Title
A single nucleotide mutation in the dual-oxidase 2 (DUOX2) gene causes some of the panda’s unique metabolic phenotypes

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Abstract
The giant panda (Ailuropoda melanoleuca) is an iconic bear native to China, famous for eating almost exclusively bamboo. This unusual dietary behavior for a carnivore is enabled by several key adaptations including low physical activity, reduced organ sizes and hypothyroidism leading to lowered energy expenditure. These adaptive phenotypes have been hypothesised to arise from a panda-unique single-nucleotide mutation in the dual-oxidase 2 (DUOX2) gene, involved in thyroid hormone synthesis. To test this hypothesis we created genome edited mice carrying the same point mutation as the panda and investigated its effects on metabolic phenotype. Homozygous mice were 27% smaller than heterozygous and wild-type ones, had 13% lower body mass-adjusted food intake, 55% decreased physical activity, lower mass of kidneys (11%) and brain (5%), lower serum thyroxine (T4: 36%), decreased absolute (12%) and mass-adjusted (5%) daily energy expenditure, and altered gut microbiota. Supplementation with T4 reversed the effects of the mutation. This work uses a state of the art genome editing approach to demonstrate the link between a single-nucleotide mutation in a key endocrine related gene leading to profound adaptive changes in the metabolic phenotype, with great importance in ecology and evolution.

MAIN TEXT
Introduction
A key aim in ecology is to understand the factors that underlie metabolic phenotypes and their ecological consequences: also known as the metabolic theory of ecology [1]. A parallel key goal in evolutionary ecology is to understand how genetic mutations build the phenotype which then is selected for in a given environment. However, linking differences in the metabolic phenotype to underlying genetic changes is challenging [2]. Most inferences come mainly from either phenotypic or genetic data alone, but studies rarely investigate associations between molecular evolution and adaptive changes in phenotypic traits [3]. The link between changes in a single gene and structural and functional phenotypic changes is difficult to demonstrate experimentally [3], especially in large protected species of animals, where laboratory manipulation of the gene is not an option. Mutations of key genes involved in endocrine function can have major phenotypic impacts [4,5]. However, the role of such mutations in species ecology and evolution is unclear.

The giant panda (Ailuropoda melanoleuca) is an iconic species for wildlife
conservation characterized by its unique biology. Pandas are bears that are endemic to China and feed almost exclusively on bamboo, which has very low nutritional value [6]. Feeding on this resource is only possible because of adaptations that have dramatically reduced panda energy demands, thereby reducing food intake requirements [7]. This low metabolism is enabled by extremely low levels of physical activity [7]. In nature they typically move less than 500 m daily, and spend about 40% of their time resting [6]. Moreover, pandas have smaller brains, kidneys, and livers compared to other large mammals, which may also contribute to their low metabolic rates [7,8]. In addition, they have low levels of thyroid hormones thyroxine (T4) and triiodothyronine (T3), which average about 50% and 60% respectively of that expected for similar sized mammals [8]. These aspects of their unusual metabolic phenotype have been hypothesized to stem from a panda-unique single nucleotide mutation in the DUOX2 gene [7], which is absent in other carnivores, mice, and humans. In pandas the mutation involves substitution of C to T, resulting in an Arginine to Termination codon in the 16-th exon of the DUOX2 gene. It is not yet known whether this premature stop codon results in no translation of the gene or whether a truncated version of the protein is produced which may have biological functions.

