UNIVERSITY OF CALIFORNIA
RIVERSIDE

Evolution of Neurobiological and Kinematic Traits in Mice Selectively Bred for High Voluntary Wheel Running

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Evolution, Ecology, and Organismal Biology

by

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December 2016

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ACKNOWLEDGEMENTS

I would like to thank my Advisor, Ted Garland, Jr., for countless hours of advice and discussion, and unending encouragement. I would like to thank my committee members, Tim Higham and Khaleel Razak, for helpful feedback and resources. Many other faculty were also instrumental in completing this dissertation, including Mark Chappell, Wendy Saltzmann, Scott Currie, and Kim Hammond.

I would also like to thank the many members of my lab during the time that I was in Riverside, Chris Oufiero, Tom Meek, Brooke Keeney, Gabe Gartner, Wendy Acosta, Jarren Kay, Layla Hiramatsu, Jennifer Singleton, and Ralph Lacerda de Albuquerque. Special thanks is due to Zoe Thompson, my coauthor on all chapters contained here, who dedicated many hours working through early manuscripts. Many other graduate students (too many to list) provided feedback, advice, and friendship, particularly, Juan Pablo Perea-Rodriguez, Brian Muir, Matt O'Neil, Keenan Morrison, and Chris Wheeler.

Finally, I would like to thank my father, for unconditional support in every endeavor that I undertake. There is no replacement for the confidence that I have gained by your encouragement.

Chapter 1 of this dissertation has been published in Physiology and Behavior and is adapted with permission from Elsevier:

Claghorn, G. C., Fonseca, I. A., Thompson, Z., Barber, C., & Garland, T., Jr. (2016). Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. *Physiol Behav, 161*, 145-154.

ABSTRACT OF THE DISSERTATION

Evolution of Neurobiological and Kinematic Traits in Mice Selectively Bred for High Voluntary Wheel Running

by

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Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, December 2016
Dr. Theodore Garland, Jr., Chairperson

Any type of locomotion requires central activation of motor systems, which can be suppressed by neurochemicals in a phenomena known as Central Fatigue (CF). Resistance to CF may evolve to support extreme locomotor abilities and may also occur in response to exercise training (phenotypic plasticity). I used the High-Runner (HR) mouse model to study CF in four replicate lines that have evolved in response to many generations of selective breeding for voluntary wheel running.

Serotonin is the most studied neurotransmitter in relation to CF. In my work, a serotonin inhibitory autoreceptor antagonist decreased endurance during forced treadmill exercise in HR mice, but not in mice from four non-selected Control (C) lines. Wheel running decreased in HR but not C mice at the highest dose of a serotonin agonist, but was unaffected by the antagonist. Therefore, serotonin signaling affects performance of both forced and voluntary exercise in a genotype-dependent manner.

I also tested effects of sports drinks and caffeine. Red Bull increased distance run voluntarily on wheels in both HR and C lines, but not did caffeine alone in water. Neither Red Bull nor caffeine significantly affected maximal aerobic capacity (VO_2max), but both reduced post-trial tiredness. Gatorade did not affect wheel running or endurance in either line type. These results suggest that caffeine increases voluntary exercise levels of mice via effects on motivation or fatigue resistance, but not VO_2max.

Finally, I analyzed strides of both sexes of HR and C mice on a motorized treadmill at speeds relevant to wheel running. Stance width was narrower in HR than C, and paw contact area and duty factor were greater in mini-muscle individuals (subset of HR mice with 50% reduced hindlimb muscle mass) than in normal-muscled HR or C. Many stride characteristics were affected by six days of wheel access. Thus, stride characteristics are responsive to selective breeding for locomotor behavior and exhibit phenotypic plasticity.

In conclusion, HR mice have evolved neurobiologically to resist serotonin-mediated CF, and morphologically and/or behaviorally to have a narrower stance. These results parallel wild animals that have evolved for, and humans that train for, extreme locomotor performance.

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INTRODUCTION TO THE DISSERTATION

Locomotion is vitally important to most animals, and animals vary greatly in the intensity and duration of locomotion that they perform. Most species that locomote at high intensity for long durations have both physical and neurobiological adaptations to support extreme locomotion. This dissertation explores a few of the adaptations related to fatigue and kinematics that have been gained by house mice, over a relatively short length of evolutionary time (tens of generations), through selective breeding for high voluntary locomotor activity on wheels (Swallow et al. 1998a; Wallace and Garland 2016).

Fatigue

Fatigue has most often been defined as the exercised-induced reduction in the ability of a muscle (or whole organism) to produce power (or maintain exercise) (reviewed in Gandevia 2001). When maximal effort expended voluntarily is less than what can be achieved through induced effort (such as by electrical stimulation of a motor nerve), then it can be said that the motor output is limited centrally (Gandevia 2001). Central fatigue limits the performance of an organism to less than the level that might be predicted by classical models of physiological maxima, such as those invoking catastrophic buildup of waste products in muscles, caused by prolonged exercise at high intensity (Noakes 2011).

This dissertation will primarily consider the monoamine hypothesis of central fatigue, which states that the neurotransmitters serotonin, dopamine, and norepinephrine control motor output by inducing feelings of tiredness, influencing thermoregulatory

function, inhibiting the motor cortex, and/or affecting the rate of circulation (Gandevia 2001; Coimbra et al. 2012 and references therein). Of these neurotransmitters, serotonin has been the most studied with specific reference to central fatigue, although the involvement of dopamine signaling in exercise is well established. Norepinephrine has received much less attention as a potential regulator of fatigue, but manipulations of norepinephrine are known to affect exercise.

Serotonin-mediate central fatigue occurs when, duing the late phase of exhaustive exercise, the ratio of serotonin to dopamine increases in certain brain regions, and in particular the striatum (Blomstrand et al. 1989; Meeusen et al. 2006). This phenomenon, and other neurobiological traits that influence fatigue, can exhibit a plastic response to training (Matsubara et al. 1998; Dwyer and Flynn 2002; Timofeeva et al. 2003; Greenwood et al. 2005; Langfort et al. 2006; Foley and Fleshner 2008; Sim et al. 2008) as well as evolutionary responses to selection, including selective breeding (Rhodes et al. 2003; Bronikowski et al. 2004; Foley et al. 2006; Mathes et al. 2010; Waters et al. 2010; Garland et al. 2011b; Waters et al. 2013). The 5-HT pathways that contribute to fatigue might be modified by training (phenotypic plasticity) or evolution in at least two ways. First, the magnitude of 5-HT release could be altered. This could be caused by neural adaptation (pheontypic plasticity) of the dorsal raphe nucleus (DRN) neurons (such that they become less likely to be hyperactivated), or plastic or evolutionary changes to the afferents of the DRN that would prevent excitation of DRN neurons, or promote inhibition of DRN neurons. Second, increased activity and/or concentration of 5-HT inhibitory autoreceptors or 5-HT transporters (SERT) could occur (Greenwood and

Fleshner 2011). Lastly, the rate-limiting step in the production of 5-HT is tryptophan transport across the blood-brain barrier (reviewed in Newsholme and Blomstrand 2006), so it is conceivable that training or evolution could result in changes in available tryptophan transport molecules. Regardless of the mechanism, it is clear that both exercise training and evolution can alter 5-HT pathways (Greenwood et al. 2005; Rhodes et al. 2005; Foley et al. 2006; Langfort et al. 2006; Girard et al. 2007; Keeney et al. 2012; Saul et al. 2016; Wallace and Garland 2016; Garland et al. 2017).

High endurance and cursoriality

In addition to neurobiological advantage, animals with great endurance have many, well defined physical adaptations that can be grouped into a suite of traits that typify a "cursorial" animal. Many cursorial animals have high endurance capacities, but not all of them do (e.g., the cheetah).

Cursorial animals are generally defined as those that can run long distances swiftly and/or easily (Gregory 1912; Stein and Casinos 1997). To quote Hildebrand (1982), "Animals that travel far, fast, or easily on the ground are said to be cursorial." Morphological features that typify a "cursor" include long, parasagittally-oriented limbs, lightening and lengthening of distal limb elements (often reported as increased metatarsal/femur length ratio), and digitigrade or unguligrade locomotion. Most of the putative cursorial adaptations are conceptually intuitive. For example, all else being equal, longer limbs increase stride length and also increase the velocity of the distal end of the limb compared to a shorter limb (Gregory 1912). Similarly, aligning the limbs in a parasagittal plane (parallel to the plane that divides the body into left and right) aligns the

long axis of limb bones with the forces of gravity and braking-propulsive forces, reducing torsional loading (Biewener 1989; Blob 2001). However, in addition to characterizing "cursors," many of the above-mentioned features are also associated with large body size and also seemingly represent inherent characteristics of particular phylogenetic lineages (clades) (Biewener 1989; Garland and Janis 1993; Carrano 1999). Furthermore, although several correlations between morphology and cursoriality have been observed at the level of broad, interspecific comparisons with mammals (Garland and Janis 1993), relatively few examples exist of apparent biomechanical adaptations that have evolved over microevolutionary time scales, with the exception of domestic dogs and horses (Morales et al. 1998; Bertram et al. 2000; Colborne et al. 2005). Small mammals are typically not thought of as cursors, with the exception of recent attention to the elephant shrew (Seckel and Janis 2008; Lovegrove and Mowoe 2014).

Cursoriality is not a binary condition, but rather one end of a spectrum of locomotor behaviors, performance, and associated morphology and kinematics (Carrano 1999). Therefore, if the characteristics that typify a cursor improve locomotor economy, performance, or safety factors (Alexander 1981), then selection for the increased expression of locomotor behaviors (high speed of locomotion, long daily movement distance, etc.) should, in general, result in some degree of cursor-like morphology and kinematics in any group. For instance, within primates, lineages leading to humans evolved long legs and a very upright and bipedal running posture, along with a suite of skeletal adaptations (Bramble and Lieberman 2004), but humans remain plantigrade and have a relatively small metatarsal/femur ratio compared to quadrupedal cursors. In

another example, elephant shrews have evolved a high metatarsal/femur length ratio, upright posture, and scapular morphology coincident with greater speed than similar-sized mammals, including closely related species (Seckel and Janis 2008; Lovegrove and Mowoe 2014).

Certain domestic animals have been bred to enhance their base cursorial traits. Examples include thoroughbred horses, racing greyhounds, and sled dogs (Poole and Erickson 2011). In horses, artificial selection and/or race training has led to a number of kinematic differences, including decreased duty factor and changes to scapula and leg joint angles during high-speed locomotion (Morales et al. 1998; Galisteo et al. 2001). Similar work in dogs has shown some evidence for kinematic divergence related to selective breeding for different locomotor traits (Bertram et al. 2000; Colborne et al. 2005). However, at least for dogs, these results are at least partially explainable by difference in body mass (Bertram et al. 2000). Thus, there remains the need to demonstrate that the characteristics that constitute a cursorial animal could evolve in disparate lineages or in small animals.

The high-runner mouse model as a study system

The Garland lab maintains an ongoing artificial selection experiment in which mice have been bred for high voluntary wheel-running activity for almost 80 generations (Swallow et al. 1998a; Rhodes et al. 2005; Garland et al. 2011a; Garland et al. 2011b; Wallace and Garland 2016). The source of the original population was an outbred strain (Hsd:ICR) from Harlan-Sprague-Dawley (Indianapolis, Indiana, USA) that was originally developed for the Institute of Cancer Research (ICR; see Hauschka and Mirand 1973). In

the Garland lab, mice were first bred randomly for two generations, then randomly separated into eight closed lines, four of which were subsequently bred for their high voluntary wheel-running behavior (high-runner or HR lines), and four that continued to be bred without regard to amount of running (control or C lines). Each generation, ~600-800 mice are weaned and, when they reach 6-8+ weeks of age, ~600 are wheel-tested for 6 days. During the wheel testing, mice are house individually in standard cages with an attached, rat-sized wheel (1.12 m circumference). Revolutions are counted automatically in 1-minute bins by a photocell counter attached to a computer with software provided by San Diego Instruments. Average wheel revolutions on days 5 and 6 serve as the criterion for selection within the HR lines by use of within-family selection, such that only the highest-running males and the highest-running females within each family are allowed to breed (sibling mating is disallowed in all lines). Mice within the control lines are similarly wheel-tested, but this information has no influence on the likelihood of being chosen to breed. Food and water are available *ad libitum* throughout the course of a generation, and the photoperiod is always set to 12 L:12 D.

HR mice run nearly three times as many wheel revolutions on average as controls, and the absolute number of wheel revolutions appears to have reached a plateau around generation 17-27, depending on line and sex (Garland et al. 2011a; Careau et al. x2013), despite continued selection. The difference in total wheel revolutions is made up of primarily an increase in average speed of running, rather than an increase in the amount of time spent running (Koteja et al. 1999), although male HR mice do run for significantly longer per night than male controls (Garland et al. 2011b).

A number of correlated responses to selection have been described in the HR mice, including increases in two whole-organism measures of exercise capacity, forced treadmill endurance (Meek et al. 2009) and maximal exercise-induced oxygen consumption (Swallow et al. 1998b; Rezende et al. 2006a; Rezende et al. 2006b). HR mice are smaller, leaner, achieve higher speeds during wheel running, and have a reduced incremental cost of transport on a whole-animal basis, although not on a mass-adjusted basis (Swallow et al. 1999; Swallow et al. 2001; Rezende et al. 2006c). Sub-organismal traits that have evolved in HR mice include increased hind limb symmetry and larger femoral heads (Garland and Freeman 2005). HR mice also have increased plasticity in response to wheel access for hemoglobin concentration and hematocrit (Swallow et al. 2005), enzyme activity in the hindlimbs (Houle-Leroy et al. 2000), glycogen storage and glucose transporter type 4 (GLUT-4) plasticity (Gomes et al. 2009), and masses of some skeletal elements (Middleton et al. 2008). HR differ from C mice in circulating hormone concentrations, having higher corticosterone (Malisch et al. 2007; Malisch et al. 2008a; Malisch et al. 2008b; Malisch et al. 2009), lower leptin (Girard et al. 2007), and higher adiponectin (Vaanholt et al. 2007) as compared to C mice (depending on sex and age).

Additionally, the mini-muscle phenotype, caused by a Mendelian recessive allele (Kelly et al. 2013) that was present at a low frequency of about 7% in the original base population, is characterized by a 50% mass reduction in the triceps surae and total hindlimb muscle mass (in homozygotes), has increased in one HR line and eventually had become fixed in another (Garland et al. 2002; Syme et al. 2005). Beyond the difference in muscle mass, the mini-muscle phenotype exhibits double the per-gram oxidative

capacity (Houle-Leroy et al. 2003), elevated heat-shock protein (Belter et al. 2004), increased glycogen in the gastrocnemius (Gomes et al. 2009), altered myosin heavy-chain composition (Guderley et al. 2006), reduced force per-cross sectional area (Syme et al. 2005), and elevated capillarization (Wong et al. 2009). Mini-muscle mice have elevated whole-animal VO_2max when tested in hypoxia (Rezende et al. 2006a).

HR mice have different reactions to diet and to some pharmaceuticals. Male HR mice that were fed Western Diet (WD, TD.88137), which is high in fat with added sucrose, ran up to 75% more than mice fed the standard diet (S), but there was no effect of the diet treatment on wheel running by C mice (Meek et al. 2010; Acosta et al. 2017). No other pharmaceutical or environmental variable had increased wheel running in HR mice to this degree within a single generation (Meek et al. 2010). Ritalin administration decreased wheel-running in HR mice while increasing wheel-running in C mice (Rhodes and Garland 2003). Administration of a type 1 cannabanoid receptor (CB_1) agonist and antagonist had sex-specific differential effects on HR vs. C mice (Keeney et al. 2008; Keeney et al. 2012). Administration of leptin decreased food consumption and body mass in both HR and C mice, but increased wheel running only in HR mice (Meek et al. 2012).

HR mice have larger brains, specifically the midbrain (Kolb 2010), and have widespread, differential (compared to C mice) elevation of brain activity as measured by immediate early genes during exercise and when prevented from exercising (Rhodes et al. 2003; Rhodes et al. 2005). HR and C mice also score differently in some other behaviors (other than wheel running). For example, HR mice exhibit decreased

thermoregulatory nest building during times of wheel access (Carter et al. 2000) and increased predatory aggression (Gammie et al. 2003), but score similarly in open-field locomotion (Bronikowski et al. 2001; Careau et al. 2012).

Dissertation chapters

Chapter 1 explores how 5-HT pharmaceuticals differentially affect endurance in HR and C mice to demonstrate how endurance can evolve with respect to central fatigue. HR mice have evolved greater endurance than their non-selected control counterparts (Meek et al. 2009), they have many described neurobiological differences (Rhodes et al. 2001; Rhodes and Garland 2003; Rhodes et al. 2005; Keeney et al. 2008; Keeney et al. 2012; Saul et al. 2016). We also explored wheel running, which is a critical component of the study, because as a self-paced behavior, it can provide unique insights into the chemical basis of central fatigue (Noakes 2011, 2012). To our knowledge, no prior experiment had used pharmacological manipulations with both voluntary and forced exercise on the same set of animals.

The purpose of chapter 2 was to test the claims of improved athletic performance for two sports drinks, as well as their ingredients alone, primarily caffeine. Caffeine has been shown to increase wheel running in gerbils and mice (Pettijohn 1979; Michna et al. 2003; Lu et al. 2007; Lou et al. 2013) and to improve some measures of endurance exercise in humans (reviewed in Kalmar and Cafarelli 2004; Doherty and Smith 2005). One psychomotor effect of caffeine is competitive inhibition of adenosine receptors in the striatum, a region that integrates signals crucial to the execution of voluntary movements (Fisone et al. 2004). There are known evolved differences in the striatum of HR mice,

namely that the striatum has been shown to respond differently between HR and C mice when wheel access is removed (Rhodes et al. 2003; Saul et al. 2016), and a preliminary study of one HR and one C line indicates differences in monoamine concentrations (Waters et al. 2013). No previous study has examined the effect of Red Bull or Gatorade specifically on an animal model that is genetically predisposed for high high motivation to exercise and also has high exercise abilities, such as the HR mice.

In the final chapter, we tested for potential microevolutionary changes in stride characteristics of mice from the long-term High Runner (HR) mouse selection experiment (Swallow et al. 1998a; Careau et al. 2013). Prior to this work, no kinematic analyses of HR mice had been published, primarily due to experimental complications of capturing quality kinematic data from large numbers of animals as small as house mice. We used the DigiGait Imaging System (Mouse Specifics, Inc.) to accomplish high-throughput phenotyping and allow comparison of the 2-dimensional stride characteristics of both sexes and all eight lines of HR and C mice running on a treadmill at speeds that are typically seen during voluntary wheel running. We obtained estimates of stride length, stride frequency, stance time, swing time, duty factor, brake time, and propel time. We also measured stance width as a proxy for running posture along the sprawled/erect spectrum, and paw contact area as an index of foot posture along the plantigrade/digitigrade or possibly crouched/erect continuum. Finally, we measured hindfoot sizes by digital photography for each individual that was treadmill tested.

References:

Acosta, W., T. H. Meek, H. Schutz, E. M. Dlugosz, and T. Garland Jr. 2017. Preference for Western diet coadapts in High Runner mice and affects voluntary exercise and spontaneous physical activity in a genotype-dependent manner. Behavioural Processes 135:56-65.

Alexander, R. M. 1981. Factors of safety in the structure of animals. Sci Prog 67:109-130.

Belter, J. G., H. V. Carey, and T. Garland, Jr. 2004. Effects of voluntary exercise and genetic selection for high activity levels on HSP72 expression in house mice. Journal of Applied Physiology 96:1270-1276.

Bertram, J. E., D. V. Lee, H. N. Case, and R. J. Todhunter. 2000. Comparison of the trotting gaits of Labrador Retrievers and Greyhounds. American Journal of Veterinary Research 61:832-838.

Biewener, A. A. 1989. Scaling body support in mammals - Limb posture and muscle mechanics. Science 245:45-48.

Blob, R. W. 2001. Evolution of hindlimb posture in nonmammalian therapsids: biomechanical tests of paleontological hypotheses. Paleobiology 27:14-38.

Blomstrand, E., D. Perrett, M. Parrybillings, and E. A. Newsholme. 1989. Effect of sustained exercise on plasma amino-acid concentrations and on 5-hydroxytryptamine metabolism in 6 different brain-regions in the rat. Acta Physiologica Scandinavica 136:473-481.

Bramble, D. M. and D. E. Lieberman. 2004. Endurance running and the evolution of Homo. Nature 432:345-352.

Bronikowski, A. M., P. A. Carter, J. G. Swallow, I. A. Girard, J. S. Rhodes, and T. Garland, Jr. 2001. Open-field behavior of house mice selectively bred for high voluntary wheel-running. Behavior Genetics 31:309-316.

Bronikowski, A. M., J. S. Rhodes, T. Garland, T. A. Prolla, T. A. Awad, and S. C. Gammie. 2004. The evolution of gene expression in mouse hippocampus in response to selective breeding for increased locomotor activity. Evolution 58:2079-2086.

Careau, V., O. R. P. Bininda-Emonds, G. Ordonez, and T. Garland, Jr. 2012. Are Voluntary Wheel Running and Open-Field Behavior Correlated in Mice? Different Answers from Comparative and Artificial Selection Approaches. Behavior Genetics.

Careau, V., M. E. Wolak, P. A. Carter, and T. Garland, Jr. 2013. Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection. Evolution 67:3102-3119.

Careau, V., M. E. Wolak, P. A. Carter, and T. Garland, Jr. x2013. Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection. Evolution.

Carrano, M. T. 1999. What, if anything, is a cursor? Categories versus continua for determining locomotor habit in mammals and dinosaurs. J. Zool. 247:29-42.

Carter, P. A., J. G. Swallow, S. J. Davis, and T. Garland, Jr. 2000. Nesting behavior of house mice (Mus domesticus) selected for increased wheel-running activity. Behavior Genetics 30:85-94.

Coimbra, C. C., D. D. Soares, and L. H. R. Leite. 2012. The Involvement of Brain Monoamines in the Onset of Hyperthermic Central Fatigue *in* K. R. Zaslav, ed. An International Perspective on Topics in Sports Medicine and Sports Injury. InTech.

Colborne, G. R., J. F. Innes, E. J. Comerford, M. R. Owen, and C. J. Fuller. 2005. Distribution of power across the hind limb joints in Labrador Retrievers and Greyhounds. American Journal of Veterinary Research 66:1563-1571.

Doherty, M. and P. M. Smith. 2005. Effects of caffeine ingestion on rating of perceived exertion during and after exercise: a meta-analysis. Scandinavian Journal of Medicine & Science in Sports 15:69-78.

Dwyer, D. and J. Flynn. 2002. Short term aerobic exercise training in young males does not alter sensitivity to a central serotonin agonist. Experimental Physiology 87:83-89.

Fisone, G., A. Borgkvist, and A. Usiello. 2004. Caffeine as a psychomotor stimulant: mechanism of action. CMLS, Cell. Mol. Life Sci. 61:857-872.

Foley, T. E. and M. Fleshner. 2008. Neuroplasticity of Dopamine Circuits After Exercise: Implications for Central Fatigue. NeuroMolecular Medicine 10:67-80.

Foley, T. E., B. N. Greenwood, H. E. W. Day, L. G. Koch, S. L. Britton, and M. Fleshner. 2006. Elevated central monoamine receptor mRNA in rats bred for high endurance capacity: Implications for central fatigue. Behavioural Brain Research 174:132-142.

Galisteo, A. M., J. L. Morales, M. R. Cano, F. Miró, E. Agüera, and J. Vivo. 2001. Inter-breed differences in equine forelimb kinematics at the walk. Journal of Veterinary Medicine Series A 48:277-285.

Gammie, S. C., N. S. Hasen, J. S. Rhodes, I. Girard, and T. Garland, Jr. 2003. Predatory aggression, but not maternal or intermale aggression, is associated with high voluntary wheel-running behavior in mice. Horm Behav 44:209-221.

Gandevia, S. C. 2001. Spinal and supraspinal factors in human muscle fatigue. Physiological Reviews 81:1725-1789.

Garland, T. and P. W. Freeman. 2005. Selective breeding for high endurance running increases hindlimb symmetry. Evolution 59:1851-1854.

Garland, T., Jr. and C. M. Janis. 1993. Does metatarsal/femur ratio predict maximal running speed in cursorial mammals? J. Zool. 229:133-151.

Garland, T., Jr., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011a. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proceedings of the Royal Society B-Biological Sciences 278:574-581.

Garland, T., Jr., M. T. Morgan, J. G. Swallow, J. S. Rhodes, I. Girard, J. G. Belter, and P. A. Carter. 2002. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267-1275.

Garland, T., Jr., H. Schutz, M. A. Chappell, B. K. Keeney, T. H. Meek, L. E. Copes, W. Acosta, C. Drenowatz, R. C. Maciel, G. van Dijk, C. M. Kotz, and J. C. Eisenmann. 2011b. The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. Journal of Experimental Biology 214:206-229.

Garland, T., Jr., M. Zhao, and W. Saltzman. 2017. Hormones and the evolution of complex traits: insights from artificial selection on behavior. Integrative and Comparative Biology 57.

Girard, I., E. L. Rezende, and T. Garland, Jr. 2007. Leptin levels and body composition of mice selectively bred for high voluntary locomotor activity. Physiol. Biochem. Zool. 80:568-579.

Gomes, F. R., E. L. Rezende, J. L. Malisch, S. K. Lee, D. A. Rivas, S. A. Kelly, C. Lytle, B. B. Yaspelkis, and T. Garland, Jr. 2009. Glycogen storage and muscle glucose transporters (GLUT-4) of mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:238-248.

Greenwood, B. N. and M. Fleshner. 2011. Exercise, Stress Resistance, and Central Serotonergic Systems. Exercise and Sport Sciences Reviews 39:140-149.

Greenwood, B. N., T. E. Foley, H. E. W. Day, D. Burhans, L. Brooks, S. Campeau, and M. Fleshner. 2005. Wheel running alters serotonin (5-HT) transporter, 5-HT1A, 5-HT1B, and alphal(1b)-adrenergic receptor mRNA in the rat raphe nuclei. Biol. Psychiatry 57:559-568.

Gregory, W. K. 1912. Notes on the priciples of quadrupedal locomotion and on the mechanism of the limbs in hoofed animals. Annals of the New York Academy of Sciences 22:267-294.

Guderley, H., P. Houleleroy, G. Diffee, D. Camp, and T. Garland, Jr. 2006. Morphometry, ultrastructure, myosin isoforms, and metabolic capacities of the "mini muscles" favoured by selection for high activity in house mice. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 144:271-282.

Hauschka, T. S. and E. A. Mirand. 1973. The "breeder: HA(ICR)" Swiss mouse, a multipurpose stock selected for fecundity. Pp. 319-331 *in* D. P. G. P. Murphy, and E. A. Mirand, ed. Perspectives in cancer research and treatment. Alan R. Liss, Inc, New York.

Hildebrand, M. 1982. Analysis of vertebrate structure. John Wiley and Sons, New York.

Houle-Leroy, P., T. Garland, Jr., J. G. Swallow, and H. Guderley. 2000. Effects of voluntary activity and genetic selection on muscle metabolic capacities in house mice Mus domesticus. Journal of Applied Physiology 89:1608-1616.

Houle-Leroy, P., H. Guderley, J. G. Swallow, and T. Garland, Jr. 2003. Artificial selection for high activity favors mighty mini-muscles in house mice. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 284:R433-R443.

Kalmar, J. M. and E. Cafarelli. 2004. Caffeine: A valuable tool to study central fatigue in humans? Exercise and Sport Sciences Reviews 32:143-147.

Keeney, B. K., T. H. Meek, K. M. Middleton, L. F. Holness, and T. Garland, Jr. 2012. Sex differences in cannabinoid receptor-1 (CB1) pharmacology in mice selectively bred for high voluntary wheel-running behavior. Pharmacology Biochemistry and Behavior 101:528-537.

Keeney, B. K., D. A. Raichlen, T. H. Meek, R. S. Wijeratne, K. M. Middleton, G. L. Gerdeman, and T. Garland. 2008. Differential response to a selective cannabinoid

receptor antagonist (SR141716: rimonabant) in female mice from lines selectively bred for high voluntary wheel-running behaviour. Behav. Pharmacol. 19:812-820.

Kelly, S. A., T. A. Bell, S. R. Selitsky, R. J. Buus, K. Hua, G. M. Weinstock, T. Garland, Jr., F. Pardo-Manuel de Villena, and D. Pomp. 2013. A novel intronic single nucleotide polymorphism in the *myosin heavy polypeptide 4* gene is responsible for the mini-muscle phenotype characterized by major reduction in hind-limb muscle mass in mice. Genetics 195:1385-1395.

Kolb, E. M. 2010. Neurobiological and Physiological Underpinnings of High Voluntary Wheel Running. Pp. xii + 228. Unpublished Ph.D. Dissertation. University of California, Riverside, Riverside, California.

Koteja, P., T. Garland, Jr., J. K. Sax, J. G. Swallow, and P. A. Carter. 1999. Behaviour of house mice artificially selected for high levels of voluntary wheel running. Animal Behaviour 58:1307-1318.

Langfort, J., E. Baranczuk, D. Pawlak, M. Chalimoniuk, N. Lukacova, J. Marsala, and J. Gorski. 2006. The effect of endurance training on regional serotonin metabolism in the brain during early stage of detraining period in the female rat. Cellular and Molecular Neurobiology 26:1327-1342.

Lou, Y., Q. Peng, T. Li, B. Nolan, J. J. Bernard, G. C. Wagner, Y. Lin, W. J. Shih, A. H. Conney, and Y. Lu. 2013. Oral Caffeine During Voluntary Exercise Markedly Inhibits Skin Carcinogenesis and Decreases Inflammatory Cytokines in UVB-Treated Mice. Nutrition and Cancer 65:1002-1013.

Lovegrove, B. G. and M. Mowoe. 2014. The evolution of micro-cursoriality in mammals. The Journal of Experimental Biology 217:1316-1325.

Lu, Y.-P., B. Nolan, Y.-R. Lou, Q.-Y. Peng, G. C. Wagner, and A. H. Conney. 2007. Voluntary exercise together with oral caffeine markedly stimulates UVB light-induced apoptosis and decreases tissue fat in SKH-1 mice. Proceedings of the National Academy of Sciences 104:12936-12941.

Malisch, J. L., C. W. Breuner, F. R. Gomes, M. A. Chappell, and T. Garland, Jr. 2008a. Circadian pattern of total and free corticosterone concentrations, corticosteroid-binding globulin, and physical activity in mice selectively bred for high voluntary wheel-running behavior. Gen. Comp. Endocrinol. 156:210-217.

Malisch, J. L., C. W. Breuner, E. M. Kolb, H. Wada, R. M. Hannon, M. A. Chappell, K. M. Middleton, and T. Garland, Jr. 2008b. Behavioral despair and home-cage activity in mice with chronically clevated baseline borticosterone concentrations. Pp. 192-201. Behavior Genetics.

Malisch, J. L., S. A. Kelly, A. Bhanvadia, K. M. Blank, R. L. Marsik, E. G. Platzer, and T. Garland, Jr. 2009. Lines of mice with chronically elevated baseline corticosterone levels are more susceptible to a parasitic nematode infection. Zoology 112:316-324.

Malisch, J. L., W. Saltzman, F. R. Gomes, E. L. Rezende, D. R. Jeske, and T. Garland, Jr. 2007. Baseline and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. Physiol. Biochem. Zool. 80:146-156.

Mathes, W. F., D. L. Nehrenberg, R. Gordon, K. Hua, T. Garland, Jr, and D. Pomp. 2010. Dopaminergic dysregulation in mice selectively bred for excessive exercise or obesity. Behavioural Brain Research 210:155-163.

Matsubara, F., M. Yoshida, and M. Tanaka. 1998. Effect of training on dopamine metabolism and appearance of c-fos in rat brain. Japanese Journal of Physical Fitness and Sports Medicine 47:333-338.

Meek, T. H., E. M. Dlugosz, K. T. Vu, and T. Garland, Jr. 2012. Effects of leptin treatment and western diet on wheel running in selectively bred high runner mice. Physiology & Behavior 106:252-258.

Meek, T. H., J. C. Eisenmann, and T. Garland. 2010. Western diet increases wheel running in mice selectively bred for high voluntary wheel running. International Journal of Obesity 34:960-969.

Meek, T. H., B. P. Lonquich, R. M. Hannon, and T. Garland. 2009. Endurance capacity of mice selectively bred for high voluntary wheel running. J. Exp. Biol. 212:2908-2917.

Meeusen, R., P. Watson, H. Hasegawa, B. Roelands, and M. F. Piacentini. 2006. Central fatigue - The serotonin hypothesis and beyond. Sports Medicine 36:881-909.

Michna, L., Y.-P. Lu, Y.-R. Lou, G. C. Wagner, and A. H. Conney. 2003. Stimulatory effect of oral administration of green tea and caffeine on locomotor activity in SKH-1 mice. Life Sciences 73:1383-1392.

Middleton, K. M., S. A. Kelly, and T. Garland, Jr. 2008. Selective breeding as a tool to probe skeletal response to high voluntary locomotor activity in mice. Integrative and Comparative Biology 48:394-410.

Morales, J., M. Manchado, J. Vivo, A. Galisteo, E. Agüera, and F. Miró. 1998. Angular kinematic patterns of limbs in elite and riding horses at trot. Equine Veterinary Journal 30:528-533.

Newsholme, E. A. and E. Blomstrand. 2006. Branched-chain amino acids and central fatigue. Journal of Nutrition 136:274S-276S.

Noakes, T. D. 2011. Time to move beyond a brainless exercise physiology: the evidence for complex regulation of human exercise performance. Applied Physiology Nutrition and Metabolism-Physiologie Appliquee Nutrition Et Metabolisme 36:23-35.

Noakes, T. D. 2012. The Central Governor Model in 2012: eight new papers deepen our understanding of the regulation of human exercise performance. British Journal of Sports Medicine 46:1-3.

Pettijohn, T. F. 1979. Effects of alcohol and caffeine on wheel running activity in the Mongolian gerbil. Pharmacology Biochemistry and Behavior 10:339-341.

Poole, D. C. and H. H. Erickson. 2011. Highly athletic terrestrial mammals: Horses and dogs. Comprehensive Physiology 1:1-37.

Rezende, E. L., T. Garland, Jr., M. A. Chappell, J. L. Malisch, and F. R. Gomes. 2006a. Maximum aerobic performance in lines of *Mus* selected for high wheel-running activity: effects of selection, oxygen availability and the mini-muscle phenotype. Journal of Experimental Biology 209:115-127.

Rezende, E. L., F. R. Gomes, J. L. Malisch, M. A. Chappell, and T. Garland, Jr. 2006b. Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. Journal of Applied Physiology 101:477-485.

Rezende, E. L., S. A. Kelly, F. R. Gomes, M. A. Chappell, and T. Garland, Jr. 2006c. Effects of size, sex, and voluntary running speeds on costs of locomotion in lines of laboratory mice selectively bred for high wheel-running activity. Physiol. Biochem. Zool. 79:83-99.

Rhodes, J., G. Hosack, I. Girard, A. Kelley, G. S. Mitchell, and T. Garland, Jr. 2001. Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology 158:120-131.

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integrative and Comparative Biology 45:438-455.

Rhodes, J. S. and T. Garland, Jr. 2003. Differential sensitivity to acute administration of Ritalin, apormorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology 167:242-250.

Rhodes, J. S., T. Garland, Jr., and S. C. Gammie. 2003. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behav. Neurosci. 117:1243-1256.

Saul, M., P. Majdak, S. Perez, M. Reilly, T. Garland, Jr., and J. S. Rhodes. 2016. High motivation for exercise is associated with altered chromatin regulators of monoamine receptor gene expression in the striatum of selectively bred mice. Genes Brain Behav.

Seckel, L. and C. Janis. 2008. Convergences in scapula morphology among small cursorial mammals: an osteological correlate for locomotory specialization. Journal of Mammalian Evolution 15:261-279.

Sim, Y.-J., H. Kim, M.-S. Shin, H.-K. Chang, M.-C. Shin, I.-G. Ko, K.-J. Kim, T.-S. Kim, B.-K. Kim, Y.-T. Rhim, S. Kim, H.-Y. Park, J.-W. Yi, S.-J. Lee, and C.-J. Kim. 2008. Effect of postnatal treadmill exercise on c-Fos expression in the hippocampus of rat pups born from the alcohol-intoxicated mothers. Brain and Development 30:118-125.

Stein, B. R. and A. Casinos. 1997. What is a cursorial mammal? J. Zool. 242:185-192.

Swallow, J. G., P. A. Carter, and T. Garland, Jr. 1998a. Artificial selection for increased wheel-running behavior in house mice. Behavior Genetics 28:227-237.

Swallow, J. G., T. Garland, Jr., P. A. Carter, W. Z. Zhan, and G. C. Sieck. 1998b. Effects of voluntary activity and genetic selection on aerobic capacity in house mice (*Mus domesticus*). Journal of Applied Physiology 84:69-76.

Swallow, J. G., T. Garland, Jr., P. Koteja, and P. A. Carter. 1999. Locomotor activity, food consumption, and body composition in house mice selected for high wheel-running activity. Faseb Journal 13:A419-A419.

Swallow, J. G., P. Koteja, P. A. Carter, and T. Garland, Jr. 2001. Food consumption and body composition in mice selected for high wheel-running activity. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 171:651-659.

Swallow, J. G., J. S. Rhodes, and T. Garland, Jr. 2005. Phenotypic and evolutionary plasticity of organ masses in response to voluntary exercise in house mice. Integrative and Comparative Biology 45:426-437.

Syme, D. A., K. Evashuk, B. Grintuch, E. L. Rezende, and T. Garland, Jr. 2005. Contractile abilities of normal and "mini" triceps surae muscles from mice (*Mus domesticus*) selectively bred for high voluntary wheel running. Journal of Applied Physiology 99:1308-1316.

Timofeeva, E., Q. Huang, and D. Richard. 2003. Effects of Treadmill Running on Brain Activation and the Corticotropin-Releasing Hormone System. Neuroendocrinology 77:388-405.

Vaanholt, L., P. Meerlo, T. Garland, Jr., G. Visser, and G. v. Dijk. 2007. Plasma Adiponectin is Increased in Mice Selectively Bred for High Wheel-running Activity, but not by Wheel Running per sé. Hormone and Metabolic Research 39:377-383.

Wallace, I. J. and T. Garland, Jr. 2016. Mobility as an emergent property of biological organization: Insights from experimental evolution. Evolutionary Anthropology: Issues, News, and Reviews 25:98-104.

Waters, R. P., R. B. Pringle, G. L. Forster, K. J. Renner, J. L. Malisch, T. Garland, Jr. , and J. G. Swallow. 2013. Selection for increased voluntary wheel-running affects behavior and brain monoamines in mice. Brain Research 1508:9-22.

Waters, R. P., K. J. Renner, C. H. Summers, M. J. Watt, G. L. Forster, L. G. Koch, S. L. Britton, and J. G. Swallow. 2010. Selection for intrinsic endurance modifies endocrine stress responsiveness. Brain Research 1357:53-61.

Wong, L. E., T. Garland, Jr., S. L. Rowan, and R. T. Hepple. 2009. Anatomic capillarization is elevated in the medial gastrocnemius muscle of mighty mini mice. Journal of Applied Physiology 106:1660-1667.

