jove

"Avatar", a Modified *Ex vivo* Work Loop Experiments Using *In vivo* Strain and Activation

Caitlin Bemis[1], **Kiisa Nishikawa**[1]

[1] Northern Arizona University

Corresponding Author

Caitlin Bemis
cmb992@nau.edu

Citation

Bemis, C., Nishikawa, K. "Avatar", a Modified *Ex vivo* Work Loop Experiments Using *In vivo* Strain and Activation. *J. Vis. Exp.* (198), e65610, doi:10.3791/65610 (2023).

Date Published

August 18, 2023

DOI

10.3791/65610

URL

jove.com/video/65610

Abstract

Movement behaviors are emergent features of dynamic systems that result from muscle force production and work output. The interplay between neural and mechanical systems occurs at all levels of biological organization concurrently, from the tuning of leg muscle properties while running to the dynamics of the limbs interacting with the ground. Understanding the conditions under which animals shift their neural control strategies toward intrinsic muscle mechanics ('preflexes') in the control hierarchy would allow muscle models to predict *in vivo* muscle force and work more accurately. To understand *in vivo* muscle mechanics, *ex vivo* investigation of muscle force and work under dynamically varying strain and loading conditions similar to *in vivo* locomotion is required. *In vivo* strain trajectories typically exhibit abrupt changes (i.e., strain and velocity transients) that arise from interactions among neural activation, musculoskeletal kinematics, and loads applied by the environment. The principal goal of our "avatar" technique is to investigate how muscles function during abrupt changes in strain rate and loading when the contribution of intrinsic mechanical properties to muscle force production may be highest. In the "avatar" technique, the traditional work-loop approach is modified using measured *in vivo* strain trajectories and electromyographic (EMG) signals from animals during dynamic movements to drive *ex vivo* muscles through multiple stretch-shortening cycles. This approach is similar to the work-loop technique, except that *in vivo* strain trajectories are scaled appropriately and imposed on *ex vivo* mouse muscles attached to a servo motor. This technique allows one to: (1) emulate *in vivo* strain, activation, stride frequency, and work-loop patterns; (2) vary these patterns to match *in vivo* force responses most accurately; and (3) vary specific features of strain and/or activation in controlled combinations to test mechanistic hypotheses.

Introduction

Moving animals achieve impressive athletic feats of endurance, speed, and agility in complex environments. Animal locomotion is particularly impressive in contrast to human-engineered machines-the stability and agility of current-legged robots, prostheses, and exoskeletons remain poor compared to animals. Legged locomotion in natural terrain requires precise control and rapid adjustments to alter the speed and maneuver environmental features that act as unexpected perturbations[1,2,3,4]. Yet, understanding non-steady locomotion is inherently challenging because the dynamics depend on complex interactions between the physical environment, musculoskeletal mechanics, and sensorimotor control[1,2]. Legged locomotion requires responding to unexpected perturbations with rapid multi-modal processing of sensory information and coordinated actuation of limbs and joints[1,5]. Ultimately, movement is made possible by muscles producing force *via* intrinsic mechanical properties of the musculoskeletal system as well as from neural control[1,5,6,7]. An outstanding question of neuromechanics is how these factors interact to produce coordinated movement in response to unexpected perturbations. The following technique utilizes muscle's intrinsic mechanical response to deformation using *in vivo* strain trajectories during controllable *ex vivo* experiments with an "avatar" muscle.

The muscle work loop technique has provided an important framework for understanding intrinsic muscle mechanics during cyclical movements[8,9,10]. The traditional work loop technique drives muscles through predefined, typically sinusoidal, strain trajectories using frequencies and activation patterns measured during *in vivo* experiments[2,8,9,11]. Using sinusoidal length trajectories can realistically estimate work and power output during flight[12] and swimming[2] under conditions where animals do not undergo rapid changes in strain trajectories due to interaction with the environment and musculoskeletal kinematics. However, *in vivo* muscle strain trajectories during legged locomotion arise dynamically from interactions among neural activation, musculoskeletal kinematics, and loads applied by the environment[5,7,13,14]. A more realistic work loop technique is needed to emulate loads, strain trajectories, and force production that corresponds to *in vivo* muscle-tendon dynamics and provides insight into how intrinsic muscle mechanics and neural control interact to produce coordinated movement in the face of perturbations.

Here, we present a novel way to emulate *in vivo* muscle forces during treadmill locomotion by using an "avatar" muscle from a laboratory rodent during controlled *ex vivo* experiments with *in vivo* strain trajectories that represent time-varying *in vivo* loads. Using the measured *in vivo* strain trajectories from a target muscle on muscles from a laboratory animal during controlled *ex vivo* experiments will emulate loads experienced during locomotion. In the experiments described here, the *ex vivo* mouse extensor digitorum longus (EDL) muscle is used as an "avatar" for the *in vivo* rat medial gastrocnemius (MG) muscle during walking, trotting, and galloping on a treadmill[13]. This approach is similar to the work-loop technique, except that *in vivo* strain trajectories are scaled appropriately and imposed on *ex vivo* mouse muscles attached to a servo motor. While mouse EDL muscles differ in size, fiber type, and architecture compared to the rat MG, it is possible to control for these differences. The "avatar" technique allows one to: (1) emulate *in vivo* strain, activation, stride frequency, and work-loop patterns; (2) vary these

patterns to match *in vivo* force responses most accurately; and (3) vary specific features of strain and/or activation in controlled combinations to test mechanistic hypotheses.

Protocol

All animal studies were approved by the Institutional Animal Care and Use Committee at Northern Arizona University. Extensor digitorum longus (EDL) muscles from male and female wild-type mice (strain B6C3Fe a/a-Ttn^{mdm}/J), aged 60-280 days, were used for the present study. The animals were obtained from a commercial source (see **Table of Materials**), and established in a colony at Northern Arizona University.

1. Selecting *in vivo* strain trajectory and preparing for use during *ex vivo* work loop experiments

NOTE: In this protocol, prior measurements from *in vivo* dynamic locomotion, provided directly to the authors (Nicolai Konow, UMass Lowell, personal communication), were used in *ex vivo* experiments. The original data was collected for Wakeling et al.[15]. Time, length or strain, EMG/activation, and force data are required to replicate the protocol.

1. Segment the entire *in vivo* trial into individual strides using any programming platform (MATLab code provided in **Suplementary Coding File 1**).
    1. Plot the length changes *vs*. time for the entire *in vivo* trial. This is used to visualize individual strides (stance to stance) and to assess among-stride variability (**Figure 1**).
    2. Calculate strain for the entire trial (Length (L) / Maximum Isometric Force at Optimal Length L_0).
    3. Select a stride from the entire trial that is representative of all strides, and that begins and ends at similar lengths. This can be done visually by graphing the lengths on top of each other to compare each stride.
    4. After a representative stride is selected, segment out strain, EMG/activation, and force data from the entire trial using any programming platform (see **Supplementary Coding File 1** for the codes used in MATLab[16]).
    5. If sampling frequency differs for strain, EMG/activation or force, interpolate the data points so that all are sampled at the same frequency.

