

RESEARCH ARTICLE

Shiver me titin! Elucidating titin's role in shivering thermogenesis

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ABSTRACT

Shivering frequency scales predictably with body mass and is 10 times higher in a mouse than a moose. The link between shivering frequency and body mass may lie in the tuning of muscle elastic properties. Titin functions as a muscle 'spring', so shivering frequency may be linked to titin's structure. The muscular dystrophy with myositis (*mdm*) mouse is characterized by a deletion in titin's N2A region. Mice that are homozygous for the *mdm* mutation have a lower body mass, stiffer gait and reduced lifespan compared with their wild-type and heterozygous siblings. We characterized thermoregulation in these mice by measuring metabolic rate and tremor frequency during shivering. Mutants were heterothermic at ambient temperatures of 20–37°C while wild-type and heterozygous mice were homeothermic. Metabolic rate increased at smaller temperature differentials (i.e. the difference between body and ambient temperatures) in mutants than in non-mutants. The difference between observed tremor frequencies and shivering frequencies predicted by body mass was significantly larger for mutant mice than for wild-type or heterozygous mice, even after accounting for differences in body temperature. Together, the heterothermy in mutants, the increase in metabolic rate at low temperature differentials and the decreased tremor frequency demonstrate the thermoregulatory challenges faced by mice with the *mdm* mutation. Oscillatory frequency is proportional to the square root of stiffness, and we observed that mutants had lower active muscle stiffness *in vitro*. The lower tremor frequencies in mutants are consistent with reduced active muscle stiffness and suggest that titin affects the tuning of shivering frequency.

KEY WORDS: Muscle, Stiffness, Thermoregulation, *mdm*, Mouse

INTRODUCTION

The natural resonating frequency of an oscillatory movement, such as tremor during shivering thermogenesis, depends on the properties of the material driving it. In the case of shivering, neural input (Stuart et al., 1966; Hohtola, 2004), animal size (Spaan and Klussmann, 1970; Günther et al., 1983; Kleinebeckel et al., 1994), and the mechanics and orientation of the body's materials (Stuart et al., 1966) could impact the frequency of oscillation. Muscle stiffness likely plays a role in determining the frequency of oscillatory motion in animals, such as tremor during shivering (Stuart et al., 1966). Muscle stiffness is determined, in part, by the molecular spring titin. We expect that changes in titin's structure could impact whole-animal movement by modulating muscle stiffness. In this study, we investigated the effect of a titin mutation on muscle stiffness and on tremor during shivering thermogenesis.

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Shivering thermogenesis is ideal for studying the effect of muscle stiffness at the whole-animal level because it is muscle driven and can be associated with oscillatory tremor (Stuart et al., 1966; Hohtola, 2004). The frequency (f) of simple harmonic motion can be modeled as:

$$f = \frac{1}{2\pi} \sqrt{\frac{k}{m}}, \quad (1)$$

where m is the mass moved by the spring and k is the spring constant. If we replace the spring constant with muscle stiffness, we would expect the frequency of oscillatory movements in animals to increase with increasing muscle stiffness and decrease with increasing body mass.

Much work on oscillatory movement has shown that body size is often correlated with frequency (Heglund and Taylor, 1988; Young et al., 1992; Lindstedt and Schaeffer, 2002 and references therein; Hurlbert et al., 2008; Sato et al., 2010; Dickerson et al., 2012). The frequency of muscle activation during shivering thermogenesis also seems to be correlated with body mass (Spaan and Klussmann, 1970). Relatively little work has related the frequency of oscillatory movements to muscle properties (Curtin and Woledge, 1993a,b) or compared stiffness with the frequency of movement (Young et al., 1992; Farley et al., 1993; Nassar et al., 2001). We can investigate the contribution of titin-based muscle stiffness to organism movement by comparing the frequency of tremor during shivering for animals with differences in titin structure and function.

The muscular dystrophy with myositis (*mdm*) mouse is characterized by a 779 bp deletion in the N2A region of the titin gene (Müller-Seitz et al., 1993; Garvey et al., 2002). This mutation is thought to contribute to changes in titin's structure (Lopez et al., 2008). The *mdm* mutants have a stiffer gait, smaller body mass and reduced lifespan (Garvey et al., 2002; Witt et al., 2004; Huebsch et al., 2005; Lopez et al., 2008). A whole-muscle study on the diaphragm reported substantial differences in the force production capabilities and elastic properties of *mdm* mutant muscle compared with wild-type muscle; of particular interest, mutant muscle has higher passive stiffness than wild-type muscle (Lopez et al., 2008).

We do not know the full extent to which titin may modulate muscle stiffness. Titin is a giant protein that is known to contribute to passive tension (Linke and Granzier, 1998; Lindstedt et al., 2002; Huebsch et al., 2005; Lopez et al., 2008; Leonard and Herzog, 2010; Herzog et al., 2012). Historically, titin was considered to have a static contribution to muscle stiffness as this protein is too compliant to substantially contribute to active muscle stiffness. However, Nishikawa et al. (2012) and Herzog (2014) have suggested that titin's contribution to muscle stiffness may increase upon muscle activation by binding to the thin filament. Leonard and Herzog (2010) and Powers et al. (2014) have shown that titin-based stiffness increases during muscle activation.

Our study aimed to investigate the effect of the *mdm* mutation on tremor during shivering thermogenesis. We tested two hypotheses:

List of symbols and abbreviations

AIC	Akaike information criterion
C	conductance
EMG	electromyography
E–O	expected–observed
F	force
f	frequency
FFT	fast Fourier transform
k	stiffness
m	mass
<i>mdm</i>	muscular dystrophy with myositis
n	number of observations
N	number of animals
N2A	region of titin between PEVK and tandem Ig domains
RMSE	root mean square error
SMR	summit metabolic rate
T	temperature
T_a	ambient temperature
T_b	body temperature
\dot{V}_{O_2}	metabolic rate
x	displacement
x_i	residual

(1) changes in titin's structure due to the *mdm* mutation will affect tremor frequency during shivering and (2) changes in the frequency of tremor during shivering will affect metabolic rate. Specifically, we expected changes in muscle stiffness in the *mdm* mutants to correlate with changes in tremor frequency during shivering.

RESULTS**Body mass**

At 30–50 days old, wild-type and heterozygous mice were significantly larger than *mdm* mutant mice ($N=6$ animals for each genotype, $P<0.0001$). Body mass did not vary significantly ($P=0.1682$) between wild-type (18.2 ± 1.0 g) and heterozygous mice (19.8 ± 0.2 g). However, both wild-type ($P<0.0001$) and heterozygous mice ($P<0.0001$) were about three times larger than age-matched mutant mice (6.3 ± 0.3 g).

Body temperature

Ambient temperature (T_a), genotype and their interaction had a significant effect on body temperature (T_b ; $P<0.0001$). Body temperature in wild-type ($P=0.149$) and heterozygous ($P=0.142$) mice did not vary with ambient temperature (Fig. 1), and body temperatures for these two genotypes were not significantly different ($P=0.985$), with a mean of $36.5\pm 0.2^\circ\text{C}$ for wild-type mice and $36.8\pm 0.2^\circ\text{C}$ for heterozygotes. In contrast, the body temperature of the mutant mice decreased at lower ambient temperature ($P<0.0001$; $T_b=20+0.48T_a$). The mutants had a relationship that was significantly different from that of wild-type ($P<0.0001$) and heterozygous mice ($P<0.0001$).

