ORIGINAL ARTICLE

Muscle health and performance in monozygotic twins with 30 years of discordant exercise habits

Katherine E. Bathgate[1] · James R. Bagley[2] · Edward Jo[3] · Robert J. Talmadge[4] · Irene S. Tobias[1] ·
Lee E. Brown[1] · Jared W. Coburn[1] · Jose A. Arevalo[1] · Nancy L. Segal[5] · Andrew J. Galpin[1]

Abstract

Introduction Physical health and function depend upon both genetic inheritance and environmental factors (e.g., exercise training).

Purpose To enhance the understanding of heritability/adaptability, we explored the skeletal muscle health and physiological performance of monozygotic (MZ) twins with > 30 years of chronic endurance training *vs.* no specific/consistent exercise.

Methods One pair of male MZ twins (age = 52 years; Trained Twin, TT; Untrained Twin, UT) underwent analyses of: (1) anthropometric characteristics and blood profiles, (2) markers of cardiovascular and pulmonary health, and (3) skeletal muscle size, strength, and power and molecular markers of muscle health.

Results This case study represents the most comprehensive physiological comparison of MZ twins with this length and magnitude of differing exercise history. TT exhibited: *(*1) lower body mass, body fat%, resting heart rate, blood pressure, cholesterol, triglycerides, and plasma glucose, (2) greater relative cycling power, anaerobic endurance, and aerobic capacity (VO_2max), but lower muscle size/strength and poorer muscle quality, (3) more MHC I (slow-twitch) and fewer MHC IIa (fast-twitch) fibers, (4) greater AMPK protein expression, and (5) greater *PAX7, IGF1Ec, IGF1Ea*, and *FN14* mRNA expression than UT.

Conclusions Several measured differences are the largest reported between MZ twins (TT expressed 55% more MHC I fibers, 12.4 ml/kg/min greater VO_2max, and 8.6% lower body fat% vs. UT). These data collectively (a) support utilizing chronic endurance training to improve body composition and cardiovascular health and (b) suggest the cardiovascular and skeletal muscle systems exhibit greater plasticity than previously thought, further highlighting the importance of studying MZ twins with large (long-term) differences in exposomes.

Keywords Fiber type · Myosin heavy chain · Maximal oxygen consumption · Endurance training · FN14 · PAX7 · Body composition · AMPK · Aerobic exercise · Aging

✉ Andrew J. Galpin
agalpin@fullerton.edu

[1] Biochemistry and Molecular Exercise Physiology Laboratory, Center for Sport Performance, Department of Kinesiology, California State University, Fullerton, 800 North State College Blvd., KHS-121, Fullerton, CA 92834, USA

[2] Muscle Physiology Laboratory, Department of Kinesiology, San Francisco State University, 1600 Holloway Ave. GYM 101, San Francisco, CA 94132, USA

[3] Human Performance Research Laboratory, California State Polytechnic University, 3801 W. Temple Ave. 66-213, Pomona, CA 91768, USA

[4] Department of Biological Sciences, California State Polytechnic University, 3801 W. Temple Ave., Pomona, CA 91768, USA

[5] Department of Psychology, California State University, Fullerton, 800 North State College Blvd., Fullerton, CA 92834, USA

Abbreviations

%BF	Body fat percentage
AMPK	5′ AMP-activated protein kinase
B2M	β-2-microglobulin

BMC	Bone mineral content
BMD	Bone mineral density
CHOL	Total cholesterol
CS	Citrate synthase
CSA	Muscular cross-sectional area
DBP	Diastolic blood pressure
DXA	Dual-energy X-ray absorptiometry
ECL	Enhanced chemiluminescent
EI	Echo intensity
FEV_1	Forced expiatory volume in the first second
FM	Fat mass
FVC	Forced vital capacity
HbA1c	Glycosylated hemoglobin
HDL	High-density lipoprotein
IGF1Ea	Insulin-like growth factor a
IGF1Ec	Mechano-growth factor
kcal	Kilocalorie
LDL	Low-density lipoprotein
LM	Lean mass
MHC	Myosin heavy chain
MSTN	Myostatin
MT	Muscle thickness
MVIC	Maximal voluntary isometric contraction
MyHC	Myosin heavy chain gene
MZ	Monozygotic
NOS3	Endothelial nitric oxide synthase
PP	Peak power
PPIA	Cyclophilin
QRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RER	Respiratory exchange ratio
RHR	Resting heart rate
RPE	Rating of perceived exertion
RPM	Rotations per minute
SBP	Systolic blood pressure
TFAM	Transcription factor A of the mitochondria
TNF	Tumor necrosis factor-α
TRIG	Triglycerides
TT	Trained twin
UT	Untrained twin
VAT	Visceral adipose tissue
VEGFA	Vascular endothelial growth factor
VL	Vastus lateralis
VO_2max	Maximal aerobic capacity
WAnT	Wingate anaerobic test
WEEE	Weekly estimated energy expenditure

Introduction

Chronic physical exercise reduces the risk of all-cause mortality and increases longevity. The efficacy of exercise training is widespread, well documented, and evident across all physiological systems. Human research studies have difficulty controlling for genetic inheritance, making it challenging to discern which specific physiological characteristics adapt to exercise, and to what extent (Boomsma et al. 2002). Fortunately, monozygotic (MZ) human twins (who share all their genes) with differing physical activity patterns alleviate this issue and are therefore preeminent research targets (Hannukainen et al. 2011; Leskinen et al. 2011; Rottensteiner et al. 2015; Boomsma et al. 2002; Segal 2017).

Previous investigations have analyzed MZ twins with either long-term (several decades) and moderate differences in physical activity (e.g., walkers vs. sedentary) *or* short-term (months-years) and large differences in physical activity (e.g., high-intensity endurance training programs vs. sedentary) (Adams et al. 1985; Fagard et al. 1991, 1987; Klissouras and Pigozzi 2009). This heterogeneity in study designs may partially explain why heritability estimates of measures, such as maximal oxygen consumption (VO_2max), range broadly from ~25 to 96% (Adams et al. 1985; Fagard et al. 1991, 1987; Klissouras and Pigozzi 2009). The plasticity of other markers of sport performance, physical fitness, muscle function, body composition, metabolic health, and the epigenome are equally difficult to interpret (Leskinen et al. 2010, 2011; Rottensteiner et al. 2015; Hannukainen et al. 2011; Segal 2017; Danis et al. 2003; Thomis et al. 1998). To date, no investigation has comprehensively assessed the physiological profiles of human MZ twins with both long-term *and* large differences in exercise habits.

We had the unique opportunity to study a pair of 52-year-old male MZ twins with similar exposomes (i.e., totality of human environmental exposures) during development (to approximately age 20 years), but significantly divergent exercise patterns for the past three decades. One brother [the exercise-trained twin (TT)] regularly engaged in rigorous endurance training, competing in triathlons and distance running events for the past 30 years; conversely, the untrained twin (UT) did not engage in regular exercise other than normal activities of daily living. The purpose of this study was to explore the effects of 30 years of endurance training on skeletal muscle health and physiological performance in these twins.

