Indiana University School of Medicine
Institutional Animal Care and Use Committee (IACUC) Animal Protocol Review Form

For IACUC Office Use Only					
Protocol Number:		Old Protocol Number:			
Approval Date:		3-Year Expiration Date:		Next Review Date:	
Amendment #:		Amendment Approval Date:			

Section A
General Information

THIS FORM MUST BE SUBMITTED AS A WORD FILE VIA EMAIL TO: somiacuc@iupui.edu

Approval is renewable annually for up to an additional two years.
Continuation of the approved animal usage beyond three years requires completion of a new application form and complete IACUC review.

Principal Investigator and General Protocol Information

Title of Project:	Example Protocol

Principal Investigator:	Dr. Example	Degree(s):	Ph.D.
Campus Address:	1234 Research Dr.	Department	Medicine
Campus Phone:	12345	IU e-mail address:	example@iu.edu

CO- Principal Investigator:		Degree(s):	
Campus Address:		Department:	
Campus Phone:		IU e-mail address:	

Study Type - Check all that apply				
x	**Research Study**		**Teaching Study**	
	Pilot Study		**Study Other (Explain)**	
	Replacement Study for an expiring protocol. Please provide the study number of the expiring study this application will replace			
x	**Amendment**	Proceed to the "**Amendment Summary of Changes**" section below		

Current Funding
This section is required because the Institution/IACUC must implement a process for ensuring what sponsored programs support is consistent with the approved activities involving animals.

Funding Sources	Grant Title(s)	Name of PI(s) on Grant
NIH	Example	Dr. Example
DOD	Example of Protocol	Dr. Example

Note: If funded by VA, you must complete the Animal Component of the Research Protocol Form instead of this form.

Amendment Summary of Changes

In the text box below; please summarize all proposed changes to the protocol and follow these guidelines:

1. Use track changes to modify the document
2. **Do not delete prior text** from this amendment summary. Place new text for the amendment summary above any previous amendment text in the box below;
3. Include the amendment number (i.e. A01, A02), and the date, to distinguish from previous approved amendments.
4. Modify the appropriate protocol sections with the information relevant to the changes requested in the amendment.
5. In section F below (Protocol Summary and AAALAC Procedures Check List), Animal Procedures, place an "X" next to each subsection. Update the corresponding subsection of the protocol.
6. The Purpose, Goals, and Hypothesis section should be updated with the added amendment changes.
7. If a new procedure is added which may cause pain or distress, a new literature search for the consideration of alternatives is required. .
8. If you are requesting additional animals, ensure that you justified the number of required animals.

A1

This amendment separates the final in vivo muscle testing timepoint from the in vitro muscle testing timepoint (updated in Section E). In the original protocol, mice were to remain under anesthesia after the final in vivo muscle test in order to undergo a non-survival surgery to excise muscles for in vitro mechanical analysis. There is some concern that prolonged exposure to isoflurane during the in vivo protocol could affect the mechanical properties of the muscle once excised. To minimize this potential effect, animals will be allowed to recover from isoflurane anesthesia from in vivo testing for approximately 24-48 hrs prior to undergoing isoflurane anesthesia for non-survival surgery to excise muscles for in vitro analysis.

Protocol Summary and AAALAC Procedures Check List

This Checklist is part of your application.

For New Studies:

The "Core Sections" and Protocol Associates Supplement are required.

For Section D, "Animal procedures", place an X next to each procedure and complete the corresponding supplemental section to be included in this application.

For Amendments:

List which of the following sections is being modified in the "Amendment Summary" section, above. If you are adding a procedure, make sure to complete the supplemental section(s) to be included in the amendment application.

