LEGENDS for SUPPLEMENTARY FIGURES and TABLES

FIGURE S1. Detailed data derived from wild type and integrin deficient female mice at 1 and 3 months. (A) Femoral length, (B) cortical bone mineral density and (C) cortical thickness (assessed by pQCT), (D) ultimate load and (E) stiffness (assessed by three-point bending test) were significantly reduced in $\alpha2\beta1$ + $\alpha11\beta1$ double deficient (dko) mice. *significant difference to wt ($p<0.05$), †significant difference to $\alpha2$ ko ($p<0.05$), #significant difference to $\alpha11$ ko ($p<0.05$). (F, G) reduced body length and (H, I) body mass in integrin mutants. (J,K) reduced IGF-1 and (L,M) IGFBP3 levels in sera of integrin deficient mice. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$.

FIGURE S2. Analyses of osteoblast cultures: Expression of integrin subunits, alkaline phosphatase activity and proliferation. (A) Primary calvarial osteoblasts were cultured as described in Methods and in legend to Fig. 3. Integrin $\alpha2$ expression increased with late differentiation, while $\alpha11$ transcripts did not change markedly. S26 transcript levels reflected similar amounts of template. (B) Alkaline Phosphatase activity and (C) osteoblast proliferation assessed by formazan production did not differ significantly between wild type and integrin deficient cells. Please note that high standard deviations arise from combining results of independent experiments.

FIGURE S3. IGF-1 signaling and growth hormone (GH) levels. (A,B) Western blot analysis of protein kinase AKT and phosphorylated (p)AKT (Ser437) in cell lysates of primary fibroblasts (A) and primary osteoblasts (B) were performed to test whether integrin-deficient cells were capable of responding to IGF-1 stimulation in vitro. Regardless of genotype, similar levels of pAKT were detected after stimulation with recombinant IGF-1 (5ng/ml) or 200nM insulin (INS) for 10 minutes. (C) GH levels were assessed in the serum of 6 weeks old wild type and double deficient (dko) mice using a rat/mouse growth hormone ELISA kit (Millipore). In both female and male mice, GH serum levels were decreased in mice lacking $\alpha2\beta1$ + $\alpha11\beta1$ integrins. *, $p<0.05$.

FIGURE S4. Hypothalamic expression of GnRH, TRH, CRH and somatostatin. Real time analysis of mRNA expression of gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and somatostatin in the hypothalamus revealed no significant differences between wild type and integrin deficient mice.

TABLE S1. Trabecular properties of femurs (pQCT). Trabecular bone parameters were measured at the age of one and three months. In contrast to cortical parameter, only $\alpha2\beta1$ + $\alpha11\beta1$ deficient animals showed significant alterations at one month, not being persistent until adulthood.

TABLE S2. Mechanical properties of femurs (3-point bending test). In addition to maximal applied load and bone stiffness, further parameters were assessed employing 3-point bending tests. Energy as well as the bending moment was significantly altered in double deficient (dko) animals, while other parameters such as elastic modulus remained unchanged.

TABLE S3. Metabolic parameters. Metabolic parameters such as dioxygen consumption, carbon dioxide production as well as heat production were evaluated by indirect calorimetry, revealing similar respiratory exchange rates in integrin deficient animals when compared to wild type controls. n=6 per genotype.

SUPPLEMENTARY METHODS

Cell culture- Primary fibroblasts were isolated from trunk skin of newborn animals and cultured as described (18). Primary chondrocytes were isolated from from rib cages of 1-4 day old mice as described previously (Budde et al., 2005).

RT-PCR- Total RNA was extracted from osteoblasts using RNeasy (Qiagen). Expression of α2 and α11 integrin subunits was detected by RT-PCR using the following primers: α2 forward 5'GCA-CCA-CAT-TAG-CAT-ACA-GA3', α2 reverse 5'GGC-ATC-ATA-CAG-GAG-AGG-AA3', α11 forward 5'CCG-CCT-TCC-TCT-GCT-TCA-TAC-CCA-T3', α11 reverse 5'GCC-GCC-TCT-CCT-CGT-TCA-CAC-ACT-C3'. S26 forward 5'AAT-GTG-CAG-CCC-ATT-CGC-TG3', S26 reverse 5'CTT-CCG-TCC-TTA-CAA-AAC-GG3'.