DUOX2 encodes a protein involved in a critical step of thyroid hormones synthesis [9]. Some previously discovered mutations in DUOX2, or its maturation factor DUOXA, in mice [4,10] and humans [5,9,11-14] have been linked to goiter, dwarfism and congenital hypothyroidism. More generally, levels of thyroid hormones are associated with variations in metabolic rate [15-17] and physical activity [18,19]. DUOX2 is also involved in antimicrobial defense of the alimentary and respiratory tract [20,21]. However, the phenotypic consequences of the single nucleotide panda-specific mutation remain unknown. Potentially, the DUOX2 mutation may affect diverse aspects of physiology, particularly metabolic characteristics, which would then be involved in shaping wide aspects of panda ecology, including its bamboo diet, its behavior, reproduction, and geographical distribution [22]. We aimed to test the hypothesis that aspects of the unusual giant panda metabolic phenotype, can be traced to the single base pair mutation in the DUOX2 gene. To identify the effects of the panda-specific mutation in this wild species, we used a genetically engineered (CrispR-cas9) mouse model carrying the same mutation than one found in the giant panda (Fig S1), i.e. substitution of C to T, 625 Arginine to Termination codon in the 15-th exon of the mouse DUOX2 gene. We investigated growth rate, and traits associated with energy balance, such as the metabolic rate, spontaneous physical activity, and food consumption, body composition, energy assimilation, water consumption, mass of the vital organs, as well as circulating levels...
of thyroid hormones, and composition of the faecal microbiota. We further demonstrate the phenotypic consequences of this mutation do not stem from the microbiota changes, and can be reversed by supplementation with exogenous T4.

Results

The panda-specific DUOX2 mutation in mouse led to changes in phenotype that mirrored the unique biology of the panda. The DUOX2 mutant homozygote animals (DUOX2^{A625T/A625T}) were much smaller than heterozygous (DUOX2^{+/A625T}) and wild-type (DUOX2^{+/+}) animals between the ages of 4 to 10 weeks, i.e. during growth (mean body mass 12.2 (DUOX2^{A625T/A625T}) vs 19.1 (DUOX2^{+/A625T}) and 19.3 (DUOX2^{+/+}) g SE±0.4, F_{2,707}=534.79, P<0.001; Fig 1.A, Table S1, S2). The DUOX2^{A625T/A625T} mice were 27% dwarfed at 10-11 weeks-old (16.0 (DUOX2^{A625T/A625T}), 21.4 (DUOX2^{+/A625T}), 21.9 (DUOX2^{+/+}) g SE±0.5, F_{2,57}=53.77, P<0.001; Table S5, S7), during which time we made physiological measurements. During seven days of measurement, the DUOX2^{A625T/A625T} mice were on average 55% less active (moving 3903 (DUOX2^{A625T/A625T}) vs 7851 (DUOX2^{+/A625T}) and 8579 (DUOX2^{+/+}) m SE±514, F_{2,399}=23.92, P<0.001; Fig 1.D, Table S1, S2), had lower daily energy intake by 25% (42.7 (DUOX2^{A625T/A625T}), 54.3 (DUOX2^{+/A625T}), 56.8 (DUOX2^{+/+}) kJ/day SE±1.1, F_{2,397}=45.51, P<0.001; Table S1, S2) and body mass-adjusted daily food intake was 13% lower (47.3 (DUOX2^{A625T/A625T}) vs 52.3 (DUOX2^{+/A625T}) and 54.2 (DUOX2^{+/+}) kJ/day SE±1.4, F_{2,396}=3.98, P=0.020; Fig 1.C, Table S1, S2). The DUOX2^{A625T/A625T} mice had lower respiratory exchange ratio (0.78 (DUOX2^{A625T/A625T}) vs 0.83 (DUOX2^{+/A625T}) and 0.82 (DUOX2^{+/+}) SE±0.01, F_{2,399}=45.54, P<0.001; Table S1, S2). The daily energy expenditure was decreased in DUOX2^{A625T/A625T} mice by 12% (40.6 (DUOX2^{A625T/A625T}), 44.9 (DUOX2^{+/A625T}), 46.3 (DUOX2^{+/+}) kJ/day SE±0.4, F_{2,399}=70.02, P<0.001), as well as daily resting energy expenditure by 10% (29.4 (DUOX2^{A625T/A625T}), 31.6 (DUOX2^{+/A625T}), 32.8 (DUOX2^{+/+}) kJ/day SE±0.4, F_{2,399}=24.02, P<0.001; Table S1, S2). These differences remained significant but were lower after adjustment for the body size (5% for DEE: 42.9 (DUOX2^{A625T/A625T}) vs 43.9 (DUOX2^{+/A625T}) and 44.9 (DUOX2^{+/+}) kJ/day SE±0.4, F_{2,399}=4.13, P=0.017; Fig 1.B, Table S1, and 6% for REE: 30.5 (DUOX2^{A625T/A625T}) vs 31.2 (DUOX2^{+/A625T}) and 32.2 (DUOX2^{+/+}) kJ/day SE±0.4, F_{2,398}=2.95, P=0.053; Table S1, S2). The females were 14% smaller than males between 4-10 weeks-old (16.6 females vs 19.3 males g SE±0.2, F_{1,714}=91.51, P<0.001; Table S3, S4), and 14% smaller at 10-11 weeks-old (18.3 vs 21.3 g SE±0.5, F_{1,58}=14.85, P<0.001). The body mass-adjusted DEE (F_{1,405}=0.13, P=0.715), REE (F_{1,405}=0.70, P=0.403), or RER (F_{1,406}=2.93, P=0.088) did not differ between
the sexes. However, females had 7% higher mass-adjusted food intake (53.1 vs 49.5 kJ/day SE±1.0, $F_{1,403}=6.26$, $P=0.013$) and 28% higher activity (7606 vs 5949 m SE±442, $F_{1,406}=7.02$, $P=0.008$) than males (Table S3, S4).

