Mild Calorie Restriction Does Not Affect Testosterone Levels and Testicular Gene Expression in Mutant Mice

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The hypothalamic-pituitary-gonadal (HPG) axis and the somatotropic axis are influenced by nutritional factors. Calorie restriction (CR) extends lifespan but suppresses both the HPG and the somatotropic axes. Since most CR studies use a fairly severe (40%–60%) reduction of calorie intake, we hypothesized that a milder CR (20%) might not be deleterious to reproduction in male mice. To test this hypothesis, we evaluated the effects of 20% CR on testicular testosterone content and on testicular expression of genes that are relevant to testicular function and reproductive competence, including insulin-like growth factor-I, cytochrome P450 aromatase (Cyp19a1), androgen receptor, luteinizing hormone receptor, follicle-stimulating hormone receptor, cytochrome P450c17 and 3-β-hydroxysteroid dehydrogenase/isomerase. To relate CR effects to the activity of the somatotropic axis, we have used growth hormone–resistant GHR knockout mice as well as transgenic mice overexpressing GH. Mild CR did not affect testosterone levels in testis homogenates and had little effect on expression of the examined genes in the reproductive organs. Altered activity of the GH/insulin-like growth factor–1 axis had a major impact on the parameters analyzed. The results also suggest that expression of several key genes involved in the control of testicular function is preserved under conditions of mild CR and encourage speculation that mild regimens of CR can produce longevity benefits without impairing reproduction. Exp Biol Med 232:1050–1063, 2007

Key words: calorie restriction; testicular testosterone levels; steroidogenesis-related genes; growth hormone; mutant mice

Introduction

The primary control center for male reproductive functions is the hypothalamus. This brain region secretes the gonadotropin-releasing hormone (GnRH), which stimulates the pituitary to secrete the gonadotropins (i.e., follicle-stimulating hormone [FSH] and luteinizing hormone [LH]). There are receptors for these two hormones in the testis, where FSH stimulates the Sertoli cells, crucial supporters of the spermatogenic process, and LH acts on the Leydig cells. Binding of LH to its receptor (LHR) on the Leydig cell membrane initiates a cascade of events that culminate in the production of testosterone from cholesterol. This process involves two organelles, mitochondria and smooth endoplasmic reticulum, and several steroidogenic enzymes, including 3-β-hydroxysteroid dehydrogenase/isomerase (3βHSD), cytochrome P450c17 (Cyp17), and cytochrome P450 aromatase (Cyp19a1-AROM). The testis is not only the main source of testosterone in the body, but it is also one of its principal targets (1–3). Three types of somatic cells—Sertoli, Leydig, and myoid cells—are present in the testis and are under the direct control of androgens, which act *via* the androgen receptor (AR; Refs. 3–5).

The state of nutrition is an important factor in the onset of puberty and continued optimal reproductive function throughout life. The hypothalamic-pituitary-gonadal (HPG) axis, described above, is influenced by nutritional factors. Undernutrition can suppress the HPG axis through its central inhibition (6). There are reports of delayed puberty (7, 8) and reduced fertility (7–10) in animals subjected to calorie restriction (CR). However, CR can also extend

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reproductive lifespan (7, 11), presumably as a result of delaying the aging process. Some information is available on the influence of CR on gene expression in the male reproductive tract (12, 13). Our group recently published a study (14) about the effects of 20% CR on fertility and levels of steroid hormones in plasma of male and female mice from different lines with disruption of the GH/insulin growth factor–1 (IGF-1) axis. Markers of the response to CR, such as hepatic expression of selected genes, and plasma levels of glucose and insulin, as well as IGF-1 were also assessed in that study. We found that, overall, the mild CR applied had no effect on the parameters analyzed. Although an improvement in health parameters was expected, 20% CR seemed not to influence them significantly. Nevertheless, reproductive parameters analyzed were not impacted as well. Of note, we report in the present study different parameters of some of the groups from the aforementioned study (14).

The somatotropic axis is also controlled by the hypothalamus, which secretes growth hormone–releasing hormone (GHRH) and GH release–inhibiting hormone (somatostatin), two neuropeptides that exert, respectively, stimulatory and inhibitory effects on the somatotrophs, the GH-producing cells localized in the pituitary gland. GH receptors can be found throughout the body, yet many actions of GH are mediated by IGF-1. This hormone is produced mainly by the liver, but it also can be produced locally by several target organs in response to GH stimulus. There is considerable evidence of the interaction between the HPG and the somatotropic axes (see reviews in Refs. 15–17). Furthermore, Childs *et al.* (Ref. 18, see also Ref. 19) described the existence in the pituitary of cells that express mRNA for FSH, LH, and GH, along with receptors for GnRH and for GHRH.

Hiney *et al.* (20) identified IGF-1 as a signal for puberty. This role of IGF-1 is supported by the observation of late onset of puberty in GH-resistant GHR-knockout (GHR-KO) mice that have very low levels of circulating IGF-1 (21) and early onset of puberty in transgenic mice that overexpress GH and have elevated IGF-1 levels (22). Zulu *et al.* (23) suggest that IGF-1 informs the hypothalamic centers that control reproduction about the status of somatic growth and nutrition. The interaction between the HPG and somatotropic axes in CR animals is relevant because IGF-1 levels are suppressed in animals subjected to CR (24, 25).

Of note, many of the physiologic characteristics seen in CR animals are shared by animals that have disruption of the GH/IGF-1 system (e.g., reduced fertility and prolonged longevity). GHR-KO mice (26) lack GH action due to the absence of GH receptors, resulting in a dwarf phenotype with remarkably extended longevity (27) but compromised fertility (16, 28). In contrast, GH-overexpressing transgenic mice have increased body size and greatly reduced lifespan (see review in Ref. 29). The transgenic mice that overexpress bovine GH (bGH) under the control of mouse metallothionein I (MT) promoter (30) or the rat phosphoenolpyruvate carboxykinase (PEPCK) promoter (31) have advanced puberty and reduced reproductive lifespan (32, 33).

CR extends lifespan in a wide range of organisms, from yeast, nematodes, and flies, to laboratory rodents and probably also monkeys (34–36). Nevertheless, the reduced reproductive performance reported in animals under CR is a source of concern, especially if the use of CR or CR mimetics to delay aging is ultimately intended as a therapeutic intervention in humans. Since nutritional status and the HPG and somatotropic axes are interrelated, and because most CR studies apply food intake restrictions of 40%–50% (34), we hypothesized that a milder CR would not be deleterious for reproduction in male mice and that the effects of CR on male reproductive system may be influenced by genetic alterations of the somatotropic axis in GHR-KO, MT-bGH, and PEPCK-bGH mice. To test these hypotheses, we evaluated the effects of 20% CR on testicular testosterone content and on testicular expression of selected genes related to the control of testicular function and production of sex steroids.

Materials and Methods

Materials. Male mice (*Mus musculus*) from our colony at the Southern Illinois University were used. Homozygous GHR-KO (–/–) mice and their normal littermates were derived from animals provided by Dr. J.J. Kopchick and were produced by crosses of homozygous males with heterozygous (+/–) females, or heterozygous animals. Transgenic (Tg) mice overexpressing bovine GH and their normal siblings were derived from animals provided by Dr. T.E. Wagner and J. Yun. The founder Tg animals were produced by microinjection of bGH gene associated with the mouse MT-1 promoter (MT-bGH line) or with the rat PEPCK promoter (PEPCK-bGH line) into recently fertilized ova. The Tg colonies were maintained by mating hemizygous transgenic males to C57BL/6 × C3H F1 hybrid females. All animal protocols were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and approved by the Southern Illinois University Laboratory Animal Care and Use Committee.

