The effects of chronic testosterone administration on body weight, food intake, and fat weight were age-dependent

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Abstract

Previously, we showed that chronic testosterone administration increased body weight (BW) and food intake (FI), but did not alter fat weight, in young female rats. To examine our hypothesis that the effects of androgens on BW, FI and body composition might be age-dependent, the effects of chronic testosterone administration were evaluated in rats of different ages; i.e., young and middle-aged rats. Although chronic testosterone administration increased BW gain, FI, and feed efficiency in both young and middle-aged rats, it increased visceral fat weight in middle-aged rats, but not in young rats. Therefore, it is possible that testosterone promotes the conversion of energy to adipose tissue and exacerbates fat accumulation in older individuals. In addition, although the administration of testosterone increased the serum leptin level, it did not alter hypothalamic neuropeptide Y mRNA expression in middle-aged rats. On the contrary, the administration of testosterone did not affect the serum leptin levels of young rats. Thus, testosterone might induce hypothalamic leptin resistance, which could lead to fat accumulation in older individuals. Testosterone might disrupt the mechanisms that protect against adiposity and hyperphagia and represent a risk factor for excessive body weight and obesity, especially in older females.

Key words

testosterone, aging, leptin, visceral fat, subcutaneous fat

Introduction

Energy balance and reproduction are closely linked in most species. The sex hormones, estrogen and androgens, are involved in the regulation of food intake (FI), body weight (BW), body composition, and energy metabolism in mammals and humans [1]. In females, estrogen plays pivotal roles in preventing excess BW gain and adiposity by suppressing FI and increasing energy metabolism. For example, ovariectomy increases FI and BW in female mammals, and these effects can be prevented by estradiol (E2) replacement [2]. Some of estrogen's effects are mediated by estrogen receptor-α (ER-α) in the hypothalamus and brainstem, and the injection of E2 into the hypothalamic paraventricular nucleus decreased BW and FI in ovariectomized (OVX) rats [3,4]. However, as most of these studies involved young animals it remains unclear whether older animals continue to be responsive to the nutritional and metabolic effects of estrogen. Recently, Santollo et al. have shown that young and middle-aged female rats exhibit similar sensitivity to the anorexigenic effects of E2, suggesting that the response to estrogen is maintained in older individuals [5].

On the other hand, the roles of androgens in female FI and BW regulation have not been fully examined, although some studies have shown that androgens increase FI and BW in females [1,6-8] and raise the risk of visceral obesity in women and experimental animals [9-13]. In our previous study, we evaluated the effects of chronic testosterone administration on BW, FI, and body composition in young female rats [14]. As a result, we found that BW gain and FI were increased by chronic testosterone administration, but the weights of visceral and subcutaneous fat were not changed. Interestingly, BW gain,

FI, and fat weight were not altered by chronic testosterone administration in OVX rats in the latter study. Therefore, these findings indicate that, similar to the actions of estrogen, the effects of androgens on BW, FI, and body composition might differ in an age-dependent manner.

To further investigate this hypothesis, we evaluated the effects of the chronic administration of testosterone in different age groups; i.e., young and middle-aged rats, in the present study. The effects of chronic testosterone administration on BW, FI, and visceral and subcutaneous fat accumulation were assessed. In addition, the levels of neuropeptide Y (NPY) and prepro-orexin (pp-orexin), which are peripheral and hypothalamic orexigenic factors, respectively, and leptin, proopiomelanocortin (POMC), and the leptin receptor (OBRb), which are anorexigenic factors, were also investigated because it has been reported that sex steroids affect the serum and hypothalamic levels and actions of these factors [15-18]. The hypothalamic mRNA expression level of the androgen receptor (AR) was also measured because the AR plays pivotal roles in the regulation of BW and FI [1].

Experimental

Animals

Eighteen young (10 weeks of age) and 16 middle-aged (12 months of age) Sprague-Dawley female rats (Charles River, Kanagawa, Japan) were housed in a room under controlled lighting (12 h light, 12 h darkness; lights turned on at 0800 and turned off at 2000) and temperature (24°C) conditions with free access to food and water. All animal

experiments were conducted in accordance with the ethical standards of the institutional animal care and use committee of the University of Tokushima. Rats were randomly assigned to either the testosterone-administered (T) or the no testosterone (control) group (n = 8 or 9 per group). In the testosterone group, a silastic tube filled with crystalline testosterone was implanted into each rat (inner diameter: 3 mm, outer diameter: 5 mm, length of the filled part: 30 mm) (As One Co., Ltd., Tokyo, Japan) (De Vries et al., 1994). In the control group, an empty tube was implanted into each rat. BW and cumulative FI were assessed every week, and the rats were sacrificed by decapitation at 4 weeks (wk) after the implantation procedure. The implantation and decapitation were carried out under sevoflurane anesthesia. The brain, blood, visceral fat (parametrial, perirenal, and mesenteric deposits), and subcutaneous fat (inguinal deposits) were collected. The weights of visceral and subcutaneous fat were assessed immediately after removal, and samples (around 300-400 mm^3) of visceral (parametrial) fat were dissected. The serum was separated by centrifugation and stored at –20°C, and the tissue samples were stored at −80°C.