B: Summary, Experimental Design, Rational, and Animal Numbers			
C: Research Sites			
D: Animal Procedures (Check all that apply)			
		D 01: Breeding, Weaning, and Genotyping	
	x	D 02: Anesthesia, Sedation, Analgesia	
		D 03: Surgical Procedures	
		x	Non-survival Surgery
			Single Survival Surgery
			Multiple Survival Surgery
	x	D 04: Agent Administration	
	x	D 05: Irradiation, Imaging with Ionizing Radiation, and Other Radioisotope Administration	
	x	D 06: Blood Sampling	
	x	D 07: Behavioral Testing	
	x	D 08: Special Caging, Husbandry, Food/Water Deprivation/Restriction	
		D 09: Immunization	
		D 10: Hybridoma	
		D 11: Prolonged Restraint	
E: Potential Experimental Complications and Emergency Management Plan			
F: Euthanasia and Disposition			
G: Justification for the Use of Animals, Unnecessary Duplication & the Three R's			
H: PI Assurance			

Protocol Associates Supplement

Abbreviations. Please list all abbreviations/acronyms in alphabetical order and include their definition.

FBA: fine branch arboreal environment
NLL: non-linear locomotion environment
IP: intraperitoneal
RPI: reference point indentation
DEXA: duel energy x-ray absorptiometry
pQCT: peripheral quantitative computed tomography

Section B
Summary, Experimental Design, Rationale, and Animal Numbers

Relevance of the proposed project to human/animal health and summary of animal work
Non-Technical Summary
(aka Lay Summary)

This section will be evaluated by non-scientists, avoid the use of terms that would be unfamiliar to non-scientists. Please define technical terms in language the general public would understand.

Describe how the proposed research addresses an underlying medical or scientific problem and how it will advance human or animal health, or scientific knowledge, for the good of society.

The musculoskeletal system is comprised of highly dynamic tissues that adapt to mechanical stimulations. Weight-bearing physical activity is often used as a non-pharmacological intervention to treat various bone- and muscle-related diseases, such as osteoporosis and sarcopenia (age-related loss of skeletal muscle mass and strength). Numerous studies have shown that high-impact exercise, resulting in higher peak musculoskeletal strains, has the potential to elicit positive responses in bone and muscle biomechanical properties. However, high-impact loading is also associated with increased risks for musculoskeletal injury, including skeletal fracture and muscle fatigue damage. This suggests that a high-impact exercise regimen may not be appropriate for all patients, especially those who already have an increased risk of fracture compared to healthy individuals. For this reason, researchers have begun examining low-intensity physical activity as a potential therapy for patients already at risk of fracture.

Describe what will happen to the animals during the studies, with particular emphasis on major procedures that may impact their welfare.

Animal studies of controlled loading environments have shown that the positive response of bone is modulated by several factors, one of which is load orientation. In some studies, mechanical loads originating from multiple, off-axis directions have elicited significant positive changes in bone structure which are correlated with a decreased risk of fracture, even when load magnitudes are relatively low. This suggests that low-intensity activities that involve loads originating from multiple directions could represent a viable alternative strategy to high-impact exercise for improving musculoskeletal biomechanical properties. Yet, we know that the musculoskeletal systems of growing and adult individuals behave differently to mechanical loading (exercise) regimens. The studies contained in this protocol will address the combined musculoskeletal effects of low-impact, multi-directional loading on the growing and adult mouse musculoskeletal systems in order to address questions of growth and timing in these low-impact, multi-directional loading regimens.

Describe potential complications that can arise from the experiments and what efforts will be done to minimize pain and distress.

The animals could potentially develop infection at the site of electrode insertion during in vivo muscle testing and/or the site of reference probe insertion during in vivo bone mechanical testing. The complications will be managed upon notification and we will work with the LARC veterinary staff to minimize pain and distress of the animal.

Please provide a rationale and hypothesis for your study.
(This section is to be more scientifically specific compared to the “Non-Technical Summary” above)
(Do not exceed 1 page)
This section will help the IACUC understand the scientific justification for the proposed research. Please address the following areas when completing this section.

1) State the global hypothesis or central hypotheses of the proposed research.
2) If there is a direct relationship with a grant, you can add the specific aims or objectives in this section.

Low-impact, multi-directional mechanical loading will result in positive changes in bone and muscle structure and mechanical properties at the tissue and organ levels.

Experimental Design Groups

Explain the experimental design and all animal procedures. This description should allow the IACUC to understand the experimental course of an animal from its entry into the experiment to the endpoint of the study.

