutive days throughout a 24 h period.

### Experiment 2

Birds ( $n = 5/\text{trt}$ ; yr 2) were randomly assigned to a  $2 \times 3$  FAT. The first factor in the FAT included the 2 forms of chicken ghrelin (**Form**): AG, and DAG. The

second factor included different doses of ghrelin: 0 nM, 1 nM, and 4 nM given in pulses every 2 h. Birds in the 0 nM group were considered control birds and received Saline via programmable pumps. The experiment was conducted for 11 consecutive days throughout a 24-h period.

### Experiment 3

Birds ( $n = 5/\text{trt}$ ; yr 3) were randomly assigned to a  $2 \times 2$  FAT. The first factor in the FAT included the 2 forms of chicken ghrelin: AG and DAG. The second factor included different doses of ghrelin: 0 nM and 1 nM given as a continuous infusion. Birds in the 0 nM group were considered control birds and received Saline via programmable pumps. The total amount of ghrelin given per day in birds receiving 1 nM was the same as the dose receiving 1 nM in Experiment 2. The experiment was conducted for 11 consecutive days throughout a 24-h period.

### Jugular Vein Cannulation

Birds were cannulated as previously described (Vizcarra et al., 2004) with modifications. Briefly, 1 wk before cannulation, birds were adapted to wear a harness (Instech Laboratories, PA) and a collar that consisted of a thin piece of cardboard with a hole that fitted the bird's neck. The purpose of the collar was to prevent birds from reaching and removing the cannula. We have previously shown that the cannulation procedure increases corticosterone concentrations during the first 3 to 4 h post cannulation (Vizcarra et al., 2004). Therefore, to avoid any potential stress-related confounding effect, birds were cannulated the day before treatments were applied. After the cannula was inserted into the jugular vein, serological suture glue was applied at the incision point to hold the cannula in place. Cannulas, swivels, needles, and tubing used during the procedure were autoclaved by the provider (Instech Laboratories, Plymouth Meeting, PA). Two KDS220 multi-syringe infusion pumps (KD, Scientific Inc, MA) were placed in a remote location that was out of sight to the birds and easily accessible to deliver treatments (Pump #1) and maintain cannula patency (Pump #2). Pump #1 was programmed to deliver treatments at specified frequencies and doses. Doses were delivered in 2 mL bolus infusions at a rate of 0.5 mL/min when birds were infused with pulses every 2 h or 4 h (Experiment 1 and 2). Pump #2 was programmed to deliver a continuous infusion of Saline at a rate of 0.4 mL/h. The cannula was connected to the harness and pumps via a Y connector (Instech Laboratories) that allowed for continuous delivery of the Saline solution and treatments. When a continuous infusion was used (Experiment 3), only Pump #1 was set to deliver the infusions at a rate of 0.4 mL/h. Fresh supplies of Saline and treatments were provided daily for 11 consecutive days. The cannulation procedure

allowed for treatment delivery in an unrestrained bird with continued free access to feed and water.

### Feed Intake and BW

Feed intake measurement was obtained every day at 08:00 h, and BW was measured at the beginning and end of the experiments. Individual daily feed intake was measured by recording the weight of feed (g) offered each day minus any unconsumed feed remaining after 24 h. Average feed consumption for each bird during the 11 d was calculated and used in the statistical model. Feed and water were provided ad libitum in all experiments. Individual ADG was calculated by subtracting the initial BW from the final BW and then dividing by 11 (i.e., the number of days between measurements).

### Blood Sampling

At the end of each experiment, a single blood sample (3 mL) was collected via the connected cannula from the jugular vein of each bird and placed into a 7-mL tube containing EDTA (Fisher Scientific, Pittsburgh, PA) to prevent clot formation. Aprotinin (500 KIU/mL of blood) was added to the blood immediately after collection to inhibit the activity of proteases. After centrifugation ( $1,800 \times g$  for 15 min at  $4^{\circ}\text{C}$ ), plasma content was collected into 2 mL cryovial tubes and stored at  $-80^{\circ}\text{C}$  until analysis. Concentrations of corticosterone were evaluated using ELISA kits following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Concentrations of GH were evaluated using ELISA kits developed for chickens (Cusabio Biotech, College Park, MD). All samples were run in duplicates in 96-well assays.

