Metabolic status and ghrelin regulate plasma levels and release of ovarian hormones in layer chicks.

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RUNNING TITLE: ghrelin, feeding and hormones in chicken

Abstract

The aim of the present study was to examine the role of nutritional status, the metabolic hormone ghrelin and their interrelationships in the control of chicken hormones involved in the regulation of reproduction. For this purpose, we identified the effect of food deprivation, administration of ghrelin 1-18 and their combination on plasma levels of testosterone (T), estradiol (E), arginine-vasotocin (AVT) and growth hormone (GH) as well as the release of these hormones by isolated and cultured ovarian fragments.

It was observed that food deprivation reduces plasma T and E and increases plasma AVT and GH levels. Food restriction also reduced the amount of E produced by isolated ovaries, but it did not affect the ovarian secretion of T and AVT. No ovarian GH secretion was detected. Ghrelin administered to *ad-libitum* fed chickens did not affect plasma T and E levels, but it did increase plasma GH and AVT concentrations. Moreover, it partially prevented the effect of food deprivation on plasma E and AVT levels, but not on T or GH levels. Ghrelin administration to control birds promoted ovarian T, but not E or AVT release and reduced T and not other hormonal outputs in birds subjected to food restriction.

Our results (1) confirmed the ovarian origin of the main plasma T and E and the extra-ovarian origin of the main blood AVT and GH; (2) showed that food deprivation-induced suppression of reproduction may be caused by suppression of T and E and the promotion of AVT and GH release; (3) suggest the involvement of ghrelin in control chicken E, AVT and GH output; and (4) indicates that ghrelin can either mimic or modify the effect of the intake of low calories on chicken plasma and ovarian hormones, i.e., it can mediate the effect of metabolic state on hormones involved in the control of reproduction.

Introduction

Reproduction is an energy-demanding process; therefore, it should be synchronized with optimal nutritional conditions. It has been postulated that the hypothetical mediator of the nutritional effect on reproduction (1) should be affected by nutrition and (2) controls reproduction, and (3) its changes should mimic and modify the effect of nutrition on reproduction. On the basis of these criteria, it is proposed that the nutritional status affects mammalian reproductive processes via the metabolic hormones, leptin, ghrelin and obestatin (see Tena-Sempere, 2008; Navarro and Kaiser, 2013; Roa and Tena-Sempere, 2014; Sirotkin, 2014 for review). In birds, malnutrition inhibits ovarian folliculogenesis, ovulation/egg laying and ovarian hormones release (Hocking, 2004; Sirotkin and Grossmann, 2015) most likely via the induction of ovarian follicular cells apoptosis (Paczoska-Eliasiewicz et al., 2003). Malnutrition is associated with changes in plasma and brain ghrelin as well as its receptors (Kaiya et al., 2007, 2013; Sirotkin et al., 2013). Ghrelin im administration in chicken is able to reduce plasma progesterone (P) levels (Sirotkin et al., 2015), which directly alters proliferation, apoptosis, steroidogenesis and protein kinases in cultured ovarian cells (Sirotkin et al., 2006; Sirotkin and Grossmann, 2007, 2008), and prevents the food restriction-induced decrease in ovarian testosterone (T), estradiol (E) and arginine-vasotocin (AVT) release (Sirotkin et al., 2015). Such data have demonstrated the importance of ghrelin in integrating nutrition and reproduction and its potential applicability for the improvement of farm avian reproduction. Nevertheless, the mediatory role of ghrelin in the metabolic control of ovarian functions has only been previously demonstrated in one study (Sirotkin et al., 2015). Moreover, in the described study, ghrelin and food restriction effects only on hormonal release by ovarian tissue in vitro, but not on plasma hormones under in-vivo conditions, were examined.

The general aim of the present study was to examine the role of the nutritional status, metabolic hormone ghrelin and their interrelationships in the control of chicken steroid and peptide hormones involved in the regulation of reproduction. For this purpose, we used both in-vivo and in-vitro approaches to identify the effect of food deprivation, administration of ghrelin 1-18 and their combination on plasma level of hormones (T, E, AVT and growth hormone, GH) whose are known autocrine/paracrine and endocrine regulators of both mammalian and avian ovarian functions (Sirotkin, 2005, 2014; Luna et al., 2014; Hrabia, 2015) as well as the release of these hormones by isolated and cultured ovarian fragments.

