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Selection for high activity-related aerobic metabolism does not alter the capacity of non-shivering thermogenesis in bank voles

Clare Stawski [a,*], Paweł Koteja [a], Edyta T. Sadowska [a], Małgorzata Jefimow [b], Michał S. Wojciechowski [b]

[a] Institute of Environmental Sciences, Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland
[b] Department of Animal Physiology, Nicolaus Copernicus University, ul. Lwowska 1, 87-100 Toruń, Poland

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ABSTRACT

An intriguing question is how the capacity of non-shivering thermogenesis (NST)—a special mechanism supporting endothermic thermoregulation in mammals—is affected by selection for high exercise metabolism. It has been proposed that high NST could be a mechanism to compensate for a low basal production of heat. On the other hand, high basal or activity metabolism is associated with physiological characteristics such as high performance of the circulatory system, which are also required for achieving a high NST. Here we tested whether selection for high aerobic exercise performance, which correlates with an increased basal metabolic rate, led to a correlated evolution of maximum and facultative NST. Therefore, we measured the NST of bank voles, *Myodes* (= *Clethrionomys*) *glareolus*, from lines selected for 13–14 generations ($n = 46$) for high aerobic metabolism achieved during swimming and from unselected, control lines ($n = 46$). Open-flow respirometry was used to measure the rate of oxygen consumption ($\dot{V}O_2$) in anesthetized bank voles injected with noradrenaline (NA). After adjusting for body mass, maximum NST (maximum $\dot{V}O_2$ recorded after injection of NA) did not differ between the selected (2.38 ± 0.08 mLO_2min^{-1}) and control lines (2.36 ± 0.08 mLO_2min^{-1}; $P = 0.891$). Facultative NST (= maximum NST minus resting metabolic rate of anesthetized animals) did not differ between the selected (1.49 ± 0.07 mLO_2min^{-1}) and control lines (1.50 ± 0.07 mLO_2min^{-1}; $P = 0.985$), either. Therefore, our results suggest that NST capacity is not strongly linked to maximum activity-related aerobic metabolic rate.

1. Introduction

Endotherms have evolved specialized mechanisms of thermogenesis to increase heat production when ambient temperatures (T_a) are low (Banet and Hensel, 1977). These primarily involve shivering thermogenesis, whereby the animal contracts antagonistic muscles to produce heat (Hohtola, 2004), and non-shivering thermogenesis (NST; Cannon and Nedergaard, 2011). NST can be either obligatory, which is mainly a by-product of the metabolism of internal organs, or facultative, which in mammals is achieved mainly, but not exclusively (Rose et al., 1999), in brown adipose tissue (BAT) by engaging uncoupling proteins (UCPs) to convert the energy of the electrochemical gradient across the mitochondrial inner membrane into heat (Cannon and Nedergaard, 2004). Further, NST is always present in many small mammals (Klaus et al., 1988). Facultative NST (fNST) provides many advantages to a homeothermic mammal. It allows for rapid increase in heat production when T_a decreases and therefore results in the widening of the thermal tolerance of a species without the need to maintain a high basal metabolic rate (BMR; Alexander, 1975). Capacity for NST also changes reversibly and it gradually increases during winter or cold acclimation, which allows for more effective heat production during adverse environmental conditions (Nespolo et al., 1999; Jefimow et al., 2004; Cannon and Nedergaard, 2011; Lichtenbelt and Schrauwen, 2011).

Because perfect homeothermy is extremely rare in most, if not in all, endotherms (e.g. Boyles et al., 2011), typically at least some degree of heterothermy is observed. Yet, heterotherms require a highly effective mechanism for the production of heat for the periodic rapid increase and maintenance of body temperature (T_b) to be able to achieve full locomotor activity (Geiser et al., 2002) and NST is one such mechanism that is known among heterothermic mammals (Haim and Izahaki, 1993; Kronfeld et al., 1994; Jefimow et al., 2000). Comparative data suggest that high NST capacity evolved as a mechanism for compensating for a low primary production of heat (Haim and Izahaki, 1993; Degen, 1997; but see: Sparti, 1992) and indicate its significant role in thermoregulation in heterothermic animals (Hayward and Lyman, 1967; Jefimow et al., 2004). Consistent with this pattern are data from a selection experiment, which demonstrated that mice selected for high BMR have a lower fNST capacity than mice selected for low BMR (Gębczyński, 2008). The result supports the idea that increased capacity for fNST compensates for low obligatory heat production. Other studies, however, indicate that high NST capacity positively

* Corresponding author at: Zoology, University of New England, Armidale, NSW 2351, Australia. Tel.: +61 2 67733756.
E-mail address: clare.stawski@gmail.com (C. Stawski).

