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A dopamine and noradrenaline reuptake inhibitor (bupropion) does not alter exercise performance of bank voles

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Abstract

Physical performance is determined both by biophysical and physiological limitations and behavioral characteristic, specifically motivation. We applied an experimental evolution approach combined with pharmacological manipulation to test the hypothesis that evolution of increased aerobic exercise performance can be triggered by evolution of motivation to undertake physical activity. We used a unique model system: bank voles from A lines, selected for high swim-induced aerobic metabolism (VO_2swim), which achieved a 61% higher mass-adjusted VO_2swim than those from unselected C lines. Because the voles could float on the water surface with only a minimum activity, the maximum rate of metabolism achieved in that test depended not only on their aerobic capacity, but also on motivation to undertake intensive activity. Therefore, we hypothesized that signaling of neurotransmitters putatively involved in regulating physical activity (dopamine and noradrenaline) had changed in response to selection. We measured VO_2swim after intraperitoneal injections of saline or the norepinephrine and dopamine reuptake inhibitor bupropion (20 mg/kg or 30 mg/kg). Additionally, we measured forced-exercise VO_2 (VO_2max). In C lines, VO_2swim (mass-adjusted mean $\pm$ standard error (*SE*): 4.0 ± 0.1 mLO$_2$/min) was lower than VO_2max (5.0 ± 0.1 mLO$_2$/min), but in A lines VO2swim (6.0 ± 0.1 mLO$_2$/min) was as high as VO_2max (6.0 ± 0.1 mLO$_2$/min). Thus, the selection effectively changed both the physiological–physical performance limit and mechanisms responsible for the willingness to undertake vigorous locomotor activity. Surprisingly, the drug had no effect on the achieved level of VO_2swim. Thus, the results did not allow firm conclusions concerning involvement of these neurotransmitters in evolution of increased aerobic exercise performance in the experimental evolution model system.

Key words: exercise performance, motivation, physical activity, selection experiment.

The physiological basis of physical activity is central to both evolutionary biology and medicine, as physical performance is closely related to Darwinian fitness (Ekkekakis et al. 2005) and has tremendous beneficial effects on many aspects of human health (Booth and Lees 2007). The actual level of physical performance of an animal is determined not only by biophysical and physiological limitations, such as muscle strength or cardiovascular efficiency, but it is also controlled by central nervous system (Knab and Lightfoot 2010; Garland et al. 2011; Loprinzi et al. 2013). Despite intensive

research, it is uncertain which neurophysiological mechanism determines whether an animal will undertake physical effort and to what physiological level the animal will use it physical abilities (Knab and Lightfoot 2010; Garland et al. 2011; Rauch et al. 2013). Similarly, it is unknown why some animals choose to perform at the upper level of their physical abilities, whereas others prefer to exercise at slower pace, far from their physiological limit (Swart et al. 2009).

The level of locomotor activity is associated with the rate of aerobic metabolism. Aerobic capacity (the maximum forced-exercise

rate of oxygen consumption: VO$_2$max) is an important trait in evolutionary-comparative physiology, because of its role in the evolution of locomotor performance. Moreover, it is widely recognized to be involved in the evolution of endothermy and hence evolution of avian and mammalian lineages, including humans (Garland and Carter 1994; Koch and Britton 2008). VO$_2$max is also considered an important trait in medical physiology as an index of physical fitness and human health (see Arena et al. 2010 for a review). Exercise behavior and the maximum rate of aerobic metabolism are genetically correlated (Waters et al. 2008). Because the level of physical performance is regulated by the central nervous system, we can expect similar influence of neuronal factors on the maximum aerobic metabolism achieved during exercise. However, despite widespread application of VO$_2$max measurements, the influence of neurobiological factors on the achieved VO$_2$max value is often neglected (Noakes 2008).

The central nervous system regulates all types of physical activity, e.g., spontaneous, voluntary, vigorous, and urgent motor behavior (Garland et al. 2011; Rauch et al. 2013). The system controls each step of exercise performance: from decision to undertake activity and regulation of its intensity, to exercise termination. Engagement in physical activity (e.g., endurance running) can have beneficial effects on reproduction, survival and health, but it is also energetically costly, time-consuming, and even risky (Ekkekakis et al. 2005). It has been proposed that neurobiological rewards (e.g., pleasurable feeling of euphoria) could have evolved as a mechanism motivating animals to engage in physical activity in order to, e.g., acquire food or avoid becoming food (Ekkekakis et al. 2005). A phenomenon known as "runners' high", i.e., the pleasurable and rewarding feeling connected with performing physical exercises, seems to correspond to the evolutionary context (Dietrich and McDaniel 2004; Garland et al. 2011). Thus, it can be hypothesized that evolution of increased aerobic exercise performance can be triggered by evolution of neurophysiological "motivation" to undertake physical activity.

To test the above hypothesis we used a unique animal model—bank voles (*Myodes glareolus;* Schreber, 1780) selected toward high swim-induced aerobic metabolism (A lines). After 19 generations of selection, animals from the A lines achieved a 61% higher mass-adjusted aerobic metabolism during swimming (VO$_2$swim, Figure 1A, B) than unselected, control lines (C lines). During selection trials, the voles did not have to swim vigorously but could float on the water surface using little energy. Thus, VO$_2$swim is not *the maximum* rate of aerobic metabolism (i.e., the aerobic capacity) in the strict sense used in exercise physiology (VO$_2$max), but a measure of *voluntary maximum* rate of oxygen consumption, which depends on the behavioral characteristics such as motivation to work. Importantly, in generation 6 of the experiment, when the difference in VO$_2$swim between the A-selected and C-control lines was about 23% (Figure 1A), VO$_2$max measured as the maximum rate of oxygen consumption during forced running on a treadmill was higher than VO$_2$swim both in the A (24%) and C lines (33%), yet VO$_2$max was more than 15% higher in A than in C lines (Sadowska 2008, Koteja et al. 2009). Thus, the selection for the increased submaximal (to some extent voluntarily achieved) aerobic performance effectively changed both the physiological–physical performance limit (VO$_2$max, i.e., the aerobic capacity), and neurophysiological mechanisms responsible for willingness of the voles to undertake vigorous locomotor activity.