Materials and Methods

2.1 Animal experiments, tissue collection and culture

Young (approximately 8 months of age) White Leghorn hens (LSL), weight 1.1-1.2 kg with an egg-laying rate of more than 95%, were housed in individual cages under standard conditions at the Experimental Station of the Institute of Animal Science on a photoperiod 12L:12D (illumination 8.00-20.00). The conditions of their care, manipulations and use corresponded the instructions of the EC no. 178/2002 and related EC documents, and the protocols were approved by the local ethics commission. After a two-month adaptation period to the conditions of the experimental farms, the hens were divided into four experimental groups: (1) the control group was fed ad libitum, no hormone treatment; (2) the group fed *ad libitum* and treated with i.m. injection of human recombinant research grade ghrelin 1-18 (Peptides International Inc., Louisville, Kentucky, USA) (this truncated ghrelin analogue ghrelin 1-18 mimicked the effect of full-length ghrelin 1-28 on chicken ovarian cells (Sirotkin and Grossmann, 2008); (3) the group subjected to food deprivation, no hormone treatment; and (4) the group subjected to food deprivation and treated with i.m. injection of human recombinant ghrelin 1-18. The animals of the food-deprivation groups had no access to food

during the entire experiment for 72 hours, whereas all of the animals had permanent access to drinking water. Hormonal treatments combined with food deprivation began together with food restriction. Ghrelin was dissolved in sterile 0.7% NaCl immediately prior to the start of the experiments and injected i.m. at doses 30 μg/animal in 1 ml of 0.7% NaCl. This consecutive injection was done for 3 days, every 10-12 hours, in the daytime (at 8.00 and 18.00). This dose, injection and sampling time (see below) were comparable to the amount of hormones in the chicken organism and those treated with previously reported experiments (Kaiya et al., 2007; Sirotkin et al., 2013, Sirotkin and Grossmann, 2015). Next, 1.5 hours after the last injection (between 9.00 and 11.00 a.m.), the animals were killed by decapitation. Their blood was collected in heparinized tubes, and the plasma was separated by 10 min centrifugation at 500 × g and frozen at -70°C until radioimmunoassay (RIA) or enzyme immunoassay (EIA). The largest (F1) follicles were isolated from the ovary. The stage of folliculogenesis was determined by recording the time of the last oviposition and by the size and position of the next ovarian follicle. Fragments of the follicular wall (5 mm in diameter, weight 24±8 mg) were isolated as previously described (Sirotkin and Grossmann, 2003, 2006). After washing three times in sterile culture medium (DMEM/F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic-antimycotic solution (all from Sigma, St. Louis, USA), these fragments were cultured without treatment for 2 d in 2 ml culture medium in Falcon 24-well plates (Becton Dickinson, Lincoln Park, USA) at 38.5°C under 5% CO_2 in humidified air. This protocol yields the maximal accumulation of ovarian hormones in the culture medium, which is the most reliable characteristic of ovarian secretory activity (Sirotkin and Grossmann, 2003).

Immunoassay

Concentrations of testosterone (T), estradiol (E), arginine-vasotocin (AVT) and growth hormone (GH) were determined in 25 µl aliquots of plasma or incubation medium by EIA and RIA, whose were previously validated for use in culture medium (Sirotkin et al., 2006; Sirotkin and Grossmann, 2007). These hormones were considered as the indices of ovarian secretory activity, stress, response to hormonal stimuli and the key regulators of both mammalian (Sirotkin, 2014) and chicken (Sirotkin and Grossmann, 2006, 2007, 2008; Luna et al., 2014; Sirotkin, 2014; Hrabia, 2015) ovarian functions.

T was assayed according to Münster (1989) using antisera against steroids (produced in the Institute of Animal Science, Neustadt, Germany). The sensitivity of the assay was 10 pg/ml. The cross-reactivity of the T antiserum was ≤96 % to dihydrotestosterone, ≤3 % to androstenedione, ≤0.01 % to P_4 and E_2, ≤0.02 % to cortisol and ≤0.001% to corticosterone. The inter- and intraassay coefficients of variation were 12.3% and 6.8%, respectively.

E concentrations were evaluated according to Münster (1989) using antisera against steroids (produced by the Institute of Animal Science, Neustadt, Germany) with an assay sensitivity of 5 pg/ml. The cross-reactivity of the E_2 antiserum was < 2 % to estrone, ≤ 0.3 % to estriol, ≤ 0.004% to T and ≤0.0001% to P_4 and cortisol. The inter- and intraassay coefficients of variation did not exceed 16.6% and 11.7%, respectively.