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correlates with BMR, both on the intraspecific (McDevitt and Speakman, 1996; Song and Wang, 2003; personal observations) as well as on the interspecific level (e.g. Wunder and Gettinger, 1996).

As obligatory NST is the result of the metabolism of internal organs it may be correlated to locomotor-related aerobic metabolism, which is dependent on the metabolism of organs in the pulmonary and circulatory systems (Gębczyński, 2008; Van Sant and Hammond, 2008). However, fNST is derived from BAT, which may not be directly involved in locomotor-related aerobic metabolism (Gębczyński, 2008; Van Sant and Hammond, 2008). A unique opportunity to test the hypotheses of a correlation, be it positive or negative, between locomotor-related aerobic capacity and obligatory NST or fNST is a selection experiment in which bank voles, *Myodes (=Clethrionomys) glareolus*, have been selected for increased rates of oxygen consumption achieved during swimming (Fig. 1; Sadowska et al., 2008). Voles from the selected lines have a higher BMR, a higher forced-exercise metabolic rate and a higher maximum total thermogenesis (Koteja et al., 2009; Sadowska et al., 2009). Here we test whether maximum NST (NST_{max}), defined as the maximum rate of oxygen consumption achieved after noradrenaline (NA) stimulation (Wunder and Gettinger, 1996; but see also: Cannon and Nedergaard, 2011), and fNST ($= NST_{max} - RMR_a$; RMR_a is the resting metabolic rate of anesthetized voles prior to NA-injection; see: Cannon and Nedergaard, 2011 for definitions and review of procedures used to measure NST) are the same in bank voles from selected lines as in bank voles from control lines, which is equivalent to testing for the presence of additive genetic correlation between the traits.

2. Materials and methods

2.1. Animals

The study was done on male bank voles, *Myodes (=Clethrionomys) glareolus*, sampled from four lines selected for a high rate of oxygen consumption achieved during swimming and four unselected, control lines (Sadowska et al., 2008). Details of the selection protocol and breeding conditions are presented in Sadowska et al. (2008). Briefly, the selection criterion was mass-independent (residual from regression) 1-minute maximum rate of oxygen consumption achieved in a 17-minute swimming trial performed at the age of 75–90 days. The water temperature was kept at 38°C to ensure that direct selection is only for locomotor-related aerobic performance and not on thermoregulatory response. To prevent inbreeding and loss of genetic variation, in each of the four selected and four control lines at least 15 breeding families were obtained in each generation, within-family selection was applied, and mating between siblings and first cousins was avoided.

Experiments reported in this study were done in two series, one in February 2012 with bank voles from generation 13 and one in July 2012 with bank voles from generation 14. The maximum rate of oxygen consumption achieved during swimming was about 50% higher in voles from the selected lines than those from unselected control lines (generation 13: 48%, generation 14: 51%; Fig. 1; $P < 0.0001$). For the final measurements a total of 92 bank voles were used, with 46 from the selected lines and 46 from the control lines. Voles used in the experiments were aged between approximately 100 and 150 days. Approval for this research was granted by the Local Committee for Ethics in Animal Research in Kraków No. 99/2006, 21/2010 and 22/2010 and 69/2012.

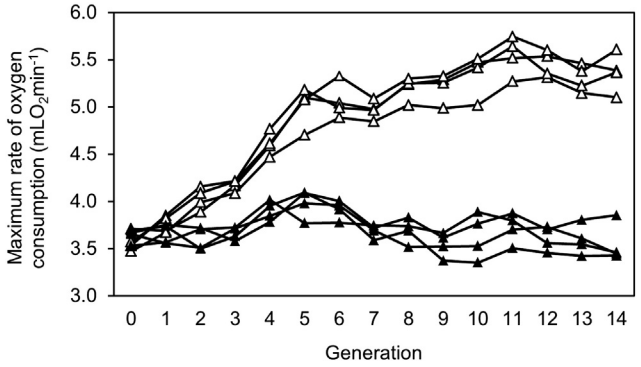

Fig. 1. Direct effect of the selection for maximum rate of oxygen consumption achieved during swimming in the bank vole. Points represent mean values of the four selected and four control lines. Selection was relaxed in generation 12, so individuals from generation 13 are offspring of non-selected parents. Selected lines are represented by open triangles and control lines by solid triangles.