Hormone assay

Serum leptin levels were measured using radioimmunoassay kits (multi-species leptin RIA kit, Linco Research Inc., MO, USA). The sensitivity of the assay was 1.0 ng/ml, and its inter- and intra-assay coefficients of variation were 3.2% and 7.8%, respectively. Serum estradiol (E2) levels were measured by a commercial laboratory (SRL, Tokyo, Japan.) using a chemiluminescence immunoassay. Detection limit of E2 kit is 10 pg/mL and co-efficient of variations is smaller than 7%.

Quantitative real-time polymerase chain reaction

Whole hypothalamic explants were dissected from the frozen brains, as described previously [14]. Briefly, the brain sections were dissected out via an anterior coronal cut at the posterior border of the mammillary bodies, parasagittal cuts along the hypothalamic fissures, and a dorsal cut 2.5 mm from the ventral surface. Total RNA was isolated from the hypothalamic explants and visceral fat using a TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy® mini kit (Qiagen Gmbh, Hilden, Germany). Then, cDNA was synthesized with oligo (deoxythymidine) primers at 50°C using the SuperScript III first-strand synthesis system for the real-time polymerase chain reaction (PCR; Invitrogen Co.). The PCR analysis was performed using the StepOnePlus™ real-time PCR system (PE Applied Biosystems, Foster City, CA, USA) and FAST SYBR® green. The hypothalamic mRNA levels of OBRb, the AR, NPY, POMC, and pp-orexin and the leptin mRNA level in visceral fat were measured. The mRNA expression level of each factor was normalized to that of GAPDH or the 18S rRNA level. Dissociation curve analysis was also performed for each gene at the end of the PCR. Each amplicon generated a single peak. The relevant primer sequences, product sizes, and annealing temperatures are shown in Table 1. The PCR conditions were as follows: initial denaturation and enzyme activation were carried out at 95°C for 20 s, followed by 45 cycles of denaturation at 95°C for 3 s, and annealing and extension for 30 s.

Statistical analysis

All results are presented as mean ± SEM values. Two-way repeated or factorial ANOVA was used to compare the effects of testosterone between young and middle-aged rats.

Tukey-Kramer *post-hoc* analysis was used to determine individual group differences following the detection of significant main or ANOVA effects (P <0.05).

Results

At the beginning of the study, the mean BW of the young groups were significantly lower than those of the middle-aged groups (young-control: 222.7 ± 3.1 g, young-T: 221.3 ± 3.3 g, middle-control: 423.7 ± 12.2 g, middle-T: 426.3 ± 8.8 g). At 4 wk after the implantation procedure, the serum E2 levels of the examined groups did not differ (young-control: 32.4 ± 3.2 pg/mL, young-T: 32.9 ± 3.6 g, middle-control: 42.4 ± 6.2 g, middle-T: 28.3 ± 6.8 g).

Repeated-measures ANOVA revealed a significant interactive effect of treatment × time on the change in BW (F = 69.3, P <0.01) (Fig. 1A). Both T (F = 58.0, P <0.01) and age (F = 4.70, P <0.01) had significant effects on the BW change seen at 4 wk after the implantation procedure (Fig. 1B). *Post-hoc* analysis showed that the BW changes observed at 4 wk after the implantation procedure in the young-T and middle-T groups were significantly greater than those seen in the young-control and middle-control groups, respectively (Fig. 1B). It also showed that the BW changes observed at 4 wk after the implantation procedure in the young-control and young-T groups were significantly greater than those seen in the middle-control and middle-T groups, respectively. Repeated-measures ANOVA revealed a significant interactive effect of treatment × time on cumulative FI (F = 163.3, P <0.01) (Fig. 1C). Both T (F = 31.0, P <0.01) and age (F = 28.0, P <0.01) had significant effects on cumulative FI at 4 wk after the implantation

procedure (Fig. 1D). *Post-hoc* analysis showed that cumulative FI at 4 wk after the implantation procedure was significantly greater in the young-T and middle-T groups than in the young-control and middle-control groups, respectively (Fig. 1D). It also showed that the young-control and young-T groups exhibited significantly greater cumulative FI at 4 wk after the implantation procedure than the middle-control and middle-T groups, respectively.