       NOTE: Researchers can determine the frequency of capture based on the time intervals between each point sampled in the entire trial. If variables are captured at the same frequency, the sampling times will be the same.
2. Calculate the frequency of segmented strides.
    1. Calculate the frequency by determining the duration of a segmented stride in seconds and dividing 1 (second) by the duration (1/duration = # strides per second).
    2. Manually determine how many data points must be acquired in *ex vivo* experiments to match the frequency.
    3. Calculate the time required for two strides. Repeat the strides at least once for estimating within muscle measurement error, which will be required for subsequent statistical analysis.
3. Determine the phase of stimulation relative to strain input using the measured EMG activity to determine the

onset and duration of the stimulation for the *ex vivo* work loops. Any programming platform can be used (see **Supplementary Coding File 1** for the code used in this study).

1. View EMG signal over the same x-axis range (time) as strain change (**Figure 1**). Enlarge the EMG signal to be visible; this can be done by multiplying the EMG signal by an arbitrary number, rescaling the strain and EMG to be on the same scale, and/or adding the EMG signal to the strain.
   NOTE: Authors rescaled the strain and EMG to be on the same scale using "rescale" function in MATLab (see **Supplementary Figure 1**).
2. Find where EMG activity starts and stops, as indicated by a change in intensity of two standard deviations[17,18].
   NOTE: Depending on the animal and muscle, EMG onset might or might not correspond with foot contact (Monica Daley, UC Irvine, personal communication) (see Discussion section).
3. Calculate the percentage of the strain cycle (e.g., 40%) at which the EMG activation onset occurs and for how long the stimulation will occur (e.g., 222 ms).
   NOTE: Researchers will need to account for an excitation-contraction coupling (ECC) delay that differs between *in vivo* movement and *ex vivo* work loops and may be different for each animal and muscle (e.g., *in vivo* ECC is 24.5 ms for rat MG, *ex vivo* ECC is ~5 ms for mouse EDL).
4. Prepare representative strain inputs for the work loop controller program. Any program that can capture force output with input for strain and stimulation can be used for the work loop controller program (see Discussion section).
   1. Take the selected stride and interpolate to the appropriate number of points necessary to have the step captured at *in vivo* frequency for two cycles (see step 1.2).
   2. Rescale the stride to start and stop at "zero strain" (e.g., L_0 or 95% L_0) after stretching by a pre-determined length excursion (see step 3.3).
   3. "Scale" selected stride, if necessary, to use as an input for strain changes in mouse EDL (see Discussion section). To scale, select a length excursion to which the mouse EDL can be stretched without damage (e.g., we typically stretch the mouse EDL by 10% L_0 regardless of the *in vivo* species). This may need to change based on preliminary results (see step 3.3).

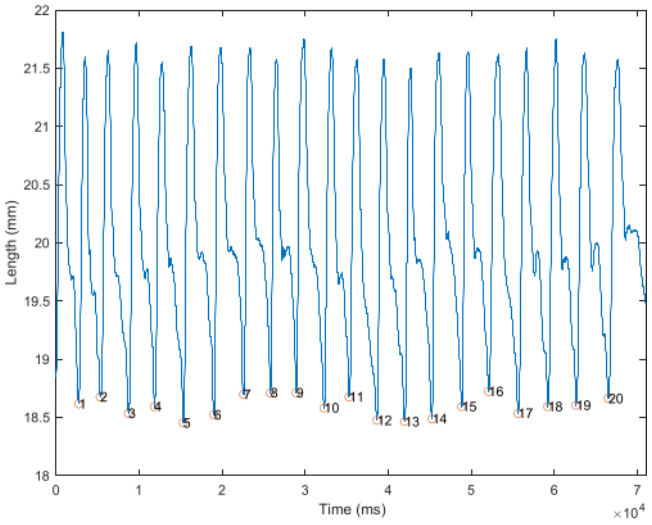

Figure 1: Length over time of *in vivo* whole trial. Length (mm) plotted against time of rat MG. Strides are demarcated by circles, from shortest length to shortest length, considered single stride. Please click here to view a larger version of this figure.

2. Evaluating maximum isometric force of mouse muscle *ex vivo*

1. Set up equipment and surgery.

   NOTE: See the Discussion section for an explanation of the equipment needed for the *ex vivo* work loop.

   1. Prepare a tissue-organ bath by inserting the oxytube needle valve into the water-jacket tissue bath (see **Table of Materials**). Connect the oxytube to a gas cylinder with 95% 0_2-5% CO_2. Allow 20 psi to fill the water-jacket tissue bath.

   2. Prepare the surgery area by running an additional oxytube from the gas line to a crystallizing dish filled with Krebs-Henseleit solution (step 2.1.3) near the surgery area. This will be used to keep the muscles aerated and hydrated during and after the surgeries.

      NOTE: Muscles can also be stored in this aerated solution up to 4 h, if more than one muscle is taken out of the mouse at a time.

   3. Prepare 1 L of Krebs-Henseleit solution containing (in mmol l^{-1}): NaCl (118); KCl (4.75); $MgSO_4$ (1.18); KH_2PO_4 (1.18); $CaCl_2$ (2.54); and glucose (10.0) at room temperature and pH to 7.4 using HCl and NaOH (see **Table of Materials**). When handling HCl and NaOH, wear the proper PPE of goggles and gloves.

   4. Fill the bath with Krebs-Henseleit solution at room temperature and pH 7.4. Submerge the muscle and the hook completely in the solution.

   5. Turn on all equipment; dual-mode muscle lever system, stimulator, and signal interface (DAQ board) (see **Table of Materials**).

2. EDL muscle dissection.

    1. Anesthetize the mouse with 1 mL of isoflurane in a bell jar and then perform cervical dislocation. Lay the mouse in either a right or left lateral recumbent position with the top hindlimb stretched and toes touching the dissection board. Remove the fur from the ankle to above the knee joint.

    2. Tent the skin with forceps and cut from the ankle joint to the hip area. Once the muscle has been exposed, cut around the ankle like a "hem" of pants. Pull the skin up to expose the leg muscles more clearly.

    3. Locate the fascia line that separates the tibialis anterior (TA) and gastrocnemius, separate using dissection scissors to expose the knee tendons. Place dissection scissors between the two exposed knee tendons. Scissors will "catch" on a pocket just below the exposed knee tendons. Blunt dissect a "pocket" while pulling the scissors away from the leg until the scissors reach the ankle to expose the EDL.

    4. Using a pre-tied loop knot in size 4-0 silk surgical suture (see **Table of Materials**), lace one end of the suture under the tendon closest to the knee. Tie a double square knot above the proximal muscle-tendon junction without placing it on the muscle or including the tendon. Cut above the knot. Gently pull the loop tied to the tendon, and the EDL will emerge from the "pocket".

    5. Tape the loop to the dissection area to create tension in the EDL. Tie a double square knot using another pre-tied loop knot at the distal muscle-tendon junction without placing it on the muscle or including the tendon. Cut the knot on the side closer to the leg to remove the whole EDL from the mouse. Cut the extra suture away from the double square knots on the proximal and distal sides of the muscle and place the muscle in the aerated bath by the surgery area.

        NOTE: Ensure to note which side is proximal and/or distal if placing the muscle in an aerated bath.

    6. To place on the servomotor lever rig, attach EDL vertically between suspended platinum electrodes. Attach the distal loop knot to the stationary hook and attach the proximal loop knot to the hook attached to the servomotor arm. Raise the tissue bath to submerge the muscle in the aerated Krebs-Henseleit solution.

        NOTE: Aeration should not disturb the muscle when it is submerged. If it does, lower the pressure of the gas. Allow muscle to equilibrate for 10 minutes before beginning stimulation.