2.2. Experimental protocol

Non-shivering thermogenesis was induced by injection of noradrenaline (NA) (Cannon and Nedergaard, 2011). Preliminary measurements on non-anesthetized and anesthetized voles in an initial pilot study were undertaken to determine the effectiveness of NA in measuring NST_{max} in bank voles from both control and selected lines. The same individual bank voles were injected subcutaneously with NA in one trial and with physiological saline in another trial. The volume of the dose of saline was the same as that required for NA and was dependent on body mass (m_b); dose (mg kg^{-1}) $= 2.53\ m_b\ (g)^{-0.4}$ (from Wunder and Gettinger 1996). The results from these pilot studies revealed that O_2 consumption rate changes after injection of NA were different to those after injection with saline, which showed that the NA dose administered was successful in inducing NST_{max}.

In pilot studies on non-anesthetized bank voles we observed unusually high locomotor activity in animals from selected lines after injection of NA. It appears that previous studies measuring NST capacity have not given enough attention to potential errors resulting from individuals being physically active during measurements and the stress caused by handling and injecting the animal (Virtue and Vidal-Puig, 2013). Physical activity during measurements of NST can create considerable noise, therefore making it difficult to obtain reliable baseline or post-injection values (Golozoubova et al., 2006; Virtue and Vidal-Puig, 2013). Thus, prior to the measurement of NST, bank voles were anesthetized intraperitoneally with sodium pentobarbital (Morbital, Biowet, ZAP, Poland) at a dose of 95 mg kg^{-1} (Golozoubova et al., 2006; Cannon and Nedergaard, 2011). This anesthetic is known to not interfere with NST measurements (Ohlson et al., 1994; Golozoubova et al., 2006; Virtue and Vidal-Puig, 2013).

Measurements were done at $T_a = 30.3 \pm 0.70$ °C, which ensured that before injection with NA anesthetized bank voles maintained a constant T_b similar to the T_b of non-anesthetized voles and equaled 37.6 ± 1.26 °C for voles from selected lines and 37.6 ± 1.08 °C for voles from control lines. During NST measurements T_b of voles was constantly monitored with a flexible rectal type-T thermocouple which was inserted at a depth of ~2 cm and secured with adhesive tape. Following administration of anesthesia voles were placed immediately into the respirometry chambers and the experiment was commenced. The initial, pre-injection measurement of RMR_a was typically ~10 min long (Fig. 2), after which NA was injected at a dose of $2.53\ m_b\ (g)^{-0.4}$ (mg kg^{-1}) (Wunder and Gettinger, 1996). Measurements of NA-induced thermogenesis were terminated soon after the individual reached peak $\dot{V}O_2$ after injection of NA (Fig. 2). Immediately after the trial voles were removed from the respirometry chamber and were placed on a wire grid above (~5 cm) an ice pack at room T_a ~20 °C, which allowed for more efficient, both radiative and convective, cooling of the body. This protocol prevented both hypothermia before injection with NA and lethal hyperthermia after injection with NA. There were no mortalities associated with NST measurements with this protocol.

2.3. Respirometry system

During each measurement of capacity for NST two (generation 13) or three (generation 14) animals were measured simultaneously using two

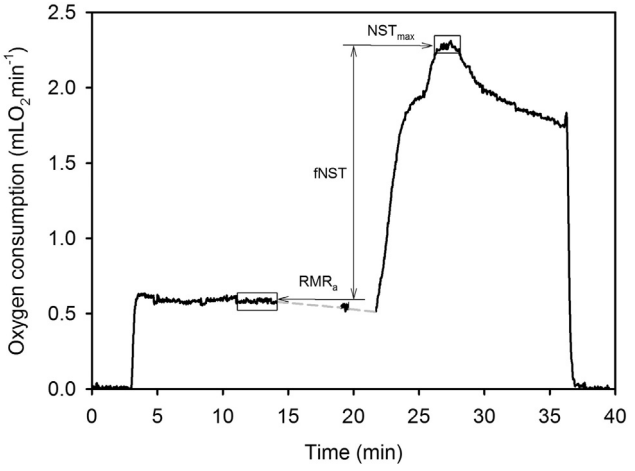

Fig. 2. An example of a $\dot{V}O_2$ measurement trace of an individual anesthetized bank vole before and after NA injection, and a graphical illustration of definitions of RMR_a, NST_{max} and fNST (see Methods for further explanation).