Metabolic rate

Because mutant body temperature varies with ambient temperature (Fig. 1), establishing a baseline metabolic rate was problematic. In studies of heterothermic mammals (e.g. Heldmaier and Ruf, 1992), comparisons are made using the relationship between metabolic rate and the temperature differential (i.e. the difference between body and ambient temperature), where slope is the conductance (Scholander et al., 1950). Fig. 2 shows the metabolic rate for the temperature differentials measured at 20–30°C for wild-type and heterozygous mice and 30–35°C for mutants (see Materials and

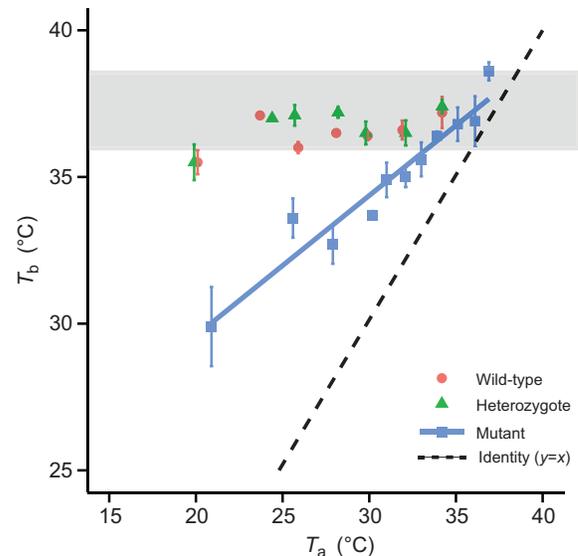


Fig. 1. The relationship between body temperature and ambient temperature. Resting body temperature (T_b) for wild-type ($N=8$), heterozygous ($N=7$) and mutant mice ($N=10$) as a function of ambient temperature (T_a). The dashed line serves as a reference line with a slope of one. The gray box shows the normal range of body temperatures for mice in typical housing conditions (i.e. ambient temperature of 23–24°C with bedding; Gordon, 2004). Points represent means for measurements from $n=2$ –6 observations. Error bars represent s.e.m.

methods for details). Within this temperature range, the metabolic rates of mutants are in the same range as the metabolic rates observed in wild-type and heterozygous mice at ambient temperatures 5–10°C lower (Fig. 2).

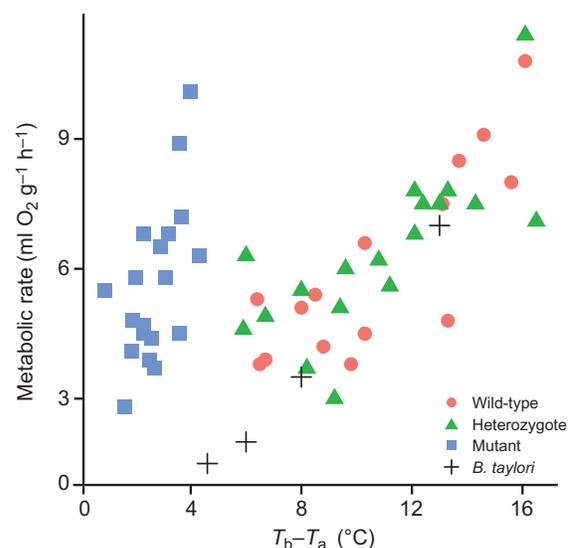


Fig. 2. Metabolic rate measurements for the *mdm* genotypes. Mass-specific metabolic rate versus the difference between body temperature and ambient temperature for the three *mdm* genotypes (wild-type: $N=8$ animals, $n=15$ total observations; heterozygote: $N=6$, $n=18$; mutant: $N=8$, $n=20$) and *B. taylori*, the pygmy mouse (Hudson, 1965). Temperatures used were within the range of temperatures over which metabolic rate increased with decreasing ambient temperature (i.e. metabolic rate does not decrease with decreasing temperature, nor were the measurements above the thermoneutral point). Metabolic rates shown here were measured at ambient temperatures between 20 and 30°C for wild-type mice, heterozygotes and *B. taylori*, and between 30 and 35°C for mutants.

The interaction between the temperature differential and genotype was not significant ($P=0.1866$), meaning that thermal conductance was not significantly different among genotypes, so this interaction was removed from the model. Both genotype ($P=0.0025$) and the temperature differential ($P<0.001$) had a significant effect on metabolic rate, with a conductance of $0.55 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ }^\circ\text{C}^{-1}$, which was significantly different from zero ($P<0.001$), and y -intercepts of $0.26 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for wild-type mice ($R^2=0.70$), $0.58 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for heterozygotes ($R^2=0.56$) and $4.3 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for mutants ($R^2=0.18$). The y -intercept for mutants was significantly different from zero ($P<0.001$) while y -intercepts for wild-type mice ($P=0.737$) and heterozygotes ($P=0.474$) were not. The relationship for mutants was significantly different from that of wild-type ($P<0.001$) and heterozygous mice ($P<0.001$) but the relationships for wild-type and heterozygous mice were not different ($P=0.499$).

Wild-type and heterozygous mice are three times larger than mutant mice, so they have reduced surface area to volume ratios and should have a lower conductance, which may explain why the mutants must generate metabolic heat at reduced temperature differentials. To account for the body size dependence of conductance, we compared the *mdm* mutants with *Baiomys taylori*, the pygmy mouse, which has an adult body mass of $6.4 \pm 1.3 \text{ g}$ (Hudson, 1965), similar to the body mass of the mutant mice ($6.3 \pm 0.3 \text{ g}$). The relationship for *B. taylori* based on data from Hudson (1965) is described by the equation $\dot{V}_{\text{O}_2} = -1.76 + 0.67(T_b - T_a)$. The relationship for *B. taylori* was not significantly different from that for wild-type ($P=0.163$) or heterozygous mice ($P=0.077$) but was different from that of the mutants ($P<0.001$).

The highest metabolic rate measured was $10.4 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for mutants, $11.1 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for wild-type mice and $11.7 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for heterozygotes (Fig. 2). The metabolic rate of mutants dropped at greater temperature differentials (K.R.T.-B., J.M., C.P., S.L. and K.C.N., personal observation), so it is likely that this represents the summit metabolic rate (i.e. the highest metabolic rate that can be elicited by cold stress) for mutants, at least under this set of experimental conditions. Metabolic rates of wild-type and heterozygous mice increased with greater temperature differentials within the range of temperatures used in this study, so there is no indication of whether the coldest temperatures used (20°C) were sufficient to achieve summit metabolic rate in these mice. Bozinovic and Rosenmann (1989) reported the following relationship for rodents:

$$\text{SMR} = 28.3 m^{-0.338}, \quad (2)$$

where SMR is summit metabolic rate ($\text{ml O}_2 \text{ consumed h}^{-1} \text{ g}^{-1}$) and m is body mass (g). Using this equation, the predicted summit metabolic rate was $14.9 \pm 0.2 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for mutants, $10.5 \pm 0.2 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for wild-type mice and $10.4 \pm 0.2 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$ for heterozygotes, where the error represents variation in body mass.

