



RESEARCH ARTICLE

Interaction of *growth hormone receptor/binding protein* gene disruption and caloric restriction for insulin sensitivity and attenuated aging [v1; ref status: indexed, <http://f1000r.es/4fk>]

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Abstract

The correlation of physiological sensitivity to insulin (*vis-à-vis* glycemic regulation) and longevity is extensively established, creating a justifiable gerontological interest on whether insulin sensitivity is causative, or even predictive, of some or all phenotypes of slowed senescence (including longevity). The *growth hormone receptor/ binding protein* gene-disrupted (GHR-KO) mouse is the most extensively investigated insulin-sensitive, attenuated aging model. It was reported that, in a manner divergent from similar mutants, GHR-KO mice fail to respond to caloric restriction (CR) by altering their insulin sensitivity. We hypothesized that maximized insulin responsiveness is what causes GHR-KO mice to exhibit a suppressed survivorship response to dietary (including caloric) restriction; and attempted to refute this hypothesis by assessing the effects of CR on GHR-KO mice for varied slow-aging-associated phenotypes. In contrast to previous reports, we found GHR-KO mice on CR to be *less* responsive than their *ad libitum* (A.L.) counterparts to the hypoglycemia-inducing effects of insulin. Further, CR had negligible effects on the metabolism or cognition of GHR-KO mice. Therefore, our data suggest that the effects of CR on the insulin sensitivity of GHR-KO mice do not concur with the effects of CR on the aging of GHR-KO mice.

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Introduction

Improvements in insulin sensitivity or blood glucose homeostatic management are hallmarks of many slow-aging mutant and dietarily restricted animals, supporting the conjectures that these endocrine and metabolic phenomena may be positive regulators of (or simply indicators of interventions that might promote) longevity [Arum *et al.*, 2009; Bartke, 2008; Bonkowski *et al.*, 2006; Lawler *et al.*, 2008; Longo & Finch, 2003; Masoro, 2003; Masoro, 2005; Mattison *et al.*, 2007; Piper & Bartke, 2008]. A recently proffered approach to biomedical ventures endeavoring to delay aging, and thus increase healthspan (the period of life during which an organism is free of substantial morbidity or physiological disability/inability), begins with studying interventions that increase lifespan [Kenyon, 2010; Miller, 2009; Olshansky *et al.*, 2007; Warner & Sierra, 2009]. Therefore, it is of high gerontological interest to study causal associations between longevity and physiological correlates that might result in anti-aging healthspan therapies based on engendering those physiological correlates, or that might serve as useful biomarkers for pharmacological or lifestyle interventions to delay the onset and/or decelerate the rate of senescence.

The *growth hormone receptor/binding protein* (*Ghr/bp*) gene-disrupted (knockout) (GHR-KO) mouse is homozygous for a targeted disruption (knock-out, KO) of the *growth hormone* (GH) *receptor* (GHR)/*binding protein* gene, and is thus GH-resistant, resulting in decreased GH hormonal signaling. GHR-KO mice were generated by insertional mutagenesis that disrupted the *Ghr/bp* gene; this results in decreased hepatic production of insulin-like growth factor 1 (IGF-1), which leads to markedly reduced levels of circulating IGF-1, a reduced growth rate, an approximately 20% reduction in adulthood length, and an approximately 40% reduction in adult body weight [Zhou *et al.*, 1997].

Of particular note, the GHR-KO mouse outlives its littermate control by approximately 40% [Coschigano *et al.*, 2000; Coschigano *et al.*, 2003].

Produced in the β -cells of the pancreatic Islets of Langerhans, the hormone insulin regulates metabolism and energy homeostasis, partly by inducing the tissue uptake of glucose from blood. The GHR-KO mouse exhibits markedly decreased plasma insulin levels, due partly to decreased proliferation of β -cells [Liu *et al.*, 2004]. As blood insulin concentration inversely mediates the systemic insulin sensitivity, insulin sensitivity is greater in the GHR-KO mouse than in its littermate control [Liu *et al.*, 2004]. This is also the case with multiple other long-lived mice [Brown-Borg *et al.*, 1996; Conover & Bale, 2007; Conover *et al.*, 2008; Dominici *et al.*, 2002; Selman *et al.*, 2008].

Results from survivorship studies reveal that aging-retarding (and thus, lifespan-increasing) dietary restriction (DR), including yet not limited to caloric restriction (CR), further increases insulin sensitivity and survivorship for some long-lived mutants, the Ames (*Prop¹^{df/df}*) Dwarf mouse [Bartke *et al.*, 2001] and the *growth hormone releasing hormone* KO (*Ghrh^{-/-}*) mouse (data not shown). However it has been reported that CR doesn't influence insulin sensitivity, and only modestly increases the survivorship of females, in the GHR-KO mouse [Bonkowski *et al.*, 2006].

As an initial hypothesis, if CR fails to exert much effect on one senescence-associated trait (longevity) of the GHR-KO mouse because its level of insulin sensitivity is already as great as permissible for a viable animal, then a GHR-KO mouse on CR should not vary from a GHR-KO mouse on an *ad libitum* (A.L.) diet in other aging-associated characteristics (namely, metabolism and cognition).

Therefore, we attempted to investigate whether insulin sensitivity is sufficient to explain the severely attenuated response to CR of slow-aging associated phenotypes in GHR-KO mice. Surprisingly, in the course of our experiments we discovered that CR actually increases blood insulin concentration and starkly reduces insulin sensitivity in GHR-KO mice. These results question the assertion that CR has no effect on GHR-KO mouse blood glucose homeostatic management and the relationship, if any, between insulin sensitivity and slowed senescence in GHR-KO mice.

Materials and methods

Animal husbandry

Ethics statement. Animal Protocol #178-02-001 was approved by the Laboratory Animal Care and Use Committee of Southern Illinois University-School of Medicine.

Ghr/bp gene-disrupted (GHR-KO) mice were generated by inserting a neomycin cassette replacing the 3'-end of the fourth exon and the 5'-end of intron 4/5 of the genomic sequence [Zhou *et al.*, 1997]. The founder population of GHR-KO mice was provided by Dr. John J. Kopchick (Ohio University, Athens, OH). GHR-KO and GHR-N (heterozygous *littermate* controls for GHR-KO mice) mice were generated by mating of GHR-KO males with females heterozygous for the *Ghr/bp*-disrupted allele (GHR-N). These breeding schemes produce *littermate* control mice that have the same genetic background and are subject to the same intra-uterine and post-natal environment as the mutants.

Abiding by service provider's instructions for sample collection and shipping, genotyping was conducted via quantitative polymerase chain reaction (q-PCR)-based technologies (Transnetyx, Inc., Cordova, TN).

The resulting mice had elements of a 129/Ola, a Balb/c, two C57Bl/6J, and two C3H/HeJ stocks; therefore, although lacking the methodological benefits of "reproducible genetic heterogeneity" [Miller, *et al.*, 1999], this stock possesses considerable genetic variation, and thus the results are likely applicable to other mouse populations.

The animals were maintained in shoebox-type cages in light- (12 hours light to 12 hours darkness) and temperature- ($22 \pm 2^\circ\text{C}$) controlled rooms with constant access to Lab Diet Formula 5001 (23% protein, 4.5% fat, 6% fiber) (Nestlé Purina, St. Louis, MO) and tap water. Littermate control pups were weaned at the age of 21–23 days, and GHR-KO pups two weeks later or at the time of weaning control pups from the next litter.

All experiments were performed in female mice, as GHR-KO stock male littermate controls are insensitive to the common dosage of insulin (0.75 U.S.P.U./kg B.W.) during insulin tolerance testing [Arum *et al.*, 2009; Bonkowski *et al.*, 2006].

Caloric restriction

Mice were 4–8 months of age at inception of restriction.

The amount of food allotted each cage of mice designated for caloric restriction was determined based on (weekly calculated) *ad libitum* food consumption for entire cages of gender-, genotype-, and birth date-matched controls; these values were averaged over the number of cages within each such group. Two hundred grams of the above-described food was placed in each A.L. cage-hopper on a weekly basis. After six days of food consumption, the remaining food was weighed on a Scout Pro Balance (Ohaus Co., Pine Brook, NJ) calibrated to weight standards on a monthly basis; food consumption values were calculated as follows: $\{(200 \text{ g.}) - [\text{food remaining after six days (in g.)}]/\text{six (days)}/\text{number of subjects in cage.}$

As a protection against dissimilar food consumption within CR cages, part of the food was broken into pieces small-enough to pass through the hopper-grate (but not crumbs). Our observations confirmed that this method allowed every restricted mouse to feed *ad libitum* during the initial surge of food consumption. Considering the valid concerns related to differential restriction resulting from a dominant cage-mate consuming more than their fair proportion, we paid particular attention to any individual mouse weight loss and health (e.g. fight wounds indicative of physical conflicts with a cage-mate) throughout our studies. It is also worth noting that our chosen level of restriction (30%) is moderate compared to the 40% level that causes considerable concerns [Liao *et al.*, 2010; Mattson, 2010]. This moderate level does not lead to an extinguishment of food supply after the initial gorge (thus, even subordinate mice have ample, albeit possibly delayed, access to food) and does not result in substantial weight loss for any sub-cohort of animal-subjects within our stocks (Figure 1A and B).