or three separate oxygen analyzers, which also recorded the baseline fractional concentration of O_2 in air. During measurements on generation 13 the analyzers used to record O_2 concentrations were FoxBox and FC-10a (both manufactured by Sable Systems Int., USA) both coupled with separate CA-10a CO_2 analyzers (Sable Systems Int., USA), while during measurements on generation 14 we used a FoxBox-C integrated CO_2 and O_2 analyzer, and two separate FC-10a analyzers (Sable Systems Int., USA). Regardless of the experimental session the animals were placed on a stainless-steel grid in air-tight respirometry chambers (made of 0.85 L plastic containers; model HPL808, Lock'n'Lock, Hana Cobi, Korea), which were housed within a temperature controlled cabinet at $T_a = 30.3 \pm 0.70$ °C (temperature measured within respirometry chambers). A constant flow rate of ~800 mL min^{-1} was used to continuously push outside air dried with Silica gel (Sigma-Aldrich, USA) through these chambers and a flow rate of ~600 mL min^{-1} of the same air was used for the reference sample. Flow rate was controlled with precise needle valves and measured upstream of the chambers with a mass flow meter (FlowBar-4, Sable Systems Int., USA). Downstream of the chambers air from each of them and from the reference line was subsampled at a rate of ~200 mL min^{-1}. A multiplexer (MUX-4, Sable Systems Int., USA) was used to manually switch between the respirometry chamber and the reference channel. The air sample, dried with magnesium perchlorate (Anhydrone, J.T. Baker, USA), was pulled through the above-mentioned gas analyzers with a small vacuum pump.

2.4. Data collection and analysis

All of the data measured by the respirometry system were recorded to a computer via an analog-to-digital interface UI2 connection (Sable Systems Int., USA) at 1 Hz. Body temperature data recorded by thermocouples were transferred to the computer via a thermocouple reading interface (USB-4718 Advantech EUROPE, Germany). ExpeData software (Sable Systems Int., USA) was used to acquire respirometry and T_a data and WaveScan 2.0 software (Advantech EUROPE, Germany) was used to acquire the T_b data. To calculate the $\dot{V}O_2$ of animals, for which both O_2 and CO_2 concentrations were measured, equation 11.7 from Lighton (2008) was used. For those animals for which only O_2 concentration was measured, equation 11.2 from Lighton (2008) was used assuming RQ = 0.8 (Koteja, 1996).

The $\dot{V}O_2$ values calculated for 1 s intervals across the entire trial were used to calculate three metabolic parameters (all expressed in mLO$_2$min^{-1}), as shown on Fig. 2: RMR_a—the resting metabolic rate of anesthetized animals, defined as a 3-min mean of stable $\dot{V}O_2$ measured before NA injection; NST_{max}—the maximum non-shivering thermogenesis, defined as the maximum 1-min $\dot{V}O_2$ achieved after NA-injection (this measure reflects the sum of obligatory and facultative thermogenesis); and fNST—facultative NST, defined as the difference between NST_{max} and RMR_a (this measure reflects solely the thermogenic effect of the NA injection).

SPSS v. 21 (IBM Corp. 2012) was used to analyze the data. Data were analyzed with a GLM procedure using the direction of selection (selected or control) and generation as fixed factors and selection line as a random factor nested within type of selection with body mass as a covariate. As we found no effect of pre-injection T_b on any of the variables it was not included in the final model. Further, we also found no differences in the slopes. Hence the mean values for NST_{max} and fNST are reported as estimated marginal means ± SE.

3. Results

Body mass of bank voles was between 16.9 and 33.2 g and did not differ between selected (26.03 ± 0.46 g) and control lines (24.87 ± 0.46 g; $F_{1,81} = 0.572$, $P = 0.478$) or between generations ($F_{1,81} = 1.945$, $P = 0.167$; Table 1). However, body mass varied significantly among replicate lines within selection directions ($F_{6,81} = 5.663$, $P < 0.001$; in one of the control lines the voles were considerably lighter than in other lines).