Chapter 1

Serotonin-Mediated Central Fatigue Underlies Increased Endurance Capacity in Mice From Lines Selectively Bred for High Voluntary Wheel Running

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) is implicated in central fatigue, and 5-HT_{1A} pharmaceuticals are known to influence locomotor endurance in both rodents and humans. We studied the effects of a 5-HT_{1A} agonist and antagonist on both forced and voluntary exercise in the same set of mice. This cohort of mice was taken from 4 replicate lines of mice that have been selectively bred for high levels of voluntary wheel running (HR) as compared with 4 non-selected control (C) lines. HR mice run voluntarily on wheels about 3x as many revolutions per day as compared with C, and have greater endurance during forced treadmill exercise. We hypothesized that drugs targeting serotonin receptors would have differential effects on locomotor behavior of HR and C mice. Subcutaneous injections of a 5-HT_{1A} antagonist (WAY-100,635), a combination of 5-HT_{1A} agonist and a 5-HT_{1A} /$_{1B}$ partial agonist (8-OH-DPAT + pindolol), or physiological saline were given to separate groups of male mice before the start of each of three treadmill trials. The same manipulations were used later during voluntary wheel running on three separate nights. WAY-100,635 decreased treadmill endurance in HR but not C mice (dose by linetype interaction, $P = 0.0014$). 8-OH-DPAT + pindolol affected treadmill endurance ($P < 0.0001$) in a dose-dependent manner, with no dose by linetype interaction. Wheel running was reduced in HR but not C mice at the highest dose of 8-OH-DPAT + pindolol (dose by linetype, $P = 0.0221$), but was not affected by WAY-100,635 treatment. These results provide further evidence that serotonin signaling is an important determinant of performance during both forced and voluntary exercise. Although the elevated wheel running of HR mice does not appear related to alterations in

serotonin signaling, their enhanced endurance capacity does. More generally, our results indicate that both forced and voluntary exercise can be affected by an intervention that acts (primarily) centrally.

INTRODUCTION

When maximal effort expended voluntarily is less than what can be achieved through induced effort (such as by electrical stimulation of a motor nerve), then it can be said that the motor output is limited centrally (Gandevia 2001). Central fatigue potentially limits the performance of an organism to less than the level that might be predicted by classical models of physiological maxima, and can be caused by reduced drive to motor neurons from the central nervous system. The monoamine hypothesis of central fatigue posits that the balance of neurotransmitters (including serotonin [5-hydroxytryptamine; 5-HT], dopamine [DA], and norepinephrine [NE]) influences thermoregulation, motor impulses, and sensations of fatigue, thus contributing to central control of the duration and intensity of exercise (reviewed in Meeusen et al. 2006). Of these neurotransmitters, serotonin has been the most studied with specific reference to central fatigue, although the effect of dopamine receptor agonists and transport inhibitors during exercise is well established (Balthazar et al. 2010; Roelands and Meeusen 2010). The predictive factor in 5-HT-generated central fatigue may not be 5-HT concentration alone, but the ratio of 5-HT:DA in such brain regions as the striatum (Meeusen et al. 2006). The relevance of that ratio may be because 5-HT appears to indirectly and directly regulate dopamine at multiple levels (reviewed in Olijslagers et al. 2006).

Although the results of pharmaceutical 5-HT manipulations in human athletes have been inconsistent (Meeusen et al. 2006), pharmacological studies with rodents support the monoamine hypothesis of central fatigue. Pharmacological manipulations of rat 5-HT receptors (Bailey et al. 1993; Ahlenius et al. 1997) and central injection of 5-HT

precursor L-Tryptophan (Soares et al. 2003; Soares et al. 2007) are sufficient to influence the performance of forced exercise at the whole-animal level. Administration of the 5-HT_3 agonist quipazine dimaleate decreased time to exhaustion in rats running on a motorized treadmill (with physical prodding for motivation), whereas 5-HT_2 antagonist LY 53857 increased time to exhaustion (Bailey et al. 1993). At exhaustion, agonist-treated animals had higher plasma glucose, liver glycogen, and muscle glycogen than vehicle-treated animals suggesting that they fatigued before they depleted energy stores. In a similar study with rats, agonists of 5-HT_{1A} receptors increased endurance, whereas an antagonist of 5-HT_{1A} receptors caused early exhaustion (Ahlenius et al. 1997). Numerous studies of rats have implicated the release of 5-HT in the onset of fatigue (Chaouloff et al. 1985; Blomstrand et al. 1989; Newsholme and Blomstrand 2006). 5-HT is released to multiple brain regions, and specific downstream effects can differ depending on brain region and receptor subtype (the functions of 5-HT in the brain are reviewed in Jacobs and Azmitia 1992; Muller and Jabcobs 2010; Deneris and Wyler 2012; Homberg 2012). 5-HT receptors in the spinal cord also have important functions related to exercise. 5-HT receptors can be found in the dorsal horn of the spinal cord, and affect the excitability of motor neurons (Bedard et al. 1987; Zhang 1991; Alvarez et al. 1998). Despite its apparent relation to fatigue, 5-HT has been shown to promote excitability in motor neurons in small concentrations (Wang and Dun 1990; Grunnet et al. 2004), but high concentrations spill over onto initial axon segments, and can inhibit action potentials in motor neurons (Cotel et al. 2013; Perrier and Cotel 2015). Finally, many authors assert

that the primary connection between 5-HT and fatigue is through its effects on thermoregulation (e.g., Coimbra et al. 2012).

The relationship between maximal exercise capacity and voluntary locomotor activity is unclear. The measures of treadmill endurance and voluntary wheel running are not correlated in rodent studies of individual variation (Friedman et al. 1992; Lambert et al. 1996) or strain variation (Lerman et al. 2002; Lightfoot et al. 2004). However, selective breeding for high voluntary wheel running in mice has resulted in increased treadmill endurance as compared with non-selected controls (Meek et al. 2009), and bidirectional selective breeding for treadmill endurance (one line selected for high endurance vs. one line selected for low endurance) has resulted in differential voluntary wheel running (rats from the high-endurance line run more on wheels than those from the low-endurance line) (Waters et al. 2008). Rodents with wheel access can effectively train themselves to perform better at forced treadmill tasks (Lambert and Noakes 1990), and can have altered 5-HT receptor mRNA expression, including a reduction in 5-HT_{1A} mRNA after 6 weeks of wheel access (Greenwood et al. 2005).

Mice from lines that have been selectively bred for high voluntary wheel running (high-runner or HR) run about three times as many wheel revolutions per day as controls (C). The absolute number of revolutions run per day reached a plateau around generation 16-28 depending on line and sex despite continued selective breeding (Careau et al. 2013). The difference in total wheel revolutions is caused primarily by an increase in average speed of running, rather than an increase in the amount of time spent running

(Koteja et al. 1999), although male HR mice do run for significantly longer per night than male controls (Garland et al. 2011b). A number of correlated responses to selection have been described in the HR mice, including increases in two whole-organism measurements of exercise capacity, forced treadmill endurance (Meek et al. 2009) and maximal exercise-induced oxygen consumption (Swallow et al. 1998b; Rezende et al. 2006a; Rezende et al. 2006b). HR differ from C mice in circulating hormone concentrations, having higher corticosterone (Malisch et al. 2007; Malisch et al. 2008a; Malisch et al. 2008b; Malisch et al. 2009), lower leptin (Girard et al. 2007), and higher adiponectin (Vaanholt et al. 2007) as compared with C mice (depending on sex and age). HR mice have larger brains, specifically the midbrains (Kolb et al. 2013), and have widespread, differential (compared to C mice) elevation of brain activity as measured by immediate early genes during exercise and when prevented from exercising (Rhodes et al. 2003; Rhodes et al. 2005). HR mice often have different reactions to environmental conditions and pharmaceuticals. Ritalin administration decreased wheel-running in HR mice while increasing wheel-running in C mice (Rhodes and Garland 2003). Administration of a type 1 cannabinoid receptor (CB_1) agonist and antagonist had sex-specific differential effects on HR vs. C mice (Keeney et al. 2008; Keeney et al. 2012). Administration of leptin increased wheel running only in HR mice (Meek et al. 2012).

The purpose of the present study was to determine if a 5-HT pharmaceutical manipulation would differentially affect endurance in mice with an innately high endurance capacity as compared with "standard" mice, and to shed light on how endurance can evolve with respect to central fatigue. The high-runner mouse model is

particularly well suited for study of the evolution of central fatigue because (1) the HR mice have evolved greater endurance than their non-selected control counterparts (Meek et al. 2009), (2) they have many described neurobiological differences, including differential responses to various pharmaceuticals (Rhodes et al. 2001; Rhodes and Garland 2003; Rhodes et al. 2005; Keeney et al. 2008; Keeney et al. 2012) and leptin (Meek et al. 2012), (3) they are well studied in terms of physiological adaptations to high activity as well as patterns of behavior, and (4) methodologically, there is an established paradigm for studying these mice as they exercise voluntarily, pacing themselves through an entire night of high-speed wheel running. Furthermore, the model contains 4 replicate selected lines and 4 non-selected control lines, which allows considerable power of inference as compared with most vertebrate selection experiments (Rhodes and Kawecki 2009; Swallow et al. 2009). To our knowledge, no experiment has used pharmacological manipulations with voluntary and forced exercise on the same set of animals. Because wheel running is self-paced, it can provide unique insights into the chemical basis of central fatigue.

METHODS

Animals

We used male mice (*Mus domesticus*) from post-selection generation 65 of the ongoing High-Runner selection experiment (Swallow et al. 1998a; Garland et al. 2011a). In brief, the experiment started with outbred Hsd:ICR stock (Harlan Sprague Dawley, Indianapolis, Indiana, USA), from which four replicate lines were selectively bred for

high voluntary wheel running on days 5 and 6 of a 6-day period of wheel access (High Runner or HR lines) while four other lines were bred without respect to running (Control or C lines). Mice are weighed, toe-clipped, and weaned at 21 days of age, and housed 4 per cage by sex and line. At 6-8 weeks of age, mice are individually housed in cages with wheel access (wheel circumference 1.12 m) for 6 days. Wheel revolutions are recorded in 1-minute intervals by a photocell counter attached to the wheel and compiled via customized software (photocells and software from San Diego Instruments, San Diego, California, USA). Breeders in the HR lines are identified as the mice that run the largest number of wheel revolutions on days 5 and 6 of the trial, whereas C mice are bred randomly, except that no sibling pairings are allowed in any line. In all selection generations and experiments, mice are kept on a 12-hour light/dark cycle with ad lib food and water.

Ninety-six male mice were obtained at weaning, with 6 mice from each of the 8 lines making 48 mice for each of the two treatment (drug) types. Where possible, no siblings were placed within the same treatment groups. Mice were housed 4 per cage from the time of weaning, through the treadmill testing, then housed singly during wheel testing. Throughout the experiment, mice were kept on a 12-hour light/dark cycle (lights on 06:00 h and lights off 18:00 h) with ad lib food and water. Assignment to each drug type, the order or dose treatments, the order of treadmill testing, and the placement on wheels were randomized, and experimenters were blind to line, linetype (HR or C), and dose where appropriate. Treadmill trials occurred when mice were approximately 6-8 weeks of age, and wheel testing occurred when mice were 8-11 weeks of age. Animal

procedures were approved by the University Institutional Animal Care and Use Committee (UCR IACUC AUP# A-20110014), and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drug protocol

Half of all of the mice were designated to receive subcutaneous injections of 5-HT_{1A} agonist 8-Hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT) + 5-$HT_{1A/1B}$ partial agonist 1-(1H-Indol-4-yloxy)-3-[(1-methylethyl)amino]-2-propanol (pindolol), and half received a 5-HT_{1A} antagonist N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate (WAY-100,635; all drugs obtained from Tocris Bioscience, Bristol, UK). The mice received a subcutaneous injection of the designated drug 15 minutes prior to the start of each of three treadmill trials, and in the middle of voluntary wheel running on three separate nights. There were more than two weeks of rest between treadmill and wheel testing, and within each set of tests, 2 days of rest between repeated injections.

8-OH-DPAT has previously been shown to increase endurance and WAY-100,635 decreased endurance measured during forced running on a motorized drum in rats (Ahlenius et al. 1997). 8-OH-DPAT is a standard drug used in studying the function of the 5-HT_{1A} receptor, but also has slight affinity for the 5-HT_7 receptor and may also act as a 5-HT reuptake inhibitor (Assie and Koek 1996). 8-OH-DPAT was previously shown to increase endurance in low doses, but decrease endurance at high doses (Ahlenius et al. 1997), but with the addition of 5-$HT_{1A/1B}$ antagonist and partial β_3 agonist pindolol, the

biphasic effect disappeared and the endurance continued to increase with increasing doses of 8-OH-DPAT. For this reason, pindolol was added to 8-OH-DPAT. WAY-100,635 has also been a standard drug used in studying the function of the 5-HT_{1A} receptor, but it was discovered that it also is a potent D_4 agonist (Chemel et al. 2006), and the behavioral effects of WAY-100,635 as a D_4 agonist have not been well defined (but see Marona-Lewicka and Nichols 2009, 2011). Systemic injection is perhaps not ideal for studying the manipulation of 5-HT receptors, given that the 5-HT receptors, including 5-HT_{1A}, are widespread and it is possible that the drugs have unintended effects beyond the central nervous system. However, in order to test our specific hypothesis regarding the effects of selective breeding, a large number of measurements from a large number of animals were required, making more targeted techniques such as central injection unfeasible.

Each drug was prepared at a constant concentration, and injection volumes were determined for each mouse based on body mass, ranging from 0.27-0.51 ml for the 8-OH-DPAT + pindolol group and 0.17-0.29 ml for the WAY-100,635 group. For 8-OH-DPAT, the dose was 0.2 mg/kg body weight of 8-OH-DPAT and 0.8 mg/kg of pindolol for the low dose. The high dose was 2.0 mg/kg 8-OH-DPAT and 8.0 mg/kg of pindolol. For WAY-100,635, the dose was 35 μg/kg for the low dose and 350 μg/kg for the high dose. These doses approximate the cited study of rats (Ahlenius et al. 1997), while accounting for the difference in average body mass between our mice (about 32 g) and the rats in the cited study (about 300 g), and assuming an allometric relationship of metabolic rate with exponent = 0.75 (Boxenbaum 1982). All drugs were diluted with physiological saline, with pindolol being first dissolved in a very small volume of glacial acetic acid to aid

dissolution, following Ahlenius (1997). Thus, saline was the vehicle for both drugs, and the appropriate amount of saline was also used for a control injection. However, we did not want to cause confusion in the figures because our non-selected mice are called "control" mice. For this reason, we have termed the "control" dose as the "vehicle" dose (short for "vehicle only").

Endurance testing protocol

Mouse endurance was assessed using a graded treadmill endurance test modified from previously published protocols (Lerman et al. 2002; Haubold et al. 2003; Meek et al. 2009). Prior to the start of exhaustive exercise trials, mice were trained on the treadmill for 15 minutes on each of three consecutive days at 10 meters/minute (m/min), 12 m/min, and 14 m/min, respectively. During testing, mice began at a treadmill speed of 14 m/min and speed was increased 1.5 m/min every 2 minutes until the mouse was unable to continue running at the belt speed (Fig. 1). For both training and testing, the treadmill was at a 25 degree incline, and a stimulation grid with adjustable-amperage (0-12 mAmp) at the end of the treadmill provided motivation. Mice were judged to be exhausted when they showed an inability to maintain speed and remained on the stimulation grid for three consecutive seconds. A single judge, who was blind to linetype and treatment, made this determination for every trial in the study. Mice completed three exhaustive exercise bouts each (with one day of rest between bouts), and received one injection per bout (dose order randomized); a vehicle injection, a low dose injection, and a high dose injection. An individual mouse thus received only two injections of one drug

(either 8-OH-DPAT + pindolol or WAY-100,635) during treadmill testing, with either 2 or 4 days between the low dose and high dose, depending on when the vehicle was administered.

Endurance was measured as time to exhaustion (TTE), distance traveled (a product of TTE and speed, which increased as the trial went on, Fig. 1), and vertical work performed, which is equal to the product of vertical distance (total distance multiplied by $\sin(25^{o})$ in meters) and the mass of the mouse in kg (Barbato et al. 1998). Each of those measurements are highly related to one another, but differ enough that we predicted that their analyses might give different results.

The testing protocol used here matches the study in which it was originally discovered that HR mice have greater endurance than C mice, and moreover in which we provided physiological evidence (blood lactate and glucose levels) indicating that it truly caused exhaustion in both HR and C mice (Meek et al. 2009). As our specific hypothesis was related to the endurance difference between HR and C mice, these methods were most appropriate.

Wheel running protocol

Following the treadmill trials, mice were housed individually in cages with wheel access. Wheel running reached a plateau after 14 days, at which point pharmaceutical manipulations began. Mice retained their treatment designations from the treadmill trials (each mouse received the same drug for both the treadmill and wheel-running trials).

Again, there were three injections (with two days of rest between injections); a vehicle injection, a low dose injection, and a high dose injection. Thus, an individual mouse received two injections of one drug (either 8-OH-DPAT + pindolol or WAY-100,635) during wheel running (in addition to the two injections given ~ 2 weeks earlier during treadmill testing), with either 3 or 6 days between the low and high dose, depending on when the vehicle was administered. Injections occurred between 2 and 3 hours after the onset of the dark period (Fig. 2). Mice were removed from the wheels under red light, injected, and returned to their cages. This took less than 5 minutes in all cases, and data from the 10 minutes before and 10 minutes after the time that the mice were injected were discarded to avoid any artifacts of handling. Wheel revolutions were counted automatically in 1-minute bins by a photocell counter attached to a computer running an automated program. The general protocols above follow several previous pharmacology studies of HR mice (e.g. Rhodes and Garland 2003; Keeney et al. 2008; Keeney et al. 2012). The first 10 minutes after injection were excluded to avoid handling effects, and the first and second hours following that period were used in separate analyses (one analysis for 10-70 and another for 70-130 minutes post-injection).

Statistical analysis

Statistical analyses were performed using SAS PROC MIXED. The primary factors of analysis were linetype (HR vs. C) and dose, and replicate line was used as a random effect nested within linetype. Individual was the factor for repeated measures. The statistical interaction dose by linetype was of prime interest because, where

significant, it indicates a differential response of the HR and control lines to the drug doses. Wheel freeness (a measure of how easy it is to turn each wheel) was measured before and after the wheel trials and temporarily included as a covariate in statistical analyses, as was age. Age and wheel freeness were not significant predictors of wheel running, and were thus left out of the final model.

The mini-muscle phenotype, caused by a Mendelian recessive allele that was present at a low frequency of about 7% in the original base population and characterized by a 50% mass reduction in the triceps surae and total hindlimb muscle mass (in homozygotes), has increased in one HR line and eventually become fixed in another (Garland et al. 2002b; Syme et al. 2005). In the one HR line that contains both genotypes, triceps surae were dissected and weighed to determine mini-muscle status, and then mini-muscle status for those mice as well as mice within the fixed mini-muscle line was considered as a factor for the 8-OH-DPAT treatment group, but not the WAY-100,635 treatment group, in which mini-muscle was fully confounded with line. The inclusion of mini-muscle in the model did not substantially change the findings, and is not included in the results presented here.

RESULTS

WAY-100,635 differentially decreased endurance in the treadmill trial in HR as compared to C mice (dose by linetype interaction, $P = .0020$, Table 1.1). HR mice exhausted more slowly (were able to run longer) than C mice while under the vehicle treatment, but exhausted at nearly the same point at either the high or low dose of the drug (Fig. 3).

Results were very similar for distance run to exhaustion and total work performed (see Table 1.1).

Time to exhaustion during the treadmill trial was significantly affected by dose in the 8-OH-DPAT + pindolol treatment ($F_{(2, 12)} = 34.57$, P = <.0001, Table 1.1), but not by linetype or the dose-by-linetype interaction (P = 0.1422, P = 0.2227, respectively). Visually, it appears that the low dose marginally increased, whereas the high dose decreased endurance in both linetypes by a roughly equal proportion (Fig. 3), but this apparent low-dose effect was not statistically significant (difference of least squares means P = 0.0566).

In all doses and drug combinations, HR mice ran significantly more wheel revolutions than C mice. For the first hour after injection, wheel running was significantly and differentially affected by 8-OH-DPAT + pindolol treatment (first hour dose by linetype, P = .0226, Table 1.2), and similarly to the treadmill results, the effect was most noticeable at the high dose, at which voluntary wheel running was dramatically reduced (Fig. 4). The effect seemed to be more strongly related to a reduction in speed (first hour $F_{(2, 12)} = 5.96$, P = 0.0160 for RPM versus $F_{(2, 12)} = 2.41$, P = 0.1321 for active minutes). In the second hour after injection, the effect mostly disappeared (Table 1.3, Fig. 4, 5). Wheel running was not significantly affected by WAY-100,635 treatment (first hour dose P = .8902, Tables 2, 3, Fig. 4, 5).

DISCUSSION

We found that a $5HT_{1A}$ antagonist (WAY-100,635) differentially decreased treadmill endurance performance in mice that have evolved high endurance capacity due to selective breeding for voluntary exercise, but did not affect endurance of their non-selected control lines. Perhaps surprisingly, a reduction in endurance capacity of HR mice was not paralleled by a reduction in voluntary wheel running. We did find that voluntary wheel running of HR mice was differentially affected by a serotonin agonist (8-OH-DPAT + pindolol) 10-70 minutes post-injection, which is the first evidence that serotonin signaling accounts for any of the behavioral differences between HR and C mice. Overall, these results demonstrate that pharmacological interventions acting on central nervous system 5-HT receptors can influence endurance and voluntary exercise in different ways, and they shed light on the microevolution of locomotor behavior.

Relevance for the High Runner selection experiment

This is the first study to provide evidence that alterations in serotonin signaling account for some of the known behavioral or performance differences between HR and C mice. For mice treated with WAY-100,635, endurance (measured as running time to exhaustion) decreased only in HR, and not C mice (Fig. 3), which surprisingly indicates that the mechanism(s) which confers increased treadmill endurance to HR mice can be completely eliminated by this drug. We do not suggest that the difference in endurance between linetypes is attributable only to differences in $5\text{-}HT_{1A}$ receptors, or even more generally neurobiological differences. HR mice have many physical qualities which

might make them more capable endurance runners (Rezende et al. 2006b; Kolb et al. 2010). However, the results here are remarkable in that the WAY-100,635-treated HR mice had virtually the same endurance as C mice (Fig. 3).

The high dose of 8-OH-DPAT had a differential effect on wheel running 10-70 minutes post-injection, with HR mice showing a greater reduction than C mice. This type of interactive effect has been found in previous studies of wheel running in HR and C mice. Drugs targeting endocannabinoid receptors (CB1 agonist WIN 55,212-2 and antagonist rimonabant, Keeney et al. 2008; Keeney et al. 2012), dopamine receptors (non-selective dopamine agonist Ritalin, D1-like agonist SCH 23390, Rhodes and Garland 2003), and dopamine transporters (DAT; cocaine and GBR 12909, Rhodes et al. 2001) have all differentially decreased wheel running in HR versus C mice. Cocaine has similar interactive effects on HR and C mice, but this drug acts on DAT in addition to the serotonin transporter (Rhodes et al. 2001). However, the SERT inhibitor fluoxetine reduced wheel running in HR and C mice equally, indicating that the differential effect of cocaine was most likely caused by its actions on DAT (Rhodes et al. 2001). Therefore, the present study provides the first strong evidence that serotonin (or possibly D_2-like receptors) may account for some of the behavioral differences between HR and C mice.

It is worth mentioning that some part of the consistent finding of drug by linetype interactions in pharmacological manipulations may be attributable to differences in pharmacokinetics between HR and C mice. HR mice are smaller (Swallow et al. 1999), which is generally associated with higher mass-specific metabolic rates, and also have

higher metabolic rates when running on wheels due to their greater speed of wheel running (Rezende et al. 2005).

High Runner mice have innately higher endurance during forced treadmill exercise (Meek et al. 2009), likely owing in part to physical and physiological traits, such as HR mice having larger hearts and higher maximal aerobic capacity (Rezende et al. 2006b; Kolb et al. 2010). In addition, HR and C mice differ in muscle properties. HR lines tend to have reduced triceps surae muscle masses (beyond mini-muscle effects - see following) (Garland et al. 2002a), and we have demonstrated differences between HR and C lines (beyond mini-muscle effects) for fiber types in the tibialis anterior muscle (Bilodeau et al. 2009). Further, HR lines show increased adaptive plasticity in gastrocnemius GLUT-4 concentrations (Gomes et al. 2009). When housed without wheel access, no differences in gastrocnemius GLUT-4 were observed. After 5 days with wheels, all mice showed elevated GLUT-4, but HR normal and mini were 2.5-fold higher than C.

In addition to these general muscle differences between the HR and C lines, other differences are specific to the subset of HR mice with the 'mini-muscle' phenotype. Depending on the muscle considered, mini-muscle individuals largely or entirely lack type IIb fibers due to a single nucleotide polymorphism (SNP) mutation in the 2b-MyHC (myh4) gene (Kelly et al. 2013). The phenotype was present at a low frequency (~7%) in the original base population, but has increased in frequency in two of the four HR lines, eventually becoming fixed in one (Garland et al. 2002b; Hannon et al. 2008; Kelly et al. 2013). In mini-muscle mice, the medial gastrocnemius has a reduced force per-cross

sectional area (Syme et al. 2005), and the gastrocnemius has elevated capillarization (Wong et al. 2009), and hindlimb muscles have double the per-gram oxidative capacity (Houle-Leroy et al. 2003). Mini-muscle mice also have increased glycogen storage in the soleus (Gomes et al. 2009). However, mini-muscle mice do not have higher treadmill endurance, so these traits appear to not be beneficial to forced-exercise endurance.

The difference in drug response between HR and C mice suggest that pharmaceutical manipulations of serotonin receptors (5-HT_{1A} antagonism, or possibly D_4 agonism by WAY-100,635) can ablate the endurance advantage of HR mice. As the receptor we targeted is found in highest concentration in the central nervous system, we hypothesize that decreased drive to the motor neurons caused the reduction in endurance of HR mice. However, the D_4 receptor is more broadly distributed in the periphery, and D_4 is associated with human hyperactivity (Faraone et al. 2005), so this interpretation is made with reservation.

The endurance values reported here are lower than those in Meek (2009), even though the testing protocol in the present study was similar and started at a lower speed. This disparity is most likely attributable to the fact that Meek (2009) allowed mice to "train" on wheels for ~10 days before (and during) the period of treadmill testing, whereas our mice had no access to wheels (and hence no opportunity for self-training) prior to endurance tests.

Voluntary versus forced exercise

The pharmacological effects we observed differed between endurance capacity during forced treadmill exercise (measured first) and voluntary wheel running (measured after endurance trials), which suggests that the two locomotor tests are not governed by identical neurobiological processes. This possibility should be noncontroversial, because endurance trials are intended to achieve maximal physical abilities through use of external motivators (Booth et al. 2010), whereas wheel running is a "voluntary" behavior in the classical sense. Voluntary wheel running and forced treadmill exercise differ in physiological and psychological conditions. For instance, in rats, forced treadmill running induces some acute psychological stress responses, as well as chronic adaptations to stress (Brown et al. 2007), whereas wheel running generally has anxiolytic effects (Duman et al. 2008). Two traditional biological markers of fatigue (blood glucose and lactate concentrations) suggest that the endurance protocol employed here is significantly more physically taxing than wheel running in both HR and C mice (Meek et al. 2009).

Nevertheless, given the sequential-test experimental design used here, we cannot rule out the possibility that something about the mice changed between the tests. For example, aspects of exercise physiology can change with as little as a few hours or a few days of wheel access (Dumke et al. 2001 and references therein; Gomes et al. 2009). Of particular relevance for the present study, training-induced differences in 5-HT_{1A} receptor expression have been reported in rats, where 6 weeks of wheel running was sufficient to increase 5-HT_{1A} mRNA in the dorsal raphe nuclei (DRN; Greenwood et al. 2005).

Although our mice had only two weeks to train on wheels before the start of injections, it seems possible that they self-trained in various ways, which in turn may have changed their drug responses. Future studies will employ a randomized testing sequence to address this possibility.

Comparison with previous findings

Our results support the findings of Ahlenius et al. (1997) in that systemic WAY-100,635 injection decreased endurance in mice, and in addition we found that the effect depended on genetic background. Specifically, in our study, WAY-100,635 decreased endurance in HR mice, but not C mice. We did not replicate (to statistical significance) the finding that 8-OH-DPAT + pindolol increased endurance. In fact, our results more closely matched the biphasic effect described by Ahlenius et al. (1997) when they used only 8-OH-DPAT, without the addition of pindolol, in that 8-OH-DPAT marginally (but not significantly) increased endurance in mice at the low dose, and significantly decreased endurance at the high dose. The biphasic effect of 8-OH-DPAT that we observed for endurance has been described before in the context of exhaustive exercise in rats (Ahlenius et al. 1997) and in other behavioral contexts in multiple species (reviewed in de Boer and Koolhaas 2005). A biphasic likely occurs because, at low doses, the agonist acts mostly presynaptically, inhibiting the release of 5-HT from the DRN, but at high doses, it acts postsynaptically, similarly to endogenous 5-HT in other parts of the brain and spinal cord (Beaudoin-Gobert and Sgambato-Faure 2014).

Implications for the serotonin central fatigue hypothesis

Our findings generally support the 5-HT central fatigue hypothesis (see Introduction) because pharmacological manipulation by a 5-HT_{1A} agonist and antagonist altered time to fatigue (treadmill endurance-running time) in mice. To our knowledge, this is the first time that pharmacological treatments during forced, exhaustive exercise have been compared with self-paced exercise (wheel running) for the same set of animals. As noted above, our results suggest that exhaustion in forced exercise and cessation of voluntary exercise are not governed by identical neurobiological processes.

Previous pharmacological studies of the 5-HT central fatigue hypothesis have yielded inconsistent results (see tables II and III in Meeusen et al. 2006). The inconsistency is often attributed to differences in ambient conditions, type of exercise, type of measurement, physical fitness, other characteristics of the study individuals, or inherent differences among species (Meeusen et al. 2006). Our study controlled most of those variables, with the exception of physical fitness, which is innately higher in HR mice (e.g., HR mice have higher maximal aerobic capacity), and in isolation, we found that genetically determined exercise capacity fundamentally changes how the endurance of an animal reacts to 5-HT pharmaceuticals.

Our study also provides direct evidence that the neurobiological mechanisms underlying exercise fatigue have evolved in response to selective breeding for high exercise output. Factors that are thought to contribute to central fatigue are known to respond to training (Nielsen et al. 1993; Greenwood et al. 2005), and genetically

determined differences in 5-HT_{1B} receptor mRNA expression has been shown in rats which were bred for high aerobic capacity (Foley et al. 2006). However, this is the first study to directly show an interactive effect of pharmaceuticals and genetic background on endurance ability.

Table 1.1. Treadmill endurance-running performance. Values are least square means ± associated standard errors from repeated-measures analysis in SAS Procedure Mixed. Different sets of mice were used for the two drugs, with sample size of 49 mice per drug. These values are after accounting for increases in performance with trial number (results not shown). The three alternative measures of exhaustion (time, distance, and vertical work) do not meaningfully change the results or interpretation. P values < 0.05 are in bold font.

	Dose					
Variable and Drug	Vehicle	Low	High	Dose effect	Linetype effect	Dose by linetype effect
Time to exhaustion (min)						
WAY-100,635						
C	18.2 ± 1.2	18.3 ± 1.2	17.9 ± 1.2	F (DF) = 13.50 (2, 12)	F (DF) = 0.40 (1, 6)	F (DF) = 10.91 (2, 12)
HR	21.5 ± 1.2	18.1 ± 1.2	17.8 ± 1.2	**P = 0.0008**	P = 0.5482	**P = 0.0020**
8-OH-DPAT + pindolol						
C	17.8 ± 1	19.4 ± 1.0	15.7 ± .1.0	F (DF) = 34.57 (2, 12)	F (DF) = 2.85 (1, 6)	F (DF) = 1.71 (2, 12)
HR	20.9 ± 1	21.5 ± 1.0	16.9 ± 1.0	**P < 0.0001**	P = 0.1422	P = 0.2227
Distance to exhaustion (m)						
WAY-100,635						
C	369.3 ± 34.0	370.5 ± 33.8	360.2 ± 33.9	F (DF) = 14.01 (2, 12)	F (DF) = 0.46 (1, 6)	F (DF) = 11.77 (2, 12)
HR	464.8 ± 34.1	369.0 ± 34.2	360.1 ± 34.5	**P = 0.0007**	P = 0.5247	**P = 0.0015**
8-OH-DPAT + pindolol						
C	358.0 ± 28.4	399.2 ± 28.4	304.2 ± 28.4	F (DF) = 31.55 (2, 12)	F (DF) = 3.06 (1, 6)	F (DF) = 2.04 (2, 12)
HR	448.9 ± 28.1	466.2 ± 28.2	336.1 ± 28.1	**P < 0.0001**	P = 0.1310	P = 0.1727
Work to exhaustion (joules)						
WAY-100,635						
C	5.2 ± 0.4	5.1 ± 0.4	5.1 ± 0.4	F (DF) = 11.36 (2, 12)	F (DF) = 0.05 (1, 6)	F (DF) = 9.20 (2, 12)
HR	5.9 ± 0.4	4.6 ± 0.5	4.5 ± 0.5	**P = 0.0017**	P = 0.8350	**P = 0.0038**
8-OH-DPAT + pindolol						
C	5.1 ± 0.4	5.7 ± 0.4	4.3 ± 0.4	F (DF) = 30.88 (2, 12)	F (DF) = 0.45 (1, 6)	F (DF) = 1.32 (2, 12)
HR	5.7 ± 0.3	5.9 ± 0.4	4.3 ± 0.3	**P < 0.0001**	P = 0.5261	P = 0.3026

Table 1.2. Voluntary wheel running over the 10-70 minutes post-injection. Values are least square means ± associated standard errors from repeated-measures analysis in SAS Procedure Mixed. Different sets of mice were used for the two drugs, with sample size of 49 mice per drug. A variable coding for test night (1-3) were also included in the analyses, and measure of wheel freeness was considered as a covariate, but was not a significant predictor and was ultimately not included (results not shown). P values < 0.05 are in bold font.

Variable and Drug	Dose			Dose effect	Line type effect	Dose by linetype effect
	Vehicle	Low	High			
Total revolutions per 10 minutes						
WAY-100,635						
C	41.9 ± 21.4	46.9 ± 21.4	33.1 ± 21.4	F (DF) = 0.12 (2, 12)	F (DF) = 32.97 (1, 6)	F (DF) = 0.71 (2, 12)
HR	191.4 ± 21.6	196.7 ± 21.6	206.6 ± 21.6	P = 0.8902	**P = 0.0012**	P = 0.5126
8-OH-DPAT + pindolol						
C	33.3 ± 19.8	24.0 ± 19.8	24.9 ± 19.8	F (DF) = 5.35 (2, 12)	F (DF) = 26.51 (1, 6)	F (DF) = 5.29 (2, 12)
HR	174.7 ± 19.7	181.2 ± 19.7	136.3 ± 19.7	**P = 0.0218**	**P = 0.0021**	**P = 0.0226**
Active minutes						
WAY-100,635						
C	3.7 ± 0.9	4.1 ± 0.9	3.2 ± 0.9	F (DF) = 0.66 (2, 12)	F (DF) = 13.19 (1, 6)	F (DF) = 1.44 (2, 12)
HR	7.6 ± 0.9	7.9 ± 0.9	8.3 ± 0.9	P = 0.5368	**P = 0.0109**	P = 0.2749
8-OH-DPAT + pindolol						
C	3.1 ± 0.6	2.4 ± 0.6	2.6 ± 0.6	F (DF) = 2.65 (2, 12)	F (DF) = 32.47 (1, 6)	F (DF) = 2.41 (2, 12)
HR	7.8 ± 0.6	7.8 ± 0.6	6.5 ± 0.6	P = 0.1114	**P = 0.0013**	P = 0.1321
Average wheel speed (RPM)						
WAY-100,635						
C	5.5 ± 2.0	5.9 ± 2.0	4.5 ± 2.0	F (DF) = 0.13 (2, 12)	F (DF) = 39.71 (1, 6)	F (DF) = 0.87 (2, 12)
HR	20.7 ± 2.0	21.2 ± 2.0	22.3 ± 2.0	P = 0.8789	P = 0.0007	P = 0.4433
8-OH-DPAT + pindolol						
C	4.9 ± 2.0	3.8 ± 2.0	3.4 ± 2.0	F (DF) = 7.26 (2, 12)	F (DF) = 31.50 (1, 6)	F (DF) = 5.96 (2, 12)
HR	19.5 ± 2.0	20.9 ± 2.0	15.9 ± 2.0	**P = 0.0086**	**P = 0.0014**	**P = 0.0160**

Table 1.3. Voluntary wheel running over the 70-130 minutes post-injection. Values are least square means ± associated standard errors from repeated-measures analysis in SAS Procedure Mixed. Different sets of mice were used for the two drugs, with sample size of 49 mice per drug. A variable coding for test night (1-3) were also included in the analyses, and measure of wheel freeness was considered as a covariate, but were not a significant predictors and were ultimately not included (results not shown). P values < 0.05 are in bold font.

Variable and Drug	Dose			Dose effect	Line type effect	Dose by linetype effect
	Vehicle	Low	High			
Total revolutions per 10 minutes						
WAY-100,635						
C	35.6 ± 17	48 ± 17	39.7 ± 17	F (DF) = 0.97 (2, 12)	F (DF) = 64.86 (1, 6)	F (DF) = 0.14 (2, 12)
HR	175.6 ± 17.2	199.3 ± 17.2	194.2 ± 17.2	P = 0.4055	**P = 0.0002**	P = 0.8679
8-OH-DPAT + pindolol						
C	22.9 ± 18.8	35.9 ± 18.8	25.3 ± 18.8	F (DF) = 5.08 (2, 12)	F (DF) = 24.04 (1, 6)	F (DF) = 2.3 (2, 12)
HR	156.8 ± 18.7	164.6 ± 18.7	122.2 ± 18.7	**P = 0.0252**	**P = 0.0027**	P = 0.1426
Active minutes						
WAY-100,635						
C	3.2 ± 0.8	4.3 ± 0.8	3.5 ± 0.8	F (DF) = 2.98 (2, 12)	F (DF) = 20.32 (1, 6)	F (DF) = 0.18 (2, 12)
HR	7.4 ± 0.8	8.5 ± 0.8	8.1 ± 0.8	P = 0.0892	**P = 0.0041**	P = 0.8377
8-OH-DPAT + pindolol						
C	2.3 ± 0.7	3.3 ± 0.7	2.5 ± 0.7	F (DF) = 3.49 (2, 12)	F (DF) = 25.63 (1, 6)	F (DF) = 1.02 (2, 12)
HR	7 ± 0.7	7.2 ± 0.7	6 ± 0.7	P = 0.0637	**P = 0.0023**	P = 0.3902
Average wheel speed (RPM)						
WAY-100,635						
C	4.7 ± 1.7	6.3 ± 1.7	5.5 ± 1.7	F (DF) = 1.24 (2, 12)	F (DF) = 72.71 (1, 6)	F (DF) = 0.06 (2, 12)
HR	19.4 ± 1.7	21.7 ± 1.7	21.2 ± 1.7	P = 0.3236	**P = 0.0001**	P = 0.9376
8-OH-DPAT + pindolol						
C	3.5 ± 1.9	5.0 ± 1.9	4.1 ± 1.9	F (DF) = 5.55 (2, 12)	F (DF) = 28.61 (1, 6)	F (DF) = 2.95 (2, 12)
HR	18.3 ± 1.9	19.3 ± 1.9	14.4 ± 1.9	**P = 0.0196**	**P = 0.0017**	P = 0.0907

Figure 1.1. Treadmill speeds and cumulative distance using a graded treadmill endurance test (modified from previously published protocols: (Lerman et al. 2002; Haubold et al. 2003; Meek et al. 2009). Top panel: Mice began at a treadmill speed of 14 m/min and speed was increased 1.5 m/min every 2 minutes until the mouse was unable to continue running. Bottom panel: Cumulative distance run in relation to time spent running. In each panel, the points indicate the maximum and minimum values observed in this study (minima: time = 10:09 (mm:ss), treadmill speed at exhaustion: 21 m/min, distance: 173.22 m; maxima: 31:08 (mm:ss), speed: 36.5 m/min, distance: 776.31 m; treatments combined).