3. Measure the maximum isometric force of EDL muscle.

    NOTE: Refer to **Table 1** for protocol on how to measure maximum isometric force using twitch and tetanus. See **Supplementary Figure 1** for an illustration of the program used by the authors.

    1. Stimulate the muscle with a supramaximal twitch to ensure the muscle has not been damaged during surgery (80 V, 1 pps, 1ms; **Table 1**; see **Supplementary Figure 2**). If no damage has occurred, use the length knob on the muscle lever system to find a muscle length using twitch stimulation at which active tension is ~1V / 0.1271 N with less than ~0.1V / 0.01271N passive tension.

    2. Record the starting length of the muscle from suture knot to suture knot in Volts and millimeters. Input measurements into the calibration portion of the

program for starting length (see **Supplementary Figure 1**).

3. Find supramaximal twitch maximum isometric force at optimal length (L_0) of EDL (**Table 1**). No rest period is technically needed, but waiting 1 min between stimulations will stabilize passive tension. Record the length (in Volts) at which the supramaximal twitch is maximum. This is the muscle optimum length (L_0) for twitch.

4. Measure the muscle with calipers at this length. Measure the muscle from suture knot to suture knot. Once L_0 has been found, shorten the muscle back to starting length (active tension ~1V / 0.1271 N).

5. Find supramaximal tetanus maximum isometric force of EDL (80 V, 180 pps, 500 ms; **Table 1**). Record the length (in Volts and millimeters) of supramaximal tetanic force at L_0 and measure the fibers from suture knot to suture knot again with calipers.

   NOTE: Increasing the muscle length in 0.5 V/0.65 mm steps will result in more accurate L_0 for both twitch and tetanus.

6. Find the submaximal isometric force of EDL (45 V, 110 pps, 500ms; **Table 1**) at L_0 before and after the experiment to ensure fatigue did not occur from the stimulation protocol. A 10% decrease in force is considered a "fatigued" muscle.

Experiment	Simulation Intensity (V)	Pulse Frequency (pps / Hz)	Stimulation Duration (ms)	Comments
1. "Warm-Up"	80	1	1	Increase or decrease length by 0.50 V to find passive tension of 1 V
2. Optimal muscle length twitch (L0)	80	1	1	Increase or decrease length by 0.50 V to find passive tension of ~1 V
3. Optimal muscle length tetanus (L0)	80	180	500	Rest 3 min between changing length by 0.50 V
4. Pre-experiment submaximal L0	45	110	500	At length of L0
6. Avatar experiments	45	110		Cyclically use representative length changes for mouse EDL
7. Post-experiment submaximal L0	45	110	500	Return to L0 after experiment and measure L0

Table 1: Stimulation protocol. Stimulation protocol for finding supramaximal and submaximal twitch and tetanus optimal length. Protocol varies by stimulation intensity, timing, and pulses per second.

3. Completing "avatar" work loop technique using selected *in vivo* strain trajectories

1. Set up the software necessary to complete "avatar" work loop techniques (see **Table of Materials**).
   NOTE: An input file (.csv or similar) that specifies the muscle length at each time step is needed (see step 1.4). Inputs for the percentage of the cycle at which the stimulation starts and for the duration of stimulation are necessary (see **Supplementary Figure 3** for example).
2. Complete "avatar" work loop technique.
   NOTE: While we use a custom LabView program, researchers can use any program that allows control of length changes in mouse EDL on a servomotor lever, control of the onset (% cycle) and duration (ms) of stimulation at specified times, and measurement of muscle force. See **Supplementary Figure 3** for an illustration of the program authors use.
   1. Upload the scaled strain changes with scaled length excursion into the program from step 1.4. See steps 1.4, 3.3, and the Discussion section for more on "scaled strain changes".
   2. Adjust the starting length of the muscle if needed (see section 3.3). Input the starting length in V and mm to calibrate results (see **Supplementary Figure 3**).
   3. Use stimulation onset and duration calculated in step 1.3.
   4. Run the muscle through the scaled length changes with determined length excursion for two cycles.
   5. Save data. If several stimulation protocols are collected on the same muscle, wait 3 min between each stimulation.
   6. Stimulate at optimal length (L_0) using submaximal activation to determine if fatigue has occurred. If force decreases by more than 10%, muscles are considered fatigued. See **Table 1** for stimulation protocols.
   7. Remove the muscle from the bath. Cut-loop knots from the muscle and dab the excess solution off the muscle. Weigh the muscle. Determine physiological cross-sectional area using the standard formula: muscle mass/(L_0*1.06)[19].
3. Tune parameters for "avatar" work loop technique (see Discussion section).
   1. Determine the starting length and length excursion by matching the *ex vivo* passive tension rise to the passive tension rise observed *in vivo* (**Figure 2**).
      NOTE: This study used percent L_0 to scale starting length (mm) and excursion (% L_0; see step 1.4 and Discussion section). For matching the tension rise in *ex vivo* mouse EDL to that of the *in vivo* rat MG, the authors found that starting length at L_0 produced the best fit (**Figure 2**).
   2. Choose three starting lengths (e.g., -5% L_0, L_0, and +5% L_0). Perform the "avatar" work loop at each of these starting lengths with a specified length excursion (e.g., 10% L_0).
      NOTE: In the present "avatar" experiments using mouse EDL, a length excursion of 10% L_0 was used.
   3. Repeat with new starting lengths and/or excursion until the rate of *ex vivo* passive tension rise is similar

to the rate of *in vivo* passive tension rise (see **Figure 2B**).

4. Depending on fiber types and activation dynamics of the muscles used, increase or decrease the duration of stimulation to optimize the match between *ex vivo* and *in vivo* force. Thus, it may be necessary to change the onset and/or duration of stimulation to best match *in vivo* force production during "avatar" experiments.

5. To decide whether this is necessary (see Discussion section), plot force over time of "avatar" and *in vivo* muscle (**Figure 3**) and calculate the coefficient of determination R^2 by squaring the scaled correlation between target and "avatar" muscle force (see Representative results).

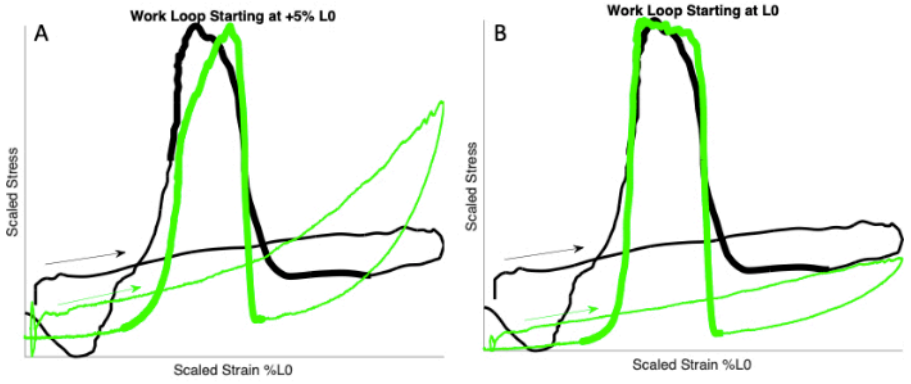

Figure 2: Matching passive tension rise. Work loops showing the *in vivo* and *ex vivo* rise in passive tension (arrows). *In vivo* scaled work loop from rat MG (black) walking at 2.9 Hz (data from Wakeling et al.[15]). *Ex vivo* scaled work loops from mouse EDL (green) at 2.9 Hz. (**A**) Starting length of mouse EDL muscle is +5% L_0. (**B**) Starting length of the mouse EDL muscle is L_0. Note that the ex vivo passive tension rise matches the *in vivo* tension rise in A but not in B. Thicker lines indicate stimulation. Please click here to view a larger version of this figure.