Methods

Participants

Two male monozygotic twins (age = 52 years) volunteered for this study. TT regularly engaged in various modes of endurance exercise and competed in multiple marathons and triathlons [logged ~63,458 running km (39,431 miles) from July 1993 to June 2015; All Word Bronze Level Ironman qualifier in 2005]. Conversely, UT did not engage in regular

exercise other than normal activities of daily living. The twins self-identified as Caucasian and originated from the Midwestern United States; TT worked as a high school track coach and UT was a truck driver. Written informed consent was obtained from subjects prior to testing; this study was approved by the University's Institutional Review Board for Human Subjects.

Experimental design and controls

The twins underwent a battery of tests (conducted over three consecutive days) to analyze (1) anthropometric characteristic and blood profiles, (2) markers of cardiovascular and pulmonary health, and (3) whole muscle size, strength, and power and molecular markers of skeletal muscle health (see detailed methods in subsequent sections). Prior to visiting the laboratory, participants completed informed consent forms, submitted DNA samples to confirm zygosity, underwent fasted blood panels from personal physicians, and tracked normal physical activity patterns and dietary intake using a cell phone application (MyFitnessPal; San Francisco, CA, USA) for seven consecutive days before the first visit. Additionally, the twins completed detailed written questionnaires and follow-up oral interviews to determine exercise histories; TT also provided proof of participation in competitive events (e.g., pictures, medals, certificates, race numbers, etc.) along with hand-written training logs from 1980-current. To determine current physical activity and dietary habits, researchers extracted exercise frequency, intensity, duration, weekly energy expenditure due to exercise (WEEE), kilocalorie (kcal) consumption, and macronutrient composition from the twin's MyFitnessPal accounts.

The twins' monozygosity was confirmed by Affiliated Genetics (now Taueret Laboratories; Salt Lake City, UT, USA) via analyses of fifteen short tandem repeat markers (STRs). The probability of the twins being MZ was greater than 99%.

During Visit 1, researchers collected medical and exercise history questionnaires and performed anthropometric, pulmonary, whole muscle size (ultrasound), strength, and power measures; additionally, the twins were familiarized with VO_2max procedures and equipment. During Visit 2, the twins completed body composition testing via dual-energy X-ray absorptiometry (DXA) and VO_2max tests on a cycle ergometer. Finally, during Visit 3 they underwent resting muscle biopsies (vastus lateralis, VL) and completed Wingate anaerobic test (WAnT). All testings were conducted at the same location/time of day, and by the same technicians; however, TT and UT were tested on separate days to avoid competition or other unnecessary interferences, and to minimize halo effects. The twins were also cautioned not to discuss any of the tests until both

had completed them and they complied fully. Furthermore, neither twin was given access to any findings until both had completed all visits.

The twins were housed in a hotel adjacent to the laboratory the day before Visit 1 through the completion of Visit 3. Participants were instructed to refrain from extraordinary physical activity, drugs, and alcohol at least 48 h prior to Visit 1, sleep ≥ 8 h before each visit, and eat their typical diets during the testing period. They were allowed to continue with normal caffeine habits (although neither twin regularly consumed coffee). Participants were asked to consume 500 ml of water the night before and 1 L of water the morning of each visit to standardize hydration.

Anthropometric measures and blood profiles

Height and body mass were measured with a stadiometer and digital Health-o-meter scale (792KL, Bridgeview, IL) and body composition [lean mass (LM), fat mass (FM), total body fat percentage (%BF), visceral adipose tissue (VAT), bone mineral content (BMC), and bone mineral density (BMD)] was assessed via DXA (Hologic Discovery-QDR Series Densitometer, Bedford, MA). A three-compartment model of body composition was applied through which FM and non-bone LM (i.e., lean mass—bone mineral content) was analyzed for the whole body. Prior to the scan, participants were made free from metallic clothing and accessories, required to empty their bladder, and refrained from consuming liquids or mineral supplements. The DXA machine was calibrated before each scan using a manufacturer-provided phantom. All DXA measurements and analyses were conducted by a single certified technologist.

Each twin also obtained fasting blood profiles prior to Visit 1 [TT was conducted by LabCorp (Dublin, OH) and UT by Kaiser Permanente (Panorama City, CA, USA)]. Analyses included total cholesterol (CHOL), low-density lipoproteins (LDL), high-density lipoproteins (HDL), triglycerides (TRIG), plasma glucose, and glycosylated hemoglobin (HbA1c).

Cardiorespiratory health

Resting heart rate (RHR), blood pressure, VO_2max, and pulmonary function were measured to assess cardiorespiratory health of the TT and UT. For resting measures, participants laid supine for 20 min before analysis of RHR via a polar heart rate monitor (Polar, Olulu, Finland) and systolic (SBP) and diastolic (DBP) blood pressures via e-sphyg 2 sphygmomanometer (American Diagnostic Corporation, Hauppauge, NY, USA).

Maximal oxygen consumption

VO_2 max was assessed using maximal graded cycling test protocols with an open-circuit indirect calorimeter (ParvoMedics TrueOne®2400, Salt Lake City, Utah, USA) and a cycle ergometer (Cosmed LC7, Monark Exercise AB, Varburg, Sweden) administered in a thermo-neutral (~ 24 °C) laboratory. Participants reported to the laboratory following at least 8 h of no strenuous activity and rested quietly seated for 10 min before testing. Subjects were then fitted with a rubber-ventilated mask with a one-way valve interfaced to the indirect calorimeter via corrugated plastic tubing as well as a wireless heart rate monitor (Polar, Oulu, Finland). Participants were instructed to cycle at a constant rate of 70 revolutions per minute (rpm) during the graded exercise test. Due to the large difference in anticipated fitness level, TT initiated the test at a constant workload of 125 W with a 25 W increase every minute until exhaustion. UT started at a constant workload of 110 W with 15 W increases each minute for the first 5 min, and a 25 W increase every minute thereafter until exhaustion. After each stage, participants were asked to report a rating a perceived exertion (RPE) using the Borg Scale. A valid VO_2 max measurement was defined as the highest VO_2 reached within the last minute of the test while satisfying the following criteria: $RPE > 18$, respiratory exchange ratio $(RER) > 1.1$, heart rate $> 90\%$ of the predicted maximum heart rate (220-age), and VO_2 plateaued within the last 30 s. Prior to each test, calibration procedures were conducted for the flow meter using a 3.0-L syringe and for the gas analyzer with verified gases of known concentrations.

Pulmonary function

Pulmonary function was assessed using a spirometer (Spirolab II, SDI Diagnostics, Easton, MA, USA). Participants wore a nose clip and sealed their lips around the disposable turbine filter. They were instructed to breathe in as hard as possible (maximal inspiration), then immediately exhale as fast, hard, and long as possible (maximal expiration). The best forced vital capacity (FVC) and forced expiatory volume in the first second (FEV_1) of five attempts was recorded.

Skeletal muscle health

Whole muscle size, strength, and power were assessed. Additionally, protein and gene expression were measured for various markers of fiber type, metabolism, growth, repair, and inflammation.