Of relevance to obviating unintended interactions between experimental factors, which might produce confusing or obfuscating variation within the data, mice were housed in genotype-, age-, and diet-specific cages.

Weekly body weight determinations and food consumption measurements

Mice were weighed weekly on a Scout Pro Balance (Ohaus Co., Pine Brook, NJ) that was calibrated to weight standards on a monthly basis. All mice were weighed in the late afternoon, approximately 20 hours after the restricted mice had been fed.

Age-grade classification

Mice were young-adults in all experiments except for the indirect calorimetry trials involving CR, the spontaneous locomotor activity experiments and the behavior (anxiety & memory) experiments, where mice had to be middle-aged in order to address gerontological queries.

Age-staging was based on a combination of 1) quantitative extrapolation from prior stock-specific survivorship data [Bonkowski *et al.*, 2006], 2) presence/appearance of aging-associated wizening (as represented quantitatively by declining body weight), and 3) spontaneous, testing-independent, (and presumably) aging-resultant mortality. Thus, young-adulthood is marked by at least 90% of

reproductively competent negative control subjects being alive; middle-age is the period between when approximately 90% of the control subjects are still alive and median survivorship; old-age is the period between median survivorship and when approx. 10% of the subjects are alive; and oldest-old age is designated as the period when $\leq 10\%$ of the controls remain.

Blood glucose regulatory assessments

All animals underwent home-cage assessments of gross health (Supplemental Table 1) and any animal exhibiting questionable health by these criteria, or which was aberrantly hypoglycemic at the inception of a test, was excluded from the testing and/or data analysis. In addition, all animals were given at-least two weeks of recuperation in-between tests.

Glucose tolerance testing [*ad libitum* (A.L.)-fed or fasted]

For A.L.-fed tests, animals had access to food for at least 16 hours before the test. For fasted tests, animals were fasted for 16 hours, although CR animals were A.L.-fed the day before the 16-hour fast commenced. Thirty minutes prior to beginning the test, each animal was weighed, had a small nick placed at the tip of its tail with a razor, and re-housed without access to food. After 30 minutes to recover from the handling stress of the weighing and tail-nicking, blood glucose concentration was assessed in each animal. Blood was obtained by applying a gentle pressure to the tail-tip, with a blood glucose monitoring system (glucometer and testing strips) (OneTouch Ultra 2, Lifescan, Inc., Milpitas, CA). Without releasing the grasp on the animal, it was manually repositioned to a nearly supine pose, and injected inter-peritoneally with 2 g. D-(+)-glucose (Sigma-Aldrich Co., St. Louis, MO) per kg of body weight. [The powdered glucose was dissolved in 0.9% sodium chloride (Sigma-Aldrich Co., St. Louis, MO)]. Subsequent blood glucose measurements were at 10, 20, 30, 40, 50, 60, 75, 90, and 120 minutes after the injection. Animals were given A.L. access to food immediately after completion of the test.

Insulin tolerance testing

Animals had access to food for at least 16 hours before the test. Animals were prepared for injection as described for glucose tolerance testing (above). Animals were injected inter-peritoneally with 0.75 U.S.P.U. of porcine insulin (Sigma-Aldrich Co., St. Louis, MO) per kg of body weight. (The lyophilized insulin was dissolved in 0.9% sodium chloride). Subsequent blood glucose measurements were at 10, 20, 30, 40, 50, 60, 75, 90, and 120 minutes after the injection. Animals were given A.L. access to food immediately after completion of testing.

Pyruvate conversion testing

Animals were fasted for 16 hours and CR animals were A.L.-fed the day before the fast commenced. Animals were prepared for injection as described for glucose tolerance testing (above). Animals were injected inter-peritoneally with 2 g of sodium pyruvic acid (Sigma-Aldrich Co., St. Louis, MO) per kg of body weight. (The lyophilized sodium pyruvate was dissolved in 0.9% sodium chloride). Subsequent blood glucose measurements were at 15, 30, 45, 60, and 120 minutes after the injection. Animals were given A.L. access to food immediately after completion of testing.

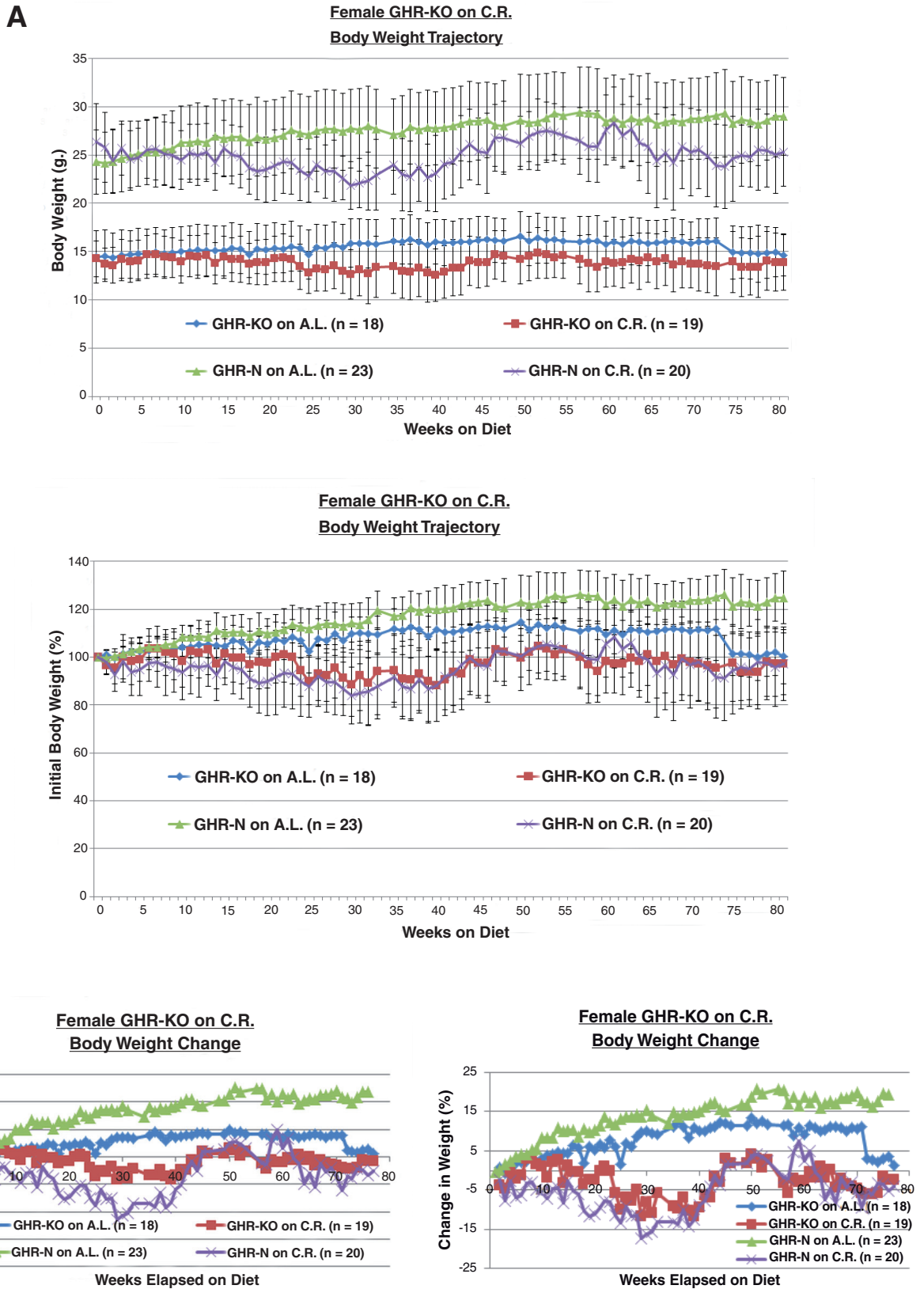


Figure 1. Effects of genotype and diet on body weight. A. 30% caloric restriction represses body weight gain (absolute or normalized-to-initial) in female GHR-KO mice and their littermate controls. **B.** 30% caloric restriction reins change in body weight (absolute or normalized-to-initial) in GHR-KO females and their littermate controls.