Neurotransmitters putatively involved in the regulation of physical activity motivation are, amongst others, dopamine and

noradrenaline (norepinephrine). Dopamine is implicated in motivation, rewarding, behavioral activation, motor movement, and physical activity (Vallone et al. 2000; Knab et al. 2009). In the central nervous system, noradrenaline plays a role in the control of level of arousal, reward mechanisms and consciousness (Roelands and Meeusen 2010). Genetic or pharmacological modifications of dopamine and noradrenaline neurotransmission result in changes of locomotor activity (Perona et al. 2008, Napolitano et al. 2010). Hence, we could expect that increased performance of voles from A lines is due to altered dopaminergic or noradrenergic signaling.

A change in particular neurotransmission can cause an altered response to a pharmacological agent; therefore, pharmacological manipulation is often used to investigate putative changes in neurotransmission (e.g., Rhodes et al. 2001, Keeney et al. 2008, Napolitano et al. 2010). For example, psychostimulants such as amphetamine increase locomotor activity in wild-type rodents by increasing the dopaminergic and noradrenergic signaling (Napolitano et al. 2010). Simultaneously, the same stimulants decrease locomotor activity in hyperactive rodents in which the basal level of dopamine is increased (Gainetdinov et al. 1999, Napolitano et al. 2010). That well-documented, paradoxical calming effect of stimulants is hypothesized to result from activation of inhibitory autoreceptors in hyperdopaminergic animals (Solanto 1998, Napolitano et al. 2010). In this study, we used pharmacological manipulation to investigate if the increased performance of bank voles
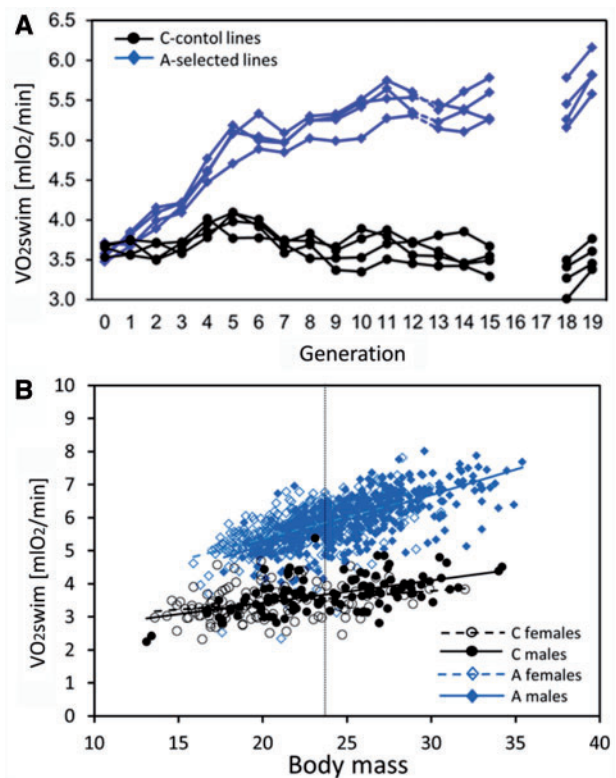
Figure 1. The direct effect of selection for high rate of swim-induced aerobic metabolism (VO$_2$swim) in bank vole. (A) Comparison of VO$_2$swim in the C-control and A-selected lines across 19 generations (replicate line means, not adjusted for body mass; in generations 12 and 15–18 the selection was relaxed); (B) the relationship between VO$_2$swim and body mass in females and males from the C and A lines in generation 19, from which individuals for this research were sampled. The dotted vertical line indicates mean body mass (23.7 g) used for calculating mass-adjusted VO$_2$swim.

from A lines is related to changes in the dopaminergic and noradrenergic signaling in the brains. We used bupropion, a dopamine and noradrenaline reuptake inhibitor, that increases physical activity in normal humans and rodents (Sidhpura et al. 2007; Mori et al. 2013) and decrease activity in Attention deficit hyperactivity disorder patients (Biederman 2005) as well as hyperactive mice (Napolitano et al. 2010). Therefore, we expected that if the selection toward high rate of aerobic metabolism resulted in changed dopamine and noradrenaline signaling, bank voles from the A and C lines will respond differently to the administration of bupropion. Specifically, bupropion injection could increase VO$_2$swim achieved by the voles from C lines and simultaneously decrease the activity of voles from A lines if the basal level of dopamine and noradrenaline is increased in those animals.

Materials and Methods

We used bank voles from generation 19 of an artificial selection experiment, in which 4 replicate lines of a nonlaboratory rodent are selected for high rate of aerobic metabolism achieved during swimming test (VO$_2$swim; "A" —aerobic lines), and 4 replicate lines are maintained as unselected control ("C" lines). The general objectives of the selection experiment, the rational of choosing the selection criterion, the breeding design, and the detailed history of experiment are described in our previous reports (Konczal et al. 2015; Sadowska et al. 2008, 2015).

