

1 **Steady-state energy balance in animal models of obesity and weight loss**

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22 **Abstract**

23

24 **Objective:** We wanted to exam the steady-state energy balance by using high fat diet-induced  
25 obese (DIO) rats and mice as models for positive energy balance, and gastric bypassed (GB)  
26 rats and gene knockout of muscarinic acetylcholine M3 receptor (M3KO) mice as models for  
27 negative energy balance. **Methods:** 132 rats and mice were used. Energy balance was  
28 measured by a comprehensive laboratory animal monitoring system. Gene expression was  
29 analysed by *in situ* hybridization in M3KO mice. **Results:** DIO rats reached the plateau of  
30 body weight 28 weeks after starting high-fat diet (25% heavier than controls), whereas DIO  
31 mice reached the plateau after 6 weeks (23% heavier than controls). At the plateau, DIO rats  
32 had higher calorie intake during light phase but not during dark phase, while mice had the  
33 same calorie intake per day as controls. DIO rats and mice had lower energy expenditure (EE)  
34 and respiratory exchange ratio (RER) than controls. GB-rats reached the plateau (15% weight  
35 loss) 2 weeks after surgery and had the same calorie intake as sham-operated controls. EE, but  
36 not RER, was higher in GB rats than controls during dark phase. The lean M3KO mice (25%  
37 lighter than wild-type (WT) mice at the plateau between 6-15 months of age) had the same  
38 calorie intake but higher EE, RER and hypothalamic mRNA expression of NPY, AgRP and  
39 leptin receptor than WT mice. **Conclusion:** When body weight gain or loss reached a plateau,  
40 the steady-state energy balance was mainly maintained by EE and/or RER rather than calorie  
41 intake.

42

43 **Keywords**

44 Eating behaviour, High-fat diet, Gastric bypass, Muscarinic acetylcholine M3 receptor  
45 knockout, Mice, Rats

## 46 **Introduction**

47           The number of overweight individuals is increasing, leading to a global obesity  
48 epidemic [1, 2]. This is of concern because obesity is a major contributor in the development  
49 of type 2 diabetes, cardiovascular diseases, certain forms of cancer, overall reduced quality of  
50 life and premature mortality [3, 4]. Currently, weight loss treatments include diet control,  
51 physical training, drug therapies, and bariatric surgery [5]. Weight loss resulting from either  
52 dieting or exercise remains usually for a shorter period of time, whereas weight loss after  
53 bariatric surgery (such as sleeve gastrectomy or gastric bypass) has much longer effect [6, 7].  
54 With intensive lifestyle interventions, a majority of obese participants in clinical trials lose  
55 7% to 10% of their initial weight at 1 year, but longer-term weight maintenance is difficult [8,  
56 9]. The development of anti-obesity drugs to target energy balance by acting on either  
57 appetite control or energy expenditure (EE) is yet without great success [9, 10, 11, 12]. For  
58 example, the pharmacological interventions reduce body weight only by 3-9% of initial  
59 weight on average at 1 year and the proportion of patients achieving clinically meaningful  
60 (>5%) weight loss ranges from 37% to 70% depending on different drugs and/or dosages [9].  
61 In order for current lifestyle interventions, drug therapies and bariatric surgeries to be  
62 maximally effective for body weight control in individual patients as well as in a patient  
63 population, better understanding of the energy balance is essential [13, 14].

64           The concept of energy balance for regulating body weight is simple in principle. When  
65 energy output equals input, i.e., energy homeostasis, the body is at steady state energy  
66 balance. A new set point for steady state balance occurs when energy intake (EI) exceeds EE  
67 over a given period of time and weight is gained, or when EE exceeds EI and weight is lost  
68 [15]. In this study, we defined the dynamic phase of energy balance as weight gain or loss of  
69 more than 5% for at least one week, and steady state as less than 5% change in body weight  
70 for at least one week. From the energy balance point of view, it has not been understood why  
71 it is easier to control the body weight during the dynamic phase of energy balance than to  
72 reverse it under the steady state condition [16]. In fact, it is not uncommon that many obese  
73 patients seek weight-loss treatments when they are under the steady state in terms of body  
74 weight. Thus, a better understanding of steady-state energy balance may help us to better  
75 understand why it is difficult to perturb a shift in EE particularly to achieve a long-term body  
76 weight loss by either dieting or exercise after obesity is established.

77           Previously, we and others have shown that dynamic development of obesity is  
78 associated with increased calorie per meal [17, 18], and that there are compensatory changes  
79 in hypothalamic gene expression of AgRP and NPY in rodents fed high-fat diet or low-energy

80 dense diet for a prolonged period of time, which is indicative of defence against changes in  
81 body weight [19, 20, 21, 22]. Gastric bypass-induced weight loss is mainly associated with  
82 increasing EE rather than reducing EI [23, 24, 25, 26, 27], which is in line with the  
83 hypothalamic gene expression of AgRP, NPY and POMC [28]. However, the initial report on  
84 M3KO mice showed a reduced food intake with increased expression of hypothalamic AgRP  
85 mRNA and reduced POMC mRNA [29], which is inconsistent with homeostatic mechanism  
86 of food intake regulation [30].

87 In the present study, we wanted to explore the steady-state energy balance to defend a  
88 change in body weight in response to either positive or negative energy balance. Thus, we  
89 used high-fat-diet fed rats and mice as models for positive energy balance displaying an obese  
90 phenotype [31], and gastric bypassed (GB) rats and gene knockout of muscarinic  
91 acetylcholine M3 receptor (M3KO) mice as models for negative energy balance [23, 32].

92

93

## 94 **Materials and Methods**

### 95 *Experimental design*

96 Four sets of experiments were conducted in rodent models, comprising two sets  
97 examining obesity development in rats and mice, and another two sets examining weight loss  
98 in rats and mice.

99 All animals were housed in groups of three or four together in individually ventilated  
100 cages (1500 cm<sup>2</sup> in size) on wood chip bedding with a 12 h light/dark cycle, room  
101 temperature of 21-22°C and 50-60% relative humidity. The standard housing conditions were  
102 specific pathogen free and in agreement with FELASA (Federation of European Laboratory  
103 Animal Science Association) recommendations. Throughout the experiment, all animals had  
104 free access to tap water and food regardless of being in metabolic or Makrolon cages. The  
105 high fat diet (D12492) was purchased from Research Diets Inc. (New Brunswick, NJ, USA),  
106 and the normal chow (RM1 811004) was purchased from Scanbur BK AS (Sweden). Animal  
107 experiments were performed according to the guidelines for the design and statistical analysis  
108 of experiments using laboratory animals after being approved by the Norwegian National  
109 Animal Research Authority (Forsøksdyrutvalget, FDU).

110 *Diet-induced obese rats:* Four weeks old male Sprague-Dawley rats were purchased  
111 from Taconic (Ejby, Denmark). The rats were divided into two groups: diet-induced obese  
112 (DIO) (n=12) and controls (n=8). To allow the DIO rats to adapt to high-fat diet, they were  
113 put on a mixture of 50% high fat diet and 50% normal chow for two weeks before being fed

114 high fat diet only (60% fat) [17]. The DIO and control rats received high fat diet or normal  
115 chow, respectively, throughout the study. Body weight was measured every week from arrival  
116 until euthanization. Food intake, eating behaviour, metabolic parameters and body  
117 composition were measured when the body weight-plateau was reached (35 weeks of age). To  
118 follow the 3R principle, this group of rats were also used in a separated study entitled “Time-  
119 restricted feeding during weekdays against obesity: a study using high-fat diet-induced obese  
120 rat models” (Olsen MK, Choi MH, Kulseng B, Zhao C-M, Chen D. unpublished data).

121 *Diet-induced obese mice:* Five weeks old male C57BL/6J mice were purchased from  
122 Jackson Laboratory (Bar Harbor, ME, USA). The mice were divided into two groups: DIO  
123 (n=10) and controls (n=10). To allow the DIO mice to adapt to the high-fat diet, they were fed  
124 a mixture of 50% high fat diet and 50% normal chow diet for two weeks before being fed  
125 high fat diet only (60% fat) for the remainder of the study. The control mice were fed normal  
126 chow for the entire study. Body weight was measured every week from arrival until  
127 euthanization. Food intake, eating behaviour, metabolic parameters and body composition  
128 was measured when the body weight-plateau was reached (11 weeks of age).

129 *Gastric bypassed rats:* 48 weeks old male Sprague-Dawley rats were purchased from  
130 Taconic (Ejby, Denmark) and were divided into two groups: GB (n=14) and sham operation  
131 (n=6). The GB was performed according to previous experiments [25] and the rats were fed  
132 normal-chow throughout the whole study. Food intake, eating behaviour and metabolic  
133 parameters were measured when the body weight-plateau had been reached (3 weeks post-  
134 surgery). To follow the 3R principle, the data from this group of rats were taken from our  
135 previous study [23] and re-analysed and re-used in the present study.

136 *M<sub>3</sub> receptor knockout (M3KO) mice:* M3KO mice were generated [33] and  
137 backcrossed 14 generations onto C57BL/6J background at Prof. Takeuchi’s laboratory (Kyoto  
138 Pharmaceutical University, Japan) and imported to Norway by Prof. D. Chen for this study.  
139 The M3KO mice were further bred through sibling matings for 2 generations. Age-matched  
140 wild-type (WT) mice on the same background (C57BL/6J) were purchased from Taconic  
141 (Ejby, Denmark). All the mice including M3KO and WT mice were fed normal chow in-  
142 house together during the study period. Body weight was measured at 2, 6, 11 and 15 months  
143 of age. At 2 months of age there were 7 mice in each group, 3:4 male:female ratio in M3KO  
144 group and 4:3 male:female ratio in WT group. At 6, 11 and 15 months of age there were 8  
145 mice in each group, with male:female ratio of 4:4 ratio in the WT group, and 4:4, 5:3 and 6:2  
146 in the M3KO group, respectively. Brain tissue from 15 months old mice was collected for *in*  
147 *situ* hybridization.

148 ***Measurements of energy balance by CLAMS***

149           The parameters for energy balance were measured by a Comprehensive Laboratory  
150 Animal Monitoring System.

151           The animals were acclimatized to the Comprehensive Laboratory Animal Monitoring  
152 System (CLAMS; Columbus Instruments International, Columbus, OH USA) for 24 h before  
153 data collection. At data collection the animals were kept in CLAMS for 48 h and data from  
154 the last 24 h were used for analysis. Before CLAMS the animals were habituated to their  
155 normal food as powder for three days, as the food in CLAMS is in powder form. The animals  
156 were placed in CLAMS when the body weight had reached a plateau. This is defined by less  
157 than 5% weight loss or gain over a period of 7 days or more. The DIO rats and mice were  
158 placed in CLAMS at 35 and 11 weeks of age, respectively, together with their respective  
159 control groups. GB and control rats were placed in CLAMS 3 weeks post-surgery, while  
160 M3KO and WT mice were 6, 11 and 15 months old when placed in CLAMS.