Non-stimulated blood glucose comparisons [*ad libitum* (A.L.)-fed or fasted]

Un-stimulated blood glucose values were obtained from young-adult mice at the beginnings of A.L.-fed and fasted glucose tolerance tests, drawn from a small nick at the tip of the tail and measured with a blood glucose monitoring system (glucometer and testing strips) (OneTouch Ultra 2, Lifescan, Inc., Milpitas, CA). A.L.-fed blood glucose values were collected after an overnight (~16 hrs.) period of A.L. feeding for all subjects; fasted blood glucose values were gathered after equivalent overnight fasting (~16 hrs.) for all subjects.

A.L.-fed blood glucose values recorded immediately preceding a sacrifice and tissue harvesting from middle-aged mice were consistent with the results obtained as above. As a control against inferences drawn from the possible effects of short-term fasting, CR mice were noted to have stomachs freighted with foodstuff upon the sacrifice that followed the blood glucose assessment.

A.L.-fed and fasted indirect calorimetry

Indirect calorimetry was conducted as previously described by Westbrook *et al.*, (2009) (Accuscan Instruments, Inc., Columbus, OH). Acclimation day testing, A.L.-fed day testing and fasted day testing were all conducted in one longitudinal stretch. Data were normalized per unit of lean body weight [Butler & Kozak, 2010] as determined by fat depot sub-dissection [Berryman *et al.*, 2010; Muzumdar *et al.*, 2008]. The values at the 17:00 hour were excluded from the statistical analyses, as this time was used for maintenance activities (*e.g.* removal of food, weighing of remaining food, and weighing of mice) during longitudinal testing paradigm. Parameters assessed are annotated in [Supplemental Table II a](#).

A.L.-fed and fasted spontaneous locomotor activity

Spontaneous locomotion was assessed using the same equipment and the indirect calorimetry procedure described above (Accuscan Instruments, Inc., Columbus, OH). The values measured at the 17:00 hour were excluded from the statistical analyses, as this time was used for maintenance activities (*e.g.* removal of food, weighing of remaining food, and weighing of mice) during longitudinal testing paradigm. Parameters assessed are annotated in [Supplemental Table II b](#).

Complete blood cell (C.B.C.) count analysis of hematopoietic cell parameters

Blood cell counting was accomplished using a VetScan HM2 Hematology System (Abaxis, Union City, CA) and $\geq 25 \mu\text{L}$ of whole blood [collected in EDTA-coated Microvette 100 μL capillary tubes (Sarstedt AG & Co., Nümbrecht, Germany)] drawn from *ad libitum*-fed subjects. The following 18 parameters were assessed: concentration of leukocytes/white blood cells (W.B.C.), concentration of lymphocytes (LYM), concentration of monocytes (MON), concentration of granulocytes (GRA), proportion of leukocytes that are lymphocytes (LYM%), proportion of leukocytes that are monocytes (MON%), proportion of leukocytes that are granulocytes (GRA%), concentration of erythrocytes/red blood cells (R.B.C.), concentration of hemoglobin (g/dL) (HGB), hematocrit (%) (HCT), mean (erythrocytic) cell volume (M.C.V.), mean corpuscular hemoglobin (pg.) (M.C.H.), mean corpuscular hemoglobin concentration (g/dL) (M.C.H.C.), red cell (erythrocytic) distribution width (%) (R.D.W.), concentration of thrombocytes/platelet cells (PLT), mean platelet

volume (M.P.V.), plateletcrit/platelet hematocrit (%) (PCT), platelet distribution width (%) (P.D.W.).

Insulin biochemical assays

Plasma insulin was measured with the multiplexed Mouse Endocrine Lincoplex ELISA kit (LINCO Research, St. Charles, MO).

One-, two-, and five-minutes open-field chamber anxiety/exploratory inclination assay

All animals underwent home-cage assessments of gross health, locomotor ability and activity ([Supplemental Table I](#)). Animals exhibiting questionable health based on these criteria were excluded from the testing.

During the light-phase of their day, the mice were individually placed in the center of a lid-less, opaque, white, $44 \times 44 \times 40$ cm. (length \times width \times height) polymer box with the floor divided into $16 \times 11 \times 11$ cm². The number of squares entered within the allotted time was noted per mouse per trial; as the experiment is contingent upon the novelty of the aberrant context, each subject was only tested once. The methods used derived from standard methodologies previously used “to analyze general activity and exploratory drive” [Crawley, 2007; Selman *et al.*, 2009].

Open-field chamber proximal long-term memory assay

All animals underwent home-cage assessments of gross health, and locomotor ability & activity ([Supplemental Table I](#)). Animals exhibiting questionable health based on these criteria were excluded from the testing.

During the light-phase of their day, mice were individually placed in the center of a lid-less, opaque, white, $44 \times 44 \times 40$ cm (length \times width \times height) polymer box with the floor divided into $16 \times 11 \times 11$ cm². The number of squares entered within one minute was noted per mouse per trial; 24 hours after the initial evaluation (acquisition), the mice were re-tested (retention). Memory index values were calculated per mouse as follows: (Retention activity/Acquisition activity); these reflect the degree to which the subject remembered the context presented 24 hours prior (with enhanced memory putatively resulting in more movement due to less anxiety). Final location scores evaluate the ultimate (after the 60-second testing interval) position of a mouse on the retention day, with a more-ensconced placement being indicative of greater anxiety (and, thus, worse memory of prior context) than a more-exposed positioning. The methods derived from standard methodologies used by other investigators [Crawley, 2007].

Data presentation and statistical analysis

Graphs were generated with Excel (Microsoft, Redmond, WA) and IrfanView Image Viewer (Irfan Skiljan, Wiener Neustadt, Austria; <http://www.irfanview.com/>). The measures of central tendency are arithmetic means, and all depictions of variation (error bars) represent the standard deviations (S.D.) [Glanz, 2002].

Pre-hoc statistical measures

In brief, experimental design approaches were taken to maximize robustness while lessening the potential need to increase sample size; utilizing 1) an *a priori* specification of a limited number of

well-defined hypotheses, 2) refinement of experimental techniques, and 3) grouping of animals so that the effect of unit variability on the treatment was minimized.

Post-hoc statistical analysis

Levene's tests (to investigate scedasticity) and Kolmogorov-Smirnov tests (to determine deviations from Gaussian distribution) were conducted to guide the choice of statistical algorithms for analysis of differences amongst groups. The combined parameters of effect size and Type 1 error probability were considered when determining phenomena meriting presentation and discussion.

Most data were contrasted with unpaired, homoscedastic Student's *t*-test, Analysis of Variance, or Analysis of Variance for Repeated Measures (ANOVA or ANOVA-R.M., resp.), as appropriate; followed by the Tukey's Honestly Significant Difference (H.S.D.) or the Dunnett's *t*-test post-hoc tests for multiple pairwise comparisons, as appropriate.

For repeatedly measured blood glucose regulatory assessments, the *p*-value for a given pairwise comparison at a given time-point represents the result of testing all of the time-points, up-to-and-including that time-point, within the repeated measures analysis; this permits testing whether both groups have experienced similar excursions in blood glucose (the null hypothesis) relative to their initial values and with consideration of all intermediate values. This mode of analysis poses more discrete and descriptive inquiries than analyzing the area under respective curves or utilizing isolated, independent blood glucose values/percentages at lone time-points. The data that are normalized to initial blood glucose values were used for the precise, time-point-specific *p*-values reported, yet the inferences of differences amongst groups do not depend on the use of these normalized data. For a particular pairwise comparison within a particular assay, the *p*-value reported in the text is the most conservative (*i.e.* highest) sub-0.05 *p*-value from the series of repeated measures analyses.

In instance of considerable variation in data confounding inferences, the data outside of 1 S.D. might have been equilaterally excluded from the data used for statistical analysis.

Statistical comparisons were conducted with PSPP for Windows (Free Software Foundation, Inc., <http://www.gnu.org/software/pspp/get.html>).

Results

Probing parameters pursuant to proliferation

A 30% CR resulted in the standard body weight (B.W.) gain attenuation, whether represented in absolute grams or in percentage-of-initial (Figure 1A) or in body weight change in grams or in percentage-of-initial (Figure 1B).

When scrutinizing proliferation on a cellular level, hematocytometric analyses of various blood cell parameters (*e.g.* erythrocytes, leukocytes, and platelets) in late-middle-aged (~25 months-of-age) revealed no effect of CR on either GHR-N mice or GHR-KO mice (Table 1).