Pre-injection T_b did not differ between selected (37.63 ± 0.19 °C) and control lines (37.54 ± 0.19 °C; $F_{1,81} = 0.057$, $P = 0.820$; Table 1). Additionally, pre-injection T_b was not affected by generation ($F_{1,81} = 1.107$, $P = 0.296$), but it was affected by body mass ($F_{1,81} = 8{,}415$, $P = 0.005$). Pre-injection T_b varied significantly among replicate lines within selection directions ($F_{6,81} = 2.360$, $P = 0.038$; in one of the selected lines and one of the control lines pre-injection T_b was only slightly higher than in other lines).

Mass-adjusted RMR_a was the same between selected (0.89 ± 0.03 mLO$_2$min^{-1}) and control lines (0.88 ± 0.03 mLO$_2$min^{-1}; $F_{1,81} = 0.055$, $P = 0.822$; Table 1; Fig. 3). However, there was a significant effect of generation ($F_{1,81} = 16.49$, $P < 0.0001$; the earlier generation had a slightly higher mass-adjusted RMR_a) and body mass ($F_{1,81} = 11.96$, $P = 0.001$).

There was a correlation between body mass and NST_{max} ($F_{1,81} = 7.603$, $P = 0.007$; Table 1). However, neither the selection direction ($F_{1,81} = 0.031$, $P = 0.866$; Fig. 4) nor generation ($F_{1,81} = 0.031$, $P = 0.866$) affected mass-adjusted NST_{max}. After adjusting for m_b, NST_{max} in bank voles from selected lines was 2.39 ± 0.07 mLO$_2$min^{-1} and in control lines was 2.41 ± 0.07 mLO$_2$min^{-1} (Table 1).

Table 1
Summary statistics. Values are adjusted means ± SE.

	Adjusted means ± SE		Significance of effects (P values from GLM)			
	Control	Selected	Generation	Selection	Lines	m_b
m_b (g)	24.87 ± 0.46	26.03 ± 0.46	0.167	0.478	0.0001	
Pre-injection T_b (°C)	37.54 ± 0.19	37.63 ± 0.19	0.296	0.820	0.038	0.005
RMR_a (mLO$_2$min^{-1})	0.88 ± 0.03	0.89 ± 0.03	0.0001	0.822	0.456	0.001
fNST (mLO$_2$min^{-1})	1.53 ± 0.07	1.50 ± 0.07	0.160	0.792	0.104	0.235
NST_{max} (mLO$_2$min^{-1})	2.41 ± 0.07	2.39 ± 0.07	0.607	0.866	0.067	0.007

m_b = body mass; RMR_a = resting metabolic rate of anesthetized animals; NST = non-shivering thermogenesis; fNST = faculatative NST; and NST_{max} = maximum NST.

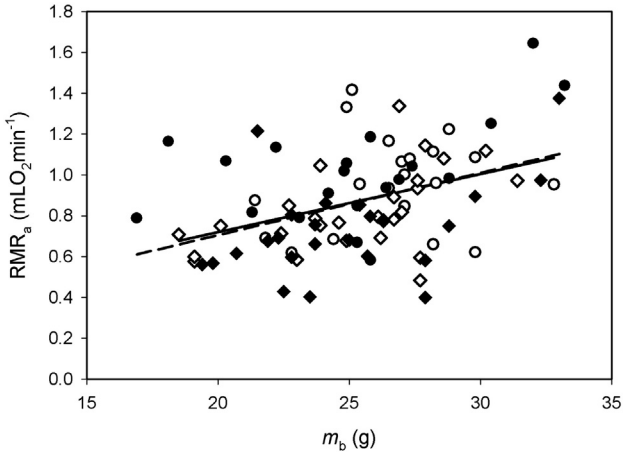

Fig. 3. RMR$_a$ (mLO$_2$min^{-1}) plotted against m_b (g) for control and selected lines. Generation 13 is represented by circles and generation 14 is represented by diamonds. Selected lines are represented by open symbols and the solid regression line, whereas control lines are represented by solid symbols and the dashed regression line.