AVT was determined using RIA according to Gray and Simon (1983). The anti-AVT antiserum was kindly provided by Dr. D.A. Gray (Max-Plank Institute for Physiological and Clinical Research, Bad Nauheim, Germany), which cross-reacted ≤1.0 % with mesotocin and angiotensin II. The sensitivity of the RIA was 0.3 pg/ml. The inter- and intraassay coefficients of variation did not exceed 8.8 % and 7.2 %, respectively.

GH was measured using the EIA based on the EIA used for porcine GH (Serpek et al., 1993) and adapted for determination of chicken GH (Zheng et al., 2007). Chicken GH for standards (AFP-9020C), iodination (AFP 7678B) and antiserum against chicken GH (AFP-551-11-1-86

dilution 1:720,000) were kindly provided by Dr. A.P.F. Parlow (National Hormones and Pituitary Program, Bethesda, USA). This antiserum has 0.7% cross-reactivity with chicken prolactin and <0.001% cross-reactivity with P_4, T, E_2 and AVT. The sensitivity of the assay was 0.2 ng/ml. The inter- and intraassay coefficients of variation did not exceed 12.9% or 10.8%, respectively.

Statistics

The data shown are the mean of the values obtained in three separate experiments performed on separate days using independent animals (8 animals per group) and their ovaries. In each in-vitro experiment, each experimental group consisted of six culture wells with ovarian fragments. Assays of the hormone levels in the incubation media were performed in duplicate. The values of the blank control were subtracted from the value determined using RIA/EIA in the cell-conditioned medium to exclude any non-specific background (less than 15% of the total values). The rates of substance secretion were calculated per mg tissue / day. Significant differences between the experiments were evaluated using two-ways ANOVA. When effects of the treatments were revealed, data obtained from the experimental and control groups were compared using the Wilcoxon-Mann-Whitney multiple range test with Sigma Plot 11.0 statistical software (Systat Software, GmbH., Erkrath, Germany). Differences compared to control were considered significant if $P < 0.05$.

Results

In chicken blood plasma. T, E, AVT and GH were detected (Fig.1). The culture medium conditioned by cultured ovarian fragments contained normal amounts of T, E and AVT, but not of measurable GH (Fig.2). These parameters were affected by food restriction, administration of ghrelin and the combination of the above factors.

Food deprivation significantly reduced the concentrations of both T (Fig. 1A), E (Fig. 1B) and increased the level of both AVT (Fig. 1C) and GH (Fig. 1D) in plasma. Ghrelin administered to ad-libitum fed chickens did not affect plasma T (Fig. 1A) or E (Fig. 1B) levels, but it did increase plasma AVT (Fig. 1C) and GH (Fig. 1D) concentrations. Moreover, ghrelin administration could partially prevent the effect of food restriction on plasma E (Fig. 1B) and AVT (Fig. 1C), but not on T (Fig. 1A) or GH (Fig. 1D) levels.

Analysis of hormones produced by ovarian tissue in vitro demonstrated that food deprivation reduced the amount of E produced by the ovary (Fig. 2B), but it did not affect the ovarian secretion of T (Fig. 1A) or AVT (Fig. 2C). Ghrelin administration to control birds promoted ovarian T (Fig. 2A), but not E (Fig. 2B) or AVT (Fig. 2C) release. Furthermore, ghrelin reduced T (Fig. 2A), but not E (Fig. 2B) or AVT (Fig. 2C) output by the ovaries of birds subjected to food deprivation.

Discussion

Our observations showed the availability of steroid hormones, AVT and GH in chicken blood plasma and the release of steroid hormones and AVT by cultured chicken ovarian tissue, but our assay failed to detect measurable GH production by chicken ovarian cells reported by other investigators (Luna et al., 2014; Hrabia, 2015) which can be explained by low GH production by chicken ovarian cells in our experiments.

A comparison of the hormones that are available in general circulation and those that are produced by isolated ovarian tissues and its changes under the effect of extra-ovarian factors demonstrates that some hormones found in blood (E, T, AVT) are mainly ovarian, but other factors (GH) are mainly extra-ovarian in origin. Furthermore, differences in the mechanisms controlling the release of these hormones were observed. For example, food deprivation reduced E in both plasma and ovarian fragment-conditioned medium. This finding suggests that the negative metabolic state directly affects ovarian E output. In contrast, the effect of

food restriction on plasma T and AVT, but not on their release by isolated ovarian tissue, suggest that the metabolic state controls T and AVT release via upstream extra-ovarian regulatory mechanisms (probably via hypothalamo-hypophysial system involved in metabolic control of gonadal functions, Roa and Tena-Sempere, 2014).