### Ghrelin Peptide Preparation

Chicken AG (GSS(O<sub>8</sub>octanoyl) FLSPTY-KNIQQQKDTRKPTARLH) and chicken DAG (GSSFLSPTYKNIQQQKDTRKPTARLH) were supplied by (Sigma Aldrich, St. Louis, MO) with a 95% purity. Approximately 100 mg of AG or DAG peptides were dissolved in 25% acetic acid. After solubilization, peptides were further diluted in ultra-pure water to a final concentration of 10 mg/mL (adjusted for purity). The resulting stock solution was aliquoted (1 mL) in cryovial tubes and stored at  $-80^{\circ}\text{C}$ . The final treatment doses were prepared in a Saline solution that consisted of sodium citrate (5 mg/mL) and gentamicin sulfate (0.5 mg/mL; Fisher Scientific) that were added to sterile physiological saline to prevent clotting and bacterial contamination.

### Statistical Analysis

The criteria used to determine the number of experimental units for the three experiments included a GLMPOWER procedure (SAS, 2009) using the

standard deviation and mean of feed intake in chickens from previous research in our laboratory (Taofeek et al., 2018). The number of experimental units was determined using a power ( $1-\beta$ ) of 90% and a probability ( $\alpha$ ) of 1%.

Data from the three experiments were analyzed as a completely randomized design with a factorial arrangement of treatment [PROC MIXED; (SAS version 9.4)]. If a significant two-way interaction existed, polynomial response curves were calculated. When polynomial responses fitted a linear or a quadratic function, significant differences in the intercept and slope were computed by applying dummy variables using a covariance analysis (Steel and Torrie, 1980). When appropriate, orthogonal polynomial contrasts were used to evaluate linear or quadratic effects. PROC IML was used to determine the polynomial coefficients for non-equally spaced doses. Due to limitations in the number of pumps, and space in the environmental controlled physiology laboratory we were able to use 6 cages (experimental units) at a time. Therefore, each experiment was replicated at least 5 times to accommodate all experimental units.

The statistical model for Experiment 1 included the effect of Frequency (2 h or 4 h), Dose (0 nM, 1 nM, or 4 nM), and the Frequency  $\times$  Dose interaction. For Experiment 2, the statistical model included the effect of ghrelin Form (AG or DAG), Dose (0 nM, 1 nM, or 4 nM) given in a pulse every 2 h, and the Form  $\times$  Dose interaction. For Experiment 3 the statistical model included the effect of ghrelin Form (AG or DAG), Dose (0 nM, or 1 nM) given as a continuous infusion, and the Form  $\times$  Dose interaction.