Blood glucose homeostatic regulation experiments

Importantly, there was no effect of the 30% CR diet on B.W. measured immediately preceding the testing for any of the blood glucose homeostasis regulation assays (Figure 2A).

In relation to our hypothesis, CR increased A.L.-fed glucose incorporation in fed female GHR-KO mice during glucose tolerance testing [$p = 0.0467$], (Figure 2B), but had no effect in fasted GHR-KO mice (Figure 2C). As for the insulin tolerance tests, CR attenuated the sensitivity of GHR-KO females to 0.75 U.S.P.U./kg B.W. of insulin [$p = 0.0483$], (Figure 2D). CR did not alter the pyruvate conversion potential in female GHR-KO mice (Figure 2E). Additionally, CR increased the plasma insulin content in GHR-KO mice [$p < 0.05$], (Figure 2F).

Therefore, our data show additive or synergistic effects of CR with the GHR-KO gene disruption on blood glucose homeostasis.

Indirect measures of metabolism

Measurements estimating the general rate of metabolic processes have long been correlated with ultimate survivorship, and have been proffered as sufficient to explain the rate of senescence [Rubner, 1908]. Whether the mechanisms by which CR retards senescence include alterations (particularly, decreases) in metabolism has been an active research hypothesis for some time [Ramsey *et al.*, 2000].

Indirect (gas exchange) calorimetric measurements of metabolism have been reported to be increased [Westbrook *et al.*, 2009], as well as decreased [Carrillo & Flouris, 2011; Mookerjee *et al.*, 2010], in animals with extended longevity. Identifying metabolic phenotypes that transcend one particular genetic background or mode of delaying and/or decelerating aging would be important for proposing or testing mechanisms of extended lifespan and healthspan.

During our analyses of oxygen consumption (VO_2), respiratory quotient (R.Q.)/respiratory exchange ratio (R.E.R.), heat production (Calories/hr), and energy expenditure (E.E.) in A.L.-fed and fasted female GHR-KO mice on CR, no genotype- or diet-based differences were detected for food consumption, changes in body weight induced by either acclimation or fasting, or thermogenesis (as crudely measured with an ambient thermometer in each chamber) while the subjects were in the indirect calorimetry chambers from the acclimation day through the A.L.-fed day to the fasted day (Table 2).

CR did not affect these A.L.-fed indirect calorimetry-based measures of metabolism effects of female littermate controls, nor that of female GHR-KO mice (Table 3).

Spontaneous locomotor activity late in life, as it can serve as a measure of the multi-factorial syndrome of frailty [Walston *et al.*, 2006] (*i.e.* frail mice would presumably be disinclined to move, or cover less area when trying) is often used as a behavioral marker of delayed and/or decelerated senescence [Ingram, 2000; Manini, 2010; Minor *et al.*, 2011; Neff *et al.*, 2013; Wilkinson *et al.*, 2012; Zhang *et al.*, 2014].

Table 1. Complete blood cell analysis reveals effects of *Ghr/bp* gene disruption, but not of caloric restriction.

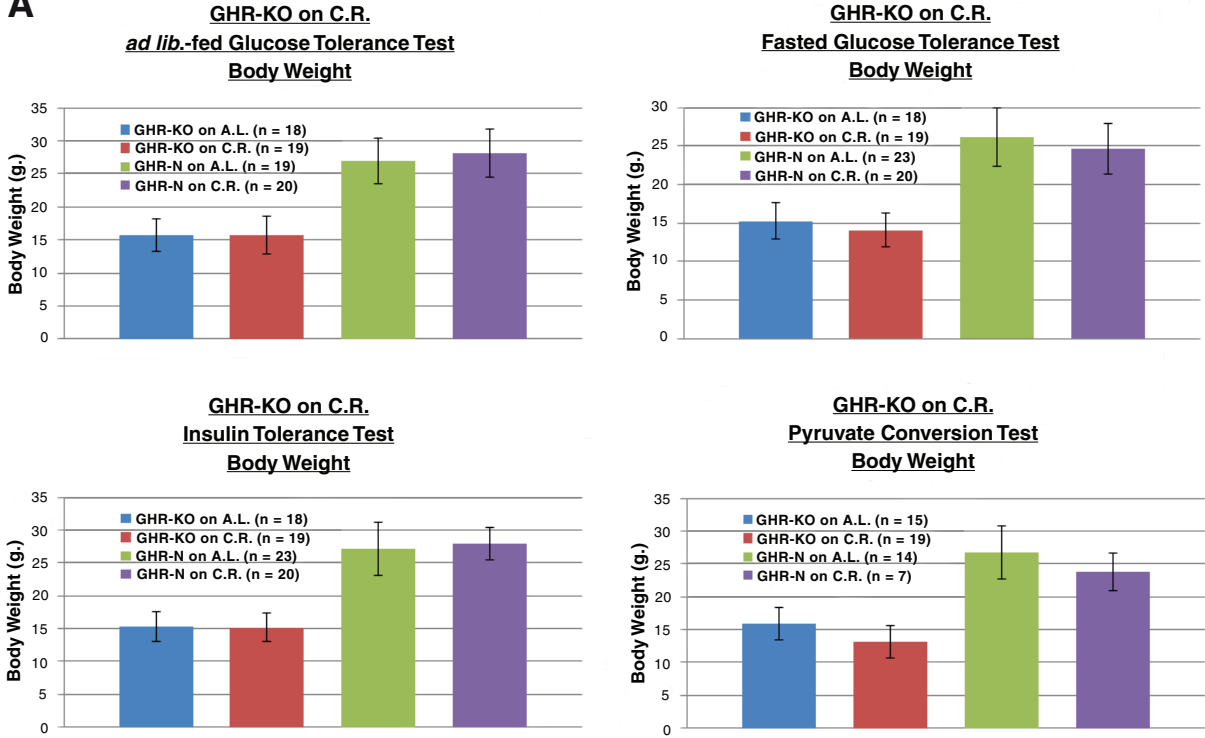
Group	Weight (g)	Blood Glucose (mg/dL)	WBC (10 ⁹ /L)	LYM (10 ⁹ /L)	MON (10 ⁹ /L)	GRA (10 ⁹ /L)	LY% (%)	MO% (%)	GR% (%)	RBC (10 ¹² /L)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDWc (%)	PLT (10 ⁹ /L)	PCT (%)	MPV (fL)	PDWc (%)
GHR-KO on A.L. (n = 15)	15.9333	110.6	9.5613	8.8153	0.3447	0.4087	92.3333	3.4067	4.4733	7.986	10.72	38.0713	48.2	13.42	27.9133	19.4333	169	0.1093	6.5533	33.8067
GHR-KO on C.R. (n = 18)	15.1267	74	6.3639	5.1517	0.1883	1.0294	81.5056	2.6778	15.9333	7.6256	10.3056	37.1589	48.7222	13.4389	27.5833	19.5778	169.7222	0.1133	6.6111	33.8389
GHR-N on A.L. (n = 13)	28.2846	123.1538	8.6477	7.0115	0.3231	1.3138	80.1	3.8154	16.0769	10.4108	15.0846	49.8562	47.8462	14.4769	30.2615	18.2538	210.3077	0.1462	6.6923	33.2769
GHR-N on C.R. (n = 7)	27.6857	93.2857	6.8171	5.0771	0.3071	1.4357	75.9	4.3714	19.7286	9.6171	13.6571	46.1057	48	14.1857	29.6143	18.3857	209.1429	0.1529	7.4429	36.3143
Comparison	Weight (g)	Blood Glucose (mg/dL)	WBC (10 ⁹ /L)	LYM (10 ⁹ /L)	MON (10 ⁹ /L)	GRA (10 ⁹ /L)	LY% (%)	MO% (%)	GR% (%)	RBC (10 ¹² /L)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDWc (%)	PLT (10 ⁹ /L)	PCT (%)	MPV (fL)	PDWc (%)
N on A.L. vs. KO on A.L.	0.0001	0.0504	0.8781	0.6014	0.3778	0.0316	0.04	0.149	0.0791	0.0001	0.0001	0.0001	0.3932	0.0001	0.0001	0.0067	0.1624	0.0766	0.0962	0.2792
N on A.L. vs. N on C.R.	0.8328	0.7483	0.491	0.3614	0.8382	0.641	0.4016	0.8645	0.399	0.7149	0.6556	0.6557	0.7437	0.5829	0.8362	0.6758	0.9446	0.9004	0.5117	0.3194
N on A.L. vs. KO on C.R.	0.0001	0.0206	0.978	0.513	0.475	0.0561	0.077	0.2159	0.1407	0.0001	0.0001	0.0001	0.1039	0.0001	0.0001	0.0006	0.2506	0.1568	0.126	0.3383
KO on A.L. vs. KO on C.R.	0.6774	0.8026	0.8524	0.9033	0.8787	0.8463	0.8021	0.865	0.8167	0.996	0.8345	0.8294	0.6563	0.4264	0.7119	0.6605	0.8007	0.7092	0.9049	0.9199

Table 2. Assessments within indirect calorimetry/spontaneous activity chambers detail lack of genotype- or diet-based differences for food consumption, body weight, or thermogenesis.