Age-matched animals were kept in "shoebox"-type cages, usually five animals per cage. The light cycle was 12:12-hr light:dark, and the temperature was set for 22°C ± 2°C. The animals had continuous free access to tap water and pelleted rodent food (Lab Diet 5001, not autoclaved, 23.4% protein, 4.5% fat, 5.8% crude fiber; PMI Nutrition International, Richmond, IN) except when submitted to CR. The CR applied was 20% reduction in food intake, which consisted of giving daily (between 1500 and 1700 hrs) to CR animals 80% of the amount of food consumed by the *ad libitum* (AL) group. The latter group had the food consumption monitored weekly during the whole experi-

ment, so that values were constantly updated. All animals were observed daily and weighed weekly.

Methods. *Protocol.* The following experimental groups were used to evaluate the effects of 20% CR on testosterone levels and testicular gene expression:

Experiment 1—CR Started Earlier in Life. *Group 1.* Male GHR-KO (KO) and N littermates (N AL, $n = 9$; N CR, $n = 10$; KO AL, $n = 9$; KO CR, $n = 8$) were submitted to 20% CR starting at 2–3 months of age and were sacrificed at 8–9 months of age.

Group 2. In male transgenic PEPCK-bGH (Tg) mice and N siblings the treatment (20% CR) was started at 3 months of age (N AL, $n = 13$; N CR, $n = 14$; Tg AL, $n = 4$; Tg CR, $n = 5$), and these animals were sacrificed at 11 months of age.

Experiment 2—CR Started Later in Life. *Group 3.* Male GHR-KO (KO) mice and N siblings were submitted to 20% CR regimen beginning at 7 months of age, with 40 animals assigned to four subgroups: N AL ($n = 8$), N CR ($n = 12$), KO AL ($n = 10$), and KO CR ($n = 10$). These animals were sacrificed at 13 months of age.

Group 4. In male transgenic MT-bGH (Tg) mice and N siblings the 20% CR regimen was started at the age of 8–9 months (N AL, $n = 6$; N CR, $n = 6$; Tg AL, $n = 12$; Tg CR, $n = 14$). These animals were sacrificed at 11–12 months of age.

Tissue Collection. After fasting for 12 hrs, animals were anesthetized using isoflurane (Aerrane; Baxter HealthCare Corp., Deerfield, IL) and were killed by cervical dislocation. Testes were quickly removed, weighed, frozen in dry ice, and kept at –80°C until processed for radioimmunoassay (RIA) or reverse transcription–polymerase chain reaction (RT-PCR). One testis per animal was used for both techniques. For testis homogenates to be tested by RIA, a fraction of about half-testicle was used (weight was recorded). For RT-PCR studies, a small fragment of about 2 mm^3 was used.

Preparation of Testis Homogenates. Testis fragments were weighed, placed in tubes containing 500 µl ice-cold phosphate buffer (50 m*M*, pH 7.4) with 0.25*M* sucrose, and homogenized. The samples were centrifuged at $600 \times g$ for 10 mins at 4°C to precipitate nuclei and cellular debris. The supernatant was collected and centrifuged at $10{,}000 \times g$ for 20 mins at 4°C to separate mitochondrial pellet. Finally, the supernatant was collected and kept at –80°C until used for RIA measurement of testicular testosterone content.

RIA. The levels of testosterone in testis homogenates were assessed by RIA (intraassay coefficient of variance < 12.0%; Diagnostic Products Corp., Los Angeles, CA). All samples were run in duplicates, following the manufacturer's instructions. The volume of homogenate used for the RIA was 50 µl, the amount recommended for plasma. All four phenotype-diet combinations were assayed in the same run in order to avoid interassay variations.

RNA Extraction. Testis total RNA was extracted using the guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (37). All RNA samples were run in 1.5% agarose gel containing ethidium bromide to check RNA quantity and quality.

Preparation of cDNA. Complementary DNA was generated from RNA samples (2 µg) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), following the manufacturer's instructions. Samples were processed in a thermal cycler (Eppendorf Mastercycler Personal; Eppendorf North America Inc., Westbury, NY) at 25°C for 5 mins, 42°C for 30 mins, then 85°C for 5 mins, and finally down to 4°C.

RT-PCR. Real-time RT-PCR amplification was carried out using iScript one-step RT-PCR kit with SYBR Green (Bio-Rad Laboratories) and SmartCycler (Cepheid, Sunnyvale CA). The housekeeping gene used was β-2-microglobulin (*B2M*; NM_009735; from Ref. 38) forward primer (FP): 5′-AAG TAT ACT CAC GCC ACC CA, reverse primer (RP): 5′-AAG ACC AGT CCT TGC TGA AG. The other primers used were: *IGF1* (NM_010512; from Ref. 38) FP: 5′-CTG AGC TG GTG GAT GCT CTT, RP: 5′-CAC TCA TCC ACA ATG CCT GT; Cytochrome P450 aromatase-Cyp19a1 (*AROM*; NM_007810) FP: 5′-TTC AAT ACC AGG TCC TGG CT, RP: 5′-AGT GTC TCC TCT CCA CTG AT; androgen receptor (*AR*; NM_013476) FP: 5′-CCA AAG GAT TGG AAG GTG AG, RP: 5′-TGT AGT AGT CGC GAT TCT GG; luteinizing hormone receptor (*LHR*; MUSLHRAA) FP: 5′-CAC TGC TGT GCT TTC AGG AA, RP: 5′-CCA CTG AGT TCA TTC TCC TC; follicle-stimulating hormone receptor (*FSHR*; NM_013523) FP: 5′-CCA TAC TAA GAG CCA GTA CC, RP: 5′-CCT TGC ATT CCA GTT GCA TG; hydroxysteroid dehydrogenase-1, delta 5–3-beta - Hsd3b1 (*HSD3*; NM_008293) FP: 5′-CAT CTT CTG CAG CTC AGT TG, RP: 5′-AGT ACT GCC TTC TCA GCC AT; and cytochrome P450, family 17, subfamily a, polypeptide 1 - Cyp17a1 (*CYP17*; NM_007809) FP: 5′-AGC ATA TCC TTG TCA CGG TG, RP: 5′-TCT TCC TCT TCA CCT CAG GA. The thermal cycler was set to perform 40 cycles, each consisting of three temperature steps: denaturing at 95°C, annealing at 62°C, and extension at 72°C. Every run contained negative controls consisting of PCR mix plus the primers and no cDNA sample. PCR products were confirmed by melting curve information and 2% agarose gel electrophoresis. The values of threshold cycle numbers (ΔCt) for each gene of interest were normalized to the housekeeping gene values by subtracting ΔCt B2M from ΔCt gene; this method was described previously (39). Results are shown as fold-change compared with N AL, the control group. All the data were processed using the softwares SmartCycler (Cepheid) and Microsoft Excel (Redmond, WA).

Statistical Analyses. Statistical analyses were performed using the softwares SPSS 10.0.1 (SPSS Inc., Chicago, IL) and GraphPad Prism 4.02 (GraphPad Software Inc., San Diego, CA). Results are shown as means ± SEM, except scatter graphs for testosterone levels in testis