Whole muscle size and quality

The right *VL* of each twin was assessed for muscular cross-sectional area (CSA), muscle thickness (MT), and echo intensity (EI; a proxy for muscle quality) using a portable brightness mode (B-mode) ultrasound machine (GE/Logic e, Wauwatosa, WI) with a 10-MHz linear array probe by the same researcher for both twins. The researchers marked a line at the midpoint of the lateral knee joint surface and the anterior superior iliac spine. Two more lines were drawn 2 and 4 cm distal to the midpoint. Constant minimal pressure was applied with the probe over the skin to avoid compression of the musculature and transmission gel was used to improve acoustic collection. Images were acquired as the transducer was manually moved mediolaterally along the marked lines on the skin. Three images from each of the three marked locations were analyzed using publicly available software. CSA, MT, and EI of each location were determined by the mean of the three measurements for each location. CSA, MT, and EI were taken as the mean values of the three locations.

Whole muscle strength

Quadriceps strength of the right limb was assessed by maximum voluntary isometric contraction (MVIC) at 90° of knee flexion. The participant was seated and fastened securely to a HUMAC NORM isokinetic dynamometer (CSMI Inc., Stoughton, MA). Three extensions at 50, 75, and 90% of maximal perceived effort were performed as a warm-up. Testing consisted of maximally extending the leg for ~ 4–6 s. One min of rest was given before repeating the trial (participants remained seated in the dynamometer). Four total attempts were given, and the highest torque was recorded as the MVIC. Additionally, handgrip strength was tested in both hands using a Jamar Plus handgrip dynamometer (Patterson Medical, Warrenville, IL). The participants performed three warm-up repetitions at 50, 75, and 90% of their perceived maximal effort, respectively. They then performed five maximal effort trials by squeezing the device as hard as possible for ~ 2–4 s. Volitional rest was taken between trials and the top score was recorded.

Whole muscle power

Muscle power was measured by a WAnT. After a self-selected warm-up (~ 5 min of light stretching and 5 min of light cycling; Monark ergometer 893E, Monark Exercise AB, Varburg, Sweden), participants performed five sprints (5 s each) at ~ 95% of perceived maximal effort (1 min rest between each sprint) prior to a maximal effort WAnT. Participants cycled between 30 and 50 rotations per minute (RPM) for 10 s at one-third of the prescribed resistance. After 10 s,

participants increased their RPM to a near-maximal rate as the technician loaded the basket to the full resistance (7.5% body mass). The basket was immediately dropped and the participants cycled for 30 s at maximum effort. The highest power produced over a 5-s period was considered the peak power (PP). Anaerobic capacity was defined by the average power throughout the 30 s. Absolute power decline was considered as PP subtracted by the lowest 5 s power output.

Muscle biopsy

During Visit 3, after an overnight fast (> 12 h) and refraining from exercise (≥ 16 h since VO_2max test), participants rested supine for 30 min. A mark was made mid-muscle belly (halfway between the greater trochanter and patella), cleaned with iodine and anesthetized with 1% lidocaine/xylocaine (without epinephrine). A small incision was made, then tissue (~ 100 mg) was obtained using the Bergström technique with applied suction as described previously (Murach et al. 2016; Bagley et al. 2017; Tobias et al. 2018). The muscle samples were immediately cleansed of excess blood, fat and connective tissue, cut into multiple ~ 15 mg bundles, and either (1) stored in cold skinning solution [(in mm): 125 K propionate, 2.0 EGTA, 4.0 ATP, 1.0 $MgCl_2$, 20.0 imidazole (pH 7.0), and 50% (v/v) glycerol] at 4 °C or (2) rapidly frozen in liquid nitrogen, and stored at − 80 °C. The incision site was cleaned and covered with sterile gauze and cohesive bandage tape.

Muscle fiber type

Muscle fiber composition was classified by myosin heavy chain protein (MHC) isoform, as well as myosin heavy chain gene (*MyHC*) expression. MHC isoforms were identified using both single fibers (to determine fiber type % distribution) and homogenized samples (to determine % area that each fiber type comprises).

Detailed methods for single muscle fiber typing via SDS-PAGE were published previously (Murach et al. 2016; Bagley et al. 2017; Tobias et al. 2018). Briefly, individual muscle fibers were randomly selected and extracted longitudinally in a physiological buffer using fine-tipped tweezers under a light microscope at room temperature and placed into 80 μl SDS buffer [10% SDS, 6 mg ml^{-1} EDTA, 0.06 m Tris (pH 6.8), 2 mg ml^{-1} bromophenol blue, 15% glycerol, and 5% b-mercaptoethanol]. For homogenate analysis, entire bundles were mechanically homogenized, and boiled for 90 s then placed in 1 ml of sodium dodecyl sulfate (SDS) buffer. Aliquots were loaded into individual wells in a 3.5% loading gel and 5% separating gel and run at 5 °C (SE 600 Series; Hoefer, San Francisco, CA, USA) and silver stained as described previously (Murach et al. 2016; Bagley et al. 2017; Tobias et al. 2018). Fiber types were identified as MHC I, I/IIa, IIa, IIa/IIx, IIx, or I/IIa/IIx for single fibers. For homogenate samples, band densitometry was analyzed via ImageJ software (National Institutes of Health, Bethesda, MD) to calculate the relative amount of MHC I, MHC IIa, and MHC IIx isoform in each sample.

The TT and UT muscle samples were also analyzed for relative levels of MyHC 1 (*MYH7*), MyHC 2a (*MYH2*), and MyHC 2x (*MYH1*) mRNAs (see Muscle gene expression section for detailed methods).

AMPK protein expression

5′AMP-activated protein kinase (AMPK) protein subunits were analyzed as markers of cellular metabolism. Primary antibodies for AMPK α1 (ab32047) and AMPK γ1 (ab32508) were purchased from AbCam (Cambridge, MA). The primary antibody for tubulin (2125) and the secondary HRP-conjugated anti-rabbit IgG antibody (7074) were from Cell Signaling Technology. Detailed methods for AMPK protein analysis were published previously (Tobias et al. 2018). Briefly, fiber bundles were homogenized using a pellet pestle in Buffer A (1% SDS, 23 mM EDTA, 10% glycerol, 400 mM β-mercaptoethanol, pH 6.8), incubated at 95 °C for 5 min, solubilized and stored at − 20 °C until analysis. Total protein concentration was measured using a Bradford assay and adjusted via dilution in Buffer A to normalize total protein loading. Fiber homogenates stored in Buffer A were combined with 20% Buffer B (1% SDS, 23 mM EDTA, 10% glycerol, 400 mM β-mercaptoethanol, pH 6.8) for western blot analysis of AMPK isoforms. Samples were run on an 8% separating polyacrylamide gel at 200 V for 90 min using Buffer C (β-mercaptoethanol, pH 6.8) and wet transferred to PVDF membrane for 3 h at 80 V using a transfer buffer containing 192 mM glycine, 25 mM Tris-base and 20% methanol. Immediately following transfer, membrane was blocked for 60 min in 5% non-fat milk powder (BioRad, Hercules, CA, USA) dissolved in Buffer D (20 mM Tris-base, 150 mM NaCl, 0.05% Tween-20). Membrane was then rinsed (3×, 5 min each) in Buffer D and rocked overnight at 4 °C in primary antibody diluted in 5% BSA in Buffer D (1:2000 for AMPK α1 and 1:1000 for tubulin) or 5% milk in Buffer D (1:1000 for AMPK γ1). Following another round of rinsing three times in Buffer D, membrane was then rocked at room temperature for 90 min in secondary antibody diluted 1:2000 in 5% non-fat milk in Buffer D. Following a final round of rinsing three times in Buffer D, membrane was developed with SuperSignal™ West Femto enhanced chemiluminescent (ECL) substrate diluted 1:10 in SuperSignal™ West Pico ECL substrate and imaged using an Omega Lum™ C imaging system (Aplegen Gel Company, San Francisco, CA, USA).