For **each** separate experiment, provide

1) the specific objective/hypothesis to be tested including the main outcome analysis/variable
2) the experimental groups and their size (n/group) **NOTE: the statistical justification for group sizes is requested in a subsequent section)**
3) a simple sequential list of all procedures performed on animals beginning with procurement/acclimation and ending with final disposition
4) a very brief statement of why any procedure is being done and provide a summary of animal numbers for each experiment (details of the procedures should be described in section D)

If new studies are proposed in an amendment, they should be added here, with the amendment number and new text at the top of the box.

Specific Aim 1: To demonstrate the osteogenic and myogenic effects of 2 experimental, continuous-activity enclosures on the growing mouse musculoskeletal system (Fine Branch Arboreal environment and Non-Linear Locomotion environment) compared to their respective controls.

Weanling C57BL/6 mice (~4 weeks) will be raised to ~4 months in both experiments, and will be subjected to baseline and monthly in vivo measurements of bone structural and material properties (dual-energy x-ray absorptiometry, reference point indentation) and muscle mechanical properties (ankle dorsiflexion and plantarflexion tetanic torque) to longitudinally track osteogenic and myogenic responses to the exercise regimen.

Approximately two weeks prior to euthanasia, in order to label active bone remodeling sites, animals will be injected with calcein (10 mg/kg, subcutaneously or IP, ~0.4 mL volume) using a 1-7-1-3 schedule meaning that label will be injected, approximately seven days will be allowed to pass (at least 5 days, no more than 10), another label will be administered, and then the animals will be euthanized approximately three days later (no less than two, no more than seven). Following euthanasia bones and muscles will be collected for later analyses, including mechanical testing (the primary outcomes of the study) and histomorphometry.

Immediately prior to sacrifice, animals will undergo a non-survival surgery where select calf muscles will be excised under deep anesthesia to permit measurement of their linear mechanical (contractile) properties in vitro.

Major outcomes for experiments of Aim 1: Assessments of bone and muscle structural and material properties in vivo and ex vivo in a mouse model of low-impact, multi-directional loading. Bone outcomes include strength, stiffness and toughness. Muscle function outcomes include length-tension, force-velocity, force-frequency, stiffness, and fatigability.

Experiment 1-1: Examination of the osteogenic and myogenic responses of the Fine Branch Arboreal (FBA) experimental enclosure in the growing mouse.

The specific objective of this experiment is to examine the longitudinal effects on bone and muscle structural and mechanical properties of an exercise regimen that either prevents (control environment) or encourages grasping with hands and feet while balancing and climbing above narrow substrates (FBA environment) (Fig 1). This exercise regimen results in low-impact, multi-directional mechanical loading on the musculoskeletal system, which may be an important treatment strategy for improving bone and muscle health in individuals with an already elevated fracture risk and who should not engage in high-impact activities.

FBA Environment: This experimental design differentiates between an environment where climbing is mandatory and an environment where climbing is not possible (Fig. 1). Experimental mice are group housed (n=10/enclosure) in an enclosure (2ft x 2ft x 2ft) that is traversed by numerous simulated fine branches that cross one another at consistent 45 degree angles. In the FBA enclosure, weanlings will be introduced to the climbing environment and food and water will be placed adjacent to the nesting site. The floor of the terrarium will be flooded with less than 2 cm of water. After subjects acclimate to these conditions for 1 week, the water

level will be raised to approximately 10 cm and the nest, food, and water will be placed in random locations within the FBA environment to encourage exploratory climbing behavior. Control mice will be group housed (n=10/enclosure) in a vertically-stratified enclosure which does not encourage climbing behaviors and prevents manual and pedal grasping, and the weanlings will be introduced in a similar manner with nest, food, and water all provided near to each other before randomizing after 1 week to encourage exploration. In both control and experimental enclosures, the location of the nest, food, and water, will be randomly changed every week.