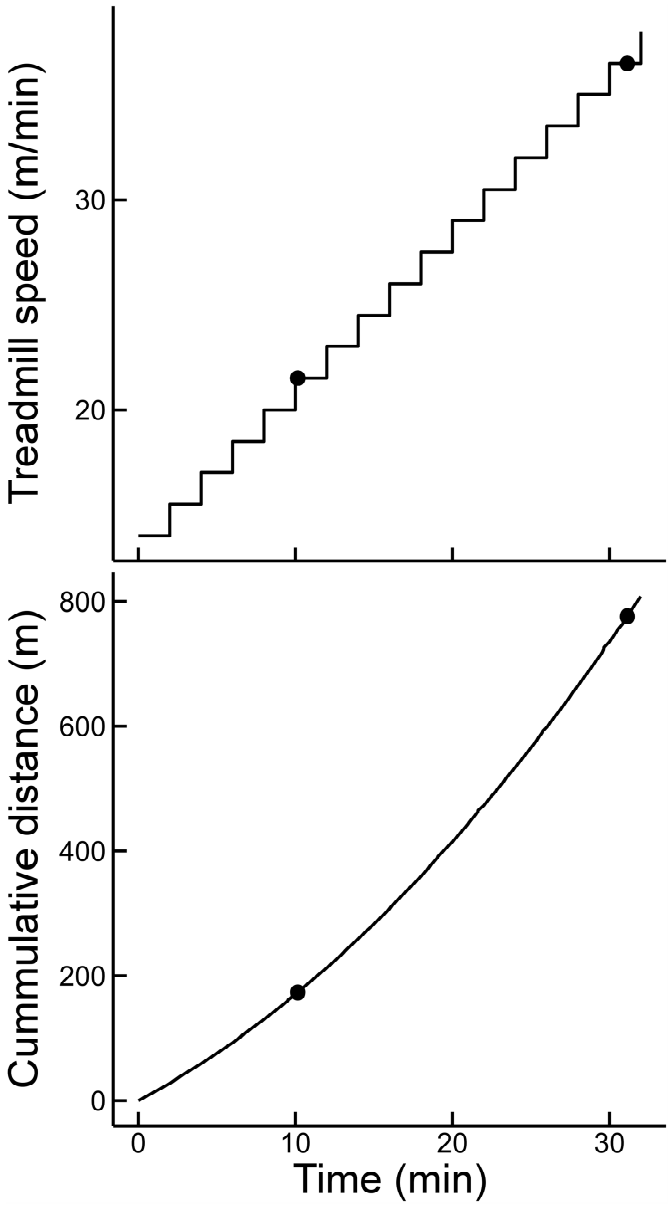


Figure 1.2. Mean revolutions per minute (binned hourly) for linetype-dose groups over three consecutive days of voluntary wheel running. Aside from an acute reduction in wheel running for mice treated with the high dose of 8-OH-DPAT, the circadian pattern of wheel running was unaffected by the drugs. In the figure, hour is hours from the start of the test on the injection day, such that time 0 represents the beginning of the wheel running period on the day that the mouse was injected. Injections began two hours after the start of the scotophase. The injection period marked in light gray. The combined time to inject all mice was less than one hour. During that hour each mouse received a subcutaneous injection of a vehicle, a low dose, or high dose of either WAY 100,635 or 8-OH-DPAT + pindolol. Mice were kept on a 12:12 light:dark cycle. The dark phase began at 18:00 and the light phase began at 6:00. Dark hours are indicated by black bars on the axis.

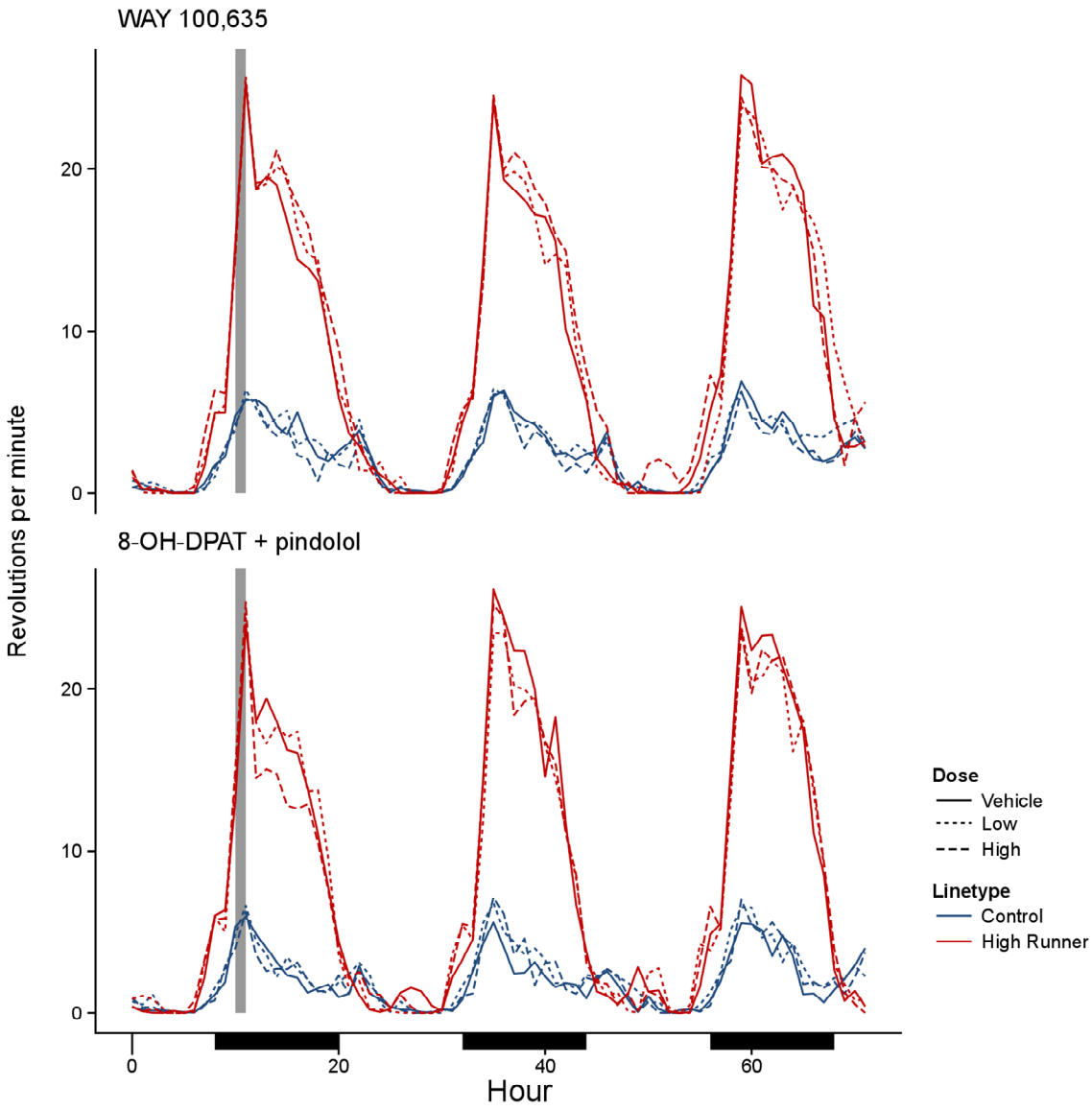

WAY 100,635
8-OH-DPAT + pindolol
Revolutions per minute
0
10
20
0
20
40
60
Hour
Dose
Vehicle
Low
High
Linetype
Control
High Runner

Figure 1.3. Time to exhaustion (min) under a graded-exercise protocol on an inclined treadmill (25º angle) with manual and electrical stimulation for HR and C mice treated with vehicle (saline), low or high doses of either WAY-100,635 (serotonin antagonist) or 8-OH-DPAT + pindolol (serotonin agonist). For both drugs, the interaction between linetype and drug is highly statistically significant (see Table 1.1). Values are least square means ± associated standard errors from repeated-measures analysis in SAS Procedure Mixed, as reported in Table 1.1.

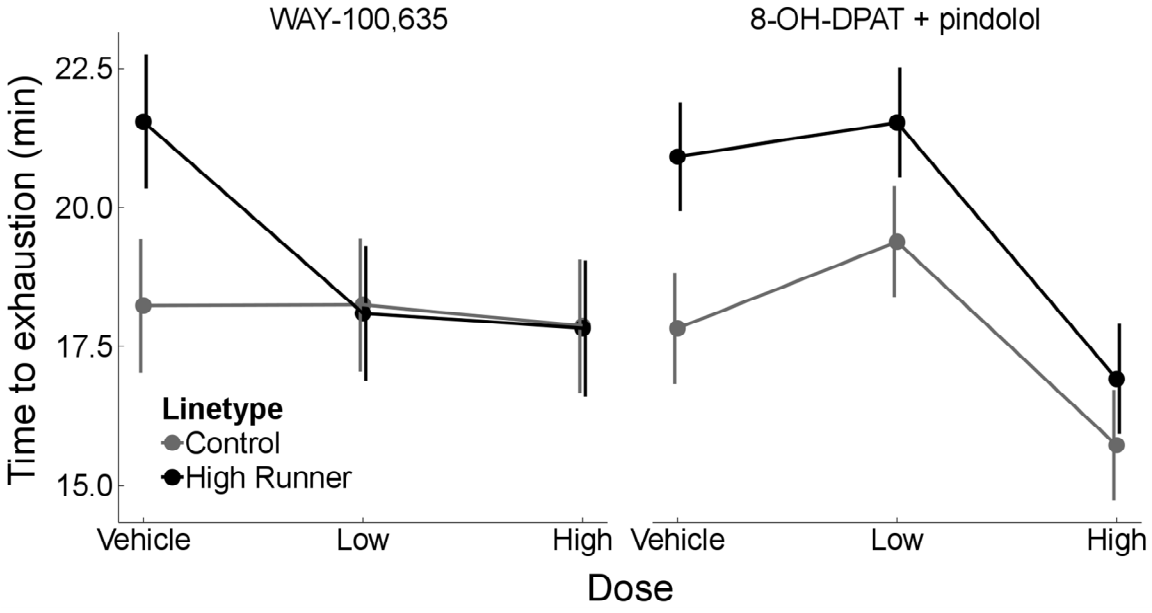
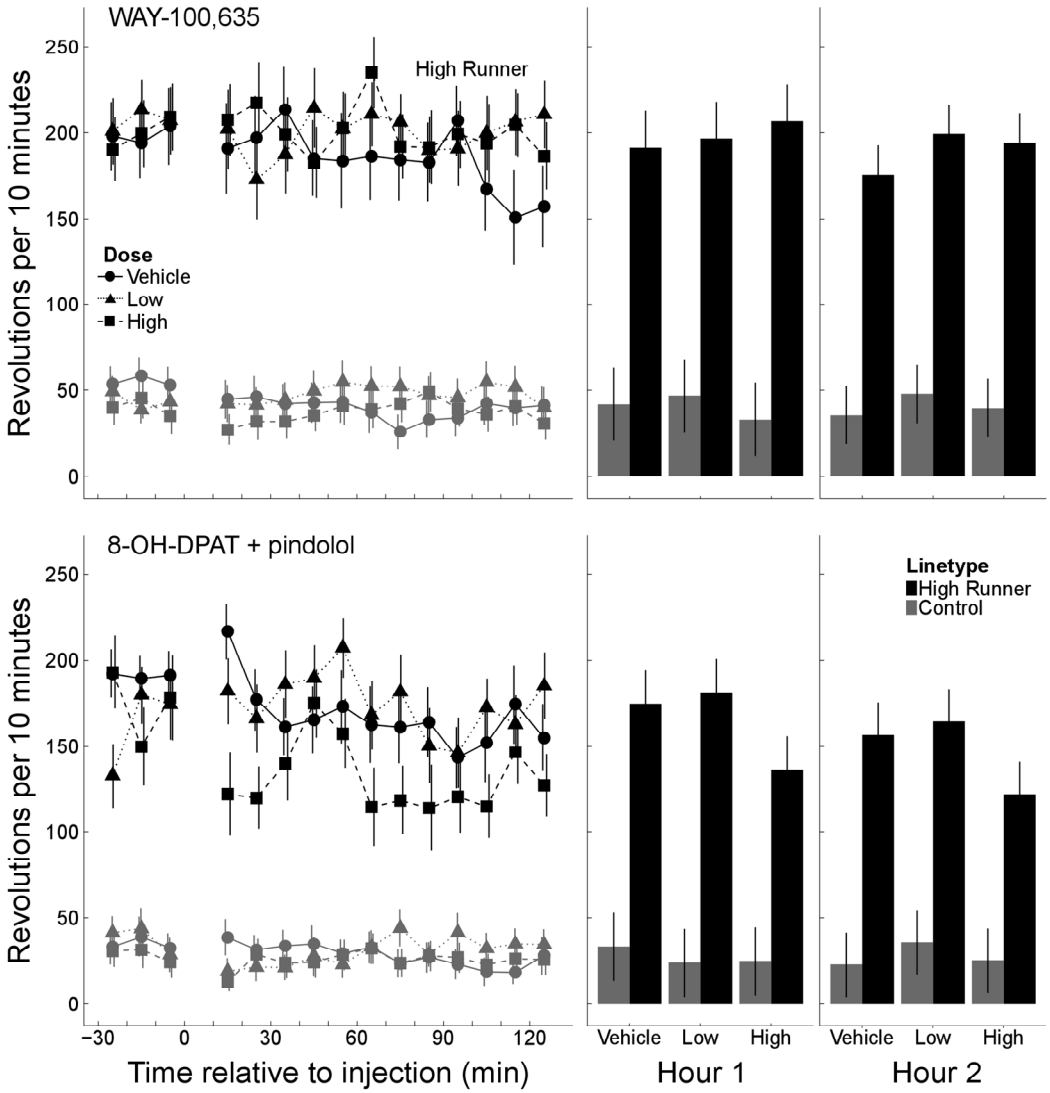


Figure 1.4. Voluntary wheel running for HR and C mice before and after systemic treatment with vehicle (saline), low or high doses of either WAY-100,635 (5-HT_{1A} antagonist) (top panels) or 8-OH-DPAT + pindolol (5-HT_{1A} agonist) (bottom panels). Left panels show wheel running in 10-min bins. Time 0 corresponds with the time of injection, and the first 10 minutes are not included due to handling effects. Right panels group data into first and second hour following injections, as analyzed statistically (10-70 and 70-130 minutes, respectively). For the serotonin agonist (bottom panels), the interaction between linetype and dose was statistically significant for the first hour (see Tables 2 and 3). Values in left panels are simple means and standard errors. Values in right panels are least square means ± associated standard errors from repeated-measures (by dose) analyses in SAS Procedure Mixed, as shown in Tables 2 and 3.



References:

Ahlenius, S., P. Kaur, and P. Salmi. 1997. Biphasic effects of 8-OH-DPAT on endurance of treadmill performance in the male rat. European Neuropsychopharmacology 7:89-94.

Alvarez, F. J., J. C. Pearson, D. Harrington, D. Dewey, L. Torbeck, and R. E. W. Fyffe. 1998. Distribution of 5-hydroxytryptamine-immunoreactive boutons on alpha-motoneurons in the lumbar spinal cord of adult cats. J. Comp. Neurol. 393:69-83.

Assie, M. B. and W. Koek. 1996. Possible in vivo 5-HT reuptake blocking properties of 8-OH-DPAT assessed by measuring hippocampal extracellular 5-HT using microdialysis in rats. British Journal of Pharmacology 119:845-850.

Bailey, S. P., J. M. Davis, and E. N. Ahlborn. 1993. Serotonergic agonists and antagonists affect endurance performance in the rat. International Journal of Sports Medicine 14:330-333.

Balthazar, C. H., L. H. Leite, R. M. Ribeiro, D. D. Soares, and C. C. Coimbra. 2010. Effects of blockade of central dopamine D1 and D2 receptors on thermoregulation, metabolic rate and running performance. Pharmacol Rep 62:54-61.

Barbato, J. C., L. G. Koch, A. Darvish, G. T. Cicila, P. J. Metting, and S. L. Britton. 1998. Spectrum of aerobic endurance running performance in eleven inbred strains of rats.

Beaudoin-Gobert, M. and V. Sgambato-Faure. 2014. Serotonergic pharmacology in animal models: From behavioral disorders to dyskinesia. Neuropharmacology 81:15-30.

Bedard, P. J., L. E. Tremblay, H. Barbeau, M. Filion, R. Maheux, C. L. Richards, and T. Dipaolo. 1987. Action of 5-Hydroxytrypamine, substance P, thyrotropin-releasing-hormone and clonside on motorneuron excitability. Canadian Journal of Neurological Sciences 14:506-509.

Bilodeau, G. M., H. Guderley, D. R. Joanisse, and T. Garland, Jr. 2009. Reduction of Type IIb Myosin and IIB Fibers in Tibialis Anterior Muscle of Mini-Muscle Mice From High-Activity Lines. J. Exp. Zool. Part A 311A:189-198.

Blomstrand, E., D. Perrett, M. Parrybillings, and E. A. Newsholme. 1989. Effect of sustained exercise on plasma amino-acid concentrations and on 5-

hydroxytryptamine metabolism in 6 different brain-regions in the rat. Acta Physiologica Scandinavica 136:473-481.

Booth, F. W., M. J. Laye, and E. E. Spangenburg. 2010. Gold standards for scientists who are conducting animal-based exercise studies. Journal of Applied Physiology 108:219-221.

Boxenbaum, H. 1982. Interspecies scaling, allometry, physiological time, and the ground plan of phamacokeneitcs. Journal of Pharmacokinetics and Biopharmaceutics 10:201-225.

Brown, D. A., M. S. Johnson, C. J. Armstrong, J. M. Lynch, N. M. Caruso, L. B. Ehlers, M. Fleshner, R. L. Spencer, and R. L. Moore. 2007. Short-term treadmill running in the rat: what kind of stressor is it?

Careau, V., M. E. Wolak, P. A. Carter, and T. Garland, Jr. 2013. Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection. Evolution 67:3102-3119.

Chaouloff, F., J. L. Elghozi, Y. Guezennec, and D. Laude. 1985. Effects of conditioned running on plasma, liver and brain tryptophan and on brain 5-hydroxytryptamin metabolism of the rat. British Journal of Pharmacology 86:33-41.

Chemel, B. R., B. L. Roth, B. Armbruster, V. J. Watts, and D. E. Nichols. 2006. WAY-100635 is a potent dopamine D-4 receptor agonist. Psychopharmacology 188:244-251.

Coimbra, C. C., D. D. Soares, and L. H. R. Leite. 2012. The Involvement of Brain Monoamines in the Onset of Hyperthermic Central Fatigue *in* K. R. Zaslav, ed. An International Perspective on Topics in Sports Medicine and Sports Injury. InTech.

Cotel, F., R. Exley, S. J. Cragg, and J.-F. Perrier. 2013. Serotonin spillover onto the axon initial segment of motoneurons induces central fatigue by inhibiting action potential initiation. Proceedings of the National Academy of Sciences 110:4774-4779.

de Boer, S. F. and J. M. Koolhaas. 2005. 5-HT1A and 5-HT1B receptor agonists and aggression: A pharmacological challenge of the serotonin deficiency hypothesis. European Journal of Pharmacology 526:125-139.

Deneris, E. S. and S. C. Wyler. 2012. Serotonergic transcriptional networks and potential importance to mental health. Nature Neuroscience 15:519-527.

Duman, C. H., L. Schlesinger, D. S. Russell, and R. S. Duman. 2008. Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice. Brain Res 1199:148-158.

Dumke, C. L., J. S. Rhodes, T. Garland, Jr., E. Maslowski, J. G. Swallow, A. C. Wetter, and G. D. Cartee. 2001. Genetic selection of mice for high voluntary wheel running: effect on skeletal muscle glucose uptake.

Faraone, S. V., R. H. Perlis, A. E. Doyle, J. W. Smoller, J. J. Goralnick, M. A. Holmgren, and P. Sklar. 2005. Molecular Genetics of Attention-Deficit/Hyperactivity Disorder. Biol. Psychiatry 57:1313-1323.

Foley, T. E., B. N. Greenwood, H. E. W. Day, L. G. Koch, S. L. Britton, and M. Fleshner. 2006. Elevated central monoamine receptor mRNA in rats bred for high endurance capacity: Implications for central fatigue. Behavioural Brain Research 174:132-142.

Friedman, W. A., T. Garland, Jr, and M. R. Dohm. 1992. Individual variation in locomotor behavior and maximal oxygen consumption in mice. Physiology & Behavior 52:97-104.

Gandevia, S. C. 2001. Spinal and supraspinal factors in human muscle fatigue. Physiological Reviews 81:1725-1789.

Garland, T., Jr., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011a. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proceedings of the Royal Society B-Biological Sciences 278:574-581.

Garland, T., Jr., M. T. Morgan, J. G. Swallow, J. S. Rhodes, I. Girard, J. G. Belter, and P. A. Carter. 2002a. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267-1275.

Garland, T., Jr., M. T. Morgan, J. G. Swallow, J. S. Rhodes, I. Girard, J. G. Belter, and P. A. Carter. 2002b. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267-1275.

Garland, T., Jr., H. Schutz, M. A. Chappell, B. K. Keeney, T. H. Meek, L. E. Copes, W. Acosta, C. Drenowatz, R. C. Maciel, G. van Dijk, C. M. Kotz, and J. C. Eisenmann. 2011b. The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. Journal of Experimental Biology 214:206-229.

Girard, I., E. L. Rezende, and T. Garland, Jr. 2007. Leptin levels and body composition of mice selectively bred for high voluntary locomotor activity. Physiol. Biochem. Zool. 80:568-579.

Gomes, F. R., E. L. Rezende, J. L. Malisch, S. K. Lee, D. A. Rivas, S. A. Kelly, C. Lytle, B. B. Yaspelkis, and T. Garland, Jr. 2009. Glycogen storage and muscle glucose transporters (GLUT-4) of mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:238-248.

Greenwood, B. N., T. E. Foley, H. E. W. Day, D. Burhans, L. Brooks, S. Campeau, and M. Fleshner. 2005. Wheel running alters serotonin (5-HT) transporter, 5-HT1A, 5-HT1B, and alphal(1b)-adrenergic receptor mRNA in the rat raphe nuclei. Biol. Psychiatry 57:559-568.

Grunnet, M., T. Jespersen, and J. F. Perrier. 2004. 5-HT1A receptors modulate small-conductance Ca2+-activated K+ channels. Journal of Neuroscience Research 78:845-854.

Hannon, R. M., S. A. Kelly, K. M. Middleton, E. M. Kolb, D. Pomp, and T. Garland, Jr. 2008. Phenotypic effects of the "mini-muscle" allele in a large HR x C57BL/6J mouse backcross. J. Hered. 99:349-354.

Haubold, K. W., D. L. Allen, Y. Capetanaki, and L. A. Leinwand. 2003. Loss of desmin leads to impaired voluntary wheel running and treadmill exercise performance. Journal of Applied Physiology 95:1617-1622.

Homberg, J. R. 2012. Serotonin and decision making processes. Neuroscience and Biobehavioral Reviews 36:218-236.

Houle-Leroy, P., H. Guderley, J. G. Swallow, and T. Garland, Jr. 2003. Artificial selection for high activity favors mighty mini-muscles in house mice. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 284:R433-R443.

Jacobs, B. L. and E. C. Azmitia. 1992. Structure and function of the brain-serotonin system. Physiological Reviews 72:165-229.

Keeney, B. K., T. H. Meek, K. M. Middleton, L. F. Holness, and T. Garland, Jr. 2012. Sex differences in cannabinoid receptor-1 (CB1) pharmacology in mice selectively bred for high voluntary wheel-running behavior. Pharmacology Biochemistry and Behavior 101:528-537.

Keeney, B. K., D. A. Raichlen, T. H. Meek, R. S. Wijeratne, K. M. Middleton, G. L. Gerdeman, and T. Garland. 2008. Differential response to a selective cannabinoid receptor antagonist (SR141716: rimonabant) in female mice from lines selectively bred for high voluntary wheel-running behaviour. Behav. Pharmacol. 19:812-820.

Kelly, S. A., T. A. Bell, S. R. Selitsky, R. J. Buus, K. Hua, G. M. Weinstock, T. Garland, Jr., F. Pardo-Manuel de Villena, and D. Pomp. 2013. A novel intronic single nucleotide polymorphism in the *myosin heavy polypeptide 4* gene is responsible for the mini-muscle phenotype characterized by major reduction in hind-limb muscle mass in mice. Genetics 195:1385-1395.

Kolb, E. M., S. A. Kelly, K. M. Middleton, L. S. Sermsakdi, M. A. Chappell, and T. Garland, Jr. 2010. Erythropoietin elevates VO_2max but not voluntary wheel running in mice. Journal of Experimental Biology 213:510-519.

Kolb, E. M., E. L. Rezende, L. Holness, A. Radtke, S. K. Lee, A. Obenaus, and T. Garland, Jr. 2013. Mice selectively bred for high voluntary wheel running have larger midbrains: support for the mosaic model of brain evolution. Journal of Experimental Biology 216:515-523.

Koteja, P., T. Garland, Jr., J. K. Sax, J. G. Swallow, and P. A. Carter. 1999. Behaviour of house mice artificially selected for high levels of voluntary wheel running. Animal Behaviour 58:1307-1318.

Lambert, M. I. and T. D. Noakes. 1990. Spontaneous running increases VO_2max and running performance in rats. Journal of Applied Physiology 68:400-403.

Lambert, M. I., C. Van Zyl, R. Jaunky, E. V. Lambert, and T. D. Noakes. 1996. Tests of running performance do not predict subsequent spontaneous running in rats. Physiology & Behavior 60:171-176.

Lerman, I., B. C. Harrison, K. Freeman, T. E. Hewett, D. L. Allen, J. Robbins, and L. A. Leinwand. 2002. Genetic variability in forced and voluntary endurance exercise performance in seven inbred mouse strains. Journal of Applied Physiology 92:2245-2255.

Lightfoot, J. T., M. J. Turner, M. Daves, A. Vordermark, and S. R. Kleeberger. 2004. Genetic influence on daily wheel running activity level. Physiol. Genomics 19:270-276.

Malisch, J. L., C. W. Breuner, F. R. Gomes, M. A. Chappell, and T. Garland, Jr. 2008a. Circadian pattern of total and free corticosterone concentrations, corticosteroid-binding globulin, and physical activity in mice selectively bred for high voluntary wheel-running behavior. Gen. Comp. Endocrinol. 156:210-217.

Malisch, J. L., C. W. Breuner, E. M. Kolb, H. Wada, R. M. Hannon, M. A. Chappell, K. M. Middleton, and T. Garland, Jr. 2008b. Behavioral despair and home-cage activity in mice with chronically clevated baseline borticosterone concentrations. Pp. 192-201. Behavior Genetics.

Malisch, J. L., S. A. Kelly, A. Bhanvadia, K. M. Blank, R. L. Marsik, E. G. Platzer, and T. Garland, Jr. 2009. Lines of mice with chronically elevated baseline corticosterone levels are more susceptible to a parasitic nematode infection. Zoology 112:316-324.

Malisch, J. L., W. Saltzman, F. R. Gomes, E. L. Rezende, D. R. Jeske, and T. Garland, Jr. 2007. Baseline and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. Physiol. Biochem. Zool. 80:146-156.

Marona-Lewicka, D. and D. E. Nichols. 2009. WAY 100635 produces discriminative stimulus effects in rats mediated by dopamine D(4) receptor activation. Behav. Pharmacol. 20:114-118.

Marona-Lewicka, D. and D. E. Nichols. 2011. Potential serotonin 5-HT1A and dopamine D-4 receptor modulation of the discriminative stimulus effects of amphetamine in rats. Behav. Pharmacol. 22:508-515.

Meek, T. H., E. M. Dlugosz, K. T. Vu, and T. Garland, Jr. 2012. Effects of leptin treatment and western diet on wheel running in selectively bred high runner mice. Physiology & Behavior 106:252-258.

Meek, T. H., B. P. Lonquich, R. M. Hannon, and T. Garland, Jr. 2009. Endurance capacity of mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:2908-2917.

Meeusen, R., P. Watson, H. Hasegawa, B. Roelands, and M. F. Piacentini. 2006. Central fatigue - The serotonin hypothesis and beyond. Sports Medicine 36:881-909.

Muller, C. P. and B. L. Jabcobs, eds. 2010. Handbook of the Behavioral Neurobiology of Serotonin. Academic Press, Elsevier, London, UK.

Newsholme, E. A. and E. Blomstrand. 2006. Branched-chain amino acids and central fatigue. Journal of Nutrition 136:274S-276S.

Nielsen, B., J. R. Hales, S. Strange, N. J. Christensen, J. Warberg, and B. Saltin. 1993. Human circulatory and thermoregulatory adaptations with heat acclimation and exercise in a hot, dry environment. The Journal of Physiology 460:467-485.

Olijslagers, J. E., T. R. Werkman, A. C. McCreary, C. G. Kruse, and W. J. Wadman. 2006. Modulation of midbrain dopamine neurotransmission by serotonin, a versatile interaction between neurotransmitters and significance for antipsychotic drug action. Current Neuropharmacology 4:59-68.

Perrier, J.-F. and F. Cotel. 2015. Serotonergic modulation of spinal motor control. Current Opinion in Neurobiology 33:1-7.

Rezende, E. L., M. A. Chappell, F. R. Gomes, J. L. Malisch, and T. Garland, Jr. 2005. Maximal metabolic rates during voluntary exercise, forced exercise, and cold exposure in house mice selectively bred for high wheel-running. Journal of Experimental Biology 208:2447-2458.

Rezende, E. L., T. Garland, Jr., M. A. Chappell, J. L. Malisch, and F. R. Gomes. 2006a. Maximum aerobic performance in lines of *Mus* selected for high wheel-running activity: effects of selection, oxygen availability and the mini-muscle phenotype. Journal of Experimental Biology 209:115-127.

Rezende, E. L., F. R. Gomes, J. L. Malisch, M. A. Chappell, and T. Garland, Jr. 2006b. Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. Journal of Applied Physiology 101:477-485.

Rhodes, J., G. Hosack, I. Girard, A. Kelley, G. S. Mitchell, and T. Garland, Jr. 2001. Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology 158:120-131.

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integrative and Comparative Biology 45:438-455.

Rhodes, J. S. and T. Garland, Jr. 2003. Differential sensitivity to acute administration of Ritalin, apormorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology 167:242-250.

Rhodes, J. S., T. Garland, Jr., and S. C. Gammie. 2003. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behav. Neurosci. 117:1243-1256.

Rhodes, J. S. and T. Kawecki. 2009. Behavior and Neurobiology *in* T. Garland, Jr., and M. D. Rose, eds. Experimental Evolution. University of California Press, Berkely, CA.

Roelands, B. and R. Meeusen. 2010. Alterations in central fatigue by pharmacological manipulations of neurotransmitters in normal and high ambient temperature. Sports Medicine 40:229-246.

Soares, D. D., C. C. Coimbra, and U. Marubayashi. 2007. Tryptophan-induced central fatigue in exercising rats is related to serotonin content in preoptic area. Neuroscience Letters 415:274-278.

Soares, D. D., N. R. V. Lima, C. C. Coimbra, and U. Marubayashi. 2003. Evidence that tryptophan reduces mechanical efficiency and running performance in rats. Pharmacology Biochemistry and Behavior 74:357-362.

Swallow, J. G., P. A. Carter, and T. Garland, Jr. 1998a. Artificial selection for increased wheel-running behavior in house mice. Behavior Genetics 28:227-237.

Swallow, J. G., T. Garland, Jr., P. A. Carter, W. Z. Zhan, and G. C. Sieck. 1998b. Effects of voluntary activity and genetic selection on aerobic capacity in house mice (*Mus domesticus*). Journal of Applied Physiology 84:69-76.

Swallow, J. G., J. P. Hayes, P. Koteja, and T. Garland, Jr. 2009. Selection experiments and experimental evolution of performance and physiology *in* T. Garland, Jr., and M. R. Rose, eds. Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments. University of California Press, Berkeley, CA.

Swallow, J. G., P. Koteja, P. A. Carter, and T. Garland, Jr. 1999. Artificial selection for increased wheel-running activity in house mice results in decreased body mass at maturity. Journal of Experimental Biology 202:2513-2520.

Syme, D. A., K. Evashuk, B. Grintuch, E. L. Rezende, and T. Garland, Jr. 2005. Contractile abilities of normal and "mini" triceps surae muscles from mice (*Mus domesticus*) selectively bred for high voluntary wheel running. Journal of Applied Physiology 99:1308-1316.

Vaanholt, L., P. Meerlo, T. Garland, Jr., G. Visser, and G. v. Dijk. 2007. Plasma Adiponectin is Increased in Mice Selectively Bred for High Wheel-running Activity, but not by Wheel Running per sé. Hormone and Metabolic Research 39:377-383.

Wang, M. Y. and N. J. Dun. 1990. 5-hydroxytryptamine responses in neonate rat motorneurons invitro. Journal of Physiology-London 430:87-103.

Waters, R. P., K. J. Renner, R. B. Pringle, C. H. Summers, S. L. Britton, L. G. Koch, and J. G. Swallow. 2008. Selection for aerobic capacity affects corticosterone, monoamines and wheel-running activity. Physiology & Behavior 93:1044-1054.

Wong, L. E., T. Garland, Jr., S. L. Rowan, and R. T. Hepple. 2009. Anatomic capillarization is elevated in the medial gastrocnemius muscle of mighty mini mice. Journal of Applied Physiology 106:1660-1667.

Zhang, L. 1991. Effects of 5-Hydroxytryptamine on cat spinal motorneurons. Canadian Journal of Physiology and Pharmacology 69:154-163.

Chapter 2

Caffeine Stimulates Voluntary Wheel Running in Mice Without Increasing Aerobic Capacity

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ABSTRACT

The "energy drink" Red Bull and the "sports drink" Gatorade are often marketed to athletes, claiming to lead to performance gains. However, both are high in sugars, and also consumed by non-athletes. Few studies have addressed the effects of these drinks or their biologically active components in rodent exercise models. We used three experiments to test effects on both voluntary and forced exercise performance in lines of mice known to differ in "athletic" traits. Mice from four replicate High Runner (HR) lines have been selectively bred for voluntary running on wheels, and run approximately three times as many revolutions per day as do mice from four non-selected Control (C) lines. HR mice also have higher endurance and maximal oxygen consumption (VO_2max) during forced treadmill exercise. In Experiment 1, we tested the hypothesis that Gatorade or Red Bull might cause or allow mice to increase their voluntary wheel running. On days 5 and 6 of 6 days of wheel access, as is used to choose breeders, HR mice ran 3.28-fold more than C, and females ran 1.21-fold more than males, with no linetype by sex interaction. On day 7, mice were administered tap water, Gatorade or Red Bull. During the subsequent 19-hour period, Gatorade had no statistical effect on running, but Red Bull significantly increased distance run by in both sexes and in both HR and C lines. The increase in distance run caused by Red Bull was attributable to time spent running, not an increase in mean (or maximum) speed. As previous studies have found that sucrose alone does not increase wheel running, we tested two other active ingredients in Red Bull, caffeine and taurine, in Experiment 2. With a similar testing protocol, caffeine alone and caffeine + taurine increased running by about half of the magnitude of Red

Bull. In Experiment 3, we tested the hypothesis that Red Bull or caffeine alone can increase physiological performance ability during aerobic exercise, measured as maximal oxygen consumption (VO_2max). In a repeated-measures design spanning 6 days, females were housed with water bottles containing water, caffeine or Red Bull in a randomized order, and tested for VO_2max twice while receiving each fluid (6 total trials). Neither Red Bull nor caffeine significantly affected either VO_2max or a subjective measure of trial quality (cooperativity, rated on a scale of 1-5), but both treatments significantly reduced tiredness (rated on a scale of 1-3) scored at the end of trials for both HR and C lines. Taken together, our results suggest that caffeine increases voluntary exercise levels of mice by delaying fatigue, rather than increasing aerobic capacity.

INTRODUCTION

Red Bull and Gatorade are marketed as energy and performance enhancers. Their marketing schemes target active individuals, with advertisements that include images of extreme sports and sponsorships granted to popular athletes. Red Bull contains a stimulant, caffeine, as well as taurine, B-group vitamins, sodium, glucose, and sucrose. Gatorade contains sucrose, dextrose, sodium, and potassium (Table 2.1).

The purpose of the present study was to test the claims of improved athletic performance and/or voluntary exercise behavior for both of these sports drinks by use of a unique animal model, selectively bred High Runner lines of mice (Swallow et al. 1998a; Wallace and Garland 2016). We used mice (*Mus domesticus,* original population from outbred Hsd:ICR strain) from an ongoing artificial selection experiment that breeds mice based on high voluntary wheel running. The mouse model consists of four replicate High Runner (HR) lines that voluntarily run up to 3-fold more revolutions per day than four non-selected Control (C) lines (Careau et al. 2013). The difference in total wheel revolutions is caused primarily by an increase in average speed of running, rather than an increase in the amount of time spent running (Koteja et al. 1999), although male HR mice do run for significantly longer per night than male controls (Garland et al. 2011). The HR mice have been viewed as animal models of elite human athletes, exhibiting elevated endurance (Meek et al. 2009) and maximal aerobic metabolic rate during exercise (Rezende et al. 2006b; Kolb et al. 2010; Dlugosz et al. 2013b), whereas the Control mice are seen as representing non-athletic humans (Meek et al. 2010). Although HR mice voluntarily run faster in the wheels (Garland et al. 2011) they are not significantly better

(or worse) sprinters (unpublished data), so no "trade-off" (Garland 2014) in locomotor abilities is apparent.

Mice in the selected HR lines reached an evolutionary plateau and have remained at this plateau for about 40 generations (Careau et al. 2013); however, previous studies show that they are physiologically capable of running more under some conditions, such as when given Western diet, high in fat and with added sucrose (Meek et al. 2010). We hypothesized that the putative performance enhancers, Red Bull and Gatorade, would increase voluntary wheel running in both HR and C mice. Further, we predicted that the effects might differ in magnitude between HR and C mice, or between males and females, because of other physiological and neurobiological differences between the linetypes and sexes that are known to exist (Rhodes et al. 2005; Girard et al. 2007; Keeney et al. 2012; Claghorn et al. 2016; Wallace and Garland 2016; Garland et al. 2017).