Determination of phosphatase activity and proliferation- Osteoblasts were seeded in microwell plates at $10^4/cm^2$ and cultured in presence of 10 mM phosphoglycerate and 5mM phosphoascorbate to induce osteogenic differentiation and mineralization (day 0) for up to 18 days. At days 4, 7, 11, 14 and 18, alkaline phosphatase activity of triplicate cultures was determined as described (57). Proliferation of primary calvarial osteoblasts was monitored in a tetrazolium-based assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega).

Western blot analysis- For detection of pAKT (Ser473), cells were stimulated with IGF-1 (5 ng/ml, Sigma) or insulin (200nM, Sigma) for 10 minutes prior to extraction in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1 % NP-40, 0.1 % SDS, 0.5 % Na-deoxycholate, 5 mM EDTA, 1 % Triton X-100, pH 8), supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Proteins were separated by SDS-PAGE and transferred to Hybond C extra membranes (Amersham Biosciences). Following blocking (5 % milk powder in TBS-T, pH 7.6), membranes were incubated with primary antibodies directed against AKT and pAKT (both 1:1000, Cell Signaling). Secondary HRP-conjugated antibodies were purchased from Dako. Signals were detected by chemiluminescence using Western Lightning (Perkin Elmer) or SuperSignal West Femto (Thermo Scientific).

Determination of circulating growth hormone (GH) levels- Blood collected by venous puncture was centrifuged (1000 x g, 30 min at 4°C). Plasma was collected, stored at -80°C, and GH levels were analyzed using a rat/mouse growth hormone ELISA kit (Millipore) according to the manufacturer's protocol.

qRT-PCR- cDNA was generated using a High-Capacity cDNA reverse transcription kit (Applied Biosystems) and amplified using TaqMan Universal PCR Master Mix, No AmpErase UNG with TaqMan Assay-on-Demand kits for GnRH, TRH, CRH, somatostatin, glucoronidase β and HPRT (Applied Biosystems). qPCR was performed as described in Experimental Procedures Section.

Analyses of metabolic parameters- Oxygen consumption, carbon dioxide production as well as heat production were measured by indirect calorimetry and the respiratory exchange rate (RER) was calculated by the ratio between CO_2 / O_2 consumption.