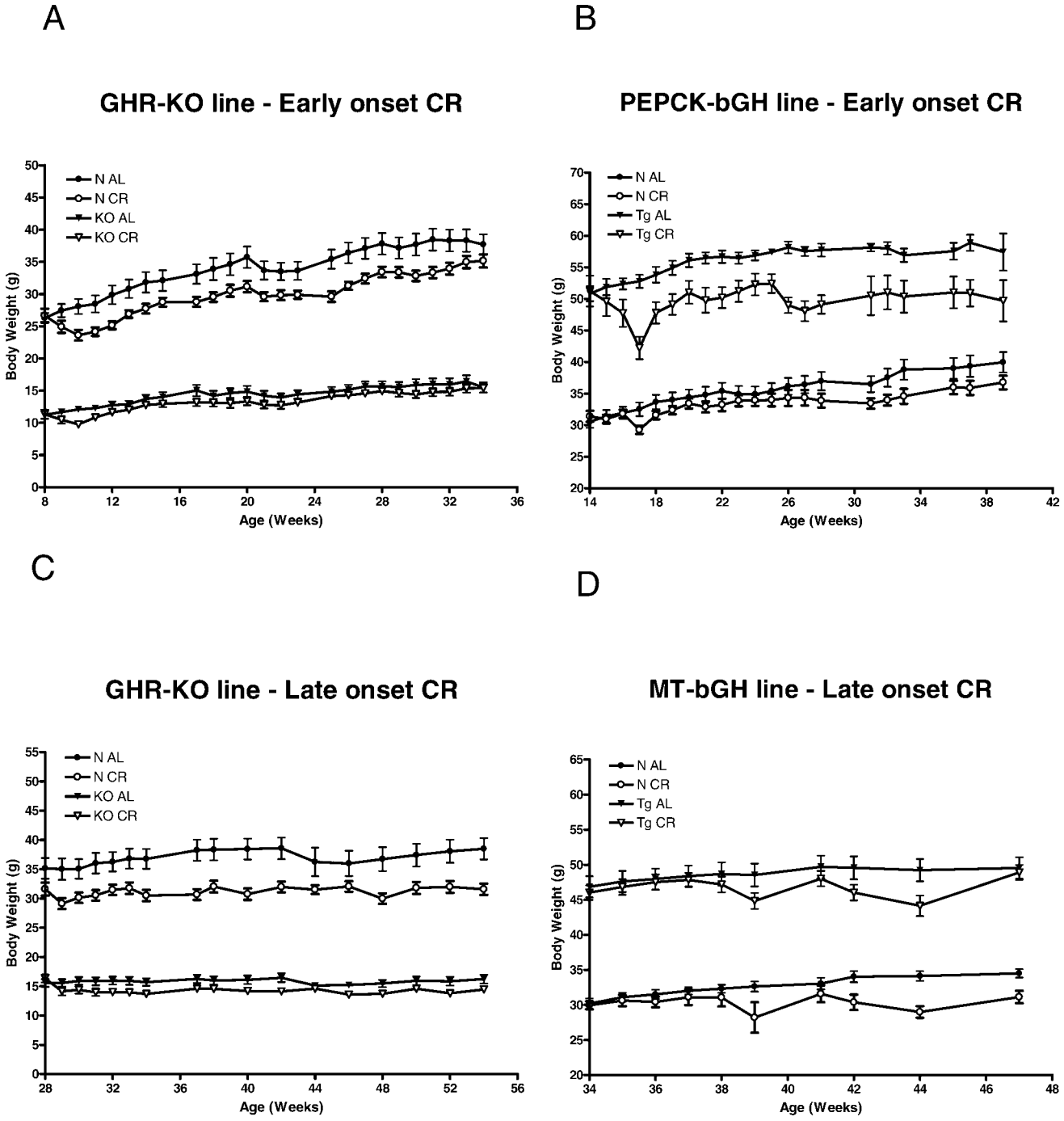

Figure 1. Growth curves of mice from the groups: (A) GHR-KO, early-onset CR; (B) PEPCK-bGH, early-onset CR; (C) GHR-KO, late-onset CR; and (D) MT-bGH, late-onset CR. Mice were fed either *ad libitum* (AL) or subjected to calorie restriction (CR). Each time point represents the average weight per subgroup, error bars are present. All comparisons were made within each group. N, normal; Tg, transgenic; KO, knockout animals.

homogenates, which show each individual measurement. Two-way ANOVA was used to evaluate the effects of phenotype, diet, and of the phenotype-diet interaction. Tukey and Games-Howell tests were used whenever appropriate as *post-hoc* tests, depending whether equal variances could be assumed or not, respectively. The level of significance was set for $P < 0.05$.

Results

The growth curves are shown in Figure 1. One can note by the error bars that the CR caused a difference in body weight for most of the duration of the study. Nevertheless, by the end of the experiment, only the males from the PEPCK-bGH line showed any effect of CR on body weight

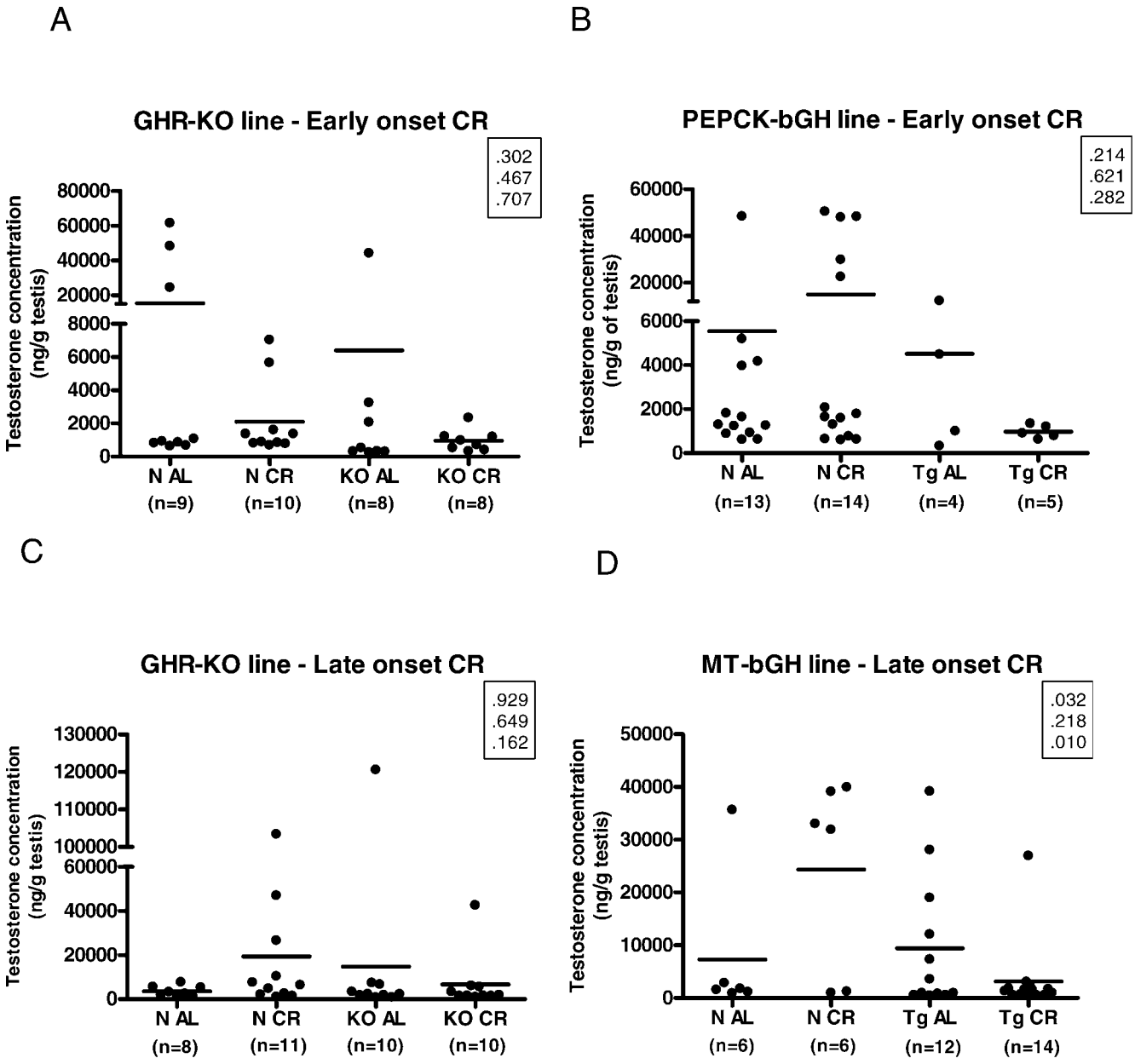

Figure 2. Effects of 20% CR on testicular testosterone levels in: (A) GHR-KO, early-onset CR; (B) PEPCK-bGH, early-onset CR; (C) GHR-KO, late-onset CR; and (D) MT-bGH, late-onset CR. Mice were fed either *ad libitum* (AL) or subjected to calorie restriction (CR). Means are represented by horizontal lines. All comparisons were made within the groups. In parentheses are the *n* numbers. The inserts along the legends show the significance (*P* value) for, from top to bottom: phenotype, diet, and the interaction between phenotype and diet. N, normal; Tg, transgenic; KO, knockout animals.