Both T ($F = 171.7$, $P < 0.01$) and age ($F = 7.16$, $P < 0.01$) had significant effects on feed efficiency (FE). *Post-hoc* analysis showed that FE was significantly higher in the young-T and middle-T groups than in the young-control and middle-control groups, respectively (Fig. 2A). It also demonstrated that FE was significantly higher in the young-control and young-T groups than in the middle-control and middle-T groups, respectively. Both T (visceral fat: $F = 5.40$, $P=0.03$; total fat: $F = 6.13$, $P=0.02$) and age (visceral fat: $F = 5.82$, $P < 0.01$; total fat: $F = 7.95$, $P < 0.01$) had significant effects on relative visceral fat weight and total fat weight. *Post-hoc* analysis showed that the relative visceral fat weight and total fat weight of the middle-T group were significantly heavier than those of the middle-control group (Figs. 2B and D). It also demonstrated that the relative visceral fat weight and total fat weight of the young-control and young-T groups were significantly lighter than those of the middle-control and middle-T groups, respectively.

Both T ($F = 6.13$, $P < 0.01$) and age ($F = 7.95$, $P < 0.01$) had significant effects on relative lean body mass. *Post-hoc* analysis showed that the relative lean body mass of the middle-T group was significantly lighter than that of the middle-control group (Fig. 2E). It also demonstrated that the relative lean body mass values of the young-control and

young-T groups were significantly heavier than those of the middle-control and middle-T groups, respectively.

Both T ($F = 11.8$, $P < 0.01$) and age ($F = 7.64$, $P < 0.01$) had significant effects on the serum leptin level. *Post-hoc* analysis showed that the serum leptin levels of the middle-T group were significantly higher than those of the middle-control group (Fig. 3A). It also demonstrated that the serum leptin levels of the young-control and young-T groups were significantly lower than those of the middle-control and middle-T groups, respectively. Neither age nor T had significant effects on the leptin mRNA level in visceral fat (Fig. 3B). Multiple comparisons tests showed that the visceral fat leptin mRNA levels of the young-control and young-T groups were significantly lower than those of the middle-control and middle-T groups, respectively.

Age ($F = 4.40$, $P < 0.01$), but not T ($F = 4.38$, $P = 0.05$), had significant effects on the hypothalamic OBRb mRNA level. *Post-hoc* analysis showed that the hypothalamic OBRb mRNA level of the middle-control group was significantly higher than that of the young-control group (Fig. 4A). Neither age nor T had any effect on the mRNA levels of other hypothalamic factors (Figs. 4B-E).

Discussion

As mentioned above, it has been shown that young and middle-aged female rats exhibit similar sensitivity to the anorexigenic effects of E2 and that older animals retain responsiveness to the nutritional and metabolic effects of estrogen [5]. On the other hand, although we have demonstrated that chronic testosterone administration promotes BW

gain and FI, but does not alter fat weight, in young female rats [14], it remains unclear whether these effects of testosterone vary according to age. In the present study, it was found that BW gain and FI were promoted by chronic testosterone administration in both young and middle-aged rats. In addition, FE was increased by testosterone administration in both groups, indicating that testosterone stimulates the conversion of energy to nutritional components. On the contrary, the effects of testosterone administration on body fat differed between young and middle-aged rats; i.e., it did not affect body fat accumulation in young rats, whereas it increased visceral fat weight in middle-aged rats. As well as the present findings, it has been reported that middle-aged (17 months) and old (31 months) rats exhibited significantly greater fat weights than young (4 months) rats [19]. Therefore, it is possible that testosterone administration promotes the conversion of energy to adipose tissue and exacerbates fat accumulation in older individuals. As far as we know, this is the first study to focus on the relationship between age and the effects of testosterone administration on BW, FI, and body composition.

In a previous study, we showed that the effects of chronic testosterone administration on BW and FI were abrogated in OVX rats, indicating that testosterone's effects depend on the estrogen milieu [14]. As the serum E2 levels of the young and middle-aged rats in the present study did not differ, the discrepancies in the effects of testosterone administration seen between these groups might not have been caused by differences in the estrogen milieu. On the other hand, it is possible that testosterone-induced increase of adiposity could be attributed to estrogen antagonism by androgens acting on estrogens. To clarify the mechanism responsible for these intergroup differences, the levels of