161           Animals were placed in CLAMS with free access to standard powder food (RM1  
162 801002, Scanbur BK AS, Sweden) or to high fat diet, and tap water. This system is composed  
163 of a four-chamber open circuit indirect calorimeter designed for continuous monitoring of  
164 individual animals. Eating behaviour and metabolic parameters were recorded automatically.  
165 High-resolution feeding data was generated by monitoring all feeder balances every 0.5  
166 seconds. The end of an eating event (meal) was determined when the balances were stable for  
167 more than 10 seconds and a minimum of 0.05 g of food were eaten. An air sample was  
168 withdrawn every 5 min. The EE (kcal/h) was calculated according to this equation:  
169  $(3.815 + 1.232 \text{ RER}) \times \text{VO}_2$ , where RER (respiratory exchange ratio) was the volume of CO<sub>2</sub>  
170 produced per volume of O<sub>2</sub> consumed. VO<sub>2</sub> was the volume of O<sub>2</sub> consumed per hour per  
171 kilogram of mass of the animal. Parameters that were obtained during light phase (7 am–7  
172 pm) and dark phase (7 pm–7 am) for each individual animal included number of meals, meal  
173 size, meal duration, accumulated food intake, intermeal interval, rate of eating, satiety ratio,  
174 drinking activity, EE and ambulatory activity. The intermeal interval was defined as the  
175 interval in minutes between two meals. Rate of eating was calculated by dividing the average  
176 meal size by the average duration of a meal, and satiety ratio, an index of the non-eating time  
177 produced by each gram of food consumed, was calculated by dividing the average intermeal  
178 interval by the average meal size.

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182 ***Measurement of body composition***

183 Body composition was determined for DIO rats and mice, and respective control  
184 groups, using dual energy X-ray absorptiometry (DXA) with small animal software (Hologic  
185 QDR 4500A, Hologic Inc., Bedford, MA, USA). Total bone area (cm<sup>2</sup>), total fat mass (g) and  
186 total fat-free mass (g) were measured, and total mass (g), lean percentage (%) and fat  
187 percentage (%) were calculated. DXA was performed under anaesthesia (isoflurane).

188

189 ***In situ hybridization***

190 Brain samples were taken at euthanization and snap-frozen in isopentane on dry ice  
191 before being stored at -80°C wrapped in aluminium foil. The frozen brains were cut (14 µm)  
192 in the region spanning the hypothalamus between Bregma -0.10 to -2.54 mm according to the  
193 Mouse Brain Atlas of Franklin & Paxinos 1997 and sections were mounted onto poly-L-  
194 lysine-coated slides. Primers for the amplification of AgRP spanned were based on Genbank  
195 sequence U89484 to amplify the sequence between bases 113-341 (forward primer 5'-  
196 TGTTCCCAGAGTTCCCAGGTC-3', reverse primer 5'-  
197 GCATTGAAGAAGCGGCAGTAGCAC-3'). Primers for the amplification of POMC were  
198 based on Genbank sequence J00162 to amplify the sequence between bases 263-665 (forward  
199 primer 5'-GGGCAAGCGTCTCTACTCCAT-3', reverse primer 5'-  
200 GCCCTTCTTGTRSRCGTTCTTGA-3'). The DNA sequence for NPY was a full-length  
201 cloned rat NPY gene sequence. Primers for the amplification of Ob-Rb (leptin receptor) were  
202 based on Genbank sequence U49107 to amplify the sequence between bases 5'-  
203 GTGTGAGCATCTCTCCTGGAG-3' (+2829 to +2849) and 5'-  
204 ACCACACCAGACCCTGAAAG-3' (+3362 to +3343)(see MCH information). Integrated  
205 optical density (IOD) was obtained by reference to the 14C microscale. Values were averaged  
206 for each animal.

207

208 ***Statistical analysis***

209 The results are expressed as means ± SEM. Statistical comparisons were performed  
210 using two-sided independent *t*-test between the groups, and paired *t*-test was used for  
211 comparisons within groups. If assumption of normal distribution was not met, Mann-Whitney  
212 U test (denoted as U test when applied) were used. A *p*-value of <0.05 was considered  
213 statistically significant. Shapiro-Wilk test was used to test for normal distribution and  
214 Levine's test for homogeneity was used to test for equal variances between the groups and *p*-  
215 values were reported accordingly. A power of >0.80 was set as minimal to ensure sufficient

216 sample sizes and were calculated using G\*Power version 3.1 and a randomization table was  
217 used to assign the animals to different groups. The data analysis was performed in SPSS  
218 version 20.0.

219

220

## 221 **Results**

### 222 ***Diet-induced obese rats***

223 The increase of body weight reached the plateau 28 weeks after starting high-fat diet  
224 feeding (33 weeks of age). DIO rats were 25% heavier than age-matched and normal diet  
225 (ND) controls at the plateau ( $p=0.001$ ) (Fig. 1a, Supplementary Table S1).

226 At 35 weeks of age, the rats were placed in CLAMS, and we found calorie intake was  
227 higher during dark phase than light phase in both ND ( $p<0.001$ ) and DIO ( $p<0.001$ ) rats  
228 indicating a good sensitivity of CLAMS. Calorie intake was slightly higher in DIO rats than  
229 ND rats during light phase ( $p=0.039$ ), but not significantly different during the dark phase  
230 ( $p=0.083$ ) (Fig. 1b). EE was higher during dark phase than light phase in ND rats ( $p<0.001$ ).  
231 However, there were no differences between ND and DIO rats during light phase ( $p=0.917$ ),  
232 but there was a trend for lower EE in DIO rats during dark phase ( $p=0.068$ ) (Fig. 1c). RER  
233 was higher during dark phase than light phase in control rats ( $p<0.001$ ). In comparison with  
234 ND rats, DIO rats had a lower RER during both light- and dark-phase ( $p=0.008$  and  $<0.001$ ,  
235 respectively)(Fig. 1d). Body composition analysis showed that total body mass ( $p<0.001$ ) and  
236 surface area ( $p<0.001$ ), fat mass ( $p<0.001$ , U test) and fat percentage ( $p<0.001$ ) were higher,  
237 whereas the lean percentage ( $p<0.001$ ) (but not lean mass ( $p=0.657$ , U test)) was lower in  
238 DIO than ND rats (Fig. S1).

239

### 240 ***Diet-induced obese mice***

241 The increase of body weight reached the plateau 6 weeks after starting high-fat diet  
242 feeding (11 weeks of age). DIO mice were 23% heavier than age-matched and normal diet  
243 controls at the plateau ( $p<0.001$ ) (Fig. 2a, Supplementary Table S2).

244 At the plateau of body weight, the mice were placed in CLAMS. We found that calorie  
245 intake was higher during dark phase than light phase particularly in ND mice ( $p<0.001$ ).  
246 There was no difference in calorie intake between DIO and ND mice neither during light  
247 phase ( $p=0.326$ ) nor during dark phase ( $p=0.943$ ) (Fig. 2b). EE was higher during dark phase  
248 than light phase in both ND ( $p<0.001$ ) and DIO ( $p<0.001$ ) mice, suggesting higher active EE.  
249 There was no difference in the EE during light phase ( $p=0.274$ ), but DIO had lower EE than



250 ND during dark phase ( $p=0.043$ ) (Fig. 2c). RER was higher during dark phase than light  
251 phase in both ND ( $p=0.001$ ) and DIO ( $p=0.008$ ) mice. In comparison with ND mice, DIO  
252 mice had a lower RER only during dark phase ( $p=0.003$ ) (Fig. 2d). Body composition  
253 analysis showed that total body mass ( $p<0.001$ ) and surface ( $p=0.004$ ), and fat mass ( $p<0.001$ ,  
254 U test) and percentage ( $p<0.001$ ) were higher, whereas the lean mass ( $p=0.020$ ) and  
255 percentage ( $p<0.001$ ) were lower in DIO than ND mice (Fig. S2).

256

### 257 ***Gastric bypassed rats***

258 GB-induced body weight loss reached the plateau, i.e., 15% weight loss, 2 weeks after  
259 surgery ( $p<0.001$ )(Fig. 3a, Supplementary Table S3), and 3 weeks post-surgery, the rats were  
260 placed in CLAMS. Calorie intake was higher during dark phase than light phase in both  
261 sham-operated and GB rats ( $p=0.001$  and  $0.003$ , respectively). There was no difference in  
262 calorie intake between the two groups neither during light phase nor during dark phase  
263 ( $p=0.289$  and  $0.870$ , respectively) (Fig. 3b). EE was higher in GB rats than sham-operated rats  
264 during dark phase ( $p=0.033$ ) (Fig. 3c). RER was higher during dark phase than light phase in  
265 both sham- and GB-operated group ( $p=0.001$  and  $0.008$ , respectively). There was no  
266 difference in RER between groups either during dark- or light-phase ( $p=0.389$  and  $0.516$ ,  
267 respectively) (Fig. 3d).

268

### 269 ***M3KO mice***

270 At the birth, the body weight did not differ between M3KO and WT mice [29]. At 2  
271 months of age there was a trend for lower body weight in M3KO than WT mice ( $p=0.065$ ).  
272 The mice was placed in CLAMS at 6, 11 and 15 months of age, and M3KO mice weighed  
273 significantly less than WT mice at all time-points ( $p<0.001$ ,  $0.001$  and  $0.001$ , respectively).  
274 There was no significant difference in body weight between 6, 11, and 15 months of age (6  
275 vs. 11:  $p=0.599$ , 6 vs. 15:  $p=0.514$  and 11 vs 15:  $p=0.292$ )(Fig. 4a). Both M3KO and WT  
276 mice had a continuous growth throughout the lifespan. (Supplementary Tables S4-6). Calorie  
277 intake was higher during dark phase than light phase in WT mice ( $p=0.002$ ,  $0.092$  and  $0.081$ ,  
278 respectively) and more so in M3KO mice ( $p=0.022$ ,  $0.004$  and  $<0.001$ , respectively). There  
279 were no differences in calorie intake between WT and M3KO mice at all time points  
280 examined, except light phase at 6 months where calorie intake was lower in M3KO compared  
281 to WT, ( $p=0.010$ ) and dark phase at 15 months of age where calorie intake was unexpectedly  
282 higher in M3KO than WT mice ( $p=0.034$ ) (Fig. 4b). Both EE and RER were higher during  
283 light- as well as dark-phase in M3KO than in WT mice at the time points measured (Light:

284 EE:  $p < 0.001$ ,  $= 0.005$  and  $= 0.005$ , respectively. RER:  $p < 0.001$ ,  $= 0.028$  and  $< 0.001$ ,  
285 respectively) (Dark: EE:  $p < 0.001$ ,  $< 0.001$  and  $= 0.003$ , respectively; RER:  $p < 0.001$ ,  $0.001$  and  
286  $0.001$ , respectively) (Fig.4c, d).