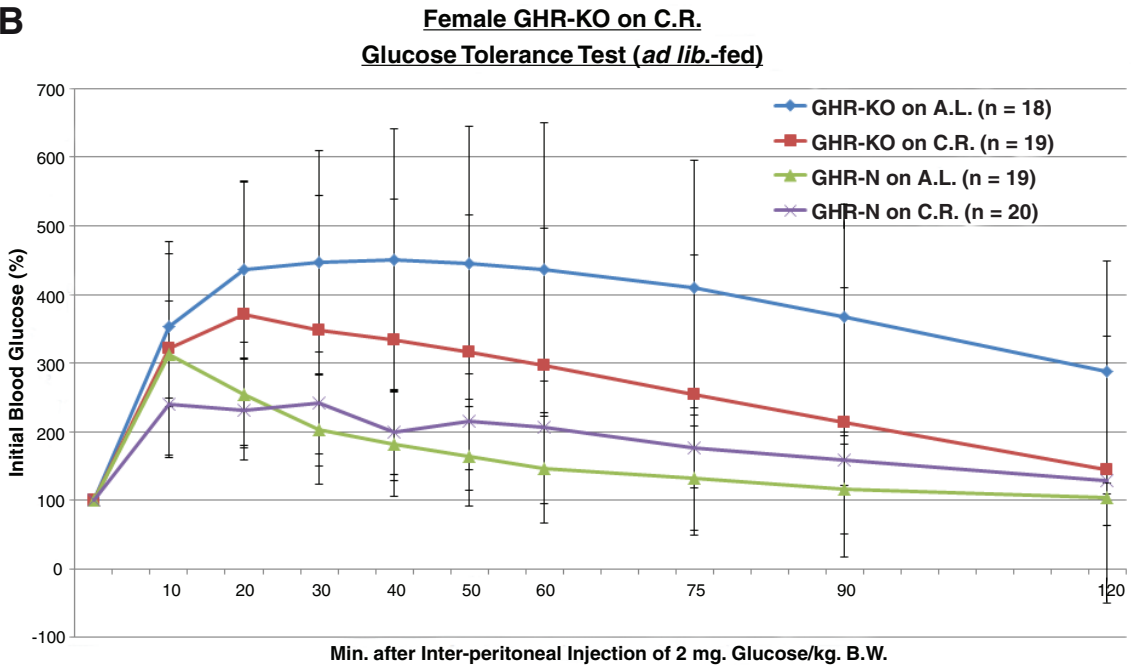
Group	Body Wt. (g.) on Day After.....			Food Remaining (g.) on Day After.....			Chamber Temp. (°C) on Day.....		
	Acclimation	A.L.-feeding	Fasting	Acclimation	A.L.-feeding	Fasting	Before Acclimation	Before A.L.-feeding	After Fasting
GHR-KO on A.L. (n = 15)	15.7133	15.48	14.7067	22.0667	19.3733		23.95	24.0111	23.9778
GHR-KO on C.R. (n = 19)	14.3737	14.1632	13.4526	21.7421	19.1053		23.8	24.0923	23.9083
GHR-N on A.L. (n = 13)	27.7	27.2923	26.0308	21.7077	18.3167		23.7111	24.0333	24.3222
GHR-N on C.R. (n = 7)	28.4429	28.4857	26.7	18.9	14.0286		23.6333	23.95	24.1667

n.b.: 25 g. of food placed in each chamber at beginning of acclimation day

A



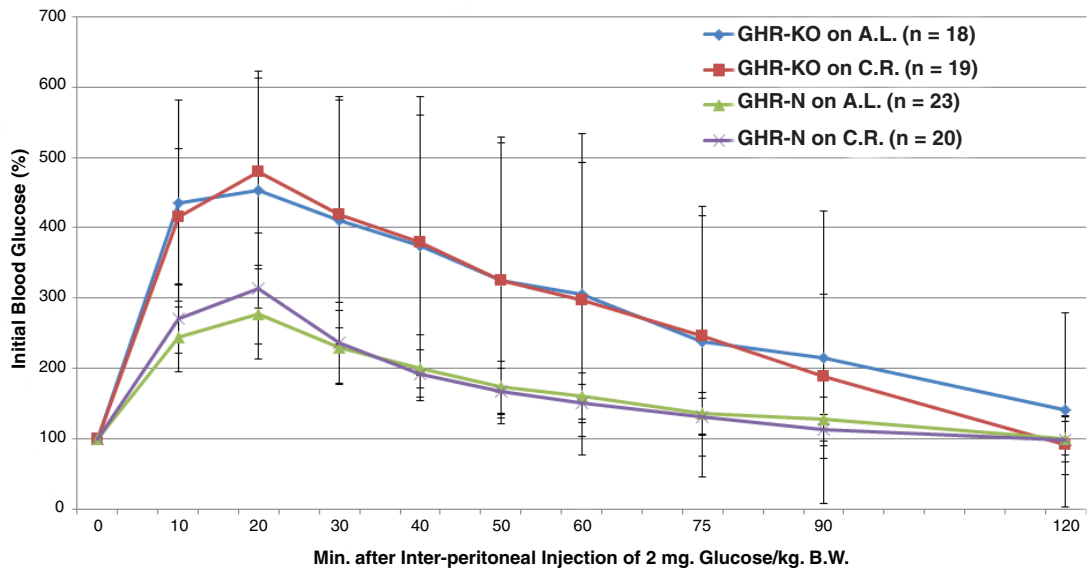
B



Comparison	0 min.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	75 min.	90 min.	120 min.
N on A.L. vs. KO on A.L.		0.3143	0.0021	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
N on A.L. vs. N on C.R.		0.8723	0.97	0.7135	0.5206	0.3764	0.2838	0.2472	0.214	0.2156
N on A.L. vs. KO on C.R.		0.4266	0.0091	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
KO on A.L. vs. KO on C.R.		0.8497	0.6251	0.4195	0.3029	0.1709	0.0912	0.0467	0.0252	0.0158

C

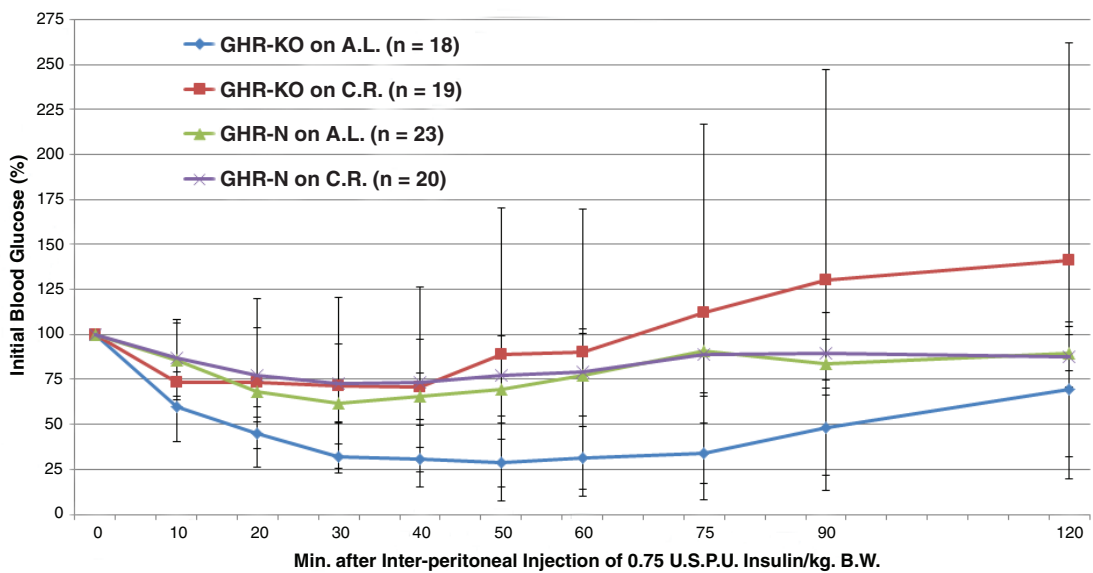
**Female GHR-KO on C.R.
Glucose Tolerance Test (Fasted)**