Figure S1

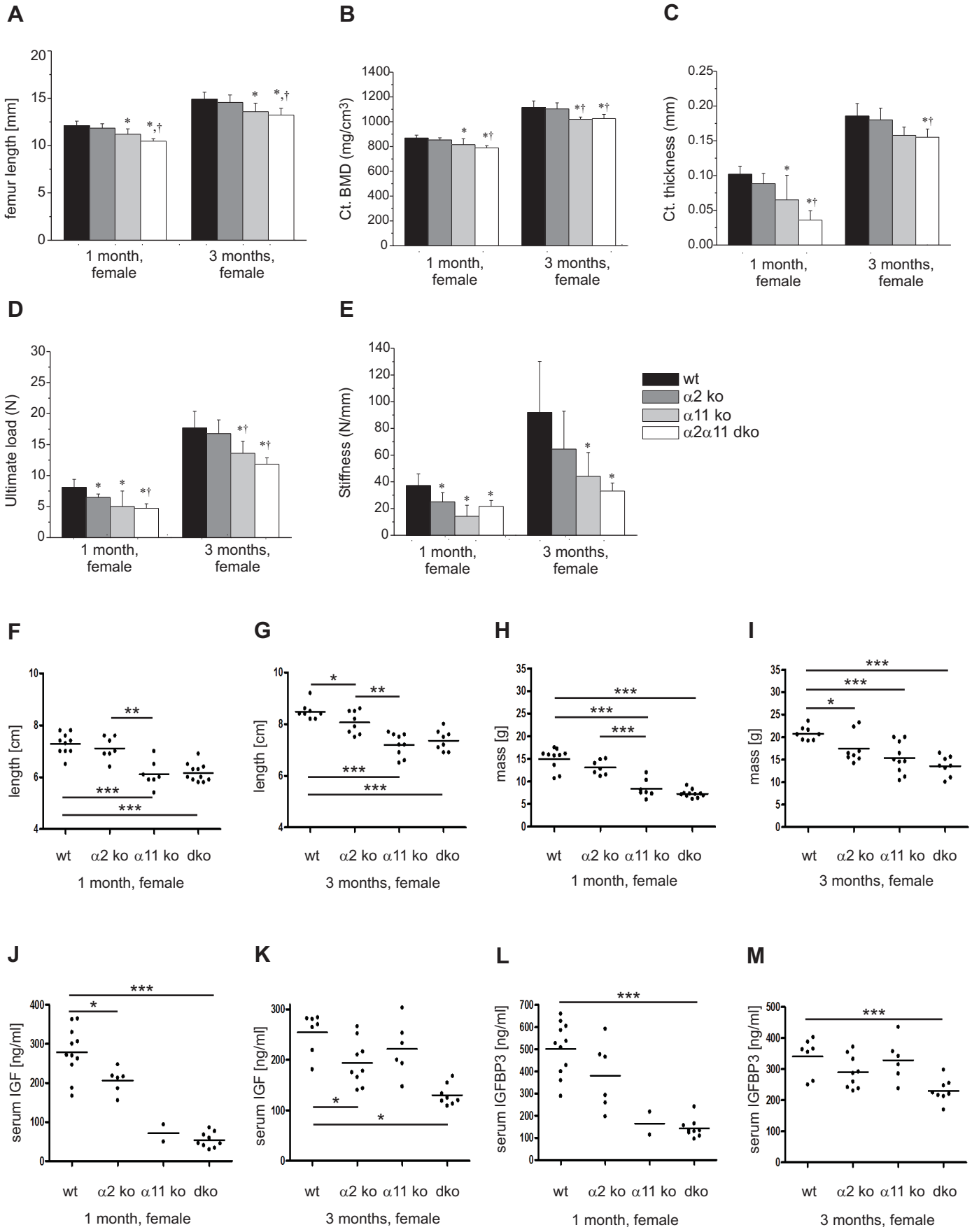
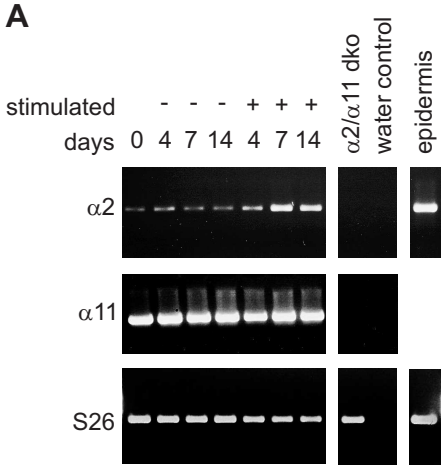
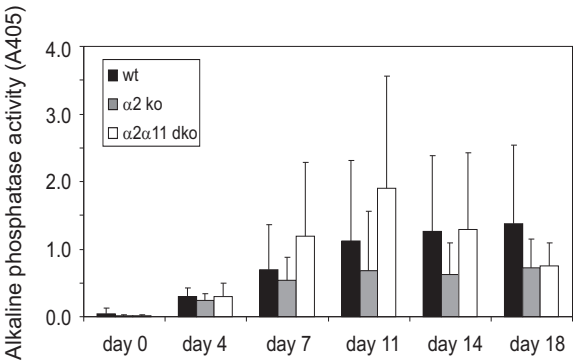

A
femur length [mm]
1 month, female
3 months, female
B
Ct. BMD (mg/cm3)
C
Ct. thickness (mm)
D
Ultimate load (N)
E
Stiffness (N/mm)
wt
α2 ko
α11 ko
α2α11 dko
F
length [cm]
wt α2 ko α11 ko dko
1 month, female
G
3 months, female
H
mass [g]
I
J
serum IGF [ng/ml]
K
L
serum IGFBP3 [ng/ml]
M