Shivering frequency

Mutants exhibited lower tremor frequencies during shivering but also had lower body temperatures than their non-mutant siblings. The mean tremor frequency during shivering was $35 \pm 2 \text{ Hz}$ for wild-type mice, $31 \pm 1 \text{ Hz}$ for heterozygotes and $19 \pm 2 \text{ Hz}$ for mutants (Fig. 3A). Genotype had a significant effect ($P<0.001$). Although shivering frequency was not significantly different between wild-type and heterozygous mice ($P>0.05$), frequencies were significantly higher for wild-type and heterozygous mice than for mutants ($P<0.05$). As was found during the measurement of metabolic rate, mutants were

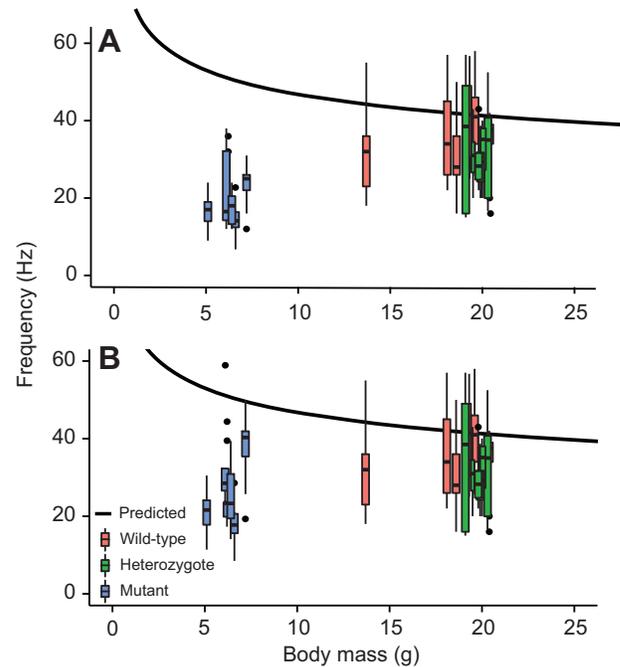


Fig. 3. Observed tremor frequency versus body mass. Tremor frequency during shivering for wild-type ($N=6$), heterozygous ($N=6$) and mutant ($N=6$) mice. Box plots represent individuals. The solid line gives the predicted frequency for a given body mass (Eqn 5; Spaan and Klussmann, 1970). (A) The observed values of tremor frequency for all three genotypes. (B) Temperature-corrected tremor frequencies for mutants. Mutant body temperatures were lower than 37°C , so mutant tremor frequencies were corrected using a Q_{10} of 2.5 and Eqn 6 (see Materials and methods).

heterothermic and body temperature varied among the three genotypes during shivering ($P=0.003$). At $12\text{--}20^\circ\text{C}$, the average body temperature was $37.3 \pm 0.6^\circ\text{C}$ with a range of $36.0\text{--}38.9^\circ\text{C}$ for wild-type mice and $37.3 \pm 0.3^\circ\text{C}$ with a range of $36.3\text{--}38.5^\circ\text{C}$ for heterozygous mice. Mutant mice had an average body temperature of $33.0 \pm 0.8^\circ\text{C}$ with a range of $28.9\text{--}35.6^\circ\text{C}$ at ambient temperatures of $20\text{--}27^\circ\text{C}$. While wild-type and heterozygous mice did not have significantly different body temperatures ($P=0.9858$), the average body temperatures of mutants were significantly lower than those of wild-type ($P=0.0138$) and heterozygous ($P=0.0138$) mice. Mutant tremor frequencies were corrected for body temperature using the Q_{10} for the rate of muscle force production in mice, which Stein et al. (1982) reported as 2.5. Using that value, the body temperature-corrected mean tremor frequency for mutants was $28 \pm 4 \text{ Hz}$ (Fig. 3B). After correcting for body temperature, there was no effect of genotype on tremor frequency ($P=0.2249$).

Mutants were much smaller than wild-type and heterozygous mice, and we know that body mass is correlated with shivering frequency. Using the relationship reported by Spaan and Klussmann (1970) between body mass and shivering frequency (based on electromyography, EMG), we calculated expected frequencies. We predicted that, on average, mutants should have tremor frequencies of about 51 Hz while wild-type and heterozygous mice should have shivering frequencies of 41–44 Hz. Our observation that mutants shivered at frequencies equal to or lower than the frequencies observed for their siblings is surprising. On average, the differences between expected and observed (E–O) frequencies for all three genotypes were positive, indicating that the observed values fell below the expected values (Fig. 4). Both the individual ($P<0.001$) and genotype ($P<0.0001$) effects on E–O values were significant.

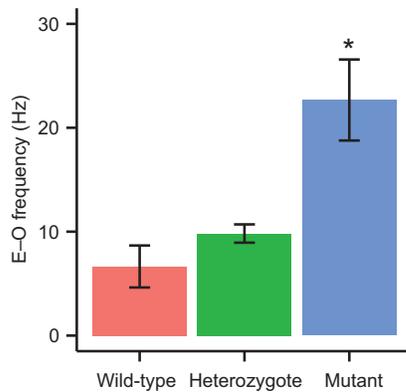


Fig. 4. Comparing shivering frequencies across genotypes. Mean temperature-corrected tremor frequency was found for each genotype ($N=6$ for each genotype). Expected shivering frequencies were found using Eqn 5 (shown as a solid line in Fig. 3). The average tremor frequency for mutants was calculated using the frequencies corrected for body temperature (Fig. 3B). The expected–observed (E–O) values for mutants were significantly greater than in wild-type and heterozygous mice ($*P<0.05$), but E–O values for wild-type and heterozygous mice did not differ significantly ($P>0.05$). Error bars represent s.e.m.

The E–O values for mutants were significantly greater than the E–O values for wild-type and heterozygous mice ($P<0.05$), but E–O values for wild-type and heterozygous mice did not differ significantly ($P>0.05$).

Predictions based on body mass alone were higher than the observed values for wild-type [root mean square error (RMSE)=19% and Akaike information criterion (AIC)=44; predicted values: 41–44 Hz, observed values: 35.4 ± 1.8 Hz] and heterozygous mice (RMSE=24% and AIC=47; predicted: 41–44 Hz, observed: 31.0 ± 0.8 Hz). However, the differences between predicted and observed frequencies were much higher for mutants (RMSE=63% and AIC=61; predicted: 50–51 Hz, observed: 19.2 ± 1.7 Hz), even when accounting for differences in body temperature (RMSE=47% and AIC=57; corrected: 28.3 ± 3.7 Hz).

Stiffness was measured in fully activated muscle *in vitro* (Fig. 5). The average stiffness was 174 ± 60 N m⁻¹ for mutant muscle and 982 ± 97 N m⁻¹ for wild-type muscle, and these values were significantly different ($U=12$, $P=0.02857$). Using these stiffness values and average body mass, we calculated the predicted frequency using Eqn 1. We predicted tremor frequencies very similar to the observed tremor frequencies for both wild-type

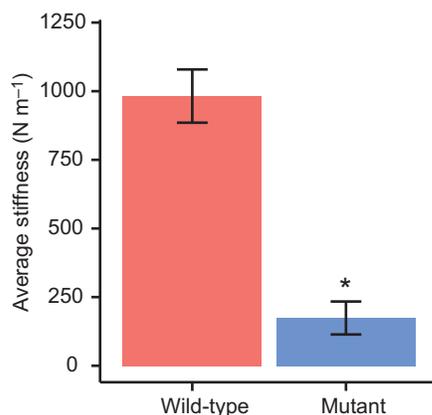


Fig. 5. Active stiffness measurement. Mean wild-type ($n=4$) and mutant ($n=3$) stiffness under maximum stimulation. Error bars represent s.e.m.