One such extra-ovarian regulator could be ghrelin. Ghrelin could influence chicken ovarian steroid hormones in both our previous (Sirotkin et al., 2006, 2015; Sirotkin and Grossmann, 2007, 2008) and present studies. In some previous (Sirotkin et al., 2006 Sirotkin and Grossmann, 2008), but not in our present experiments, ghrelin promoted chicken ovarian AVT output, which can be explained by variations in initial state of AVT producing cells between the experiments. Furthermore, our present observations of ghrelin-induced increase in plasma GH confirmed previous findings (Baudet and Harvey, 2003) that ghrelin is a physiological GH secretagogue not only in the mammalian, but also in the avian pituitary. The observed ghrelin-induced changes could be due to influence of ghrelin on hormonal regulators of reproduction at the level of the ovary (steroids), the upstream hypothalamo-hypophysial system (GH) or on the differentiation of CNS and ovarian tissue. The site and fine mechanisms of ghrelin influence on organisms require further studies, but the present observations suggest the action of ghrelin at both CNS and gonadal level. In our experiments, the pronounced effect of ghrelin on testosterone release by ovarian tissue (Fig.2A) was not associated with the corresponding changes in plasma testosterone (Fig.1A). It suggests that the direct action of ghrelin on the ovary could be masked by additional factors in CNS or general circulation affecting steroid transport, binging or degradation.

Our observations confirmed previous reports that food deprivation reduces ovarian steroid hormones (Paczoska-Eliasiewicz et al., 2003, Sirotkin and Grossmann, 2015) and promotes GH (Buyse et al., 2000, 2002) levels in chicken plasma. The food deprivation-induced increase in blood AVT levels observed in our experiments and the fasting-induced reduction in ovarian AVT release observed in our previous (Sirotkin and Grossmann, 2015) but not our

present studies indicated that the metabolic state can affect blood and maybe ovarian AVT. Variations in initial state of ovarian AVT producing cells between the experiments could influence ovarian AVT response not only to ghrelin (see above) but also to food restriction. The steroid hormones GH and AVT are known regulators of both mammalian and avian reproductive processes including ovarian steroidogenesis (Sirotkin, 2005, 2014; Luna et al., 2014; Hrabia, 2015). Thus, it is possible that food deprivation can affect reproductive processes via changes in the release of these peptide and steroid hormones. In addition, the metabolic state can affect these hormonal regulators of reproduction via ghrelin. Food consumption affects the production of chicken ghrelin, ghrelin acylation and ghrelin receptor (Kaiya et al., 2007, 2013; Sirotkin et al., 2013; Sintubin et al., 2014). Furthermore, this is the first evidence that ghrelin can mimic the effect of food deprivation on plasma GH and AVT, and that ghrelin can modify the effect of food deprivation on chicken plasma hormones. Taken together, combined with the data on the importance of ghrelin in the control of basic ovarian functions (see above), these evidence suggest that ghrelin can be the key hormone mediating the effect of the metabolic state on downstream hormonal regulators of avian ovarian functions.

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FIGURE LEGENDS

Fig. 1.

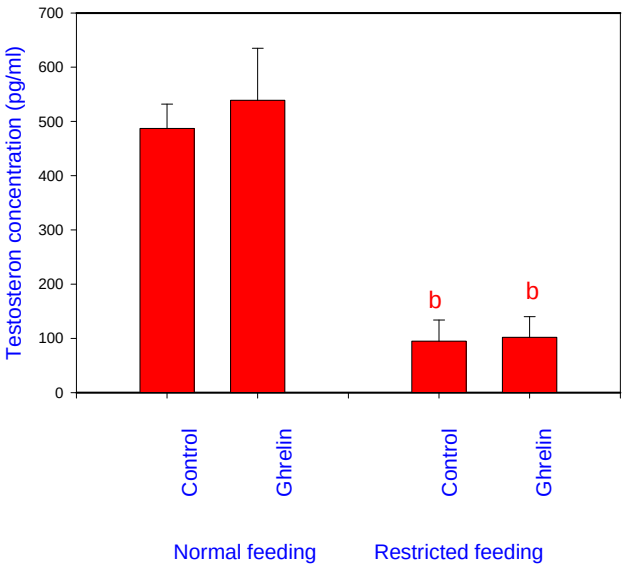
Effect of food restriction and administration of ghrelin 1-18 (in vivo) on the levels of testosterone (A), estradiol (B), arginine-vasotocin (C) and growth hormone (D) in chicken blood plasma. Data are the mean ± S.E.M. Differences between the groups at $P < 0.05$ were considered significant: a) effect of hormones administration (differences between control and hormone-treated chicken); b) effect of food restriction (differences between corresponding groups of chickens subjected and not subjected to food restriction).