We measured VO$_2$swim with 2, custom-built, open-flow respirometric systems (Koteja 1996). In most instances, measurements were conducted simultaneously in 2 animals. Details of the systems' construction changed across the course of the selection experiment. In generation 19, we used FC-10 oxygen analyzer and CA2-2A carbon dioxide analyzer in 1 system, and FC-10 a oxygen analyzer (without carbon dioxide analyzer) in the second (Sable Systems, Las Vegas, NV, United States). The 3-dm^3 respirometric chamber (a cylindrical, transparent glass jar, 15 cm diameter) was partly filled with water (6-cm space between the water surface and the top of the chamber was maintained), so the voles could swim freely. The nominal air flow through respirometric chamber was 2000 mL/min, regulated by a mass-flow controller (GFC17: Aalborg, Orangeburg, NY, United States; or ERG3000: Beta-Erg, Warszawa, Poland). A sample of excurrent air was dried first with either ND2 nafion tube or DG-1 cold-trap (Sable Systems) and then with chemical absorber (magnesium perchlorate), and directed to gas analyzers, which recorded the gas concentrations every second. The selection criterion was 1-min VO$_2$swim corrected for effective volume (calculated according to appropriate equations in Koteja 1996). The water temperature was 38°C to ensure that the animals were using energy

only for locomotor activity and not for thermoregulation. We added a drop of dog shampoo to the water to ensure soaking of the entire fur.

In generation 19, we measured VO$_2$swim of 371 females and 443 males from A lines and 116 females and 112 males from C lines at the age of 80–85 days, as a part of our regular selection program. Based on the results of these whole-generation measurements, called later a "selection trial", we chose 6 males and 6 females from each of the A and C lines for further pharmacological experiment (96 animals in total). The sample could not include voles that achieved the best results during the trial, as those animals were used for producing the next generation. Therefore, to avoid a bias, voles with the lowest results, as well as those that could not swim or were diving, were also excluded. Thus, the animals used in the pharmacological experiment represented the medium values in the distribution of VO$_2$swim within a particular line. To maximize independence of the observations (and simplify statistical models) we included only 1 individual from a full-sibling family. In each family, the single individual was sampled from 1 of 4 subsequent litters (1st to 4th) and that grouping was included in statistical analyses. The descriptive statistics concerning both the whole generation and the sample of animals used in pharmacological trials are presented in Table 1.

The animals were kept in standard plastic mice cages with sawdust bedding, under long photoperiod (16:8 h light:dark; light phase was starting at 2 AM) and temperature 20 ± 1°C. We kept 3 animals in a cage, but 4 days before the pre-trial the animals were separated to individual cages. Standard rodent food (Labofed H, Kcynia, Poland) and water were available ad libitum.

All procedures associated with the breeding scheme, the selection protocol as well as experimental procedures was approved by the Local Bioethical Committee in Krakow, Poland (No. 68/2012 and No. 61/2014).

Pharmacological experiment

About 15 weeks after the selection trial we started a series of 5 VO$_2$swim trials on each individual, according to the following schedule:

- pre-trial: after saline injection,
- 3 main pharmacological trials, in random order: after injection of either saline or bupropion in 1 of 2 doses (low: 20 mg/kg, high: 30 mg/kg), and
- post-trial: after saline injection.

We maintained 1-week breaks between each trial to minimize a carry-over effect. During the pre- and post-trials, performed to test

Table 1. Body mass (in grams) and the rate of swim-induced oxygen consumption (VO$_2$swim [mLO$_2$/min]) achieved by all bank voles from the 19th generation that underwent selection trial and the sub-sample of voles used in the pharmacological experiment: simple means ± standard deviations (SDs) and the least squares mean (LSM) of VO$_2$swim ANCOVA-adjusted for mean body mass (23.7 g) ± standard error (*SE*)

Analysis	Selection	Sex	N	Body mass [g] average ± *SD*	VO2swim [mLO2/min] average ± *SD*	LSM ± *SE*
All animals generation 19th	C	Female	116	20.4 ± 3.8	3.4 ± 0.5	3.5 ± 0.08
		Male	112	24.2 ± 4.2	3.7 ± 0.5	3.6 ± 0.07
	A	Female	371	22.5 ± 2.9	5.7 ± 0.6	5.8 ± 0.06
		Male	443	25.5 ± 3.3	6.0 ± 0.7	5.7 ± 0.05
Animals used in pharmacological experiment	C	Female	24	20.1 ± 3.8	3.4 ± 0.3	3.4 ± 0.07
		Male	24	24.3 ± 4.0	3.7 ± 0.3	3.6 ± 0.06
	A	Female	24	22.5 ± 2.9	5.7 ± 0.6	5.7 ± 0.07
		Male	24	25.4 ± 4.1	6.0 ± 0.7	5.6 ± 0.07

for putative effect of memory, training or age, we administered only saline. All the injections were administered intraperitoneally (i.p.) in volume of 10 mL/kg (i.e., about 0.2 mL per individual vole), 30 min before the swimming test, i.e., around the expected time of peak brain dopamine concentration (Sidhpura et al. 2007). All the measurements were performed in 3 blocks, comprising 32 individuals (2 individuals from each sex and replicate line) measured on the same day. We applied the same procedures to all blocks during 3 consecutive measurement days. We prepared the fresh drug's solutions on each measurement day, an hour before the first injection. After dissolving bupropion hydrochloride (Biokom, Janki, Poland) in 0.9% saline, the solution was filtered with syringe filters with micropores 0.2 μm (Rotilabo, Linegal Chemicals, Warsaw, Poland).