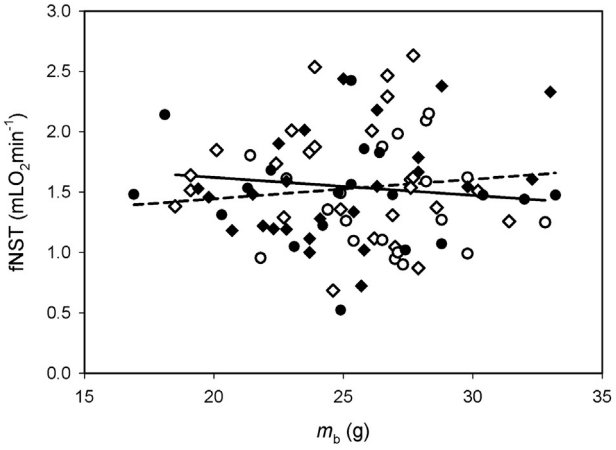

Fig. 5. fNST (mLO$_2$min^{-1}) plotted against m_b (g) for control and selected lines. Generation 13 is represented by circles and generation 14 is represented by diamonds. Selected lines are represented by open symbols and the solid regression line, whereas control lines are represented by solid symbols and the dashed regression line.

There was no difference in mass-adjusted fNST between selected (1.50 ± 0.07 mLO$_2$min^{-1}) and control lines (1.53 ± 0.07 mLO$_2$min^{-1}; $F_{1,81} = 0.076$, $P = 0.792$; Table 1; Fig. 5). Also there was no effect of generation ($F_{1,81} = 2.011$, $P = 0.160$) or body mass ($F_{1,81} = 1.431$, $P = 0.235$) on fNST.

4. Discussion

Results from the current study leave no doubt that neither NST$_{max}$ nor fNST differs between bank voles from lines selected for high aerobic metabolism obtained during exercise and bank voles from unselected control lines. This result suggests that there is no additive genetic correlation, either positive or negative, between exercise metabolism and NST capacity. From previous research we know that BMR in voles is genetically positively correlated with maximum swim-induced aerobic metabolism (Sadowska et al., 2005) and that BMR is indeed higher in the selected than in the control lines (Koteja et al., 2009). Thus, the present results do not support a correlation between BMR and fNST, either.

A similar study undertaken on laboratory mice selected for high BMR and low BMR found no differences in NST$_{max}$ between the selection lines (Gębczyński, 2008), which is consistent with the findings of the current study. However, fNST was significantly lower in the high BMR mice in comparison to the low BMR mice (Gębczyński, 2008), which is inconsistent with our data as we found no difference in fNST between selected and control voles. Yet, in his study, Gębczyński (2008) analyzed the capacity of NST in laboratory mice specifically selected for high and low BMR. It is likely that different selection pressures act when rodents are selected for low BMR, and the result of this selection is the compensatory role of fNST in animals showing normally low RMR.

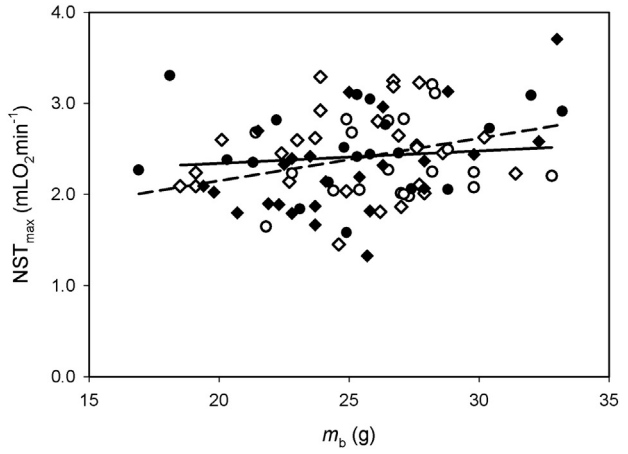

Fig. 4. NST$_{max}$ (mLO$_2$min^{-1}) plotted against m_b (g) for control and selected lines. Generation 13 is represented by circles and generation 14 is represented by diamonds. Selected lines are represented by open symbols and the solid regression line, whereas control lines are represented by solid symbols and the dashed regression line.