peripheral and hypothalamic orexigenic/anorexigenic factors and the AR were evaluated because it has been reported that these factors mediate the effects of gonadal steroids on BW, FI, and energy metabolism [1,15-18] and that the effects of E2 administration on the gene expression levels of these factors change according to age [5]. As a result, it was found that the serum leptin level was increased by testosterone administration in middle-aged rats, but not in young rats. In contrast, leptin mRNA expression was not affected by the administration of testosterone in either group, although the middle-aged rats displayed higher leptin mRNA expression than the young rats. Thus, the increase in the serum leptin level seen in the testosterone-treated middle-aged rats might have been due to greater fat deposition, rather than changes in leptin mRNA expression. It has been reported that leptin suppresses appetite and increases energy expenditure, and thereby, reduces FI and BW gain in non-obese conditions [20]. These effects of leptin on FI and BW are partially mediated by hypothalamic NPY; i.e., leptin downregulates hypothalamic NPY gene expression and suppresses appetite [21]. On the other hand, although obese humans and animals frequently exhibit elevated serum leptin levels [21,22], their hypothalamic sensitivity, including the sensitivity of NPY expression, to leptin are decreased, and this condition is called ‘leptin resistance’ [21]. Similarly, in the current study although the serum leptin level was increased by testosterone administration in the middle-aged rats, FI and BW gain were not suppressed, and the hypothalamic NPY mRNA expression level was not changed. In addition, the hypothalamic OBRb mRNA expression level was increased in the control middle-aged rats, which might be one of the counter-regulatory mechanisms that helps to prevent obesity, whereas no such changes in OBRb expression

were observed in the testosterone-treated middle-aged rats. Thus, it is possible that testosterone inhibits the mechanisms that protect against adiposity and obesity, and testosterone administration induced fat accumulation in middle-aged rats in the current study. On the other hand, we could not detect any testosterone-induced changes in the levels of other hypothalamic appetite regulatory factors; i.e., pp-orexin, POMC, and the AR. It has been reported that metabolic disturbances, such as obesity and insulin resistance, are predominant in older women with PCOS, which often associated with hyperandrogenism [23]. These results indicate that androgen might play important roles in the development of body weight gain and adiposity in older ages in women. Limitation of this study; because serum testosterone level in testosterone-administered groups were around 30-fold higher than that in control-groups (data not shown), indicating that the testosterone-administration induced supra-physiological testosterone levels in present study. Thus, another protocol, which induces lower testosterone level, would be needed to reproduce hyper-androgenic women.

There is one limitation in present study. Because serum estradiol levels were measured using chemiluminescence immunoassay, but not accurate methods such as LC/MS/MS, in this study, it is difficult to be sure whether changes in estradiol levels could be accurately detected between the groups. Therefore, further studies using more accurate measurement methods would be needed to clarify our hypothesis that testosterone’s effects on adiposity could be attributed to estrogen antagonism by androgens.

In summary, BW gain, FI, and FE were increased by chronic testosterone

administration in both young and middle-aged rats in the present study. These results indicate that testosterone stimulates the conversion of energy to nutritional components. Conversely, testosterone administration increased visceral fat weight in middle-aged rats, whereas it did not affect visceral fat weight in young rats. Therefore, testosterone administration might promote the conversion of energy to adipose tissue and exacerbate fat accumulation in older individuals. In addition, in the middle-aged rats although the serum leptin level was increased by testosterone administration, hypothalamic NPY mRNA expression was not affected. On the contrary, testosterone administration did not affect the serum leptin level in young rats. Thus, it is possible that testosterone induces hypothalamic leptin resistance, which might lead to the accumulation of fat in older individuals. Testosterone might disrupt the mechanisms that protect against adiposity and hyperphagia and could be a risk factor for excessive body weight and obesity, especially in older females.

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Figure legends

Fig. 1.

Body weight (BW) change (% of initial BW) (A, B) and cumulative food intake (FI) (g/100 g BW) (C, D) in each group. Data are expressed as mean ± SEM values. Values with different letters (a-c) are significantly different (P <0.05).

Fig. 2.

Feed efficiency (BW gain/cumulative FI) (A); relative visceral, subcutaneous, and total fat weight (g/100 g BW) (B-D); and relative lean body mass (g/100 g BW) (E) in each group. Data are expressed as mean ± SEM values. Values with different letters (a-c) are significantly different (P <0.05).

Fig. 3.

Serum leptin level (A) and leptin mRNA level in visceral fat (B) in each group. Data are expressed as mean ± SEM values. Values with different letters (a-c) are significantly different (P <0.05).

Fig. 4.

Hypothalamic mRNA levels of the leptin receptor (OBRb) (A), the androgen receptor (AR) (B), neuropeptide Y (NPY) (C), pp-orexin (D), and proopiomelanocortin (POMC)

(E) in each group. Data are expressed as mean ± SEM values. Values with different letters (a, b) are significantly different (P <0.05).