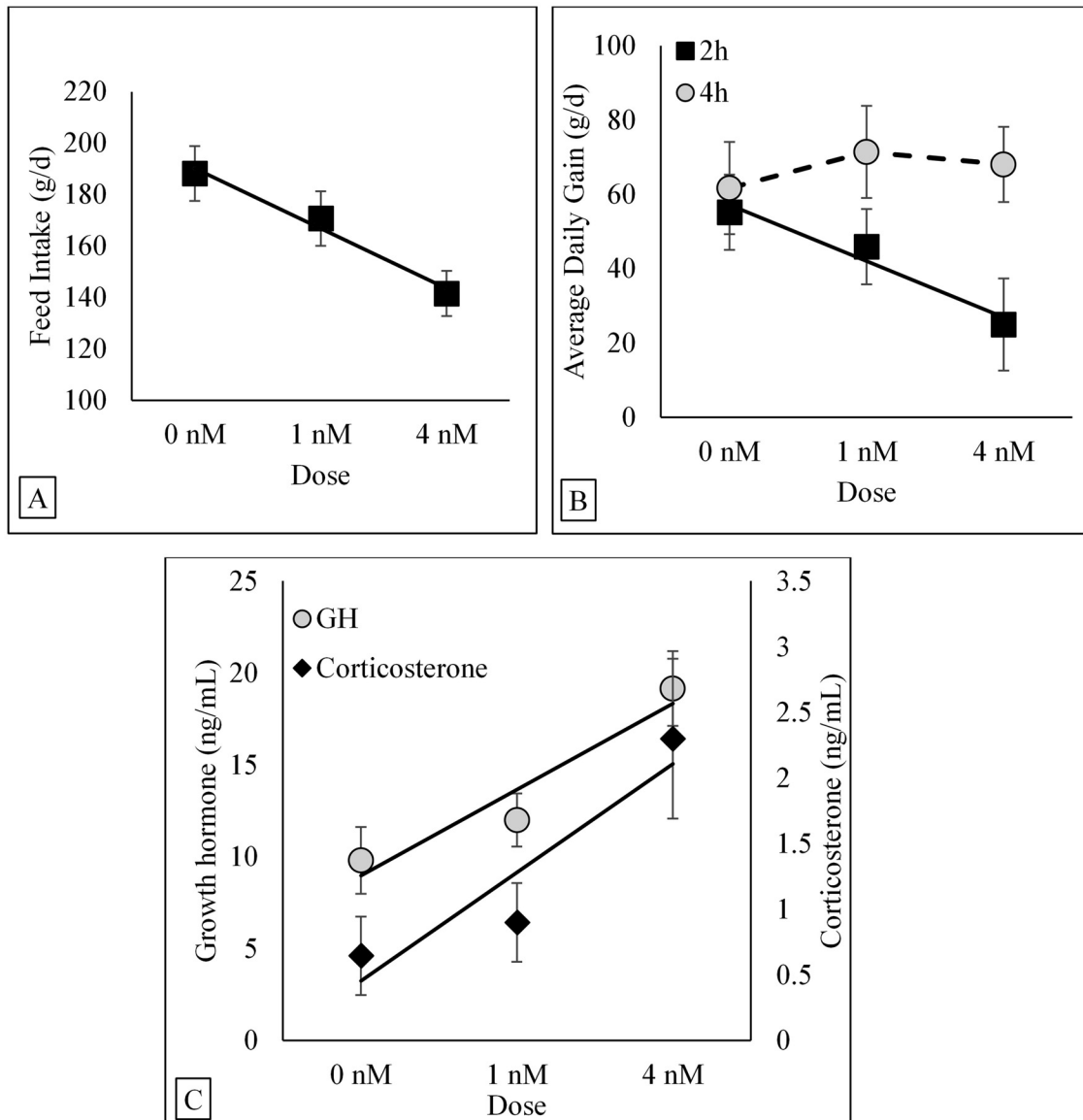
Doses used in all experiments were carefully selected using information from previous reports (Table 1). Jugular cannula patency ranged from 3 to 15 d. Only birds that completed 11 d of treatment were used in the analysis. Initially, we also tested doses of 0.5 nM/100 g BW/d. However, only a few birds reached the pre-established 11-d treatment. Therefore, data for the 0.5 nM treatment was not included in the statistical analysis and is not reported.

## RESULTS

### Experiment 1

There was no significant Frequency or Frequency  $\times$  Dose interactions for FI. However, there was a Dose effect ( $P = 0.003$ ; Figure 1A). Linear orthogonal contrast best described the relationship between AG doses and FI ( $P = 0.002$ ). Results from the linear regression indicated that there was a decrease of  $10.8 \pm 3.1$  g of FI per each nM/100 g BW/d of exogenous AG systemically infused.

There was a Frequency  $\times$  Dose interaction ( $P = 0.086$ ) for ADG (Figure 1B). When polynomial response curves were evaluated, a linear model best described ( $P = 0.011$ ) the association between Dose and ADG when AG was given at a frequency of 1 pulse every 2 h. Results from the



**Figure 1.** Least square regressions (solid line) and means (symbols)  $\pm$  SE for FI (A), ADG (B) and corticosterone and GH concentrations (C) in broiler chickens after systemic infusion of exogenous acylated ghrelin at different doses (0 nM, 1 nM, and 4 nM) and pulse frequencies (2 h or 4 h). There was a Dose effect on FI ( $P = 0.003$ ). The solid line indicates a dose-dependent decrease of FI. There was a Frequency  $\times$  Dose interaction on ADG ( $P = 0.086$ ). The solid line indicates a linear dose-dependent decrease in average daily gain, whereas the broken line indicates the absence of a significant linear or quadratic function. There was a Dose effect on corticosterone ( $P = 0.033$ ) and GH ( $P = 0.011$ ) concentrations. The solid lines indicate a linear dose-dependent increase of corticosterone and GH concentrations. Treatments were delivered via programmable pumps for eleven consecutive days throughout a 24 h period. Abbreviations: 2h, infused (i.v.) once every 2 h; 4h, infused (i.v.) once every 4 h; GH, growth hormone.