Non-shivering thermogenesis makes up a large component of an animal's thermogenic capacity, to which BMR and shivering thermogenesis also contribute (Merritt, 1995; Wunder and Gettinger, 1996; Nespolo et al., 2001; Van Sant and Hammond, 2008). Of each of these metabolic variables, NST appears to be the most flexible as it changes rapidly in response to changes in the environment (Kronfeld-Schor et al., 2000; Nespolo et al., 2001, 2002; Wang et al., 2006; Nowack et al., 2013). In a previous study, it was found that while activity related aerobic capacity was genetically correlated with BMR, the total thermogenic capacity was not (Sadowska et al., 2005). Additionally, another study found that thermogenic capacity did not differ between laboratory mice selected for high BMR and low BMR (Książek et al., 2004). These studies (Książek et al., 2004; Sadowska et al., 2005) and our current study suggest that an increase in BMR would not result from a selection for an increase in thermogenic capacity or NST and that these two traits are genetically independent. Additionally, since we found no difference between selected and control animals neither in NST$_{max}$ nor in fNST, it is clear that voles in the present study also did not differ in their obligatory heat production. The major difference between the results of the present study and the studies by Labocha et al. (2004), Sadowska et al. (2005), Koteja et al. (2009) and Sadowska et al. (2009) was that we measured oxygen consumption of anesthetized animals (RMR$_a$), while they measured conscious animals (BMR). However, the BMR values presented by Labocha et al. (2004; 52–55 mLO$_2$h^{-1}) and Sadowska et al. (2005; 52–55 mLO$_2$h^{-1}) are only slightly higher than the RMR$_a$ values reported in the current study. Additionally, it is justified that RMR$_a$ is lower than BMR, as maintaining consciousness costs some energy that the anesthetized animals would not be spending. This clearly suggests that the main contributor to the differences observed between selected and control voles in the previous studies was metabolism of muscles, which in our study was attenuated by the action of anesthesia. Another possibility could be also an effect of non-specific, pharmacological metabolic attenuation of metabolic rate by barbiturates (Ashcraft and Frankenfield, 2013). Nevertheless, sodium pentobarbital, which we used, does not interfere with NST (Ohlson et al., 1994; Golozoubova

et al., 2006; Virtue and Vidal-Puig, 2013) and thus should not affect results of the present study and our conclusions. The fact that we did not find differences in NST_{max} nor in fNST supports the idea that differences in metabolic performance between selected and control voles come rather from a correlated effect of selection on muscular metabolism (c.f. Kemp, 2006), rather than of upregulation of the performance of other metabolically active organs, like BAT, or with the upregulation of oxygen or substrate delivery.

Our results clearly show that selection for high aerobic metabolism has not resulted in an overall change in NST capacity, defined as the maximum response to NA injection. However, in addition to inducing UCP-1-related thermogenic response in BAT, NA can also induce a thermogenic response through other mechanisms (Golozoubova et al., 2006). Thus, one could speculate that both of the mechanisms were affected by the selection, but in the opposite way: NST capacity could be increased in the selected lines as a part of up-regulation of the metabolic capacity of metabolically active organs (c.f. Houle-Leroy et al., 2000; Kemp, 2006) while UCP-1-related thermogenic capacity of BAT could be decreased (as predicted by the hypothesis of the compensatory role of fNST; Haim and Izahaki, 1993; Degen, 1997). Such a scenario seems to be unlikely, because—in light of our results—it would require a nearly perfect matching of the opposite effects, but it provides an interesting perspective for further research.

While the evolution of endothermy in mammals and birds allowed for the development of many ecological and evolutionary advantageous traits (Bergman and Casadevall, 2010; Clarke and Pörtner, 2010), endothermy itself is an energetically costly trait (Nagy et al., 1999; Speakman and Thomas, 2003). Therefore, homeothermy is often abandoned by many species, and as a result heterothermy is common among both mammals and birds (McKechnie and Lovegrove, 2002; Wojciechowski and Pinshow, 2009; Dausmann, 2014; Riek and Geiser, 2014; Stawski et al., 2014). Heterothermic animals are able to save large amounts of energy by periodically decreasing metabolic rate and T_b, but they need to be able to return to normothermic levels and NST can provide a rapid increase in both metabolic rate and T_b to aid these arousals (Geiser et al., 2002; Jefimow et al., 2004; Nowack et al., 2013). Our finding suggests that fNST is not genetically correlated to exercise or, indirectly, to basal metabolism and is likely to be regulated flexibly. Together with results of Gębczyński's studies (2008), our results point toward different scenarios of the correlated evolution of thermogenic capacity in mammals. On the one hand, selection for low BMR led to the correlated, compensatory increase (in evolutionary terms) of fNST. This scenario would be favored in unpredictable environments, e.g. desert, where low maintenance costs compensated by effective facultative heat production are crucial for the survival of small endotherms, as suggested earlier (Haim and Izahaki, 1993; Degen, 1997). On the other hand, under more predictable conditions selection for high exercise MR led to a correlated increase of basal heat production, which ultimately did not require to be compensated by efficient facultative heat production. Under this scenario, the flexible, long-term adjustments of the capacity for NST would be evolutionarily advantageous.

Acknowledgments

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