The average percent of lean mass corrected for body mass was higher in DUOX2$^{A625T/A625T}$ mice than in DUOX2$^{+/A625T}$ and DUOX2$^{+/+}$ ones (90.8 (DUOX2$^{A625T/A625T}$) vs 87.8 (DUOX2$^{+/A625T}$) and 87.8 (DUOX2$^{+/+}$) % SE±0.35, $F_{2,56}=3.53$, $P=0.036$), but the percent of fat was not significantly different ($F_{2,56}=2.05$, $P=0.138$; Table S5, S7). Adjusted daily water intake in metabolic cages was lower in DUOX2$^{A625T/A625T}$ mice (3.4 (DUOX2$^{A625T/A625T}$) vs 6.4 (DUOX2$^{+/A625T}$) and 7.3 (DUOX2$^{+/+}$) g SE±1.4, $F_{2,53}=10.47$, $P<0.001$), and the daily urine production tended to be higher, but was not significant (2.1 (DUOX2$^{A625T/A625T}$), 1.3 (DUOX2$^{+/A625T}$), 1.4 (DUOX2$^{+/+}$) g SE±0.2, $F_{2,53}=2.87$, $P=0.066$; Table S5, S7). The energy assimilation did not differ between genotypes (90.9 (DUOX2$^{A625T/A625T}$), 90.8 (DUOX2$^{+/A625T}$), 90.9 (DUOX2$^{+/+}$) % SE±0.2, $F_{2,57}=0.14$, $P=0.873$; Table S5, S7). After adjustment for body mass (organ masses, not dimensional or histological analysis), the DUOX2$^{A625T/A625T}$ mice had smaller kidneys ($F_{2,56}=4.44$, $P=0.016$; Fig 2.B), smaller brains ($F_{2,56}=4.70$, $P=0.013$; Fig 2.C), and spleens ($F_{2,56}=21.08$, $P<0.001$; Table S8) compared to DUOX2$^{+/A625T}$ and DUOX2$^{+/+}$, but adjusted liver size was not significantly different ($F_{2,56}=0.43$, $P=0.652$; Fig 2.A, Table S6, S8). Moreover, the DUOX2$^{A625T/A625T}$ mice tended to have smaller lungs ($P=0.061$), and had smaller tails ($P<0.001$), skin ($P=0.029$), less mesenteric white adipose tissue ($P=0.001$), but more subcutaneous white adipose tissue ($P=0.001$) and bigger stomachs ($P=0.030$; Table S6, S8). Masses of the other organs did not differ between the three genotypes (Table S6, S8).