287 In comparison with age-matched WT mice, the M3KO mice had higher mRNA  
288 expression of NPY, AgRP and leptin receptor in the arcuate nucleus in the hypothalamus  
289 ( $p = 0.001$ ,  $< 0.001$  and  $= 0.003$ , respectively) (Fig. 5). Pro-opiomelanocortin (POMC) in the  
290 ARC and melanin-concentrating hormone (MCH) in ventromedial hypothalamus were  
291 unchanged in terms of mRNA expression ( $p = 0.356$  and  $0.200$ , respectively) (Fig. 5).

292

293

## 294 Discussion

295 The results of the present study using 4 different animal models have demonstrated a  
296 common phenotype, i.e. steady-state energy balance at the plateau of body weight  
297 development. EE was measured during light- and dark-phases separately to indicate basal and  
298 active metabolic rates, respectively. Interestingly, the basal metabolic rate was unchanged in  
299 both obese rats and mice compared to normal chow-fed controls, but the active metabolic rate  
300 seemed to be reduced significantly in mice and non-significantly in rats (probably due to the  
301 sample size). The results also showed a higher RER in dark phase than light phase during the  
302 steady-state energy balance, indicating a higher oxidative capacity of the muscle for the  
303 provision of energy in the dark phase [34]. Furthermore, the obese rats had a low RER during  
304 both light-and dark- phases, suggesting a lower muscle's oxidative capacity.

305 These results may help us to explain what we often see in clinic, i.e., the large  
306 variability in individual responses to weight loss interventions in patients could be because  
307 the differences in the time frame over which a steady-state energy balance establishes  
308 between individuals. This is in line with a recent study showing that the longer it took to reach  
309 a steady state following GB surgery, the greater the weight loss was. In addition, the majority  
310 of patients undergoing GB surgery did not lose significant weight after reaching steady state  
311 [35].

312 Previously it has been reported that NPY and AgRP mediate complementary functions  
313 of hyperphagia and reduce EE in leptin receptor deficient mice [36]. In addition, it has been  
314 demonstrated that there are compensatory changes in hypothalamus, particularly NPY and  
315 AgRP gene expression, in normal rats and mice fed either with high-fat diet or low-energy  
316 dense diet for long period, indicative of a compensation to defend the body weight changes

317 [19, 20, 21, 22]. Previously, we and others have shown that GB-induced weight loss in rats  
318 was not due to decreased calorie intake, but due to an increase in EE [23, 24, 25, 26, 27],  
319 which is in line with the hypothalamic gene expression of NPY, AgRP, or POMC [28].  
320 Therefore, we did not analyse the hypothalamic gene expression in obese rats, obese mice and  
321 GB rats in the present study.

322         However, we further analysed the M3KO mouse model with respect to both energy  
323 balance and the hypothalamic gene expression. This was because in the initial publication  
324 [29] reported that M3KO mice had reduced food intake with increased expression of  
325 hypothalamic AgRP mRNA and decreased expression of POMC mRNA, which appeared to  
326 be inconsistent with the homeostatic mechanism of food intake regulation associated with the  
327 regulatory neuropeptides encoded by these genes[30]. In contrast to this previous study we  
328 found M3KO mice did not have significantly overall reduced calorie intake as measured using  
329 a more precise method (the state-of-the-art CLAMS) than one used in the study by Yamada et  
330 al. [29]. Furthermore, we found that reduced calorie intake during the light phase and  
331 increased calorie intake during the dark phase were accompanied by increase EE during both  
332 light phase and dark phases throughout all stages of growth and maturity. Additionally, in  
333 contrast to this previous study, we found that RER was increased in M3KO mice, implicating  
334 use of carbohydrates as the predominant fuel for cellular respiration. This may also suggest  
335 high rates of *de novo* lipogenesis in these mice as carbohydrates are partially oxidized and  
336 directed towards fatty acid synthesis. This would have a considerable effect on the fat mass of  
337 these mice and is highly relevant to their steady state. Similar to EE, RER was higher in both  
338 the light and dark phases. Taken together with the findings of equal body weights at birth and  
339 during the initial post-natal development, we suggest that the lean phenotype of the M3KO  
340 mice is not caused by prenatal factors or environment, but develops as the mice grow and is  
341 maintained as the mice achieve a new steady-state of energy balance. Since the absence of M3  
342 receptor has beneficial effects, which protect mice against obesity, some studies with new  
343 classes of M3-receptor selective antagonists have been initiated but still in its preliminary  
344 phases, and the lack of specific antagonists or agonists for M3 receptors in CNS, make it  
345 impossible to validate this anti-obesity target at present [37]. In addition, there are several  
346 other possible pharmacological treatments for obesity targeting EE, such as thyroid hormone  
347 receptor  $\beta$  agonists (GC-1 and GC-24) and NADPH:quinone reductase 1 (NQO1) activators  
348 (MB12066) [38, 39]. Some of these compounds have shown promising results as obesity  
349 treatment in animal studies, with MB12066 having completed Phase I clinical trial [40, 41,  
350 42].

351 The results of the present study suggest that when a body weight plateau is reached  
352 following either weight gain or loss, a new steady-state energy homeostasis occurs which is  
353 mainly due to alterations in EE and RER. The decreased EE in obese animals and increased  
354 EE in lean animal models suggest that interventions for reduction in body weight or for  
355 continuation of weight loss should target EE.

356

#### 357 **Disclosure of interest**

358 No conflicts of interest, financial or otherwise, are declared by the authors

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365

#### 366 **Author contributions**

367 MKO, HJ, NC, PB, KT, BK, DC, and C-MZ conceived and designed the experiments. MKO,  
368 HJ, NC, PB, DC and C-MZ performed the experiments and analysed the results. KT provided  
369 animal models. MKO, HJ, NC, PB, KT, BK, DC and C-MZ wrote the manuscript. All authors  
370 reviewed the manuscript.

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542



543 **Figures Captions**

544

545 **Figure 1** | (a) Body weight of high-fat-diet (HFD) induced obese (DIO) (n=12) rats relative to  
546 normal diet (ND) (n=8) rats from 5 to 35 weeks of age. Grey area represents body weight  
547 plateau (less than 5% increase or decrease in BW over last couple of weeks). CLAMS was  
548 performed at 35 weeks of age for both DIO (n=11) and ND (n=7) rats. (b) Calorie intake  
549 (g/100g body weight) during light phase and dark phase of DIO and ND rats. (c) EE  
550 (kcal/h/100g body weight) during light phase and dark phase of DIO and ND rats. (d) RER  
551 during light phase and dark phase of DIO and ND rats. White background indicates light  
552 phase and grey background indicates dark phase. Data is expressed as mean  $\pm$  SEM. \*, \*\*,  
553 \*\*\*:  $p < 0.05$ ,  $0.01$ ,  $0.001$  between DIO and ND. Independent  $t$ -test was used between groups  
554 and paired  $t$ -test within groups (light vs. dark phase).

555

556 **Figure 2** | (a) Body weight of high-fat-diet (HFD) induced obese (DIO) (n=10) mice relative  
557 to normal diet (ND) (n=10) mice from 5 to 12 weeks of age. Grey area represents body weight  
558 plateau (less than 5% increase or decrease in BW over last couple of weeks). CLAMS was  
559 performed at 11 weeks of age for both DIO (n=10) and ND (n=10) mice. (b) Calorie intake  
560 (g/100g body weight) during light phase and dark phase of DIO and ND mice. (c) EE  
561 (kcal/h/100g body weight) during light phase and dark phase of DIO and ND mice. (d) RER  
562 during light phase and dark phase of DIO and ND mice. White background indicates light  
563 phase and grey background indicates dark phase. Data is expressed as mean  $\pm$  SEM. \*\*, \*\*\*:  
564  $p < 0.01$ ,  $0.001$  between DIO and ND. Independent  $t$ -test was used between groups and paired  
565  $t$ -test within groups (light vs. dark phase).

566

567 **Figure 3** | (a) Body weight of GB-operated (n=14) rats relative to sham-operated (n=6) rats up  
568 to 5 weeks post-surgery. Grey area represents body weight plateau (less than 5% increase or  
569 decrease in BW over last couple of weeks). CLAMS was performed at 3 weeks after surgery  
570 for both GB (n=8) and sham-operated (n=6) rats. (b) Calorie intake (g/100g body weight)  
571 during light phase and dark phase of GB and sham rats. (c) EE (kcal/h/100g body weight)  
572 during light phase and dark phase of GB and sham rats. (d) RER during light phase and dark  
573 phase of GB and sham rats. White background indicates light phase and grey background  
574 indicates dark phase. Data is expressed as mean  $\pm$  SEM. \*, \*\*  $p < 0.05$ ,  $0.01$  between GB and  
575 sham. Independent  $t$ -test was used between groups and paired  $t$ -test within groups (light vs.  
576 dark phase).

577

578 **Figure 4** | (a) Body weight of M3KO mice relative to wild-type (WT) (n=7) at 2 (n=8), 6  
579 (n=8), 11 (n=8) and 15 (n=8) months of age. Grey area represents body weight plateau (less  
580 than 5% increase or decrease in BW over last couple of weeks). CLAMS was performed at 6,  
581 11 and 15 months of age for both M3KO (n=8) and WT (n=8) mice. (b) Calorie intake  
582 (g/100g body weight) during light phase and dark phase of WT and M3KO mice at 6, 11 and  
583 15 months of age. (c) EE during light phase and dark phase of WT and M3KO mice at 6, 11  
584 and 15 months of age. (d) RER during light phase and dark phase of WT and M3KO mice at  
585 6, 11 and 15 months of age.

586

587 **Figure 5** | mRNA expression (integrated optical density, %IOD) of AgRP, NPY, POMC and  
588 leptin receptor (*Lepr*) in arcuate nucleus and of MCH in ventromedial hypothalamus of WT  
589 (n=8) and M3KO (n=8) mice at 15 months of age. Data is expressed as mean  $\pm$  SEM. \*, \*\*,  
590 \*\*\*:  $p < 0.05, 0.01, 0.001$  between M3KO and WT. Independent *t*-test was used between  
591 groups.