Both Gatorade and Red Bull contain glucose, sucrose, and fructose (Ventura et al. 2011), and additionally Red Bull contains the psychologically and/or physiologically active compounds caffeine (0.32 mg/ml), taurine (4 mg/ml), and a mixture of B vitamins (Table 2.1). Of the advertised ingredients, caffeine has received the most study in the exercise literature, and has been shown to increase wheel running in gerbils (Pettijohn 1979) and mice (Michna et al. 2003; Lu et al. 2007; Lou et al. 2013) and to improve some measures of endurance exercise in humans (reviewed in Kalmar and Cafarelli 2004; Doherty and Smith 2005). The primary psychomotor effect of caffeine is apparently competitive inhibition of adenosine receptors in the striatum, a region that integrates

signals crucial to the execution of voluntary movements (Fisone et al. 2004). Of relevance here, the striatum has been shown to respond differently between HR and C mice when wheel access is removed (Rhodes et al. 2003), and a preliminary study indicates differences in monoamine concentrations in this region (Waters et al. 2013). Adenosine receptors are widespread in the body (Lynge and Hellsten 2000), so caffeine likely affects multiple systems contributing to performance. Taurine at doses of 100-500 mg/kg (two weeks of daily treatment by lavage) increased forced treadmill endurance-running capacity in rats (Miyazaki et al. 2004) (the doses used for mice in the present study [see Methods] are somewhat higher than this, and were consumed freely over a period of ~19 hours). Combinations of the active ingredients in Red Bull have been shown to increase aerobic endurance performance in non-athlete humans (Alford et al. 2001). The psychomotor effect of taurine is also likely related to its actions on the striatum, as large doses of taurine increase extracellular dopamine in the striatum (Salimäki et al. 2003). However, no previous study has examined the effect of Red Bull or Gatorade specifically on mammals that are genetically predisposed for high exercise abilities and high motivation to exercise, such as the HR mice.

METHODS

Experimental animals

Mice were sampled from multiple generations of an ongoing artificial selection experiment that breeds mice for high levels of voluntary wheel running (Swallow et al. 1998a). Briefly, mice from four replicate High Runner (HR) lines have been selected for

the behavior of voluntary running on wheels (1.12 m circumference), and, at an apparent selection limit, run approximately three times as many revolutions per day as do mice from four non-selected Control (C) lines (Careau et al. 2013). Mice are housed on a 12:12 photoperiod, with lights off 19:00-07:00. All procedures were approved by the University of California, Riverside, Institutional Animal Care and Use Committee, which follows the National Research Council Guide for the Care and Use of Laboratory Animals (revised 2011).

Experiments 1 and 2: effects of Red Bull, its components, and Gatorade on wheel running

We studied mice of both sexes from generation 70 for Experiment 1 (mean age at start of wheel testing = 58 days), and generation 71 for Experiment 2 (mean age = 54 days). In Experiment 1, after 6 days of wheel access as part of the routine testing to select breeders, 250 mice (both sexes) were randomly assigned to one of three treatments: tap water, Gatorade or Red Bull. In Experiment 2, after 6 days of wheel access, 587 mice (both sexes) were randomly assigned to one of four treatments: tap water, Red Bull, caffeine in water, or caffeine + taurine in water (caffeine and taurine each matching the concentration in water of Red Bull, Table 2.1). In both experiments, bottles were filled with 50 ml and provided to mice between 15:00 and 17:00, and left for the duration of the wheel test (19 hours, 17:00-12:00; lights off 19:00-07:00).

Fluid consumption was measured by weighing water bottles as they were placed on cages on day 6 and when they were taken off cages on day 7, a period of 19 hours.

The difference in mass between days 6 and 7 cannot simply be interpreted as fluid consumption because of spilling and evaporation. Therefore, 10 bottles per treatment were placed on empty mouse cages at the same time we were recording wheel data. Any difference in these bottles could only be interpreted as spillage or evaporation. The average spillage for each drink type was subtracted from the apparent fluid consumption before statistical analyses. Any values of less than zero were set to zero for statistical analyses. During Experiment 1, we discovered that the carbonation of Red Bull caused some leakage, so for Experiments 2 and 3, we poured cans of Red Bull into a beaker with a stir bar at high speed for 15 minutes; as expected, this reduced the amount of leakage as compared with Experiment 1.

Wheel revolutions were measured automatically in one-minute bins using photocells attached to wheels from 17:00-12:00, i.e., over a 19-hour period. From the revolutions recorded every minute, total revolutions, number of active minutes (minutes with revolutions greater than 0), average speed (revolutions per active minute), and maximum speed (highest revolutions during a single minute) were calculated.

Experiment 3: effects of Red Bull and caffeine on VO_2max

Retired female breeders (N = 63) from generation 71 were chosen (mean age = 138 days). Females were used because they show less of a decline in voluntary wheel running with age as compared to males (Bronikowski et al. 2006). As mice are active on wheels almost entirely during the dark period (Girard et al. 2001; Malisch et al. 2009), they were put on a reversed photoperiod to allow measurements during normal work

hours. The light cycle was set to 12:12, with lights off at 11:00 and lights on at 23:00 for all mice 5 days prior to the first VO_2max test (see below). At 09:00 on the day of the VO_2max test (similar time-before-dark as in the wheel running experiments), bottles were provided with one of the three treatments: water, caffeine or Red Bull. The Red Bull was at room temperature and stirred to remove dissolved CO_2 from the drink for approximately 15 minutes using a magnetic stir bar. This was done to reduce bottle leakage caused by carbonation. Nonetheless, sufficient leakage sometimes occurred to prevent gathering of accurate data on fluid consumption. The treatment regimens were structured in different permutations of the treatments to maximize diversity in order of treatments given and minimize any possible carry-over effects (e.g., mice were given treatments in balanced, random orders). Each mouse was tested twice for each treatment for a total of six tests per mouse, across six, non-consecutive, testing days with 4 days between trials.

At 12:00, mice were moved to the adjacent testing room and placed in the testing wheel one at a time over the period of 12:00 to 16:00 in the dark room, minimally lit with red light. The timing was chosen to match the peak wheel-running activity of mice (~1-3 hours after the onset of darkness: Girard et al. 2001; Malisch et al. 2009). The O_2 analysis apparatus consisted of an incurrent H_2O scrubber (Drierite), mass flow controller, measurement wheel (effective volume 900 mL), H_2O and CO_2 scrubber (Drierite and indicating soda lime), followed by the O_2 sensor and O_2 analyzer (Figure 2.1). The incurrent CO_2 was not scrubbed. Flow through the wheel metabolic chamber was set to 2,000 mL/min, and instantaneous corrections were applied (Bartholomew et al.

1981). The O_2 consumption data were collected with Warthog Systems LabHelper X software (Mark A. Chappell and the Regents of the University of California, Riverside, CA, USA). This wheel apparatus for measuring VO_2max was chosen over the more traditional treadmill-based test because the former method obtains equivalent values (Dlugosz et al. 2013a) and more closely mimics the behavior for which the High Runner mice have been bred.

At the beginning of each run, a baseline oxygen concentration was recorded for approximately one minute. Each mouse was then placed into the wheel metabolic chamber and forced to run until O_2 consumption plateaued and remained steady for ~75 seconds (trials averaged 6 minutes in length). At the end of each trial, each mouse was rated objectively for tiredness on a scale from 1-3. Tiredness was based on time spent motionless (typically prone) before the mouse resumed spontaneous locomotion, with the rating of 3 indicating greater than 5 seconds before spontaneous locomotion and 1 indicating less than one second before locomotion. Mice were also rated for trial quality, based on cooperativeness while being forced to run (Swallow et al. 1998b). The scale was from 1-5, with 1 indicating no cooperation (the mouse would not attempt to run) and 5 being fully cooperative (mouse would continue to attempt to run even when pushed past the speed at which VO_2max was attained).

VO_2max values were obtained by processing and analyzing the $\%O_2$ data with Warthog LabHelper X software. The program recorded two channels: $\%O_2$ and flow. A typical graph for $\%O_2$ would contain the atmospheric baselines at the beginning and end of the tests, the resting O_2 consumption rate of the mouse placed on the wheel, followed

by a steady increase in O_2 consumption as the mouse was forced to exercise until it reached VO_2max. The graphs were processed by creating a duplicate of the $\%O_2$ channel and creating a baseline collected from atmospheric air at the beginning and end of each trial. The $\%O_2$ samples were smoothed by the Warthog LabAnalyst X software over six consecutive measurements, with measurements recorded once per second. VO_2max was calculated from the $\%O_2$ (compared to baseline) and flow measurements, as the highest 60-second interval. Each mouse was tested twice per treatment, and the higher of the two measurements was used in the analysis.

Statistical analysis

For Experiments 1 and 2, we used the Mixed Procedure in SAS 9.1.3 (SAS Institute, Cary, NC, USA) to apply nested analysis of covariance (ANCOVA) to our data for wheel running. The main factors were drink type (treatment), sex, and linetype (HR vs. C), with replicate lines nested within linetype. The statistical interactions of sex by linetype, sex by drink, drink by linetype, and sex by drink by linetype were also tested, but were typically not significant. Degrees of freedom were 1 and 6 for testing the effects of linetype, drink type, sex, and the interaction terms (see tables in Results for full d.f.). Running data were analyzed in two ways. First, we analyze proportional responses by computing a ratio of running on day 7 (the day of treatment, which was shorter by 4 hours due to experimental setup) to the last night before treatment (day 6). Second, we analyzed absolute responses, using only the data obtained on day 7. Outliers were removed if the standardized residual was > |3|.

The HR lines of mice have been bred for the amount of running over an entire daily cycle, on a 12:12 photoperiod as used in the present experiments, not the amount of wheel running restricted to the dark hours. As we wished to make the wheel measurements as relevant as possible to the conditions of this long-term selection experiment, we analyzed wheel running for as many hours as possible, which was 19 hours, given the time required to administer the fluids. The number of revolutions run during the photophase is typically small for both HR and C lines of mice (e.g., see Girard et al. 2001; Malisch et al. 2009).

For Experiment 3, we used the Mixed Procedure in SAS 9.1.3 to apply repeated-measures (individual mouse as the unit of repeated measures) analysis of covariance (ANCOVA) models to the VO_2max values. The primary factors were linetype (HR or C) and drink type, as well as their interaction. The covariates of body mass, time spent running, and age were also used in the model (though time spent running and age were not predictive, and thus only body mass was included in the data presented here). Tiredness and run quality were considered as covariates for the analysis of VO_2max, but they had little predictive value and ultimately were not included in the reported model. An outlier was removed if the standardized residual was > |3|, and this procedure was repeated as necessary (in the case of VO_2max, four times). In four repetitions, a total of seven outliers from a total of 196 observations were removed (p-values were not viewed prior to the removal of outliers).

RESULTS

Experiment 1: effects of Red Bull and Gatorade on wheel running

Averaging values for days 5 and 6 of wheel access, consistent with many previous studies of these lines of mice (Garland et al. 2011) HR mice ran 3.3-fold more revolutions than C ($p < 0.0001$), and females ran 1.2-fold more revolutions than males ($p = 0.0290$), with no linetype by sex interaction ($p = 0.2821$) (results not shown).

On the 7th day, based on analysis of proportional responses, drink type had a significant effect on total wheel revolutions (+14% for Red Bull, +4% for Gatorade, and -5% for water; treatment $P = 0.0053$, Table 2.2, Figure 2.2) in both High Runner and Control lines, with no statistical interaction between drink and linetype, drink and sex, or drink by sex by linetype (all $P > 0.17$). (Remember that values recorded on day 7 were for only 19 hours, versus 23 hours on day 6, so the apparent reduction with water is an artifact of the shorter amount of time over which wheel revolutions were recorded.) The effects of drink type were through increases in the amount of time spent running ($p = 0.0004$), with no statistically significant effect on the average speed of wheel running or the maximum running speed (highest 1-minute interval) (Table 2.2, Figure 2.3).

Based on analyses of absolute amounts of wheel running on day 7 (Table 2.3, Figure 2.4), drink treatment again affected both total revolutions run (+21% for Red Bull, -1% for Gatorade) and the number of active minutes (+23% for Red Bull, +2% for Gatorade), with no interactions.

The amount of fluid consumed (adjusted for spillage and evaporation) depended on drink type ($P = 0.0321$), but was not significantly affected by any other factor or

interaction term. On average, mice consumed 7.4 $\pm$ 0.88 g of water, 10.4 $\pm$ 0.84 g of Gatorade, and 8.4 $\pm$ 0.89 g of Red Bull. (Results were similar when body mass was used as a covariate.)

Experiment 2: effects of Red Bull, caffeine, and taurine on wheel running

Based on proportional responses, on the 7th day, drink type interacted with linetype in its effect on total wheel revolutions (P = 0.0259, Table 2.2, Figure 2.2). For both HR (-9%) and C (-1%) lines, mice receiving water had a small decrease in the number of wheel revolutions, compared to night 6, attributable to the shorter duration of data recording on day 7. In contrast, Red Bull increased revolutions in both HR (11%) and C (20%) mice, caffeine increased revolutions by 2% and 7%, respectively, and caffeine + taurine increased revolutions by 5% in HR mice while decreasing revolutions by 1% in C mice. The effect of drink type was through changes in the amount of time spent running (linetype by treatment interaction P = 0.0516), rather than through effects on running speed (Table 2.2).

Based on analyses of absolute amounts of wheel running on day 7 (Table 2.3), revolutions run was not significantly affected by drink type (P = 0.1484), but HR mice ran more than C (P < 0.0001) and females ran more than males (P = 0.0303), with no significant interactions. We performed additional analyses to elucidate effects of caffeine. First, we computed an a priori contrast of water versus the three caffeine-containing fluids and found a marginally non-significant effect of caffeine ($F_{1,18}$ = 3.98, P = 0.0615). Second, we tested whether the amount of fluid containing caffeine, consumed

during the 19-hour wheel trial, had an effect. This variable indicating caffeine dose was taken as the amount of Red Bull or caffeine or caffeine plus taurine solution consumed (the latter two were matched to Red Bull in terms of grams caffeine per ml of fluid), or given a value of zero for mice that had water. This covariate was a significant positive predictor of wheel revolutions ($F_{1,536} = 4.75$, $P = 0.0297$) and the significance levels of the other factors were little changed (P for drink type = 0.1623, P for linetype < 0.0001, P for sex = 0.0205, all other P = n.s.).

Drink treatment interacted with linetype in its effect on the number of minutes with any wheel revolutions (P = 0.0087). Inspection of the values shown in Figure 2.4 indicates that all three fluid treatments increased the number of active intervals, relative to water, regardless of sex or linetype, although this differential varied somewhat among the subgroups. In addition, HR mice of both sexes always had more active intervals than their C counterparts, regardless of the fluid being consumed.

The amount of fluid consumed (adjusted for spillage and evaporation) depended on drink type (P < 0.0001), was higher in males (P = 0.0014), which are larger than females, but was not significantly affected by linetype or any interaction term. On average, mice consumed 8.0 $\pm$ 0.24 g of water, 5.1 $\pm$ 0.25 g of Red Bull, 8.0 $\pm$ 0.23 g of caffeine solution, and 8.4 $\pm$ 0.24 g of caffeine plus taurine solution. When body mass was included as a covariate (P < 0.0001), the effect of drink type was still highly significant (P < 0.0001), but the effect of sex was eliminated (P = 0.8878), and again no other factor or interaciton term was significant. Adjusted for body mass (grand mean =

25.4 g), mice consumed 7.9 $\pm$ 0.26 g of water, 5.1 $\pm$ 0.27 g of Red Bull, 8.0 $\pm$ 0.25 g of caffeine solution, and 8.3 $\pm$ 0.26 g of caffeine plus taurine solution.

Experiment 3: effects of Red Bull and caffeine on VO_2max

The effect of treatment on VO_2max was not statistically significant (Table 2.4, Figure 2.5). Linetype had a significant effect on VO_2max (P = 0.0242: Table 2.4, Figure 2.5), consistent with previous findings that HR mice have an increased maximal aerobic capacity (Swallow et al. 1998b; Rezende et al. 2006a; Dlugosz et al. 2009; Kolb et al. 2010). Trial duration was not a significant predictor of VO_2max (weak positive effect, results not shown). Tiredness and run quality scores did not affect VO_2max, nor did their inclusion in the model have an appreciable effect on the effect sizes or significance level of other factors (results not shown). Thus, these scores were not included in the reported model. Cooperativeness was not significantly affected by treatment (Table 2.4, Figure 2.6). HR mice tended to be more cooperative than C mice, but not significantly so (P = 0.0535). Body mass was used as a covariate for VO_2max and was highly predictive (P < 0.0001, Table 2.4).

Although tiredness did not affect VO_2max, tiredness was significantly affected by treatment (P = 0.0039; Table 2.4, Figure 2.6), with caffeine-treated mice being rated as less tired than water-treated mice, and Red Bull-treated mice even less tired than those receiving caffeine. Test number (each mouse was tested six times for VO_2max) was also considered as a covariate, but was not a significant predictor (P = 0.3) and so was not

included in the final model. Linetype, body mass, trial length, and age did not affect tiredness ($P > 0.05$; results not shown).

We also analyzed the amount of fluid consumed immediately prior to initiation of VO_2max trials, adjusted for spillage and evaporation as described above. The amount of time between placing bottles on the cages and the start of the VO_2max trial averaged 293 minutes, but varied from 178 to 456 minutes among individual trials, so we used this time interval as a covariate. Adjusting for elapsed time ($P < 0.0001$), fluid consumption depended on drink type ($P = 0.0007$), but was not affected by linetype ($P = 0.6350$) nor the drink type by linetype interaction ($P = 0.7154$). On average, mice consumed 4.7 ± 0.51 g of water, 5.3 ± 0.51 g of Red Bull, 8.2 ± 0.51 g of caffeine solution. (Results were similar when body mass was used as a covariate.)

Finally, we tested whether the amount of fluid containing caffeine, consumed prior to VO_2max trials, had an effect. This variable indicating caffeine dose was taken as the amount of Red Bull or caffeine solution consumed (the latter was matched to Red Bull in terms of grams caffeine per ml of fluid), or given a value of zero for mice that had water. This covariate was not a significant predictor of VO_2max ($P = 0.6719$) and the significance levels of the other factors and body mass were scarcely changed. Results for analyses of cooperativeness and tiredness as dependent variables were also unaffected by inclusion of caffeine dose as a covariate.

DISCUSSION

As compared with mice drinking tap water, Red Bull increased voluntary wheel running (total revolutions/day) in both Experiment 1 (differences of least squares means, $P = 0.0243$) and Experiment 2 ($P = 0.0292$). The increase in total distance run was caused by a greater amount of time spent running (more 1-minute intervals showing any revolutions), not an increase in average (or maximum) running speed. When caffeine or caffeine + taurine was given in water, in the same concentrations as found in Red Bull (Experiment 2), the increase in wheel running relative to water was only about half that of Red Bull (Figure 2.4) and not statistically significant ($P = 0.1305$ and $P = 0.3772$, respectively). If caffeine is the only ingredient affecting wheel running duration, then the differential effects versus Red Bull observed in Experiment 2 potentially could be explained if mice drank circa twice as much Red Bull as compared with the caffeine-containing solutions. However, the difference was opposite to this, with mice consuming, on average, about 8 ml of caffeine-containing solutions versus only 5 ml of Red Bull. (Previous studies have also showed that mice will consume more fluid when given caffeine solutions vs water Michna et al. 2003; Lu et al. 2007; Lou et al. 2013.) Thus, some other ingredient(s) in Red Bull (i.e., one of the sugars or B vitamins) appears to be having an additive or possibly interactive effect on voluntary activity levels, but whether this effect is acting through central or peripheral mechanisms is unknown. Here, it is worth noting that in similar experiments performed on females at generation 42, sucrose solutions increased wheel running revolutions per day in C but not HR mice (Kolb 2010).

Maximal oxygen consumption (VO_2max) was not significantly affected by the fluid treatments (Table 2.4, Figure 2.5). However, both Red Bull and caffeine reduced tiredness ratings of both HR and C mice, so the finding that Red Bull and caffeine caused an increase in wheel-running behavior may be attributable to decreased or delayed fatigue by affecting central nervous system fatigue (reviewed in Kalmar and Cafarelli 2004). Such an effect could be attributable to caffeine's known effects as an adenosine receptor antagonist (Davis et al. 2003), and/or it's complex effects on other neurotransmitters (Fredholm et al. 1999).

Post-trial tiredness ratings did not significantly differ between HR and C lines, but tiredness was significantly reduced by caffeine and Red Bull (Table 2.4, Figure 2.6). This effect might also be caused by the action of caffeine on the perception of fatigue, as caffeine acts as a stimulant on the central nervous system and can delay fatigue by blocking adenosine receptors (Davis et al. 2003). Cooperativeness scored during VO_2max trials tended to be higher in HR than C lines of mice (but not significantly so, $P = 0.0535$), supporting previous evidence of a motivational difference between the linetypes (Rhodes et al. 2005; Belke and Garland 2007; Saul et al. 2016). Studies of these lines at generation 10 (Swallow et al. 1998b) and 35 (Rezende et al. 2005) did not find a significant difference in cooperativity between the HR and C lines, so if there is indeed a difference in cooperativity, it may have evolved in subsequent generations.

The mechanism by which caffeine increased wheel running and decreased tiredness in mice is unclear, but one or more of many proposed mechanisms may be involved. Caffeine has been shown to delay fatigue in a variety of studies on humans

(Costill et al. 1978; Graham and Spriet 1995; Cole et al. 1996; Alford et al. 2001), but fewer studies have been conducted on rodents. In mice, ad-lib access to bottles containing caffeine solution (similar to our method) or caffeinated tea increased wheel running and fluid consumption (Michna et al. 2003; Lu et al. 2007; Lou et al. 2013). In gerbils, caffeine increased wheel revolutions when animals were placed inside a wheel and measured for 10 minutes, 30 minutes after receiving an intraperitoneal injection of caffeine (Pettijohn 1979). If and how this sort of wheel-running test relates to the prolonged (19 hour) test used in the present study is unknown. Ryu et al. (2001) showed that rats which received caffeine ran significantly longer in a treadmill endurance trial and had significantly lower blood glucose, higher blood lactate, and higher liver glycogen at exhaustion than those receiving placebo, and attributed this to the effect of caffeine on lipolysis and "glycogen sparing". However, the evidence for changes in substrate utilization as a result of caffeine is mixed (Graham et al. 2000; Cruz et al. 2015). Lim et al. (2001) also showed that rats injected subcutaneously with caffeine ran significantly longer in a treadmill endurance trial, and that caffeine-treated animals had lower tryptophan hydroxylase in the raphe nuclei, suggesting that caffeine suppressed serotonin-related central fatigue. Serotonin-induced central fatigue has been suggested to have been altered by selective breeding in HR mice, but in this case, caffeine seems to have affected both HR and C mice in the same way (Claghorn et al. 2016). Davis et al. (2003) showed that caffeine injected intracerebroventricularly increased spontaneous activity and time to exhaustion in a forced treadmill exercise trial, whereas intraperitoneal caffeine injection had no such effect, and concluded that a substantial part of the effect of

caffeine on endurance was due to central nervous system effects. Central effects of caffeine in humans have been well reviewed (Graham and Spriet 1995; Kalmar and Cafarelli 2004; Kalmar 2005).

In conclusion, Red Bull (and to a lesser extent, caffeine in water) increased voluntary wheel running by mice, and decreased tiredness following maximal metabolic rate trials, while not increasing aerobic capacity itself. The mechanism of action is unknown, but could be a combination of central and peripheral effects. In any case, positive effects on exercise behavior, if they can be shown to occur in humans, could have important implications for promoting voluntary exercise.

Table 2.1. Active ingredients in Red Bull and Gatorade. All units given are mg/ml. Caffeine, taurine, sodium, and potassium were taken from the nutrition label or product websites, and sugar information were measured values from Ventura et al., (2011).

	Caffeine	Glucose	Sucrose	Fructose	Total Sugar	Taurine	Sodium	Potassium
Red Bull	0.32	36	51	19	106	4	0.4	0
Gatorade	-	24	14	21	59	-	0.45	0.13

Table 2.2. ANOVA results for experiments 1 and 2: proportional responses. Red Bull increased wheel running in both experiments, in roughly the same magnitude. The increase in wheel running was caused by an increase in the time spent running, rather than the average or max speed of running.

Experiment 1		Proportional Change in Revolutions		Proportional Change in Max Speed		Proportional Change in Mean Speed		Proportional Change in Active Minutes	
Factor	df	F	P	F	P	F	P	F	P
Sex	(1, 6)	0.51	0.5026	1.5	0.2661	0.96	0.3658	0.01	0.9361
Linetype	(1, 6)	3.02	0.1328	0.49	0.511	2.96	0.136	1.98	0.2094
Treatment	(2, 12)	6.14	**0.0145**	0.2	0.8242	0	0.9997	11.74	**0.0015**
Sex*Treatment	(2, 214)	0.07	0.9278	1.52	0.2205	0.59	0.5579	0.18	0.8317
Linetype*Treatment	(2, 12)	0.67	0.5318	0.04	0.9579	0.11	0.893	1.02	0.3899
Sex*Linetype	(1, 6)	0	0.9515	0.23	0.6492	0.03	0.8615	0.03	0.8721
Sex*Linetype*Treatment	(2, 214)	1.78	0.1715	1.73	0.1791	1.12	0.3275	1.22	0.2965
Experiment 2									
Factor	df	F	P	F	P	F	P	F	P
Sex	(1, 6)	0.64	0.4554	2.62	0.1566	0.1	0.7605	0.58	0.4738
Linetype	(1, 6)	0	0.9684	3.26	0.1211	0.97	0.3637	0.15	0.712
Treatment	(3, 18)	15.49	**<.0001**	2.26	0.1161	0.85	0.4841	22.17	**<.0001**
Sex*Treatment	(3, 540)	0.95	0.4165	1.89	0.1309	0.71	0.5458	0.59	0.6233
Linetype*Treatment	(3, 18)	3.91	**0.0259**	1.75	0.1932	1.72	0.1981	3.12	0.0516
Sex*Linetype	(1, 6)	0.23	0.6503	0.16	0.7039	0	0.9872	0.37	0.5642
Sex*Linetype*Treatment	(3, 540)	1.14	0.3311	0.62	0.6049	1.77	0.1527	1.11	0.3456

Table 2.3. ANOVA results for experiments 1 and 2: absolute responses. Drink type (Treatment) had a significant effect on revolutions run per day and number of active minutes in Experiment 1, as well as minutes run in Experiment 2 (Figure 2.4). As compared with mice drinking tap water, Red Bull increased voluntary wheel running (total revolutions/day) in both Experiment 1 (differences of least squares means, P = 0.0243) and Experiment 2 (P = 0.0292). This increase in wheel running was caused by an increase in the time spent running, rather than the average or max speed of running.

Experiment 1		Revolutions		Max Speed		Mean Speed		Active Minutes	
Factor	df	F	P	F	P	F	P	F	P
Sex	(1, 6)	4.12	0.0886	0.11	0.7490	0.61	0.4655	10.98	**0.0161**
Linetype	(1, 6)	185.79	**<.0001**	150.42	**<.0001**	98.38	**<.0001**	12.36	**0.0126**
Treatment	(2, 12)	4.87	**0.0283**	0.23	0.7960	0.96	0.4088	15.07	**0.0005**
Sex*Treatment	(2, 214)	0.10	0.9087	0.19	0.8303	0.01	0.9881	0.25	0.7829
Linetype*Treatment	(2, 12)	0.07	0.9299	0.56	0.5869	0.78	0.4807	1.05	0.3793
Sex*Linetype	(1, 6)	0.60	0.4679	0.28	0.6140	0.73	0.4257	0.65	0.4504
Sex*Linetype*Treatment	(2, 214)	0.29	0.7516	0.74	0.4781	0.34	0.7156	0.18	0.8391
Experiment 2									
Factor	df	F	P	F	P	F	P	F	P
Sex	(1, 6)	7.96	**0.0303**	4.06	0.0905	3.35	0.1171	13.98	**0.0096**
Linetype	(1, 6)	166.48	**<.0001**	141.81	**<.0001**	107.68	**<.0001**	4.71	0.0730
Treatment	(3, 18)	2.01	0.1484	1.39	0.2776	0.83	0.4954	13.22	**<.0001**
Sex*Treatment	(3, 540)	0.22	0.8819	0.55	0.6476	0.47	0.7037	0.71	0.5455
Linetype*Treatment	(3, 18)	1.05	0.3955	0.49	0.6922	0.26	0.8513	5.27	**0.0087**
Sex*Linetype	(1, 6)	0.77	0.4147	1.93	0.2145	1.30	0.2978	1.99	0.2085
Sex*Linetype*Treatment	(3, 540)	0.87	0.4547	0.13	0.9409	0.65	0.5845	1.56	0.1987

Table 2.4. Repeated measures ANOVA results for experiment 3. The higher of the two measurements for an individual mouse and treatment combination were used in the analysis. Nested, repeated-measures ANCOVA indicated that body mass was a significant predictor of VO_2max ($P < 0.0001$), and HR mice had greater VO_2max than control mice ($P = 0.0242$), but treatment did not significantly affect VO_2max and there was no linetype by treatment interaction. HR mice tended to be more cooperative, but not significantly so ($P = 0.0535$).

		VO_2max		Tiredness		Cooperativity	
Factor	df	F	P	F	P	F	P
Linetype	(1, 6)	8.96	**0.0242**	0.78	0.4121	5.74	0.0535
Treatment	(2, 12)	1.01	0.3916	9.11	**0.0039**	0.89	0.4367
Linetype*Treatment	(2, 12)	0.44	0.6540	0.31	0.7390	0.41	0.6737
Body mass	(1, 164)	28.14	<.0001				

Figure 2.1. Simplified schematic of apparatus to measure maximal oxygen consumption (VO_2max) during forced exercise in a wheel metabolic chamber (see Dlugosz et al. 2013a; Andrew et al. 2016). CO_2 was not scrubbed from the incurrent air. The wheel metabolic chamber had an effective volume of 900 ml.

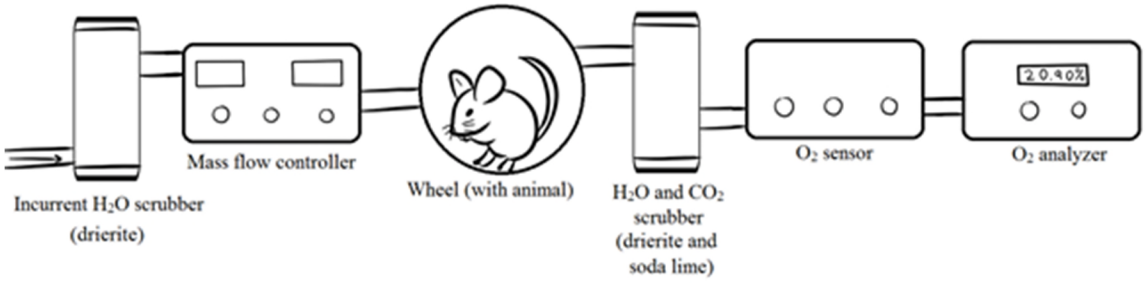


Figure 2.2. Wheel revolutions as proportional responses were significantly increased by caffeine treatment. Values are least squares means and associated standard errors from an ANOVA conducted in SAS Procedure Mixed. Asterisks denote values significantly different from zero at $P < 0.05$. **Left:** In experiment 1, Red Bull significantly (Table 2.2, $p = 0.0053$) increased voluntary wheel running as measured over 19 hours (17:00-12:00) in both High Runner (HR) and Control (C) lines, with no statistical interaction between drink and linetype, or drink and sex. Drink type affected total wheel revolutions by increasing the average time spent running ($p=0.0004$). This difference is primarily driven by an increase in wheel running in the Red Bull group. Drink type did not have a statistically significant effect on the average or maximum speed of wheel running (Table 2.2). **Right:** In experiment 2, caffeine-containing beverages significantly ($P < 0.0001$) increased voluntary wheel running over 19 hours (17:00-12:00) in both High Runner (HR) and Control (C) lines, with Red Bull having about twice the effect of Caffeine or Caffeine + Taurine. Values shown are proportional differences in running between days 6 and 7. Drink type affected total wheel revolutions by increasing the average time spent running ($p<0.0001$). Drink type did not have a statistically significant effect on the average speed of wheel running (Table 2.2). Results for maximum running speed (highest 1-minute interval) were similar to those for average speed (see Fig 3 and Table 2.2).

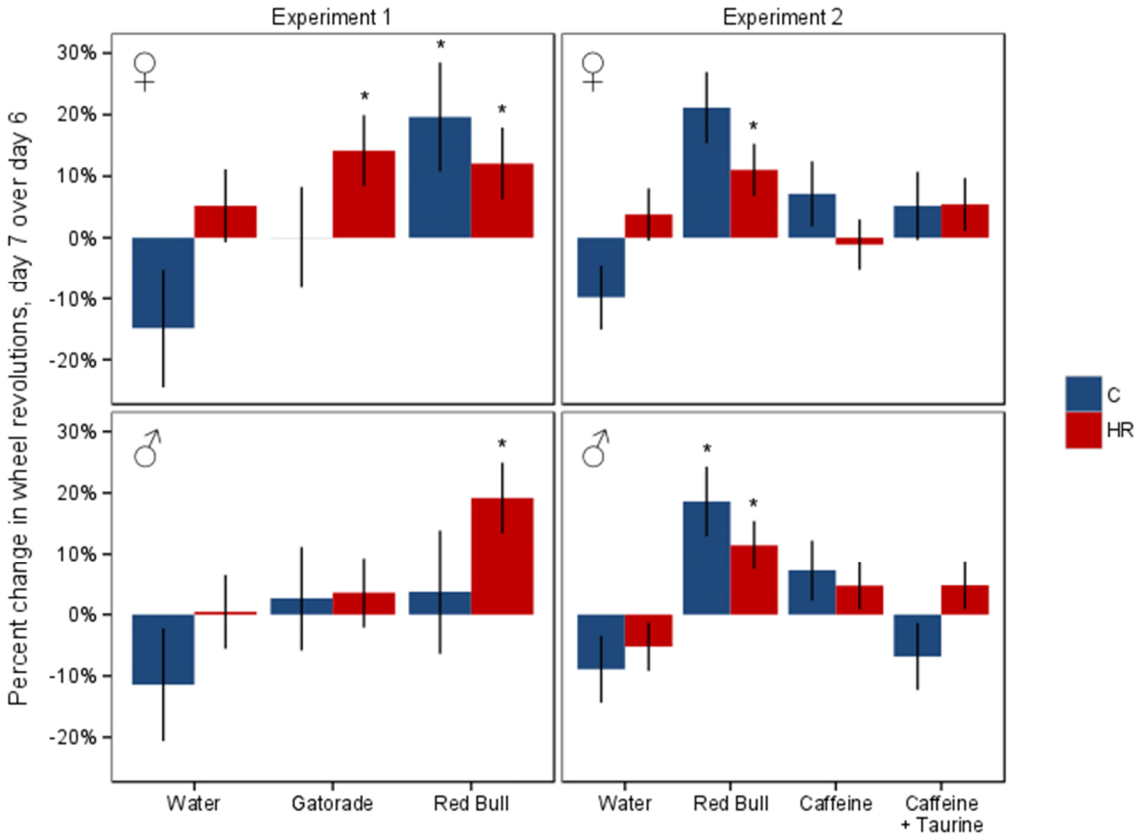


Figure 2.3. Maximum wheel speed as proportional responses was not affected by caffeine treatment. Values are least squares means and associated standard errors from an ANOVA conducted in SAS Procedure Mixed. Asterisks denote values significantly different from zero at $P < 0.05$. **Left:** Experiment 1 change in maximum wheel speed (highest revolutions in a 1-minute interval, as monitored over 19 hours) between days 6 and 7 of wheel access (treatment was given on day 7). **Right:** Experiment 2 change in maximum wheel speed between days 6 and 7 of wheel access (treatment was given on day 7).

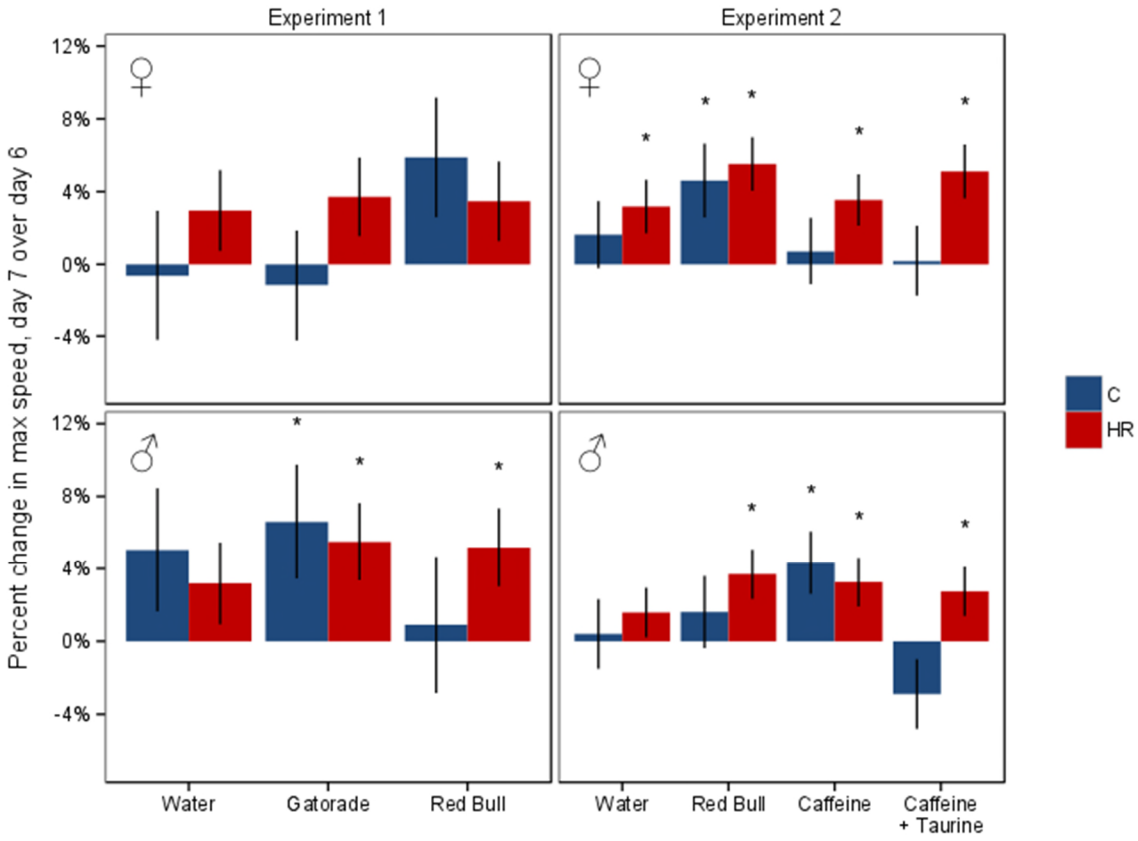


Figure 2.4. Absolute responses for wheel revolutions and the number of active minutes. In experiment 1, both total revolutions run and the number of active minutes were significantly increased by Red Bull (see Table 2.3). In experiment 2, revolutions were increased by Red Bull. Wheel circumference = 1.12 m. Values are least squares means and associated standard errors from an ANOVA conducted in SAS Procedure Mixed.