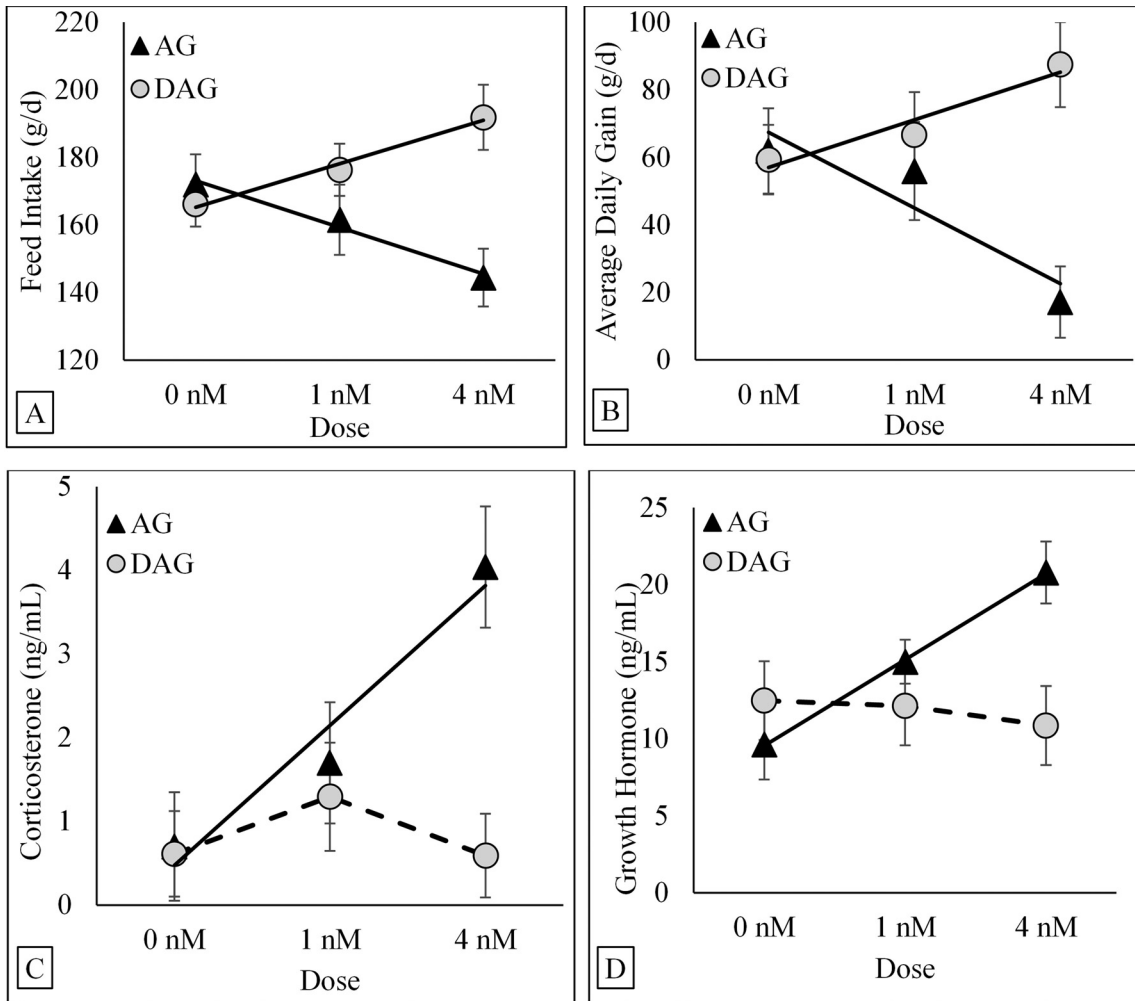
linear regressions indicated that there was a decrease of  $8.3 \pm 2.3$  g in ADG per each nM/100 g BW/d of exogenous AG systemically infused. However, there was no significant polynomial response (linear or quadratic) between Dose and ADG when AG was given at a frequency of 1 pulse every 4 h.