Fig. 2.

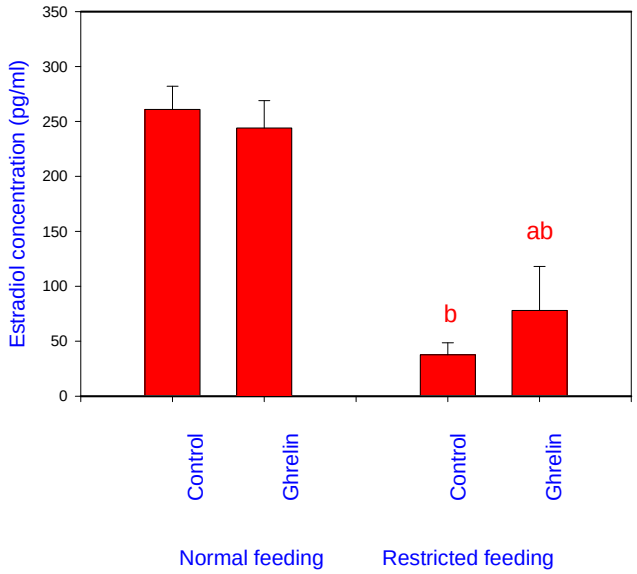
Effect of food restriction and administration of ghrelin in vivo on the release of testosterone (A), estradiol (B) and arginine-vasotocin (C) by isolated chicken ovarian fragments. Legends are similar to those presented in Fig. 1.

Fig. 1

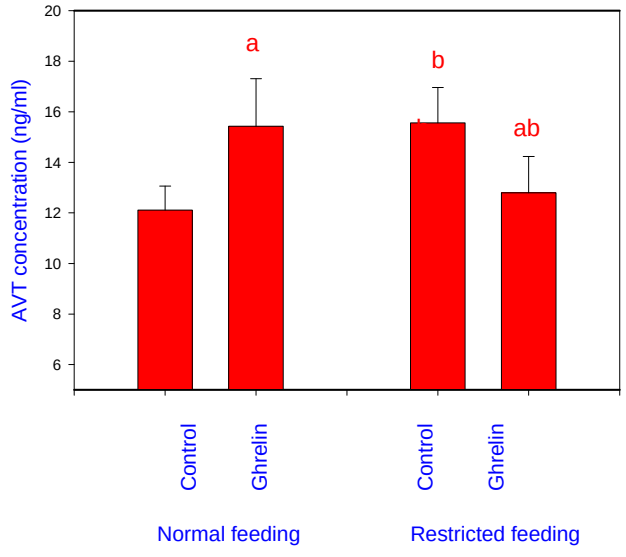
A


Testosteron concentration (pg/ml)
700
600
500
400
300
200
100
0
b
b
Control
Ghrelin
Control
Ghrelin
Normal feeding
Restricted feeding

B


Estradiol concentration (pg/ml)
350
300
250
200
150
100
50
0
b
ab
Control
Ghrelin
Control
Ghrelin
Normal feeding
Restricted feeding

C


AVT concentration (ng/ml)
20
18
16
14
12
10
8
6
a
b
ab
Control
Ghrelin
Control
Ghrelin
Normal feeding
Restricted feeding