(predicted: 35–36 Hz; RMSE=11% and AIC=38) and mutant mice (predicted: 24–25 Hz; RMSE=30% and AIC=48). Adding stiffness resulted in substantially improved (i.e. lower) RMSE and AIC values for both wild-type and mutant mice, which indicates that muscle stiffness is a useful parameter in predicting tremor frequency.

DISCUSSION

We have documented altered thermoregulation and shivering in *mdm* mutants compared with wild-type and heterozygous mice. In particular, the mutants are heterothermic and increase their metabolic rates at lower temperature differentials than age-matched siblings and the size-matched pygmy mouse. The mutant mice shiver with a lower tremor frequency than expected and have a lower active stiffness. A model of body mass alone predicts a higher tremor frequency in mutants than in their larger siblings, while a model that accounts for both lower body mass and lower active stiffness in mutants predicts a lower tremor frequency. Thus, one consequence of the *mdm* mutation is a decrease in tremor frequency, which is likely linked to decreased active muscle stiffness.

Body temperature and metabolic rate

We observed heterothermy in mutant mice but not in wild-type or heterozygous mice. The change in body temperature in mutants could reflect a torpor-based thermoregulation strategy, which is common among small mammals (Hudson, 1965; Heldmaier and Ruf, 1992). This seems an unlikely explanation because prior to a drop in body temperature, these animals increased their metabolic rates in response to decreased ambient temperatures, an opposite response to entering torpor. The plausible alternative hypothesis is that body temperature decreases because the rate of heat loss exceeds the rate of heat production, which could be the result of a reduced thermogenic capacity, a greater rate of heat loss, or both.

Heat for thermoregulation is produced through muscle-driven shivering and brown adipose-driven non-shivering thermogenesis. We may expect a reduction in heat production through shivering thermogenesis in the mutants because the *mdm* mutation affects muscle. However, we cannot rule out the possibility of differences in non-shivering thermogenesis in brown adipose tissue (Lončar, 1991; Klaus et al., 1998; Hu et al., 2010). While it is difficult to distinguish between the importance of muscle-driven shivering thermogenesis, brown adipose-based non-shivering thermogenesis and heat produced through other metabolic pathways, it is clear that the mice are heterothermic because heat production is quickly surpassed by heat loss.

The metabolic rate of *mdm* mutants increased at much lower temperature differentials ($T_b - T_a$) than that of age-matched siblings and sized-matched *B. taylori*. Thus, the rate of heat loss exceeded the rate of heat production more readily in mutants, resulting in a higher critical temperature (i.e. the lowest ambient temperature at which the metabolic cost of thermoregulation is minimal). According to the Scholander equation (Scholander et al., 1950), mutants must have higher conductance than their non-mutant siblings and *B. taylori* (see Materials and methods for more detail), although we did not find significant differences between the slopes (i.e. conductance) of the relationships between the temperature differential and metabolic rate. The small size and resulting high surface area to volume ratio is expected to cause a higher conductance and narrower thermoneutral zone in mutants; however, size cannot account for differences between *mdm* mutants and *B. taylori*. The lower body mass and emaciated appearance of these animals suggest that conductance could be

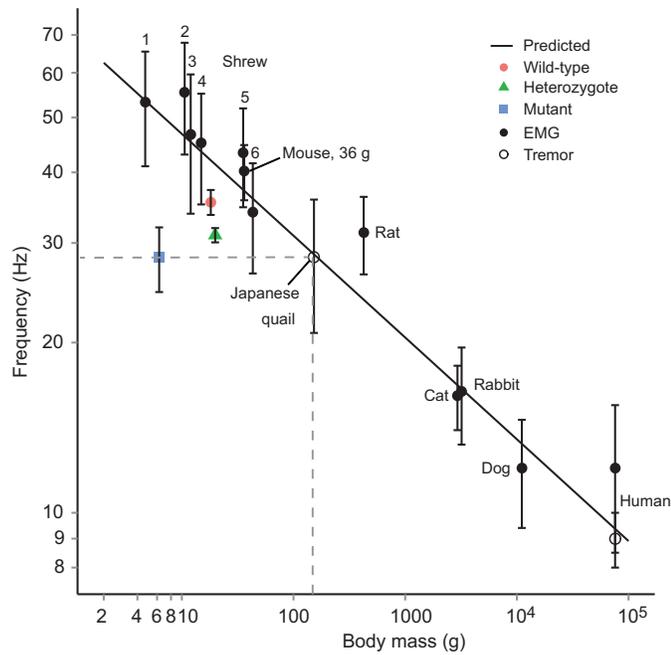


Fig. 6. Allometric relationship between body mass and shivering frequency. The line is the relationship reported by Spaan and Klussmann (1970) (Eqn 5). The colored symbols represent the data from the current study, and error bars represent s.e.m. Black circles are data from other studies. Filled circles represent shivering frequencies measured using electromyography. The open circles represent the frequency of tremor during shivering. Error bars represent s.d. or range, whichever was reported. The dashed lines show that the mean tremor frequency for the *mdm* mutants (28 ± 4 Hz) is a frequency we would expect to observe for an animal of >100 g. This is surprising considering that the mutants are only 6–8 g. The numbers indicate different shrew species: 1, 3 and 4 are in the Soricinae subfamily (*Sorex minutes*, *Sorex coronet* and *Neomys fodiens*, respectively) and 2, 5 and 6 are in the Crocidurinae subfamily (*Crocidura russula*, *Suncus murinus* and *Crocidura olivieri*, respectively). Sources for individual data points: shrews, Kleinebeckel et al. (1994); mouse, rat and rabbit, Günther et al. (1983); Japanese quail, Hohtola and Stevens (1986); human (both EMG and tremor frequencies), Schneider and Brooke (1979) and references therein; cat and dog, Spaan and Klussmann (1970).

higher due to diminished levels of insulating white adipose tissue. The stress of heterothermy and the metabolic cost associated with high conductance may play a role in limiting the growth of these animals and decreasing their lifespan.

Our highest measured metabolic rate ($10.4 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$) fell short of the predicted value ($14.9 \text{ ml O}_2 \text{ h}^{-1} \text{ g}^{-1}$) for mutants, but observed values were similar to, if not higher than, predicted values for wild-type and heterozygous mice. Mutants are subject to the Q_{10} effects of dropping body temperatures (Fig. 1), which coincide with increases in the temperature differential. As body temperature falls, so must the summit metabolic rate. Therefore, we expect that summit metabolic rate in mutants is limited by body temperature and that the calculated mutant conductance may, consequently, be an underestimate.

Reduced heat production or increased conductance could cause heterothermy in the *mdm* mutants. A reduction in shivering thermogenesis due to altered muscle function is a simple explanation. If thermogenesis is reduced as a result of low shivering frequencies, metabolic rate may be limited by Q_{10} effects, which could in turn affect other types of thermogenesis. However, the mutants increased metabolic rate at lower temperature differentials than their siblings, indicating that they are also losing heat faster. Thus, the heterothermy in mutants is likely due to high

conductance but could also be linked to lower heat production. More studies are needed to distinguish the contributions of lower thermogenesis and higher conductance to mutant heterothermy.