Figure S2

A



B



C

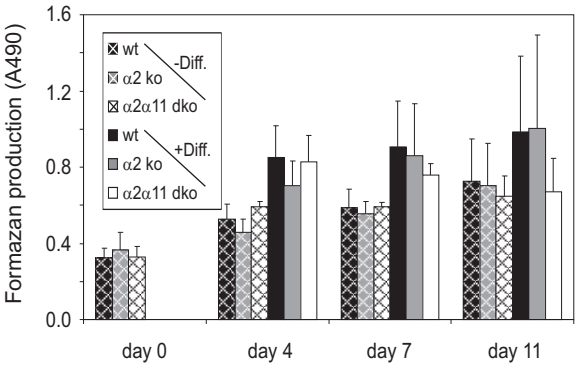


Figure S3

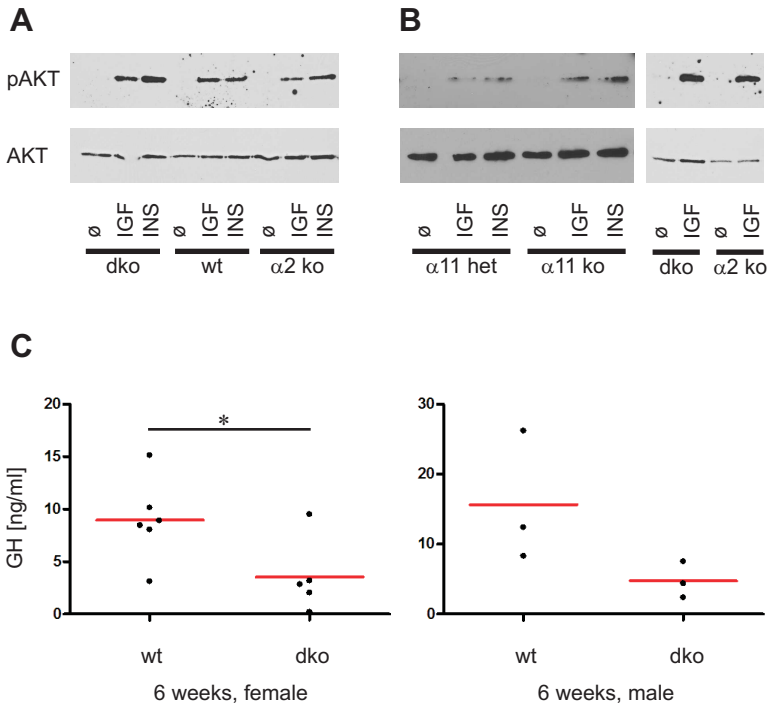

A
pAKT
AKT
ø IGF INS ø IGF INS ø IGF INS
dko wt α2 ko
B
ø IGF INS ø IGF INS ø IGF ø IGF
α11 het α11 ko dko α2 ko
C
GH [ng/ml]
20
15
10
5
0
*
wt dko
6 weeks, female
30
20
10
0
wt dko
6 weeks, male

Figure S4

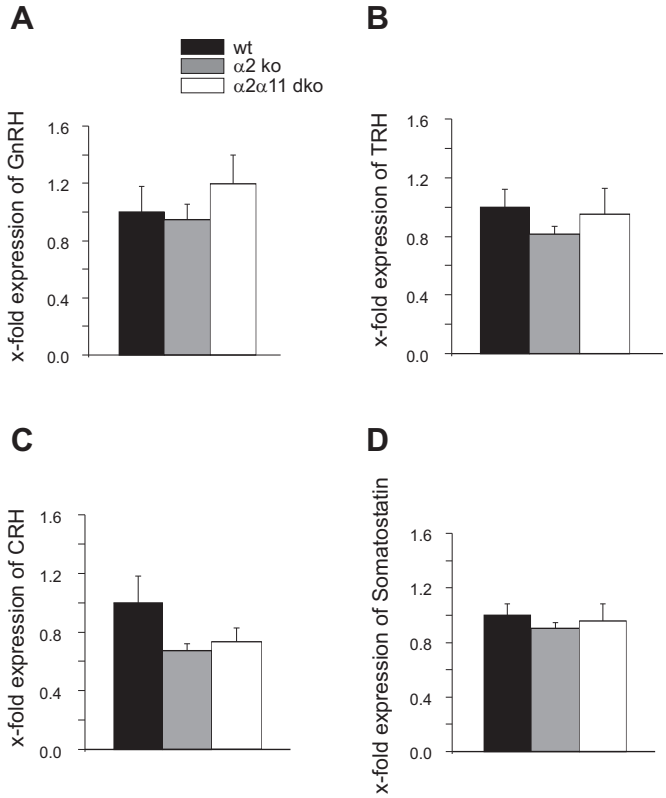

A
wt
α2 ko
α2α11 dko
x-fold expression of GnRH
1.6
1.2
0.8
0.4
0.0
B
x-fold expression of TRH
1.6
1.2
0.8
0.4
0.0
C
x-fold expression of CRH
1.6
1.2
0.8
0.4
0.0
D
x-fold expression of Somatostatin
1.6
1.2
0.8
0.4
0.0