Forced running

Three weeks after the post-trial, we measured the maximum rate of oxygen consumption achieved during forced running (VO_2max) in a motorized treadmill enclosed within a respirometric chamber (type BTU-100-1U-M, Bio-Sys-Tech, Bialystok, Poland), according to a generally applied protocol (e.g., Rezende et al. 2005; Arena et al. 2010). In previous experiments, we have already successfully applied the protocol to our voles (Sadowska 2008). The tested vole was placed in the chamber while the treadmill was stopped, and after 1 min the treadmill was started at an initial speed of 6 m/min. Subsequently the speed was increased every 45 s by 6 m/min. The voles were forced to run by mild electric shocks (160 V, 0.5–1.5 mA) provided through bars at the end of the moving belt. Before each trial, we wetted the legs and abdomens of the voles in order to increase electrical conductivity. Without this procedure some animals lie on the bars and ignore the electric stimulation. We performed the trial till exhaustion, thus we ended the trial when the vole failed to keep pace with the treadmill. The measurement lasted for up to 15 min. Before the actual measurement, we performed 2 training trials to familiarize the voles with the novel situation and let them learn continuous running on the treadmill.

Statistical analysis

The main analyses were performed by means of nested analyses of covariance (ANCOVA) mixed models, implemented in SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) mixed procedure (with REML estimation method). Body mass was analyzed with ANCOVA including fixed effects of line Type (selected vs. control), Sex, and line Type × Sex interaction and random effects of replicate Line (nested in line Type), Family nested in Line, and Line × Sex interaction. The models included also Litter Size, Number of the Litter of the same female and type of Analyzer as cofactors. The rate of swim-induced aerobic metabolism (VO_2swim) during the selection trial was analyzed with ANCOVA including the same effects listed above and Body Mass as covariate. We tested the significance of random factors (replicate Lines, their interactions with other factors, Family) with likelihood ratio test (only if the variance estimation was positive).

We performed repeated measures ANCOVAs to compare: 1) pre- and post-trial VO_2swim (training effect), 2) the pre-trial VO_2 swim with VO_2max (voluntary vs. forced performance), and 3) the VO_2swim achieved during 3 pharmacological trials (the focal analysis in this work: drug effect). These models included the same between-subject effects as in the model described above except Family, because families were represented by only 1 individual. In the analysis of Exercise type, the effect of Analyzer was not included, either,

because all measurements of VO_2max were performed on the same analyzer. The within-subject (within-individual) fixed effects, appropriate for the particular model, were: 1) Trial (pre vs. post), 2) Exercise type (swim vs. run), or 3) Drug (saline vs. bupropion low dose vs. bupropion high dose). The models included also fixed interaction between line Type and the appropriate within-subject factor (line Type × Trial, line Type × Exercise type or line Type × Drug) as well as a respective random interaction (Line × Trial, Line × Exercise type or Line × Drug). In the analysis of the drug effect, we considered 2 models in which a repeated measure factor was Drug or Number of a trial (the trial order). Based on Akaike information criterion (AIC), we selected the model with Number of a trial as a repeated measure factor. For all repeated measure models, we selected the unstructured covariance structure as the most appropriate.

Preliminary models also included interactions between Sex and other fixed effects. These interactions, however, were not of the main interest from the perspective of the study, and—when nonsignificant ($P < 0.05$)—were excluded from the final analysis. Preliminary ANCOVAs included also interactions between Body Mass and the main categorical effects (tests of homogeneity of slopes). We excluded these interactions from final models if they were not significant. However, the slopes of the relationships between VO_2 and Body Mass were always higher in A than in C lines, and the difference was significant in most of the analyses. To investigate whether the levels of VO_2 differ between the line Types in the range of observed body mass, we coded Body Mass by subtracting from the actual body mass the mass of the lightest animal in the dataset: 13 g in the analyses of VO_2swim achieved during selection trial, and 15 g in the other analyses. Because the slope of the relation between VO_2 and body mass was always steeper in A than in C lines, demonstrating a significantly higher level of VO_2 in A lines at the minimal body mass implies that the difference is significant also for the entire range of body mass. For a more transparent presentation of the results, we performed also the analyses for the mean body masses (23.7 g in the analysis of the selection trial VO_2swim, and about 25 g in the other analyses).

We excluded statistical outliers from the analysis when the studentized residual was higher than 3.0. In that way, we excluded 2 outliers from the analysis of pre- and post-trial, 2 outliers from the analysis of Exercise type effect, and 4 outliers from drug effect analysis. In the analysis of selection-trial VO_2swim for all voles in generation 19, i.e., a much larger sample, we increased the threshold to 3.5 (3 outliers were excluded). In all models, we used the Satterthwaite's approximation to calculate the effective degrees of freedom in the denominator of the F-test. Tukey–Kramer adjustment was used for pairwise a posteriori comparisons.