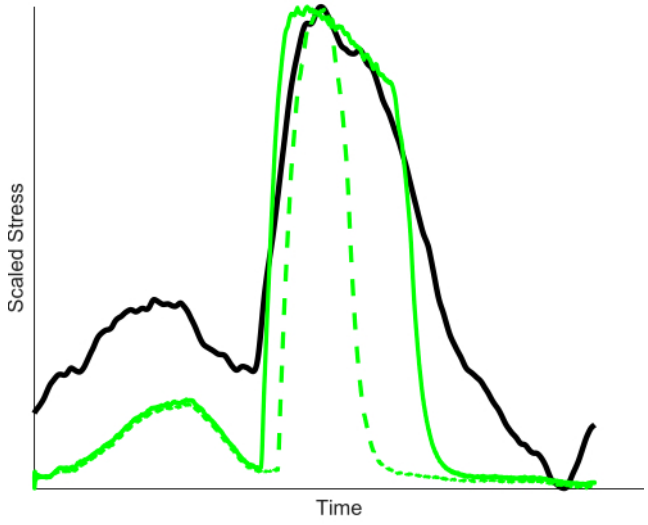

Figure 3: Optimizing stimulation duration of mouse EDL to match *in vivo* force of rat MG (black line). The force generated by the mouse EDL using the EMG-based stimulation (green dashed line) decreases earlier than the *in vivo* force, likely due to faster deactivation of the mouse EDL compared to rat MG. To optimize the fit between the *in vivo* and *ex vivo* forces, the mouse EDL was stimulated for a longer duration (solid green line). EMG-based stimulation R^2 = 0.55, Optimized stimulation R^2 = 0.91. Please click here to view a larger version of this figure.

Representative Results

The goal of the "avatar" experiments is to replicate *in vivo* force production and work output as closely as possible during *ex vivo* work loop experiments. This study chose to use mouse EDL as an "avatar" for rat MG because mouse EDL and rat MG are both comprised of mostly of fast-twitch muscles[20,21]. Both muscles are primary movers of the ankle joint (EDL ankle dorsiflexor, MG ankle plantarflexor) with similar pennation angles (mouse EDL 12.4 + 2.12°[22], rat MG 20° used in this study[15]). Scaled representative work loops of rat MG[15] were compared to *ex vivo* "avatar" experiments (**Figure 4**) using two different stimulation protocols (one from measured EMG activity and one optimized as in step 3.3). R^2 values presented here were calculated using the entire scaled stretch-shortening cycle (2 cycles/condition), with each cycle having more than 2000 points corresponding to the locomotor speed (walk = 5521 points, trot = 5002, gallop = 2502 points). Work loops were scaled to account for differences in muscle size, P0, and PCSA. Scaling was done by linearly mapping force and strain onto a similar scale (0-1) to compare rat MG and mouse EDL. Visually, it is apparent that optimizing the stimulation protocol (**Figure 4B**) to account for different activation dynamics of the mouse EDL and rat MG muscles improves the fit to the *in vivo* rat MG force compared to the EMG-based activation (see Discussion section). For the mouse EDL, approximately doubling the stimulation duration for slower strain trajectories (walk and trot) increased the R^2 by 62% in walking and 109% in trot. For the faster strain

trajectory (gallop), increasing stimulation time by half the observed time increased the R^2 by 22%.

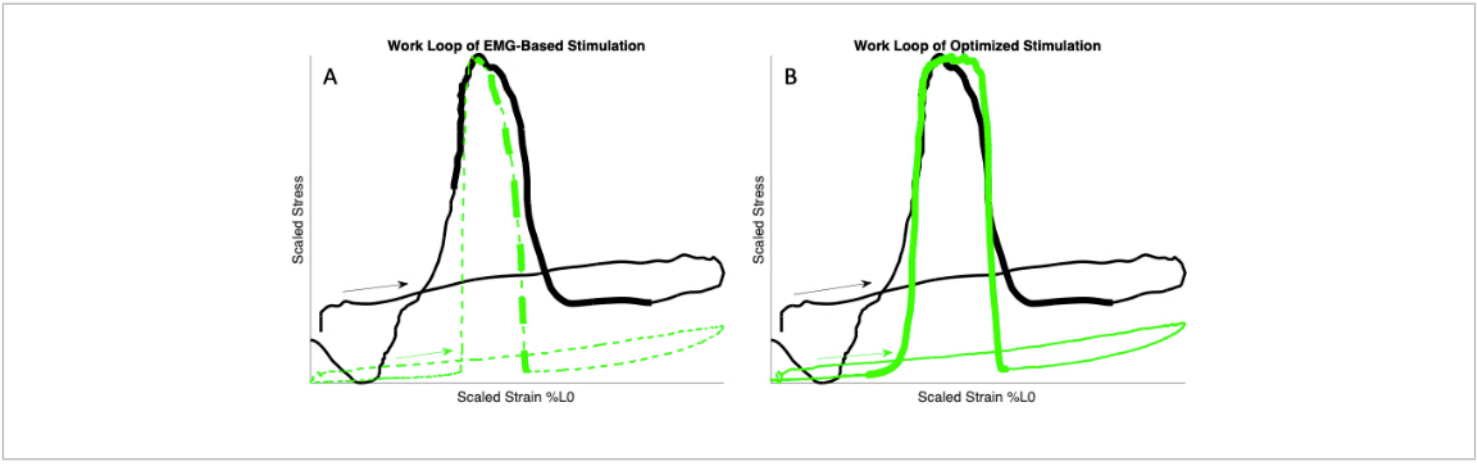

Figure 4: Comparison of *in vivo* and *ex vivo* work loops. Work loops of *in vivo* rat MG (black) and *ex vivo* mouse EDL (green) during walking (2.9Hz) using *in vivo* strain trajectories. The thicker line indicates stimulation in both *in vivo* and *ex vivo* work loops. (**A**) Work loop of *in vivo* rat MG (black) and *ex vivo* mouse EDL (dashed green) during walking using EMG-based stimulation protocol. (**B**) Work loop of *in vivo* rat MG (black) and *ex vivo* mouse EDL (solid green) during walking (2.9Hz) using optimized stimulation. Please click here to view a larger version of this figure.

High R^2 between mouse EDL *ex vivo* force production and *in vivo* force production of rat medial gastrocnemius (MG)[15] indicates good replication (**Figure 5**). In EMG-based stimulation experiments, average R^2 values were 0.535, 0.428, and 0.77 for walk, trot, and gallop, respectively. In optimized stimulation experiments, average R^2 values were 0.872, 0.895, and 0.936 in walk, trot, and gallop, respectively. As previously discussed (step 3.3, **Figure 5**), depending on the activation dynamics of the muscles used, the stimulation protocol may also need to be optimized. Prediction of *in vivo* MG force using *ex vivo* mouse EDL was improved across all locomotor speeds by optimizing stimulation, increasing R^2 (**Figure 5A**,**B**), and decreasing root mean square error (RMSE). RMSE decreased after optimization for all speeds (**Figure 6**). Averaged RMSE for EMG-based stimulation was 0.31, 0.43, and 0.158 for walk, trot, and gallop. Averaged RMSE for optimized stimulation was 0.181, 0.116, 0.101 for walk, trot, and gallop.