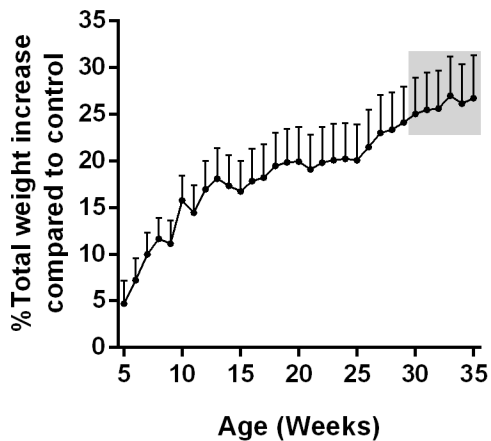
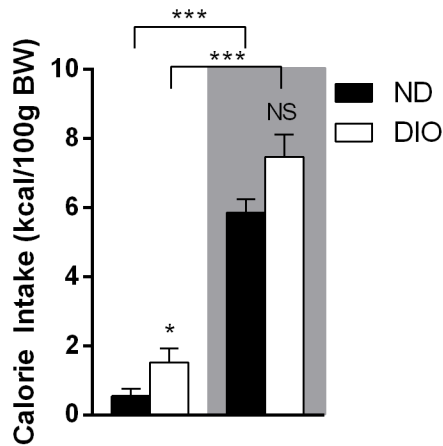
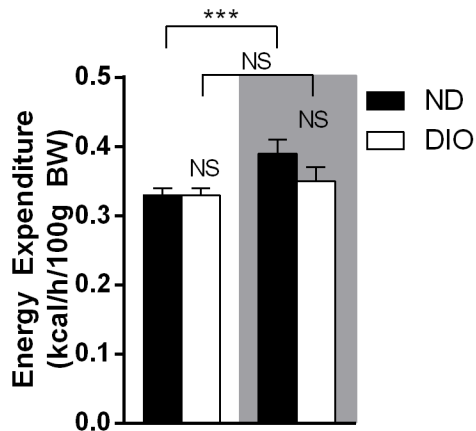
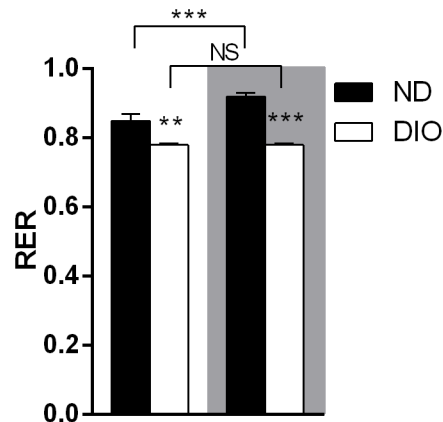
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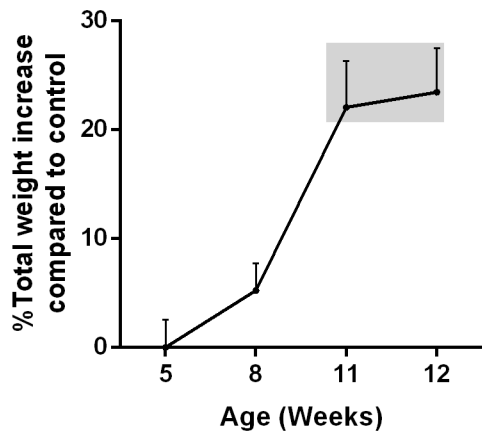
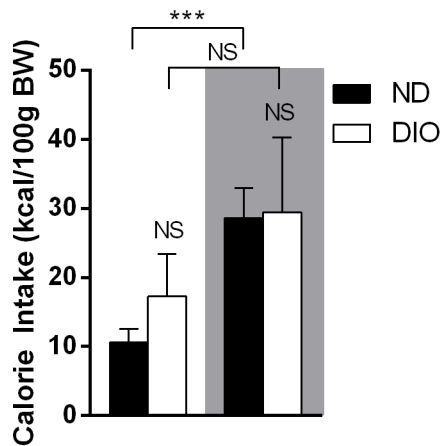
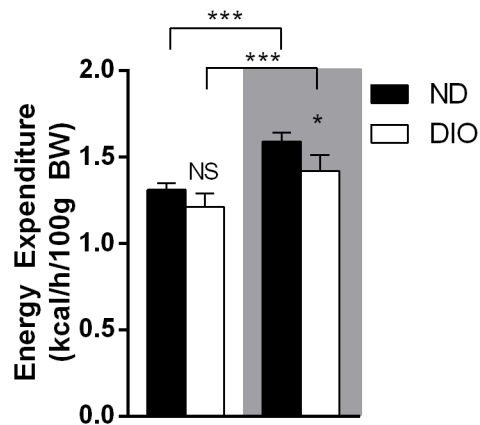
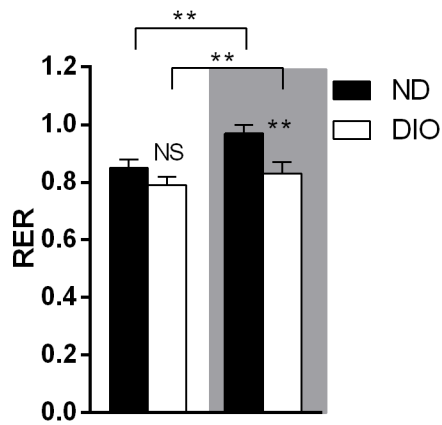
593 **Figure S1** | Body composition of DIO (n=11) and normal diet (ND) (n=8) rats at 35 weeks of  
594 age. (a) Total mass (g), (b) Fat mass (g), (c) Fat (%), (d) Surface area of the rats (cm<sup>2</sup>), (e)  
595 Lean mass (g) and (f) Lean (%). Data is expressed as mean  $\pm$  SEM. \*\*, \*\*\*:  $p < 0.01, 0.001$   
596 between DIO and ND. Independent *t*-test was used for all comparisons except for fat and lean  
597 mass where U test was used.

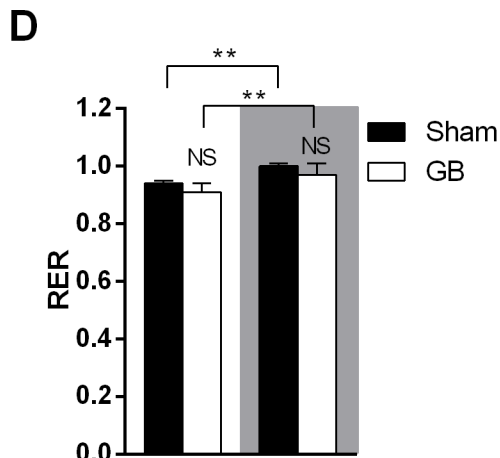
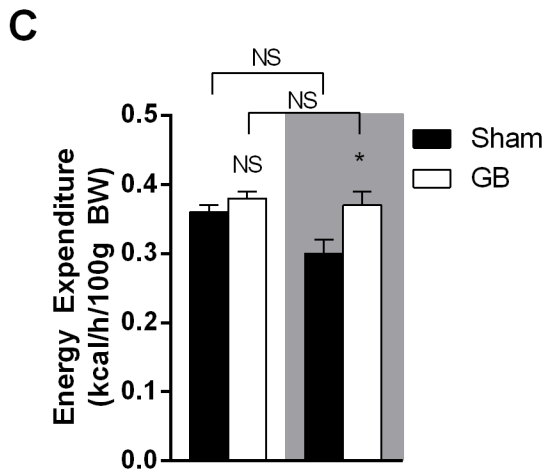
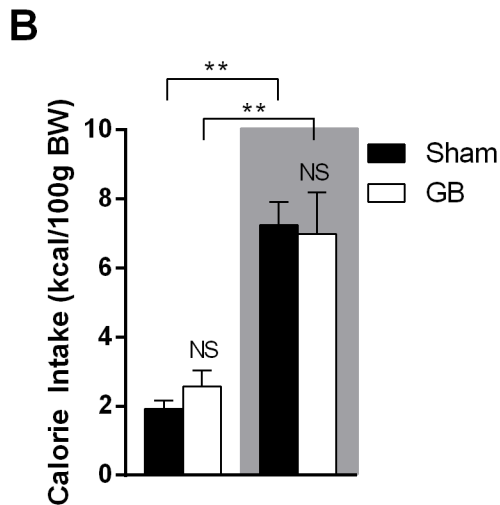
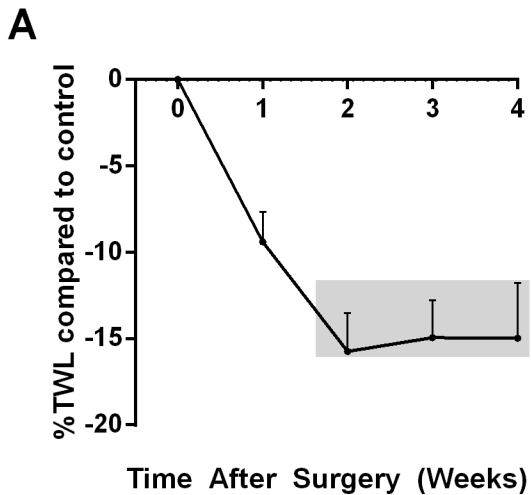
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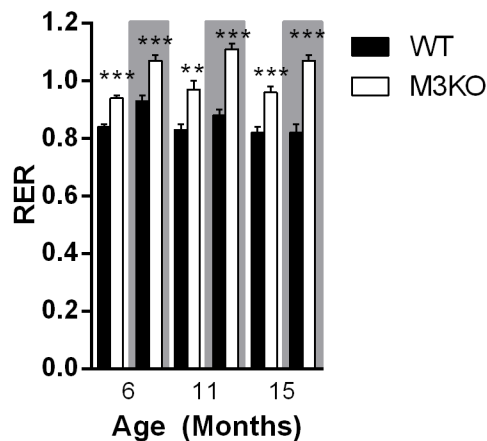
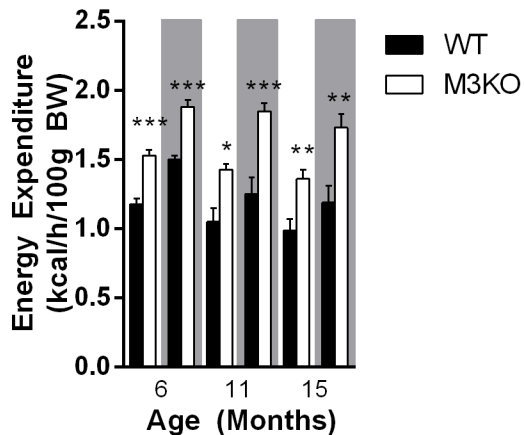
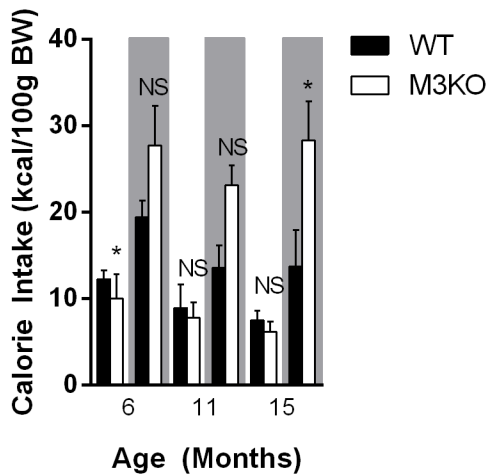
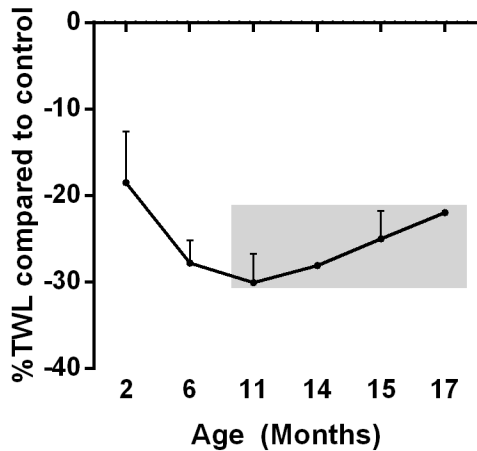
599 **Figure S2** | Body composition of DIO (n=10) mice and normal diet (ND) (n=10) mice at 11  
600 weeks of age. (a) Total mass (g), (b) Fat mass (g), (c) Fat (%), (d) Surface area of the rats  
601 (cm<sup>2</sup>), (e) Lean mass (g) and (f) Lean (%). Data is expressed as mean  $\pm$  SEM. \*, \*\*, \*\*\*:  $p <$   
602  $0.05, 0.01, 0.001$  between DIO and ND. Independent *t*-test was used for all comparisons  
603 except for fat mass where U test was used.