There was no significant Frequency or Frequency  $\times$  Dose interactions for corticosterone and GH concentrations. However, there were Dose effects for corticosterone ( $P = 0.033$ ) and GH ( $P = 0.011$ ) concentrations (Figures 1C). Linear orthogonal contrast best described the relationship between AG doses and corticosterone and GH concentrations. Polynomial contrasts showed that AG doses linearly increased corticosterone and GH. Results from the linear regression indicated that AG

increased concentrations of corticosterone ( $0.6 \pm 0.2$  ng/mL) and GH ( $2.2 \pm 0.5$  ng/mL) per each nM/100 g BW/d of exogenous AG systemically infused.

## Experiment 2

A Form  $\times$  Dose interaction ( $P < 0.001$ ) for FI was best described by linear regression equations (Figure 2A). When comparing both regression lines using dummy variables, FI in birds receiving 0 nM/g BW/d (linear intercept) was not influenced by ghrelin forms ( $166.1 \pm 6.4$ , and  $172.0 \pm 8.6$  g/d; DAG and AG, respectively). However, there were significant differences in the slopes ( $P = 0.004$ ). Feed intake linearly increased with doses in birds receiving



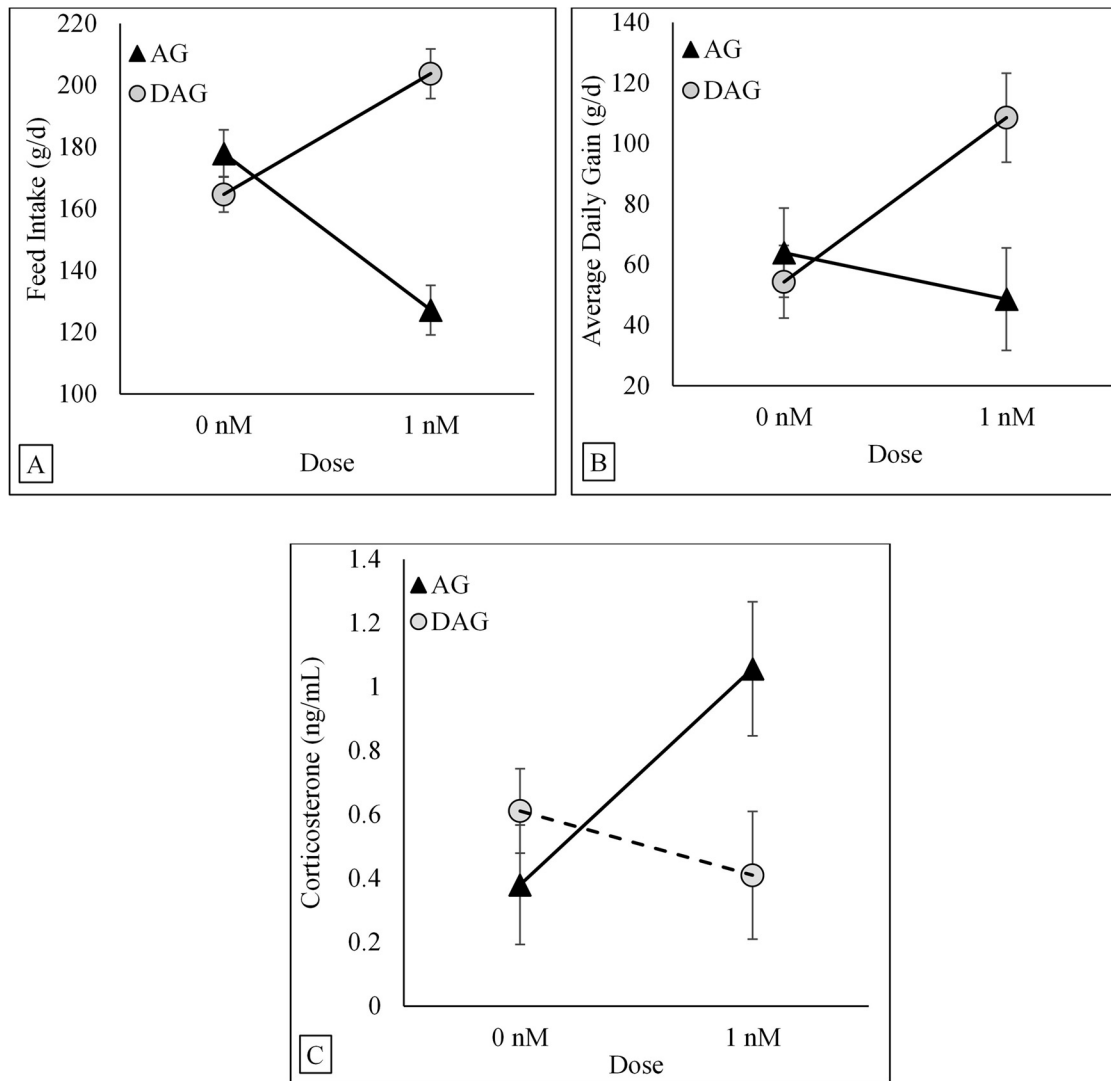
**Figure 2.** Least square regression (solid lines) and mean (symbols)  $\pm$  SE for FI (A), ADG (B), corticosterone (C), and GH concentrations (D) in broiler chickens after systemic infusion of two ghrelin forms (AG and DAG) at different doses (0 nM, 1 nM, and 4 nM) given at a frequency of 1 pulse every 2 h. There was a Form  $\times$  Dose interaction on FI ( $P < 0.001$ ) and ADG ( $P = 0.034$ ). The solid lines indicate a linear dose-dependent increase of FI and ADG when DAG was infused and a linear dose-dependent decrease of FI and ADG when AG was infused. There was a Form  $\times$  Dose interaction on corticosterone concentrations ( $P = 0.008$ ). The solid line indicates a linear dose-dependent increase in corticosterone concentrations when AG was infused, whereas the broken line indicates the absence of a significant linear or quadratic function when DAG was infused. There was a Form  $\times$  Dose interaction on GH concentrations ( $P < 0.001$ ). The solid line indicates a linear dose-dependent increase in GH concentrations when AG was infused, whereas the broken line indicates the absence of a significant linear or quadratic function when DAG was infused. Treatments were delivered via programmable pumps for eleven consecutive days throughout a 24 h period. Abbreviations: AG, chicken acylated-ghrelin; DAG, chicken desacylated-ghrelin.