Muscle gene expression

Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) methods were used to quantify selected mRNAs associated with: (1) skeletal muscle fiber type [MyHC 1 (*MYH7*), MyHC 2a (*MYH2*), MyHC 2× (*MYH1*)] (Welle et al. 1999), (2) oxidative metabolism [transcription factor A of the mitochondria (*TFAM*), citrate synthase (*CS*)] (Mancini et al. 2017; Scribbans et al. 2017), (3) angiogenesis [endothelial nitric oxide synthase (*NOS3*), vascular endothelial growth factor (*VEGFA*)] (Mancini et al. 2017), (4) muscular growth and repair [myostatin (*MSTN*), Pax7 (*PAX7*), mechano-growth factor (*IGF1Ec*), insulin-like growth factor a (*IGF1Ea*), MyoD (*MYOD1*)] (Hameed et al. 2003; Pessina et al. 2010; Silvennoinen et al. 2015), and (5) inflammatory responses [TWEAK (*TNFSF12*), FN14 TWEAK receptor (*TNFRSF12A*), tumor necrosis factor-α (*TNF*)].

Briefly, a portion of each sample (stored at − 80 °C) was used for total RNA isolation using Trizol, purified with RNAqueous micro kit reagents and minicolumns (Invitrogen, ThermoFisher, Waltham, MA, USA) and stored at − 80 °C. Reverse transcription was performed using Vilo Superscript IV reverse transcriptase (Invitrogen, ThermoFisher, Waltham, MA, USA) and random primers. The relative levels of each mRNA were quantified using the specific primer pairs (see Table 1) in a real-time Sybr Green-based RT-PCR procedure using the Fast SYBR™ Green Master Mix (Applied Biosystems, ThermoFisher, Waltham, MA, USA). For QRT-PCR quantification, the cycle thresholds were obtained for β-2-microglobulin (B2M) and cyclophilin (PPIA) as normalizing genes (Li et al. 2015) and the ∆∆CT method was used for calculation of relative expression levels (Livak and Schmittgen 2001). Cycle threshold levels were set at the mid-exponential phase for each gene from all samples (in triplicate) in a single run.

Data analysis

Percent differences between TT and UT were analyzed for select variables using MS Office Excel 2013 (Microsoft, Redmond, WA, USA). Relative gene expression data were calculated as described above (presented in arbitrary units).

Results

Exercise histories

The twins exhibited divergent physical patterns since the mid-1980s. Both TT and UT participated in recreational sports (e.g., baseball and basketball) from ages 10–20 (1973–1983). UT remained recreationally active in basketball and light cycling (~ 4× per week) from ages 20–39 (1983–1999), but has not been involved in recreational sports and been otherwise relatively inactive since ~ 1999 (around this time, UT sustained a moderate ankle injury with pain that has persisted to date).

TT began running cross-country/track competitively in high school, competed in collegiate cross-country/track, and was selected to All-Conference Team in 1985. After college,

Table 1 Primers for selected genes used for polymerase chain reaction (PCR)

Human gene name (common name)	Forward primer (5′–3′)	Reverse primer (5′–3′)
MYH1 (MyHC 2×) (38)	TTTATCTAACTGCTGAAAGGTGAC	TCTCCAAAAGTCATAAGTACAAATG
MYH2 (MyHC 2a) (38)	ATGTCCTGATGCCATGG	CAAACTACCCTATGCTTTATTTCC
MYH7 (MyHC 1) (38)	CTTTGCCACATCTTGATCTG	TGCTTTATTCTGCTTCCTCC
TFAM (T_{Fam}) (24)	CGCTCCCCCTTCAGTTTTGT	CACTCCGCCCTATAAGCATC
CS (citrate synthase) (31)	ACTGTGGACATGATGTATGGTG	GTAGCAGTTTCTGGCATTCAG
NOS3 (eNOS) (24)	CGGGGGATTCTGGCAGGAGC	CGTAGGTCTTGGGGGTTGTCA
VEGFA (VEGF) (24)	GAGGAAAGGGAAAGGGGCA	CTCGGCTTGTCACATCTGC
MSTN (myostatin) (33)	CTACAACGGAAACAATCATTACCA	GTTTCAGAGATCGGATTCCAGTAT
PAX7 (Pax7) (27)	CACTGTGACCGAAGCACTGT	GTCAGGTTCCGACTCCACAT
TNFSF12 (TWEAK)	CGATCGCAGCCCATTATGAAG	TGTTGATTCTGGCTTCCTCCC
TNFRSF12A (FN14)	CTCTGAGCCTGACCTTCGTG	GTCTCCTCTATGGGGGTGGT
TNF (TNA-α)	GCTGCACTTTGGAGTGATCG	CTTGTCACTCGGGGTTCGAG
IGF1Ec (MGF) (12)	CGAAGTCTCAGAGAAGGAAAGG	ACAGGTAACTCGTGCAGAGC
IGF1Ea (IGF-1) (12)	GCCTGCTCACCTTCACCAGC	TCAAATGTACTTCCTTCTGGGTCTTG
MYOD1 (MyoD) (12)	GCAGGTGTAACCGTAACC	ACGTACAAATTCCCTGTAGC
PPIA (cyclophilin) (22)	TCCTGGCATCTTGTCCAT	TGCTGGTCTTGCCATTCCT
B2M (β-2-microglobulin) (22)	CTATCCAGCGTACTCCAAAG	GAAAGACCAGTCCTTGCTGA

Primers for *TWEAK, FN14* and *TNF* were designed for this study using published sequences in the NCBI database

TT continued endurance training and began competing in running road races (best marathon time: 3:01:07 in 1993). His best marathon times were relatively consistent over 20 years, with only a 6.9% increase in race time from 1985 (3:03:10) to 2005 (3:15:05). From ages 30–40 (1993–2003) TT continued endurance training and began resistance training (2–3× per week). From ages 40–50 (2003–2013), TT recorded ~ 34,195 running kilometers (~ 21,248 miles). He also engaged in cycling, resistance training, and swimming 2–3× per week. He competed in one Ironman triathlon (time: 12:33:59), two half Ironman triathlons, two Olympic triathlons, two marathons, and 45 other running races.