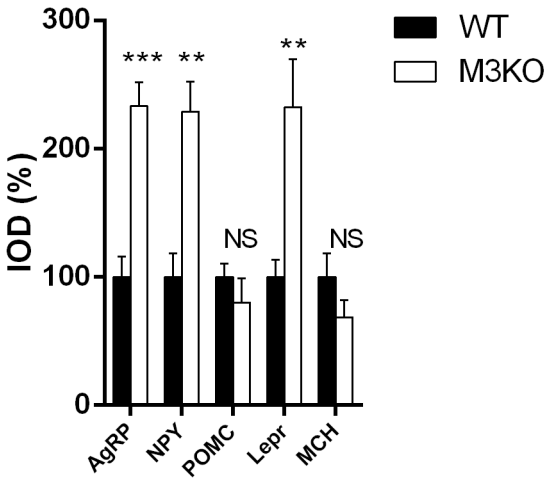
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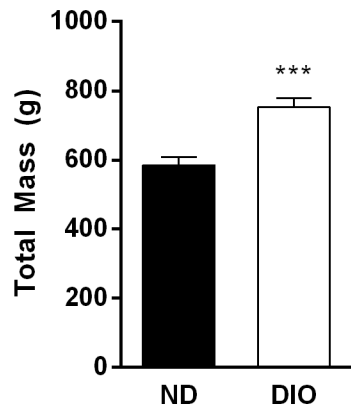
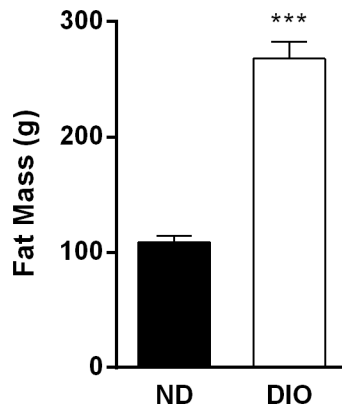
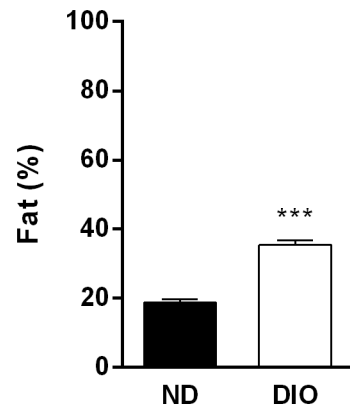
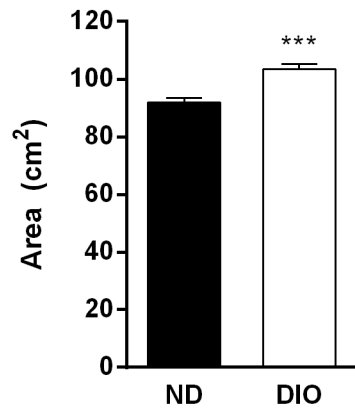
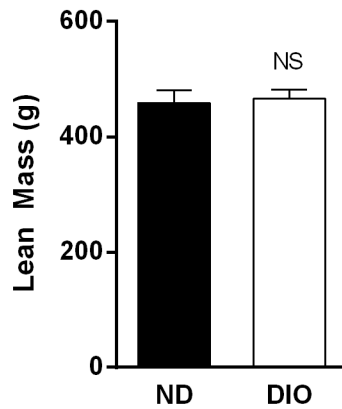
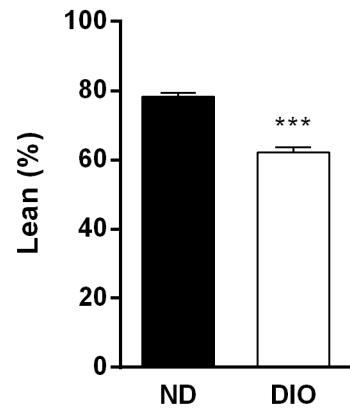
**A****B****C****D**

**A****B****C****D**







**A****B****C****D****E****F**



**Supplementary Table S1** | Energy balance in normal diet (ND) and diet-induced obese (DIO) rats at 35 weeks of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U –test were used if normality assumptions were not met (denoted U).

	<b>Parameters</b>	<b>ND (n=7)</b>	<b>DIO (n=11)</b>	<b>p-value (two-tailed)</b>
24 Hours	Food Intake (g)	15.05±1.05	12.84±0.97	0.155
	Food Intake (g/100g body weight)	2.49±0.18	1.73±0.13	0.004 <sup>u</sup>
	Calories intake (kcal)	38.67±2.71	67.3±5.07	0.001
	Calories intake (kcal/100g body weight)	6.41±0.45	9.05±0.69	0.008 <sup>u</sup>
	Number of meals	64.29±7.61	24.64±1.92	0.002
	Meal size (g/meal)	0.25±0.03	0.54±0.05	0.000
	Meal size (kcal/meal)	0.64±0.07	2.85±0.27	0.000
	Meal duration (min)	82.22±5.1	33.72±1.85	0.000
	Meal duration (min/meal)	1.39±0.16	1.44±0.12	0.771
	Intermeal interval (min)	22.84±3.08	58.31±4.73	0.000
	Satiety ratio (min/g)	91.27±6.15	115.6±14.95	0.246 <sup>u</sup>
	Rate of eating (g/min)	0.19±0.02	0.38±0.02	0.000
	Water intake (mL)	14.86±0.65	11.56±0.77	0.009
	Water intake (ml/100g body weight)	2.47±0.14	1.54±0.1	0.000
	Water intake during one interval (ml/time)	0.96±0.14	0.96±0.11	0.988
	Energy expenditure (kcal/h)	2.19±0.13	2.53±0.12	0.084
	Energy expenditure (kcal/h/100g body weight)	0.36±0.01	0.34±0.01	0.219
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.004±0	0.004±0	0.970
	RER	0.89±0.01	0.78±0.01	0.000
	VO <sub>2</sub>	733.04±25.46	702.51±25.88	0.438
	VCO <sub>2</sub>	653.15±29.62	549.67±21.47	0.011
Ambulatory activity	8059.8±225.77	5736±257.01	0.104 <sup>u</sup>	
	<b>Parameters</b>	<b>ND (n=7)</b>	<b>DIO (n=11)</b>	<b>p-value (two-tailed)</b>
Light Phase	Food Intake (g)	1.26±0.49	2.23±0.52	0.328 <sup>u</sup>
	Food Intake (g/100g body weight)	0.21±0.08	0.3±0.07	0.423
	Calories intake (kcal)	3.23±1.27	11.67±2.75	0.035 <sup>u</sup>
	Calories intake (kcal/100g body weight)	0.55±0.2	1.52±0.38	0.039
	Number of meals	6.57±1.85	5.09±0.96	0.445
	Meal size (g/meal)	0.17±0.04	0.39±0.07	0.033
	Meal size (kcal/meal)	0.45±0.09	2.04±0.36	0.001
	Meal duration (min)	6.08±1.93	6.1±1.52	0.996
	Meal duration (min/meal)	0.89±0.13	1.08±0.12	0.322
	Intermeal interval (min)	141.98±40.4	155.39±27.7	0.780
	Satiety ratio (min/g)	1265.18±653.95	3650.66±3232.5	0.285 <sup>u</sup>
	Rate of eating (g/min)	0.19±0.02	0.36±0.04	0.004
	Water intake (mL)	0.15±0.08	0.9±0.28	0.044 <sup>u</sup>
	Water intake (ml/100g body weight)	0.03±0.01	0.11±0.03	0.085 <sup>u</sup>
	Water intake during one interval (ml/time)	0.07±0.04	0.4±0.11	0.027 <sup>u</sup>
	Energy expenditure (kcal/h)	1.99±0.11	2.45±0.1	0.009
	Energy expenditure (kcal/h/100g body weight)	0.33±0.01	0.33±0.01	0.917
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.003±0	0.003±0	0.044 <sup>u</sup>
	RER	0.85±0.02	0.78±0.01	0.008
	VO <sub>2</sub>	673.28±19.97	681.08±21.43	0.807
	VCO <sub>2</sub>	572.11±26.3	531.86±18.05	0.210
Ambulatory activity	1486.4±94.14	1516.89±88.74	0.596 <sup>u</sup>	
	<b>Parameters</b>	<b>ND (n=7)</b>	<b>DIO (n=11)</b>	<b>p-value (two-tailed)</b>
Dark Phase	Food Intake (g)	13.79±0.95	10.62±0.9	0.033
	Food Intake (g/100g body weight)	2.28±0.15	1.43±0.12	0.000
	Calories intake (kcal)	35.44±2.45	55.63±4.72	0.002
	Calories intake (kcal/100g body weight)	5.86±0.4	7.47±0.64	0.083
	Number of meals	57.71±6.75	19.55±2.23	0.001
	Meal size (g/meal)	0.26±0.03	0.6±0.07	0.000
	Meal size (kcal/meal)	0.66±0.07	3.14±0.35	0.000
	Meal duration (min)	76.13±4.81	27.62±1.5	0.000
	Meal duration (min/meal)	1.43±0.17	1.6±0.18	0.536
	Intermeal interval (min)	12.04±1.66	38.94±5.23	0.000 <sup>u</sup>
	Satiety ratio (min/g)	47.09±3.15	67.18±7.04	0.020 <sup>u</sup>
	Rate of eating (g/min)	0.19±0.02	0.38±0.02	0.000
	Water intake (mL)	14.71±0.61	10.67±0.62	0.001 <sup>u</sup>
	Water intake (ml/100g body weight)	2.44±0.13	1.43±0.09	0.000
	Water intake during one interval (ml/time)	1.05±0.16	1.08±0.13	0.897
	Energy expenditure (kcal/h)	2.39±0.16	2.61±0.14	0.345
	Energy expenditure (kcal/h/100g body weight)	0.39±0.02	0.35±0.02	0.068
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.004±0	0.004±0	0.429
	RER	0.92±0.01	0.78±0.01	0.000
	VO <sub>2</sub>	792.8±32.26	723.93±32.88	0.177
	VCO <sub>2</sub>	734.19±34.67	567.49±26.79	0.001
Ambulatory activity	6573.4±296.45	4219.11±267.14	0.104 <sup>u</sup>	

**Supplementary Table S2** | Energy balance in normal diet (ND) and diet-induced obese (DIO) mice at 11 weeks of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U –test were used if normality assumptions were not met (denoted U).