D

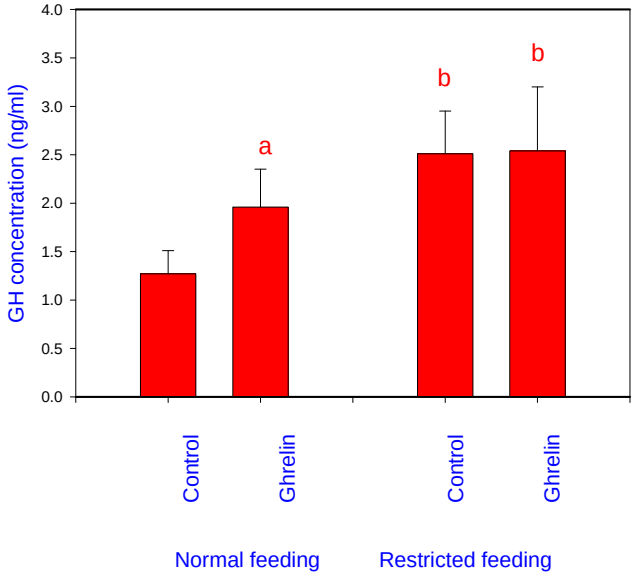
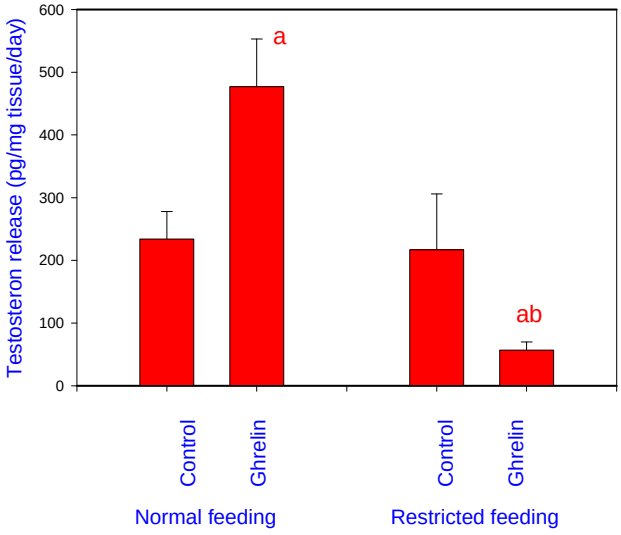
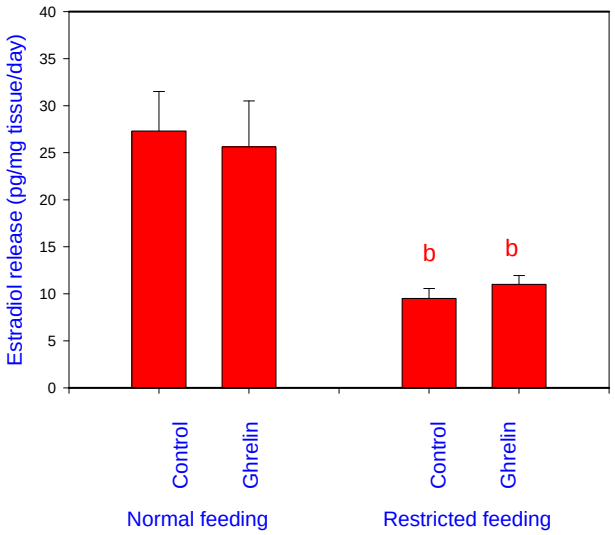

GH concentration (ng/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
Control
Ghrelin
Control
Ghrelin
Normal feeding
Restricted feeding

Fig. 2

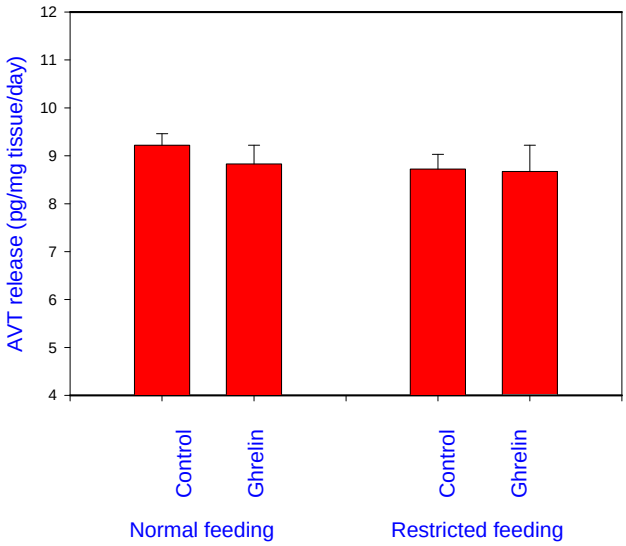
A


Testosteron release (pg/mg tissue/day)
600
500
400
300
200
100
0
a
ab
Control
Ghrelin
Control
Ghrelin
Normal feeding
Restricted feeding

B


Estradiol release (pg/mg tissue/day)
40
35
30
25
20
15
10
5
0
b
b
Control
Ghrelin
Control
Ghrelin
Normal feeding
Restricted feeding

C


AVT release (pg/mg tissue/day)
12
11
10
9
8
7
6
5
4
Control
Ghrelin
Control
Ghrelin
Normal feeding
Restricted feeding