DAG. Results from linear regressions indicated that there was an increase of  $6.7 \pm 2.5$  g of FI per each nM/100 g BW/d of exogenous DAG systemically infused. However, FI linearly decreased with doses in birds receiving AG. Results from linear regressions indicated that there was a decrease of  $7.6 \pm 3.2$  g of FI per each nM/100 g BW/d of exogenous AG systemically infused.

In congruence with FI data, there was also a Form  $\times$  Dose interaction ( $P = 0.034$ ) for ADG. Linear regression equations best described the association between ghrelin forms and doses (Figure 2B). When comparing both regression lines using dummy variables, ADG in birds receiving 0 nM/g BW/d (linear intercept) was not influenced by ghrelin forms ( $59.3 \pm 10.3$ , and  $61.9 \pm 12.6$  g/d; DAG and AG, respectively). However, there were significant differences in the slopes ( $P = 0.002$ ). Body weight gain linearly increased with doses in birds receiving DAG. Results from linear regressions indicated that there was an

increase of  $8.6 \pm 4.5$  g/d of ADG intake per each nM/100 g BW/d of exogenous DAG systemically infused. On the other hand, ADG linearly decreased with doses in birds receiving AG. Results from linear regressions indicated that there was a decrease of  $12.2 \pm 2.8$  g/d of ADG per each nM/100 g BW/d of exogenous AG systemically infused.

There was a Form  $\times$  Dose interaction ( $P = 0.008$ ) for corticosterone concentrations (Figure 2C). When polynomial response curves were evaluated, a linear model best described ( $P = 0.017$ ) the association between doses and corticosterone concentrations when exogenous AG was given. Results from the linear regression indicated that AG increases the concentration of corticosterone by  $0.9 \pm 0.3$  ng/mL per each nM/100 g BW/d of exogenous AG systemically infused. However, there was no significant polynomial response (linear or quadratic) between doses and corticosterone concentrations when exogenous DAG was given.