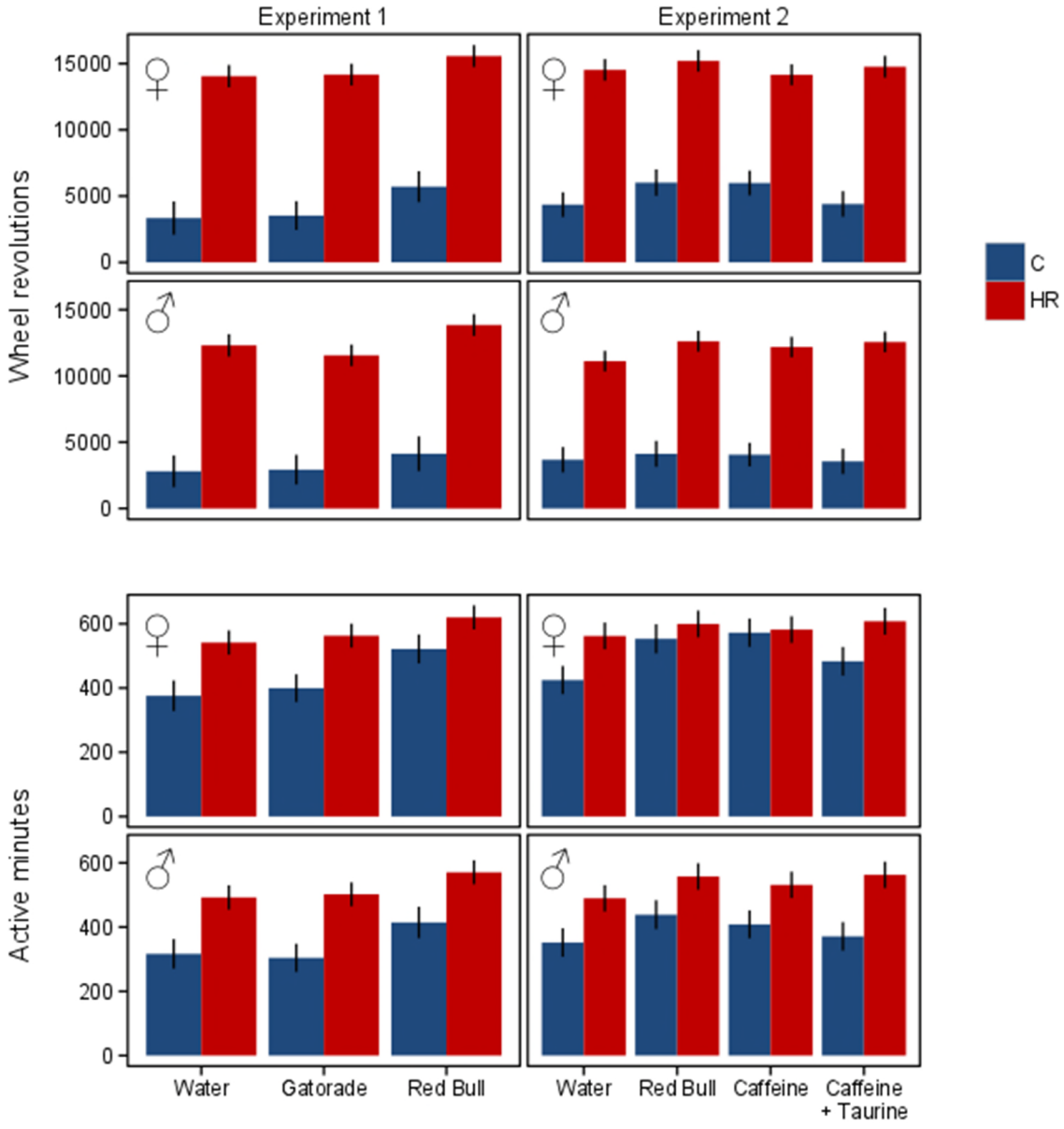


Figure 2.5. VO_2max is greater in HR mice, but not affected by treatment. **Left:** VO_2max vs. body mass, separated by treatment and linetype. Symbols are individual trials (the higher of two trials per mouse per treatment). Lines indicate least squares regressions within treatment and linetype, and the shaded area indicates 95% confidence intervals about the lines. **Right:** Average VO_2max values over the 3 treatments for HR and C mice. Values are least squares means and associated standard errors from a repeated-measures ANCOVA conducted in SAS Procedure Mixed.

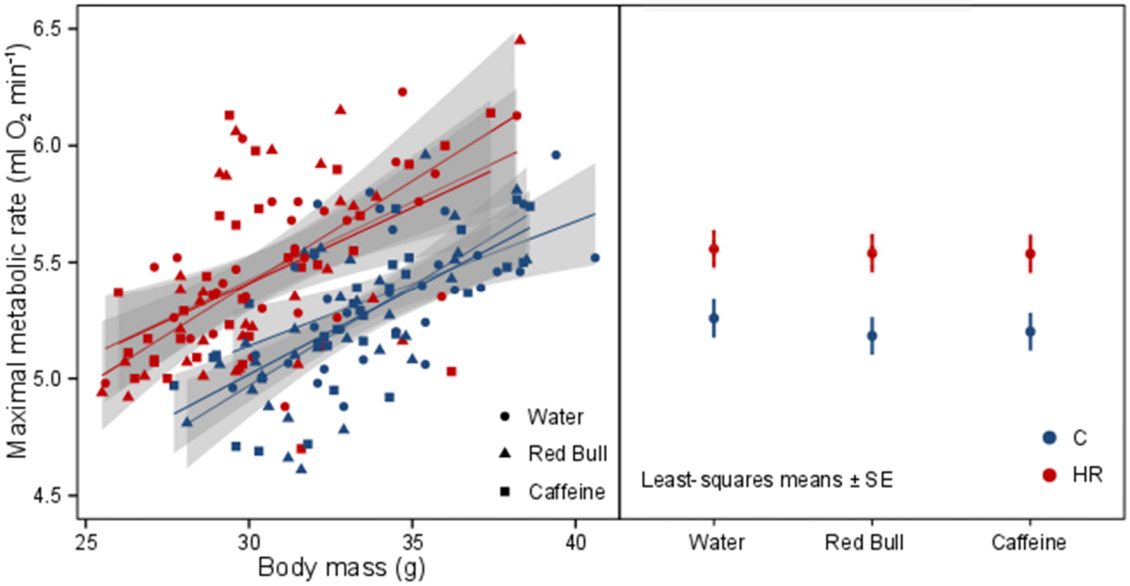
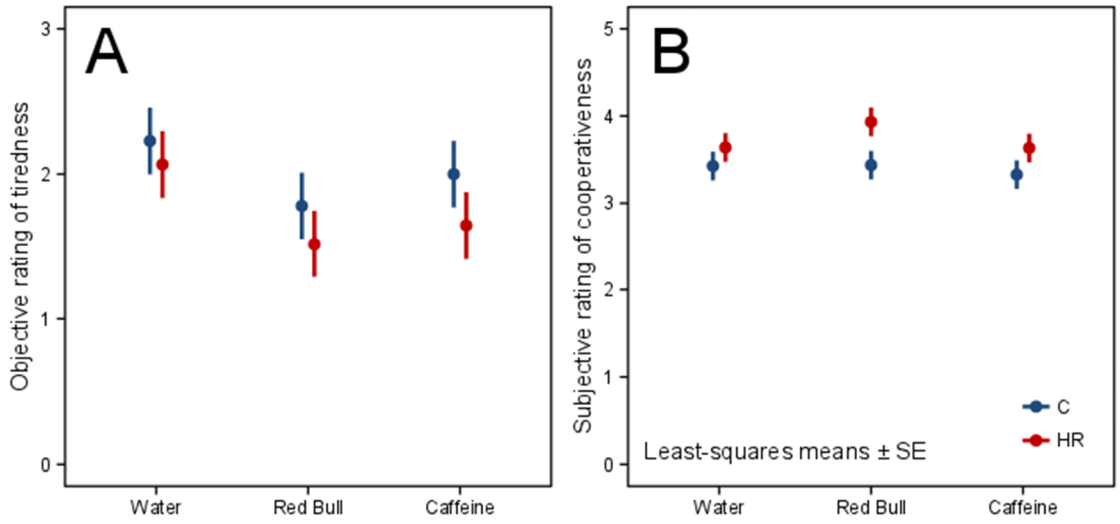


Figure 2.6. Tiredness and cooperativity in VO_2max trial. Values are least squares means and associated standard errors from a repeated-measures ANOVA conducted in SAS Procedure Mixed. **A) Left:** Average tiredness ratings at the end of VO_2max trials for the linetypes across treatment. Treatment significantly affected tiredness scores (P = 0.0039), with Red Bull-treated mice being the least tired, and water-treated mice being the most tired. HR mice scored lower on average, but not significantly, and there was no linetype by treatment interaction (P = 0.74, Table 2.4). **B) Right:** Average cooperativeness ratings during tests of VO_2max for the linetypes across treatment. HR mice tended to be more cooperative, but not significantly so (P = 0.0535). Treatment did not affect these scores and the interaction was not significant (P = 0.67, Table 2.4).



References:

Alford, C., H. Cox, and R. Wescott. 2001. The effects of Red Bull Energy Drink on human performance and mood. Amino Acids 21:139-150.

Andrew, J. R., W. Saltzman, M. A. Chappell, and T. Garland, Jr. 2016. Consequences of fatherhood in the biparental california mouse (*Peromyscus californicus*): Locomotor performance, metabolic rate, and organ masses. Physiol Biochem Zool 89:130-140.

Bartholomew, G. A., D. Vleck, and C. M. Vleck. 1981. Instantaneous measurements of oxygen consumption during pre-flight warm-up and post-flight cooling in Sphingid and Saturniid moths. Journal of Experimental Biology 90:17-32.

Belke, T. W. and T. Garland, Jr. 2007. A brief opportunity to run does not function as a reinforcer for mice selected for high daily wheel-running rates. J. Exp. Anal. Behav. 88:199-213.

Bronikowski, A. M., T. J. Morgan, T. Garland, Jr., and P. A. Carter. 2006. The evolution of aging and age-related physical decline in mice selectively bred for high voluntary exercise. Evolution 60:1494-1508.

Careau, V., M. E. Wolak, P. A. Carter, and T. Garland, Jr. 2013. Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection. Evolution 67:3102-3119.

Claghorn, G. C., I. A. Fonseca, Z. Thompson, C. Barber, and T. Garland, Jr. 2016. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiol Behav 161:145-154.

Cole, K. J., D. L. Costill, R. D. Starling, B. H. Goodpaster, S. W. Trappe, and W. J. Fink. 1996. Effect of caffeine ingestion on perception of effort and subsequent work production. Int J Sport Nutr 6:14-23.

Costill, D. L., G. P. Dalsky, and W. J. Fink. 1978. Effects of caffeine ingestion on metabolism and exercise performance. Med Sci Sports 10:155-158.

Cruz, R. S. d. O., R. A. de Aguiar, T. Turnes, L. G. A. Guglielmo, R. Beneke, and F. Caputo. 2015. Caffeine Affects Time to Exhaustion and Substrate Oxidation during Cycling at Maximal Lactate Steady State. Nutrients 7:5254-5264.

Davis, J. M., Z. Zhao, H. S. Stock, K. A. Mehl, J. Buggy, and G. A. Hand. 2003. Central nervous system effects of caffeine and adenosine on fatigue. Am J Physiol Regul Integr Comp Physiol 284:R399-404.

Dlugosz, E. M., M. A. Chappell, D. G. McGillivray, D. A. Syme, and T. Garland, Jr. 2009. Locomotor trade-offs in mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:2612-2618.

Dlugosz, E. M., M. A. Chappell, T. H. Meek, P. A. Szafrańska, K. Zub, M. Konarzewski, J. H. Jones, J. E. P. W. Bicudo, R. F. Nespolo, V. Careau, and T. Garland, Jr. 2013a. Phylogenetic analysis of mammalian maximal oxygen consumption during exercise. The Journal of experimental biology 216:4712-4721.

Dlugosz, E. M., H. Schutz, T. H. Meek, W. Acosta, C. J. Downs, E. G. Platzer, M. A. Chappell, and T. Garland, Jr. 2013b. Immune response to a *Trichinella spiralis* infection in house mice from lines selectively bred for high voluntary wheel running. J Exp Biol 216:4212-4221.

Doherty, M. and P. M. Smith. 2005. Effects of caffeine ingestion on rating of perceived exertion during and after exercise: a meta-analysis. Scandinavian Journal of Medicine & Science in Sports 15:69-78.

Fisone, G., A. Borgkvist, and A. Usiello. 2004. Caffeine as a psychomotor stimulant: mechanism of action. CMLS, Cell. Mol. Life Sci. 61:857-872.

Fredholm, B. B., K. Bättig, J. Holmén, A. Nehlig, and E. E. Zvartau. 1999. Actions of Caffeine in the Brain with Special Reference to Factors That Contribute to Its Widespread Use. Pharmacological Reviews 51:83-133.

Garland, T., Jr. 2014. Quick guide: Trade-offs. Current Biology 24.

Garland, T., Jr., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proceedings of the Royal Society B-Biological Sciences 278:574-581.

Garland, T., Jr., M. Zhao, and W. Saltzman. 2017. Hormones and the evolution of complex traits: insights from artificial selection on behavior. Integrative and Comparative Biology 57.

Girard, I., M. W. McAleer, J. S. Rhodes, and T. Garland, Jr. 2001. Selection for high voluntary wheel-running increases speed and intermittency in house mice (*Mus domesticus*). Journal of Experimental Biology 204:4311-4320.

Girard, I., E. L. Rezende, and T. Garland, Jr. 2007. Leptin levels and body composition of mice selectively bred for high voluntary locomotor activity. Physiol. Biochem. Zool. 80:568-579.

Graham, T. E., J. W. Helge, D. A. MacLean, B. Kiens, and E. A. Richter. 2000. Caffeine ingestion does not alter carbohydrate or fat metabolism in human skeletal muscle during exercise. The Journal of Physiology 529:837-847.

Graham, T. E. and L. L. Spriet. 1995. Metabolic, catecholamine, and exercise performance responses to various doses of caffeine. J Appl Physiol (1985) 78:867-874.

Kalmar, J. M. 2005. The influence of caffeine on voluntary muscle activation. Medicine and Science in Sports and Exercise 37:2113-2119.

Kalmar, J. M. and E. Cafarelli. 2004. Caffeine: A valuable tool to study central fatigue in humans? Exercise and Sport Sciences Reviews 32:143-147.

Keeney, B. K., T. H. Meek, K. M. Middleton, L. F. Holness, and T. Garland, Jr. 2012. Sex differences in cannabinoid receptor-1 (CB1) pharmacology in mice selectively bred for high voluntary wheel-running behavior. Pharmacology Biochemistry and Behavior 101:528-537.

Kolb, E. M. 2010. Neurobiological and Physiological Underpinnings of High Voluntary Wheel Running. Pp. xii + 228. Unpublished Ph.D. Dissertation. University of California, Riverside, Riverside, California.

Kolb, E. M., S. A. Kelly, K. M. Middleton, L. S. Sermsakdi, M. A. Chappell, and T. Garland, Jr. 2010. Erythropoietin elevates VO_2max but not voluntary wheel running in mice. Journal of Experimental Biology 213:510-519.

Koteja, P., T. Garland, Jr., J. K. Sax, J. G. Swallow, and P. A. Carter. 1999. Behaviour of house mice artificially selected for high levels of voluntary wheel running. Animal Behaviour 58:1307-1318.

Lim, B. V., M. H. Jang, M. C. Shin, H. B. Kim, Y. J. Kim, Y. P. Kim, J. H. Chung, H. Kim, M. S. Shin, S. S. Kim, E. H. Kim, and C. J. Kim. 2001. Caffeine inhibits exercise-induced increase in tryptophan hydroxylase expression in dorsal and median raphe of Sprague-Dawley rats. Neurosci Lett 308:25-28.

Lou, Y., Q. Peng, T. Li, B. Nolan, J. J. Bernard, G. C. Wagner, Y. Lin, W. J. Shih, A. H. Conney, and Y. Lu. 2013. Oral Caffeine During Voluntary Exercise Markedly Inhibits Skin Carcinogenesis and Decreases Inflammatory Cytokines in UVB-Treated Mice. Nutrition and Cancer 65:1002-1013.

Lu, Y.-P., B. Nolan, Y.-R. Lou, Q.-Y. Peng, G. C. Wagner, and A. H. Conney. 2007. Voluntary exercise together with oral caffeine markedly stimulates UVB light-induced apoptosis and decreases tissue fat in SKH-1 mice. Proceedings of the National Academy of Sciences 104:12936-12941.

Lynge, J. and Y. Hellsten. 2000. Distribution of adenosine A(1), A(2A) and A(2B) receptors in human skeletal muscle. Acta Physiologica Scandinavica 169:283-290.

Malisch, J. L., C. W. Breuner, E. M. Kolb, H. Wada, R. M. Hannon, M. A. Chappell, K. M. Middleton, and T. Garland, Jr. 2009. Behavioral despair and home-cage activity in mice with chronically clevated baseline borticosterone concentrations. Behavior Genetics 39:192-201.

Meek, T. H., J. C. Eisenmann, and T. Garland, Jr. 2010. Western diet increases wheel running in mice selectively bred for high voluntary wheel running. International Journal of Obesity 34:960-969.

Meek, T. H., B. P. Lonquich, R. M. Hannon, and T. Garland, Jr. 2009. Endurance capacity of mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:2908-2917.

Michna, L., Y.-P. Lu, Y.-R. Lou, G. C. Wagner, and A. H. Conney. 2003. Stimulatory effect of oral administration of green tea and caffeine on locomotor activity in SKH-1 mice. Life Sciences 73:1383-1392.

Miyazaki, T., Y. Matsuzaki, T. Ikegami, S. Miyakawa, M. Doy, N. Tanaka, and B. Bouscarel. 2004. Optimal and effective oral dose of taurine to prolong exercise performance in rat. Amino Acids 27:291-298.

Pettijohn, T. F. 1979. Effects of alcohol and caffeine on wheel running activity in the Mongolian gerbil. Pharmacology Biochemistry and Behavior 10:339-341.

Rezende, E. L., M. A. Chappell, F. R. Gomes, J. L. Malisch, and T. Garland, Jr. 2005. Maximal metabolic rates during voluntary exercise, forced exercise, and cold exposure in house mice selectively bred for high wheel-running. Journal of Experimental Biology 208:2447-2458.

Rezende, E. L., T. Garland, Jr., M. A. Chappell, J. L. Malisch, and F. R. Gomes. 2006a. Maximum aerobic performance in lines of *Mus* selected for high wheel-running activity: effects of selection, oxygen availability and the mini-muscle phenotype. Journal of Experimental Biology 209:115-127.

Rezende, E. L., S. A. Kelly, F. R. Gomes, M. A. Chappell, and T. Garland, Jr. 2006b. Effects of size, sex, and voluntary running speeds on costs of locomotion in lines

of laboratory mice selectively bred for high wheel-running activity. Physiol. Biochem. Zool. 79:83-99.

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integrative and Comparative Biology 45:438-455.

Rhodes, J. S., T. Garland, Jr., and S. C. Gammie. 2003. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behav. Neurosci. 117:1243-1256.

Ryu, S., S.-K. Choi, S.-S. Joung, H. Suh, Y.-S. Cha, S. Lee, and K. Lim. 2001. Caffeine as a lipolytic food component increases endurance performance in rats and athletes. Journal of Nutritional Science and Vitaminology 47:139-146.

Salimäki, J., G. Scriba, T. P. Piepponen, N. Rautolahti, and L. Ahtee. 2003. The effects of systemically administered taurine and N-pivaloyltaurine on striatal extracellular dopamine and taurine in freely moving rats. Naunyn-Schmiedeberg's Arch Pharmacol 368:134-141.

Saul, M., P. Majdak, S. Perez, M. Reilly, T. Garland, Jr., and J. S. Rhodes. 2016. High motivation for exercise is associated with altered chromatin regulators of monoamine receptor gene expression in the striatum of selectively bred mice. Genes Brain Behav.

Swallow, J. G., P. A. Carter, and T. Garland, Jr. 1998a. Artificial selection for increased wheel-running behavior in house mice. Behavior Genetics 28:227-237.

Swallow, J. G., T. Garland, Jr., P. A. Carter, W. Z. Zhan, and G. C. Sieck. 1998b. Effects of voluntary activity and genetic selection on aerobic capacity in house mice (*Mus domesticus*). Journal of Applied Physiology 84:69-76.

Ventura, E. E., J. N. Davis, and M. I. Goran. 2011. Sugar Content of Popular Sweetened Beverages Based on Objective Laboratory Analysis: Focus on Fructose Content. Obesity 19:868-874.

Wallace, I. J. and T. Garland, Jr. 2016. Mobility as an emergent property of biological organization: Insights from experimental evolution. Evolutionary Anthropology: Issues, News, and Reviews 25:98-104.

Waters, R. P., R. B. Pringle, G. L. Forster, K. J. Renner, J. L. Malisch, T. Garland, Jr. , and J. G. Swallow. 2013. Selection for increased voluntary wheel-running affects behavior and brain monoamines in mice. Brain Research 1508:9-22.

Chapter 3

Selective Breeding and Short-Term Access to a Running Wheel Alter Stride Characteristics in House Mice

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ABSTRACT

Mice from 4 replicate High Runner (HR) lines selectively bred for high levels of voluntary wheel running show many differences in locomotor behavior and morphology as compared with 4 non-selected control (C) lines, but stride characteristics have not been studied. We hypothesized that HR mice would show stride alterations that have coadapted with locomotor behavior, morphology, and physiology. More specifically, we predicted that HR mice would have stride characteristics that differed from C mice in ways that parallel some of the adaptations seen in highly cursorial animals. For example, we predicted that limbs of HR mice would swing closer to the parasagittal plane, resulting in a 2-dimensional measurement of narrowed stance width. We also expected that some differences between HR and C mice might be amplified by six days of wheel access, as is used to select breeders each generation. We used the DigiGait Imaging System (Mouse Specifics, Inc.) to capture high-speed videos in ventral view as mice ran on a motorized treadmill across a range of speeds, and then automatically calculate several aspects of strides. Young adults of both sexes were tested both before and after 6 days of wheel access. Stride length, stride frequency, stance width, stance time, brake time, propel time, swing time, duty factor, and paw contact area were analyzed using a nested analysis of covariance, with body mass as a covariate. As expected, body mass and treadmill speed affected nearly every analyzed metric. Six days of wheel access also affected nearly every measure, indicating pervasive training effects, in both HR and C mice. As predicted, stance width was significantly narrower in HR than C mice. Paw contact area and duty factor were significantly greater in mini-muscle individuals (subset of HR mice with 50% reduced hindlimb muscle mass) than in normal-muscled HR or C. We conclude that stride characteristics of house mice are adaptable in response to both selective breeding and changes in daily locomotor behavior (activity levels) that occur during as few as six days. These results have important implications for understanding the evolution and coadaptation of locomotor behavior and performance.

INTRODUCTION

In the present study, we test for potential microevolutionary changes in stride characteristics of mice from the long-term High Runner (HR) mouse selection experiment (Swallow et al. 1998a; Careau et al. 2013b). The four replicate HR mouse lines have been bred for high voluntary wheel-running activity for nearly 70 generations, and have a variety of behavioral, morphological, and physiological adaptations as compared with four non-selected control (C) lines (Rhodes et al. 2005; Swallow et al. 2009; Garland et al. 2011a; Garland et al. 2011b; Wallace and Garland 2016). However, kinematic analyses of HR mice have not yet been attempted, primarily due to experimental complications of capturing quality kinematic data from large numbers of animals as small as house mice. For example, digitization of markers affixed to skin can involve significant error in the characterization relative to bones (Filipe et al. 2006; Bauman and Chang 2010), and the use of biplanar x-ray motion analysis (i.e., XROMM; Brainerd et al. 2010) requires equipment and expertise that is only available at a few sites.

In the present study, we use the DigiGait Imaging System (Mouse Specifics, Inc.) to accomplish high-throughput phenotyping and allow comparison of the 2-dimensional stride characteristics of both sexes and all eight lines of HR and C mice running on a treadmill at speeds that are typically seen during voluntary wheel running. We hypothesized that HR mice would show stride alterations that have coadapted with locomotor behavior, morphology, and physiology (see below). Specifically, we obtained

estimates of stride length, stride frequency, stance time, swing time, duty factor, brake time, and propel time (Table 3.1). We measured stance width as a proxy for running posture along the sprawled/erect spectrum, and predicted that HR mice should have narrower stances. We also measured paw contact area as an index of foot posture along the plantigrade/digitigrade or possibly crouched/erect continuum, predicting that HR mice would run more on their toes, thus having a smaller paw contact area. To separate functional from morphological paw size, we measured hindfoot sizes by digital photography for each individual that was treadmill tested (N = 100).

The starting population for the HR selection experiment was 224 individuals from a widely used outbred strain (Hsd:ICR), purchased from Harlan-Sprague-Dawley (Indianapolis, Indiana, USA). Mice were bred randomly for two generations, then separated into eight closed lines, four of which were subsequently bred for high voluntary wheel-running behavior on days 5 & 6 of a 6-day period of wheel access as young adults (Swallow et al. 1998a). Four additional control lines have been bred without regard to amount of running. Mice from HR lines run, on average, nearly three times as many wheel revolutions per day as controls (Figure 3.1). The difference in total wheel revolutions between HR and C mice is caused primarily by an increase in average speed of running, rather than an increase in the amount of time spent running (Koteja et al. 1999), although male HR mice do run for significantly longer per day than male controls (Garland et al. 2011b). A selection limit of unknown origin was reached around generation 16-28, depending on line and sex (Garland et al. 2011a; Careau et al. 2013b), and wheel running has not increased in the HR lines for at least the next 40 generations of

selective breeding. This result may indicate that HR mice are running as far as they are physically capable, although no physical, physiological or psychological limits to wheel running have been explicitly identified (Rhodes et al. 2005; Rezende et al. 2009; Kolb et al. 2010; Meek et al. 2010; Claghorn et al. 2016).

A number of correlated evolutionary responses to selection have been observed in the HR mice, including increases in two whole-organism indicators of exercise capacity, endurance during forced treadmill exercise (Meek et al. 2009; Claghorn et al. 2016) and maximal exercise-induced oxygen consumption (Swallow et al. 1998b; Rezende et al. 2006a; Rezende et al. 2006b; Kolb et al. 2010; Dlugosz et al. 2013). HR mice are also smaller, leaner, achieve higher maximum speeds during wheel running, and have a reduced incremental cost of transport on a whole-animal basis, although not on a mass-adjusted basis (Swallow et al. 1999; Swallow et al. 2001; Rezende et al. 2006c). Relevant sub-organismal traits that have evolved in HR mice include increased symmetry of hindlimb bone lengths, larger femoral heads, heavier foot bones, and altered semicircular canal shape (Garland and Freeman 2005; Kelly et al. 2006; Schutz et al. 2014).

A subset of HR mice have the mini-muscle phenotype, which is characterized by a 50% mass reduction in the triceps surae and total hindlimb muscle mass (in homozygotes), caused by a drastic reduction of type IIb muscle fibers (Guderley et al. 2006; Talmadge et al. 2014). The mini-muscle phenotype is caused by a Mendelian recessive allele that was present at a low frequency (~7%) in the original base population,

but has increased in frequency in two of the four HR lines, eventually becoming fixed in one (Garland et al. 2002a; Hannon et al. 2008; Kelly et al. 2013). In mini-muscle individuals, the medial gastrocnemius has a reduced force per-cross sectional area (Syme et al. 2005), but hindlimb muscles have double the per-gram oxidative capacity (Houle-Leroy et al. 2003). The gastrocnemius also has elevated capillarization (Wong et al. 2009) and increased glycogen storage (Gomes et al. 2009). Mini-muscle mice have elevated whole-animal VO_2max when tested in hypoxia (Rezende et al. 2006a), but have reduced maximal sprint speeds (as measured on a photocell-timed racetrack) and elevated cost of locomotion on wheels (Dlugosz et al. 2009). Several of these differences might lead one to expect differences in stride characteristics between mini-muscle and wild type individuals, although the direction is difficult to predict.

METHODS

Animals

Fifty male and fifty female mice from generation 68 of the HR selective breeding experiment (Swallow et al. 1998a) were studied, with 6 males and 6 females from each of seven of the eight lines, and 8 males and 8 females from line 6, an HR line that remains polymorphic for the mini-muscle phenotype (e.g., Syme et al. 2005, unpublished results) unpublished results. Mice were raised under the typical conditions of the selection experiment (Swallow et al. 1998b; Careau et al. 2013b), with the exception that they were not toe-clipped for identification, and were housed singly from the time of weaning. Mice did not have access to wheels at any point before the first measurements. Food and

water were available *ad libitum* throughout the course of the project, and the photoperiod was 12 L:12 D, with the light phase beginning at 7:00 and the dark phase beginning at 19:00. The UCR IACUC approved all experimental conditions and protocols.

DigiGait imaging system

At approximately 6 weeks of age, all mice were tested on the DigiGait Imaging System (Mouse Specifics, Inc.). This system uses high speed (148 frames/s), ventral plane videography to image the subject as their limbs advance to and retreat from a motorized transparent treadmill belt. Software determines the area of the paws for each of the four limbs to determine spatial and kinematic indices throughout sequential strides. Over 30 metrics of posture and locomotion are reported to characterize the gait. Based on our initial hypotheses, we chose to analyze stride length, stride frequency, duty factor, swing time, stance time, propel time, brake time, stance width, and paw contact area (contact surface area with treadmill).

Tests were conducted during the light phase, between 8:00 and 18:00. Each mouse was removed from its cage, weighed, and immediately placed onto the treadmill inside of a transparent acrylic enclosure, which was adjusted in length such that the mouse had enough room to run, but was kept in frame of the camera. The mouse had about 30 seconds to acclimate while the software controlling the camera was being prepared. The treadmill was started suddenly, and stopped immediately if the mouse failed to orient or keep pace with the treadmill. This was repeated until the mouse was able to maintain pace in frame for 1-3 seconds, with the minimum requirement for a

"successful" trial being ten or more in-frame strides. Nearly every mouse in the sample was able to successfully run at all test speeds. Mice were tested at successive speeds of 30, 50, 70, and 90 cm/s with no incline, and only rested for the time that it took to start the next trial (< 2 minutes). Speeds were chosen to bracket the speeds that mice run on wheels (Figure 3.1). Total handling time for each animal was 6-15 minutes. Each mouse was tested once (per speed) before and once immediately after 6 days of wheel access. In total, we analyzed 8 videos each of almost all of the 100 mice, for 797 total videos analyzed. All mice used a trot gait at all measured speeds, with the exception of a small number of mice which used 1-3 strides of a galloping gait when adjusting to the highest two test speeds. Because this behavior was so rare, these strides were excluded from our analysis.

Wheel access

Following the initial DigiGait testing, mice were individually housed in cages with access to the same wheels used in the routine selective breeding protocol (Swallow et al. 1998a: wheel circumference 1.12 m) for 6 days. Mice had no wheel exposure prior to this. As during routine wheel testing for the selection experiment, revolutions were recorded in 1-minute intervals by a photocell counter attached to the wheel and compiled via customized software (San Diego Instruments, San Diego, California, USA) for 23 hours per day over 6 days.

Photographs were taken to measure paw size in all mice. Mouse feet were gently pressed against a glass slide post-mortem, and photographed ventrally with a consumer-

grade digital SLR camera (Nikon D60 with a Nikon 50mm lens). The paw photographs were outlined in ImageJ once each for size analysis. In keeping with the rest of this report, left and right paws were averaged for each mouse, and only hind paw data are shown.

Dissection

Males were dissected about 6 weeks after the second DigiGait trial, and, for logistical reasons, females were dissected about 3 weeks after the males. Of chief interest was the mass of the triceps surae, to verify the mini-muscle phenotype of mice from the mini-muscle-fixed line and to determine the phenotype of mice from the polymorphic line.

Statistics

Group comparisons were performed using SAS PROC MIXED (SAS 9.4, SAS Institute, Cary, NC, USA). The four primary factors of analysis were line type (HR vs. C), sex, training (before vs. after wheel access), and treadmill speed. All possible interaction terms were also included, except where noted, and we mostly reported least squares means ± standard errors from the full models. For the one HR line that contains both mini-muscle and normal-muscle mice phenotypes (lab designation HR#6), mini-muscle status was determined by dissection (Garland et al. 2002b), and then mini-muscle status for those mice as well as mice within the fixed mini-muscle line (HR#3) was used as an additional explanatory variable. As in previous analyses of these lines of mice,

replicate line was used as a random effect nested within line type. Individual was the factor for repeated measures. Body mass was used as a covariate in all analyses. Wheel freeness (a measure of how many revolutions the wheel spins after being accelerated to a standard speed) was measured before and after the wheel trials, but was not predictive of wheel running and thus was not included as a covariate (models which included freeness did not yield different results from what is reported here).

RESULTS

Additional tables and figures, as well as some results not described here (e.g., for repeatability of stride characteristics before versus after six days of wheel access), can be found in appendices A-C.

Body mass

Female HR mice were nearly the same body mass as female C mice, but HR males were significantly smaller than C males, as indicated by a significant sex by line type interaction ($P = 0.0250$; Table A.1, Figure 3.4). On average, mice from all groups gained mass during the period of wheel access (training $P = 0.0045$, Figure 3.4). Mini-muscle mice were significantly smaller than normal-muscled mice (mini-muscle $P = 0.0173$, Figure 3.4). Body mass was used as a covariate in the analysis of all stride characteristics, and it had a statistically significant effect on stance width, paw contact area, swing time, stance time, duty factor, and propel time ($P < 0.05$ for all; Figure 3.4, Tables 2, A.5).

Wheel running

On days 5 and 6 of the 6 days of wheel access (the values used in the routine selective breeding protocol), HR mice ran significantly more wheel revolutions per day than C ($P < 0.0001$, Figure 5), females ran significantly more than males ($P = 0.0088$, Figure 5), and the line type * sex interaction was also significant ($P = 0.0412$, Figure 5). The HR vs. C fold difference in daily running was 3.1 for females and 2.7-fold for males (Tables A.3, A.4, Figure 5). HR mice ran for significantly more time than C mice ($P = 0.0346$), and significantly faster ($P < 0.0001$). The differences between HR and C speeds were larger for females than for males (sex by linetype interaction for average speed P = 0.0151) and for maximum speed P = 0.0132). Female HR ran about 150% faster on average, and reached top speeds that were 104% faster than female C mice. Male HR mice ran about 89% faster on average, and reached top speeds 61% faster than male C mice. The ranges of speeds observed were comparable to previous generations and to the treadmill speeds used in the kinematic analysis (see Materials and Methods and Figure 3.1). On average, mini-muscle individuals ran significantly faster on wheels (Table A.3, Figure 5).

Effects of speed on stride

As expected, treadmill speed greatly affected nearly every metric of stride that we analyzed. As treadmill speed increased, both stride length and frequency increased, stance, swing, brake, and propel times decreased, duty factor decreased, and stance width decreased (all $P < 0.0001$; Table 3.2, Figure 3.2). Paw contact area was the only

measurement not significantly influenced by treadmill speed. In a few cases, there was a significant interaction between speed and line type, sex, or training. In most cases, this indicated that the measurement was different between line types, sexes, or training status at low speeds, but then converged at high speeds. For example, brake time was significantly affected by training, but at the highest speed, brake time was nearly identical before and after wheel access (Table 3.2, Figure 3.2).

Effect of selective breeding on stride

Adjusted for body mass and as predicted, stance width was significantly narrower in HR than in C mice (effect of line type $P = 0.0143$; Figure 3.2). However, stride length, stride frequency, paw contact area, swing time, stance time, brake time, and propel time were not significantly affected by selective breeding (effect of line type $P > 0.05$ for all; Tables 3.2, A5).

Effects of sex on stride

Females showed a trend for shorter, more frequent strides ($P = 0.0902$, 0.0758 for sex effect on stride length and stride frequency, respectively; Table 3.2, Figure 3.2). The increase in frequency was related to a trend for decreased stance time ($P = 0.0701$), which was in turn attributable to a significantly smaller amount of time spent in the propel phase ($P = 0.0201$). Females had significantly wider stances ($P = 0.0157$) and smaller paw contact areas ($P = 0.0488$) than males, regardless of line type. No sex by line type interactions were found for any measurement, suggesting that selection for

wheel running has not affected the sexes differently in terms of gait during these treadmill tests.

Effects of wheel access on stride

Following 6 days of wheel access, stride length significantly increased (P = 0.0001; Table 3.2, Figure 3.2), stride frequency decreased ($P < 0.0001$), stance width decreased ($P = 0.0040$), paw contact area decreased ($P = 0.0022$), and stance, swing, brake, and propel time increased ($P = 0.0001$, $P = 0.0087$, $P = 0.0050$, $P = 0.0004$, respectively). One interaction of interest was a line type by training effect on brake time ($P = 0.0197$), indicating that while HR did not significantly differ from C overall, they were relatively more different from one another after wheel access (Table A.6).

Effects of mini-muscle phenotype on stride

Paw contact area was significantly greater in the subset of HR mice with the mini-muscle phenotype (8.46% larger paw area, $P = 0.0058$, Figure 3.3). However, digitized photographs of hind paws of these same individual mice did not reveal any statistical difference in paw area for mini-muscle individuals, nor between HR and C mice (see appendix B, Tables B.1-B.2). Duty factor was also significantly greater in mini-muscle mice ($P = 0.0266$, Table 3.2), owing, at least partly, to a significantly longer brake time ($P = 0.0064$).

DISCUSSION

Overview of results

In spite of the fact that the run voluntarily at much higher speeds on wheels (Figure 3.1), mice from the selectively bred HR lines were quite similar to C mice in most stride characteristics that we quantified during treadmill locomotion. The one notable difference between linetypes was that, as predicted, HR mice had narrower stance widths (ran with feet closer to one another and to the sagittal plane) than non-selected controls. We also found that mini-muscle individuals (a subset of the HR mice with a genetically determined 50% reduction in hindlimb muscle mass) have larger paw contact areas -- but not larger paws -- and higher duty factor across running speeds. We found no statistically significant effect of selective breeding on any of the temporal measurements of stride.

Although relatively few of the traits tested herein have evolved in HR mice, almost every measured stride characteristic was significantly affected by 6 days of wheel access, thus demonstrating pervasive "training," "conditioning," "learning" or "plasticity" (Garland and Kelly 2006; Kelly et al. 2012), as well as the power of the methods employed to detect even relatively small variations. In addition, sex affected a number of stride characteristics, even after accounting statistically for the effects of body size (males are larger than females). Interestingly, even though Garland and Freeman (2005) showed that, at generation 11, males had shorter leg bones than females (adjusted for body mass), we found a seemingly opposite trend of females taking shorter, more frequent strides (*P*

= 0.0902, 0.0758 for sex effect on stride length and stride frequency, respectively). Thus, motor behavior can mask morphological differences in some cases.

Stance width

All mice narrowed their stance in response to increased speeds, but HR stances were narrower at all speeds (Figure 3.2). All else being equal, a narrower stance should result in a more vertical alignment of limbs, and therefore a more "erect" posture (Biewener 1989; Carrano 1999). Such alignment is purported to be an adaptation to align the long bones with the direction of the ground reaction force, thus reducing strain (Carrano and Biewener 1999). Therefore, the narrowed stance in HR mice could be related to the increased wheel-running speeds (Figure 3.1) that have evolved as the primary mechanism by which HR mice have increased wheel running, because this change should increase ground reaction forces. Force plate and 3D kinematic data would be required to test this idea.