(Fig. 1). The levels of testosterone in testis homogenates were highly variable among individuals from the same phenotype-diet subgroup (Fig. 2). Generally, no significant differences regarding phenotype, diet or the onset of diet were detected among the four phenotype-diet combinations in each of the four groups studied. In the MT-bGH line (Fig. 2D), there was a significant interaction between phenotype and diet.

The testicular gene expression of *IGF1* was significantly different in the PEPCK-bGH line, as we observed a phenotype effect in this group: transgenic animals had 1.3-fold higher levels of *IGF1* mRNA compared with their normal (N) counterparts, when AL and CR animals were pooled (Fig. 3B). In the MT-bGH line, there was a significant interaction between phenotype and diet affecting the testicular expression of the *IGF1* gene in this group (Fig. 3D). No phenotype (KO vs. N) or diet effects were detected on testicular *IGF1* gene expression in mice from the GHR-KO line, both early- and late-onset CR groups (Fig. 3A and C).

In the mice from the GHR-KO line, early-onset CR, the testicular *AROM* gene expression was higher (1.9-fold) in

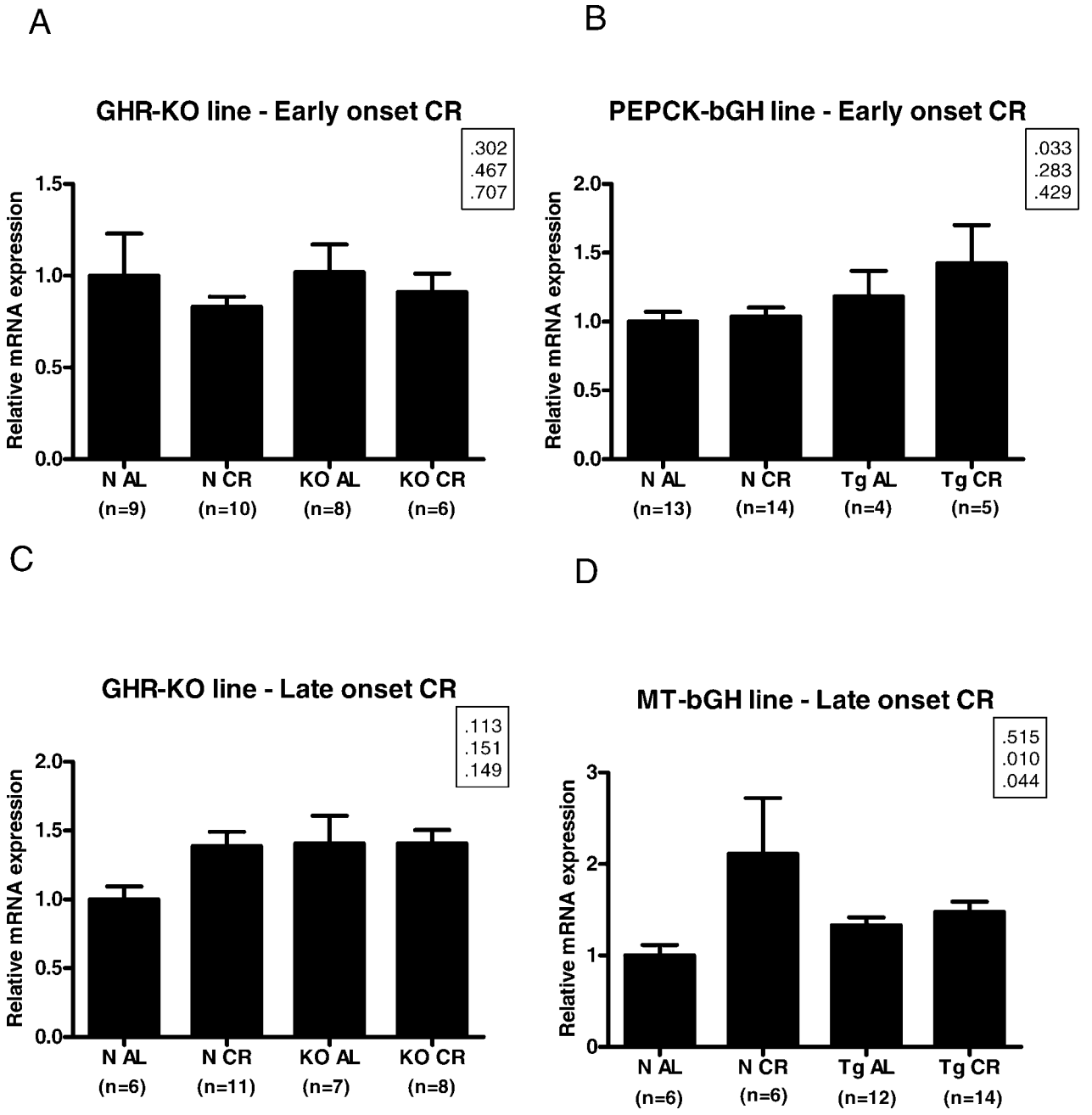

Figure 3. Effects of 20% CR on testicular *IGF1* gene expression levels in: (A) GHR-KO, early-onset CR; (B) PEPCK-bGH, early-onset CR; (C) GHR-KO, late-onset CR; and (D) MT-bGH, late-onset CR. Mice were fed either *ad libitum* (AL) or subjected to calorie restriction (CR). Values shown are mean ± SEM. All comparisons were made within the groups. In parentheses are the *n* numbers. The insets along the legends show the significance (*P* value) for, from top to bottom: phenotype, diet, and the interaction between phenotype and diet. N, normal; Tg, transgenic; KO, knockout animals.

KO animals from both diets compared with N animals from both diets (Fig. 4A). We found no differences in testis *AROM* gene expression in the remaining groups analyzed, regardless of phenotype or diet (Fig. 4B, C, and D).

We observed a phenotype effect on testicular *AR* gene expression in mice from the GHR-KO line, early-onset CR (Fig. 5A). KO animals on both diets had 1.6-fold higher levels of *AR* mRNA compared with N animals on these diets. There was a significant interaction between phenotype and diet in the GHR-KO line, late-onset CR (Fig. 5C). No differences were seen in *AR* gene expression in the testes of mice from the PEPCK-bGH line (Fig. 5B). We observed a diet effect in the mice from the MT-bGH line: CR caused a 1.3-fold increase of the *AR* mRNA levels in the testis of mice from both phenotypes compared with AL animals from both phenotypes. When comparing individual means, we observed a significant ($P < 0.018$) difference in the N