The level of serum T4 (thyroxine) was 36% lower in DUOX2$^{A625T/A625T}$ mice than in DUOX2$^{+/A625T}$ and DUOX2$^{+/+}$ ones (22.4 (DUOX2$^{A625T/A625T}$) vs 34.3 (DUOX2$^{+/A625T}$) and 35.1 (DUOX2$^{+/+}$) nmol/L SE±2.2, $F_{2,56}=9.98$, $P<0.001$; Fig 2.E, Table S5, S7). However, serum T3 (triiodothyronine) did not differ between genotypes ($F_{2,56}=0.28$, $P=0.757$; Fig 2.D). Analyses of faecal microbiota revealed presence of 15 genera (Fig 3.A, Table S9). The analyses of scores from the PCA showed significant differences between genotypes ($PC_2$: $F_{2,58}=13.48$, $P=0.001$; Table S10, S11), indicating the DUOX2$^{A625T/A625T}$ mice had lower abundance of commensal *Bifidobacterium* (correlation coefficient: 0.903) and slightly lower of *Akkermansia* (0.123), but slightly higher of *Desulfovibrio* (-0.268; Fig 3.B, Table S9).

To show if the effects of the DUOX2 mutation were a direct result of the mutation, or rather consequential of changes in the gut microbiota, four week-old DUOX2$^{+/+}$ mice were exposed to an antibiotic mix in drinking water [23], and then gavaged with either DUOX2$^{+/+}$,
DUOX2^{A625T/A625T}, or giant panda feces. The mice did not adopt the panda microbiome, but the microbiome from DUOX2^{+/+} and DUOX2^{A625T/A625T} treated mice was not distinct from the donors (Fig S2.A-B). In the DUOX2^{A625T/A625T} treated mice commensal *Akkermansia* were lower and Lachnospiraceae slightly higher (PC1: F_{2,45}=3.94, P=0.027; PC2: F_{2,45}=4.66, P=0.015; Fig S2.C-D, Table S14-S16). The treatment did not affect the body mass (22.8 (DUOX2^{+/+}) vs 22.7 (DUOX2^{A625T/A625T}) g SE±0.3, F_{1,72}=0.01, P=0.922), or the activity (2968 vs 2772 m SE±177, F_{1,72}=0.61, P=0.438), mass-adjusted DEE (44.68 vs 44.46 kJ/day SE±0.62, F_{1,72}=0.061, P=0.805), and REE (32.45 vs 31.82 kJ/day SE±0.56, F_{1,71}=0.65, P=0.422), however, adjusted food intake (71.70 vs 64.00 kJ/day SE±1.21, F_{1,71}=20.17, P<0.001) was lower in the mice with the DUOX2^{A625T/A625T} microbiota (Table S12-S13).

To investigate if treatment with T4 would reverse the effects caused by the DUOX2 mutation, DUOX2^{A625T/A625T} mice were exposed to 5 µg/mL of T4 in drinking water starting from 4 weeks-old [24]. However, using this dose, serum T3 and T4 were increased by about ten times above wild-type levels (Table S17), so we used a lower dose of 0.5 µg/mL T4, generating a more physiological effect. The T4 supplemented mice grew larger between 4 and 10 weeks-old compared with un-supplemented mice (mean body mass 15.3 vs 12.9 g SE±0.5, F_{1,70}=46.06, P<0.001; Fig 4.A, Table S18, S19). Moreover, they had higher activity by 53% (5400 vs 2557 m SE±268, F_{1,63}=56.07, P<0.001; Fig 4.D) and higher adjusted food intake by 14% (48.49 vs 41.51 kJ/day SE±1.70, F_{1,62}=7.72, P=0.007; Fig 4.C). Mass-adjusted DEE was also increased by 12% (44.17 vs 38.69 kJ/day SE±0.65, F_{1,62}=32.57, P<0.001; Fig 4.B), and REE by 14% (30.89 vs 26.53 kJ/day SE±0.69, F_{1,62}=18.59, P<0.001; Table S18, S19). The mass of their kidneys was higher (F_{1,7}=73.55, P<0.001; Fig 4.E), and they tended to have a higher percent of fat (F_{1,8}=5.12, P=0.053; Table S20, S21). Under supplementation serum thyroid hormones were both increased (T3: 1.8 vs 0.4 SE±0.3, F_{1,8}=12.81, P=0.007; Fig 4.F, and T4: 139.9 vs 15.7 SE±19.8 nmol/L, F_{1,8}=19.70, P=0.002; Fig 4.G, Table S20, S21).