Tremor frequency

Body mass, body temperature and muscle stiffness should all be considered when measuring tremor frequency. We used Eqn 6 (see Materials and methods) to normalize for body temperature. We accounted for differences in body mass and muscle stiffness using two models. (1) First, we used a model for body mass alone (Eqn 5; see Materials and methods) and found that the difference between expected and observed tremor frequencies was significantly larger for mutants than for wild-type and heterozygous mice. (2) Second, we measured active muscle stiffness and found that mutant muscle had lower active stiffness than wild-type muscle. A model that included both body mass and muscle stiffness (Eqn 1; see Introduction) produced better predictions for tremor frequency for both wild-type and mutant mice, with an especially large improvement in the predictions for mutants.

Mass-only model

Spaan and Klussmann (1970) produced an allometric relationship between body mass and shivering frequency using animals with a wide range of body masses, from mice with an average body mass of 40 g to dogs with an average body mass of over 11 kg. This relationship has since been shown to hold for larger animals (i.e. humans; Schneider and Brooke, 1979) as well as smaller animals (i.e. shrews; Kleinebeckel et al., 1994), the smallest of which have a body mass of 4–5 g, smaller than the *mdm* mutants. Fig. 6 includes our data, studies that reported shivering frequencies measured using EMG (i.e. the frequency of changes in electrical activity within the muscle; Spaan and Klussmann, 1970; Schneider and Brooke, 1979; Günther et al., 1983; Kleinebeckel et al., 1994), and studies that reported the frequency of tremor (i.e. the frequency of movement of the body or a part of the body; Schneider and Brooke, 1979; Hohtola and Stevens, 1986). All three genotypes had lower than expected shivering frequencies, likely due to differences in EMG and tremor frequencies. However, the difference between expected and observed values for mutants was significantly larger than for wild-type and heterozygous mice as we would expect the smaller mutants to have a higher tremor frequency rather than a tremor frequency similar to or lower than that for the larger wild-type and heterozygous mice. In fact, the 6.3 g mutants shivered at a frequency that we would expect for an animal of more than 100 g. Thus, this model based on body mass provided a poor prediction of shivering frequency for mutants.

Although we are making an intraspecific comparison, we cannot ignore the effect that body mass is known to have on shivering frequency in mammals (Spaan and Klussmann, 1970; Günther et al., 1983; Kleinebeckel et al., 1994), particularly because the *mdm* mutants are far outside the normal range of body mass for adult laboratory mice. Spaan and Klussmann (1970) reported that similarity in body mass, not relatedness of species, resulted in similar shivering frequencies. Furthermore, Kleinebeckel et al. (1994) measured shivering frequency for several species in the Crocidurinae and Soricinae shrew subfamilies. They found there was substantial variation in shivering frequency within the subfamily Crocidurinae, with smaller species exhibiting shivering frequencies more similar to small soricines than to other crocidurine species. Our use of the interspecific allometric relationship is appropriate in this case because (1) all of the animals we studied fall within the range of body masses to which this relationship applies, (2) mice

with the *mdm* genotype have significantly different body masses, and (3) body mass is a stronger predictor of shivering frequency than phylogeny.

The EMG frequency reflects muscle stimulation during shivering, but there is likely damping from viscoelastic structures and the mass of the body (Stuart et al., 1966). Thus, while they are related, we expect small differences between the EMG frequency and tremor frequency. In fact, our data support damping because the observed tremor frequency was lower than the EMG-based prediction for all three genotypes. However, the difference between expected and observed frequencies for mutant mice was significantly larger than for the other *mdm* genotypes, suggesting that it was not damping alone that caused the reduced tremor frequency we observed in the mutants. Furthermore, we were able to model shivering as a simple harmonic oscillation and make very good predictions of shivering frequency based on body mass and active muscle stiffness.

Mass and stiffness model

We predicted tremor frequencies by using Eqn 1 and *in vitro* measurements of active muscle stiffness. Our measurements show that mutants have a lower active muscle stiffness than wild-type mice. By using a model that included stiffness, not just body mass, we were able to make better predictions for shivering frequency for both wild-type and mutant mice. This suggests that muscle stiffness may play an important role in shivering thermogenesis. It may also indicate that the larger difference between expected and observed shivering frequencies for mutants could be accounted for by differences in active muscle stiffness.

The lower tremor frequency observed in *mdm* mutants could also be caused by slower contraction rates and/or lower passive stiffness. Although Lopez et al. (2008) found a higher slow:fast ratio for the myosin heavy chain isoform in mutant muscle than in non-mutant control muscles, these authors reported that mutant muscle actually had a shorter time to peak force and a shorter half-relaxation time compared with non-mutant controls. Therefore, if tremor frequency were dependent on muscle contraction kinetics, we would expect that mutants would shiver at or above expected tremor frequencies, not below, as we observed.

Passive stiffness may also affect shivering frequency. Passive stiffness and passive tension are higher in mutant muscle than in wild-type muscle for whole-muscle experiments (Lopez et al., 2008), although Witt et al. (2004) did not observe differences in passive tension in single muscle fibers. Higher passive stiffness could result from the presence of more collagen and/or from a shorter titin spring (Lopez et al., 2008). An increase in muscle stiffness should result in an increase in tremor frequency for *mdm* mutants, not a decrease in tremor frequency, as we observed.

Thus, titin may be implicated as simultaneously contributing to reduced active stiffness and increased passive stiffness in *mdm* mutants. Leonard and Herzog (2010) and Powers et al. (2014) observed that titin-based muscle stiffness increases upon activation. It is plausible that the mechanism by which titin increases active muscle stiffness is affected by the *mdm* mutation (Nishikawa et al., 2012; Herzog, 2014). Our observations of lower tremor frequency and reduced active stiffness in mutant muscle are consistent with the hypothesis that titin is an important modulator of active muscle stiffness.

Conclusions

The combination of the higher metabolic cost of thermoregulation and the inability of mutants to remain homeothermic suggests that the *mdm* mutation may be an ideal model to study the limitations of

thermoregulation. Differences in shivering frequency cannot be explained by changes in contraction kinetics or by changes in passive muscle stiffness. Our results demonstrate that mutants shiver at lower frequencies than predicted, which may be best explained by the lower active muscle stiffness that we observed *in vitro*. Titin may provide the tuning of shivering frequency through its role in setting active muscle stiffness.

MATERIALS AND METHODS

Mice

Breeding pairs of B6C3Fe *a/a-mdm* mice (*Mus musculus*, Linnaeus) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and used to establish a colony. The *mdm* mutation is recessive, so heterozygous mice were bred to obtain pups that had homozygous wild-type, heterozygous and homozygous mutant genotypes. Heterozygous and wild-type mice were indistinguishable, so ear punches and tail snips were used for genotyping following the methods of Lopez et al. (2008). The mice were fed *ad libitum* and housed at 23–24°C with a 14 h:10 h light:dark cycle. Mutants were housed in cages that rested partially on a heating pad to allow these animals to select a warmer environment. All animal procedures were approved by the Institutional Animal Care and Use Committee at Northern Arizona University.