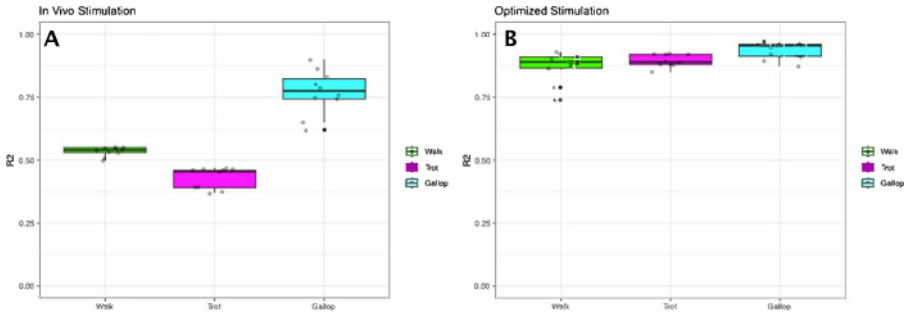

Figure 5: R^2 Values for *in vivo* and *ex vivo* force production: Box and whisker plot of R^2 values for *in vivo* and *ex vivo* force comparisons. Individual observations plotted, median, 25th, and 75th percentile indicated. (**A**) R^2 values for *in vivo* and *ex vivo* force production using stimulation protocol based on measured *in vivo* EMG signal during walking at 2.9 Hz (green), trotting at 3.2 Hz (magenta), and galloping at 6.2 Hz (cyan). (**B**) R^2 values for *in vivo* and *ex vivo* force production using optimized stimulation (see **Figure 2**). Optimizing the stimulation onset and duration increased R^2 for all gaits. EMG-based stimulation: walk R^2 = 0.50-0.55, trot R^2 = 0.37-0.47, gallop R^2= 0.62-0.90; optimized stimulation: walk R^2 = 0.74-0.93, trot R^2 = 0.85-0.92, gallop R^2 = 0.87-0.97. Please click here to view a larger version of this figure.

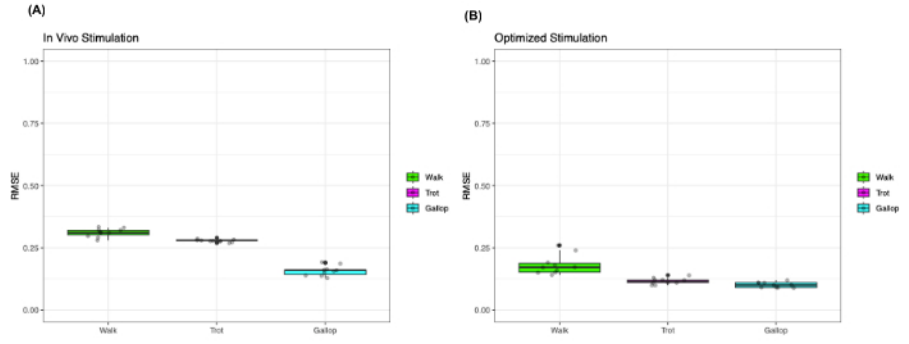

Figure 6: Root-mean square error (RMSE) for *in vivo* and *ex vivo* force production. Box and whisker plot of RMSE values for *in vivo* and *ex vivo* force comparisons. Individual observations plotted, median, 25th, and 75th percentile indicated. (**A**) RMSE values for *in vivo* and *ex vivo* force production using EMG-based stimulation protocol. (**B**) RMSE values for *in vivo* and *ex vivo* using optimized stimulation protocol. Optimizing the stimulation onset and duration reduced RMSE for all gaits. Walking at 2.9 Hz (green), trot at 3.2 Hz (magenta), and gallop at 6.4 Hz (cyan). Please click here to view a larger version of this figure.

To test the performance of traditional work loop methods at predicting *in vivo* muscle forces, sinusoidal work loops were also performed for the mouse EDL at the same frequency, length excursion, starting length, stimulation onset, and duration as for the "avatar" experiments using *in vivo* rat MG strain trajectories. R^2 values were significantly lower than for the *in vivo* strain trajectories for both EMG-based and optimized stimulation protocols (**Figure 7**). Averaged R^2 values for EMG-based stimulation using sinusoidal length trajectories were 0.062, 0.067, and 0.141 at walk, trot, and gallop frequencies. Averaged R^2 values for optimized stimulation using sinusoidal length trajectories were 0.09, 0.067, and 0.141 at walk, trot, and gallop frequencies.