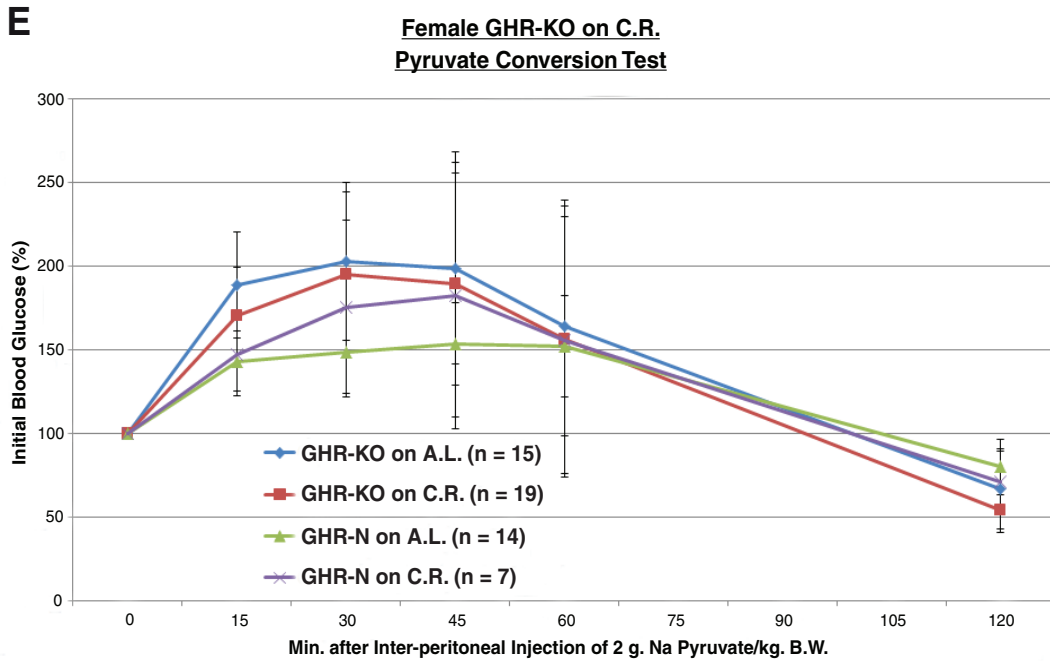
Comparison	0 min.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	75 min.	90 min.	120 min.
N on A.L. vs. KO on A.L.		0.0009	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
N on A.L. vs. N on C.R.		0.8231	0.6807	0.6584	0.6551	0.6866	0.7078	0.7232	0.7527	0.7539
N on A.L. vs. KO on C.R.		0.0011	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
KO on A.L. vs. KO on C.R.		0.9567	0.8924	0.7556	0.6409	0.5668	0.5152	0.4893	0.49	0.5414

D

**Female GHR-KO on C.R.
Insulin Tolerance Test**



Comparison	0 min.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	75 min.	90 min.	120 min.
N on A.L. vs. KO on A.L.		0.0037	0.0025	0.0007	0.0001	0.0002	0.0001	0.0003	0.0138	0.1293
N on A.L. vs. N on C.R.		0.6039	0.3707	0.2337	0.1146	0.0599	0.0394	0.0364	0.0234	0.018
N on A.L. vs. KO on C.R.		0.006	0.0024	0.0006	0.0001	0.0001	0.0001	0.0001	0.0034	0.0296
KO on A.L. vs. KO on C.R.		0.0453	0.0479	0.0483	0.0466	0.0450	0.0421	0.0403	0.0362	0.0314



Comparison	0 min.	15 min.	30 min.	45 min.	60 min.	120 min.
N on A.L. vs. KO on A.L.		0.0214	0.0005	0.0002	0.001	0.0265
N on A.L. vs. N on C.R.		0.7385	0.4374	0.3545	0.3878	0.5149
N on A.L. vs. KO on C.R.		0.0288	0.0005	0.0001	0.0002	0.007
KO on A.L. vs. KO on C.R.		0.828	0.9841	0.6618	0.5143	0.5676

F Female GHR-KO on C.R.
Plasma Insulin

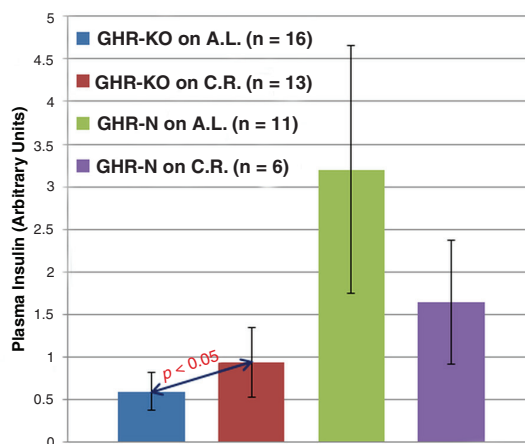


Figure 2. Endocrinological assessment of effect of 30% caloric restriction on GHR-KO mouse. A. 30% caloric restriction does not affect body weight immediately preceding a tolerance or conversion test for GHR-N or GHR-KO females. **B.** 30% caloric restriction partially corrects the glucose intolerance of female GHR-KO mice under A.L.-fed conditions (including repeated-measures statistical analysis table). **C.** 30% caloric restriction does not affect the glucose intolerance of GHR-KO females under fasted conditions (including repeated-measures statistical analysis table). **D.** 30% caloric restriction corrects the enhanced insulin sensitivity of female GHR-KO mice (including repeated-measures statistical analysis table). **E.** 30% caloric restriction does not significantly alter the heightened *de novo* hepatic glucose production of GHR-KO females (including repeated-measures statistical analysis table). **F.** 30% caloric restriction increases plasma insulin in female GHR-KO mice.

Table 3. Caloric restriction did not affect A.L.-fed indirect calorimetry-based measures of metabolism.

Group	VO ₂	VCO ₂	R.Q. (R.E.R.)	Heat Prod.	Energy Exp.
GHR-KO on A.L. (n = 15)	91.6681	56.0253	0.6533	412.9019	1772.5304
GHR-KO on C.R. (n = 17)	94.9652	59.5818	0.7117	375.2352	1843.8404
GHR-N on A.L. (n = 12)	46.0284	39.6722	0.8636	385.5301	948.1727
GHR-N on C.R. (n = 7)	54.6331	51.8225	0.8973	434.147	1149.1911
Comparison	VO ₂	VCO ₂	R.Q. (R.E.R.)	Heat Prod.	Energy Exp.
N on A.L.vs.KO on A.L.	0.0001	0.0001	0.0001	0.194	0.0001
N on A.L.vs.N on C.R.	0.0262	0.0001	0.0601	0.0966	0.0027
N on A.L.vs.KO on C.R.	0.0001	0.0001	0.0001	0.6373	0.0001
KO on A.L.vs.KO on C.R.	0.4652	0.0139	0.0026	0.0248	0.3517

Similarly, CR had no germane effect on the spontaneous locomotion of female littermate control mice, or female GHR-KO mice (Table 4).

Cognitive assessments

The retention of cognitive capability (in particular, memory function) into middle-age and beyond in the GHR-KO mice further supports that the effects of the *Ghr/bp* disruption extend beyond increasing survivorship, to include ameliorating senescence and its resultant functional decrements [Bartke, 2005; Kinney *et al.*, 2001a; Kinney *et al.*, 2001b; Kinney-Forshee *et al.*, 2004; O. Arum & A. Bartke, (unpublished data)]. With noteworthy exceptions, which are partly due to varied methodologies of caloric restriction or cognitive assessment amongst scientists, CR is broadly considered to be beneficial for retarding aging-resultant cognitive decline [Arslan-Ergul *et al.*, 2013; Joseph *et al.*, 2009].

Regarding the cognitive assessments, CR had no effect on the anxiety of either littermate control or GHR-KO females (Figure 3A). The memory index results derived from open-field activity (Figure 3B) did not support the initial hypothesis that the greater insulin sensitivity of GHR-KO mice precludes their full benefits from CR (as the GHR-KO mice on A.L. and the GHR-KO mice on CR, which have differing insulin responsiveness, did not differ in memory performance). Similar inferences were concluded for the open-field activity-based memory tests regarding the final location of the mice (Figure 3C).

Data set 1. Experimental data showing the effect(s) of growth hormone receptor/binding protein (*Ghr/bp*) gene disruption and/or caloric restriction on the various outcomes.