Fig. 1.

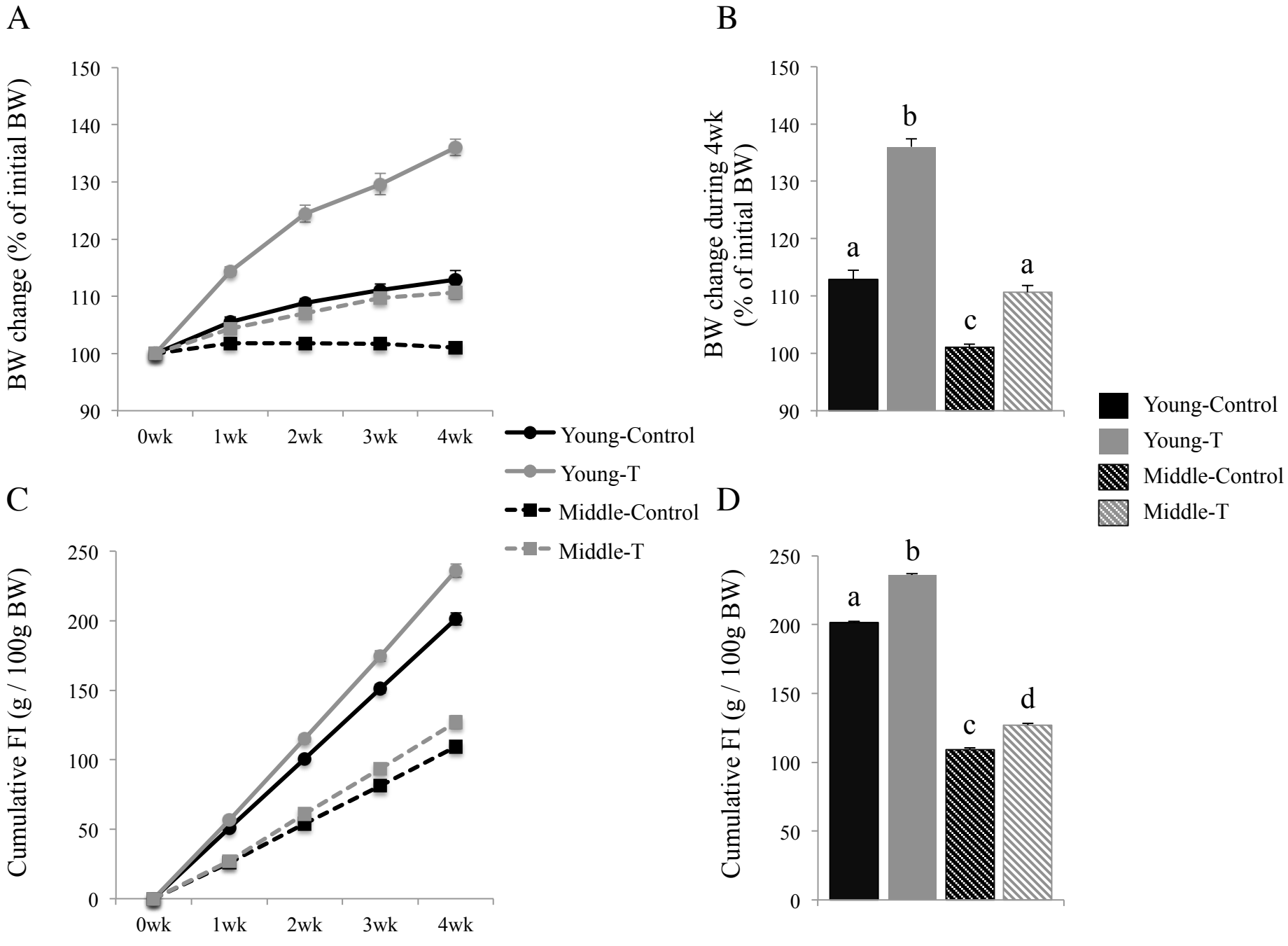