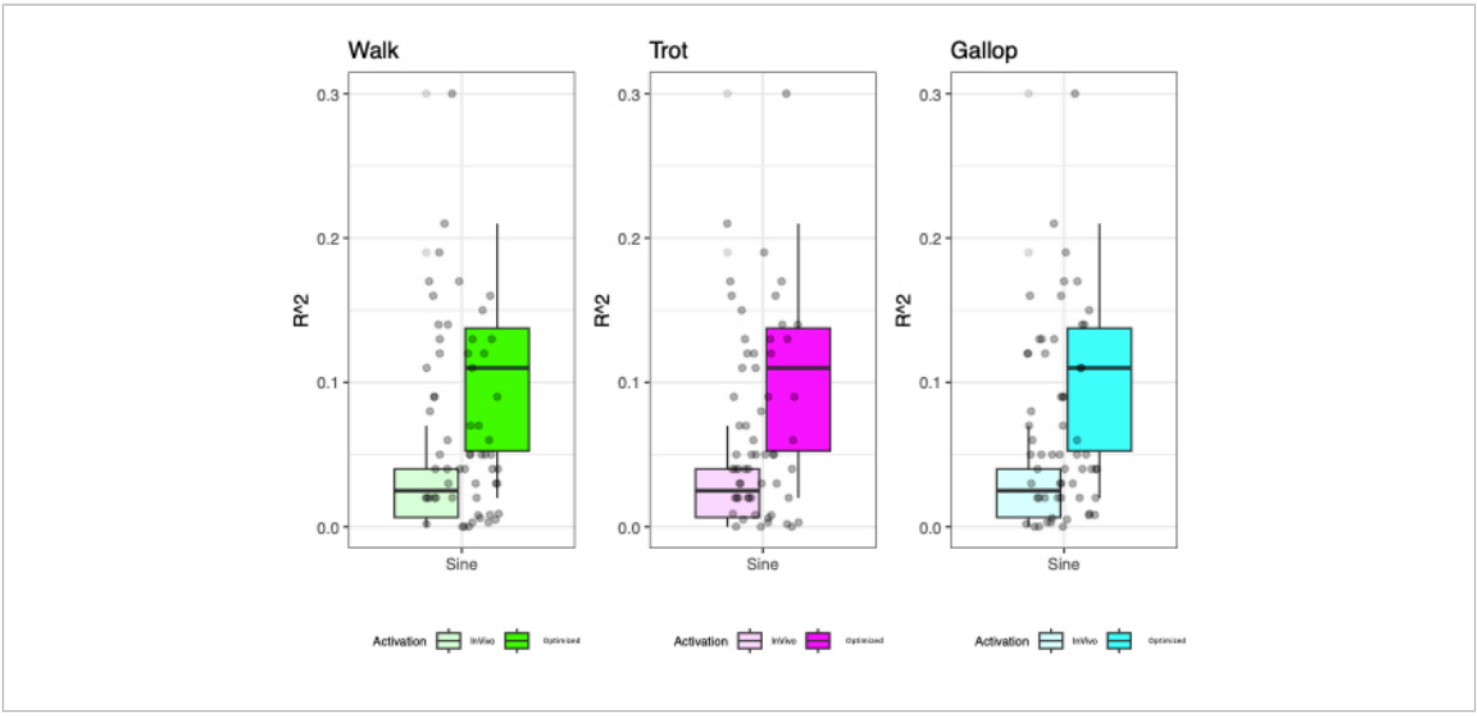

Figure 7: R^2 Values for *in vivo* and *ex vivo* force production using sinusoidal length changes. Box and whisker plot of RMSE values for *in vivo* and *ex vivo* force comparisons. Individual observations plotted, median, 25^{th}, and 75^{th} percentile indicated. R^2 values for walk (green, 2.9 Hz), trot (magenta, 3.2 Hz), and gallop (cyan, 6.2 Hz) using sinusoidal length changes with EMG-based (translucent) and optimized (opaque) stimulation protocols. For both EMG-based and optimized stimulation, the R^2 values were lower for the sinusoidal length changes than for *in vivo* length changes. EMG-based stimulation: walk R^2 = 0.00 - 0.30, trot R^2 = 0.00 - 0.02, gallop R^2= 0.03 - 0.07; optimized stimulation: walk R^2 = 0.02 - 0.21, trot R^2 = 0.02 - 0.12, gallop R^2 = 0.12 - 0.17. Please click here to view a larger version of this figure.

Work loops produced by the *ex vivo* mouse EDL muscle using sinusoidal length trajectories do not as accurately emulate *in vivo* rat MG force compared to *in vivo* strain trajectories (**Figure 8**). The change in work produced by sinusoidal vs. *in vivo* strain trajectories can be explained by the absence of strain and velocity transients in the sinusoidal trajectory (**Figure 9**). While the muscles were stimulated at similar lengths during the active shortening phase of the contractions

in both sinusoidal trajectories and *in vivo*-based strain trajectories, the onset of stimulation occurred at different phases of the cycle (e.g., stimulation onset occurred at a phase of 74% for trot EMG-based stimulation, but at a phase of 43% for walking EMG-based stimulation; see Discussion section).

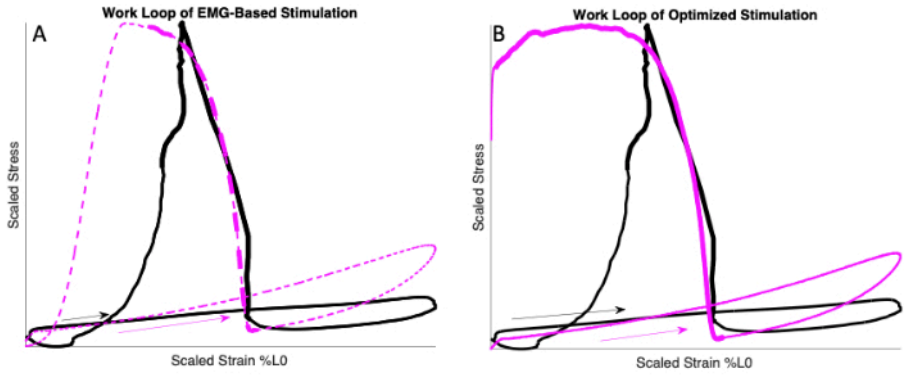

Figure 8: Comparing *in vivo* and *ex vivo* sinusoidal work loops. (**A**) *In vivo* work loop (black) from rat MG and *ex vivo* work loop (dashed magenta) from mouse EDL using sinusoidal strain trajectory and EMG-based stimulation. (**B**) *In vivo* work loop (black) from rat MG and *ex vivo* work loop (solid magenta) from mouse EDL using sinusoidal strain trajectory and optimized stimulation. Note that the sinusoidal work loops overestimate the *in vivo* work due to the absence of strain and velocity transients in the sinusoidal trajectory. EMG-based stimulation R^2 = 0.0003, optimized stimulation R^2 = 0.084. Please click here to view a larger version of this figure.

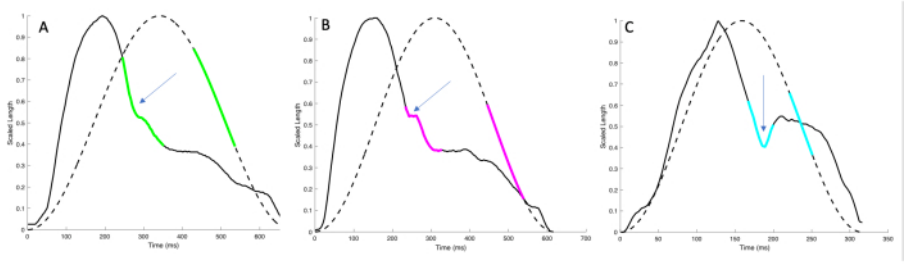

Figure 9: Comparison of *in vivo* strain and *ex vivo* sinusoidal length trajectories. Comparison of *in vivo* strain and *ex vivo* sinusoidal length trajectories at walk (green), trot (magenta), and gallop (blue). The solid line is *in vivo* strain trajectory. Dashed line *ex vivo* sinusoidal length trajectory. The highlighted portion is stimulation. Stimulation started at the same length during the shortening phase of the stride. Arrows indicating strain and velocity transients. Deviations from sinusoidal are impedance from outside forces on muscle. Please click here to view a larger version of this figure.

Supplementary Figure 1: Program used to collect isometric maximal force at optimal length. The program used to determine optimal length during supramaximal and submaximal twitch and tetanic stimulation. Please click here to download this File.

Supplementary Figure 2: Viable twitch response. Twitch response of mouse EDL. Twitch force rises and falls quickly and should reach active tension of ~1 V. "Noise" should be minimal after the peak active tension has been reached. Please click here to download this File.

Supplementary Figure 3: Program used to collect work loop data. The program used to control muscle length of and timing of stimulation in *ex vivo* work loops. Please click here to download this File.

Supplementary Coding File 1: MATLab code used to segment and create an experimental protocol for the work loop. MATLab code that was used to segment target step information (length, EMG activation, and force) into individual strides. Code includes scaling and interpolating target animal steps into lengths that *ex vivo* mouse EDL can stretch. Additionally, includes code to smooth EMG signal and compare activation to select onset and duration of stimulation in *ex vivo* work loop experiments. Please click here to download this File.

Discussion

While organisms move seamlessly across landscapes, the underlying loads and strains that the muscles experience vary drastically[1,6,23]. During both *in vivo* locomotion[1,24] and in "avatar" experiments, muscles are stimulated submaximally under cyclical, non-steady conditions. The isometric force-length and isotonic force-velocity relationships are not well suited for predicting muscle force under these conditions[2]. Understanding the effects of non-steady strain (i.e., transients) and loading is essential for predicting force production during *in vivo* movement, and therefore is the main rationale for developing these "avatar" experiments[2]. "Avatar" experiments allow us to control muscle loading and strain trajectories while measuring force output. The "avatar" technique investigates the force response of muscles under *in vivo*-like conditions, without confounding factors of neural control and tendon compliance. To perform the "avatar" experiments, researchers will need a program that allows a muscle to go through prescribed length changes with the ability to stimulate at different starting lengths and for varying durations (see **Supplementary Figure 3** for the program the authors use). Researchers need to specify starting muscle length (mm), length of excursion (mm), onset of stimulation (% of cycle duration) and duration of stimulation (ms) before doing experiments (see steps 1.3-1.4to obtain values for these parameters). In general, it is often desirable to select strides that are representative of all strides in the trial (e.g., start and end at a similar length, reach a similar peak force, have average EMG activity, etc.). Determining whether EMG/activation and force data from a selected stride is representative of other strides in the same trial can be helpful for "tuning" later, which can be done by plotting work loops (force versus length) of the entire trial using the target animal's muscle. During bipedal and quadrupedal locomotion, the shortest length to shortest length generally demarcates an entire stride (toe-off to toe-off), but EMG activation can vary. In some animals and muscles, EMG activation is closely correlated to foot contact, such as the rat MG shown here[22]. In other animals, such as the guinea fowl lateral gastrocnemius, EMG activation generally occurs at the longest length to achieve more stability during unknown terrain[25].