	<b>Parameters</b>	<b>ND (n=10)</b>	<b>DIO (n=10)</b>	<b>p-value (two-tailed)</b>
24 Hours	Food Intake (g)	4.22±0.61	3.07±1.13	0.063 <sup>u</sup>
	Food Intake (g/100g body weight)	15.25±2.27	8.91±3.18	0.035 <sup>u</sup>
	Calories intake (kcal)	10.84±1.56	16.07±5.91	0.353 <sup>u</sup>
	Calories intake (kcal/100g body weight)	39.19±5.83	46.69±16.67	0.280 <sup>u</sup>
	Number of meals	85.3±7.71	61.7±8.45	0.054
	Meal size (g/meal)	0.05±0	0.04±0.012	0.105 <sup>u</sup>
	Meal size (kcal/meal)	0.13±0.01	0.11±0.03	0.105 <sup>u</sup>
	Meal duration (min)	105.27±11.07	32.23±5.66	0.000
	Meal duration (min/meal)	1.24±0.07	0.51±0.044	0.000
	Intermeal interval (min)	16.54±1.4	27.7±4.78	0.048
	Satiety ratio (min/g)	361.8±42.88	970.82±208.87	0.017
	Rate of eating (g/min)	0.04±0.01	0.09±0.022	0.029 <sup>u</sup>
	Water intake (mL)	2.21±0.05	1.68±0.18	0.018
	Water intake (ml/100g body weight)	7.99±0.26	5.15±0.72	0.002
	Water intake during one interval (ml/time)	0.05±0	0.07±0.021	0.280 <sup>u</sup>
	Energy expenditure (kcal/h)	0.4±0.01	0.44±0.026	0.023 <sup>u</sup>
	Energy expenditure (kcal/h/100g body weight)	1.45±0.04	1.31±0.085	0.165 <sup>u</sup>
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0±0	0.05±0	0.796 <sup>u</sup>
	RER	0.91±0.03	0.81±0.031	0.016
	VO <sub>2</sub>	2941.72±89.33	2718.82±170.02	0.529 <sup>u</sup>
	VCO <sub>2</sub>	2702.743±92.59	2210.76±182.3	0.027
Ambulatory activity	11262.8±2522.14	10998.4±1933.35	0.436 <sup>u</sup>	
	<b>Parameters</b>	<b>ND (n=10)</b>	<b>DIO (n=10)</b>	<b>p-value (two-tailed)</b>
Light Phase	Food Intake (g)	1.14±0.2	1.15±0.42	0.165 <sup>u</sup>
	Food Intake (g/100g body weight)	4.12±0.75	3.29±1.18	0.075 <sup>u</sup>
	Calories intake (kcal)	2.94±0.52	6±2.21	0.684 <sup>u</sup>
	Calories intake (kcal/100g body weight)	10.59±1.93	17.25±6.17	1.000 <sup>u</sup>
	Number of meals	28.1±3.03	17.4±2.32	0.012
	Meal size (g/meal)	0.04±0	0.06±0.02	0.739 <sup>u</sup>
	Meal size (kcal/meal)	0.1±0.01	0.15±0.05	0.739 <sup>u</sup>
	Meal duration (min)	31.15±2.92	6.87±1.37	0.000 <sup>u</sup>
	Meal duration (min/meal)	1.15±0.1	0.38±0.04	0.000
	Intermeal interval (min)	25.83±2.49	43.87±4.7	0.005
	Satiety ratio (min/g)	685.11±73.44	1420.31±335.79	0.059
	Rate of eating (g/min)	0.04±0.01	0.19±0.08	0.001 <sup>u</sup>
	Water intake (mL)	0.67±0.04	0.62±0.11	0.692
	Water intake (ml/100g body weight)	2.41±0.15	1.93±0.39	0.264
	Water intake during one interval (ml/time)	0.05±0	0.05±0.01	0.436 <sup>u</sup>
	Energy expenditure (kcal/h)	0.36±0.01	0.41±0.02	0.140
	Energy expenditure (kcal/h/100g body weight)	1.31±0.04	1.21±0.08	0.274
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0±0	0±0	0.684 <sup>u</sup>
	RER	0.85±0.03	0.79±0.03	0.190 <sup>u</sup>
	VO <sub>2</sub>	2701.94±87.25	2521.09±161.52	0.338
	VCO <sub>2</sub>	2303.14±96.38	1994.48±161.82	0.105 <sup>u</sup>
Ambulatory activity	2780.08±659.06	3022.8±570.81	0.785	
	<b>Parameters</b>	<b>ND (n=10)</b>	<b>DIO (n=10)</b>	<b>p-value (two-tailed)</b>
Dark Phase	Food Intake (g)	3.07±0.45	1.92±0.72	0.035 <sup>u</sup>
	Food Intake (g/100g body weight)	11.13±1.68	5.62±2.06	0.007 <sup>u</sup>
	Calories intake (kcal)	7.9±1.16	10.07±3.8	0.315 <sup>u</sup>
	Calories intake (kcal/100g body weight)	28.59±4.31	29.44±10.78	0.280 <sup>u</sup>
	Number of meals	57.2±5.44	44.3±6.67	0.151
	Meal size (g/meal)	0.05±0.01	0.04±0.01	0.052 <sup>u</sup>
	Meal size (kcal/meal)	0.14±0.01	0.1±0.03	0.052 <sup>u</sup>
	Meal duration (min)	74.12±10.46	25.36±5	0.001
	Meal duration (min/meal)	1.28±0.1	0.55±0.05	0.000
	Intermeal interval (min)	12.24±1.45	20.42±4.43	0.247 <sup>u</sup>
	Satiety ratio (min/g)	253.23±42.66	741.89±156.87	0.013
	Rate of eating (g/min)	0.05±0.01	0.07±0.02	0.165 <sup>u</sup>
	Water intake (mL)	1.54±0.05	1.06±0.08	0.000 <sup>u</sup>
	Water intake (ml/100g body weight)	5.58±0.24	3.23±0.36	0.000 <sup>u</sup>
	Water intake during one interval (ml/time)	0.06±0	0.08±0.03	0.190 <sup>u</sup>
	Energy expenditure (kcal/h)	0.44±0.02	0.48±0.03	0.075 <sup>u</sup>
	Energy expenditure (kcal/h/100g body weight)	1.59±0.05	1.42±0.09	0.043 <sup>u</sup>
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.01±0	0.01±0	0.796 <sup>u</sup>
	RER	0.97±0.03	0.83±0.04	0.003
	VO <sub>2</sub>	3181.38±94.72	2916.54±186.14	0.579 <sup>u</sup>
	VCO <sub>2</sub>	3102.16±108.17	2427.04±207.7	0.010
Ambulatory activity	8482±1911.33	7975.6±1433.72	0.436 <sup>u</sup>	

**Supplementary Table S3** | Energy balance in sham- and gastric bypass (GB)-operated rats 3 weeks post-surgery. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U –test were used if normality assumptions were not met (denoted U).

	<b>Parameters</b>	<b>Sham (n=6)</b>	<b>GB (n=8)</b>	<b>p-value (two-tailed)</b>
24 Hours	Food Intake (g)	20.51±1.17	18.63±3.09	0.852 <sup>U</sup>
	Food Intake (g/100g body weight)	3.57±0.24	3.72±0.6	0.345 <sup>U</sup>
	Calories intake (kcal)	52.72±3	47.88±7.94	0.852 <sup>U</sup>
	Calories intake (kcal/100g body weight)	9.18±0.63	9.56±1.53	0.345 <sup>U</sup>
	Number of meals	44.83±4.48	44±6.95	0.928
	Meal size (g/meal)	0.48±0.04	0.39±0.06	0.323
	Meal size (kcal/meal)	1.22±0.11	1±0.16	0.323
	Meal duration (min)	58.43±7.37	65.15±11.08	0.648
	Meal duration (min/meal)	1.3±0.1	1.41±0.11	0.505
	Intermeal interval (min)	31.64±3.15	84.44±56.47	0.755 <sup>U</sup>
	Satiety ratio (min/g)	66.85±3.71	914.52±847.79	0.852 <sup>U</sup>
	Rate of eating (g/min)	0.37±0.03	0.28±0.05	0.184
	Water intake (mL)	20.22±1.83	17.85±2.38	0.472
	Water intake (ml/100g body weight)	3.5±0.31	3.61±0.49	0.865
	Water intake during one interval (ml/time)	2.09±0.2	1.32±0.17	0.013
	Energy expenditure (kcal/h)	1.92±0.07	1.86±0.05	0.852 <sup>U</sup>
	Energy expenditure (kcal/h/100g body weight)	0.33±0.01	0.38±0.01	0.003
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.003±0	0.003±0	0.055
	RER	0.97±0.01	0.94±0.04	0.852 <sup>U</sup>
	VO <sub>2</sub>	663.32±16.09	755.31±15.33	0.005 <sup>U</sup>
VCO <sub>2</sub>	638.31±17.4	707.19±27.26	0.020 <sup>U</sup>	
Ambulatory activity	6423.33±375.13	6186±623.55	0.770	
Light Phase	<b>Parameters</b>	<b>Sham (n=6)</b>	<b>GB (n=8)</b>	<b>p-value (two-tailed)</b>
	Food Intake (g)	4.35±0.54	4.99±0.91	0.593
	Food Intake (g/100g body weight)	0.75±0.09	1±0.18	0.289
	Calories intake (kcal)	11.18±1.39	12.82±2.35	0.593
	Calories intake (kcal/100g body weight)	1.93±0.23	2.57±0.46	0.289
	Number of meals	12.67±1.8	16.13±3.04	0.389
	Meal size (g/meal)	0.37±0.07	0.29±0.03	0.260
	Meal size (kcal/meal)	0.96±0.17	0.74±0.09	0.260
	Meal duration (min)	12.3±2.07	15.76±3.29	0.429
	Meal duration (min/meal)	1.02±0.19	0.9±0.1	0.552
	Intermeal interval (min)	57.05±8.2	80.3±40.2	0.414 <sup>U</sup>
	Satiety ratio (min/g)	168.48±31.65	674.16±546.43	0.573 <sup>U</sup>
	Rate of eating (g/min)	0.38±0.04	0.32±0.02	0.172
	Water intake (mL)	0.36±0.26	1.67±0.81	0.081 <sup>U</sup>
	Water intake (ml/100g body weight)	0.06±0.04	0.34±0.16	0.008 <sup>U</sup>
	Water intake during one interval (ml/time)	0.21±0.12	0.6±0.23	0.491 <sup>U</sup>
	Energy expenditure (kcal/h)	2.08±0.03	1.9±0.06	0.029
	Energy expenditure (kcal/h/100g body weight)	0.36±0.01	0.38±0.01	0.126
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.003±0	0.003±0	0.824
	RER	0.94±0.01	0.91±0.03	0.389
VO <sub>2</sub>	726.68±15.56	776.37±20.45	0.094	
VCO <sub>2</sub>	682.5±12.08	705.98±29.9	0.531	
Ambulatory activity	1636.17±103.11	1591.63±86.9	0.746	
Dark Phase	<b>Parameters</b>	<b>Sham (n=6)</b>	<b>GB (n=8)</b>	<b>p-value (two-tailed)</b>
	Food Intake (g)	16.16±1.3	13.64±2.44	0.426
	Food Intake (g/100g body weight)	2.82±0.26	2.72±0.47	0.870
	Calories intake (kcal)	41.54±3.34	35.06±6.28	0.426
	Calories intake (kcal/100g body weight)	7.24±0.67	6.99±1.2	0.870
	Number of meals	32.17±4.17	27.88±4.64	0.521
	Meal size (g/meal)	0.53±0.04	0.45±0.08	0.464
	Meal size (kcal/meal)	1.35±0.11	1.16±0.2	0.464
	Meal duration (min)	46.13±6.52	49.39±8.17	0.772
	Meal duration (min/meal)	1.43±0.06	1.77±0.16	0.086
	Intermeal interval (min)	22.2±3.06	63.6±42.28	0.852 <sup>U</sup>
	Satiety ratio (min/g)	41.7±3.46	787.62±742.99	0.662 <sup>U</sup>
	Rate of eating (g/min)	0.37±0.03	0.27±0.06	0.043 <sup>U</sup>
	Water intake (mL)	19.86±1.77	16.18±1.86	0.108 <sup>U</sup>
	Water intake (ml/100g body weight)	3.44±0.31	3.27±0.39	0.750
	Water intake during one interval (ml/time)	2.43±0.32	1.49±0.2	0.013 <sup>U</sup>
	Energy expenditure (kcal/h)	1.75±0.14	1.82±0.1	0.414 <sup>U</sup>
	Energy expenditure (kcal/h/100g body weight)	0.3±0.02	0.37±0.02	0.033
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.003±0	0.003±0	0.113
	RER	1±0.01	0.97±0.04	0.516
VO <sub>2</sub>	600.02±36.7	734.01±37.61	0.029	
VCO <sub>2</sub>	594.19±37.54	708.2±40.76	0.070	
Ambulatory activity	4787.17±284.06	4594.38±555.18	0.785	