Table S1. Trabecular properties of femurs (pQCT).

	wt	α2 ko	α11 ko	α2α11 dko
	1 month males			
BMD (mg/cm³)	389 ± 10	367 ± 23	373 ± 14	323 ± 28 *,†,#
Tb.BMD (mg/cm³)	265 ± 11	247 ± 31	271 ± 13	207 ± 42 *,†,#
BMC (mg)	1.54 ± 0.08	1.40 ± 0.12	1.46 ± 0.06	1.22 ± 0.21 *,#
Tb.BMC (mg)	0.32 ± 0.02	0.28 ± 0.04	0.32 ± 0.01	0.24 ± 0.06 *,#
CSA (mm²)	3.96 ± 0.17	3.80 ± 0.16	3.92 ± 0.12	3.74 ± 0.35
Tb.CSA (mm²)	1.19 ± 0.05	1.14 ± 0.05	1.18 ± 0.04	1.12 ± 0.10
	3 months males			
BMD (mg/cm³)	481 ± 40	455 ± 48	445 ± 60	450 ± 47
Tb.BMD (mg/cm³)	227 ± 25	203 ± 51	221 ± 63	222 ± 42
BMC (mg)	1.95 ± 0.28	1.75 ± 0.36	1.69 ± 0.25	1.65 ± 0.31
Tb.BMC (mg)	0.28 ± 0.04	0.24 ± 0.08	0.26 ± 0.07	0.25 ± 0.06
CSA (mm²)	4.03 ± 0.41	3.80 ± 0.41	3.78 ± 0.17	3.63 ± 0.46
Tb.CSA (mm²)	1.21 ± 0.12	1.14 ± 0.12	1.14 ± 0.05	1.09 ± 0.14
	1 month females			
BMD (mg/cm³)	383 ± 17	348 ± 31	344 ± 20	319 ± 29 *
Tb.BMD (mg/cm³)	263 ± 20	223 ± 41	238 ± 8	203 ± 33 *
BMC (mg)	1.50 ± 0.10	1.32 ± 0.17	1.31 ± 0.12	1.16 ± 0.19 *
Tb.BMC (mg)	0.31 ± 0.03	0.26 ± 0.05	0.28 ± 0.02	0.23 ± 0.05 *
CSA (mm²)	3.93 ± 0.19	3.79 ± 0.30	3.79 ± 0.13	3.61 ± 0.32
Tb.CSA (mm²)	1.18 ± 0.06	1.14 ± 0.09	1.14 ± 0.04	1.08 ± 0.10
	3 months females			
BMD (mg/cm³)	451 ± 37	450 ± 30	413 ± 18 *	409 ± 21 *,†
Tb.BMD (mg/cm³)	154 ± 25	167 ± 49	170 ± 13	178 ± 35
BMC (mg)	1.60 ± 0.15	1.57 ± 0.17	1.42 ± 0.19	1.39 ± 0.17
Tb.BMC (mg)	0.17 ± 0.03	0.18 ± 0.06	0.18 ± 0.02	0.19 ± 0.05
CSA (mm²)	3.55 ± 0.18	3.48 ± 0.19	3.42 ± 0.36	3.39 ± 0.32
Tb.CSA (mm²)	1.07 ± 0.05	1.05 ± 0.06	1.03 ± 0.11	1.02 ± 0.10

Values are mean ± SD
*significant difference to wt ($p < 0.05$)
†significant difference to α2 ko ($p < 0.05$)
#significant difference to α11 ko ($p < 0.05$)

Table S2. Mechanical properties of femurs (3-point bending test).