Results

The effect of body mass

In the selection trial, body mass of bank voles ranged from 13 to 35 g (Figure 1B) and mean body mass was 23.7 g (Table 1). The body mass did not differ between voles from A and C lines ($F_{1,6} = 1.8$, $P = 0.230$). Females were significantly lighter than males ($F_{1,366} = 204$, $P < 0.0001$), but the line Type × Sex interaction was not significant ($F_{1,362} = 2.7$, $P = 0.102$). The body mass was affected by the Litter Number in which the animal was reared (LSM ± SE: 1st litter 22 ± 0.8, 2nd litter 23 ± 0.7, 3rd and 4th litter combined 23 ± 0.7; $F_{2,997} = 12.4$, $P < 0.0001$) and number of pups in the litter (slope ± SE: −0.3 ± 0.06, $t_{957} = -4.2$, $P < 0.0001$). Similarly, in the sample of 96 voles used later in pharmacological experiment there

was no difference in body mass between line Types ($F_{1,7} = 0.6$, $P = 0.477$), females had lower body mass than males ($F_{1,81} = 24.3$, $P < 0.0001$) and the interaction line Type × Sex was not significant ($F_{1,81} = 0.6$, $P = 0.449$). Here the cofactors were not significant (the Litter Number: $F_{2,83} = 2.7$, $P = 0.074$, number of pups in a litter: $F_{1,85} = 0.7$, $P = 0.787$).

The rate of oxygen consumption measured either during all the swimming trials (VO$_2$swim) or the forced running trial (VO$_2$max) increased with body mass ($P < 0.0002$), and the slope of the relationship was always higher in A than in C lines (positive Line Type × Body mass interaction; Table 2, Figures 1B, 2A, B, 3A). At the minimal observed body mass, voles from the A lines achieved significantly higher VO$_2$ than those from C lines (Table 2, Figures 1B, 2A, B, 3A). Therefore, because the difference always increased with body mass (slopes higher in A than in C lines), we can infer that the difference was significant for the whole range of voles' body mass. Unless otherwise stated, further results concern models for mean body mass.

The direct effect of selection

In the selection trial, voles from the A lines achieved a significantly higher mass-adjusted VO$_2$swim than voles from C lines (adjusted for a mean body mass of 23.7 g; $F_{1,7.5} = 827$, $P < 0.0001$, Figure 1B, Table 1). Analyzing the effect of sex was complicated because the regression slope was steeper for males than for females (Sex × Body mass interaction: $F_{1,953} = 8.0$, $P = 0.005$; Figure 1). At the minimal observed body mass (13 g), females achieved a higher VO$_2$swim than males ($F_{1,906} = 8.0$, $P = 0.006$), but the difference between sexes decreased with body mass and in the middle of body mass ranged it reversed, so that at the maximal observed body mass males had higher VO$_2$swim than females ($F_{1,889} = 6.0$, $P = 0.014$). For the VO$_2$swim adjusted for a mean body mass, the effect of Sex was not significant ($F_{1,567} = 0.04$, $P = 0.87$), but line Type × Sex interaction was highly significant ($F_{1,525} = 7.7$, $P = 0.006$): in A lines VO2swim was higher in females than in males (Tukey–Kramer: $t_{243} = 2.6$, $P = 0.04$), whereas in C lines the difference was reversed, although not significant ($t_{681} = -1.6$, $P = 0.36$, Table. 1). The level of VO$_2$swim was affected by the Analyzer type ($F_{1,994} = 9.3$, $P = 0.002$), the Litter Number (LSM ± SE: 1st litter 4.67 ± 0.05, 2nd

litter 4.65 ± 0.04, 3rd and 4th litter combined 4.75 ± 0.04; $F_{2,1010} = 4.0$, $P = 0.019$), and number of pups in a litter (slope ± SE: − 0.02 ± 0.01, $t_{908} = -1.8$, $P = 0.071$). Likelihood ratio test indicated that random effects of replicate Line ($\chi^2 = 9.5$, $P = 0.002$) and Family ($\chi^2 = 28.1$, $P = 0.000$) contributed significantly to the variation of VO$_2$swim.

Results for the sub-sample of voles used in pharmacological trials ($N = 96$) were similar to those for the whole dataset: selection-trial VO$_2$swim (adjusted for mean body mass) was higher in A lines than in C lines ($F_{1,6} = 669$, $P < 0.0001$). The effect of Sex ($F_{1,81} = 0.2$, $P = 0.7$) was not significant, but the line Type × Sex interaction was significant ($F_{1,80} = 11.8$, $P = 0.001$): in A lines females achieved a higher VO$_2$swim than males did (Tukey–Kramer: $t_{79} = 2.9$, $P = 0.022$, Table 1), whereas in C lines the difference was reversed, although not significant ($t_{82} = -2.0$, $P = 0.189$, Table 1). Cofactors had significant effect on the VO$_2$swim (analyzer: $F_{1,78} = 4.7$, $P = 0.033$, the number of the pups in a litter: slope ± SE: $-0.02 ± 0.01$, $t_{82} = -2.1$, $P = 0.037$).

Pre- and Post-trials

In the tests performed ≥15 weeks after the selection trial, voles from A lines achieved significantly higher VO$_2$swim than voles from C lines (for mean body mass = 25 g: $F_{1,17} = 157$, $P < 0.0001$; Figure 2A, C). The effect of Sex ($F_{1,11} = 0.7$, $P = 0.405$) was not significant, but the interaction term line Type × Sex was significant ($F_{1,11} = 10.6$, $P = 0.007$). This was because in A lines females tended to have higher VO$_2$swim than males, whereas the trend was reversed in C lines, although these differences were not significant (Tukey–Kramer, A lines: $t_{10} = 1.7$, $P = 0.345$; C lines: $t_{13} = -2.8$, $P = 0.068$). VO$_2$swim did not differ between the pre- and post-trials ($F_{1,7} = 4.5$, $P = 0.073$; Figure 2A, C) and the interaction term line Type × Trial was not significant, either ($F_{1,7} = 0.6$, $P = 0.481$).