**Supplementary Table S4** | Energy balance in wild-type (WT) and M3KO mice at 6 months of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U – test were used if normality assumptions were not met (denoted U).

	<b>Parameters</b>	<b>WT (n=8)</b>	<b>M3KO (n=8)</b>	<b>p-value (two-tailed)</b>
24 Hours	Food Intake (g)	4±0.24	3.49±0.51	0.380
	Food Intake (g/100g body weight)	12.33±0.8	14.69±1.79	0.249
	Calories intake (kcal)	10.28±0.62	8.97±1.31	0.380
	Calories intake (kcal/100g body weight)	31.68±2.06	37.75±4.61	0.249
	Number of meals	80.25±8.76	24±6.17	0.000
	Meal size (g/meal)	0.05±0.01	0.19±0.04	0.005
	Meal size (kcal/meal)	0.14±0.02	0.5±0.09	0.005
	Meal duration (min)	69.29±11.55	23.51±4.71	0.005
	Meal duration (min/meal)	0.86±0.09	1.5±0.51	0.130 <sup>U</sup>
	Intermeal interval (min)	18.28±1.92	86.49±18.76	0.008
	Satiety ratio (min/g)	346.19±19.64	430.65±51.52	0.160
	Rate of eating (g/min)	0.07±0.01	0.18±0.03	0.002
	Water intake (mL)	2.61±0.2	3.46±0.68	0.267
	Water intake (ml/100g body weight)	8.03±0.63	14.94±2.9	0.050
	Water intake during one interval (ml/time)	0.07±0.01	0.06±0.01	0.539
	Energy expenditure (kcal/h)	0.44±0.01	0.4±0.01	0.028
	Energy expenditure (kcal/h/100g body weight)	1.34±0.03	1.71±0.04	0.000
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.005±0	0.005±0	0.000
	RER	0.89±0.01	1±0.01	0.000
	VO <sub>2</sub>	2742±67.57	3370.29±96.59	0.000
VCO <sub>2</sub>	2440±57.88	3417.59±84.93	0.000	
Ambulatory activity	14323.75±3489.96	16439.88±4172.16	0.703	
	<b>Parameters</b>	<b>WT (n=8)</b>	<b>M3KO (n=8)</b>	<b>p-value (two-tailed)</b>
Light Phase	Food Intake (g)	1.55±0.11	0.93±0.27	0.005 <sup>U</sup>
	Food Intake (g/100g body weight)	4.78±0.37	3.9±1.08	0.010 <sup>U</sup>
	Calories intake (kcal)	3.98±0.28	2.38±0.71	0.005 <sup>U</sup>
	Calories intake (kcal/100g body weight)	12.28±0.96	10.02±2.79	0.010 <sup>U</sup>
	Number of meals	28.25±4.14	7.25±2.96	0.001
	Meal size (g/meal)	0.06±0.01	0.31±0.1	0.039
	Meal size (kcal/meal)	0.16±0.02	0.8±0.26	0.039
	Meal duration (min)	17.94±3.24	6.48±2.07	0.010
	Meal duration (min/meal)	0.65±0.09	2.57±1.4	0.878 <sup>U</sup>
	Intermeal interval (min)	27.23±3.64	196.09±51.27	0.013
	Satiety ratio (min/g)	448.53±25.91	717.32±100.33	0.032
	Rate of eating (g/min)	0.11±0.03	0.35±0.12	0.084
	Water intake (mL)	0.65±0.09	0.62±0.1	0.859
	Water intake (ml/100g body weight)	2.01±0.31	2.71±0.43	0.212
	Water intake during one interval (ml/time)	0.06±0.01	0.05±0.01	0.431
	Energy expenditure (kcal/h)	0.38±0.01	0.36±0.01	0.045
	Energy expenditure (kcal/h/100g body weight)	1.18±0.04	1.53±0.04	0.000
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.004±0	0.005±0	0.000
	RER	0.84±0.01	0.94±0.01	0.000
	VO <sub>2</sub>	2457.6±77.21	3073.19±99.3	0.000
VCO <sub>2</sub>	2059.68±72.3	2903.66±61	0.000	
Ambulatory activity	3964.83±401.03	4951.83±617.33	0.210	
	<b>Parameters</b>	<b>WT (n=8)</b>	<b>M3KO (n=8)</b>	<b>p-value (two-tailed)</b>
Dark Phase	Food Intake (g)	2.45±0.25	2.56±0.49	0.843
	Food Intake (g/100g body weight)	7.55±0.76	10.79±1.78	0.117
	Calories intake (kcal)	6.3±0.63	6.59±1.27	0.843
	Calories intake (kcal/100g body weight)	19.4±1.96	27.73±4.58	0.117
	Number of meals	52±5.69	16.75±3.44	0.000
	Meal size (g/meal)	0.05±0.01	0.18±0.03	0.003
	Meal size (kcal/meal)	0.13±0.02	0.47±0.08	0.003
	Meal duration (min)	51.35±8.6	17.03±3.25	0.005
	Meal duration (min/meal)	0.99±0.12	1.39±0.45	0.402
	Intermeal interval (min)	13.75±1.54	52.23±9.71	0.005
	Satiety ratio (min/g)	287.66±30.5	303.25±40.23	0.762
	Rate of eating (g/min)	0.06±0.01	0.18±0.03	0.005
	Water intake (mL)	1.96±0.17	2.84±0.6	0.198
	Water intake (ml/100g body weight)	6.02±0.52	12.23±2.53	0.044
	Water intake during one interval (ml/time)	0.07±0.01	0.07±0.01	0.582
	Energy expenditure (kcal/h)	0.49±0.01	0.44±0.02	0.034
	Energy expenditure (kcal/h/100g body weight)	1.5±0.03	1.88±0.05	0.000
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.005±0	0.006±0	0.002
	RER	0.93±0.02	1.07±0.02	0.000
	VO <sub>2</sub>	3026.41±63.52	3667.4±102.96	0.000
VCO <sub>2</sub>	2820.32±65.79	3931.53±129.81	0.000	
Ambulatory activity	15133.5±1779.52	16968±2483.48	0.562	

**Supplementary Table S5 | Energy balance in wild-type (WT) and M3KO mice at 11 months of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U – test were used if normality assumptions were not met (denoted U).**