	wt	α2 ko	α11 ko	α2α11 dko
	1 month males			
Deformation (mm)	0.50 ± 0.05	0.47 ± 0.06	0.52 ± 0.11	0.46 ± 0.08
Energy (mJ)	3.0 ± 0.4	2.2 ± 0.4 *	2.8 ± 0.8	1.4 ± 0.4 *,†,#
Bending moment (Nmm)	11.1 ± 1.6	8.6 ± 1.2 *	9.3 ± 1.2	5.9 ± 1.4 *,†,#
Ultimate stress (MPa)	61.9 ± 8.8	58.6 ± 4.1	58.0 ± 5.4	55.8 ± 9.7
Strain	0.16 ± 0.01	0.14 ± 0.02	0.16 ± 0.03	0.12 ± 0.02 *,#
Elastic modulus (GPa)	0.40 ± 0.06	0.44 ± 0.08	0.39 ± 0.14	0.45 ± 0.07
	3 months males			
Deformation (mm)	0.43 ± 0.11	0.44 ± 0.12	0.54 ± 0.14	0.41 ± 0.11
Energy (mJ)	6.1 ± 1.7	5.2 ± 2.0	5.4 ± 1.1	3.9 ± 1.4 *
Bending moment (Nmm)	28.9 ± 5.2	24.0 ± 4.0 *	21.9 ± 2.1 *	20.0 ± 4.4 *
Ultimate stress (MPa)	104.7 ± 17.6	101.2 ± 8.8	91.1 ± 5.8	101.0 ± 18.4
Strain	0.14 ± 0.04	0.14 ± 0.04	0.17 ± 0.04	0.12 ± 0.04
Elastic modulus (GPa)	0.81 ± 0.31	0.80 ± 0.27	0.55 ± 0.12	0.92 ± 0.40
	1 month females			
Deformation (mm)	0.48 ± 0.07	0.46 ± 0.05	0.42 ± 0.06	0.43 ± 0.09
Energy (mJ)	2.6 ± 0.6	2.0 ± 0.4	1.4 ± 0.8 *	1.3 ± 0.4 *
Bending moment (Nmm)	10.1 ± 1.6	8.1 ± 0.7 *	6.3 ± 3.1 *	5.9 ± 0.9 *,†
Ultimate stress (MPa)	61.8 ± 9.3	61.0 ± 7.0	40.8 ± 16.4	60.3 ± 13.1
Strain	0.14 ± 0.02	0.13 ± 0.01	0.13 ± 0.02	0.12 ± 0.02
Elastic modulus (GPa)	0.44 ± 0.06	0.49 ± 0.09	0.32 ± 0.09	0.54 ± 0.17
	3 months females			
Deformation (mm)	0.40 ± 0.07	0.39 ± 0.07	0.44 ± 0.08	0.42 ± 0.12
Energy (mJ)	4.7 ± 1.1	3.7 ± 0.5	3.8 ± 0.8	2.9 ± 0.8 *
Bending moment (Nmm)	22.1 ± 3.3	21.0 ± 2.8	17.0 ± 2.4 *,†	14.8 ± 1.3 *,†
Ultimate stress (MPa)	99.1 ± 14.9	104.1 ± 4.6	89.2 ± 16.9	90.4 ± 15.3
Strain	0.12 ± 0.02	0.12 ± 0.02	0.13 ± 0.03	0.12 ± 0.03
Elastic modulus (GPa)	0.83 ± 0.25	0.90 ± 0.13	0.71 ± 0.24	0.81 ± 0.31

Values are mean ± SD
*significant difference to wt ($p < 0.05$)
†significant difference to α2 ko ($p < 0.05$)
#significant difference to α11 ko ($p< 0.05$

Table S3. Metabolic parameters.

	wt	α2 ko	α11 ko	α2α11 dko
O_2 consumption (ml/h/kg)	2700 ± 244	3076 ± 411	3246 ± 788	2857 ± 397
CO_2 production (ml/h/kg)	2447 ± 193	2738 ± 445	2925 ± 774	2599 ± 423
Heat production (kcal/h/kg)	13.35 ± 1.16	15.15 ± 2.10	16.03 ± 3.96	14.13 ± 2.02
Respiratory exchange rate (RER)	0.91 ± 0.04	0.89 ± 0.04	0.89 ± 0.03	0.91 ± 0.04