**Discussion**

In ecological and evolutionary studies, genotypic and phenotypic data have traditionally been investigated independently, which hinders identification of specific genetic mutations the presence of which can be mechanistically linked to adaptive changes in the phenotype [3]. The phenotypic effects of single nucleotide mutations have been mainly inferred from predicted protein structures [2] or in the evolution of simple organisms [25,26], where laboratory manipulation of the genes can be used to show the phenotypic changes, but are challenging to investigate in large protected animals. Here, we inferred the links between a
single genotypic trait and multiple phenotypic characteristics relevant to a wild species of rare mammal with unique biology, using a genetically manipulated laboratory mouse model. The genetic mutation we considered was a single nucleotide variant of a single gene involved in endocrine function, which lead to disproportionally large changes to the metabolic and behavioral phenotype.

Among other hypothyroid mouse strains, a spontaneous mutation in DUOX2 has been reported to cause decreased body size and serum T4 levels [4]. Similar to this strain, the DUOX2A625T/A625T were also dwarfed, which in hypothyroid animals is a direct consequence of the effects of TSH on bone and soft tissues growth, particularly TSH effects on the hepatic expression of GHR and consequent IGF1 production [4,27]. Moreover, thyroid hormones strongly influence neonatal growth especially in species with low maturity at birth, which includes rodents [27], but also pandas [28]. In fact, new-born panda offspring are relatively the smallest among all placental mammals [28], which seems a life history strategy to decrease the high energetic demands of reproduction. Consistently, also our DUOX2A625T/A625T mice were much smaller than their littermates as pups, and they remained dwarfed as adults. Interestingly, in evolutionary time the giant panda seems to be dwarfed as well. The current giant panda, Ailuropoda melanoleuca, evolved in the Holocene and shrunk in size relative to the extinct panda, A. baconi which was considerably larger, and which was preceded by the small A. microta [6]. However, the role of the DUOX2 mutations in the evolution of panda body size remains unknown.

Our previous work indicated that giant pandas have lower than expected daily energy expenditure [7], and the daily and resting energy expenditures of the DUOX2A625T/A625T mice were also significantly decreased, even though the effect was reduced after correction for their small body mass. Therefore, it seems that the mutation in the DUOX2 gene may have contributed to this unusual metabolic trait of the pandas. However, the impact of the mutation on DEE in mice was much lower than that observed in pandas [7]. Similar to pandas, the T4 level of the DUOX2A625T/A625T mice was decreased, but pandas also have lowered T3 levels, which was not observed in the DUOX2A625T/A625T mice. The mutation in DUOX2 affects both T4 and T3 synthesis, but T3 is largely generated by conversion of T4 to T3 by DIO [29]. We therefore explored whether this difference between the mice and pandas, might be due to additional mutations in the panda DIO enzymes. We investigated amino acid sequences of DIO enzymes (Supplementary Materials), but although we found some variation among different species, no pattern between the species with low and high T4 to T3 ratios was found (Fig S3.A-C). Therefore, mutations in DIO genes are unlikely to influence T4 to T3
conversion in the giant panda. Hence, in addition to the DUOX2 mutation, the full metabolic phenotype of giant panda likely depends on yet unknown genetic mechanisms affecting T4 to T3 conversion, or TSH levels. Other possible mutations affecting panda metabolic phenotype may include genomic mitochondrial genes. Two such mutations have been identified in pandas, one in cytochrome c oxidase (COX), a rate limiting enzyme of electron transport chain [30], and another in ATP8, encoding and affecting post translational modification of ATP synthase [31]. The effects caused by DUOX2 mutation reported by Johnson et al. [4] appear larger than those observed in our mice, including 90% decrease in T4 levels, versus 36% decrease in DUOX2\textsuperscript{A625T/A625T} mice. However, after recalculating the units used in both studies, the T4 of wild-type mice from Johnson is twice as high as those in our study. Also, the methodology used to assess T4 levels was different, as well as the age of the mice. Moreover, the T4 level in pandas is about 50% of that expected for similar sized mammal, which is close to the level we found. However, it is also possible that the panda-unique mutation leads to translation of a truncated protein, which still retains some biological functions.