Running versus swimming

The analysis of covariance showed that the slopes of relationship between the rate of metabolism and body mass differed not only between the A and C lines (Table 2, discussed already above), but also between VO$_2$swim and VO$_2$max (Exercise type × Body mass interaction: $F_{1,70} = 3.5$, $P = 0.65$) and combinations of the selection and

Table 2. Partial results of the ANCOVA for the swim-induced (VO$_2$swim) and forced-running (VO$_2$max) aerobic metabolism, in which the covariate was "body mass minus minimal observed body mass" (13 g in case of selection trial and 15 g in the other analyses; see "Materials and Methods" for the rationale of the analysis)

Analysis	line Type	Body mass		line Type × Body Mass	
	F value (Ddf, Ndf) P value	Slopes ± SE [mLO$_2$/(min × g)]	t value (df) P value	Difference in slopes ± SE [mLO$_2$/(min × g)]	t value (df) P value
VO$_2$swim selection trial (whole generation)	101 (1,47) < 0.0001	0.07 ± 0.01	6.4 (394) < 0.0001	0.08 ± 0.01	7.2 (434) < 0.0001
VO$_2$swim selection trial (voles used in experimental trials)	85 (1,84) < 0.0001	0.04 ± 0.01	4.9 (29) < 0.0001	0.10 ± 0.01	8.4 (32) < 0.0001
VO$_2$swim pre- vs. post-trial (training effect)	5.4 (1,79) 0.022	0.01 ± 0.02	0.4 (26) 0.662	0.12 ± 0.03	4.9 (24) < 0.0001
Pre-trial VO2swim vs. VO2max (voluntary vs. forced performance)	10.7 (1,35) 0.002	0.10 ± 0.02	6.4 (29) < 0.0001	0.02 ± 0.02	1.1 (33) 0.295
VO$_2$swim 3 pharmacological trials (drug effect)	19.2 (1,20) 0.0003	0.05 ± 0.03	1.7 (20) 0.097	0.06 ± 0.03	1.9 (16) 0.071

The line Type effect provides a test of significance of the difference adjusted means of C and A lines at the minimum body mass, and the line Type × Body Mass interaction describes the difference in regression slopes.
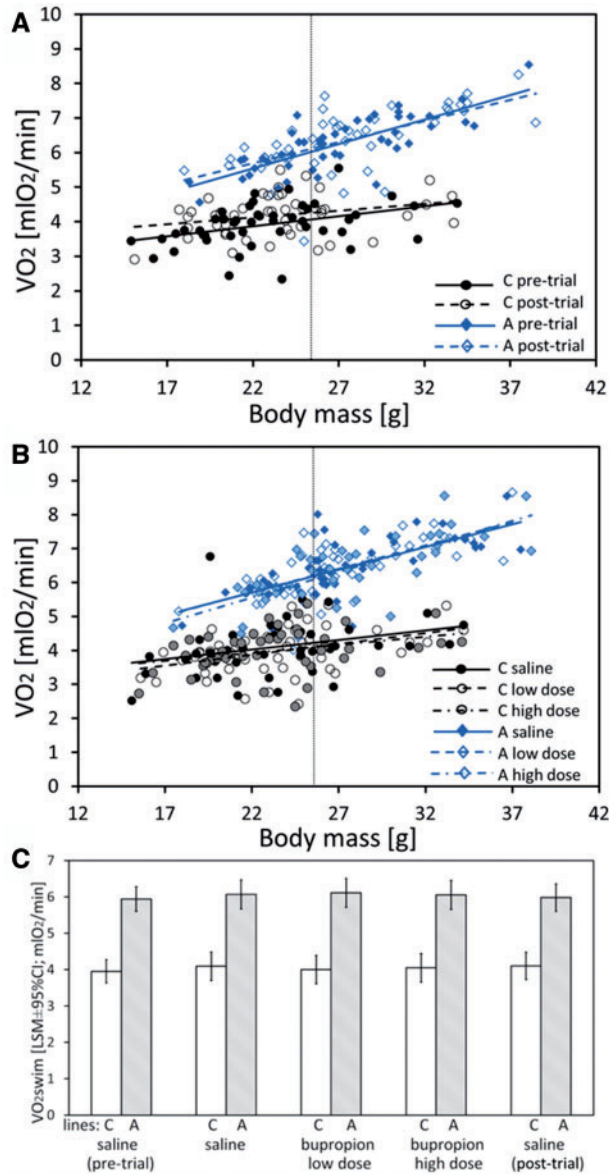
Figure 2. The rate of aerobic metabolism achieved by bank voles from C-control ($N = 48$) and A-selected ($N = 48$) lines during repeated swimming trials (VO_2swim) that were preceded by injections (saline or bupropion). (A–B) The relationship between VO_2swim achieved in the pre- and post-trials (saline injection; A) or in the 3 pharmacological trials (with injection of saline or bupropion; B) and body mass. Dotted lines indicate the mean body mass (25 g) for which the adjusted mean was calculated. (C) The adjusted least squares mean (LSM) with 95% confidence limits (LSM [95% CL]) from 2 separate mixed ANCOVA models (for pre- post-trials, and for the 3 in-between pharmacological trials).