<http://dx.doi.org/10.5256/f1000research.5378.d37530>

Data showing the effects of either the senescence-retarding *Ghr/bp* mutation, the senescence-retarding 30% caloric restriction, or both factors on the following (groups of) traits: 1) body weight, 2) complete blood cell content, 3) food consumption, 4) ad libitum-fed glucose tolerance, 5) fasted glucose tolerance, 6) 0.75 U.S.P.U. insulin tolerance, 7) 0.3 U.S.P.U. insulin tolerance, 8) pyruvate conversion, 9) open-field activity, 10) open-field memory, 11) various characteristics peripheral to the indirect calorimetry and spontaneous activity chambers-based experiments, 12) ad libitum-fed indirect calorimetry and spontaneous activity, and 13) fasted indirect calorimetry and spontaneous activity.

Discussion

The initial aim of this study was to investigate if the very limited response of the GHR-KO mouse to a 30% CR diet in terms of longevity [Bonkowski *et al.*, 2006] is related to the inability of these mutants to respond to a 30% CR diet with regards to insulin sensitivity [Bonkowski *et al.*, 2006]. This was based on the hypothesis that it is the maximization of the response to CR in the insulin sensitivity test that acts as a “ceiling/floor” effect limiting the survivorship response to CR [Bonkowski *et al.*, 2006]. Our insulin sensitivity results in GHR-KO mice on 30% CR differed from those obtained in a previous study showing that caloric restriction promotes euglycemia in GHR-KO mice (Figure 2C). These differences might have been due to the difference in ages of the animals {12 months in [Bonkowski *et al.*, 2006] vs. 8–13 months in the present report}, or different durations of CR (10 or 12 months vs. 4–6 months, respectively). Those *caveats emptor* notwithstanding, that blood insulin content is increased by CR in GHR-KO mice (Figure 2E) dovetails with the improved performance in glucose bolus assimilation (Figure 2A), decreased insulin sensitivity (Figure 2C), and (statistically indistinguishable) decreased gluconeogenic capability (Figure 2D) of GHR-KO mice on CR relative to their A.L. counterparts. Moreover, data from macromolecular analysis of insulin signaling in GHR-KO mice on CR, including decreased insulin receptor (INSR) and thymoma viral proto-oncogene 1/protein kinase b (AKT1/PKB) concentrations in the skeletal musculature of GHR-KO’s on CR, and decreased phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) subunits content in the livers of GHR-KO’s on CR (relative to GHR-KO’s on AL), also corroborate and portend decreased insulin sensitivity in GHR-KO mice on CR [Bonkowski *et al.*, 2009]. Additionally, it is worth noting that a tight regulation of euglycemia would be more consistent with health and survival than a predilection for hypoglycemia [Tan & Flanagan, 2013], thus “improving health”, as CR has been broadly documented as doing, might mean preventing the innate endocrinological/metabolic derangements that are merely coincident with the longevity of the GHR-KO mouse. Finally, to the best of our knowledge, published reports on CR-mediated induction of insulin sensitivity (*vis-à-vis* increased blood glucose assimilation dynamics) using insulin tolerance tests or hyperinsulinemic-euglycemic clamping assays on healthy mice are either lacking or are not consistently reproduced. This is an important limitation, and *caveat emptor*, given that mutant mice with abnormal growth and adult body composition have been documented to have insulin

Table 4. Caloric restriction did not affect A.L.-fed measures of spontaneous locomotion.

Group	Total Distance	Hor. Activity Ct.	Amb. Activity Ct.	Rest Time	Rest Episode Ct.	Mvmt. Time	Mvmt. Episode Ct.	Stereo. Time	Stereo. Episode Ct.	Stereo. Activity Ct.	Vert. Episode Ct.	Vert. Activity Ct.	Vert. Activity Time	Loco. Clock. Revs.	Loco. Counter. Revs.
GHR-KO on A.L. (n = 15)	56.3851	192.6097	163.1155	556.6244	38.1217	43.3094	37.3903	7.9026	12.5841	29.4942	3.8304	6.8155	2.5093	0.2681	0.229
GHR-KO on C.R. (n = 17)	64.2217	216.6341	182.1955	553.7476	41.5477	46.1707	40.8561	8.9517	14.3607	34.4386	7.1078	6.7298	15.9952	0.3376	0.2122
GHR-N on A.L. (n = 12)	56.9752	204.6205	171.0504	554.4677	41.1762	45.4566	40.4714	9.177	13.7315	33.5702	8.971	25.8674	11.2301	0.2286	0.2367
GHR-N on C.R. (n = 7)	68.7457	231.3768	191.912	550.7739	42.2298	49.1525	41.528	10.6875	15.7495	39.4648	9.2143	25.2443	9.9293	0.2557	0.3302
Comparison	Total Distance	Hor. Activity Ct.	Amb. Activity Ct.	Rest Time	Rest Episode Ct.	Mvmt. Time	Mvmt. Episode Ct.	Stereo. Time	Stereo. Episode Ct.	Stereo. Activity Ct.	Vert. Episode Ct.	Vert. Activity Ct.	Vert. Activity Time	Loco. Clock. Revs.	Loco. Counter. Revs.
N on A.L.vs. KO on A.L.	0.9371	0.5322	0.6443	0.5747	0.1661	0.576	0.1659	0.0299	0.2379	0.0867				0.4116	0.8377
N on A.L.vs. N on C.R.	0.1801	0.2782	0.3389	0.4354	0.724	0.4345	0.7255	0.0603	0.1187	0.0688	0.8449	0.8843	0.4361	0.5075	0.094
N on A.L.vs. KO on C.R.	0.3753	0.5288	0.5084	0.837	0.8632	0.8381	0.8595	0.7026	0.5453	0.7347				0.1661	0.3991
KO on A.L.vs. KO on C.R.	0.3584	0.211	0.2654	0.4124	0.0766	0.4144	0.0756	0.0496	0.0743	0.0396	0.0002	0.923	0.0001	0.3678	0.6131