Physical activity data for captive and wild pandas indicate that they spend only about 30-50% of their time on physical activity, which is lower than in other bears [7]. The difference in activity, i.e. daily distance moved, between our DUOX2\textsuperscript{A625T/A625T} mice and other two genotypes was very strong, which indicates that the DUOX2 gene mutation may be largely responsible for the panda’s low physical activity. The mutation may also affect the range of panda distribution, as pandas tend to choose parts of the forest with gentle slopes, allowing more energetically efficient travel [32]. The mutation also affected absolute and body mass-corrected food intake. A reduction in physical activity, metabolic rate and food requirements would have been a major advantage to pandas when they started to consume the low calorie bamboo, because it would have significantly reduced the amount of time per day they would need to spend feeding. Hence, the DUOX2 mutation may have been a key innovation in the panda lineage enabling survival on their nutritionally poor bamboo diet.

In giant pandas some organs are smaller compared to similar-sized mammals [7], and similarly, kidneys and brains of DUOX2\textsuperscript{A625T/A625T} mice were smaller. It is not known whether the spleen and lungs are also smaller in pandas, like in the DUOX2\textsuperscript{A625T/A625T} mice. However, the vital organs account for about 60% of resting energy expenditure, but their weight is only about 5% of total body mass [33]. Therefore, low mass of kidneys, brain and lungs, probably contributed to the low metabolic rate of our DUOX2\textsuperscript{A625T/A625T} mice, despite the liver mass being unchanged. The DUOX2\textsuperscript{A625T/A625T} mice were overall leaner, and adipose tissue has low
energy expenditure [33]. The brain size has previously been shown to be associated with physical activity, as several structural parts of the brain are enlarged in physically active humans [34], and consistently, brains of low active DUOX2^{A625T/A625T} mice and giant pandas are smaller. Whereas, smaller kidneys of DUOX2^{A625T/A625T} mice seem to be associated with their increased urination. This may be due to retarded kidneys, or a direct effect of hypothyroidism leading to kidney injury [35]. Pandas also frequently urinate, which has been interpreted as social communication rather than a physiological dysfunction, but this may not be a correct interpretation. Despite feeding with bamboo, pandas have short carnivore digestive system, simple stomach and degenerated cecum [6], and although the DUOX2^{A625T/A625T} mice had enlarged stomach, the other sections of their gut remained unchanged.

The gut microbiota plays a critical role in health and physiology [36,37], and we observed that the DUOX2 mutation had a significant impact on the faecal microbiota. The faecal microbiota of DUOX2^{A625T/A625T} mice was depleted in *Bifidobacterium* and *Akkermensia*. *Bifidobacterium* can be detected in adult pandas [21], but is especially abundant in panda infants [37], mirroring many animals. *Bifidobacterium* affects health, immunity and metabolism, due to its role in milk digestion, and defense against pathogenic microbes [37]. In human adolescence, *Bifidobacterium* is gradually replaced by *Firmicutes/Bacteroidetes*. After panda cubs begin to eat bamboo the *Bifidobacterium* is replaced with other communities too. So, the DUOX2^{A625T/A625T} mice microbiota was more similar to bamboo-eating adult pandas, yet not enriched with any cellulose-digesting genus, such as *Bacillus* or *Pseudomonas* [21]. However, as pandas and mice consume very different types of food, any extrapolation from mice to pandas should be done with caution. The DUOX2 expression is regulated by two signaling pathways, induced by normal gut microbiota and is further upregulated in state of dysbiosis [20,38]. Expression of DUOX2 and several other antimicrobial genes was increased in gut epithelium of mice fed low-protein diet, previously subjected to microbiota transplant from mice fed high-fat diet [38]. Although *Bifidobacterium* or *Akkermensia* are not reported to induce DUOX2 expression as a defense mechanism of the gut [20,38], they have anti-inflammatory effects, and in DUOX2^{A625T/A625T} mice we observed mild increase of pathogenic *Desulfovibrio*. The microbiota of DUOX2^{+/+} mice gavaged with microbiota from DUOX2^{A625T/A625T} mice was poorer in *Akkermensia*, which like *Bifidobacterium* is common in healthy mice. This treatment led to decreased food intake. Thus, the DUOX2 mutation may induce changes in the gut microbiome contributing to the overall reduction in food intake, but did not contribute to the other phenotypes observed.
Treatment with T4 at doses previously used in the literature [24,39] caused hyperthyroidism, but supplementation with the lower dose elevated serum T4 to a level which can be considered euthyroid [39]. The treatment also led to increased T3, likely because of its tissue conversion from T4 by DIO. Therefore, the treated mice did not completely resemble the wild-type mice. However, even so, we confirmed that by manipulating the T4 level, i.e. reversing the effect of the DUOX2 mutation on serum T4, the main phenotypic effects on growth, metabolism, activity, food intake, and kidney mass were reversed and the magnitude of reversal almost exactly mirrored the deficit caused by the mutation. This confirms that physiological consequences of the single nucleotide panda-specific mutation come almost exclusively from its effects on endocrine system.