exercise groups (second-order line Type × Exercise type × Body mass interaction: $F_{1,70} = 18.9$, $P < 0.0001$). This complex pattern appeared because the slope for VO_2swim in C lines was lower than the 3 other slopes: for VO_2max in C lines and both the VO_2swim and VO_2max in A lines (Figure 3A). At the minimum observed body mass (15 g), the effect of selection was significant (Table 2), but the effect of Exercise type not ($F_{1,44} = 2.3$, $P = 0.14$). The effect of Exercise type was significant for body mass above 17 g ($F_{1,36} = 4.7$, $P = 0.036$), indicating a generally higher level of VO_2max compared with VO_2swim, but the difference depended on both the body mass

and line Type (Figure 3). The analysis of covariance for the mean body mass revealed not only significant main effects of line Type ($F_{1,6} = 185$, $P < 0.0001$) and Exercise type ($F_{1,6} = 10.3$, $P < 0.018$), but also significant interaction between the factors ($F_{1,7} = 68$, $P < 0.0001$). The interaction appeared because in A lines the levels of VO_2max and VO_2swim did not differ (Tukey–Kramer: $t_7 = 0.6$, $P = 0.92$), whereas in C lines VO_2max was much higher than VO_2swim ($t_7 = -12.3$, $P < 0.0001$, Figure 3).

As in the analysis of VO_2swim alone, the effect of Sex was not significant ($F_{1,89} = 1.8$, $P = 0.185$), but line Type × Sex interaction was significant ($F_{1,88} = 13.8$, $P = 0.0004$): in A lines females achieved higher mass-adjusted VO_2 (averaged across VO_2swim and VO_2max) than males, but the difference was not significant (Tukey–Kramer: $t_{83} = 1.8$, $P = 0.285$), whereas in C lines the difference was reversed and significant ($t_{88} = -3.3$, $P = 0.006$).

The effect of the drug

During the 3 main trials voles from the A lines achieved a higher VO_2swim than those from C lines (at mean body mass $= 25$ g: $F_{1,6} = 79$, $P < 0.0001$, Figure 2B, C). There was no differences between sexes ($F_{1,7} = 1.0$, $P = 0.341$), and the interaction term line Type × Sex was not significant, either ($F_{1,7} = 4.4$, $P = 0.075$). The Drug had no effect on VO_2swim ($F_{2,146} = 0.7$, $P = 0.475$, Figure 2B, C) and the interaction term line Type × Drug was not significant, either ($F_{2,145} = 0.9$, $P = 0.394$). Number of pups in a litter had significant effect on the VO_2swim (slope $\pm$ SE: -0.09 ± 0.03, $t_{77} = -2.8$, $P = 0.006$).

In addition, for the drug effect we analyzed also VO_2swim calculated for 5-min maximum. The results were similar to those described above (for 1-min-maximum VO_2swim), with the significant difference between A and C lines ($F_{1,6} = 80$, $P < 0.0001$), but no effect of Drug ($F_{2,13} = 1.6$, $P = 0.239$) or line Type × Drug interaction ($F_{2,13} = 0.6$, $P = 0.547$).

Discussion

According to a general model of the evolution of complex adaptations, natural selection in the first place operates on behavior. However, because the range of behaviors available to an individual is constrained by its organismal performance, the selection forces subsequent changes in the morpho-physiological and biochemical characteristics underlying the performance (Garland and Carter 1994). We reported that in generation 6 of the selection experiment bank voles from both A and C lines achieved a higher 1-min maximum rate of oxygen consumption during forced running on a treadmill (VO_2max) than during swimming (VO_2swim; Sadowska 2008, Koteja et al. 2009). Thus, apparently, the level of VO_2swim was not limited by the voles' aerobic capacity at that time. However, in subsequent generations the level of VO_2swim continued to increase in the A lines, and in generation 19 it approached that of VO_2max (Figure 3). On the other hand, in C lines VO_2max was much higher than VO_2swim, but lower than VO_2max in A line voles (Figure 3). The results indicated that selection for high rate of voluntarily achieved aerobic metabolism changed both the physiological abilities (aerobic capacity) and behavioral trait (willingness to undertake physical activity). It seems that evolution of the behavioral component in our model has already pushed the vole's performance in the swimming test up to the limit imposed by physiological or biochemical mechanisms. However, this latter conclusion should be treated with caution, because VO_2max was measured a few weeks after VO_2swim, whereas for a methodologically strong comparison both
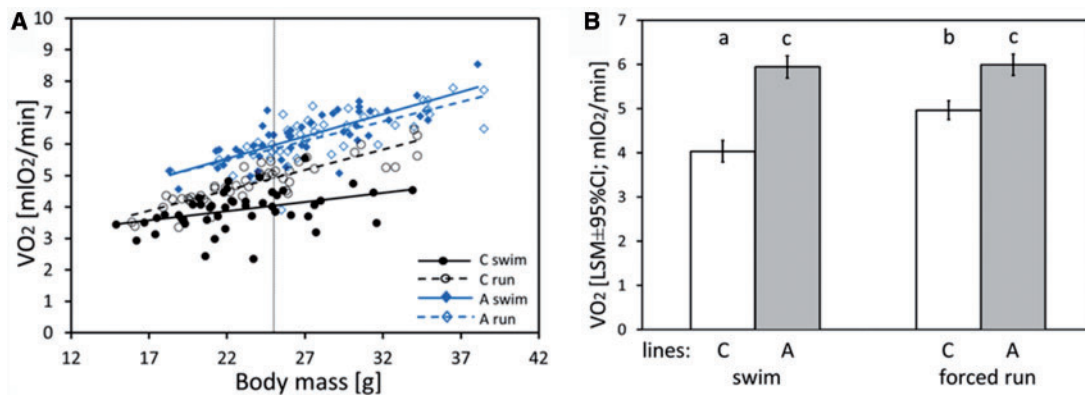
Figure 3. The aerobic metabolism (VO$_2$) achieved by control ($N = 48$) and selected ($N = 48$) bank voles during swimming (VO$_2$swim) and forced running (VO$_2$max). (A) The relationship between VO$_2$ and body mass. Dotted line indicate the mean body mass (25 g) used for calculating mass-adjusted VO$_2$. (B) Adjusted least squares mean with 95% confidence limits (LSM [95% CL]) from mixed ANCOVA models for selection (C lines vs. A lines) and exercise type (swim vs. forced run) groups. Different lowercase letters (a, b, c) indicate significantly different groups (post hoc Tukey–Kramer comparisons).

traits should be measured at the same age and in a randomized order. At any rate, the observed pattern supports the general concept of triggering the evolution of complex physiological adaptions by selection acting on behavioral traits (e.g., Garland and Carter 1994, Swallow et al. 2009).