Pre-study physical activity and dietary habits

Self-recorded physical activity patterns differed between TT and UT during the week before testing. TT reported averaging 69 min of endurance exercise per day [running at 5.3 min/km (8.5 min/mile) pace] and three days of upper body resistance training per week. UT recorded an average of 22 min of walking each day. This resulted in an estimated WEEE of 7594 kcal for TT and 204 kcal for UT (189.5% difference). TT reported consuming an average of 3517 ± 515 kcal per day (51% carbohydrate, 33% fat, and 15% protein), while UT consumed $1,741 \pm 787$ kcal per day (31% carbohydrate, 42% fat, and 27% protein). TT consumed ~ 173 kcal over his estimated total daily energy expenditure (TDEE), while UT consumed ~ 208 kcal over his estimated TDEE (TDEE includes estimated resting metabolic rate and energy expenditure from physical activity).

Anthropometric measures

TT was 186 cm tall and weighed 94.0 kg (BMI: 27.2 kg/m^2, overweight), while UT was 183 cm tall and weighed 104.5 kg (BMI: 31.2 kg/m^2, obese class I).

Body composition

Total LM was the same between twins (74.72 vs. 74.64 kg), but UT had 47.3% greater total body fat mass. In addition, UT demonstrated 43.3% greater estimated VAT mass and area than TT (551 vs. 355 g). Both total BMC and BMD were similar between twins (see Table 2).

Resting heart rate, blood pressure, and blood profiles

RHR was 30.3% higher in UT (42 vs. 57 bpm). Resting BP for the TT was 122/57 mmHg (Category: Elevated) and 132/77 mmHg for UT (Category: Stage 1 hypertension) (Whelton et al. 2017). TT displayed lower total cholesterol,

Table 2 Body composition and blood profiles of male monozygotic (MZ) twins with 30 years of discordant exercise training histories

Body composition	Trained twin (TT)	Untrained twin (UT)	% Difference
BF%	19.2	27.8	− 36.6
BMC (g)	3.65	3.58	1.9
BMD (g/cm^2)	1.39	1.35	2.9
Fasted blood profile			
Total cholesterol (mg/dl)	172	202	− 16.0
LDL (mg/dl)	92	124	− 29.6
HDL (mg/dl)	64	57	11.6
Triglycerides (mg/dL)	81	107	− 27.7
Plasma glucose (mg/dL)	86	102	− 17.0
HbA1c (%)	5.8	5.7	1.7

*NF% body fat percentage, *BMC* bone mineral content, *BMD* bone mineral density, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *HbA1c* glycosylated hemoglobin

LDL, triglycerides, plasma glucose, and cholesterol/HDL (2.7 vs. 3.5) (see Table 2).

Maximal oxygen consumption

TT had a 30.1 and 23.8% higher relative (47.5 vs. 35.1 ml/kg/min) and absolute (4.66 vs. 3.67 L/min) VO$_2$max, respectively. The final completed stages were 350 W for TT and 300 W for UT. Maximum HR during the test was 157 bpm for TT and 178 bpm for UT (11.8% difference). Peak RER was 1.17 for TT and 1.25 for UT (6.4% difference) and maximum ventilation (VE) was of 124.2 L/min for TT and 118.7 for UT (4.4% difference). Final RPE was 18 for TT and 19 for UT (on the 6–21 Borg Scale).

Pulmonary function

FVC was similar (6.01 vs. 5.88 l) for TT and UT, respectively. However, UT slightly exceeded TT in FEV$_1$ (4.47 vs. 4.76 l, 6.3% difference) and FEV$_1$% (74.4 vs. 81.0%, 8.5% difference).

Muscle size and performance

Average CSA and MT of the vastus lateralis differed by 4.5% (larger in UT; 1,050 vs. 1098 cm^2 and 15.4 vs. 16.1 cm, respectively). EI was also higher in the UT (64.1 vs. 77.1 AU), but to a much larger magnitude (18.4%).

Muscle strength

The TT knee extensors produced 60% less torque during the IMVC (137 vs. 254 N m). When normalized to LM, TT still produced less torque (1.83 vs. 3.40 N m/kg, 60% difference).

Similarly, TT produced 24 and 17% less force in his right (44.3 vs. 56.5 kg) and left hand (43.7 vs. 51.7 kg) than UT, respectively. When normalized to LM TT still displayed less force in both hands (right = 59 vs. 76% of LBM, 25.2% difference; left = 58 vs. 69% of LM, 17.3% difference).

TT and UT displayed similar absolute peak power during the WAnT (788 vs. 784 W). However, TT had a greater relative peak power (8.38 vs. 7.53 W/kg, 10.7% difference) and 43.7% less absolute power decline (237 vs. 370 W) and power drop (34.2% vs. 48.9%, 5.33% difference). TT displayed higher average absolute power (665 vs. 585 W, 12.9% difference) and higher average relative power (7.08 vs. 5.62 W/kg, 23.0% difference). Despite this, TT took 46.7% more time to reach peak power than UT (3.20 vs. 1.99 s).

Muscle fiber type

Single fiber MHC

A total of 213 single fibers were analyzed for MHC isoform. Fiber-type distributions for TT and UT are shown in Fig. 1a. TT had 2.4× more MHC I fibers, 13.3× fewer MHC II fibers, and 10× fewer hybrid fibers (i.e., MHC I/IIa, IIa/IIx, and I/IIa/IIx).

Homogenate MHC

The composition of MHC content (i.e., from homogenate muscle samples) displays the total myofibrillar protein area that each MHC-type encompasses. These data mirrored the single fiber MHC findings (see Fig. 1b). TT muscle had 1.5 times more MHC I, 5 times less MHC IIa and 14 times less MHC IIx protein vs. UT.
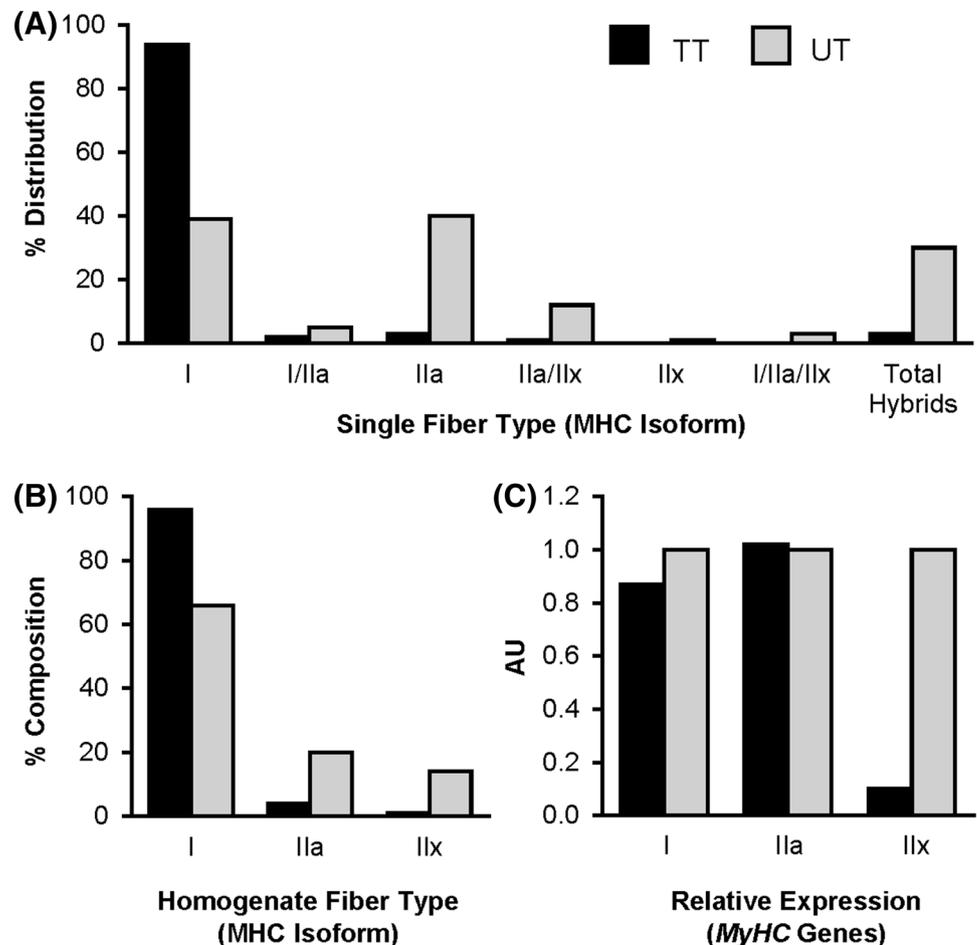
MHC gene expression