To perform "avatar" experiments, it is important to minimize the noise in the *ex vivo* force data. Force measurements are sensitive to several issues, including but not limited to tearing of the muscles during surgery, compliance of the sutures if the loop-knots are too long, improper scaling of the length inputs and excursion, and muscle fatigue. Tearing of the muscles often occurs when "dissecting the pocket" (step 2.2.3) and tying the loop-knot around the proximal portion of the tendon (step 2.2.4). While "dissecting the pocket", keeping the dissection scissors flat and horizontal to the muscle will prevent the tips from nicking the EDL. Additionally, pulling the dissection scissors away and distally while blunt dissecting will also limit contact between the dissection scissors and the EDL muscles. Additionally, muscles should be kept damp with Krebs-Henseliet solution during the surgery preparation and when being used on the rig.

Properly scaling length inputs is more complicated. Muscle passive and active force can be affected if the starting length and/or excursion are not scaled properly. The *ex vivo* rise in passive tension should match the *in vivo* rise in passive tension (see **Figure 1**). One scaling issue that has been observed in previous experiments is that both passive and active tension can be affected if the length excursion (starting length to longest length) is too small or too large. Theoretically, muscles should reach peak force near their optimal length (L_0)[26], which is why we use optimal length (L_0) to scale *in vivo* muscle lengths in *ex vivo* "avatar" experiments to accurately replicate *in vivo* force production. Architectural differences between muscles will play a role in determining the starting length and length excursion parameters. Although optimal length (L_0) is found during supramaximally stimulated isotonic and isometric conditions, using it as a scaling metric in "avatar" experiments can potentially highlight limitations of the force-length and force-velocity relationships during cyclical movement that need more investigation. In most steady-state conditions, muscle's instantaneous length, velocity, and activation (i.e., force-length and force-velocity properties) can be used to predict force and work output with reasonable accuracy[12,24,27]. Under dynamic conditions with variable loading, the force increases as a function of velocity[28] and has a complex relationship with strain and activation[29,30]. This contradicts the isotonic force-velocity and isometric force-length properties of muscles[28]. In the rat MG, strain and velocity transients are evidence of loading, such as foot contact or interaction with the environment (i.e., rough terrain, wind, sudden change in direction for predation avoidance) (**Figure 9**). These rat MG strain trajectories, like most realistic conditions, have sudden changes in the applied load, force production, and work output[2,28]. This experimental method aims to highlight these complex interactions among strain, velocity, and activation dynamics under *in vivo* conditions that are not well explained by traditional force-length and force-velocity relationships.

Other issues can occur when the muscle starting length is too short or long. A too-short starting length will result in a reduced rate of rise in tension during the passive and active stretch (not shown), whereas a too-long starting length will result in an increased rate of rise in passive tension (see **Figure 1B**). Using the ratio of active to passive tension can be helpful. For example, in rat MG, passive tension (N) is generally around half the active tension (**Figure 2**). If a muscle starts at too long a length and/or is stretched to a length that is too long, the passive tension may be too high relative to the active tension (see **Figure 1B**), and the force may decrease quickly due to overstretching. Also, stretching to a length that is too long will potentially damage the muscle and can cause the muscle to fatigue more quickly. Additionally, active tension may appear

unfused if the starting length is too short and/or the muscle is not stretched to a long enough length.

Preliminary experiments are necessary to determine starting length and excursion based on L_0. Additional preliminary experiments may be needed to adjust the duration of stimulation if the activation dynamics of the muscles used are different. These optimizations are needed because the fiber type composition and/or activation dynamics of *in vivo* and *ex vivo* muscles may be different. In our representative results (**Figure 4** and **Figure 5**), we used two stimulation protocols for mouse EDL during *ex vivo* experiments to replicate *in vivo* rat MG force production. To optimize force production in the mouse EDL to best fit *in vivo* rat MG, stimulation duration was increased (**Figure 2** and **Figure 3**). Rat MG is comprised of slower fiber types than mouse EDL[31,32,33]. This was evident in "avatar" experiments because *ex vivo* mouse EDL muscles produced force faster after excitation, and force decreased at a faster rate after deactivation than observed *in vivo* in rat MG[15] (**Figure 2**), even after accounting for excitation-contraction delay differences between *in vivo* and *ex vivo* conditions[34]. Depending on the *ex vivo* and *in vivo* target muscles, optimization of stimulation might be needed in other "avatar" experiments as well. Either the mouse EDL or soleus (SOL) muscles may be used in this *ex vivo* work loop technique. EDL was chosen as an "avatar" for the rat MG due to the similarities in muscle fiber type and pennation structure. It is possible that some muscles may have a complex structure and cannot be emulated using muscles from laboratory rodents as an "avatar".

While "avatar" experiments do need some manual optimization to best replicate *in vivo* force production, the technique is applicable to a variety of different animals and locomotor modes. The "avatar" technique can be especially useful to understand *in vivo* force production in animals whose muscles are too large or otherwise inaccessible for *ex vivo* experiments. While only preliminary work has been done on larger animals[35], this work has shown potential for the applicability of this technique across animals, muscles, and locomotor gait using laboratory mice as "avatars". The usefulness of "avatar" experiments depends on how accurately a convenient, inexpensive, readily available, and well-characterized laboratory rodent model (i.e., mouse EDL) can be used for understanding *in vivo* mechanics of different muscles from varying species of vertebrates. Results from preliminary "avatar" experiments presented here (rat MG) and elsewhere (guinea fowl LG[19]), suggest this technique can be used to accurately predict *in vivo* forces and could be applied to other animals. Future applications of this method should expand the types of muscles and animals that have been used as both targets and "avatar" during *ex vivo* and *in vitro* experiments. "Avatar" experiments allow us to examine factors that affect muscle force and work output during *in vivo* locomotion when muscle loading and strain vary abruptly[1,2,19]. Specifically, the "avatar" method allows us to examine the effects of strain and velocity transients on muscle force that are not captured by traditional muscle models or sinusoidal work loop experiments.

Disclosures

All authors acknowledge there is no conflict of interest.

Acknowledgments

We thank Dr. Nicolai Konow for providing the data used in this study. Funded by NSF IOS-2016049 and NSF DBI-2021832.