In another experimental evolution model system, laboratory mice selected for long-distance voluntary wheel running achieved a higher VO$_2$ during the voluntary running than control mice (Rezende et al. 2005). All animals from that experiment achieved higher VO$_2$max during forced running, but there was no difference in VO$_2$max between selected and control mice (Rezende et al. 2005). Rezende et al. (2015) argued that selection for voluntary wheel running resulted in the evolution of behavior (willingness to undertake physical activity) independently of physical performance abilities (aerobic capacity). However, laboratory mice were unintentionally selected for over 400 generations toward decreased physical activity (Garland et al. 2011), and this casts a doubt on the validity of applying the inferences to wild animals and humans (Rezende et al. 2005; Garland et al. 2011). Furthermore, wheel-running behavior is difficult to interpret because it reflects not only general activity, but also other behavioral traits such as addiction and anxiety-like behavior (Novak et al. 2012). Therefore, utilizing running wheels in research concerning general physical activity requires careful interpretation (Novak et al. 2012).

The study was designed to investigate if dopamine and noradrenaline signaling regulate the motivation to be physically active and if the motivation had changed due to selection for achieving high rate of aerobic metabolism. We used a pharmacological manipulation approach that has been successfully applied in previous studies on correlated response to artificial selection (Rhodes et al. 2001; Rhodes et al. 2003; Keeney et al. 2008; Keeney et al. 2012). As the experiment required repeated trials on the same animals, in order to control for putative effect of memory or training, we performed additional trials, with only saline injections before and after the set of pharmacological trials (pre- and post-trials). We found that the effect of training was negligible, as there was no difference in the VO$_2$swim achieved by the voles in the pre- and post-trials (Figure 2A, C). Surprisingly, the dopamine and noradrenaline reuptake inhibitor (bupropion) had no effect on the VO$_2$swim achieved by either the selected or control bank voles. We hypothesized that even if bupropion did not influence the VO$_2$swim per se, it could change the swimming characteristics. For example, the animals could achieve the same 1-min VO$_2$swim after

bupropion and saline, but the drug could influence their motivation to swim vigorously for a longer time, as the increase in brain dopamine can delay fatigue (Davis and Bailey 1997) However, we did not find a significant effect of the drug on VO$_2$swim calculated for 5-min periods, either. Thus, the results did not allow firm conclusions concerning involvement of dopamine and noradrenaline in evolution of increased aerobic exercise performance in the experimental evolution model system.

Our model animals are not commonly used in pharmacological studies and the data concerning pharmacology and pharmacokinetics of different drugs administered to bank voles are scarce. This raises the question whether the kind of drug, dose, and timing of administration before measuring the response variable were appropriate. However, we chose the drug, dose, and timing that, according to the literature, increases the level of physical activity in a variety of animal species and humans (Cryan et al. 2001; Watson et al. 2005; Sidhpura et al. 2007; Mori et al. 2013). For example, immobility time in forced swimming test performed on mice decreased after intraperitoneal injection (i.p.) of 30 mg/kg bupropion (the drug was administered 30 min before the test; Cryan et al. 2001). In another experiment, locomotor activity of rats increased 20 min after 30 mg/kg bupropion i.p., when compared with saline injection, and the effect lasted for 80 min (Sidhpura et al. 2007). In the same experiment, dopamine concentration in the rat brain (nucleus accumbens) increased after 30 mg/kg bupropion i.p., when compared with base values, achieved a peak after 40 min and returned to basal values over next 20–40 min (Sidhpura et al. 2007). Therefore, we expected that the drug should affect the physical performance of the bank vole, too.

Finally, one could argue that behavior of the voles during the swimming trial is determined by an ability to cope with the stressful situation (cf. Kott et al. 2016). Inadequate coping with stress can, as well as the lack of sufficient motivation, set the actual performance well below the physiological or physical limits. Thus, in our selection model the selection could act on the ability to cope with stress rather than on motivation—or on both of those behavioral mechanisms. Interestingly, female rats showed a stronger stress response to the forced swimming test than males did (Dalla et al. 2008), and in our experiment female voles from the unselected C lines achieved a lower VO$_2$swim compared with males, whereas the pattern was reversed in the A-selected lines (Table 1). Thus, it can be speculated that the stress response indeed limited exercise performance of the unselected voles and the limitation was stronger in females, but

selection resulted in improved stress-coping, which revealed that females have actually a superior aerobic exercise performance. As the selection experiment on bank voles is continued, further investigation of the putative neurophysiological mechanisms underlying the evolution of high VO_2swim is possible, and we will pursue the opportunity both in the direction of stress-coping mechanisms (functioning of hypothalamus–pituitary–adrenals axis) and the motivation mechanisms aspects (using different pharmacological substances as well as direct measurements of monoamines in the brain).

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