As shown in Fig. 1-C, *MyHC* 2x was expressed at a lower level in TT relative to UT. No differences were observed in *MyHC* I or IIa expression between subjects.

AMPK protein expression

Relative isoform expression was measured for AMPK α1 (catalytic subunit) and AMPK γ1 (regulatory subunit) with tubulin as a standard loading control in the fiber

Fig. 1 Vastus lateralis fiber type in monozygotic (MZ) twins [trained twin (TT) vs. untrained twin (UT)]. **a** Single muscle fiber myosin heavy chain (MHC) fiber-type percent (%) distribution (total hybrids: MHC I/IIa, IIa/IIx, and I/IIa/IIx fibers). **b** Homogenate MHC fiber-type % composition (unable to identify hybrids with homogenate method). **c** Relative myosin heavy chain (MyHC) mRNA expression at rest (arbitrary units, AU). MyHC I (*MYH7*), MyHC IIa (*MYH2*), MyHC IIx (*MYH1*)

homogenates (Fig. 2). TT exhibited higher expression of both isoforms.

Muscle gene expression

No differences were observed between TT and UT in markers of oxidative metabolism or angiogenesis gene products (Fig. 3a, b). Some differences were observed in the expression of genes related to muscle growth and repair, including elevations in *PAX7, IGF1Ec* and *IGF1Ea*, and lower *MYOD1* in the TT (Fig. 3c). The only difference in the expression of inflammatory response markers was an elevation in *FN14* in TT (Fig. 3d).

Discussion

This study represents the most comprehensive physiological comparison of MZ twins with this length and magnitude of differing exercise histories to date. Major findings were that TT exhibited: (1) lower total body mass and %BF, (2) lower RHR, BP, total cholesterol, LDL, triglycerides, and plasma glucose levels, (3) a higher aerobic capacity (VO2max), (4) lower quadriceps muscle size and strength, poorer muscle quality, but greater relative cycling power and anaerobic endurance, (5) more MHC I (slow-twitch) and fewer MHC IIa (fast-twitch) and hybrid muscle fibers, (6) greater muscle AMPK α1 and γ1 protein expression, and (7) elevated expression of muscle growth, repair, and inflammatory mRNA markers (i.e., *PAX7, IGF1Ec, IGF1Ea, FN14*) compared to the UT. The magnitude of difference in several of the health, cardiovascular, and muscular strength measures are larger than research on MZ twins with 3–30 years of discordant physical activity patterns (Hannukainen et al. 2011; Rottensteiner et al. 2015; Leskinen et al. 2010) (see Table 3). The variables with the largest differences between twins (i.e., > 24%) were TRIG, LDL, WEEE, body composition, VO2max, grip strength, and leg strength; all of which are independent and significant predictors of mortality (Volaklis et al. 2015).

Body composition differed to a greater extent than similar twin research (Leskinen et al. 2010) (see Table 3). Total muscle mass was similar between TT and UT, meaning the differences in total body mass (10.5 kg) were almost entirely explained by fat mass (17.8 vs. 28.8 kg). When compared to age- and sex-specific normative data for %BF, UT was placed in the 38th percentile, while TT was in the second percentile (Kelly et al. 2009). These body composition differences may not be explained solely by the divergent exercise habits as other lifestyle factors likely contributed as well. For example, our short-term dietary tracking data indicate UT consumed 35 more kcals over his estimated TDEE than TT. This small difference may account for ~7 kg of additional body mass [(35 kcal*52 weeks*30 years)/7700 kcal/kg] when extrapolated over 30 years. These might explain why UT demonstrated ~43% greater VAT mass than TT.

As anticipated, our data show that chronic endurance training improves most markers of cardiovascular health. Previous studies have reported an average decrease of 3–5 bpm in RHR after 10–20 weeks of endurance training (Cornelissen et al. 2010; Wilmore et al. 1996), which is far less than the 15 bpm difference found in the current study. In concert, a meta-analysis concluded that on average, exercise reduces SBP by 3.84 mmHg and DBP by 2.58 mm Hg (Whelton et al. 2002). Our data suggest the physiological potential for a much larger difference as TT was 10 mmHg (SBP) and 20 mm Hg (DBP) lower than UT. The length of the discordance in activity (30 years) could likely explain the dissimilarity as most of the studies included in the meta-analysis only ranged from 3 weeks to 2 years. TT also demonstrated healthier CHOL, TRIG, LDL, HDL, and fasting blood glucose concentrations, which agrees with other research (Leskinen et al. 2010; Rottensteiner et al. 2015). However, the magnitude of difference between our twins was similar (HDL), less than (TRIG), and greater (fasting glucose and CHOL) than comparable research (Leskinen et al. 2010; Rottensteiner et al. 2015) (see Table 3).

VL size (CSA and MT) was similar between TT and UT. Yet, *VL* quality (EI) and overall strength were better in UT. This has important health implications as quadricep