Paw contact areas

Interestingly, paw contact area was the only characteristic unaffected by treadmill speed. The mini-muscle phenotype may cause larger paw contact areas during loading because individuals with this phenotype rely more on two-legged support, as in rat aging models (Horner et al. 2011). Alternatively, because mini-muscles have reduced total-force generating capacity (see Syme et al. 2005), affected individuals may use a relatively crouched posture and longer stance time to generate the work necessary for push-off

(Usherwood 2013). Either possibility is consistent with the finding that mini-muscle mice also have significantly greater duty factor (Figure 3.2, Table 3.2). However, the present data indicate that the significant difference is derived from an increase in the time spent in the braking phase of stance, rather than the propulsive stage (see Table A.5). Force plate data would clarify these findings, but photographic evidence indicates that although the effective paw area during stance is larger, the actual paws of mini-muscle mice are not larger than those of non-mini-muscle mice, nor do HR mice differ from C (see Appendix B).

Training effects

Training effects were pervasive, with mice using longer, less frequent (Figure 3.2) strides following six days of wheel access, and spending more time in both the stance and swing phases. Furthermore, stance width (Figure 3.2) and paw contact area decreased following wheel access. Training effects from wheel access have been observed in numerous rodent studies, including several involving the HR and C mice, but we have typically used much longer training periods, usually 6-10 weeks (e.g., Rhodes et al. 2003; Kelly et al. 2006; Middleton et al. 2008; Meek et al. 2014). However, training effects at the molecular level have been observed in as few as four days of wheel access (Gomes et al. 2009).

Effects of running speed

As expected, treadmill speed had widespread effects (Herbin et al. 2007), with increasing speed eliciting longer, more frequent strides, owing to both decreased stance and swing times (see online supplemental Table A.5). Stance time decreased more dramatically than swing time with increasing speed, and thus, duty factor decreased. Increasing speed also resulted in decreased stance width. The stance width for C mice at the highest speed was smaller than, or comparable to, that of the HR mice at low speeds (Figure 3.2), which shows that C mice are physically capable of a narrower stance, but "choose" to use a wider stance than HR mice across all tested speeds. Treadmill speeds were chosen to capture the range of speeds that are normally seen during wheel running (Figure 3.1, also see online supplemental appendix, Table A.4), so we are confident that the chosen treadmill speeds were sufficient to compare stride characteristics at speeds relevant to voluntary wheel running. However, as discussed in the next section, we do recognize that treadmill running likely differ somewhat from wheel running.

Potential effects of methodology on stride characteristics

Running HR and C mice on a treadmill most likely yielded somewhat different results than would be obtained if strides were measured during running either overground or on a wheel. For instance, Herbin et al. (2007) found that, at the same speeds, mice took shorter, more frequent strides when running on a treadmill as compared with running overground, though changes with speed were consistent under either condition.

No detailed kinematic description of wheel running currently exists, but it would likely be somewhat different from both treadmill and overground locomotion.

Selection experiments and the DigiGait system as approaches to study locomotion

Studying the evolution of locomotor behavior and performance is a difficult task, for several reasons. For example, getting wild animals to behave under controlled conditions is fraught with complications, ranging from obtaining collecting permits to outright safety for both subject and investigator. Moreover, broad-scale comparisons, even if conducted within a phylogenetically informed context (e.g., Albuquerque et al. 2015; Higham et al. 2015), are always based on correlational evidence (Garland et al. 2017). In addition, sometimes the precise nature of selection in the wild is difficult to determine, even in the case of well-known animal athletes (Wilson et al. 2013). Therefore, modeling (e.g., Cespedes and Lailvaux 2015) or experimental evolution (Swallow et al. 2009; Wallace and Garland 2016) can provide useful alternatives. In the present study, we used simplified methodology and evolutionary scope for the sake of generating more powerful inferences about kinematic responses to a known type of selection related to a locomotor behavior that, although artificial, is at least one that wild rodents will perform (Meijer and Robbers 2014).

Biomechanics methods are generally laborious, typically requiring manual, frame-by-frame digitization of hundreds or thousands of points for a single animal within a single trial. In this study, we used the DigiGait treadmill videography system to rapidly characterize two-dimensional, footfall-based measurements such as stride length, stride

frequency, braking and propel times, stance width, and duty factor (Cops et al. 2013) over a wide range of speeds (30-90 cm/s). This instrumentation uses one high speed camera positioned ventrally, in combination with dedicated software, to capture numerous postural and kinematic metrics, and this enabled us to find meaningful differences in gaits between HR and C mice. Two-dimensional gait differences identified in this study will inform future studies of the evolution of kinematics in HR mice using 3D techniques.

Much of the existing literature on kinematic and morphological adaptations for high locomotor performance focus on so-called "cursors" (Carrano 1999). Most "cursors" are relatively large animals. Comparing the kinematics of animals of disparate size and shape has the added complication of transferability of kinematics and posture between different limb morphologies (Gatesy and Pollard 2011). Apparent broad-scale trends linking cursoriality (i.e. aspects of enhanced locomotor behavior and performance) with morphology can break down when comparing animals of different size within a clade (Day and Jayne 2007). Attempts to describe the evolution of cursoriality are also confounded by phylogeny, because the vast majority of (mammalian) cursors are members of a small number of clades, namely Carnivora, Perissodactyla, and Artiodactyla. Morphological characteristics that appear to be correlated with high locomotor activity are sometimes more closely tied to phylogeny. For example, metatarsal/femur ratio is well correlated with sprint speed among species in the broad sense (after accounting for phylogeny and body size), but when the pool is limited to "cursors," the pattern is greatly diminished (Garland and Janis 1993).

We view the High Runner mouse model is reasonably well suited for study of the evolution of kinematics because (1) HR mice have been bred for, and have evolved, great voluntary daily movement distances, (2) HR mice are better endurance runners in forced treadmill exercise tests (Meek et al. 2009; Claghorn et al. 2016), (3) HR mice have some described anatomical differences relevant to kinematics, including increased hindlimb symmetry and larger femoral heads (Garland and Freeman 2005; Kelly et al. 2006), and (4) they are well studied in terms of physiological adaptations to high activity as well as patterns of wheel-running behavior (Girard et al. 2001; Rezende et al. 2009; Garland et al. 2011a; Garland et al. 2011b). Furthermore, HR and C mice have a known evolutionary history, and differ only slightly in body mass and do not differ meaningfully in such skeletal dimensions as femur length (Garland and Freeman 2005; Kelly et al. 2006), so the model does not share the same confounding effects of disparate body size, extreme morphological differences, or phylogenetic effects that plague many other comparisons. Known morphological differences between HR and C mice did not translate into detectable differences in stride in the present study. Mini-muscle mice have longer hind limbs (longer tibiafibula and sum of hindlimb bones [femur, tibiafibula, and metatarsals], Kelly et al. 2006), but did not have significantly longer strides. HR mice have heavier foot bones, with no statistically significant effect of mini-muscle beyond the HR effect (Kelly et al. 2006), but here the mini-muscle mice had significantly greater paw contact areas, whereas paw contact area did not differ between HR and C mice. These results serve as a cautionary tale for attempts to infer function solely from form.

Table 3.1: Stride characteristics measured

Stride characteristic	Description
Stride length (cm)	Distance between contacts of the same paw in one limb cycle
Stride frequency (Hz)	Number of complete limb cycles within a second of running
Duty factor (%)	Percent of stride time which is spent in stance
Brake time (s)	Time from initial paw contact to maximal paw contact
Propel time (s)	Time from maximal paw contact to paw liftoff
Paw area (cm^2)	Maximal paw contact area with the treadmill during stance
Stance width (cm)	Lateral distance between left and right paws during stance
Stance time (s)	Time within a limb cycle in which the paw is in contact with the treadmill
Swing time (s)	Time within a limb cycle in which the paw is not in contact with the treadmill

Table 3.2: Effects of line type, sex, training, treadmill speed, mini-muscle, and body mass on key stride characteristics: Results of nested ANCOVAs. Graphs of these stride characteristics can be found in Figure 3.2.

	Stride Frequency			Stance Width			Duty Factor		
Factor	d.f.	F	p	d.f.	F	p	d.f.	F	p
Line type	(1, 6)	0.01	0.9139	(1, 6)	11.64	**0.0143**	(1, 6)	0.5	0.5045
Sex	(1, 6)	4.59	0.0758	(1, 6)	11.12	**0.0157**	(1, 6)	0.02	0.8975
Training	(1, 6)	87.54	**<.0001**	(1, 6)	20.41	**0.0040**	(1, 6)	0.97	0.3632
Speed	(3, 18)	6177.67	**<.0001**	(3, 18)	114.12	**<.0001**	(3, 18)	894.83	**<.0001**
Mini-muscle	(1, 655)	0.01	0.9283	(1, 655)	1.39	0.2394	(1, 655)	4.94	**0.0266**
Line type*sex	(1, 6)	3.66	0.1042	(1, 6)	0.57	0.4775	(1, 6)	0.95	0.3679
Line type*training	(1, 6)	0.18	0.6898	(1, 6)	0.63	0.4568	(1, 6)	2.61	0.1571
Line type*speed	(3, 18)	0.39	0.7614	(3, 18)	2.49	0.0934	(3, 18)	0.97	0.4275
Sex*training	(1, 6)	0.94	0.3704	(1, 6)	1.46	0.2718	(1, 6)	0.03	0.8608
Sex*speed	(3, 18)	2.46	0.0955	(3, 18)	0.6	0.6206	(3, 18)	3.54	**0.0358**
Training*speed	(3, 18)	1.35	0.2889	(3, 18)	3.42	**0.0397**	(3, 18)	5.58	**0.0069**
Line type*sex*training	(1, 6)	2.27	0.1824	(1, 6)	0.01	0.9272	(1, 6)	0.04	0.8571
Line type*sex*speed	(3, 18)	0.39	0.7633	(3, 18)	0.91	0.4545	(3, 18)	1.23	0.3263
Line type*training*speed	(3, 18)	0.5	0.6859	(3, 18)	1.99	0.1515	(3, 18)	1.51	0.2448
Sex*training*speed	(3, 18)	0.48	0.7011	(3, 18)	0.19	0.9030	(3, 18)	0.02	0.9971
(4-way interaction)	(3, 18)	1.77	0.1892	(3, 18)	0.33	0.8067	(3, 18)	0.92	0.4488
Body mass	(1, 655)	0.14	0.7108	(1, 655)	39.41	**<.0001**	(1, 655)	28.3	**<.0001**

Table 3.2, continued:

	Stride Length			Paw Contact Area			Swing time		
Factor	d.f.	F	p	d.f.	F	p	d.f.	F	p
Line type	(1, 6)	0.04	0.8481	(1, 6)	0.01	0.9140	(1, 6)	0.04	0.8413
Sex	(1, 6)	4.07	0.0902	(1, 6)	6.07	**0.0488**	(1, 6)	2.83	0.1434
Training	(1, 6)	78.79	**0.0001**	(1, 6)	26.17	**0.0022**	(1, 6)	14.65	**0.0087**
Speed	(3, 18)	3805.22	**<.0001**	(3, 18)	1.28	0.3128	(3, 18)	57.96	**<.0001**
Mini-muscle	(1, 655)	0.01	0.9043	(1, 550)	7.67	**0.0058**	(1, 728)	1.93	0.1647
Line type*sex	(1, 6)	2.07	0.2004	(1, 6)	0.28	0.6179	(1, 6)	2.85	0.1422
Line type*training	(1, 6)	0.09	0.7779	(1, 6)	0	0.9958	(1, 6)	1.31	0.2968
Line type*speed	(3, 18)	0.19	0.9048	(3, 18)	0.18	0.9095	(3, 18)	0.29	0.8311
Sex*training	(1, 6)	1.63	0.2487	(1, 6)	0	0.9571	(1, 728)	0.73	0.3929
Sex*speed	(3, 18)	1.82	0.1805	(3, 18)	0.73	0.5461	(3, 728)	4.02	**0.0074**
Training*speed	(3, 18)	1.55	0.2360	(3, 18)	5.1	**0.0099**	(3, 728)	1.26	0.2878
Line type*sex*training	(1, 6)	1.75	0.2338	(1, 6)	0.01	0.9261	*did not converge*		
Line type*sex*speed	(3, 18)	0.16	0.9190	(3, 18)	0.17	0.9168	*did not converge*		
Line type*training*speed	(3, 18)	0.62	0.6132	(3, 18)	0.7	0.5648	*did not converge*		
Sex*training*speed	(3, 18)	0.37	0.7729	(3, 18)	0.61	0.6198	*did not converge*		
(4-way interaction)	(3, 18)	1.7	0.2035	(3, 18)	1.02	0.4090	*did not converge*		
Body mass	(1, 655)	0.3	0.5858	(1, 550)	26.7	**<.0001**	(1, 728)	12.89	**0.0004**

Figure 3.1: HR mice have evolved to run more wheel revolutions than C mice primarily by running faster. A: Revolutions per day for female mice from all 8 lines averaged between days 5 and 6 of a 6-day period of wheel access. Once reaching their selection limits, HR mice run (solid lines) about 3 times as many wheel revolutions as C mice (dotted lines), and total wheel revolutions for HR mice has not appreciably increased since generation 31 (Kolb et al. 2013). The differential between HR and C is similar for males, but all males run less on average. B: Time spent running has not diverged significantly between female HR and C mice. Male HR mice do spend more time running, but the differential in total wheel revolutions is primarily due to increased speed, and not time spent running, for both males and females. C: Maximum wheel running speed, extrapolated from the highest number of wheel revolutions in a single minute, has also significantly diverged. Black horizontal bars represent the four testing speeds at which we characterized gait. A-C: Data for genertations 0-31 compiled by Careau (2013a); subsequent generations represent unpublished data. The gap in data from generation 32-35 was from when the lab moved from Madison, WI to Riverside, CA. Seasonal variation is apparent, with mice tending to run more during winter generations and less during the summer (see Careau et al. 2013a).

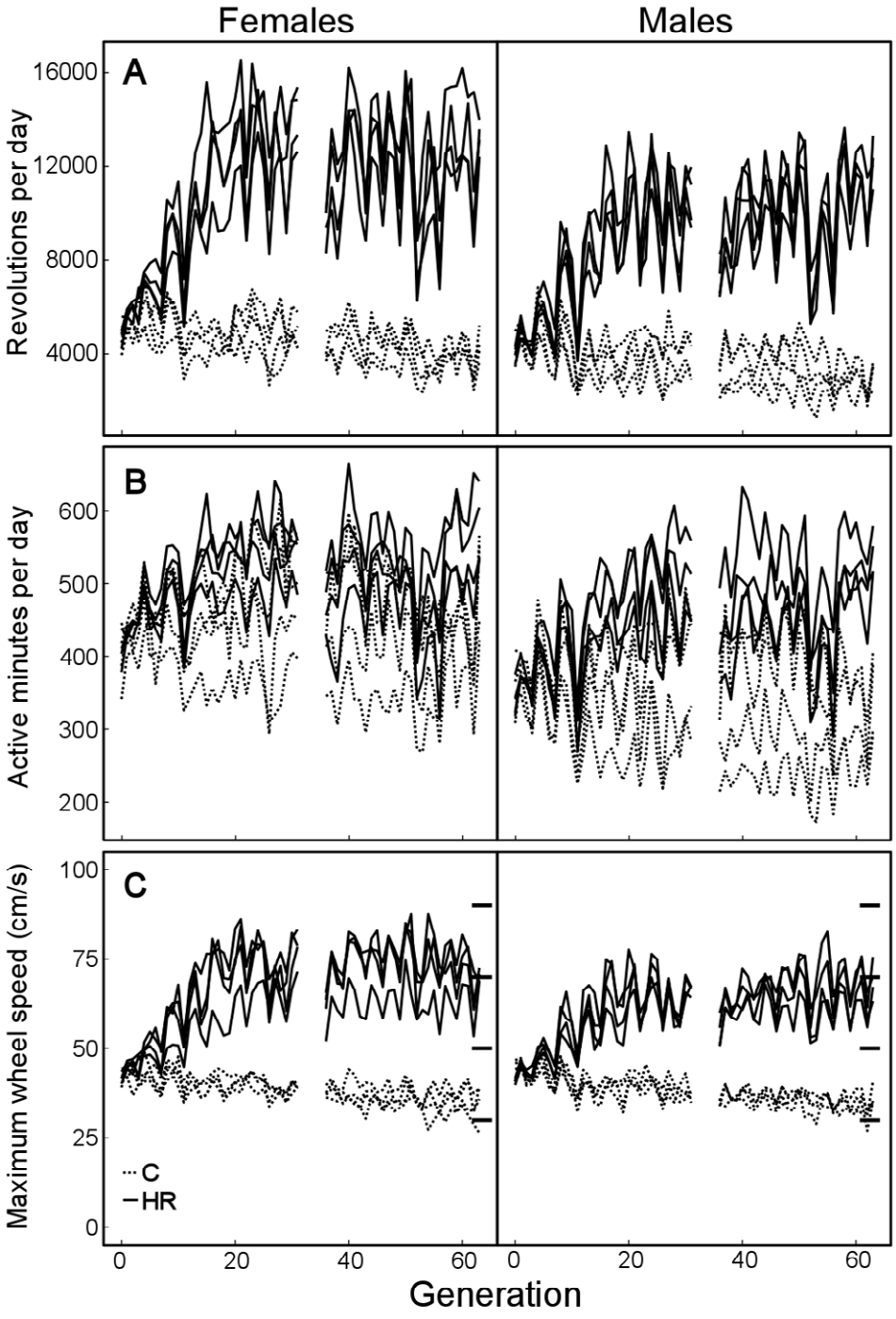

Females
Males
A
B
C
Revolutions per day
16000
12000
8000
4000
Active minutes per day
600
500
400
300
200
Maximum wheel speed (cm/s)
100
75
50
25
0
C
HR
0
20
40
60
Generation

Figure 3.2: High Runner mice use narrower stances while running on a treadmill, but do not differ from Control mice in stride frequency or duty factor. Speed was tightly controlled, but graphs are offset along the x-axis for clarity of the error bars. The results of nested ANCOVA are shown in Table 3.2, and least-squares (LS) means are available in Appendix 1. None of the characteristics shown was affected by training (six days of wheel access). Top row: Stride frequency is not affected by line type, but strides become more frequent with increasing treadmill speed, and longer (stride length not shown) and less frequent following wheel access. Middle row: Stance width is significantly smaller in HR mice under all test conditions and for both sexes, as indicated by asterisks. Stance width also becomes smaller with increasing speed and decreases following wheel access. Bottom row: Duty factor is not significantly different between HR and C mice, but decreases with increasing speed.

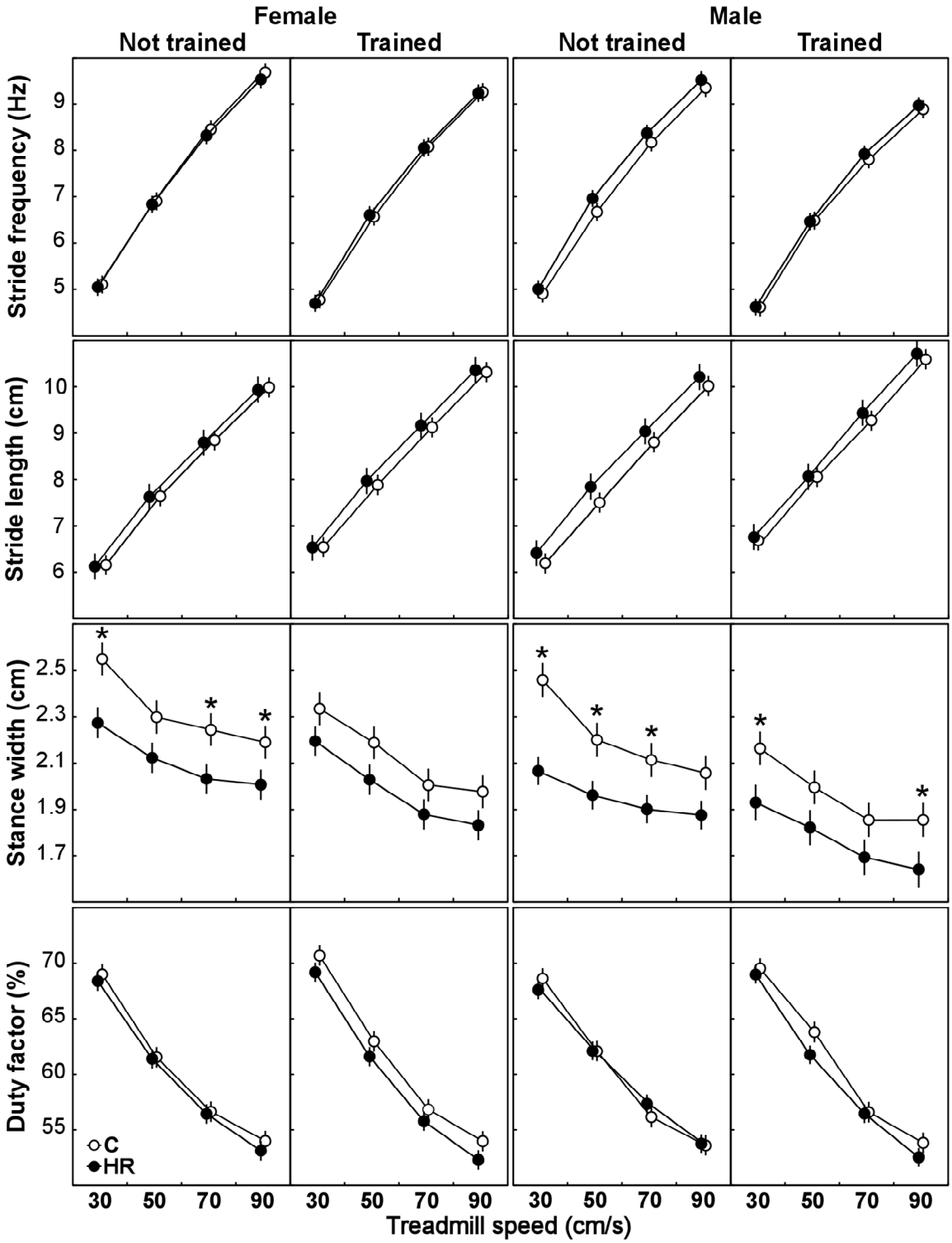

Female
Male
Not trained
Trained
Not trained
Trained
Stride frequency (Hz)
9
8
7
6
5
Stride length (cm)
10
9
8
7
6
Stance width (cm)
2.5
2.3
2.1
1.9
1.7
*
Duty factor (%)
70
65
60
55
C
HR
30
50
70
90
Treadmill speed (cm/s)

Figure 3.3: Mini-muscled mice have larger paw contact area while running on a treadmill, but the paw contact area of normal-muscled HR mice does not differ significantly from Controls. Simple means and SE (not accounting for repeated measures) for paw contact area are shown. Speed was tightly controlled, but graphs are offset along the x-axis for clarity. The results of nested ANCOVA are shown in Table 3.2, and LS means are available in Appendix 1.

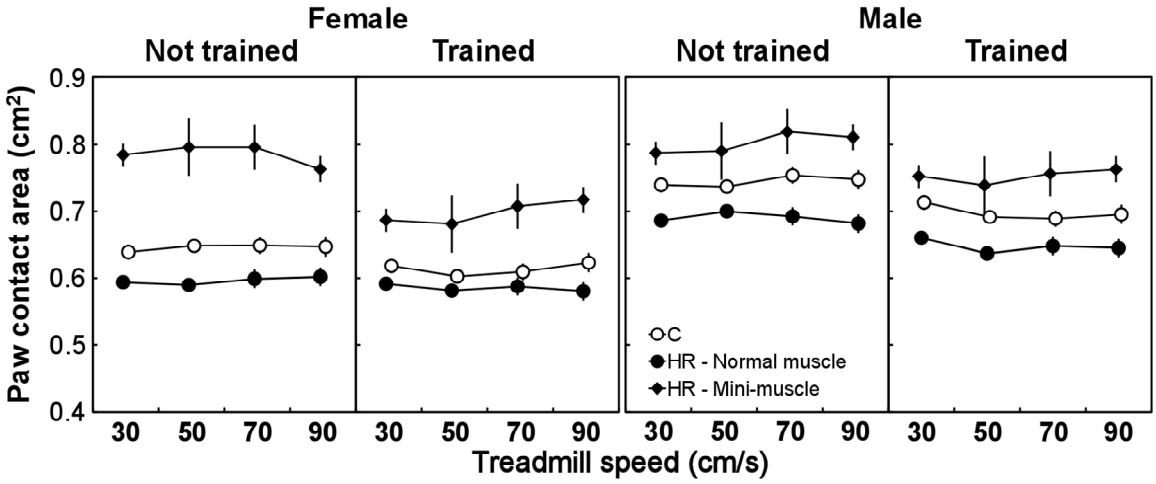


Figure 3.4: Body mass for HR and C mice. Least-squares means ± SE from a nested ANCOVA, as shown in are shown in Table A.2.

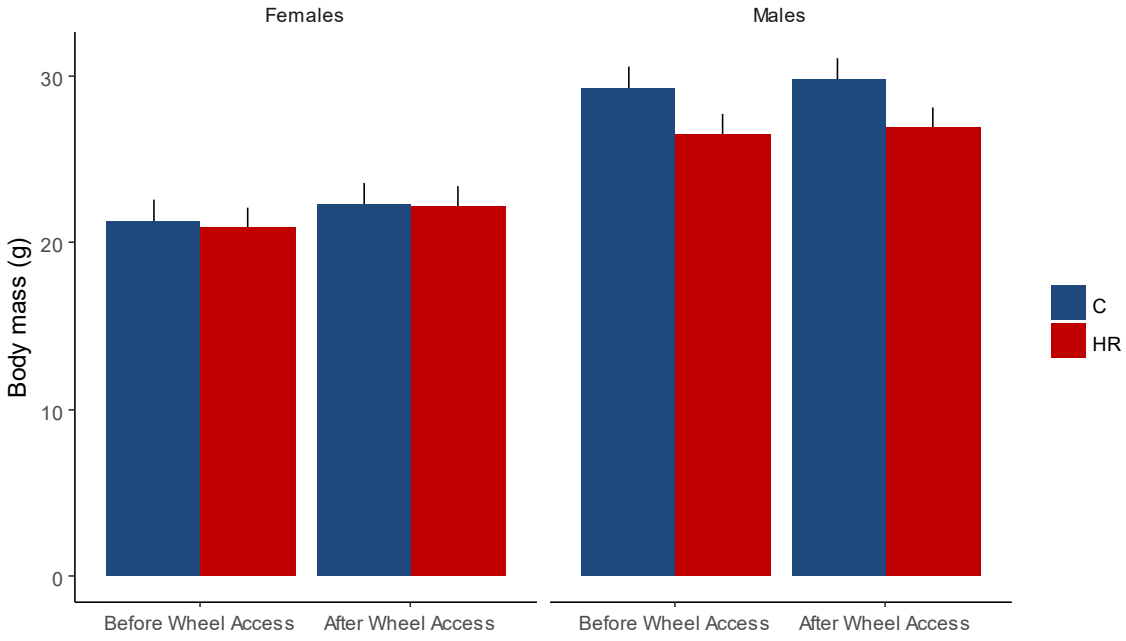
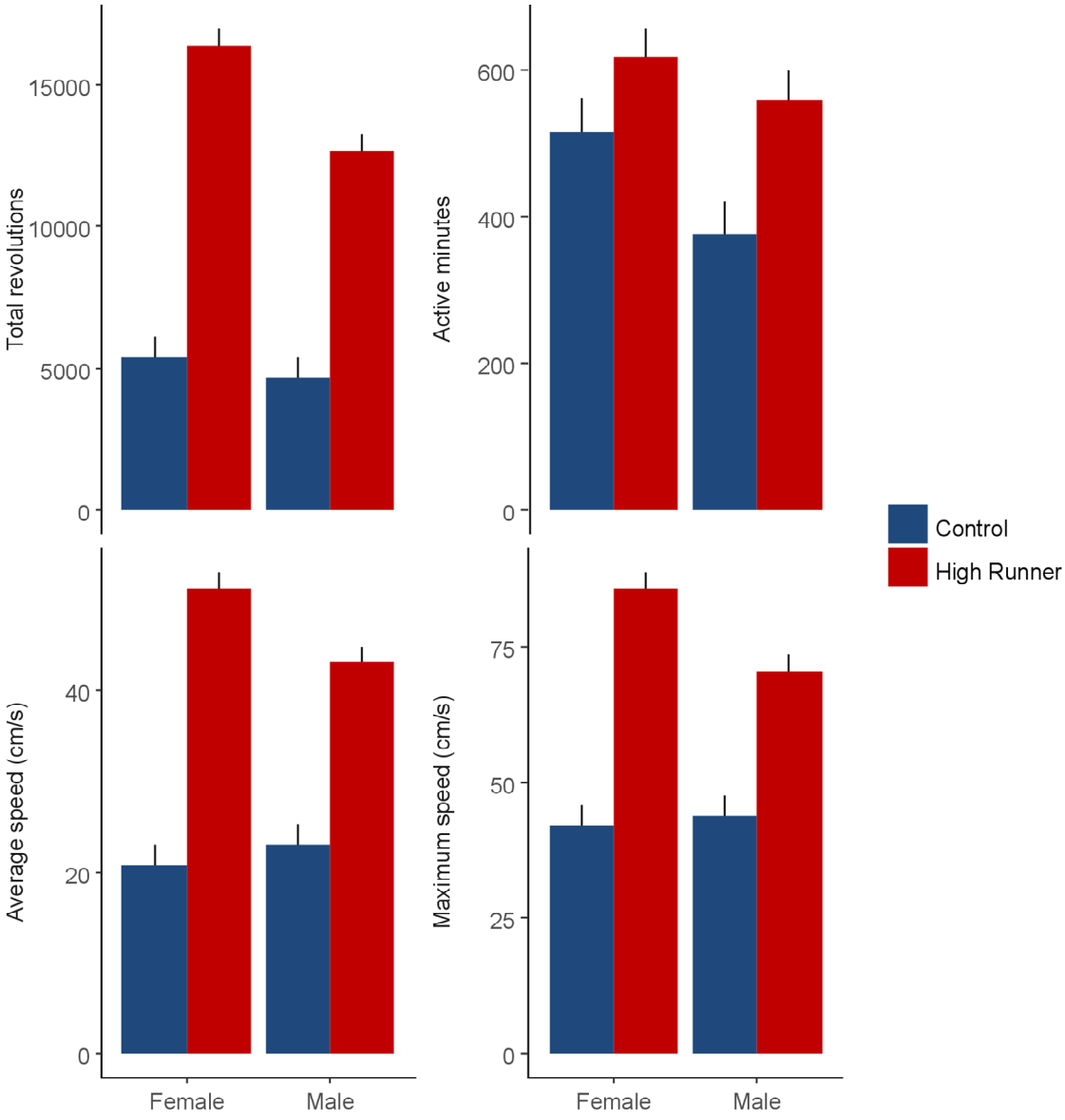


Figure 3.5: Wheel running for HR and C mice. Least-squares means ± SE from a nested ANCOVA, as shown in are shown in Table A.3.



References:

Albuquerque, R. L., de, K. E. Bonine, and T. Garland, Jr. 2015. Speed and Endurance Do Not Trade Off in Phrynosomatid Lizards. Physiol Biochem Zool 88:634-647.

Bauman, J. M. and Y.-H. Chang. 2010. High-speed X-ray video demonstrates significant skin movement errors with standard optical kinematics during rat locomotion. Journal of Neuroscience Methods 186:18-24.

Biewener, A. A. 1989. Scaling body support in mammals - Limb posture and muscle mechanics. Science 245:45-48.

Brainerd, E. L., D. B. Baier, S. M. Gatesy, T. L. Hedrick, K. A. Metzger, S. L. Gilbert, and J. J. Crisco. 2010. X-ray reconstruction of moving morphology (XROMM): precision, accuracy and applications in comparative biomechanics research. J Exp Zool A Ecol Genet Physiol 313:262-279.

Careau, V., M. E. Wolak, P. A. Carter, and T. Garland, Jr. 2013a. Data from: Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection. Dryad Data Repository.

Careau, V., M. E. Wolak, P. A. Carter, and T. Garland, Jr. 2013b. Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection. Evolution 67:3102-3119.

Carrano, M. T. 1999. What, if anything, is a cursor? Categories versus continua for determining locomotor habit in mammals and dinosaurs. J. Zool. 247:29-42.

Carrano, M. T. and A. A. Biewener. 1999. Experimental alteration of limb posture in the chicken (Gallus gallus) and its bearing on the use of birds as analogs for dinosaur locomotion. J Morphol 240:237-249.

Cespedes, A. M. and S. P. Lailvaux. 2015. An individual-based simulation approach to the evolution of locomotor performance. Integrative and Comparative Biology 55:1176-1187.

Claghorn, G. C., I. A. Fonseca, Z. Thompson, C. Barber, and T. Garland, Jr. 2016. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiol Behav 161:145-154.

Cops, E. J., M. Sashindranath, M. Daglas, K. M. Short, C. da Fonseca Pereira, T. Y. Pang, R. H. Lijnen, I. M. Smyth, A. J. Hannan, A. L. Samson, and R. L. Medcalf. 2013. Tissue-type plasminogen activator is an extracellular mediator of Purkinje cell damage and altered gait. Experimental Neurology 249:8-19.

Day, L. M. and B. C. Jayne. 2007. Interspecific scaling of the morphology and posture of the limbs during the locomotion of cats (Felidae). Journal of Experimental Biology 210:642-654.

Dlugosz, E. M., M. A. Chappell, D. G. McGillivray, D. A. Syme, and T. Garland, Jr. 2009. Locomotor trade-offs in mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:2612-2618.

Dlugosz, E. M., H. Schutz, T. H. Meek, W. Acosta, C. J. Downs, E. G. Platzer, M. A. Chappell, and T. Garland, Jr. 2013. Immune response to a *Trichinella spiralis* infection in house mice from lines selectively bred for high voluntary wheel running. J Exp Biol 216:4212-4221.

Filipe, V. M., J. E. Pereira, L. M. Costa, A. C. Maurício, P. A. Couto, P. Melo-Pinto, and A. S. P. Varejão. 2006. Effect of skin movement on the analysis of hindlimb kinematics during treadmill locomotion in rats. Journal of Neuroscience Methods 153:55-61.

Garland, T., Jr. and P. W. Freeman. 2005. Selective breeding for high endurance running increases hindlimb symmetry. Evolution 59:1851-1854.

Garland, T., Jr. and C. M. Janis. 1993. Does metatarsal/femur ratio predict maximal running speed in cursorial mammals? J. Zool. 229:133-151.

Garland, T., Jr., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011a. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proceedings of the Royal Society B-Biological Sciences 278:574-581.

Garland, T., Jr., M. T. Morgan, J. G. Swallow, J. S. Rhodes, I. Girard, J. G. Belter, and P. A. Carter. 2002a. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267-1275.

Garland, T., Jr., M. T. Morgan, J. G. Swallow, J. S. Rhodes, I. Girard, J. G. Belter, and P. A. Carter. 2002b. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267-1275.

Garland, T., Jr., H. Schutz, M. A. Chappell, B. K. Keeney, T. H. Meek, L. E. Copes, W. Acosta, C. Drenowatz, R. C. Maciel, G. van Dijk, C. M. Kotz, and J. C. Eisenmann. 2011b. The biological control of voluntary exercise, spontaneous

physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. Journal of Experimental Biology 214:206-229.

Garland, T., Jr., M. Zhao, and W. Saltzman. 2017. Hormones and the evolution of complex traits: insights from artificial selection on behavior. Integrative and Comparative Biology 57.

Gatesy, S. M. and N. S. Pollard. 2011. Apples, oranges, and angles: Comparative kinematic analysis of disparate limbs. Journal of Theoretical Biology 282:7-13.

Girard, I., M. W. McAleer, J. S. Rhodes, and T. Garland, Jr. 2001. Selection for high voluntary wheel-running increases speed and intermittency in house mice (*Mus domesticus*). Journal of Experimental Biology 204:4311-4320.

Gomes, F. R., E. L. Rezende, J. L. Malisch, S. K. Lee, D. A. Rivas, S. A. Kelly, C. Lytle, B. B. Yaspelkis, and T. Garland, Jr. 2009. Glycogen storage and muscle glucose transporters (GLUT-4) of mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:238-248.

Guderley, H., P. Houle-Leroy, G. M. Diffee, D. M. Camp, and T. Garland, Jr. 2006. Morphometry, ultrastructure, myosin isoforms, and metabolic capacities of the "mini muscles" favoured by selection for high activity in house mice. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 144:271-282.

Hannon, R. M., S. A. Kelly, K. M. Middleton, E. M. Kolb, D. Pomp, and T. Garland, Jr. 2008. Phenotypic effects of the "mini-muscle" allele in a large HR x C57BL/6J mouse backcross. J. Hered. 99:349-354.

Herbin, M., R. Hackert, J.-P. Gasc, and S. Renous. 2007. Gait parameters of treadmill versus overground locomotion in mouse. Behavioural Brain Research 181:173-179.

Higham, T. E., A. V. Birn-Jeffery, C. E. Collins, C. D. Hulsey, and A. P. Russell. 2015. Adaptive simplification and the evolution of gecko locomotion: Morphological and biomechanical consequences of losing adhesion. Proceedings of the National Academy of Sciences 112:809-814.

Horner, A. M., D. W. Russ, and A. R. Biknevicius. 2011. Effects of early-stage aging on locomotor dynamics and hindlimb muscle force production in the rat. The Journal of Experimental Biology 214:3588-3595.

Houle-Leroy, P., H. Guderley, J. G. Swallow, and T. Garland, Jr. 2003. Artificial selection for high activity favors mighty mini-muscles in house mice. American

Journal of Physiology-Regulatory Integrative and Comparative Physiology 284:R433-R443.

Kelly, S. A., T. A. Bell, S. R. Selitsky, R. J. Buus, K. Hua, G. M. Weinstock, T. Garland, Jr., F. Pardo-Manuel de Villena, and D. Pomp. 2013. A novel intronic single nucleotide polymorphism in the myosin heavy polypeptide 4 gene is responsible for the mini-muscle phenotype characterized by major reduction in hind-limb muscle mass in mice. Genetics 195:1385-1395.

Kelly, S. A., P. P. Czech, J. T. Wight, K. M. Blank, and T. Garland, Jr. 2006. Experimental evolution and phenotypic plasticity of hindlimb bones in high-activity house mice. J. Morphol. 267:360-374.

Kolb, E. M., S. A. Kelly, K. M. Middleton, L. S. Sermsakdi, M. A. Chappell, and T. Garland, Jr. 2010. Erythropoietin elevates VO_2max but not voluntary wheel running in mice. Journal of Experimental Biology 213:510-519.

Kolb, E. M., E. L. Rezende, L. Holness, A. Radtke, S. K. Lee, A. Obenaus, and T. Garland, Jr. 2013. Mice selectively bred for high voluntary wheel running have larger midbrains: support for the mosaic model of brain evolution. Journal of Experimental Biology 216:515-523.

Koteja, P., T. Garland, Jr., J. K. Sax, J. G. Swallow, and P. A. Carter. 1999. Behaviour of house mice artificially selected for high levels of voluntary wheel running. Animal Behaviour 58:1307-1318.

Meek, T., J. Eisenmann, B. Keeney, R. Hannon, E. Dlugosz, and T. Garland. 2014. Effects of early-life exposure to Western diet and wheel access on metabolic syndrome profiles in mice bred for high voluntary exercise. Genes, Brain and Behavior 13:322-332.

Meek, T. H., J. C. Eisenmann, and T. Garland, Jr. 2010. Western diet increases wheel running in mice selectively bred for high voluntary wheel running. International Journal of Obesity 34:960-969.