References

1. Dickinson, M. H. How Animals move: an integrative view. *Science.* **288** (5463), 100-106 (2000).
2. Sponberg, S., Abbott, E., Sawicki, G. S. Perturbing the muscle work loop paradigm to unravel the neuromechanics of unsteady locomotion. *Journal of Experimental Biology.* **226** (7), jeb243561 (2023).
3. Daley, M. A., Biewener, A. A. Running over rough terrain reveals limb control for intrinsic stability. *Proceedings of the National Academy of Sciences.* **103** (42), 15681-15686 (2006).
4. Daley, M. A., Usherwood, J. R., Felix, G., Biewener, A. A. Running over rough terrain: guinea fowl maintain dynamic stability despite a large unexpected change in substrate height. *Journal of Experimental Biology.* **209** (1), 171-187 (2006).
5. Daley, M. A. Understanding the agility of running birds: Sensorimotor and mechanical factors in avian bipedal locomotion. *Integrative and Comparative Biology.* **58** (5), 884-893 (2018).
6. Biewener, A. A. *Animal locomotion.* Oxford University Press. Oxford ; New York. (2003).
7. Robertson, B. D., Sawicki, G. S. Unconstrained muscle-tendon workloops indicate resonance tuning as a mechanism for elastic limb behavior during terrestrial locomotion. *Proceedings of the National Academy of Sciences.* **112** (43), E5891-E5898 (2015).
8. Josephson, R. K. Mechanical power output from striated muscle during cyclic contraction. *The Journal of Experimental Biology.* **114**, 493-512 (1985).
9. Ahn, A.N. How muscles function - the work loop technique. *Journal of Experimental Biology.* **215** (7), 1051-1052 (2012).
10. Sawicki, G. S., Robertson, B. D., Azizi, E., Roberts, T. J. Timing matters: tuning the mechanics of a muscle-tendon unit by adjusting stimulation phase during cyclic contractions. *Journal of Experimental Biology.* **218** (19), 3150-3159 (2015).
11. Libby, T., Chukwueke, C., Sponberg, S. History-dependent perturbation response in limb muscle. *Journal of Experimental Biology.* **223** (1), jeb199018 (2020).
12. Askew, G. N., Marsh, R. L., Ellington, C. P. The mechanical power output of the flight muscles of blue-breasted quail (*Coturnix chinensis*) during take-off. *Journal of Experimental Biology.* **204** (21), 3601-3619 (2001).
13. Sponberg, S., Libby, T., Mullens, C. H., Full, R. J. Shifts in a single muscle's control potential of body dynamics are determined by mechanical feedback. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences.* **366** (1570), 1606-1620 (2011).
14. Loeb, G. E., Brown, I. E., Cheng, E. J. A hierarchical foundation for models of sensorimotor control. *Experimental Brain Research.* **126** (1), 1-18 (1999).
15. Wakeling, J. M., Tijs, C., Konow, N., Biewener, A. A. Modeling muscle function using experimentally determined subject-specific muscle properties. *Journal of Biomechanics.* **117**, 110242 (2021).
16. The Mathworks, Inc. *MATLAB:R2021a.* (2021).
17. Tenan, M. S., Tweedell, A. J., Haynes, C. A. Analysis of statistical and standard algorithms for detecting muscle

onset with surface electromyography. *PLOS ONE.* **12** (5), e0177312 (2017).

18. Roberts, T. J., Gabaldón, A. M. Interpreting muscle function from EMG: lessons learned from direct measurements of muscle force. *Integrative and Comparative Biology.* **48** (2), 312-320 (2008).
19. Rice, N., Bemis, C. M., Daley, M. A., Nishikawa, K. Understanding muscle function during perturbed *in vivo* locomotion using a muscle avatar approach. *Journal of Experimental Biology.* **226** (13), jeb244721 (2023).
20. Silva Cornachione, A., Cação Oliveira Benedini-Elias, P., Cristina Polizello, J., César Carvalho, L., Cláudia Mattiello-Sverzut, A. Characterization of Fiber types in different muscles of the hindlimb in female weanling and adult wistar rats. *Acta Histochemica Et Cytochemica.* **44** (2), 43-50 (2011).
21. Hämäläinen, N., Pette, D. The histochemical profiles of fast fiber types IIB, IID, and IIA in skeletal muscles of mouse, rat, and rabbit. *Journal of Histochemistry & Cytochemistry.* **41** (5), 733-743 (1993).
22. Charles, J. P., Cappellari, O., Spence, A. J., Hutchinson, J. R., Wells, D. J. Musculoskeletal geometry, muscle architecture and functional specialisations of the mouse hindlimb. *PLOS ONE.* **11** (4), e0147669 (2016).
23. Nishikawa, K. Titin: A Tunable spring in active muscle. *Physiology (Bethesda, Md.).* **35** (3), 209-217 (2020).
24. Dick, T. J. M., Biewener, A. A., Wakeling, J. M. Comparison of human gastrocnemius forces predicted by Hill-type muscle models and estimated from ultrasound images. *The Journal of Experimental Biology.* **220** (Pt 9), 1643-1653 (2017).
25. Daley, M. A., Biewener, A. A. Leg muscles that mediate stability: mechanics and control of two distal extensor muscles during obstacle negotiation in the guinea fowl. *Philosophical Transactions of the Royal Society B: Biological Sciences.* **366** (1570), 1580-1591 (2011).
26. Seth, A., Sherman, M., Reinbolt, J. A., Delp, S. L. OpenSim: a musculoskeletal modeling and simulation framework for in silico investigations and exchange. *Procedia IUTAM.* **2**, 212-232 (2011).
27. Sandercock, T. G., Heckman, C. J. Doublet potentiation during eccentric and concentric contractions of cat soleus muscle. *Journal of Applied Physiology (Bethesda, Md.: 1985).* **82** (4), 1219-1228 (1997).
28. Marsh, R. L. How muscles deal with real-world loads: the influence of length trajectory on muscle performance. *The Journal of Experimental Biology.* **202** (Pt 23), 3377-3385 (1999).
29. Hessel, A. L., Monroy, J. A., Nishikawa, K. C. Non-cross bridge viscoelastic elements contribute to muscle force and work during stretch-shortening cycles: evidence from whole muscles and permeabilized fibers. *Frontiers in Physiology.* **12**, 648019 (2021).
30. Lindstedt, S., Nishikawa, K. Huxleys' missing filament: form and function of titin in vertebrate striated muscle. *Annual Review of Physiology.* **79**, 145-166 (2017).
31. Abbate, F., De Ruiter, C. J., Offringa, C., Sargeant, A. J., De Haan, A. In situ rat fast skeletal muscle is more efficient at submaximal than at maximal activation levels. *Journal of Applied Physiology.* **92** (5), 2089-2096 (2002).
32. Eng, C. M., Smallwood, L. H., Rainiero, M. P., Lahey, M., Ward, S. R., Lieber, R. L. Scaling of muscle

architecture and fiber types in the rat hindlimb. *Journal of Experimental Biology.* **211** (14), 2336-2345 (2008).

33. Manuel, M., Chardon, M., Tysseling, V., Heckman, C.J. Scaling of motor output, from mouse to humans. *Physiology (Bethesda, Md.).* **34** (1), 5-13 (2019).
34. Zajac, F. E. Muscle and tendon: properties, models, scaling, and application to biomechanics and motor control. *Critical Reviews in Biomedical Engineering.* **17** (4), 359-411 (1989).
35. Rice, N. Understanding muscle function during in vivo locomotion using a novel muscle avatar approach. *ProQuest Dissertations and Theses.* at <https://libproxy.nau.edu/login?url=https://www.proquest.com/dissertationstheses/understanding-muscle-function-during-em-vivo/docview/2444890224/se-2?accountid=12706> (2020).