In summary, we demonstrated here that the decrease in T4 levels directly caused by the panda-unique mutation in the DUOX2 gene, led to several changes in the metabolic phenotype, similar to those observed in the giant panda, and these changes were reversible by T4 supplementation. Hence, the mutation of this single nucleotide in a key endocrine gene resulted in disproportional changes to various adaptive physiological and anatomical traits. The mutation appears to underlie the low energetic requirements of the panda which likely played an important role in the evolution of the panda unique metabolic phenotype, affecting its ability to exploit bamboo, its life history strategy, aspects of its behavior, and relation with its environment. Moreover we demonstrated how links between genetic factors and adaptive changes in phenotypic characteristics of wild protected species can be investigated using laboratory model animals modified using state of the art genome editing techniques.
Figure 1. (A) Body mass between 4 and 10 weeks-old [g/week], (B) body mass-adjusted daily energy expenditure [kJ/day] and (C) daily food intake [kJ/day] in respirometry chamber, and (D) accumulated daily distance moved [m/day] in mutant homozygous (DUOX2\textsuperscript{A625T/A625T}), heterozygous (DUOX2\textsuperscript{+/A625T}) and wild-type homozygous (DUOX2\textsuperscript{+/+}) mice carrying Giant panda-specific mutation in DUOX2 gene; LSM with SE.
Figure 2. (A) Body mass-adjusted liver, (B) kidneys and (C) brain mass, and (D) levels of T3-triiodothyronine and (E) T4-thyroxine [nmol/L] in mutant homozygous (DUOX2\textsuperscript{A625T/A625T}), heterozygous (DUOX2\textsuperscript{+/A625T}) and wild-type homozygous (DUOX2\textsuperscript{+/+}) mice carrying a Giant panda-specific mutation in DUOX2 gene.
Figure 3. Principal Component (PC) analysis of fecal microbiota in mutant homozygous (DUOX2<sup>A625T/A625T</sup>), heterozygous (DUOX2<sup>+/A625T</sup>) and wild-type homozygous (DUOX2<sup>++</sup>) mice carrying Giant panda-specific mutation in DUOX2 gene at the age of 10-11 weeks old: (A) PC1 and PC2 with microbiota genus; (B) PC1 and PC2 with genotype.
Figure 4. Reversal of the impact of the DUOX2 mutation by supplementation with T4. (A) Body mass between 4 and 10 weeks-old [g/week], (B) body mass-adjusted daily energy expenditure [kJ/day] and (C) daily food intake [kJ/day] in respirometry chamber, and (D) accumulated daily distance moved [m/day], (E) body mass-adjusted kidneys mass, and (F) levels of T3- triiodothyronine and (G) T4- thyroxine [nmol/L] in mutant homozygous (DUOX2_{A625T/A625T}) mice carrying Giant panda-specific mutation in DUOX2 gene, exposed (T4) and non-exposed (Co) to 0.5 ug/mL of T4 supplementation; LSM with SE.
Methods