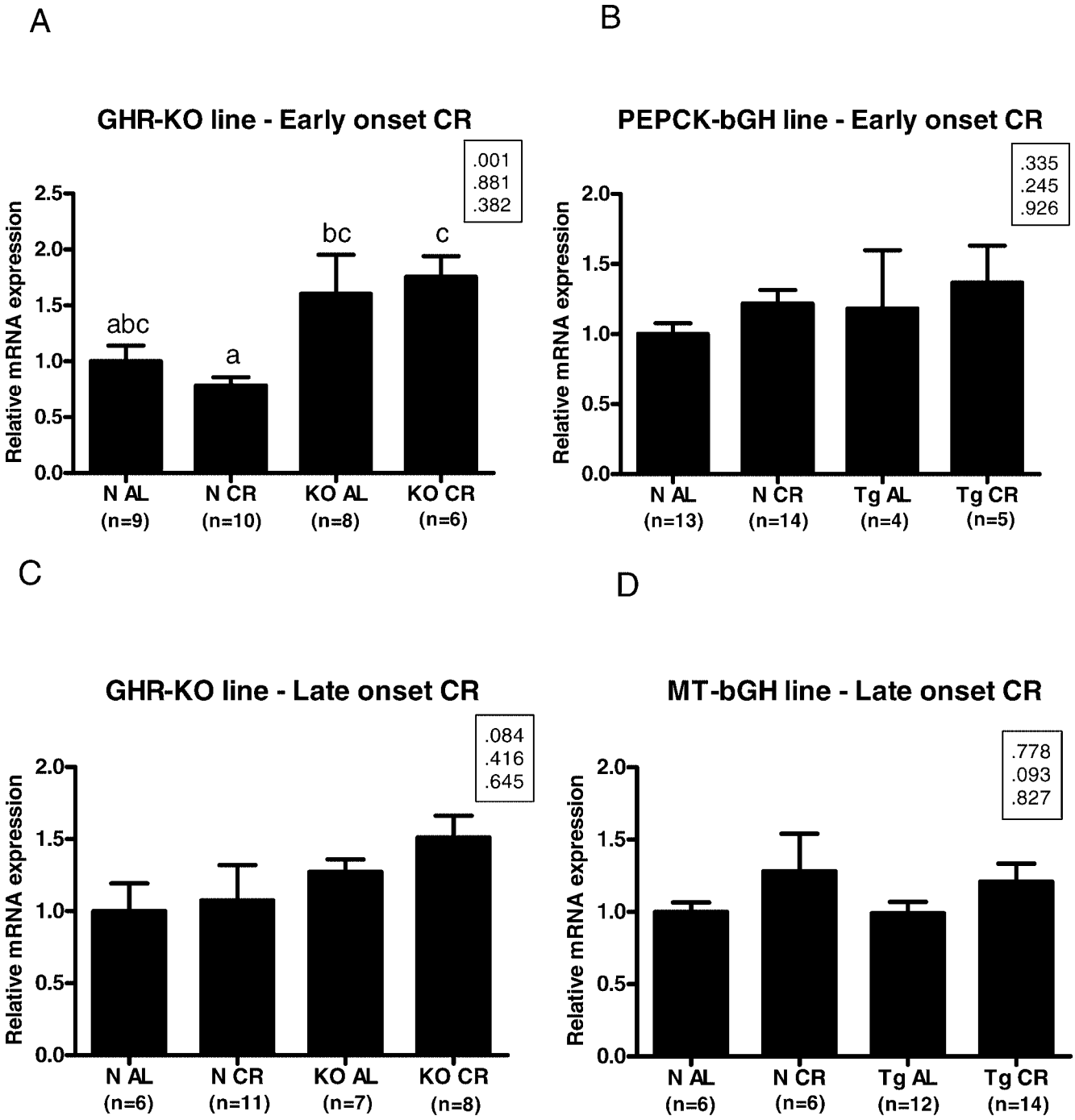

Figure 4. Effects of 20% CR on testicular *AROM* gene expression levels in: (A) GHR-KO, early-onset CR; (B) PEPCK-bGH, early-onset CR; (C) GHR-KO, late-onset CR; and (D) MT-bGH, late-onset CR. Mice were fed either *ad libitum* (AL) or subjected to calorie restriction (CR). Values shown are mean ± SEM. All comparisons were made within the groups. Bars that do not share same letter are statistically different at $P < 0.05$ or less. In parentheses are the *n* numbers. The inserts along the legends show the significance (*P* value) for, from top to bottom: phenotype, diet, and the interaction between phenotype and diet. N, normal; Tg, transgenic; KO, knockout animals.

animals: N CR mice had 1.6-fold higher *AR* gene expression compared with N AL mice (Fig. 5D).

No significant phenotype or CR-related differences were detected in testicular *LHR* mRNA levels in the examined groups of mice (data not shown).

There was a striking phenotype effect on testicular *FSHR* gene expression in the GHR-KO line, early-onset CR (Fig. 6A). KO animals had 2.9-fold higher levels of *FSHR* mRNA in the testis compared with N animals, diets pooled. We observed both a phenotype effect and a diet effect in the GHR-KO line, late-onset CR (Fig. 6C): KO animals had 2.1-fold higher expression of the *FSHR* gene in the testis

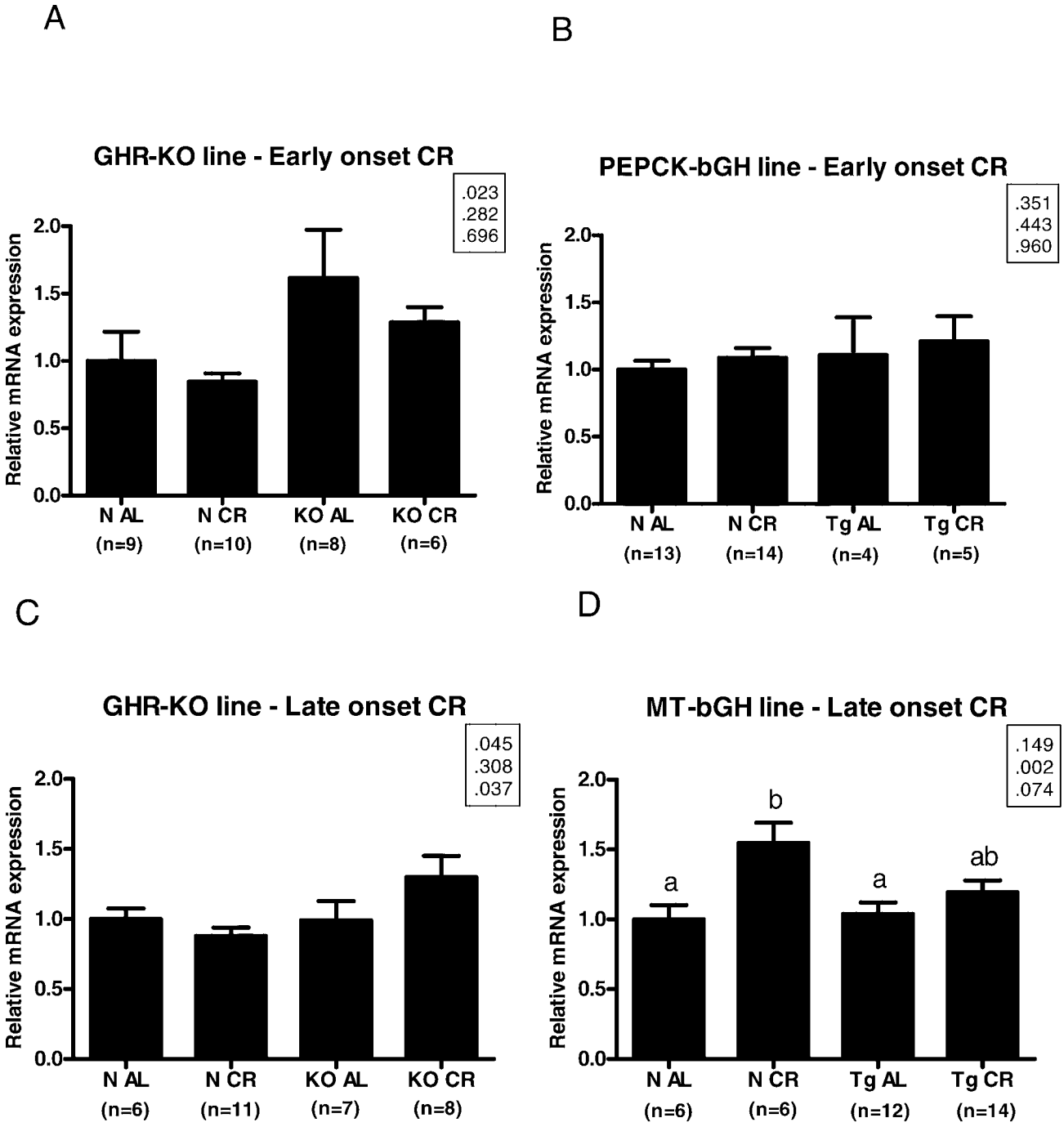

Figure 5. Effects of 20% CR on testicular *AR* gene expression levels in: (A) GHR-KO, early-onset CR; (B) PEPCK-bGH, early-onset CR; (C) GHR-KO, late-onset CR; and (D) MT-bGH, late-onset CR. Mice were fed either *ad libitum* (AL) or subjected to calorie restriction (CR). Values shown are mean ± SEM. All comparisons were made within the groups. Bars that do not share same letter are statistically different at $P < 0.05$ or less. In parentheses are the *n* numbers. The insets along the legends show the significance (*P* value) for, from top to bottom: phenotype, diet, and the interaction between phenotype and diet. N, normal; Tg, transgenic; KO, knockout animals.