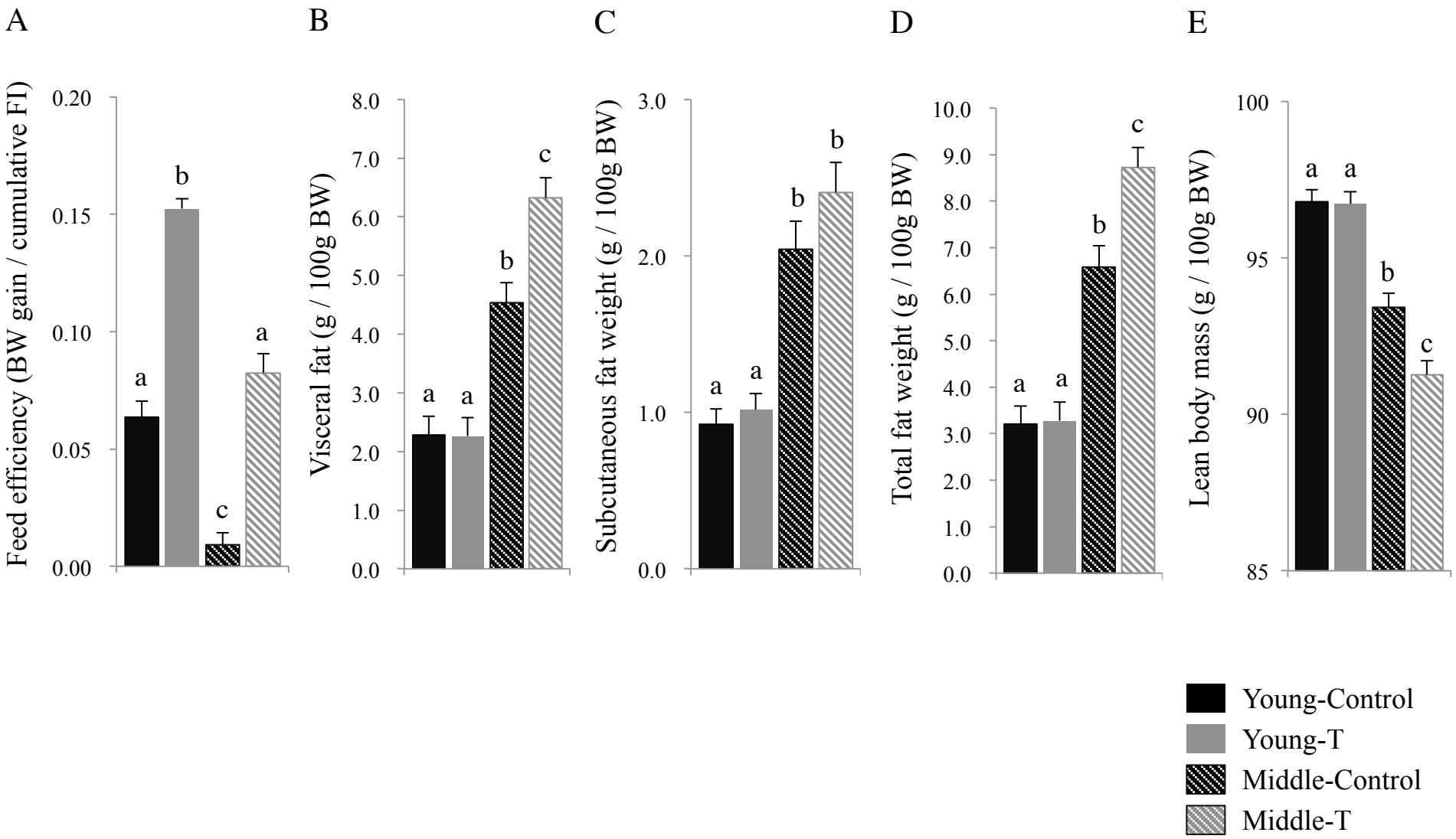