Meek, T. H., B. P. Lonquich, R. M. Hannon, and T. Garland. 2009. Endurance capacity of mice selectively bred for high voluntary wheel running. J. Exp. Biol. 212:2908-2917.

Meijer, J. H. and Y. Robbers. 2014. Wheel running in the wild. Proceedings of the Royal Society B: Biological Sciences 281:20140210.

Middleton, K. M., S. A. Kelly, and T. Garland, Jr. 2008. Selective breeding as a tool to probe skeletal response to high voluntary locomotor activity in mice. Integrative and Comparative Biology 48:394-410.

Rezende, E. L., T. Garland, Jr., M. A. Chappell, J. L. Malisch, and F. R. Gomes. 2006a. Maximum aerobic performance in lines of *Mus* selected for high wheel-running activity: effects of selection, oxygen availability and the mini-muscle phenotype. Journal of Experimental Biology 209:115-127.

Rezende, Enrico L., Fernando R. Gomes, Mark A. Chappell, and T. Garland, Jr. 2009. Running behavior and ts energy cost in mice selectively bred for high voluntary locomotor activity. Physiol. Biochem. Zool. 82:662-679.

Rezende, E. L., F. R. Gomes, J. L. Malisch, M. A. Chappell, and T. Garland, Jr. 2006b. Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. Journal of Applied Physiology 101:477-485.

Rezende, E. L., S. A. Kelly, F. R. Gomes, M. A. Chappell, and T. Garland, Jr. 2006c. Effects of size, sex, and voluntary running speeds on costs of locomotion in lines of laboratory mice selectively bred for high wheel-running activity. Physiol. Biochem. Zool. 79:83-99.

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integrative and Comparative Biology 45:438-455.

Rhodes, J. S., H. van Praag, S. Jeffrey, I. Girard, G. S. Mitchell, T. Garland, Jr., and F. H. Gage. 2003. Exercise increases hippocampal neurogenesis to high levels but does not improve spatial learning in mice bred for increased voluntary wheel running. Behav. Neurosci. 117:1006-1016.

Schutz, H., H. A. Jamniczky, B. Hallgrimsson, and T. Garland, Jr. 2014. Shape-shift: semicircular canal morphology responds to selective breeding for increased locomotor activity. Evolution 68:3184-3198.

Swallow, J. G., P. A. Carter, and T. Garland, Jr. 1998a. Artificial selection for increased wheel-running behavior in house mice. Behavior Genetics 28:227-237.

Swallow, J. G., T. Garland, Jr., P. A. Carter, W. Z. Zhan, and G. C. Sieck. 1998b. Effects of voluntary activity and genetic selection on aerobic capacity in house mice (*Mus domesticus*). Journal of Applied Physiology 84:69-76.

Swallow, J. G., T. Garland, Jr., P. Koteja, and P. A. Carter. 1999. Locomotor activity, food consumption, and body composition in house mice selected for high wheel-running activity. Faseb Journal 13:A419-A419.

Swallow, J. G., J. P. Hayes, P. Koteja, and T. Garland, Jr. 2009. Selection experiments and experimental evolution of performance and physiology *in* T. Garland, Jr., and

M. R. Rose, eds. Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments. University of California Press, Berkeley, CA.

Swallow, J. G., P. Koteja, P. A. Carter, and T. Garland, Jr. 2001. Food consumption and body composition in mice selected for high wheel-running activity. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 171:651-659.

Syme, D. A., K. Evashuk, B. Grintuch, E. L. Rezende, and T. Garland, Jr. 2005. Contractile abilities of normal and "mini" triceps surae muscles from mice (*Mus domesticus*) selectively bred for high voluntary wheel running. Journal of Applied Physiology 99:1308-1316.

Talmadge, R. J., W. Acosta, and T. Garland. 2014. Myosin heavy chain isoform expression in adult and juvenile mini-muscle mice bred for high-voluntary wheel running. mechanisms of development 134:16-30.

Usherwood, J. R. 2013. Constraints on muscle performance provide a novel explanation for the scaling of posture in terrestrial animals. Biology Letters 9:20130414.

Wallace, I. J. and T. Garland, Jr. 2016. Mobility as an emergent property of biological organization: Insights from experimental evolution. Evolutionary Anthropology: Issues, News, and Reviews 25:98-104.

Wilson, A. M., J. Lowe, K. Roskilly, P. E. Hudson, K. Golabek, and J. McNutt. 2013. Locomotion dynamics of hunting in wild cheetahs. Nature 498:185-189.

Wong, L. E., T. Garland, Jr., S. L. Rowan, and R. T. Hepple. 2009. Anatomic capillarization is elevated in the medial gastrocnemius muscle of mighty mini mice. Journal of Applied Physiology 106:1660-1667.

CONCLUDING REMARKS

This dissertation used the I used the High-Runner (HR) mouse model to study central fatigue and running dynamics in four replicate lines that have evolved in response to many generations of selective breeding for voluntary wheel running. In chapter 1, we presented first study to provide evidence that alterations in serotonin signaling account for some of the known differences between HR and non-selected Control (C) lines of mice with respect to locomotor behavior (voluntary wheel running) and performance (endurance capacity during forced treadmill exercise). We do not suggest that the difference in endurance between linetypes is attributable only to differences in 5-HT_{1A} receptors, or even more generally to neurobiological differences alone. However, the results here are remarkable in that the WAY-100,635-treated HR mice had virtually the same endurance as C mice (Fig. 1.3). The observed differences in drug response between HR and C mice suggest that pharmaceutical antagonism of the serotonin receptors 5-HT_{1A} (or possibly D_4 agonism by WAY-100,635) can ablate the endurance advantage of HR mice. As the receptor we targeted is found in highest concentration in the central nervous system, we hypothesize that decreased drive to the motor neurons caused the reduction in endurance of HR mice. However, the D_4 receptor is more broadly distributed in the periphery, and D_4 is associated with human hyperactivity (Faraone et al. 2005), so the serotonin interpretation is made with reservation.

The pharmacological effects we observed differed between endurance capacity during forced treadmill exercise (measured first) and voluntary wheel running (measured

after endurance trials), which suggests -- unsurprisingly -- that the two locomotor tests are not governed by identical neurobiological processes. Given the sequential-test experimental design used here, we cannot rule out the possibility that something about the mice changed between the tests. For example, aspects of exercise physiology can change with as little as a few hours or a few days of wheel access (Dumke et al. 2001 and references therein; Gomes et al. 2009). Our results support the findings of Ahlenius et al. (1997) in that systemic WAY-100,635 injection decreased endurance in mice, and in addition we found that the effect depended on genetic background.

More generally, our findings support the 5-HT central fatigue hypothesis (see Introduction) because pharmacological manipulation by a 5-HT_{1A} agonist and antagonist altered time to fatigue (treadmill endurance-running time). To our knowledge, this is the first time that pharmacological treatments during forced, exhaustive exercise have been compared with self-paced exercise (wheel running) for the same set of animals. As noted above, our results suggest that exhaustion during forced exercise and cessation of voluntary exercise are not governed by identical neurobiological processes.

In chapter 2, we showed that the sports drink Red Bull (but not Gatorade) increased the voluntary wheel running (total revolutions/day) for both sexes of both HR and C lines. However, maximal oxygen consumption (VO_2max) was not significantly affected by any of the fluid treatments (Table 2.4, Fig. 2.5), indicating that this aspect of physical performance ability may not be enhanced by Red Bull or one of its constituents, caffeine. Red Bull and caffeine each reduced tiredness ratings of both HR and C mice,

scored at the end of VO_2max tests, so the finding that Red Bull and caffeine caused an increase in wheel-running behavior may be attributable to decreased or delayed fatigue by affecting central nervous system fatigue (reviewed in Kalmar and Cafarelli 2004). This effect might also be caused by the action of caffeine on the perception of fatigue, as caffeine acts as a stimulant on the central nervous system and can delay fatigue by blocking adenosine receptors (Davis et al. 2003). We concluded that Red Bull (and to a lesser extent, caffeine in water) increased voluntary wheel running by mice, and decreased tiredness following maximal aerobic metabolic rate trials, while not increasing aerobic capacity itself. Genetic background, as represented by HR and C lines of mice, did not cause differential effects of caffeine in either voluntary or forced exercise metrics. The mechanism of action is unknown, but could be a combination of central and peripheral effects. In any case, positive effects on exercise behavior, if they can be shown to occur in humans, could have important implications for promoting levels of voluntary exercise.

In chapter 3, adjusting for variation in body mass,we found that HR mice were quite similar to C mice in most stride characteristics that we recorded with an automated, treadmill-based system, while they ran at speeds bracketing those exhibited during voluntary wheel running. The one notable difference between linetypes was that, as predicted, HR mice had narrower stance widths (ran with feet closer to one another and to the sagittal plane) than did mice from the non-selected control lines. We also found that mini-muscle individuals (a subset of the selected mice with hindlimb muscle masses reduced by ~50%) have larger paw contact areas and higher duty factors across running

speeds. We found no statistically significant effect of selective breeding on any of the temporal measurements of stride. Almost every measured stride characteristic was significantly affected by 6 days of wheel access (i.e., "training," "conditioning," "learning" or "plasticity"). In addition, sex affected a number of stride characteristics, even after accounting statistically for the effects of body size (males are larger than females). Interestingly, even though Garland and Freeman (2005) showed that, at generation 11, males had shorter leg bones than females (adjusted for body mass), we found a seemingly opposite trend of females taking shorter, more frequent strides (P = 0.0902, 0.0758 for sex effect on stride length and stride frequency, respectively)

All mice narrowed their stance in response to increased speeds, but HR stances were narrower at all speeds (Figure 3.2). All else being equal, a narrower stance should result in a more vertical alignment of limbs, and therefore a more “erect” posture (Biewener 1989; Carrano 1999). Such alignment is purported to be an adaptation to align the long bones with the direction of the ground reaction forces, thus reducing strain. Therefore, the narrowed stance in HR mice could be related to the increased wheel-running speeds (Figure 3.1), and thus, increased ground reaction forces (we presume), that have evolved as the primary mechanism by which HR mice have increased daily wheel-running distances. Force plate and 3D kinematic data would be required to resolve this hypothesis.

The mini-muscle phenotype may cause larger paw contact areas during loading because individuals with this phenotype rely more on two-legged support, as in rat aging

models (Horner et al. 2011), or because the mini-muscles have reduced total-force generating capacity (Syme et al. 2005), so the mice use a relatively crouched posture and longer stance time to generate the work necessary for push-off (Usherwood 2013). Either possibility is consistent with the finding that mini-muscle mice also have significantly greater duty factors (Figure 3.2, Table 3.2). However, the present data indicate that the significant difference is derived from an increase in the time spent in the braking phase of stance, rather than the propulsive stage (see Table A.5). Force plate data would clarify these findings, but photographic evidence shows that though the effective paw area during stance is larger, the actual paws of mini-muscle mice are not larger than those of non-mini-muscle mice, nor do HR mice differ from C (see Appendix B).

We view the High Runner mouse model as reasonably well suited for study of the evolution of kinematics because (1) HR mice have been bred for, and have evolved, great voluntary daily movement distances, (2) HR mice have evolved increased endurance during forced treadmill exercise (Meek et al. 2009; Claghorn et al. 2016), (3) HR mice have some described anatomical differences relevant to kinematics, including increased hindlimb symmetry and larger femoral heads (Garland and Freeman 2005; Kelly et al. 2006), and (4) they are well studied in terms of physiological adaptations to high activity as well as patterns of wheel-running behavior (Girard et al. 2001; Rezende et al. 2009; Garland et al. 2011a; Garland et al. 2011b). Furthermore, HR and C mice have a known evolutionary history, and differ only slightly in body mass and do not differ meaningfully in such skeletal dimensions as femur length (Garland and Freeman 2005; Kelly et al. 2006), so the model does not share the same confounding effects of disparate body size,

extreme morphological differences, or phylogenetic signal (Gartner et al. 2010) that plague many other comparisons. Known morphological differences between HR and C mice did not translate into detectable differences in stride in the present study. Mini-muscle mice have longer hind limbs (longer tibiafibula and sum of hindlimb bones [femur, tibiafibula, and metatarsals], Kelly et al. 2006), but did not have significantly longer strides. HR mice have heavier foot bones, with no statistically significant effect of mini-muscle beyond the HR effect (Kelly et al. 2006), but here the mini-muscle mice had significantly greater paw contact areas, whereas paw contact area did not differ between HR and C mice. These results serve as a cautionary tale for attempts to infer function solely from form.

References:

Ahlenius, S., P. Kaur, and P. Salmi. 1997. Biphasic effects of 8-OH-DPAT on endurance of treadmill performance in the male rat. European Neuropsychopharmacology 7:89-94.

Biewener, A. A. 1989. Scaling body support in mammals - Limb posture and muscle mechanics. Science 245:45-48.

Carrano, M. T. 1999. What, if anything, is a cursor? Categories versus continua for determining locomotor habit in mammals and dinosaurs. J. Zool. 247:29-42.

Claghorn, G. C., I. A. Fonseca, Z. Thompson, C. Barber, and T. Garland, Jr. 2016. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiol Behav 161:145-154.

Davis, J. M., Z. Zhao, H. S. Stock, K. A. Mehl, J. Buggy, and G. A. Hand. 2003. Central nervous system effects of caffeine and adenosine on fatigue. Am J Physiol Regul Integr Comp Physiol 284:R399-404.

Dumke, C. L., J. S. Rhodes, T. Garland, Jr., E. Maslowski, J. G. Swallow, A. C. Wetter, and G. D. Cartee. 2001. Genetic selection of mice for high voluntary wheel running: effect on skeletal muscle glucose uptake.

Faraone, S. V., R. H. Perlis, A. E. Doyle, J. W. Smoller, J. J. Goralnick, M. A. Holmgren, and P. Sklar. 2005. Molecular Genetics of Attention-Deficit/Hyperactivity Disorder. Biol. Psychiatry 57:1313-1323.

Garland, T., Jr. and P. W. Freeman. 2005. Selective breeding for high endurance running increases hindlimb symmetry. Evolution 59:1851-1854.

Garland, T., Jr., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011a. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proceedings of the Royal Society B-Biological Sciences 278:574-581.

Garland, T., Jr., H. Schutz, M. A. Chappell, B. K. Keeney, T. H. Meek, L. E. Copes, W. Acosta, C. Drenowatz, R. C. Maciel, G. van Dijk, C. M. Kotz, and J. C. Eisenmann. 2011b. The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. Journal of Experimental Biology 214:206-229.

Gartner, Gabriel E. A., James W. Hicks, Paulo R. Manzani, Denis V. Andrade, Augusto S. Abe, T. Wang, Stephen M. Secor, and T. Garland, Jr. 2010. Phylogeny, Ecology, and Heart Position in Snakes. Physiol. Biochem. Zool. 83:43-54.

Girard, I., M. W. McAleer, J. S. Rhodes, and T. Garland, Jr. 2001. Selection for high voluntary wheel-running increases speed and intermittency in house mice (*Mus domesticus*). Journal of Experimental Biology 204:4311-4320.

Gomes, F. R., E. L. Rezende, J. L. Malisch, S. K. Lee, D. A. Rivas, S. A. Kelly, C. Lytle, B. B. Yaspelkis, and T. Garland, Jr. 2009. Glycogen storage and muscle glucose transporters (GLUT-4) of mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:238-248.

Horner, A. M., D. W. Russ, and A. R. Biknevicius. 2011. Effects of early-stage aging on locomotor dynamics and hindlimb muscle force production in the rat. The Journal of Experimental Biology 214:3588-3595.

Kalmar, J. M. and E. Cafarelli. 2004. Caffeine: A valuable tool to study central fatigue in humans? Exercise and Sport Sciences Reviews 32:143-147.

Kelly, S. A., P. P. Czech, J. T. Wight, K. M. Blank, and T. Garland, Jr. 2006. Experimental evolution and phenotypic plasticity of hindlimb bones in high-activity house mice. J. Morphol. 267:360-374.

Meek, T. H., B. P. Lonquich, R. M. Hannon, and T. Garland, Jr. 2009. Endurance capacity of mice selectively bred for high voluntary wheel running. Journal of Experimental Biology 212:2908-2917.

Rezende, Enrico L., Fernando R. Gomes, Mark A. Chappell, and T. Garland, Jr. 2009. Running behavior and ts energy cost in mice selectively bred for high voluntary locomotor activity. Physiol. Biochem. Zool. 82:662-679.

Usherwood, J. R. 2013. Constraints on muscle performance provide a novel explanation for the scaling of posture in terrestrial animals. Biology Letters 9:20130414.

Appendix A: Stride Characteristics (Chapter 3) Supplemental Tables

Table A.1: Body mass in grams: LS means ± SE.

	Before Wheel Access		After Wheel Access	
	C	HR	C	HR
Females	21.3 ± 1.28	20.9 ± 1.18	22.3 ± 1.28	22.2 ± 1.18
Males	29.3 ± 1.28	26.6 ± 1.16	29.8 ± 1.29	27.0 ± 1.16

Table A.2: Results of nested analysis of variance (ANOVA) of body mass.

	Body mass		
Factor	d.f.	F	p
Line type	(1, 6)	0.87	0.3875
Sex	(1, 6)	246.89	**<.0001**
Training	(1, 6)	19.44	**0.0045**
Mini-muscle	(1, 165)	5.78	**0.0173**
Line type*sex	(1, 6)	8.81	**0.0250**
Line type*training	(1, 6)	0.04	0.8552
Sex*training	(1, 6)	3.59	0.1070
Line type*sex*training	(1, 6)	0.26	0.6288

Table A.3: Results of nested ANOVA of wheel running.

		Wheel running days 5 & 6		Active minutes days 5 & 6		Average speed days 5 & 6		Max speed days 5 & 6	
Factor	d.f.	F	p	F	p	F	p	F	p
Line type	(1, 6)	14.54	**<.0001**	19.4	**0.0346**	3.63	**<.0001**	7.75	**0.0001**
Sex	(1, 6)	222.39	**0.0088**	7.4	**0.0045**	130.13	0.1055	80.78	**0.0319**
Mini-muscle	(1, 81)	2.9	0.0924	0.06	0.802	7.57	**0.0073**	3.76	0.0559
Line type*sex	(1, 6)	6.71	**0.0412**	3.33	0.1179	11.33	**0.0151**	12.09	**0.0132**

Table A.4: Wheel running: LS means ± SE

		Total revolutions	Active minutes	Average speed (cm/s)	Maximum speed (cm/s)
Female	Control	5348 + 740	514 ± 45.6	20.7 ± 2.3	42 ± 3.9
	High Runner	16410 + 616	616 ± 40.6	51.3 ± 1.9	85.9 ± 3.3
Male	Control	4635 + 730	375 ± 45.4	23 ± 2.3	43.9 ± 3.9
	High Runner	12679 + 571	559 ± 39.0	43.1 ± 1.8	70.6 ± 3.1

Table A.5: Effects of line type, sex, training, treadmill speed, mini-muscle, and body mass on stride characteristics: Results of nested ANCOVAs. SAS PROC MIXED failed to converge for stance time and swing time using the full model, so all three-way and four-way interactions were excluded**.**

	Stride Length			Paw Contact Area			Swing time		
Factor	d.f.	F	p	d.f.	F	p	d.f.	F	p
Line type	(1, 6)	0.04	0.8481	(1, 6)	0.01	0.9140	(1, 6)	0.04	0.8413
Sex	(1, 6)	4.07	0.0902	(1, 6)	6.07	**0.0488**	(1, 6)	2.83	0.1434
Training	(1, 6)	78.79	**0.0001**	(1, 6)	26.17	**0.0022**	(1, 6)	14.65	**0.0087**
Speed	(3, 18)	3805.22	**<.0001**	(3, 18)	1.28	0.3128	(3, 18)	57.96	**<.0001**
Mini-muscle	(1, 655)	0.01	0.9043	(1, 550)	7.67	**0.0058**	(1, 728)	1.93	0.1647
Line type*sex	(1, 6)	2.07	0.2004	(1, 6)	0.28	0.6179	(1, 6)	2.85	0.1422
Line type*training	(1, 6)	0.09	0.7779	(1, 6)	0	0.9958	(1, 6)	1.31	0.2968
Line type*speed	(3, 18)	0.19	0.9048	(3, 18)	0.18	0.9095	(3, 18)	0.29	0.8311
Sex*training	(1, 6)	1.63	0.2487	(1, 6)	0	0.9571	(1, 728)	0.73	0.3929
Sex*speed	(3, 18)	1.82	0.1805	(3, 18)	0.73	0.5461	(3, 728)	4.02	**0.0074**
Training*speed	(3, 18)	1.55	0.2360	(3, 18)	5.1	**0.0099**	(3, 728)	1.26	0.2878
Line type*sex*training	(1, 6)	1.75	0.2338	(1, 6)	0.01	0.9261	*did not converge*		
Line type*sex*speed	(3, 18)	0.16	0.9190	(3, 18)	0.17	0.9168	*did not converge*		
Line type*training*speed	(3, 18)	0.62	0.6132	(3, 18)	0.7	0.5648	*did not converge*		
Sex*training*speed	(3, 18)	0.37	0.7729	(3, 18)	0.61	0.6198	*did not converge*		
(4-way interaction)	(3, 18)	1.7	0.2035	(3, 18)	1.02	0.4090	*did not converge*		
Body mass	(1, 655)	0.3	0.5858	(1, 550)	26.7	**<.0001**	(1, 728)	12.89	**0.0004**

Table A.5 continued:

	Stance time			Brake Time			Propel Time		
Factor	d.f.	F	p	d.f.	F	p	d.f.	F	p
Line type	(1, 6)	0.47	0.5197	(1, 6)	2.28	0.1817	(1, 6)	0.04	0.8550
Sex	(1, 6)	4.84	0.0701	(1, 6)	2.44	0.1690	(1, 6)	9.84	**0.0201**
Training	(1, 6)	78.23	**0.0001**	(1, 6)	18.58	**0.0050**	(1, 6)	49.16	**0.0004**
Speed	(3, 18)	1814.96	**<.0001**	(3, 18)	796.77	**<.0001**	(3, 18)	991.09	**<.0001**
Mini-muscle	(1, 728)	1.96	0.1620	(1, 655)	7.49	**0.0064**	(1, 655)	0.01	0.9294
Line type*sex	(1, 6)	1.48	0.2688	(1, 6)	1.8	0.2286	(1, 6)	0.15	0.7105
Line type*training	(1, 6)	0.42	0.5390	(1, 6)	1.95	0.2124	(1, 6)	0.05	0.8315
Line type*speed	(3, 18)	0.31	0.8204	(3, 18)	4.24	**0.0197**	(3, 18)	0.44	0.7296
Sex*training	(1, 728)	1.69	0.1945	(1, 6)	0.09	0.7704	(1, 6)	0.88	0.3856
Sex*speed	(3, 728)	6.39	**0.0003**	(3, 18)	1.79	0.1859	(3, 18)	10.49	**0.0003**
Training*speed	(3, 728)	48.22	**<.0001**	(3, 18)	4.42	**0.0169**	(3, 18)	21.92	**<.0001**
Line type*sex*training	*did not converge*			(1, 6)	2.16	0.1925	(1, 6)	0.45	0.5258
Line type*sex*speed	*did not converge*			(3, 18)	2.33	0.1086	(3, 18)	0.33	0.8048
Line type*training*speed	*did not converge*			(3, 18)	0.2	0.8923	(3, 18)	0.18	0.9114
Sex*training*speed	*did not converge*			(3, 18)	0.87	0.4738	(3, 18)	0.87	0.4734
(4-way interaction)	*did not converge*			(3, 18)	3.39	**0.0407**	(3, 18)	1.22	0.3311
Body mass	(1, 728)	12.86	**0.0004**	(1, 655)	0.02	0.8904	(1, 655)	13.16	**0.0003**

Table A.6: Gait characteristics (LS Means ± SE).

Females		Before wheel access		After wheel access	
Trait	Speed (cm/s)	C	HR	C	HR
Stride length	30	6.09 ± 0.215	6.14 ± 0.208	6.48 ± 0.214	6.53 ± 0.206
(cm)	50	7.58 ± 0.215	7.64 ± 0.208	7.92 ± 0.214	7.87 ± 0.206
	70	8.75 ± 0.215	8.85 ± 0.208	9.12 ± 0.214	9.10 ± 0.206
	90	9.90 ± 0.215	10.0 ± 0.209	10.31 ± 0.214	10.29 ± 0.207
Stride frequency	30	5.11 ± 0.195	5.05 ± 0.189	4.78 ± 0.194	4.70 ± 0.188
(Hz)	50	6.90 ± 0.195	6.83 ± 0.189	6.56 ± 0.194	6.60 ± 0.188
	70	8.45 ± 0.195	8.32 ± 0.189	8.08 ± 0.194	8.05 ± 0.188
	90	9.69 ± 0.195	9.53 ± 0.190	9.26 ± 0.194	9.24 ± 0.188
Duty factor	30	69.0 ± 0.92	68.4 ± 0.88	70.7 ± 0.91	69.2 ± 0.87
(%)	50	61.6 ± 0.92	61.4 ± 0.88	63.0 ± 0.91	61.6 ± 0.87
	70	56.6 ± 0.92	56.4 ± 0.88	56.8 ± 0.91	55.8 ± 0.87
	90	54.0 ± 0.92	53.1 ± 0.89	54.0 ± 0.92	52.3 ± 0.87
Propel time	30	100.2 ± 2.5	100.4 ± 2.4	108.3 ± 2.5	110.2 ± 2.4
(ms)	50	70.6 ± 2.5	70.5 ± 2.4	74.3 ± 2.5	71.9 ± 2.4
	70	53.2 ± 2.5	53.7 ± 2.4	54.4 ± 2.5	54.6 ± 2.4
	90	44.7 ± 2.5	43.9 ± 2.4	45.4 ± 2.5	44.1 ± 2.4
Brake time	30	38.3 ± 1.12	38.3 ± 1.05	43.2 ± 1.10	39.4 ± 1.03
(ms)	50	22.5 ± 1.12	23.1 ± 1.05	25.1 ± 1.10	24.7 ± 1.03
	70	17.8 ± 1.12	17.9 ± 1.05	19.8 ± 1.10	18.1 ± 1.03
	90	15.2 ± 1.12	15.9 ± 1.06	16.9 ± 1.11	16.0 ± 1.04
Paw contact area	30	0.69 ± 0.029	0.68 ± 0.028	0.66 ± 0.029	0.64 ± 0.028
(cm^2)	50	0.70 ± 0.029	0.68 ± 0.028	0.64 ± 0.029	0.64 ± 0.028
	70	0.70 ± 0.029	0.69 ± 0.028	0.65 ± 0.029	0.65 ± 0.028
	90	0.70 ± 0.029	0.68 ± 0.028	0.67 ± 0.029	0.65 ± 0.028
Stance width	30	2.55 ± 0.072	2.27 ± 0.067	2.34 ± 0.071	2.2 ± 0.065
(cm)	50	2.30 ± 0.072	2.12 ± 0.067	2.19 ± 0.071	2.03 ± 0.065
	70	2.24 ± 0.072	2.03 ± 0.067	2.01 ± 0.071	1.88 ± 0.065
	90	2.19 ± 0.072	2.01 ± 0.067	1.98 ± 0.071	1.83 ± 0.066

Table A.6 continued:

Males		Before wheel access		After wheel access	
Trait	Speed (cm/s)	C	HR	C	HR
Stride length	30	6.34 ± 0.214	6.19 ± 0.202	6.73 ± 0.215	6.67 ± 0.202
(cm)	50	7.81 ± 0.214	7.50 ± 0.202	8.03 ± 0.215	8.05 ± 0.202
	70	9.01 ± 0.214	8.80 ± 0.203	9.41 ± 0.215	9.26 ± 0.202
	90	10.19 ± 0.214	10.02 ± 0.202	10.69 ± 0.215	10.59 ± 0.202
Stride frequency	30	4.91 ± 0.194	5.01 ± 0.184	4.61 ± 0.195	4.62 ± 0.184
(Hz)	50	6.67 ± 0.194	6.96 ± 0.184	6.48 ± 0.195	6.46 ± 0.184
	70	8.17 ± 0.194	8.37 ± 0.184	7.80 ± 0.195	7.92 ± 0.184
	90	9.35 ± 0.194	9.52 ± 0.184	8.89 ± 0.195	8.97 ± 0.184
Duty factor	30	68.6 ± 0.92	67.6 ± 0.84	69.5 ± 0.93	69.0 ± 0.84
(%)	50	62.1 ± 0.92	62.1 ± 0.84	63.8 ± 0.93	61.8 ± 0.84
	70	56.2 ± 0.92	57.3 ± 0.84	56.6 ± 0.93	56.5 ± 0.84
	90	53.6 ± 0.92	53.8 ± 0.84	53.8 ± 0.93	52.5 ± 0.84
Propel time	30	107.0 ± 2.5	107.0 ± 2.3	114.8 ± 2.5	115.0 ± 2.3
(ms)	50	75.4 ± 2.5	71.8 ± 2.3	78.1 ± 2.5	76.5 ± 2.3
	70	54.9 ± 2.5	54.5 ± 2.3	57.3 ± 2.5	57.7 ± 2.3
	90	45.2 ± 2.5	44.4 ± 2.3	46.9 ± 2.5	46.4 ± 2.3
Brake time	30	39.0 ± 1.13	32.6 ± 0.98	42.0 ± 1.16	38.6 ± 0.98
(ms)	50	21.8 ± 1.13	21.4 ± 0.99	24.5 ± 1.16	23.0 ± 0.98
	70	17.2 ± 1.13	17.6 ± 0.99	18.7 ± 1.16	17.1 ± 0.98
	90	15.4 ± 1.13	15.4 ± 0.99	16.6 ± 1.16	15.4 ± 0.98
Paw contact area	30	0.72 ± 0.029	0.73 ± 0.027	0.70 ± 0.029	0.70 ± 0.027
(cm^2)	50	0.72 ± 0.029	0.73 ± 0.027	0.68 ± 0.029	0.68 ± 0.027
	70	0.74 ± 0.029	0.74 ± 0.027	0.68 ± 0.029	0.69 ± 0.027
	90	0.73 ± 0.029	0.73 ± 0.027	0.68 ± 0.029	0.69 ± 0.027
Stance width	30	2.46 ± 0.074	2.07 ± 0.061	2.16 ± 0.075	1.93 ± 0.061
(cm)	50	2.20 ± 0.073	1.96 ± 0.062	2.00 ± 0.075	1.82 ± 0.061
	70	2.11 ± 0.073	1.90 ± 0.062	1.85 ± 0.075	1.69 ± 0.061
	90	2.06 ± 0.073	1.88 ± 0.062	1.85 ± 0.075	1.64 ± 0.061

Appendix B: Photographic measurement of paw area

Photographs were taken to measure paw size in all mice. Mouse feet were gently pressed against a glass slide post-mortem, and photographed ventrally with a consumer-grade digital SLR camera (Nikon D60 with a Nikon 50mm lens). The paw photographs were outlined in ImageJ once each for size analysis. In keeping with the rest of this report, left and right paws were averaged for each mouse, and only hind paw data are shown.

Males showed a trend towards larger feet than females ($F_{(1,6)} = 5.22$, $P = 0.0624$) and body size was a good predictor of paw area ($F_{(1.74)} = 26.96$, $P < 0.0001$), but we did not find a significant foot size difference between mini-muscle and non-mini-muscle mice ($F_{(1,74)} = 0.28$, $P = .5963$). We also found no evidence of an effect of line type ($F_{(1,6)} = 0.43$, $P = 0.5354$) or a line type by sex interaction ($F_{(1,6)} = 0.03$, $P = 0.8755$).

Table B.1: Effects of line type, sex, and body mass on paw size measured from photographs: Results of nested ANCOVA.

	df	F	P
Sex	(1, 6)	5.22	0.0624
Linetype	(1, 6)	0.43	0.5354
Mini-muscle	(1, 74)	0.28	0.5963
Sex by Lintype	(1, 6)	0.03	0.8755
Body Mass	(1, 74)	26.96	<.0001

Table B.2: Paw size measured from photographs. Lest-squares means from nested ANCOVA.

		Measured area of paw (mm^2)
Females	C	68.0 ± 1.7
	HR	69.4 ± 1.5
Males	C	69.6 ± 1.7
	HR	70.9 ± 1.5

Appendix C: Repeatability of gait characteristics

We analyzed repeatability for each trait, in groups defined by line type, sex, and treadmill speed, by calculating the correlation (Pearson's r) using base R 3.1.1 (2014) and the plyr package (Wickham 2011) between measurements taken before and after wheel access.

The majority of measurements, broken down by line type, sex, and treadmill speed, were highly repeatable before and after access to wheels (median repeatability = 0.510, Table C.1, Figs A.1-8). Exceptions were found mostly where there was little variation (e.g. stance time at high treadmill speeds, Fig. A.4), and where there was a large training effect of the variable in question (e.g., stride frequency for HR mice, Table C.2, where there was a trend toward a line type by sex interaction).

Table C.1: Group-wise repeatability between measurements taken before and after wheel access

		Pearson's r (* indicates 2-tailed $P < 0.05$)							
	Speed (cm/s)	Stride length	Stride frequency	Stance time	Swing time	Brake time	Propel time	Stance width	Paw area
Females									
	(n)	(23-26)	(23-26)	(23-26)	(23-26)	(23-26)	(23-26)	(23-26)	(21-22)
C	30	0.847	0.853	0.735	0.722	0.595	0.814	0.737	0.655
	50	0.812	0.805	0.724	0.804	0.419	0.564	0.325	0.521
	70	0.867	0.870	0.708	0.865	0.577	0.698	0.503	0.692
	90	0.806	0.803	0.663	0.651	0.434	0.620	0.258	0.715
HR	30	0.323	0.328	0.148	0.519	0.232	0.091	0.669	0.802
	50	0.055	0.110	0.298	0.104	0.593	0.325	0.642	0.881
	70	0.149	0.141	0.307	0.061	0.538	0.500	0.322	0.867
	90	-0.273	-0.230	0.332	-0.241	0.495	0.633	0.347	0.861
Males									
	(n)	(24-26)	(24-26)	(24-26)	(24-26)	(24-26)	(24-26)	(24-26)	(18-23)
C	30	0.767	0.692	0.682	0.790	0.276	0.529	0.725	0.532
	50	0.831	0.816	0.603	0.670	0.461	0.482	0.548	0.324
	70	0.802	0.768	0.366	0.639	-0.035	0.411	0.422	0.251
	90	0.628	0.636	0.101	0.481	0.023	0.104	0.588	0.557
HR	30	0.427	0.410	0.331	0.468	0.467	0.226	0.500	0.863
	50	0.745	0.662	0.536	0.666	0.670	0.696	0.331	0.777
	70	0.583	0.590	0.436	0.418	0.368	0.665	0.092	0.849
	90	0.422	0.425	0.340	0.549	0.350	0.364	0.355	0.669

Table C.2: Group-wise effect of training, i.e., before versus after six days of wheel access

		Group-wise effect of training by group (p-value and sign of paired t-test within group)							
	Speed (cm/s)	Stride length	Stride frequency	Stance time	Swing time	Brake time	Propel time	Stance width	Paw area
Females									
	(d.f.)	(22-25)	(22-25)	(22-25)	(22-25)	(22-25)	(22-25)	(22-25)	(20-21)
C	30	**<.0001+**	**<.0001-**	**<.0001+**	0.7736	**0.0003+**	**<.0001+**	**<.0001-**	0.0511
	50	**<.0001+**	**<.0001-**	**<.0001+**	0.8255	**0.0006+**	**0.001+**	0.1644	**0.0001-**
	70	**<.0001+**	**<.0001-**	**<.0001+**	**0.0029+**	**0.0002+**	**0.0225+**	**0.0003-**	**0.0004-**
	90	**<.0001+**	**<.0001-**	**0.0005+**	**0.0199+**	**<.0001+**	0.1134	**0.0024-**	0.0685
HR	30	**0.0001+**	**<.0001-**	**<.0001+**	0.2736	0.3802	**<.0001+**	0.3958	**0.0198-**
	50	**0.0066+**	**0.0033-**	**0.0008+**	0.5060	**0.0246+**	**0.0418+**	0.1257	**0.0074-**
	70	**0.0014+**	**0.0012-**	**0.0150+**	**0.0323+**	0.7187	**0.0258+**	0.0519	**0.0043-**
	90	**0.0209+**	**0.0195-**	0.0843	0.1163	0.4732	0.0826	**0.0159-**	**0.0105-**
Males									
	(d.f.)	(23-25)	(23-25)	(23-25)	(23-25)	(23-25)	(23-25)	(23-25)	(17-22)
C	30	**0.0003+**	**0.0016-**	**<.0001+**	0.3429	**0.0683+**	**0.0018+**	**<.0001-**	0.0694
	50	**0.0029+**	**0.0066-**	**<.0001+**	0.1857	**0.0002+**	**0.0078+**	**0.0025-**	**0.0171-**
	70	**<.0001+**	**<.0001-**	**0.0001+**	0.5634	**0.0041+**	**0.0009+**	**0.0029-**	**0.0060-**
	90	**<.0001+**	**<.0001-**	**0.0181+**	**0.0329+**	**0.0035+**	0.1489	**0.0049-**	**0.0059-**
HR	30	**<.0001+**	**<.0001-**	**<.0001+**	0.2451	**0.0002+**	**0.0004+**	**0.0133-**	**0.0050-**
	50	**<.0001+**	**<.0001-**	**<.0001+**	**<.0001+**	**0.0049+**	**<.0001+**	**0.0060-**	**<.0001-**
	70	**<.0001+**	**<.0001-**	**0.0037+**	**0.0003+**	0.4473	**<.0001+**	**0.0014-**	**0.0002-**
	90	**<.0001+**	**<.0001-**	**0.0030+**	**<.0001+**	0.4088	**0.0048+**	**0.0014-**	**0.0265-**

Figure C.1: Repeatability of stride length before and after 6 days of wheel access separated by line type, sex, and treadmill speed. Pearson's r and the result of a paired t-test can been found in tables C.1 and C.2, respectively.