Wild-type pups reach a body mass comparable to adult mutant body mass (i.e. ~6 g) within 2 weeks of birth, which is near the time when shivering thermogenesis is observed but before these animals are weaned and capable of independently thermoregulating for long periods (Chew and Spencer, 1967). Thus, size-matching the animals was not appropriate as differences in metabolic rate and shivering frequency between neonatal wild-type mice and adult mutant mice could reflect developmental rather than genotypic differences. We chose to instead measure metabolic rate and shivering frequency in age-matched mice.

Because of the short lifespan of mutants, their general fragility and the need to use these animals in terminal experiments within a certain age range, we used different animals for each set of experiments and could not collect all data from a given individual. The genotype of wild-type and heterozygous mice was often not known until after the animal was killed, so controlling these sample sizes was difficult. Large changes in body temperature caused concern for animal welfare, so sample sizes for mutants at very low ambient temperatures were limited. As a result, the sample size varies from experiment to experiment and from data point to data point. Details about sample sizes are given in supplementary material Table S1.

Temperature measurements

Body and ambient temperatures were measured using small T-type thermocouples (copper-constantan, Omega Engineering, Inc., Stamford, CT, USA). Mice were anesthetized using isoflurane USP (MWI Veterinary Supply, Boise, ID, USA), which was administered in vapor form using a Forane vaporizer (Ohio Medical Corporation, Gurnee, IL, USA). During anesthesia, the animal was placed on a heating pad and provided with a continuous flow of anesthetic (1–2% isoflurane:oxygen mixture for wild-type and heterozygous mice; 0.5–1% for mutants) through a nose cone. While the mouse was under anesthesia, a thermocouple was inserted into the rectum (Good, 2005) and secured by attaching the lead to the tail with cloth medical tape. The thermocouple was inserted 2 cm for wild-type and heterozygous mice (Good, 2005) but only 1 cm (Dirks et al., 2002) for the much smaller mutant mice; these depths were reported by others to provide accurate body temperature measurements while avoiding injury. A similar thermocouple was placed in the animal's chamber to measure ambient temperature. Body and ambient temperature were monitored using the HH127 TC Datalogger (Omega Engineering, Inc.) and were sampled at a rate of 1 sample min⁻¹. Body temperatures displayed in Fig. 1 were taken after the ambient temperature was ramped from room temperature to the test temperature over 20 min and held constant for at least 5 min.

Metabolic rate measurements

Metabolic rates were measured over a range of temperatures (20–37°C) for mice that were 29–60 days old (wild-type mice: *N*=8; heterozygotes: *N*=7; mutants: *N*=10) with 2–6 animals measured at each temperature. Each

animal was anesthetized and a thermocouple was inserted rectally, as described above. All animals recovered for a minimum of 20 min before measurements were taken. Animals were placed in a small, air-tight chamber (4.7×10×7 cm). A pump (Model R2 Flow Control; Applied Electronics, Sunnyvale, CA, USA) applied positive pressure to ambient air to push it through the chamber at a minimum of 300 ml min⁻¹. The air passed through a copper coil prior to entering the chamber, and both the copper coil and the chamber were placed in a water bath in order to manipulate the chamber temperature. Air was scrubbed of water using a Drierite column both before entering the chamber and after leaving the chamber before oxygen measurements. The animal was placed in the chamber and allowed to acclimate for a minimum of 20 min before any measurements were made. A Matheson Mass Flow Readout (Model 8102-1452-FM, Tokyo, Japan) recorded flow rate, and an FC-10 Oxygen Analyzer (Sable Systems, Las Vegas, NV, USA) measured oxygen levels. A NI USB-6009 data acquisition device relayed these data to a custom-written LabView program (National Instruments, Austin, TX, USA; see supplementary material Fig. S1 for an example). Data were collected at 4 Hz. Metabolic rate was the average oxygen consumption over a minimum of 60 s that was measured when the animal was still and oxygen consumption showed little variability. Oxygen uptake was measured in this open flow system using the equation from Lighton (2008):

$$\dot{V}_{O_2} = \frac{\dot{V}_I \times (F_{I_{O_2}} - F_{E_{O_2}})}{1 - (F_{I_{O_2}} \times (1 - RQ))}, \quad (3)$$

where \dot{V}_I is the rate of air flow (ml min⁻¹), $F_{I_{O_2}}$ is the volume fractional concentration of oxygen entering the chamber (or ambient oxygen level), $F_{E_{O_2}}$ is the volume fractional concentration of air exiting the chamber and RQ is the respiratory quotient, which was assumed to be 0.85. Note that a 10% error in RQ would result in a 1.8% error in the measurement. Measurements were taken after the animal had been in the chamber for at least 20 min which is a full 18 ‘washout’ cycles, as the total volume of the system was calculated to be 329 ml³. Mass-specific metabolic rate was calculated in units of ml O₂ h⁻¹ g⁻¹.

Here, we define thermal conductance as the overall heat transfer coefficient, which incorporates heat lost by radiation and convection as well as conduction. In the following equation where \dot{V}_{O_2} is metabolic rate in ml O₂ g⁻¹, T_b is body temperature in °C and T_a is ambient temperature in °C:

$$\dot{V}_{O_2} = C(T_b - T_a), \quad (4)$$

conductance (C) has units of ml O₂ g⁻¹ °C⁻¹ (Scholander et al., 1950). This equation, first used by Scholander et al. (1950), has been referred to as ‘Newton’s law of cooling’. In order to determine the conductance (i.e. rate of heat loss), we found the relationship between metabolic rate and the temperature differential between body and ambient temperature. We did not include measurements above the thermoneutral zone. We used the temperature range of 20–30°C for wild-type and heterozygous mice because 30°C can be considered the point of thermoneutrality (Hussein, 1991) or the center of the thermoneutral zone (Gordon, 2004). Mutant mice did not maintain a constant body temperature (Fig. 1), so determining their thermoneutral zone was difficult. Metabolic rate seemed to reach a minimum at 34–35°C, but when ambient temperature dropped below 30°C, the metabolic rate of the mutant mice tended to drop with decreasing ambient temperature (K.R.T.-B., J.M., C.P., S.L. and K.C.N., personal observation). Therefore, temperatures ranging from 30 to 35°C were used for the mutants.

Shivering frequency

Six age-matched (30–50 day old) mice of each genotype were used for these experiments. In order to affix an accelerometer, the animals were anesthetized, as described above, and a chemical depilator (Nair, Church & Dwight Co., Princeton, NJ, USA) was used to remove a patch of fur on the dorsal side, posterior to the head. Animals were permitted to recover for 2–24 h prior to any other manipulation.

An accelerometer (model ADXL213, Analog Devices, Norwood, MA, USA) was used to determine tremor frequency during shivering with a sampling frequency of 1000 Hz. A NI USB-6009 data acquisition device (National Instruments) provided a 5 V power source for the accelerometer and relayed the signal to a custom-written LabView (version 10.0, National Instruments) data acquisition program. Fine wires (0.13 mm copper wire, California Fine Wire, Grove Beach, CA, USA) provided lightweight leads, and thicker wire (1.3 mm, tin–copper wire, Consolidated Electronic Wire & Cable, Franklin Park, IL, USA) served as a point of attachment to the accelerometer. The accelerometer was wrapped in electrical tape to secure wire attachments. The total mass of the accelerometer, including wires and the electrical tape, was 0.45 g, which is <8% of the body mass of the smallest animals used.