Fig. 2 Relative expression of 5′AMP-activated protein kinase (AMPK) protein isoforms, AMPK α1 (catalytic subunit) and AMPK γ1 (regulatory subunit) with tubulin as a standard loading control in vastus lateralis homogenate samples from the trained twin (TT) vs. untrained twin (UT). AMPK γ1 was below the limit of detection in UT
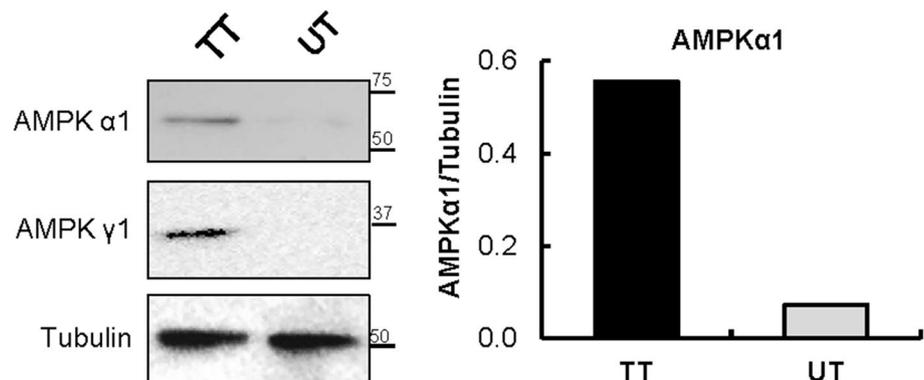
Fig. 3 Resting *vastus lateralis* mRNA expression of select genes related to skeletal muscle: **a** oxidative metabolism [transcription factor A of the mitochondria (*TFAM*), citrate synthase (*CS*)], **b** angiogenesis [endothelial nitric oxide synthase (*eNOS*), vascular endothelial growth factor (*VEGF*)], **c** growth and repair [myostatin (*MSTN*), Pax7 (*PAX7*), mechano-growth factor (*MGF*), insulin-like growth factor a (*IGF-1*), MyoD (*MYOD1*)], and **d** inflammatory responses [*TWEAK* (*TNFSF12*), *FN14* (TWEAK receptor; *TNFRSF12A*), tumor necrosis factor-α (*TNF-α*)] in the trained twin (TT) *vs.* untrained twin (UT). Relative gene expression represented in arbitrary units
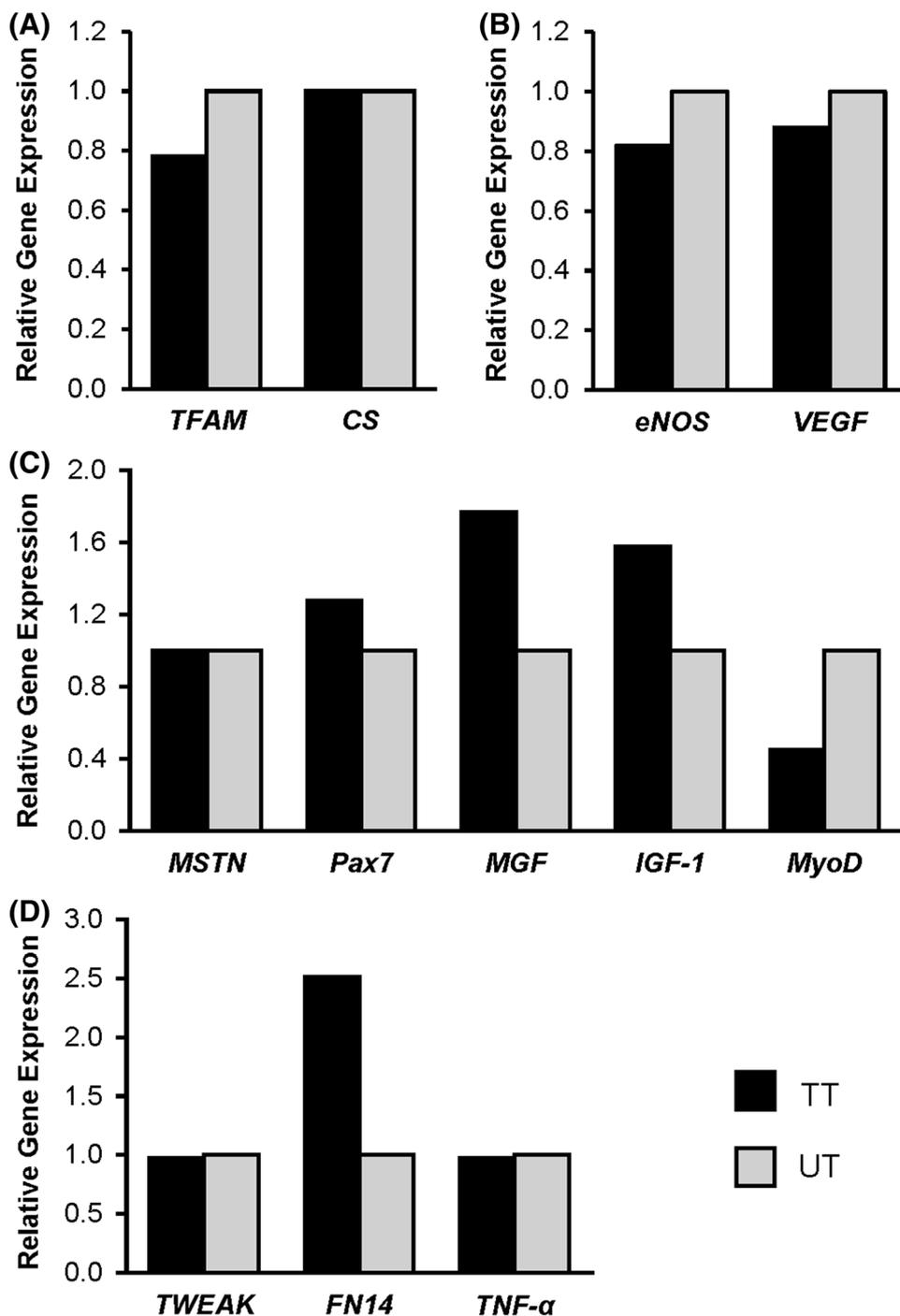
EI correlates with functional strength in middle-aged adults (Fukumoto et al. 2012). The lower muscle quality in TT differs from previous findings (Leskinen et al. 2009), where inactive twins exhibited more intramuscular thigh fat compared to their trained counterparts. This difference could be explained by our use of ultrasound *vs.* magnetic resonance imaging (MRI) used by Leskinen et al. 2009. While MRI is considered a "gold standard" to measure intramuscular fat and muscle size, there is no general consensus on standard assessment tools for muscle quality. Diagnostic ultrasound is gaining popularity as an imaging modality due to its portability and relative affordability (Correa-de-Araujo et al. 2017).

The stronger grip of UT is possibly a result of his delivery truck occupation, yet this remains speculative. Explanation for the differences in *VL* quality and strength is also not clear, and counter to previous research which suggested the more active twin should be favored (Leskinen et al. 2010).

Table 3 Percent differences (%) in body composition, muscular strength, and cardiorespiratory fitness variables between the trained twin (TT) and untrained twin (UT) with 30 years of discordant exercise training histories (current study) compared to the literature (13, 20, 29)

Variable	Current study	3 years (Hannukainen et al. 2011)	3 years (Rottensteiner et al. 2015)	30 years (Leskinen et al. 2010)
Body mass	↓ 10.6	03.8	02.6	08.7
BMI	↓ 13.7	04.1	03.6	07.4
FM	↓ 47.3	16.4	18.2	32.3
FFM	↓ 00.1	00.8	02.5	00.9
BF%	↓ 36.6	10.8	14.8	22.7
IMVC	↓ 59.9	–	04.6	17.6
Grip strength	↓ 16.8	00.6	–	01.3
Rel. VO2max	↑ 30.1	15.9	15.6	20.7

↑ TT greater than UT, ↓ TT less that UT, *3 years* 3 years of discordance between twins, *30 years* 30 years of discordance between twins, *BMI* body mass index, *FM* fat mass, *FFM* fat free mass, *BF%* body fat percentage, *IMVC* isometric voluntary contraction (quadriceps), *Rel. VO_2max* relative maximal oxygen consumption (ml/kg/min)

One difference between our study and similar research (Leskinen et al. 2010) is our TT utilized much higher training volume and a different exercise mode. In regard to muscle power, PP during the WAnT did not differ between TT and UT, but it took TT almost twice (47%) as long to reach PP. A previous study by Jacob and colleagues also found endurance-trained individuals produced significantly less power during a WAnT than both untrained and sprint-trained individuals (Jacob et al. 2002). Thus, it is unclear if TT's decades of endurance-running training, or other lifestyle factors, compromised the quadriceps quality, torque production, and time to PP.