	<b>Parameters</b>	<b>WT (n=8)</b>	<b>M3KO (n=8)</b>	<b>p-value (two-tailed)</b>
24 Hours	Food Intake (g)	3.04±0.56	3.1±0.25	0.003 <sup>U</sup>
	Food Intake (g/100g body weight)	8.78±1.99	12.03±0.73	0.000 <sup>U</sup>
	Calories intake (kcal)	7.82±1.43	7.97±0.64	0.003 <sup>U</sup>
	Calories intake (kcal/100g body weight)	22.56±5.12	30.91±1.87	0.000 <sup>U</sup>
	Number of meals	72.63±9.47	46.38±16.07	0.181
	Meal size (g/meal)	0.04±0.01	0.32±0.18	0.328 <sup>U</sup>
	Meal size (kcal/meal)	0.11±0.02	0.83±0.47	0.328 <sup>U</sup>
	Meal duration (min)	38.1±3.65	36.63±13.44	0.918
	Meal duration (min/meal)	0.61±0.1	1.09±0.34	0.442 <sup>U</sup>
	Intermeal interval (min)	21.91±3.23	112.61±56.13	0.505 <sup>U</sup>
	Satiety ratio (min/g)	529.99±62.39	438.87±46.99	0.263
	Rate of eating (g/min)	0.08±0.01	0.4±0.22	0.574 <sup>U</sup>
	Water intake (mL)	2.28±0.27	3.74±0.41	0.010
	Water intake (ml/100g body weight)	6.48±1	14.62±1.71	0.001
	Water intake during one interval (ml/time)	0.06±0.01	0.06±0	0.515
	Energy expenditure (kcal/h)	0.42±0.04	0.42±0.02	0.970
	Energy expenditure (kcal/h/100g body weight)	1.15±0.11	1.64±0.03	0.003
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.004±0	0.005±0	0.023
	RER	0.85±0.02	1.04±0.02	0.000 <sup>U</sup>
	VO <sub>2</sub>	2363.17±228.09	3199.54±61.23	0.008
VCO <sub>2</sub>	2021.22±198.3	3357.79±66.95	0.000	
Ambulatory activity	17856.83±976.98	17046.83±2190.65	0.743	
	<b>Parameters</b>	<b>WT (n=8)</b>	<b>M3KO (n=8)</b>	<b>p-value (two-tailed)</b>
Light Phase	Food Intake (g)	1.19±0.31	0.77±0.17	0.263
	Food Intake (g/100g body weight)	3.48±1.06	3.02±0.7	0.723
	Calories intake (kcal)	3.06±0.81	1.99±0.45	0.263
	Calories intake (kcal/100g body weight)	8.95±2.72	7.77±1.81	0.723
	Number of meals	26.5±5.95	22.25±7.98	0.676
	Meal size (g/meal)	0.05±0.01	0.18±0.09	0.199
	Meal size (kcal/meal)	0.13±0.02	0.45±0.23	0.199
	Meal duration (min)	12.07±1.84	14.7±7.58	0.028 <sup>U</sup>
	Meal duration (min/meal)	0.64±0.13	0.73±0.25	0.753
	Intermeal interval (min)	39.93±10.26	151.88±61.13	0.028 <sup>U</sup>
	Satiety ratio (min/g)	774.76±132	1399.58±662.91	0.007 <sup>U</sup>
	Rate of eating (g/min)	0.1±0.03	0.34±0.17	0.219
	Water intake (mL)	0.76±0.14	0.78±0.2	0.950
	Water intake (ml/100g body weight)	2.14±0.42	2.97±0.72	0.340
	Water intake during one interval (ml/time)	0.05±0.01	0.05±0.01	0.717
	Energy expenditure (kcal/h)	0.38±0.03	0.37±0.02	0.737
	Energy expenditure (kcal/h/100g body weight)	1.05±0.1	1.43±0.04	0.005
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.004±0	0.005±0	0.328 <sup>U</sup>
	RER	0.83±0.02	0.97±0.03	0.028 <sup>U</sup>
	VO <sub>2</sub>	2167.93±221.84	2841.45±79.9	0.013
VCO <sub>2</sub>	1794.32±182.51	2755.23±105.23	0.000	
Ambulatory activity	3857.33±425.65	4110.5±656.7	0.753	
	<b>Parameters</b>	<b>WT (n=8)</b>	<b>M3KO (n=8)</b>	<b>p-value (two-tailed)</b>
Dark Phase	Food Intake (g)	1.85±0.27	2.33±0.24	0.645 <sup>U</sup>
	Food Intake (g/100g body weight)	5.3±0.99	9±0.88	0.442 <sup>U</sup>
	Calories intake (kcal)	4.75±0.69	5.98±0.63	0.645 <sup>U</sup>
	Calories intake (kcal/100g body weight)	13.61±2.54	23.14±2.26	0.442 <sup>U</sup>
	Number of meals	46.13±5.85	24.13±8.36	0.049
	Meal size (g/meal)	0.05±0.01	0.52±0.36	0.083 <sup>U</sup>
	Meal size (kcal/meal)	0.12±0.02	1.33±0.92	0.083 <sup>U</sup>
	Meal duration (min)	26.03±3.47	21.93±6.37	0.581
	Meal duration (min/meal)	0.65±0.12	1.33±0.45	0.328 <sup>U</sup>
	Intermeal interval (min)	16.26±1.8	82.67±40.89	0.195 <sup>U</sup>
	Satiety ratio (min/g)	424.69±65.51	298.51±48.29	0.143
	Rate of eating (g/min)	0.07±0.01	0.49±0.29	0.328 <sup>U</sup>
	Water intake (mL)	1.52±0.17	2.97±0.42	0.007
	Water intake (ml/100g body weight)	4.34±0.67	11.65±1.9	0.028 <sup>U</sup>
	Water intake during one interval (ml/time)	0.06±0	0.06±0	0.366
	Energy expenditure (kcal/h)	0.46±0.05	0.48±0.03	0.745
	Energy expenditure (kcal/h/100g body weight)	1.25±0.12	1.85±0.06	0.000 <sup>U</sup>
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.005±0	0.006±0	0.009
	RER	0.88±0.02	1.11±0.02	0.000 <sup>U</sup>
	VO <sub>2</sub>	2558.41±238.86	3557.62±107.81	0.000 <sup>U</sup>
VCO <sub>2</sub>	2248.11±216.3	3960.36±137.2	0.000	
Ambulatory activity	13999.5±762.25	12936.33±1838.05	0.610	

**Supplementary Table S6** | Energy balance in wild-type (WT) and M3KO mice at 15 months of age. Data expressed as mean±SEM. Independent *t*-test was used for all comparisons. U – test were used if normality assumptions were not met (denoted U).

	Parameters	WT (n=8)	M3KO (n=8)	<i>p</i> -value (two-tailed)
24 Hours	Food Intake (g)	2.98±0.51	3.73±0.5	0.304
	Food Intake (g/100g body weight)	8.25±1.85	13.41±2.17	0.092
	Calories intake (kcal)	7.65±1.3	9.59±1.28	0.304
	Calories intake (kcal/100g body weight)	21.2±4.75	34.46±5.57	0.092
	Number of meals	93±21.96	42.88±13.88	0.959 <sup>u</sup>
	Meal size (g/meal)	0.04±0.01	0.22±0.1	0.015 <sup>u</sup>
	Meal size (kcal/meal)	0.1±0.02	0.57±0.26	0.015 <sup>u</sup>
	Meal duration (min)	53.23±12.81	58.31±20.9	0.015 <sup>u</sup>
	Meal duration (min/meal)	0.76±0.2	2.48±0.88	0.092
	Intermeal interval (min)	20.42±4.11	82.2±32.6	0.234 <sup>u</sup>
	Satiety ratio (min/g)	611.73±149.57	413.72±82.69	0.021 <sup>u</sup>
	Rate of eating (g/min)	0.07±0.02	0.11±0.03	1.000 <sup>u</sup>
	Water intake (mL)	2.37±0.38	4.67±0.94	0.040
	Water intake (ml/100g body weight)	6.49±1.35	16.83±3.84	0.000 <sup>u</sup>
	Water intake during one interval (ml/time)	0.08±0.02	0.08±0.02	0.905
	Energy expenditure (kcal/h)	0.42±0.04	0.44±0.02	0.679
	Energy expenditure (kcal/h/100g body weight)	1.09±0.1	1.54±0.08	0.003
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.004±0	0.005±0	0.000 <sup>u</sup>
	RER	0.82±0.03	1.01±0.02	0.001 <sup>u</sup>
	VO <sub>2</sub>	2263.56±213.94	3041.98±161.91	0.012
VCO <sub>2</sub>	1837.37±157.2	3114.74±180.56	0.000	
Ambulatory activity	18779±3267.54	14149.83±3576.57	0.362	
	<b>Parameters</b>	<b>WT (n=8)</b>	<b>M3KO (n=8)</b>	<b><i>p</i>-value (two-tailed)</b>
Light Phase	Food Intake (g)	1.09±0.15	0.66±0.12	0.043
	Food Intake (g/100g body weight)	2.9±0.45	2.39±0.46	0.440
	Calories intake (kcal)	2.8±0.39	1.7±0.31	0.043
	Calories intake (kcal/100g body weight)	7.47±1.16	6.15±1.19	0.440
	Number of meals	35.75±10.68	19.38±6.89	0.219
	Meal size (g/meal)	0.04±0.01	0.12±0.05	0.180
	Meal size (kcal/meal)	0.11±0.02	0.3±0.13	0.180
	Meal duration (min)	15.11±5	16.64±6.38	0.021 <sup>u</sup>
	Meal duration (min/meal)	0.74±0.23	2.33±1.02	0.166
	Intermeal interval (min)	30.78±7.92	118.35±45.17	0.095
	Satiety ratio (min/g)	733.59±130.19	1281.43±340.48	0.050 <sup>u</sup>
	Rate of eating (g/min)	0.15±0.05	0.07±0.01	0.328 <sup>u</sup>
	Water intake (mL)	0.82±0.16	0.78±0.28	0.908
	Water intake (ml/100g body weight)	2.24±0.51	2.83±1.06	0.626
	Water intake during one interval (ml/time)	0.08±0.02	0.05±0.01	0.279 <sup>u</sup>
	Energy expenditure (kcal/h)	0.38±0.04	0.39±0.02	0.946
	Energy expenditure (kcal/h/100g body weight)	0.99±0.08	1.36±0.07	0.005
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.004±0	0.005±0	0.034
	RER	0.82±0.02	0.96±0.02	0.000
	VO <sub>2</sub>	2056±180.32	2716.36±141.6	0.012
VCO <sub>2</sub>	1664.28±131.51	2608.84±144.57	0.000	
Ambulatory activity	4753±486.8	4258±1153.65	0.701	
	<b>Parameters</b>	<b>WT (n=8)</b>	<b>M3KO (n=8)</b>	<b><i>p</i>-value (two-tailed)</b>
Dark Phase	Food Intake (g)	1.88±0.46	3.07±0.4	0.072
	Food Intake (g/100g body weight)	5.34±1.64	11.02±1.77	0.034
	Calories intake (kcal)	4.84±1.18	7.89±1.03	0.072
	Calories intake (kcal/100g body weight)	13.74±4.22	28.32±4.55	0.034
	Number of meals	57.25±11.66	23.5±8.4	0.034
	Meal size (g/meal)	0.03±0.01	0.32±0.15	0.001 <sup>u</sup>
	Meal size (kcal/meal)	0.09±0.02	0.83±0.39	0.001 <sup>u</sup>
	Meal duration (min)	38.12±9.03	41.68±15.35	0.845
	Meal duration (min/meal)	0.79±0.19	2.62±0.8	0.057
	Intermeal interval (min)	15.16±2.79	69.65±27.16	0.105 <sup>u</sup>
	Satiety ratio (min/g)	586.3±170.24	231.18±42.9	0.002 <sup>u</sup>
	Rate of eating (g/min)	0.06±0.02	0.14±0.03	0.105 <sup>u</sup>
	Water intake (mL)	1.56±0.28	3.89±0.72	0.014
	Water intake (ml/100g body weight)	4.24±0.95	14±2.99	0.028 <sup>u</sup>
	Water intake during one interval (ml/time)	0.08±0.02	0.09±0.02	0.955
	Energy expenditure (kcal/h)	0.46±0.04	0.49±0.02	0.500
	Energy expenditure (kcal/h/100g body weight)	1.19±0.12	1.73±0.1	0.003
	Energy expenditure (kcal/h/cm <sup>2</sup> body surface)	0.005±0	0.006±0	0.015
	RER	0.82±0.03	1.07±0.02	0.000 <sup>u</sup>
	VO <sub>2</sub>	2471.12±250.41	3367.61±185.81	0.012
VCO <sub>2</sub>	2010.47±185	3620.64±222.35	0.000	
Ambulatory activity	14026±2998.34	9891.83±2435.21	0.310	