A
BW change (% of initial BW)
150
140
130
120
110
100
90
0wk
1wk
2wk
3wk
4wk
Young-Control
Young-T
Middle-Control
Middle-T
B
BW change during 4wk (% of initial BW)
a
b
c
a
C
Cumulative FI (g / 100g BW)
250
200
150
100
50
0
D
d

Fig. 2.


A
B
C
D
E
Feed efficiency (BW gain / cumulative FI)
Visceral fat (g / 100g BW)
Subcutaneous fat weight (g / 100g BW)
Total fat weight (g / 100g BW)
Lean body mass (g / 100g BW)
0.00
0.05
0.10
0.15
0.20
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
0.0
1.0
2.0
3.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
85
90
95
100
a
b
c
a
a
b
c
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b
c
Young-Control
Young-T
Middle-Control
Middle-T

Fig. 3.

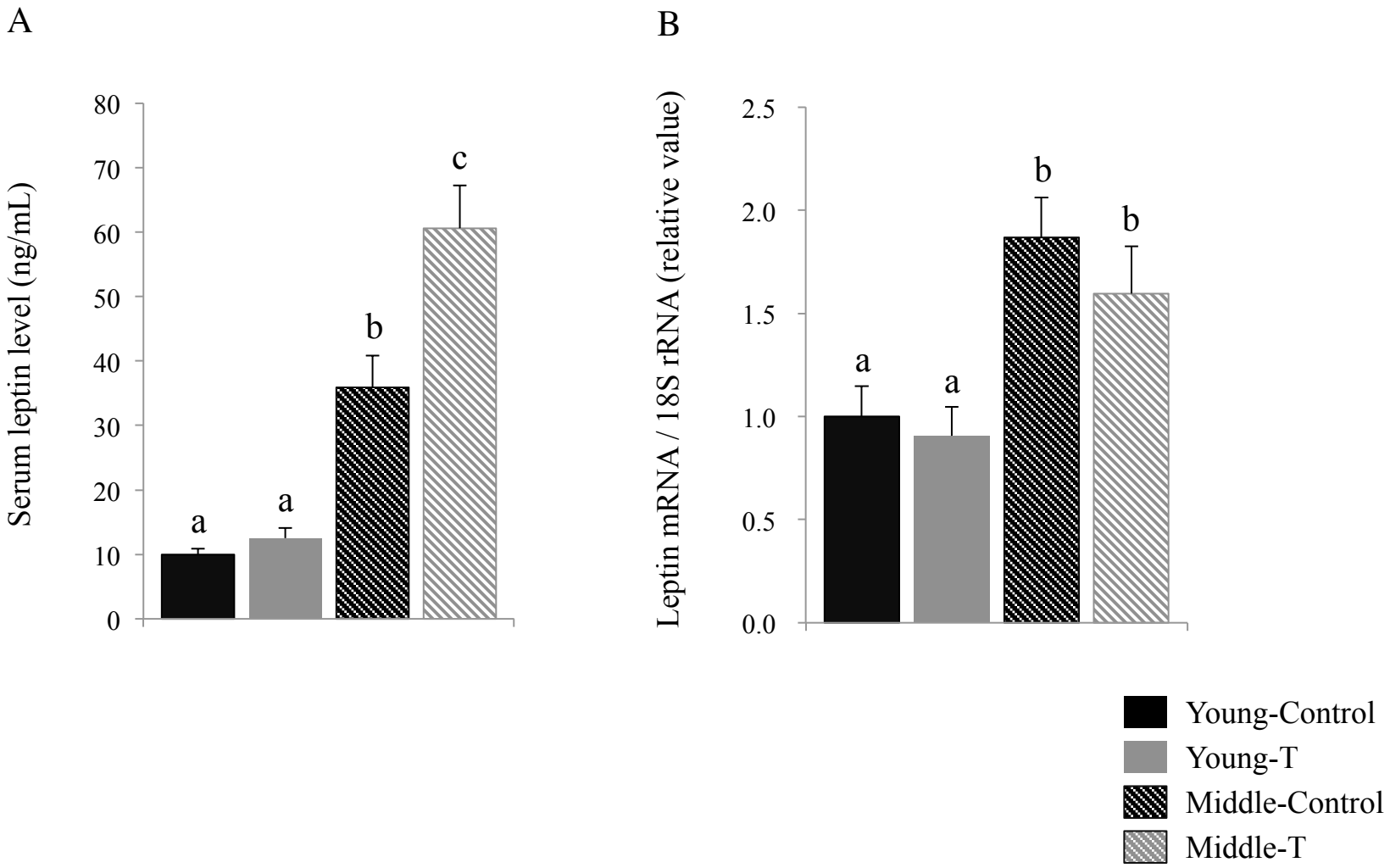
A
Serum leptin level (ng/mL)
80
70
60
50
40
30
20
10
0
a
a
b
c
B
Leptin mRNA / 18S rRNA (relative value)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
b
Young-Control
Young-T
Middle-Control
Middle-T

Fig. 4.