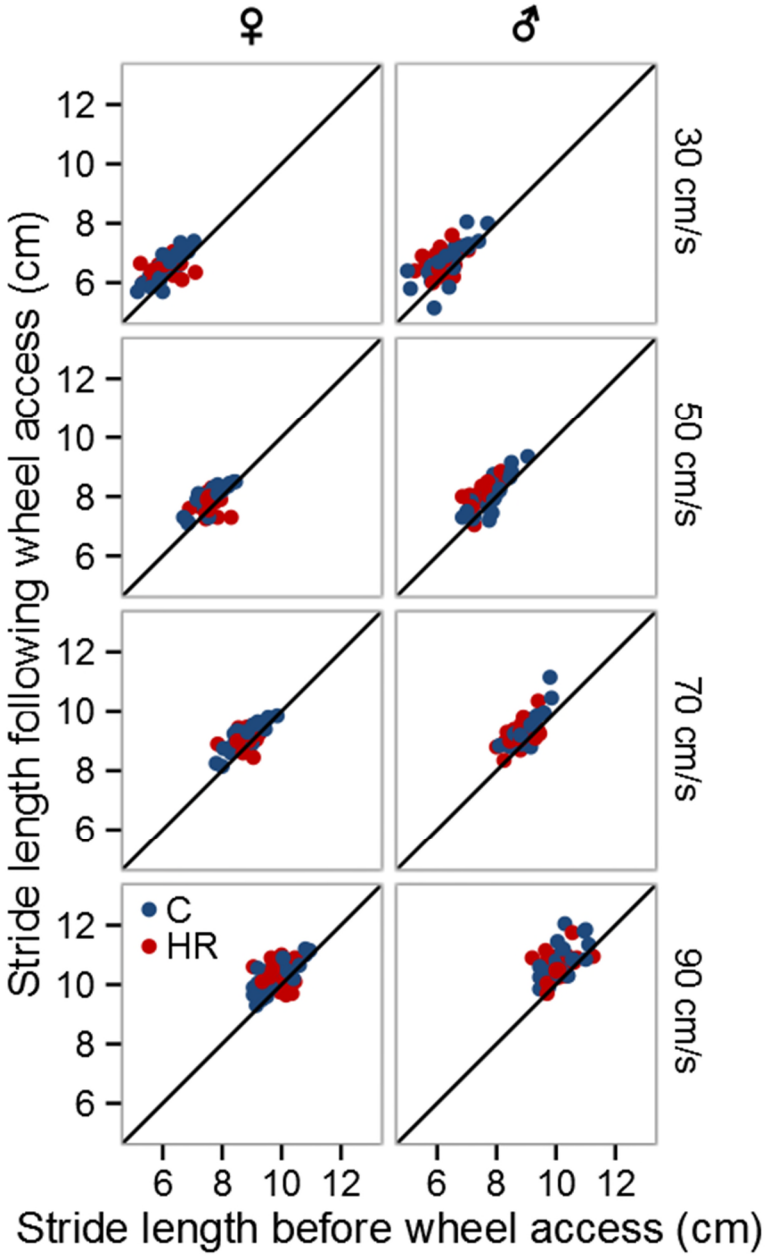


Figure C.2: Repeatability of stride frequency before and after 6 days of wheel access separated by line type, sex, and treadmill speed. Pearson's r and the result of a paired t-test can been found in tables C.1 and C.2, respectively.

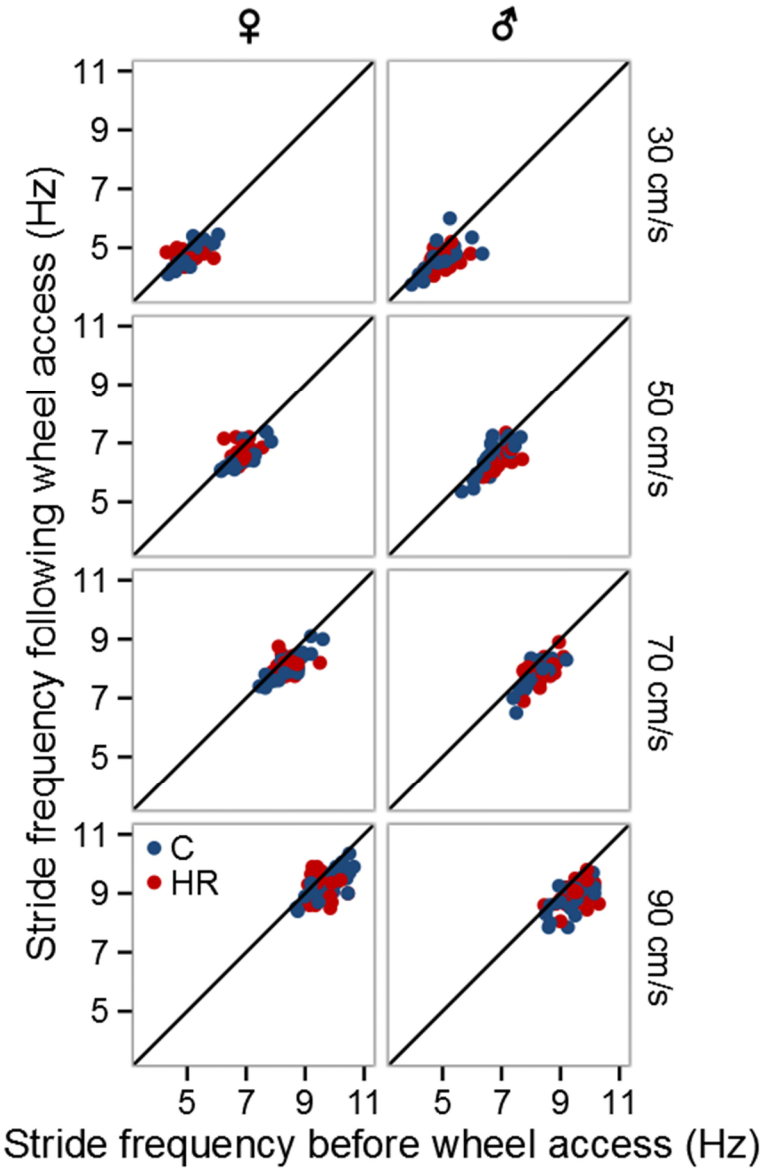


Figure C.3: Repeatability of swing time before and after 6 days of wheel access separated by line type, sex, and treadmill speed. Pearson's r and the result of a paired t-test can been found in tables C.1 and C.2, respectively.

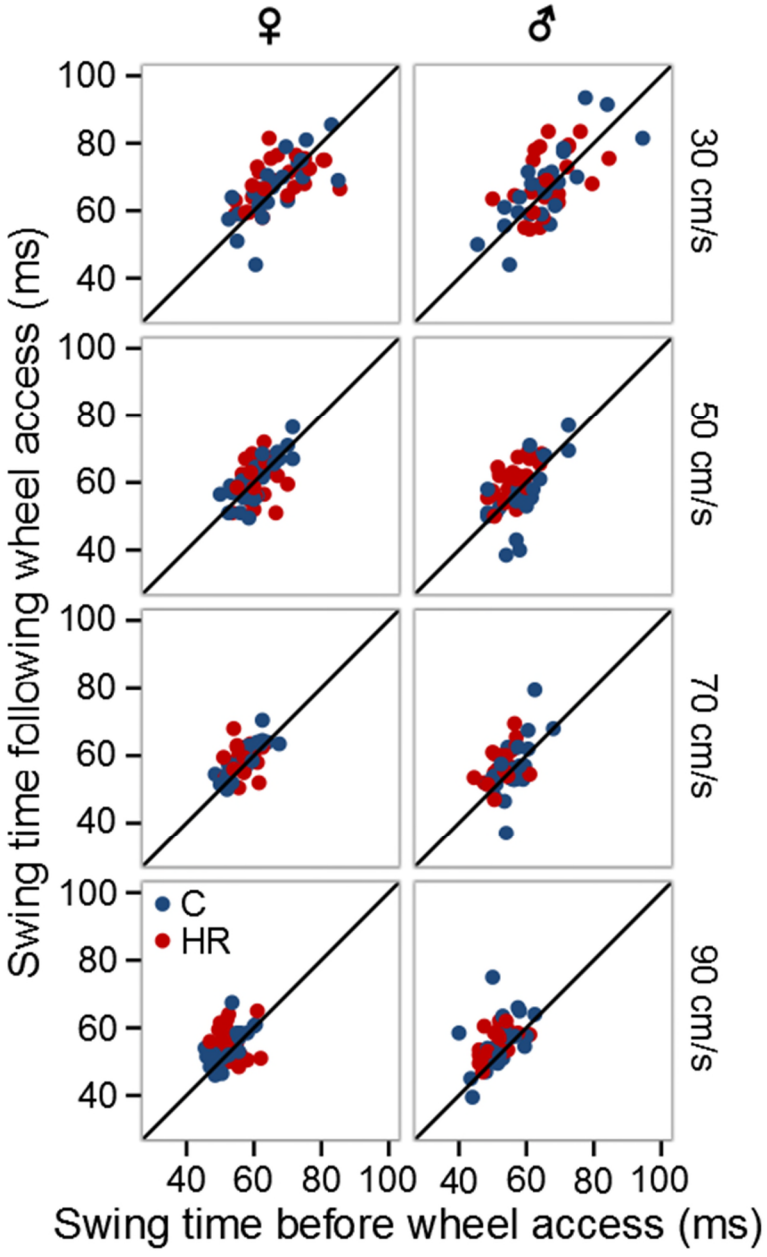


Figure C.4: Repeatability of stance time before and after 6 days of wheel access separated by line type, sex, and treadmill speed. Pearson's r and the result of a paired t-test can been found in tables C.1 and C.2, respectively.

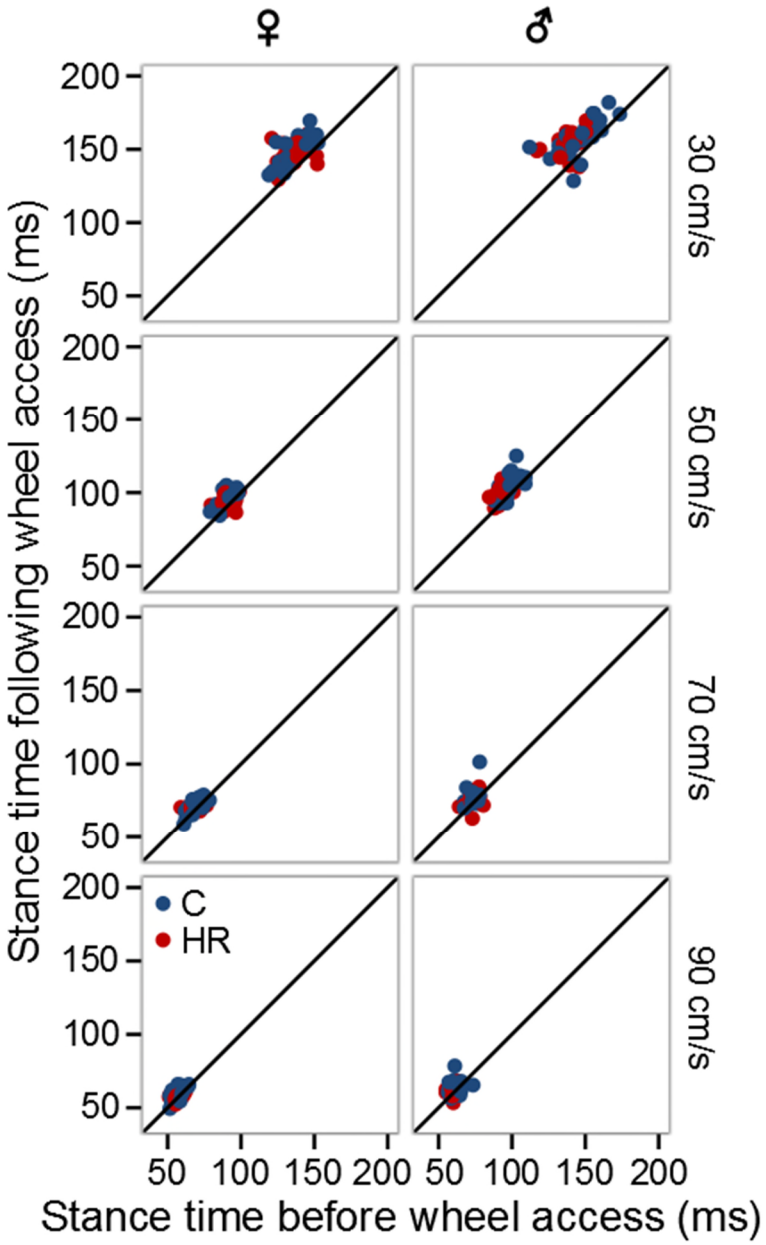


Figure C.5: Repeatability of propel time before and after 6 days of wheel access separated by line type, sex, and treadmill speed. There is no evidence of measurement error which would explain the discrepant point in top left panel, therefore, we did not treat it as an outlier. Pearson's r and the result of a paired t-test can been found in tables C.1 and C.2, respectively.

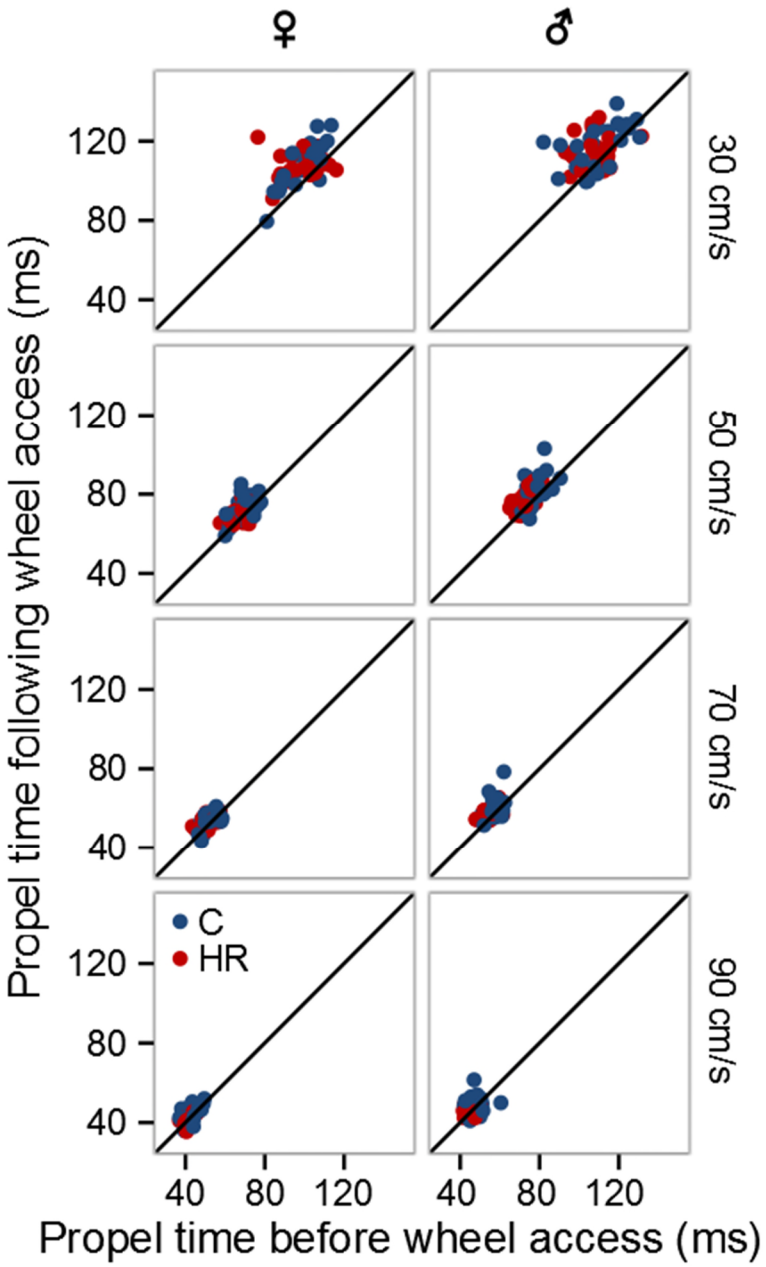


Figure C.6: Repeatability of brake time before and after 6 days of wheel access separated by line type, sex, and treadmill speed. There is no evidence of measurement error which would explain the discrepant point in top left panel, therefore, we did not treat it as an outlier. Pearson's r and the result of a paired t-test can been found in tables C.1 and C.2, respectively.

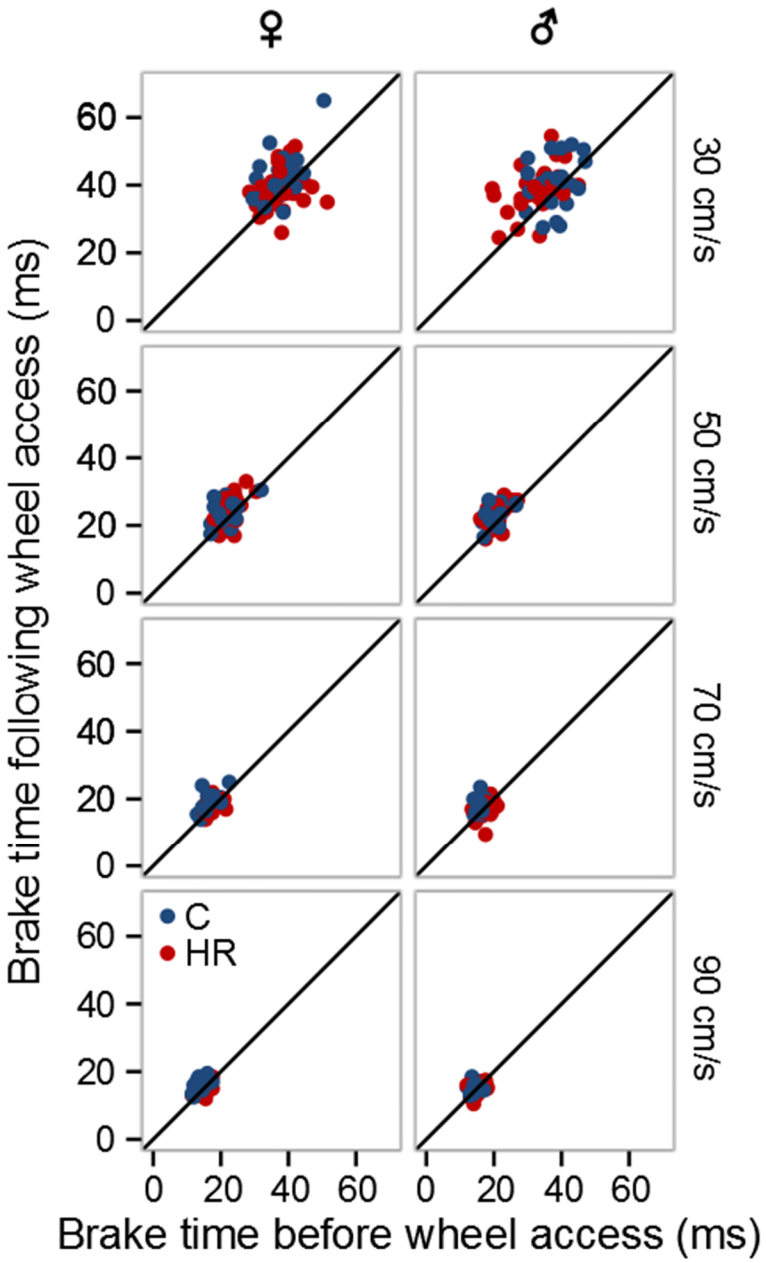


Figure C.7: Repeatability of paw contact area before and after 6 days of wheel access separated by line type, sex, and treadmill speed. Pearson's r and the result of a paired t-test can been found in tables C.1 and C.2, respectively.

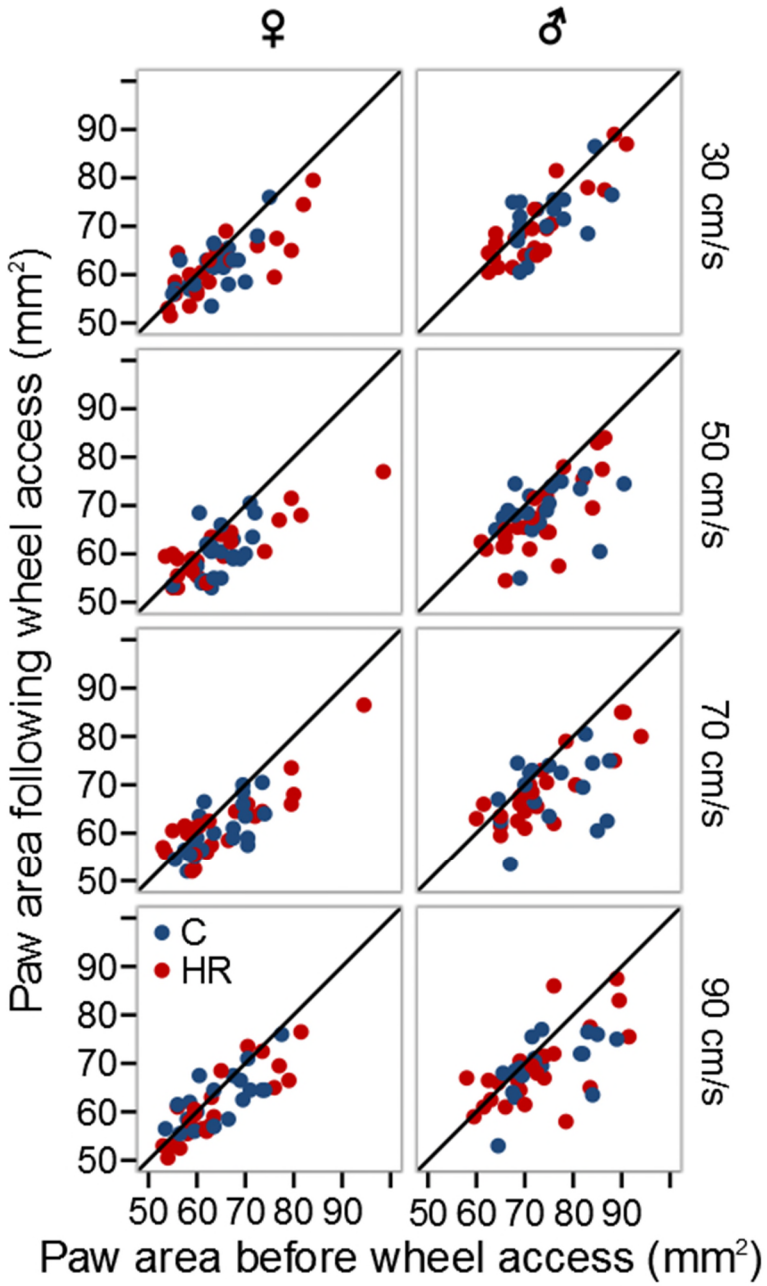
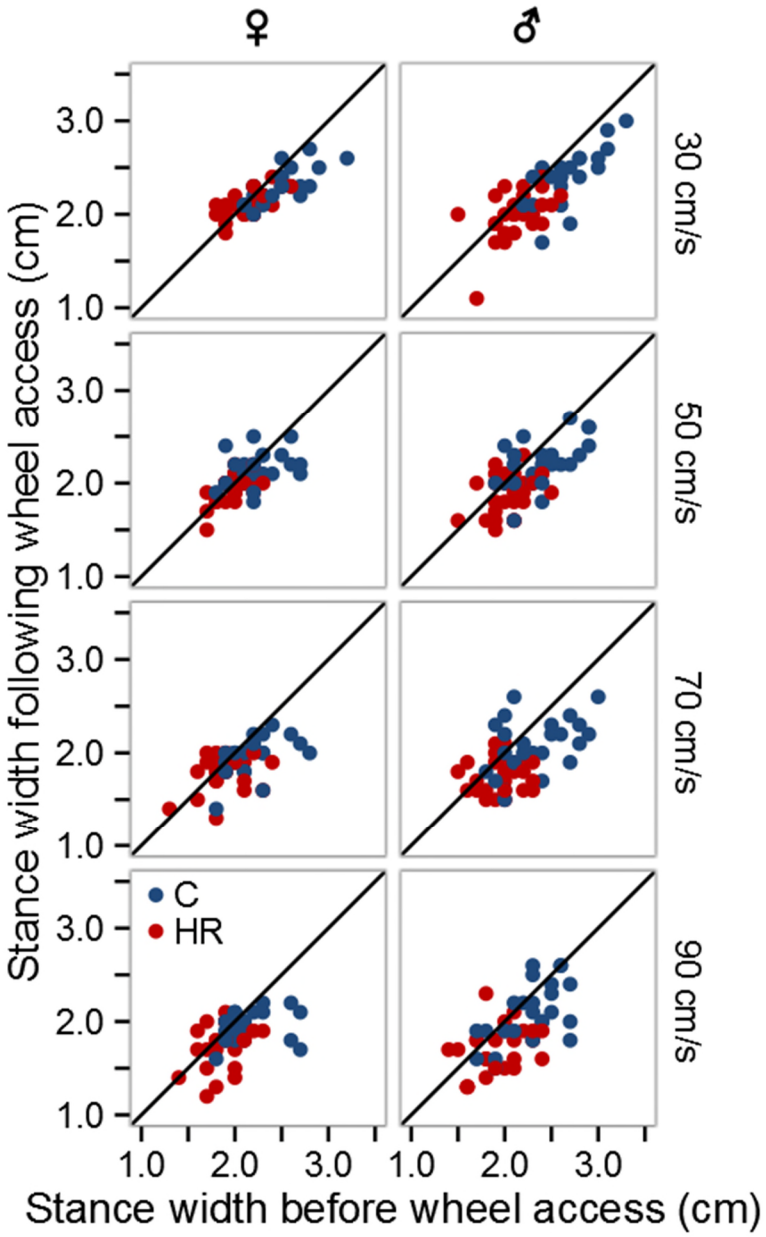


Figure C.8: Repeatability of stance width before and after 6 days of wheel access separated by line type, sex, and treadmill speed. Pearson's r and the result of a paired t-test can been found in tables C.1 and C.2, respectively.



APPENDIX D. Notes Added After Publication

After publication of this chapter (Claghorn et al. 2016), we noticed two sentences in the abstract seemingly at odds with one another. First: "Wheel running was reduced in HR but not C mice at the highest dose of 8-OH-DPAT + pindolol (dose by linetype, P = 0.0221), but was not affected by WAY-100,635 treatment." Then: “Although the elevated wheel running of HR mice does not appear related to alterations in serotonin signaling...” We should have been more explicit in the abstract, but we intended to indicate that whereas WAY-100,635 completely eliminated the HR/C difference in endurance, 8-OH-DPAT only slightly reduced the difference in wheel running. Also, we were skeptical of the reduction in endurance at the high dose of 8-OH-DPAT, because we suspected that it was most likely acting post-synaptically (as per the description of the biphasic effect by Ahlenius 1997).

Also, after publication of this chapter (Claghorn et al. 2016), we received an email from Chris Perry (29 May 2016) in which he argued that our results could be interpreted as peripheral effects of the 5-HT drugs. His argument was essentially something that we had discussed among ourselves as a possible explanation of the findings. He asked, if the drug causes feelings of tiredness, centrally, then wouldn't that manifest in reduced *voluntary* exercise before it reduced *maximal* exercise performance?

We settled on the explanation in the paper based partly on the findings of Ahlenius et al. (1997), who state (& cite several papers) that the effects of 8-OH-DPAT "produced by local brain infusions are similar to those seen after systemic administration." Thus, we presumed from the start that the effect would be central, rather than peripheral. Second, and perhaps more importantly, we worked under the presumption that the drugs would effectively lower the endurance "ceiling" (e.g., maximal aerobic speed), which the mice rarely approach while running on wheels. (To be more precise, mice from the non-selected control lines do not approach the ceiling. HR get closer (see Girard et al. 2001; Fig. 1 in Rezende et al. 2005), but still do not exhaust themselves on wheel.) Therefore, you could lower the "ceiling" without affecting wheel running. In further support of this idea, Kolb et al. (2010) found that treatment with erythropoietin increased blood hemoglobin and maximal aerobic capacity of both HR and C mice, without significantly increasing their wheel running.

The full correspondence is included below:

From: Chris Perry [chrisperry72@hotmail.com]

Sent: Sunday, May 29, 2016 3:05 PM

To: geraldclaghorn@gmail.com; Theodore Garland Jr

Subject: Serotonin in Central Fatigue

Hi,

I really enjoyed your recent article in Physiology and Behavior on serotonin and central fatigue (Claghorn GC, Fonseca IA, Thompson Z, Barber C, Garland T Jr. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiol Behav. 2016 Jul 1;161:145-54). I am a physician (critical care and emergency medicine), but have an interest in sports science and the mechanisms of fatigue in endurance competition. The research that has been done in recent years on neurotransmitters and their role in central fatigue is very exciting to me, both in terms of the application to sports and also potentially the application to the care of critically ill patients.

I was a little confused about one thing, though, and I was hoping you could help clear it up for me. I noticed that the effects of the antagonist primarily were seen on the HR mice during forced treadmill testing, but not in wheel running. Maybe I am misunderstanding, but wouldn't this lead you to conclude more that the effect is peripheral, rather than central? It would seem to me that if the effects were more on central fatigue, the results would be the opposite, i.e. the HR mice would be affected more in the voluntary running than in the forced running. Am I wrong in thinking this?

Anyways, great article!

Chris Perry, MD, CISSN, CNSC

Guttenberg, NJ

From: Gerald Claghorn [geraldclaghorn@gmail.com]

Sent: Friday, June 03, 2016 6:39 PM

To: Chris Perry; Theodore Garland Jr

Cc: Zoe Thompson; Ivana Fonseca

Subject: Re: Serotonin in Central Fatigue

Hi Chris,

Thank you for reading our paper, and for your comments.

Your line of thinking, if I understand it correctly, is something that the authors and our colleagues discussed as a possible explanation of the findings. If the drug causes feelings of tiredness, centrally, wouldn't that manifest in reduced voluntary exercise before it reduced maximal exercise?

We settled on the explanation in the paper based partly on the findings of Ahlenius et al. (1997), who state (& cite several papers) that the effects of 8-OH-DPAT "produced by local brain infusions are similar to those seen after systemic administration". Thus, we presumed from the start that the effect would be central, rather than peripheral. Second, and perhaps more importantly, we worked under the presumption that the drugs would effectively lower the endurance 'ceiling', which the mice do not approach while running on wheels. (At least, the control mice do not approach the ceiling. HR get closer, but still

do not exhaust themselves on wheel.) Therefore, you could lower the 'ceiling' without affecting wheel running.

If you have more questions, or disagree with these arguments, I would be happy to discuss it further.

Thanks again,

Gerald

From: Chris Perry [chrisperry72@hotmail.com]

Sent: Monday, June 06, 2016 9:20 AM

To: Gerald Claghorn; Theodore Garland Jr

Cc: Zoe Thompson; Ivana Fonseca

Subject: Re: Serotonin in Central Fatigue

Hi,

Thanks so much for the response!

I agree with your presumption that the effects would be central rather than peripheral, based on the reasoning you gave. But it would still seem to me that the effects of the treatments would be manifested more in the wheel running trial if this were truly affecting central fatigue. I understand that if it were just the "ceiling" being lowered, that the effect wouldn't be seen if the rats were not reaching the ceiling in either group. But wouldn't the idea be that if we were looking at rats (HR) with improved central fatigue, that they would be getting closer to their "ceiling" (i.e. pushing themselves closer to their maximum capacity) compared to control rats in normal situations, but when the theoretical mechanism of their improved central fatigue was abolished that they would then be just like the control rats in how close they get to their ceiling?

That was what I was getting at. Obviously, the results are what they are. It's just that I was a little surprised that the results were not manifested more in what I thought was the

central fatigue test, rather than in the test that would be a combination of central and peripheral fatigue.

Very interesting study, though, and fascinating topic!

By the way, I recently started a blog and cited your article in my recent post (https://medsportsnutrition.com/2016/05/31/its-whats-inside-that-counts-but-whats-inside-the-role-of-serotonin-and-central-fatigue/). Please feel free to review it and let me know if you think I am off base.

Take care,

Chris

“

It’s what’s inside that counts. But what’s inside? The role of serotonin and central fatigue. #serotonin #centralfatigue

A 20th century philosopher, by the name of Vince Lombardi, once observed that “Fatigue makes cowards of us all”, and never were wiser words spoken. Fatigue is truly your greatest enemy in endurance competition. But what is fatigue? And how can we combat it?

To begin with, let’s make the distinction between peripheral and central fatigue.

Peripheral fatigue basically refers to the tiredness in your muscles as competition wears on, where, deep down inside, your brain is saying it still wants to give more, but your body just won’t deliver. Central fatigue, on the other hand, refers to fatigue of the brain. In other words, “peripheral” is your body quitting, “central” is your brain quitting.

Central fatigue seems to, in general, receive much less attention from a scientific standpoint. Probably because there is a long standing dogma in sports that, with regards to competitive drive or inner fire, you either have it or you don’t.

However, the science says differently. The reality is that central fatigue has as much of a physiological basis as peripheral fatigue. But, as with anything related to the physiology of the brain, the mechanisms of central fatigue are incredibly complex and very poorly understood. One thing that is known, though, is that the levels and interactions of the neurotransmitters serotonin, dopamine and norepinephrine play a significant role (2, 3).

Today's article that I will be reviewing, by Claghorn et al., in the upcoming July edition of Physiology & Behavior (1), delves specifically more into the role of serotonin in central fatigue.

It is a very interesting article which examined the effects of manipulating the serotonergic pathways in rats who had been selectively bred for high levels of running (HR), looking at both voluntary and forced endurance exercise, and compared these effects to control rats (C) who had not been so selectively bred.

The HR rats had been selectively bred for high voluntary wheel running. The idea being that these were rats that had a mitigated central fatigue response as compared to the C group rats. By testing the responses of serotonin antagonism and agonism in these rats, they sought to further examine the role of serotonin in central fatigue.

What they found was that administration of an antagonist to the 5-HT_{1A} receptor (which itself normally inhibits serotonin release) led to decreased endurance on forced running in the HR group, down to the level of the C group rats. The intervention, however, had no effect on endurance in the C group. This would appear to support the theory that the HR group's higher baseline endurance was mediated through lower serotonin output, since abolishing this response brought their endurance down to that of the C group.

They also found, though, that the response to a 5-HT_{1A} *agonist*, on the other hand, had a dose-dependent effect on forced endurance, with low doses leading to a mildly improved performance, but higher doses actually having a negative effect on performance. And these effects were similar in both the HR and C groups. They speculate that this biphasic effect occurs because at low doses the agonist acts pre-synaptically, inhibiting serotonin release, and at high doses it acts post-synaptically, mimicking serotonin at the effector regions in the brain and spinal cord.

Finally, they found no difference between groups using either of these manipulations during the voluntary wheel running portion of the testing. This difference in results between the forced running and voluntary running lends support to the notion that voluntary running and forced running are governed by different neurobiological processes.

Their conclusion was that these results showed the importance of serotonin signaling in performance during both forced and voluntary exercise. And, more generally, that both forced and voluntary exercise can be affected by an intervention that acts primarily centrally.

Serotonin has received a good deal of attention in recent years with regards to its effects on central fatigue (2, 3). The evidence appears to show a consistent relationship between higher levels of serotonin in the brain and increased central fatigue (3). Of course, as with anything related to neurophysiology, the answer is obviously much more complex than just "more" or "less". For example, much speculation has also been given to the concept that what is more important is the ratio of serotonin to dopamine rather than simply the levels of serotonin (2). But clearly serotonin is a big player in this process.

But how does that translate into helping our training?

Much of the focus has been given to the role of tryptophan (2, 3, 12). Tryptophan is a rate-limiting precursor in the synthesis of serotonin. Tryptophan is heavily protein bound in the bloodstream, with only the "free" (unbound) portion able to cross into the brain. By decreasing the amount of free tryptophan entering the brain, the theory goes, less serotonin should be produced and central fatigue should be mitigated.

To accomplish this, one approach is to increase the amount of branched chain amino acids, or BCAAs, in the bloodstream during endurance exercise. BCAAs (leucine, valine, and isoleucine) compete with free tryptophan for entry into the brain. A lower ratio of free tryptophan to BCAAs should lead to less tryptophan in the brain and, consequently, less serotonin being produced.

The evidence in human studies for BCAA administration and endurance performance, though has been equivocal, with some studies showing a benefit (5, 7) and others not (8, 9, 10, 11).

Various potential explanations have been given for the disparate results. There may not be a true benefit, or maybe the benefit is too small to be consistently seen. Often, small scale studies fail to show a benefit as the numbers of participants do not provide enough statistical power to detect a difference. Absence of evidence, as the old saying goes, isn't evidence of absence.

Personally, though, I feel that differences in ammonia production may be a significant part of the explanation.

Ammonia, like serotonin, also is a contributor to central fatigue (6) and is a byproduct of the metabolism of BCAAs. Research protocols using higher doses of BCAAs will also lead to higher ammonia production. Many of the negative studies had a relatively broad spectrum of BCAA dosing and they did not make it clear which study participants benefited and which didn't, only that, overall, no benefit was found. There appears to be a relationship between the BCAA dosing and the likelihood of a performance benefit (3). It could very well be that a sweet spot needs to be found, with enough BCAAs to give the benefit of less serotonin production, but not too much to lead to excessive ammonia production.

Another approach to decrease the amount of tryptophan entering the brain is to decrease the proportion of free tryptophan in relation to that which is protein bound. Fatty acids compete with tryptophan for protein binding (4). As exercise wears on and your body runs out of carbohydrate sources for oxidation, you begin to tap more into your fat stores, leading to more fatty acids in the blood stream. This, as the theory goes, leads to displacement of bound tryptophan from protein, and thus higher proportions of free tryptophan, which subsequently enter the brain leading to increased serotonin synthesis. By delaying this process of fat mobilization as much as possible through the administration of carbohydrates, central fatigue can theoretically be attenuated. This is why strategies aimed at increasing earlier fatty acid mobilization through low-glycemic index carbs may be ill-advised.

Regardless of the various controversies, central fatigue is undeniably a very complex physiological process. But the research continues to teach us more every day. And with every bit of new knowledge learned comes new questions. When it comes to competitive drive, "what's inside" may count, but what actually IS inside is really the much bigger question.

References:

1. Claghorn GC, Fonseca IA, Thompson Z, Barber C, Garland T Jr. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiol Behav. 2016 Jul 1;161:145-54.
2. Meeusen R, Watson P, Hasegawa H, Roelands B, Piacentini MF. Central fatigue: the serotonin hypothesis and beyond. Sports Med. 2006;36(10):881-909.
3. Blomstrand E: A role for branched-chain amino acids in reducing central fatigue. J Nutr 2006, 136(2):544S-547S.

4. Curzon G, Friedel J, Knott PJ. The effect of fatty acids on the binding of tryptophan to plasma protein. Nature. 1973;242:198–200.

5. Blomstrand E, Hassmen P., Ekblom B., and Newsholme E. A. Administration of branched-chain amino acids during sustained exercise – effects on performance and on plasma concentration of some amino acids. Eur J Appl Physiol Occup Physiol. 1991;63(2):83-88

6. Mutch BJ, Banister EW. Ammonia metabolism in exercise and fatigue: a review. Med Sci Sports Exerc. 1983;15(1):41-50.

7. Mittleman KD, Ricci MR, Bailey SP Branched-chain amino acids prolong exercise during heat stress in men and women. Med Sci Sports Exerc 1998 30:83–91

8. van Hall G, Raaymakers J S, Saris W H, and Wagenmakers A J. Ingestion of branched-chain amino acids and tryptophan during sustained exercise in man: failure to affect performance. J Physiol. 1995 Aug 1; 486(Pt 3): 789–794.

9. Madsen K, MacLean DA, Kiens B, Christensen D. Effects of glucose, glucose plus branched-chain amino acids, or placebo on bike performance over 100 km. J Appl Physiol (1985). 1996 Dec;81(6):2644-50.

10. Watson P, Shirreffs SM, Maughan RJ. The effect of acute branched-chain amino acid supplementation on prolonged exercise capacity in a warm environment. Eur J Appl Physiol. 2004 Dec;93(3):306-14.

11. Cheuvront SN, Carter R 3rd, Kolka MA, Lieberman HR, Kellogg MD, Sawka MN. Branched-chain amino acid supplementation and human performance when hypohydrated in the heat. J Appl Physiol (1985). 2004 Oct;97(4):1275-82.

12. Newsholme EA, Blomstrand E. Branched-chain amino acids and central fatigue. J Nutr. 2006 Jan;136(1 Suppl):274S-6S.

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From: Theodore Garland Jr [theodore.garland@ucr.edu]

Sent: Monday, June 06, 2016 1:36 AM

To: Chris Perry; Gerald Claghorn

Cc: Zoe Thompson; Ivana Fonseca

Subject: Re: Serotonin in Central Fatigue

Chris, thanks so much for all of this! We really appreciate the feedback.

Cheers,

Ted