compared with N animals, diets combined, whereas the 20% CR promoted a 1.2-fold increase in the *FSHR* mRNA levels in the testis of both KO and N animals compared with AL animals, phenotypes pooled. No effects of phenotype or diet were detected on *FSHR* gene expression in the testis of mice from the PEPCK-bGH or the MT-bGH lines (Fig. 6B and D).

No effects of the disruption of the *GHR* gene or subjection of the animals to CR on *HSD3* gene expression were detected in the testis of mice from the GHR-KO line, both early- and late-onset CR groups (Fig. 7A and C). We observed a phenotype effect in the PEPCK-bGH line: transgenic animals had 27% lower levels of testicular *HSD3* mRNA compared with their N counterparts, diets pooled

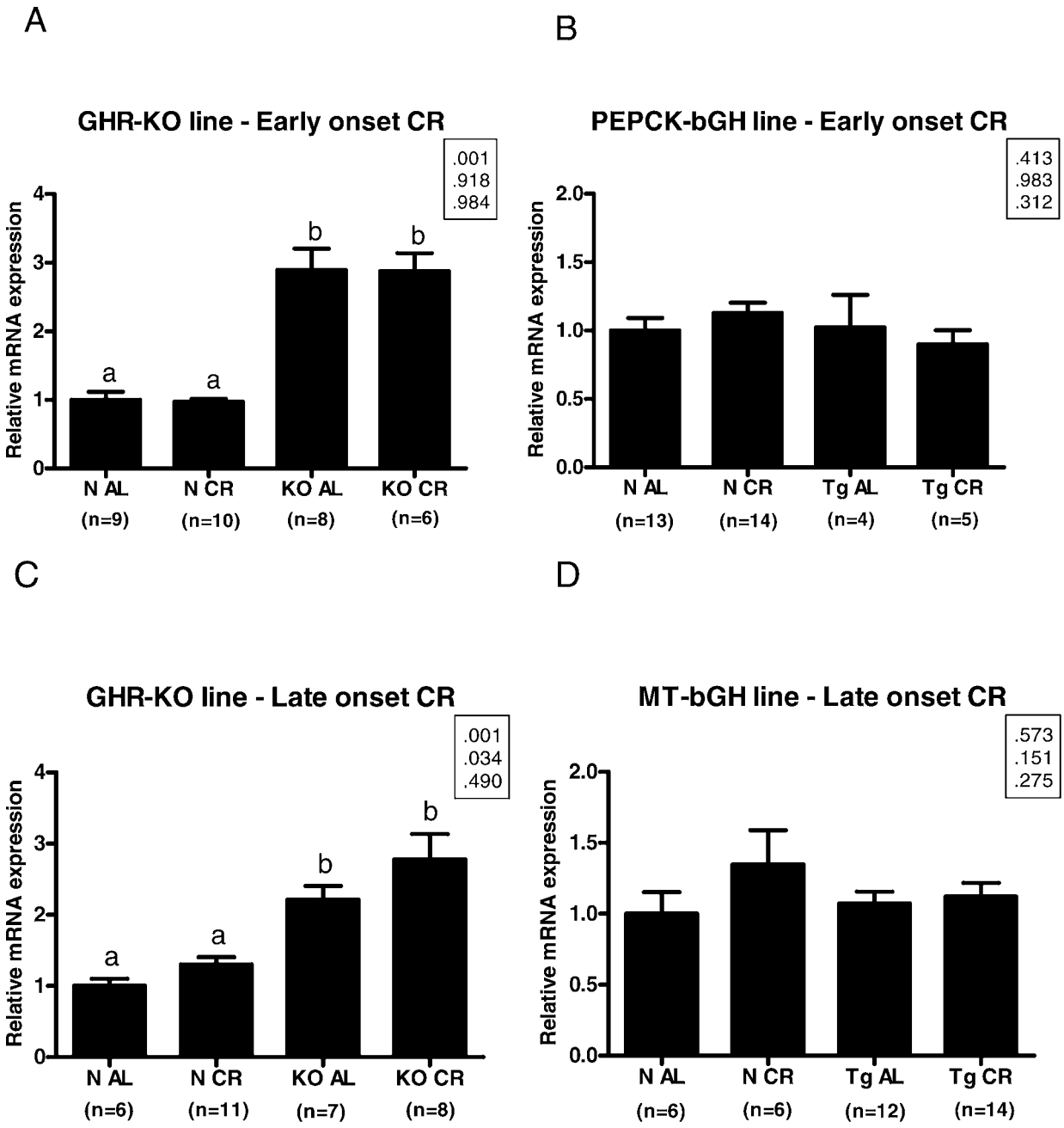

Figure 6. Effects of 20% CR on testicular *FSHR* gene expression levels in: (A) GHR-KO, early-onset CR; (B) PEPCK-bGH, early-onset CR; (C) GHR-KO, late-onset CR; and (D) MT-bGH, late-onset CR. Mice were fed either *ad libitum* (AL) or subjected to calorie restriction (CR). Values shown are mean ± SEM. All comparisons were made within the groups. Bars that do not share same letter are statistically different at $P < 0.05$ or less. In parentheses are the *n* numbers. The inserts along the legends show the significance (*P* value) for, from top to bottom: phenotype, diet, and the interaction between phenotype and diet. N, normal; Tg, transgenic; KO, knockout animals.

(Fig. 7B). In the MT-bGH line, there was also a significant influence of the phenotype on the testicular expression of the *HSD3* gene: transgenic animals had 24% lower levels of *HSD3* mRNA compared with their N counterparts, diets pooled (Fig. 7D).