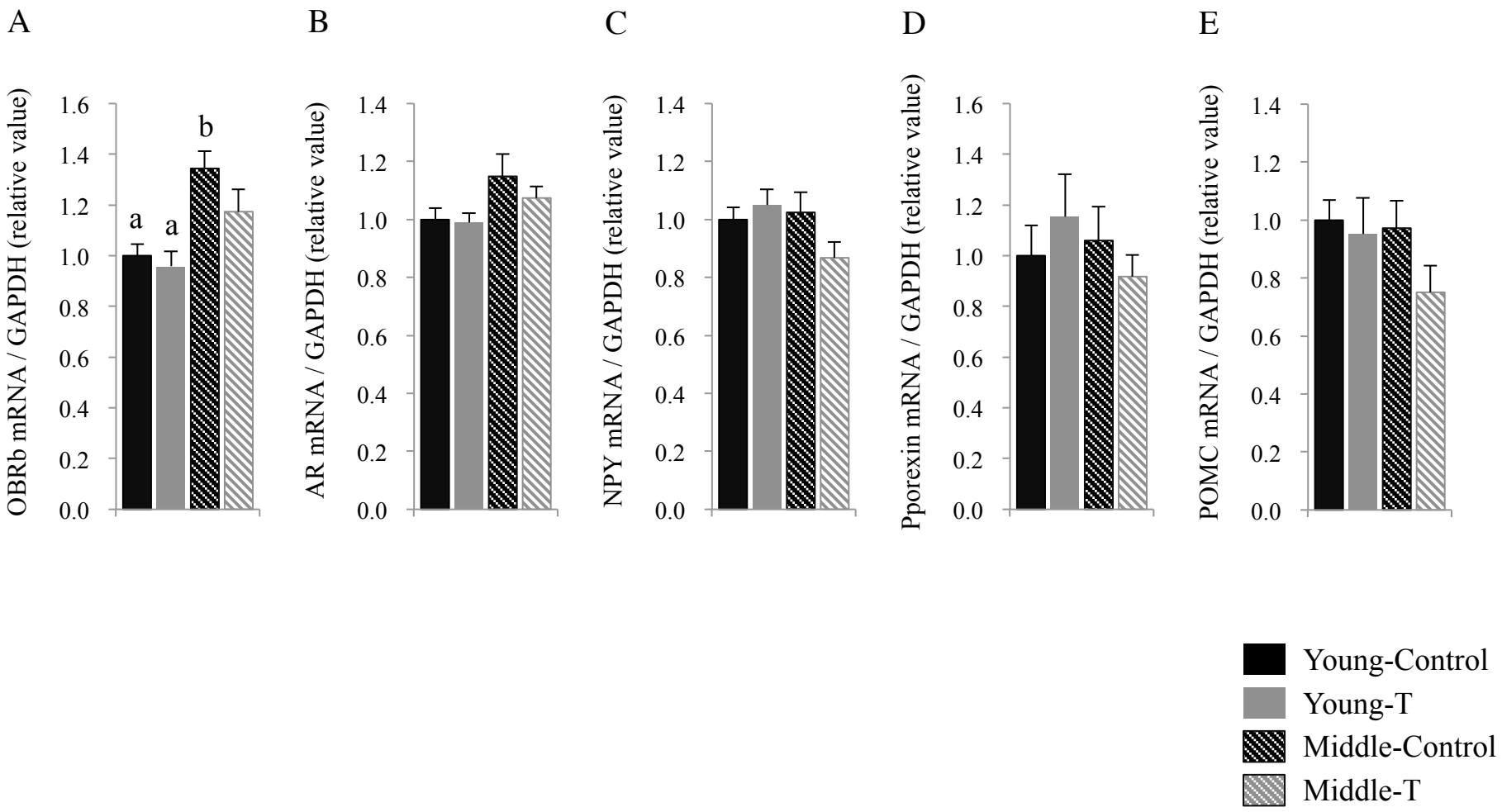
A
B
C
D
E
OBRb mRNA / GAPDH (relative value)
AR mRNA / GAPDH (relative value)
NPY mRNA / GAPDH (relative value)
Pporexin mRNA / GAPDH (relative value)
POMC mRNA / GAPDH (relative value)
a
a
b
Young-Control
Young-T
Middle-Control
Middle-T

Table 1 Primer sequences, product sizes and annealing temperature

Primer	Sequence	Product size (bp)	Annealing T (℃)
AR forward	CCC ATC GAC TAT TAC TTC CC	58	63
AR reverse	TTA CGA GCT CCC AGA GTC AT		
NPY forward	GGG GCT GTG TGG ACT GAC CCT	148	66
NPY reverse	GAT GTA GTG TCG CAG AGC GGA G		
POMC forward	CCT CAC CAC GGA AAG CA	104	66
POMC reverse	TCA AGG GCT GTT CAT CTC C		
OBRb forward	GCA GCT ATG GTC TCA CTT CTT TTG	113	63
OBRb reverse	GTT CCC TGG GTG CTC TGA		
pporexin forward	GCC GTC TCT ACG AAC TGT TG	303	60
pporexin reverse	CGA GGA GAG GGG AAA GTT AG		
leptin forward	GGT CAC CGG TTT GGA CTT CAT	64	66
leptin reverse	CTG GTC CAT CTT GGA CAA ACT CA		
GAPDH forward	ATG GCA CAG TCA AGG CTG AGA	64	70
GAPDH reverse	CGC TCC TG GAA GAT GGT GAT		
18S rRNA forward	GAC GGA CCA GAG CGA AAG C	<65	64
18S rRNA reverse	AAC CTC CGA CTT TCG TTC TTG A		