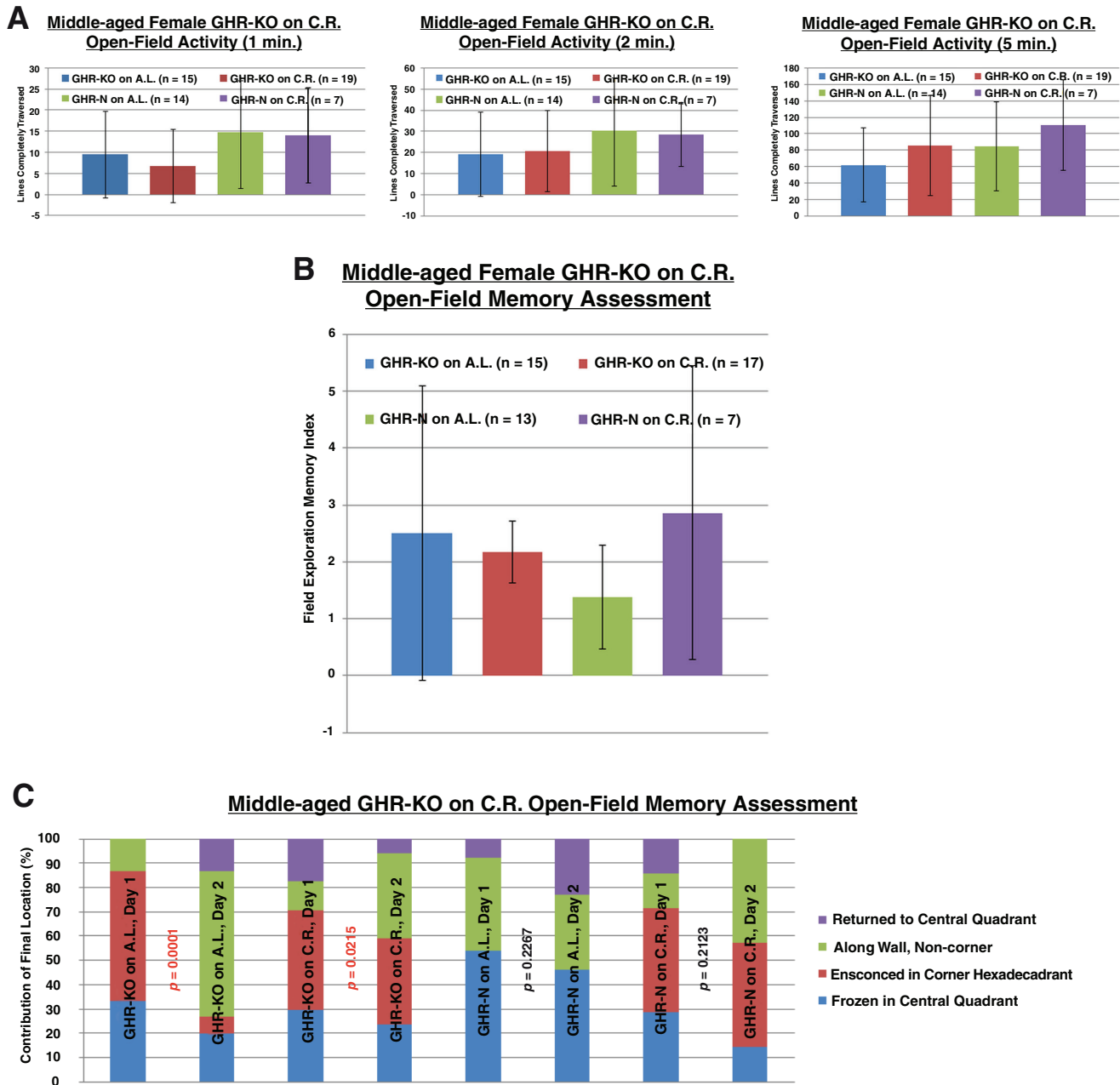


Figure 3. Failure of 30% caloric restriction to influence cognition. A. Neither *Ghr/bp* disruption nor 30% caloric restriction alters anxiety-betraying activity in an open field for female mice. **B.** 30% caloric restriction does not change the (memory index) performance of female GHR-KO mice in the open-field paradigm. **C.** 30% caloric restriction does not change the (final location) performance of female GHR-KO mice in the open-field paradigm.

tolerance testing results in disagreement with the molecular biology-based assumptions of their insulin sensitivity [Boparai *et al.*, 2010].

We also investigated the effects of CR on the performance of GHR-KO mice in other gerontologically associated measures. We discovered that CR did not alter the metabolism or spontaneous activity of GHR-KO mice and also revealed that CR has no effect on the anxiety or memory function of GHR-KO mice. This documentation of lacking amenability of GHR-KO mice

to effects of CR further underscore a seeming epistasis of the genetic effect of *Ghr/bp* disruption to the environmental effect of dietary restriction.

In summary, our results question the notion of maximized insulin sensitivity obviating further lifespan increase in GHR-KO mice. Future studies aimed at elucidating concordant physiological, and ultimately (macro)molecular, underpinnings of disparate instances of longevity would benefit from heeding analyses that reduce or eliminate the likelihood of suspected mechanisms.

Data availability

Dataset 1. Experimental data showing the effect(s) of *growth hormone (GH) receptor (GHR)/binding protein (Ghr/bp)* gene disruption and/or caloric restriction on the various outcomes, <http://dx.doi.org/10.5256/f1000research.5378.d37530> [Arum *et al.*, 2014].

Author contributions

O.A., R.K.K., and A.B. acquired funding for this study; O.A. and A.B. conceived and designed this study; J.G.T. provided accommodations for the blood glucose regulatory dynamics, and technical training for the anxiety and cognitive assessments in this study; O.A., R.K.B., & J.K.S. methodologically executed this study; O.A. statistically analyzed the data from this study; and O.A. & A.B. prepared the

manuscript for this study. J.J.K. provided the founder population of the *growth hormone receptor/binding protein* gene-disrupted mice bred for this study. All authors approved the final content of the manuscript.

Competing interests

No competing interests were disclosed.

Grant information

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Supplemental Tables

Supplemental Table I. Home-Cage assessment rubric. Characteristics of interest in home-cage assessments are succinctly detailed.

Condition	Rating	Description
Initial Posture	1	Sitting or standing normally, rearing or asleep
	2	Crouching over or lying low
	3	Slight sway while in standing position
	4	Excessive sway or head bobbing in standing position
	5	Flattened, limbs may be spread out
	6	Lying on side, limbs in air
Salivation	1	None
	2	Slight
	3	Severe
Lacrimation	1	None
	2	Slight
	3	Severe
Fur	1	Normal, silky and smooth
	2	Pilo-erection
	3	Over groomed
Vocalization	0	No, spontaneous vocals
	1	Yes, spontaneous vocals

Supplemental Table II a. Legend for indirect calorimetry-based metabolic dependent variables.

VO₂ (Volume of oxygen consumed per hour, normalized to body weight.)
VCO₂ (Volume of carbon dioxide produced per hour, normalized to body weight.)
Respiratory Quotient (Respiratory Exchange Ratio) (VCO ₂ /VO ₂)
Heat Production [((4.33 × VO ₂) + (0.67 × VCO ₂)) + (Wt.(kg.) × (60 Min./Hr.))]
Energy Expenditure [VO ₂ × (364 + 113 × R.Q.)/22.4]

Supplemental Table II b. Legend for voluntary locomotion dependent variables.

Total distance [The total distance that the subject has traveled. For the purpose of this variable, the location of the subject is defined as the centroid (center of mass) of the subject.]
Horizontal activity count [A count of sensor changes (beam breaks).]
Ambulatory activity count (Number of beam breaks while animal is ambulating. Does not include stereotypy behavior.)
Rest time (The length of time that the subject spent at rest. A resting period is defined as a period of inactivity greater than or equal to 1 second.)
Rest episode count (The total number of resting periods. A resting period is defined as a period of inactivity greater than or equal to 1 second.)
Movement time (The length of time that the subject spent in activity. Activity is defined as a period in which ambulation or stereotypy occurred.)
Movement episode count (The total number of locomotor episodes. Episodes are separated by rest periods of at least 1 second.)
Stereotypy time [The total amount of time that stereotypic behavior is exhibited. A break in stereotypy of 1 second or more is required to separate one stereotypic episode from the next. If the animal breaks the same beam (or set of beams) repeatedly then the monitor considers that the animal is exhibiting stereotypy. This typically happens during grooming, head bobbing, <i>etc.</i>]
Stereotypy episode count [This corresponds to the number of times that stereotypic behavior was observed in the animal. A break in stereotypy of 1 second or more is required to separate one stereotypic episode from the next. If the animal breaks the same beam (or set of beams) repeatedly then the monitor considers that the animal is exhibiting stereotypy. This typically happens during grooming, head bobbing, <i>etc.</i>]
Stereotypy activity count [Number of beam breaks that occur during a period of stereotypic activity. If the animal breaks the same beam (or set of beams) repeatedly then the monitor considers that the animal is exhibiting stereotypy. This typically happens during grooming, head bobbing, <i>etc.</i>]
Vertical episode count (Each time the animal rears up, this is incremented by 1. The animal must go below the level of the vertical sensor for at least 1 second before the next rearing can be registered.)
Vertical activity count (Cumulative vertical beam breaks.)
Vertical activity time (When the animal rears up, this timer starts incrementing.)
Locomotor clockwise revolutions (Counts the number of clockwise revolutions that the subject travels in an open field.)
Locomotor counter-clockwise revolutions (Counts the number of counter-clockwise revolutions that the subject travels in an open field.)

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The study addresses a long running question in aging research, which concerns the interaction of the two regimes known to consistently extend mammalian (mouse) lifespan: caloric restriction and manipulation of the GH/IGF1 axis.

The study provides a wealth of very useful endocrinological and metabolic data and the authors must be commended their extensive work. The interpretation with regards to the effects of insulin sensitivity and caloric restriction are plausible, though ultimately, whether this is truly the mechanism by which the lifespan-extending effects are mediated (and account for the differential response to CR in GHRko mice), will require direct experimental demonstration.

I would like the authors to discuss the metabolic results a little more. For example, it is indicated that the total caloric output of GHRKO mice is higher, considerably so, than WT (regardless of CR). However, the heat production is more or less the same. At the same time, CR has opposite effects on heat production in GHRko versus WT mice. It would seem to me overall, that as a whole the metabolic effects of CR in table 3, are much attenuated in the GHRko mice, i.e. WT mice have much more profound alterations in metabolic parameters in response to CR than do GHRko mice to CR.

These are just suggestions. Overall, the data and paper are very valuable as is.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

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Caloric restriction prolongs longevity in experimental animals via altering insulin sensitivity. The growth hormone receptor knockout (GHR KO) mice live longer and have increased insulin sensitivity. In this

study, the authors tested the hypothesis that the modest effect of caloric restriction on longevity of GHR KO mouse is because of the failure of caloric restriction to further increase the already maximally elevated insulin sensitivity in this mouse model. Surprisingly, the authors found that caloric restriction increased blood insulin concentration and reduced insulin sensitivity in GHR KO mice. These findings refute the established assertion between insulin sensitivity and slowed senescence in GHR KO mice. The paper is well written and the data analyses are appropriate. The conclusions are supported by experimental data. The number of animals per group are considerably large except for GHR-N on caloric restricted group. I recommend indexing the paper as submitted.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.