We observed a phenotype effect on testicular *CYP17* gene expression in the GHR-KO line, early-onset CR (Fig. 8A): KO animals had 49% lower levels of *CYP17* mRNA in the testis compared with N animals, diets combined. The GHR-KO line mice from the late-onset CR group also presented influence of the phenotype on *CYP17* mRNA levels in testis (Fig. 8C): KO animals had *CYP17* gene expression reduced by 40% compared with N animals, diets pooled. The phenotype effect was also observed in the PEPCK-bGH line (Fig. 8B). Transgenic animals had reduced levels (38%) of testicular *CYP17* mRNA compared

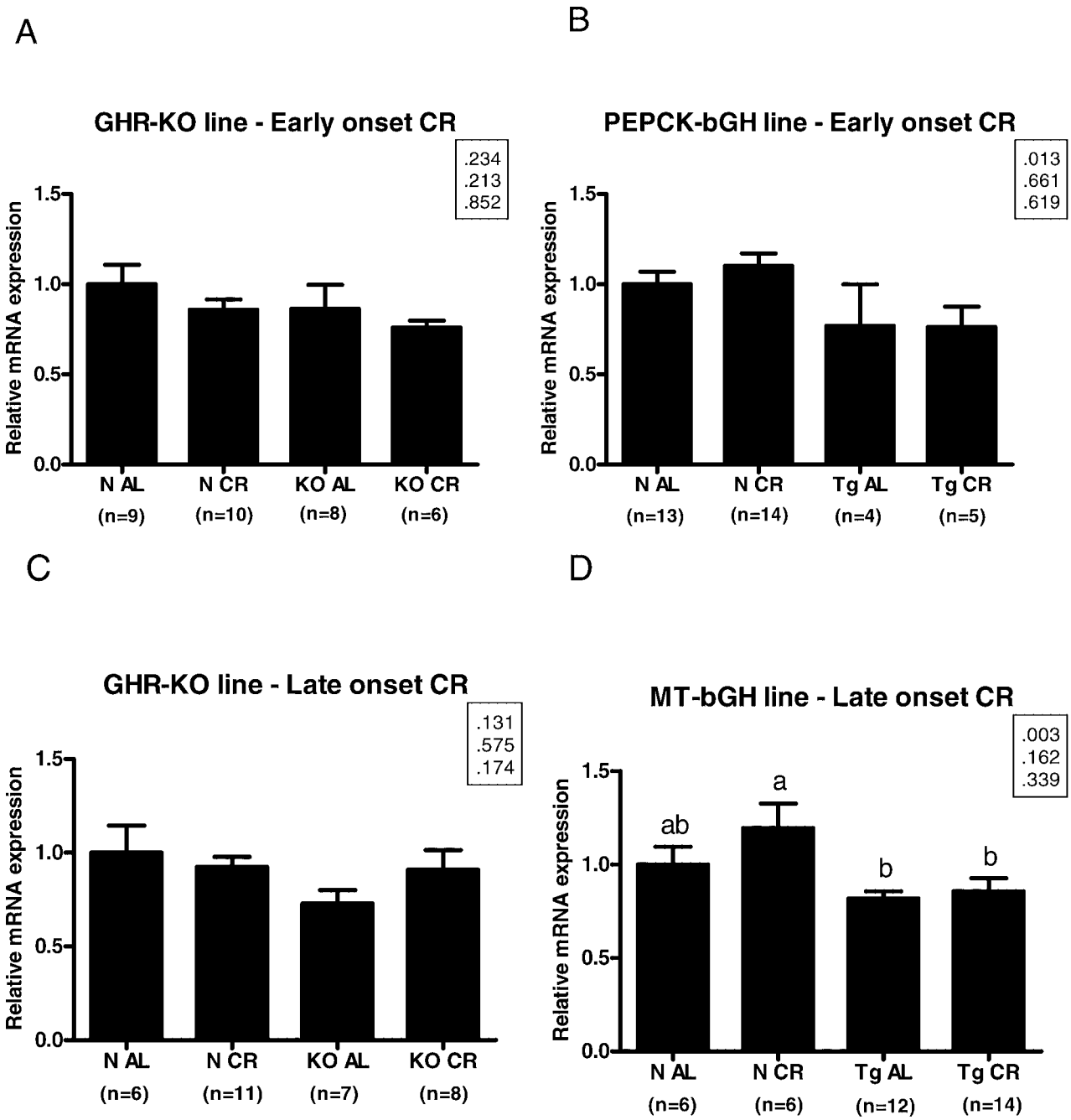

Figure 7. Effects of 20% CR on testicular *HSD3* gene expression levels in: (A) GHR-KO, early-onset CR; (B) PEPCK-bGH, early-onset CR; (C) GHR-KO, late-onset CR; and (D) MT-bGH, late-onset CR. Mice were fed either *ad libitum* (AL) or subjected to calorie restriction (CR). Values shown are mean ± SEM. All comparisons were made within the groups. Bars that do not share same letter are statistically different at $P < 0.05$ or less. In parentheses are the *n* numbers. The insets along the legends show the significance (*P* value) for, from top to bottom: phenotype, diet, and the interaction between phenotype and diet. N, normal; Tg, transgenic; KO, knockout animals.

with N animals, diets combined. However, no differences were seen in *CYP17* gene expression in the testes of mice from the MT-bGH line (Fig. 8D).

Discussion

Because of the pulsatile fashion of testosterone release to plasma (as a consequence of pulsatile LH release; Refs. 40, 41), it was felt that testosterone levels in testis homogenates may provide a more reliable measure of the androgenic status in experimental animals. However, the high variability characteristic of plasma testosterone levels is frequently found also in testicular testosterone levels (41). We observed no significant effects of diet or phenotype on this parameter in all groups examined. Although our ability to detect differences may have been compromised by inherently high variability of this parameter, this finding