All experimental procedures were approved by the ethical committee numbers HP2018007, HP2019021, HP2019030. To investigate the effects of a DUOX2 mutation, analogous to one discovered in giant panda [7], a mouse model carrying this mutation (DUOX2^{+/A625T} mice) has been genetically engineered using a conditional strategy CrispR-Cas9. The mouse was based on the C57BL/6 background, and contained heterozygous mutation to the DUOX2 gene, where 625\(^{th}\) Arginine was changed to a Termination codon (A625T). Next, the heterozygous population (DUOX2\(^{+/A625T}\)) was expanded to achieve also the mutant homozygous (DUOX2\(^{A625T/A625T}\)) and wild-type homozygous (DUOX2\(^{++}\)) genotypes.

All newborn animals were genotyped before the experimental procedures using PCR. A balanced number of animals from both sexes and three genotypes was randomly assigned to the experiment. A series of anatomical and physiological traits were investigated in 10 male and 10 female individuals for each of three genotypes: DUOX2\(^{++}\), DUOX2\(^{+/A625T}\), DUOX2\(^{A625T/A625T}\). To measure energy balance, the animals were maintained in individual cages and fed with baseline diet (D12450B, Research Diets, Inc.). After one week, the resting metabolic rate, spontaneous activity, and food consumption were measured using TSE PhenoMaster system (TSE PhenoMaster, Germany) for seven subsequent days. The body composition was measured before and after the metabolic rate measurement by magnetic resonance spectroscopy (EchoMRI-3N1-100\(^{TM}\)). Next, the energy assimilation, water consumption and urine production were measured in metabolic cages (Tecniplast, USA) for three days. Finally, the animals were killed by exposure to CO\(_2\) and dissected. The mass of various organs was measured, and the blood was collected for analyses of thyroid hormones T4 and T3 levels. The analysis was performed using RIA radioimmunoassay. The fecal samples were collected for analyses of the composition of microorganisms. The composition was investigated using DNA sequencing of 16S rRNA gene.

To investigate if the effect of the DUOX2 mutation was a direct result of the mutation, or an indirect effect mediated by changes in gut microbiota, a subpopulation of juvenile DUOX2\(^{+/+}\) mice were exposed to antibiotics mix in the drinking water [23]. The gut microbiota was then repopulated by gavage with either DUOX2\(^{+/+}\), DUOX2\(^{A625T/A625T}\), or giant panda feces. Next, the metabolic rate, activity and food intake was measured, and the fecal samples were analyzed.

To investigate if treatment with T4 would reverse the phenotypic effects caused by the DUOX2 mutation, a separate population of juvenile DUOX2\(^{A625T/A625T}\) mice was exposed to
T4 in drinking water. The animals were subjected to the same measurement protocols as the animals in the main part of the experiment. The experiment was initially performed using 5 µg/mL concentration of T4 [24,39], and was later repeated on a different set of individuals using 0.5 µg/mL concentration.

Statistical analyses were performed with IBM SPSS 23. One-way, or two-way ANOVA and ANCOVA were used, and results were presented as least square means (LSM) ±SE and P values at alpha 0.05. The fecal microbiota was analysed with PCA, followed by one-way ANOVA. Detailed description of the methods has been included in the Supplementary Materials.

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Author Contributions
J.R.S., F.W. and Y.N. contributed to study conception; J.R.S. and L.L. managed construction of the mouse model; J.R.S. and A.M.R. designed the study; A.M.R., L.L., Y.H. and M.L. established the mouse line; A.M.R., J.T., C.L. and C.N. collected the data; Q.W. analysed the DIO sequence; J.W. analysed the microbiome; A.M.R and J.R.S analysed the data and wrote the paper; all authors added and commented on the manuscript.

Competing interests
The authors declare no conflicts of interests.

Data Accessibility
The data are available from the corresponding author John R. Speakman upon request, and are deposited in a public repository Zenodo, accession number . . .