**Figure 3.** Least square regression (solid lines) and mean (symbols)  $\pm$  SE for FI (A), ADG (B), and corticosterone concentrations (C) in broiler chickens after systemic infusion of two ghrelin forms (AG and DAG) at two doses (0 nM and 1 nM) infused continually. There was a Form  $\times$  Dose interaction on FI ( $P < 0.001$ ) and ADG ( $P = 0.034$ ). The solid lines indicate a linear increase of FI and ADG when DAG was infused and a linear decrease of FI and ADG when AG was infused. There was a Form  $\times$  Dose interaction on corticosterone concentrations ( $P = 0.038$ ). The solid line indicates a linear increase in corticosterone concentrations when AG was infused, whereas the broken line indicates the absence of a significant linear function when DAG was infused. Treatments were delivered via programmable pumps for eleven consecutive days throughout a 24 h period. Abbreviations: AG, chicken acylated-ghrelin; DAG, chicken desacylated-ghrelin.

Similarly, there was a Form  $\times$  Dose interaction ( $P < 0.001$ ) for GH concentrations (Figure 2D). When polynomial response curves were evaluated, a linear model best described ( $P < 0.001$ ) the association between doses and GH concentrations when exogenous AG was given. Results from the linear regression indicated that AG increased concentrations of GH by  $2.8 \pm 0.5$  ng/mL per each nM/100 g BW/d of exogenous AG systemically infused. However, there was no significant polynomial response (linear or quadratic) between doses and GH concentrations when exogenous DAG was given.

### Experiment 3

A Form  $\times$  Dose interaction ( $P < 0.001$ ) for FI was described by linear regression equations (Figure 3A). When comparing both regression lines using dummy

variables, FI in birds receiving 0 nM/g BW/d (linear intercept) was not influenced by ghrelin forms ( $164.6 \pm 5.7$  and  $177.8 \pm 7.7$  g/d; DAG and AG, respectively). However, there were significant differences in the slopes ( $P < 0.001$ ). Feed intake linearly increased with doses in birds receiving a continuous infusion of exogenous DAG (slope =  $39.1 \pm 9.9$  g) whereas FI decreased linearly in birds receiving a continuous infusion of exogenous AG (slope =  $-50.7 \pm 10.9$  g).

A Form  $\times$  Dose interaction ( $P = 0.034$ ) for ADG was described by linear regression equations (Figure 3B). When comparing both regression lines using dummy variables, ADG in birds receiving 0 nM/g BW/d (linear intercept) was not influenced by ghrelin forms ( $54.3 \pm 12.0$  and  $63.9 \pm 14.7$  g/d; DAG and AG, respectively). However, there were significant differences in the slopes ( $P = 0.034$ ). Average daily gain linearly increased with doses in birds receiving a continuous infusion of

exogenous DAG (slope =  $54.3 \pm 22.3$  g/d) whereas FI decreased linearly in birds receiving a continuous infusion of exogenous AG (slope =  $-15.4 \pm 13.9$  g/d).

There was a Form  $\times$  Dose interaction ( $P = 0.038$ ) for corticosterone concentrations (Figure 3C). A linear model described ( $P = 0.022$ ) the association between doses and corticosterone concentrations when exogenous AG was given (slope =  $0.68 \pm 0.19$ ). However, there was no linear response (slope not different from zero) between doses and corticosterone concentrations when exogenous DAG was given.

There was no significant Form, Dose, or Form  $\times$  Dose interactions for GH concentrations. On average, concentrations of GH were  $16.6 \pm 3.6$  ng/mL.

## DISCUSSION

The data presented in the current study clearly shows that exogenous AG doses resulted in an inhibitory effect of FI when infused as a pulse every 2 h or continuously. When 0.6 nM/100g BW of AG was given (i.v.) to layer chickens, or when 0.1 to 0.5 nM/bird of AG were given (i.p.) to migratory birds, no differences in FI were observed (Kaiya et al., 2007; Goymann et al., 2017). The relative lower doses, treatment delivery, age, and type of bird can explain differences with the present experiment. Nevertheless, the vast majority of authors that reported the effect of exogenous AG on feed intake (Table 1) agree with our data. The anorexigenic effect of AG in chicken broilers was evident in the present experiment and it was associated with decreased ADG. Additionally, there were several orders of magnitude differences in the relationship between AG and FI when exogenous AG was infused continuously (regression slope =  $-50.7 \pm 10.9$  g/d) compared with pulsatile infusions (regression slope  $-10.8 \pm 3.1$  and  $-7.6 \pm 3.2$  g/d; Experiment 1 and 2, respectively). A sustained increase in AG appears to be necessary to elicit a physiological response to the anorexic effect of acylated-ghrelin and the associated decrease of ADG.