Mice were weighed just before the shivering trial. The accelerometer was glued to a small strip of the adhesive portion of a sterile bandage. Animals were anesthetized, as described above, and the bandage and accelerometer were applied to the region where fur had been removed. The accelerometer was oriented to measure acceleration along the long axis of the animal. The animals were permitted to recover for at least 20 min prior to data collection.

Shivering trials took place in a hibernation chamber (Hotpack Corp., Philadelphia, PA, USA), which was capable of both heating and cooling. Animals were placed in a small glass cage inside the chamber. The thermoneutral zone for wild-type mice is 27–32°C (Gordon, 2004). Tremor measurements were made when the animal was below its thermoneutral zone and when shivering was visually confirmed. The chamber temperature was maintained in the range 12–20°C for wild-type and heterozygous mice and 19–27°C for mutants in order to elicit shivering. The temperature range differed between genotypes because mutants tended to shiver at higher temperatures than non-mutant mice and because mutants were unable to maintain body temperature at lower chamber temperatures. We computed a linear model for shivering frequency with genotype and ambient temperature as fixed effects in order to determine the effect of the wide range of ambient temperatures used to elicit shivering. Ambient temperature did not have a significant effect on shivering frequency for the range of temperatures used in this study ($P=0.682$), so ambient temperature was not considered in further analysis of shivering frequency.

Body temperature was the average of the rectal temperatures recorded during tremor events. For each animal, 6–32 independent tremor events were analyzed. The accelerometer data for each tremor event were filtered using a bandpass filter (5–70 Hz). The lower limit (5 Hz) eliminated low frequency movement artifacts and the upper limit (70 Hz) eliminated high frequency noise above the highest expected tremor frequencies (about 50 Hz). The filtered data then underwent a fast Fourier transform (FFT) using a custom-written LabView program (National Instruments; supplementary material Fig. S2). FFTs can be used to calculate the relative abundance of each frequency that occurs in the signal. The frequency with the largest relative amplitude (i.e. the most prevalent frequency) from each trial was included in the calculation of the average shivering frequency for a given animal.

The relationship between shivering frequency and body mass is well established empirically (Spaan and Klussmann, 1970; Günther et al., 1983; Kleinebeckel et al., 1994). Spaan and Klussmann (1970) reported the relationship between shivering frequency and body mass in mammals to be:

$$\log(f) = 1.85 - 0.18 \log(m), \quad (5)$$

where f is the mean shivering frequency (s⁻¹) and m is mean body mass (g). Despite age matching the mice, the body masses of the mutant mice (6.3±0.3 g) were much lower than the body masses of the wild-type (18.2±1.0 g) and heterozygous mice (19.8±0.2 g). Using Eqn 5, we were able to calculate a predicted tremor frequency for each genotype and, thereby, take into account the differences in body mass.

Correcting for body temperature

Like other rates and the frequencies of other movements, tremor frequencies depend on body temperature (Schaeffer et al., 1996). Mutant mice often had body temperatures below the normal body temperature for mice (i.e. 37°C; Gordon, 2004). To account for these temperature differences, shivering frequencies were normalized in order to estimate the frequencies that would

be expected if the mutants had a body temperature of 37°C. Stein et al. (1982) reported that the rate of force production for mouse muscle was temperature dependent with a Q_{10} of 2.5; metabolic rate in mice has a Q_{10} of 2.4 (Geiser, 2004). These values are very similar, so we opted to use the muscle-specific value. Thus, we calculated expected tremor frequencies at a body temperature of 37°C using a Q_{10} of 2.5 and the equation:

$$f_{\text{corrected}} = f_{\text{observed}} \times Q_{10}^{(37 - T_{\text{observed}})/10}, \quad (6)$$

where $f_{\text{corrected}}$ is the tremor frequency expected if body temperature is 37°C, f_{observed} is the observed tremor frequency and T_{observed} is the observed body temperature.

Stiffness measurements and modeling

Soleus muscles were extracted from anesthetized wild-type ($N=4$) and *mdm* mutant ($N=3$) mice. The distal end of the muscle was attached to an inflexible hook and the proximal end was attached to a dual servomotor force lever (Aurora Scientific, Inc., Series 300B, Aurora, ON, Canada) that measured position and force. In load–clamp experiments, muscles are rapidly unloaded and the change in muscle length due to the change in load can be used to measure active muscle stiffness during unloading. A series of load–clamp experiments was used to estimate the active stiffness of wild-type and *mdm* soleus muscles at stimulus frequencies corresponding to the shivering frequency, following the methods of Lappin et al. (2006). For each muscle, data from 6–8 load clamp tests were collected to model the active stress–strain relationship during active unloading using the equation for an exponential spring,

$$F = F_0(e^{(x/d)} - 1), \quad (7)$$

where F is force, F_0 and d are constants that control the shape of the curve, and x is strain.

Statistical analysis

ANOVA and Tukey's honestly significant difference tests were used to compare body mass among genotypes. Body temperature and muscle stiffness values showed unequal variances among genotypes, so the non-parametric Wilcoxon test and Steel–Dwass comparisons were used to compare body temperatures and the Mann–Whitney test was used to compare stiffness values.

The relationship between body temperature and ambient temperature, as well as the relationship between metabolic rate and the temperature differential (body temperature–ambient temperature), were compared using an ANCOVA with genotype as a main effect. The ANCOVA model for metabolic rate and the temperature differential did not include the temperature differential–genotype interaction term because this term did not significantly improve the model.

Average tremor frequency for each genotype was determined, and expected tremor frequency at 37°C was calculated using Eqn 5. The E–O frequencies were compared using a nested ANOVA that accounted for within-individual and within-genotype variation.

To compare models for predicting tremor frequency (i.e. body mass alone given by Eqn 5 versus body mass and stiffness given by Eqn 1), we calculated RMSE and AIC. RMSE is defined by:

$$\text{RMSE} = \sqrt{\frac{\sum_N (f_{\text{expected}} - f_{\text{observed}})^2}{\sum_N f_{\text{expected}}^2}} \times 100\%, \quad (8)$$

where f_{expected} is the expected frequency and f_{observed} is the observed frequency (Perreault et al., 2003). AIC values are given by:

$$\text{AIC} = 2p - 2\ln(L), \quad (9)$$

where p is the number of parameters. Here, $\ln(L)$ is defined such that:

$$\ln(L) = 0.5 \times \left(-N \times \left(\ln(2\pi) + 1 - \ln(N) + \ln \sum_{i=1}^N x_i^2 \right) \right), \quad (10)$$

where x_i is the residual and N is the number of residuals (Spiess and Neumeyer, 2010).

$P < 0.05$ was considered to be significant. Values are reported as means \pm s.e.m. Statistical analysis was performed using JMP (v. 9 or 11, SAS Institute, Inc., Cary, NC, USA) and RStudio (v. 0.97.310).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.R.T.-B., K.C.N. and S.L. contributed to the conceptual development and experimental design of this work. K.R.T.-B. collected data for the shivering frequency and metabolic rate experiments, analyzed the data and drafted the manuscript. J.M. collected and analyzed the stiffness measurement data. C.P. was responsible for genotyping the mice. S.L. helped to develop the protocols used for collecting the metabolic rate data. K.C.N. contributed funding. All authors contributed to the interpretation and presentation of the data and edited the manuscript.