As expected, TT displayed markedly higher anaerobic and aerobic endurances. However, the magnitude of difference between the twins (> 30%) was larger than most previous reports for VO_2max (Table 3), albeit still within the range proposed by previous heritability research (Schutte et al. 2016). In our study, TT's relative VO_2max placed him in the 90th percentile for males ages 50–59, while UT's value placed him in the 60th percentile (Riebe et al. 2018). The divergence in maximal cardiovascular fitness has important health implications for the twins as each metabolic equivalent of task (1 MET) decrease all-cause mortality by 13–15% (Kodama et al. 2009). The minimal differences in maximal spirometry found here is consistent with the prior conclusion that these measures change little with exercise training in healthy individuals. However, previous studies report elite marathon runners possess ~ 7% higher FVC and FEV1% than age-matched controls (Eastwood et al. 2001), indicating a greater reliance on genetic inheritance than exercise habits.

MHC fiber-type distribution is highly variable among individuals, and it has been suggested that ~ 45% of one's fiber-type variance is solely determined by genetic factors (Simoneau and Bouchard 1995). To provide a comprehensive view of muscle fiber type, we analyzed both MHC fiber-type distribution (i.e., fiber type of single muscle fibers) and homogenate MHC fiber-type composition (i.e.,

fiber type of homogenized muscle samples). As shown in Fig. 1 (A, B), compared to UT, TT's *VL* had a greater percent composition of MHC I muscle fibers, as well as more total MHC I fibers and fewer hybrid fibers. The UT fiber-type distribution (~ 40% MHC I, ~ 40% MHC IIa, ~ 30% hybrids) was similar to previous research in healthy/non-exercise-trained males (~ 36% MHC I, ~ 32% MHC IIa, ~ 32% hybrids) (Dickinson et al. 2010). The predominance of MHC I fibers (and fewer hybrid and MHC IIx fibers) in TT was expected, but 94% MHC I fibers is amongst the highest documented in the literature. Succinctness among the single fiber, homogenate, and gene data support the pronounced bias away from MHC IIx expressing fibers in TT. Interestingly, basal *MyHC* I and IIa gene expression levels were similar between twins, suggesting that downstream mechanisms may dictate MHC protein isoform synthesis. This further highlights the sometimes limited syncing of single acute measures of molecular mechanisms with long-term eventual protein expression. Another striking finding was the magnitude of difference between fiber-type compositions in the twins. Previous estimates suggest ~ 30% of differences in MHC I fiber type between individuals may be explained exclusively by environment factors (Simoneau and Bouchard 1995). Here we report a much larger difference in MHC I fibers between twins, highlighting the magnitude of muscle adaptability with exercise training may be more reliant on exposomal factors than previously thought.

AMPK regulates cellular metabolism and is activated during acute exercise (Kjobsted et al. 2018). The catalytic subunit isoform (AMPK α1) and regulatory subunit isoform (AMPK γ1) are known to form a complex with AMPK β2 that is only activated during long duration, low-intensity exercise (Kjobsted et al. 2018). We found higher expression of both AMPK α1 and γ1 isoforms in TT, which parallels previous research showing elevated α1 and γ1 protein concentrations after chronic endurance

training (Frosig et al. 2004). Thus, these two isoforms may be particularly associated with long-term endurance exercise adaptations.

Differences in key mRNA markers of muscle growth, repair, and inflammation were also observed (Fig. 3). Curiously, TT did not show higher expression of genes related to oxidative capacity (*TFAM* or *CS*) or angiogenesis (*NOS3* or *VEGFA*). These results were unexpected, considering elevations in oxidative capacity and muscle capillarization are hallmark adaptations with endurance training. Three possible reasons could explain these findings. One, TT may have reached a new steady state for mitochondrial and capillary density and no longer required elevated expression of the related genes. Second, it is possible, yet unlikely, that TT was in a detraining state and mRNAs associated with oxidative capacity and angiogenesis returned to baseline. Third, since the muscle biopsies were obtained approximately 48 h after the muscle performance testing, it is possible that UT would have a greater response to the muscle activity required for the performance analyses resulting in elevations in *TFAM, CS, NOS3* (eNOS), and *VEGFA* expression that approached and superseded those of TT.

A clear elevation existed in the expression of two splice variants of the *IGF-1* gene in TT. Both mechano-growth factor (IGF-1Ec) and IGF-1Ea were elevated more than 50% in TT. As both of these isoforms are secreted by skeletal muscle and thought to activate muscle satellite cells (Hameed et al. 2003), we evaluated the expression of Pax7 (a marker for satellite cell number). A modest elevation in Pax7 was observed for TT, suggesting elevated satellite cell content, which would be expected with endurance training and more MHC I fibers (slow muscle fibers have more myonuclei than fast fibers) (Allen et al. 1995). Thus, as fibers were transformed from fast to slow a greater number of myonuclei would likely be required to maintain the elevated expression for nuclear DNA-encoded mitochondrial proteins. Additionally, MyoD (a marker for muscle precursor cell differentiation) was decreased in TT, likely reflecting the smaller proportion of MHC IIa muscle fibers as relative levels of MyoD are highly correlated with fast muscle fibers (Hughes et al. 1993).

TT showed an elevated *FN14* gene expression, a receptor for *TWEAK* (Tajrishi et al. 2014). The proinflammatory cytokine system involving *TWEAK, TNFa*, and *FN14* have been implicated in inducing catabolic changes in muscle with sarcopenia (Tajrishi et al. 2014). While the precise roles of TWEAK-FN14 signaling in human skeletal muscle require more research, early investigations suggest moderate intensity endurance exercise elevates *FN14* (Raue et al. 2015). The modest elevation in *FN14* mRNA in TT in our study likely reflects his primarily MHC I fiber-type composition, as resting *FN14* levels have been shown to be higher in MHC I *vs*. IIa fibers (Trappe et al. 2015).

This is the first study to perform a comprehensive analysis of skeletal muscle health and physiological performance in MZ twins with both long-term and large divergences in exercise habits. Magnitudes of difference in several measures are the largest reported between MZ twins (i.e., TT expressed 55% more MHC I fibers, 12.4 ml/kg/min greater VO_2max, and 8.6% lower %BF vs. UT). Our findings support utilizing chronic endurance exercise training to improve body composition and cardiovascular health and suggest these physiological systems exhibit greater plasticity than previously thought. These results highlight the need to further study the exposome's role on physiological adaptations across the lifespan.

Compliance with ethical standards

Conflict of interest The authors declared no conflicts of interest.

Ethical standards All procedures performed in this study were in accordance with the ethical standards of the University's Institutional Review Board for Human Subjects and with the 1964 Declaration of Helsinki and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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