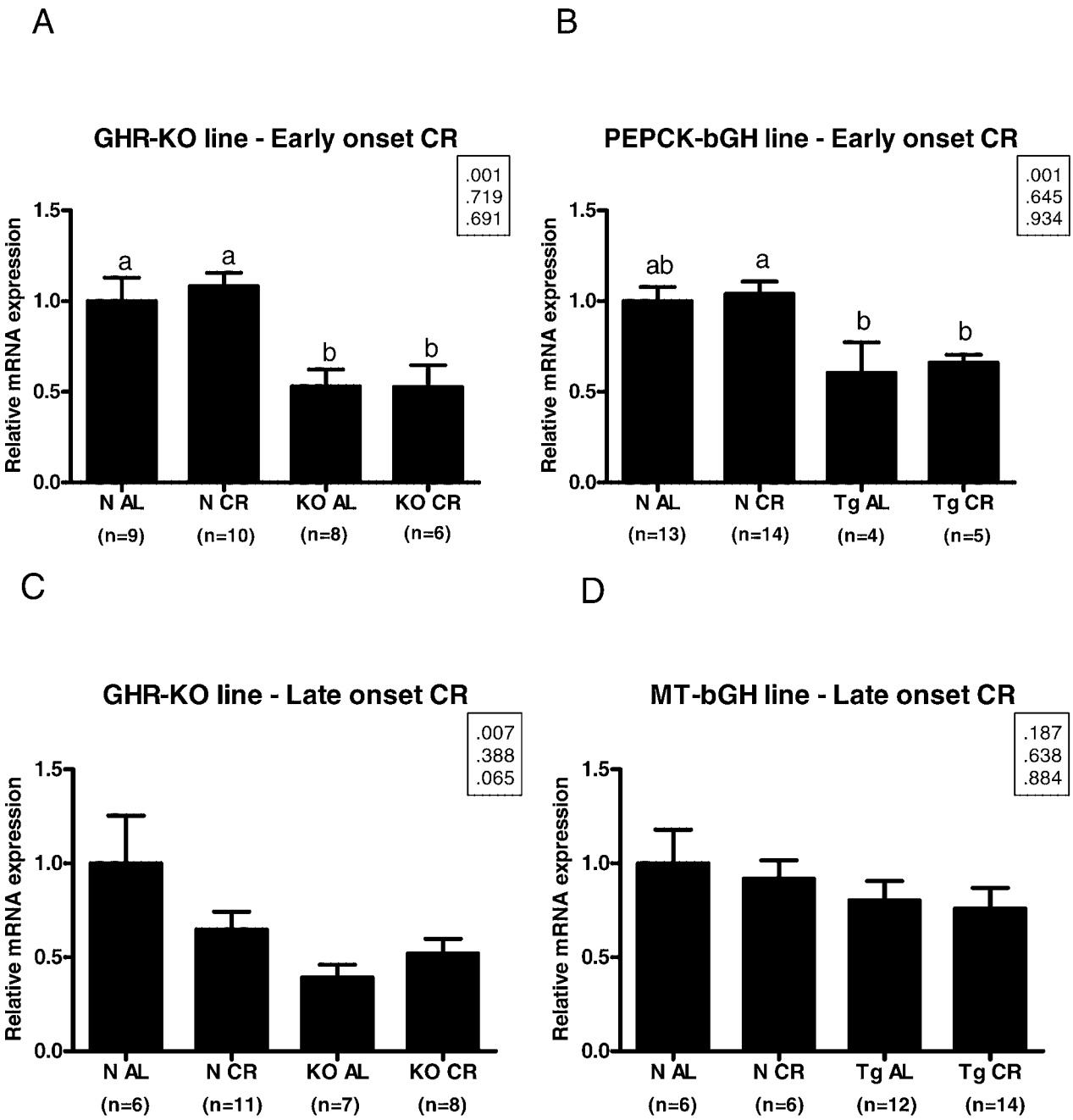

Figure 8. Effects of 20% CR on testicular *CYP17* gene expression levels in: (A) GHR-KO, early-onset CR; (B) PEPCK-bGH, early-onset CR; (C) GHR-KO, late-onset CR; and (D) MT-bGH, late-onset CR. Mice were fed either *ad libitum* (AL) or subjected to calorie restriction (CR). Values shown are mean ± SEM. All comparisons were made within the groups. Bars that do not share same letter are statistically different at $P < 0.05$ or less. In parentheses are the *n* numbers. The insets along the legends show the significance (*P* value) for, from top to bottom: phenotype, diet, and the interaction between phenotype and diet. In panel C, although phenotype effect is significant, there are no superscripts in the graph. That is because statistical difference was not reached on the *post-hoc* tests, when the subgroups were compared one to another. N, normal; Tg, transgenic; KO, knockout animals.

likely reflects the mild intensity of the CR used in the present study. Other studies applying more severe CR (42, 43) showed reduced levels of testosterone in the CR animals. The levels of testosterone in the testis reported in this study correlate to the values for plasma testosterone reported in our previous study (14) in a way that in homogenates, testosterone levels are roughly 50 times higher than in plasma.

Even though CR is reported to reduce plasma levels of IGF-1 (24), we observed no alterations in *IGF1* mRNA levels in the testes in all groups studied. Once again, it is possible that 20% CR is too mild to cause alteration in this parameter. Plasma IGF-1 levels were also not altered by diet in these animals (14). When comparing phenotypes, we found a significant difference in testicular *IGF1* gene expression in only one group, the mice from PEPCK-bGH

line. This is not surprising, since regulation of gonadal *IGF1* levels differs from the regulation of its hepatic expression and, consequently, plasma levels. Mice with nondetectable IGF-1 levels in plasma (GHR-KO line) have normal or nearly normal *IGF1* gene expression in the testis.[2] Perhaps the same is true for animals with elevated plasma levels of IGF-1 (i.e., the transgenic mice in this study). In the PEPCK-bGH line—the only group in which a significant difference in testicular *IGF1* expression was detected—the *IGF1* mRNA levels in Tg animals were just 30% higher than in N animals. This is a modest difference compared with the discrepancies reported for IGF-1 plasma levels between Tg and N mice from this line (32). Additional data on the animals reported in the present study, such as plasma testosterone levels, plasma IGF-1 levels, and fertility test performance among others, have already been reported (14).

The cytochrome P450 aromatase enzyme (AROM) is the product of the gene Cyp19a1 and is responsible for the conversion of androgens into estrogens (44). This enzyme is present in virtually all cell types in the rodent adult testis, including Sertoli cells, Leydig cells, spermatocytes, spermatids, and spermatozoa (45, 46). In the present study, the regimen of 20% CR did not affect the levels of *AROM* mRNA in the testis. Only one group, GHR-KO line, early-onset CR, showed statistical differences in the testicular expression of the *AROM* gene, and that difference was due to a phenotype effect: KO animals had higher levels of this gene compared with N animals. These KO animals had clearly impaired reproductive performance compared with their N counterparts. Perhaps, besides the already known factors contributing to the reduced fertility seen in GHR-KO mice (28, 16), one more could be added: higher expression of testicular *AROM* mRNA, probably leading to a higher rate of conversion of testosterone to estradiol. Estrogens are well established as important hormones in male reproductive function (see review in Ref. 47) but, most importantly, the correct balance between estrogens and androgens is necessary for optimal male reproduction (see review in Ref. 48).

Interestingly, the homozygous mutants from the GHR-KO line, early-onset CR, had higher expression of the *AR* gene in testis compared with normal animals. This may reflect a compensatory effect, since they have smaller testes and somewhat compromised reproduction (16, 28). Of note, the 20% CR induced an increase in *AR* mRNA levels in the animals from the MT-bGH line. This may have also been a compensatory mechanism, attempting to maintain normal function in the testis despite CR.

No significant differences in testicular *LHR* mRNA levels were detected in any of the groups examined. This indicates that 20% CR may be sufficiently mild to not compromise this parameter of testicular function in the groups studied. This finding also suggests that the levels of pituitary hormones known to regulate testicular LHR levels may have not been altered by this regimen of CR. To our knowledge, there are no reports on the effects of CR on LHR levels.

Tg mice overexpressing bGH did not exhibit differences in testicular *FSHR* gene expression. However, in both groups of mice from the GHR-KO line, the homozygous mutants had significantly higher levels of *FSHR* mRNA than N mice. Besides this phenotype effect, group 3 also presented a diet effect: 20% CR promoted an increase in *FSHR* gene expression in the testis, phenotypes combined. Increased expression of *FSHR* in the homozygous mutants compared with N mice from the GHR-KO line presumably reflects reduced circulating FSH levels in these animals (49). FSH negatively regulates the levels of its own receptor (50). The functional consequences of increased expression of *FSHR* in the testis likely include partial compensation for the suppression of FSH release by phenotype or phenotype in combination with CR.

The testicular steroidogenesis takes place mainly in Leydig cells and involves a series of biochemical events that culminate in sex steroid production from cholesterol. One of the enzymes that are crucial for this process is 3βHSD, which converts pregnenolone into progesterone (51). This enzyme can be found in mitochondrial and microsomal membranes, depending on the cell type and species considered (see review in Ref. 52). The animals from the GHR-KO line in our study did not exhibit any differences in *HSD3* gene expression in the testis related to diet or phenotype. Interestingly, in both transgenic lines—PEPCK-bGH and MT-bGH—there was a phenotype effect: animals overexpressing GH had lower levels of testicular *HSD3* mRNA compared with their N counterparts.

Another enzyme with a pivotal role in steroidogenesis is Cyp17, a single enzyme with two different functions, acting as a 17α-hydroxylase and as a $C_{17,20}$-lyase (51, 53). This microsomal enzyme, therefore, catalyzes two distinct reactions: first it converts progesterone into 17α-hydroxyprogesterone by hydroxylation of the former molecule at carbon 17, then it cleaves the two-carbon side chain, which results in the formation of androstenedione (51). In humans, this enzyme is expressed in adrenals and gonads but not in the placenta, whereas in rodents Cyp17 is expressed in placenta and gonads but not in the adrenals (53). We found differences in the levels of *CYP17* mRNA in the testis in three of our four experimental groups (i.e., the mice from GHR-KO line, from both early- and late-onset CR groups, and the ones from the PEPCK-bGH line); only the animals from MT-bGH line presented no significant differences. In the remaining comparisons, the mutants or transgenics had reduced testicular *CYP17* gene expression compared with their normal counterparts. As these mutants usually show reduced fertility compared with normal animals, the results were not entirely unexpected. Nevertheless, 20% CR did not impact this parameter in the animals studied.

In summary, 20% CR did not affect testosterone levels in testis homogenate and had little effect on expression of

[2] Chandrashekar and Bartke. Unpublished data, 2007.

the examined genes in the reproductive organs. It appears that the altered activity of the GH/IGF-1 axis in the mice studied had a major impact on the parameters analyzed. This preliminary study of selected parameters of reproductive function encourages speculation that mild regimens of CR can produce longevity benefits without impairing reproduction.

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