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Supplementary material

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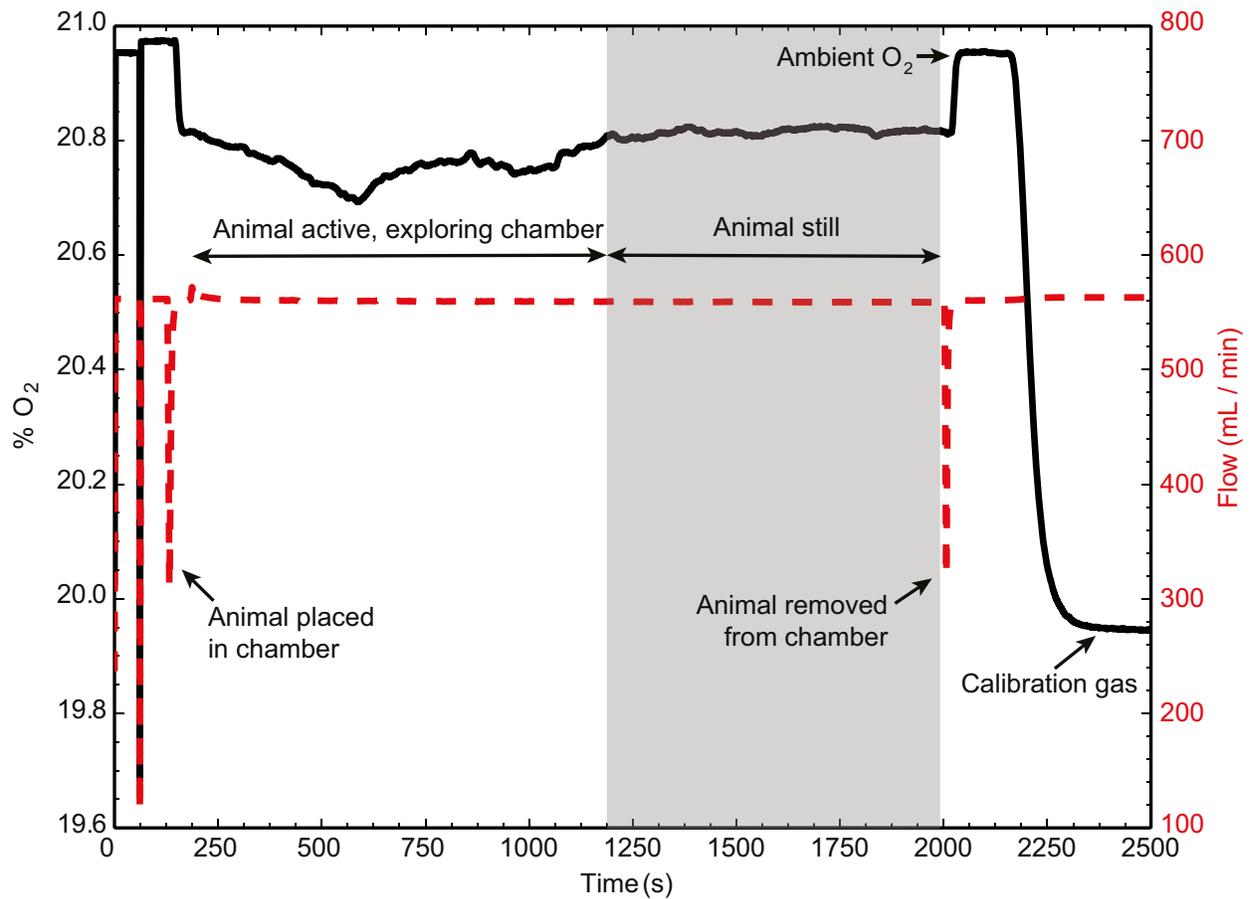


Fig. S1. Representative oxygen and flow measurement. In this example, the animal was placed in the chamber at 100 s and removed at 2000 s, at which times the chamber was opened and flow dropped. When the animal was in the chamber, the oxygen level dropped due to the animal's oxygen consumption. Measurements were taken when the animal was resting and after adjusting to the new environment for a minimum of 20 min; the data that fit these parameters are indicated by the gray box. Oxygen levels were calibrated using ambient oxygen levels (20.95%) and a calibration gas with a known level of oxygen. Combining oxygen levels and flow allowed us to determine oxygen consumption per unit time.

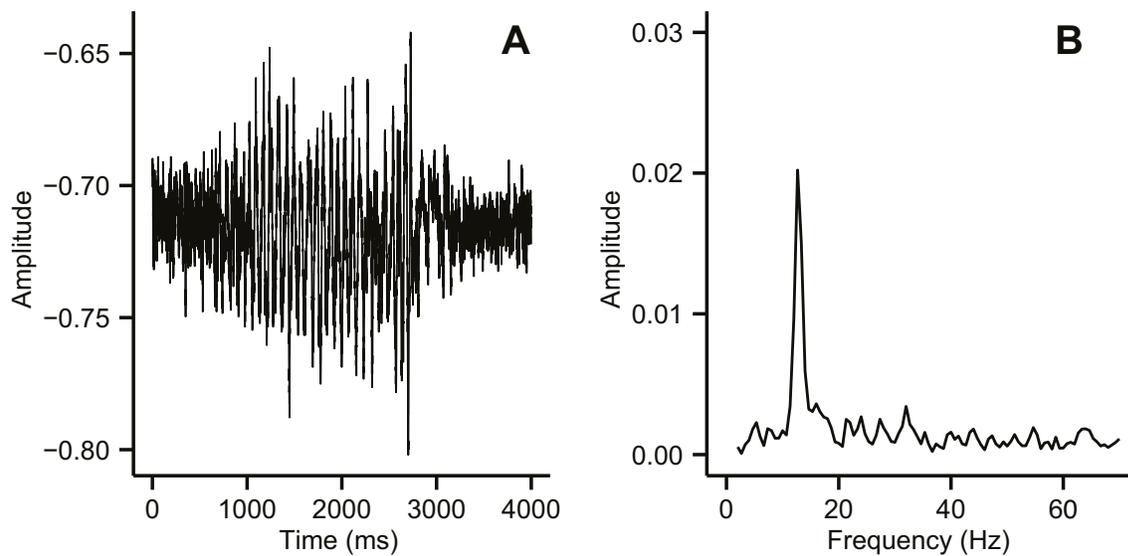


Fig. S2. Measurement of tremor frequency during shivering. (A) These representative accelerometer data show one bout of shivering from an *mdm* mutant. (B) A fast Fourier transform was used to find the most prevalent tremor frequency in each bout of shivering. In this example, the most prevalent frequency was about 12 Hz.

Table S1. Samples Sizes by Experiment.

Note: T_a are in $^{\circ}\text{C}$ and all measurements were taken within $\pm 1^{\circ}\text{C}$ of given temperature.

Genotype	Shivering	Metabolic Rate and T_b Measurement												
		T_a :	20	24	26	28	30	31	32	33	34	35	36	37
Wildtype	6		3	3	3	3	3	–	3	–	4	–	–	–
Heterozygous	6		3	4	3	4	4	–	6	–	4	–	–	–
Mutant	6		2	–	3	3	4	3	4	3	3	4	3	3