In contrast to the AG results, DAG doses resulted in increased FI when given as a pulse every 2 h or continuously. Our results are in conflict with the 2 other publications that reported the use of DAG infusions in birds. When 0 to 0.1 nM/bird of DAG were given (ICV) to layer chickens, or when 0.1 to 0.5 nM/bird of DAG were given (i.p.) to migratory birds, no differences (Tachibana et al., 2011) or a decrease (Goymann et al., 2017) in FI was observed. The route of administration, type of bird, treatment delivery, and age of birds (among others) can explain differences between results. Nevertheless, co-injection of DAG and AG attenuated the AG-induced anorexia in chickens (Tachibana et al., 2011). The differential effect DAG and AG on feed intake has also been reported in rats and goldfishes (Chen et al., 2005; Matsuda et al., 2006). In fact, it has been shown that DAG can independently have other physiological effects in mammals, including vasodilation and apoptosis (Chung et al., 2008; Ku et al., 2015).

The orexigenic effect of DAG in chicken broilers was clear in the present experiment and was also associated with increased ADG. We also noted that there were several orders of magnitude differences in the slope of ADG when DAG was infused continuously (slope =  $39.1 \pm 9.9$  g/d) compared with pulsatile infusions (slope =  $6.7 \pm 2.5$  g/d, Experiment 2). Given the low affinity of DAG to GHSR-1a, our data and that from others (Cassoni et al., 2004; Toshinai et al., 2006), points to the presence of a still unknown DAG specific receptor (or another elusive mechanism) that requires a sustained increase in DAG to elicit an effective physiological response.

Corticosterone concentrations were increased when AG was infused with a pulse every 2 h (Experiment 2) or continuously (Experiment 3). These data agree with the direct effect of AG on the secretion of CRH as previously reported (Kaiya et al., 2002; Saito et al., 2005). In turn, adrenocorticotrophic hormone stimulates the secretion of corticosterone from the adrenal glands. However, no changes in corticosterone were found when DAG was infused at any dose or frequency (Experiments 2 and 3). As noted above, the incorporation of an octanoic acid in ghrelin is required for the activation of the GHSR-1a receptor, while the affinity of des-acyl-ghrelin to GHSR-1a is minimal. Data suggest that DAG's modus operandi does not involve the HPA axis.

The ability of AG to stimulate GH release is by binding to the GHSR-1a that, in turn, activates the phospholipase C and protein kinase C pathway leading to calcium influx, resulting in GH secretion (Kohno et al., 2003). Findings from this study on the stimulatory effect of AG on GH are in line with other studies (Ahmed and Harvey, 2002). However, the increased secretion of GH was observed only when AG was given as pulse every 2 h (Experiment 2) but not when it was infused continuously (Experiment 3) suggesting a potential downregulation of the receptor as previously reported (Geelissen et al., 2003). Collectively, data from the present experiments indicate that the pulsatile nature of acylated ghrelin is necessary to elicit a physiological response for the release of GH, whereas a sustained increase in AG is more effective in decreasing feed intake. On the other hand, DAG did not elicit an increase in GH when it was infused with a pulse every 2 h (Experiment 2) or continuously (Experiment 3). Taken together, the inability of DAG to trigger the release of corticosterone and GH suggests a mechanism of action independent of AG.

In conclusion, our data demonstrate that doses, pulse frequency, and ghrelin forms differentially affect feed intake, body weight gain, and concentrations of corticosterone and GH in broiler chickens. The endocrine control of appetite in chickens by ghrelin appears to be governed by the 2 forms of ghrelin (i.e., AG and DAG) working in opposite directions. However, only AG has the ability to modulate GH (when infused in pulses every 2 h) and corticosterone secretion, whereas DAG is not involved in the secretion of corticosterone or GH.



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## DISCLOSURES

No personal, professional or financial conflicts of interest are associated with any of the authors.

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