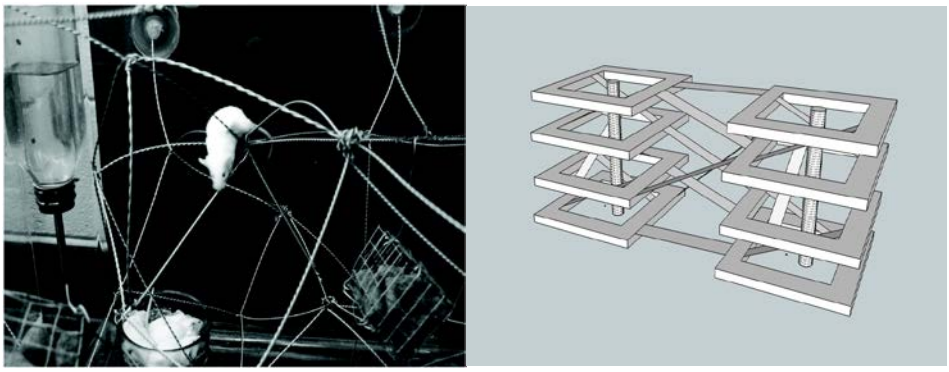


Figure 1. Fine Branch Arboreal Environment. Experimental enclosure on left; control on right.

Groups:

- Mice group housed in FBA experimental environment
- Mice group housed in the control environment

Sequential list of procedures:

- Acquisition from vendor
- Acclimation at IUSM for minimum of 3 days
- Random assignment to FBA or control environment groups (water flood level in floor of FBA environment < 2 cm)
- Anesthesia for tail vein blood draw and kinematic marker placement, recovery, locomotor kinematics analysis, ear punching/notching for identification
  - At baseline and monthly for up to ~4 months
- Recovery period of at least 1 day
- Anesthesia and in vivo musculoskeletal imaging (pQCT) followed immediately by bone densitometry scanning (DEXA)
  - At baseline and monthly for up to ~4 months
- Recovery period of at least 1 day
- Anesthesia and in vivo muscle function analysis (ankle torque) of one leg followed by SC injection of buprivacaine and lidocaine (2 separate injections) prior to in vivo bone material property analysis (BioDent) of the other leg
  - At baseline and monthly for up to 5 months
- At the end of Week 1, water flood level in floor of FBA environment raised to ~10 cm
- 1^{st} IP injection of calcein for dynamic histomorphometric analysis ex vivo ~12 days prior to sacrifice
- 2^{nd} IP injection of calcein for dynamic histomorphometric analysis ex vivo ~4 days prior to sacrifice
- Approximately 24-48 hrs after the final in vivo testing timepoint, mice will be anesthetized for a non-survival surgery to isolate muscles for in vitro testing followed immediately by euthanasia.

Total animals for Experiment 1-1 = 40 C57BL/6 mice (2 groups x 20 mice/group). Two experiments of 10 mice/group will be conducted in series in order to reduce error associated with building multiple FBA experimental enclosures.

Experiment 1-2: Examination of the osteogenic and myogenic responses of the Non-Linear Locomotion (NLL) experimental enclosure in the growing mouse.

The specific objective of this experiment is to examine the longitudinal effects on bone and muscle structural and mechanical properties of an exercise regimen that encourages only linear locomotion (control environment) or encourages only non-linear locomotion (NLL environment). This exercise regimen results in low-impact, multi-directional mechanical loading on the musculoskeletal system, which may be an important treatment strategy for improving bone and muscle health in individuals with an already elevated fracture risk and who should not engage in high-impact activities.

NLL Environment: This experimental design differentiates between non-linear locomotion (diverse-orientation loading) and linear locomotion (stereotypic-orientation loading) (Fig. 2). Mice are housed individually in modified "shoebox" experimental and control enclosures. Enclosures are constructed from standard mouse cages with modified tunnel apparatuses installed. The wire top of each enclosure is replaced with standard acrylic to prevent climbing behaviors. Food and water sources are placed at opposite ends of the enclosure, encouraging animals to traverse the enclosure numerous times a day.

Groups:

- Mice individually housed in NLL experimental environment
- Mice individually raised in the control environment

Sequential list of procedures:

- Acquisition from vendor
- Acclimation at IUSM for minimum of 3 days
- Random assignment to NLL or control environment groups
- Anesthesia for tail vein blood draw and kinematic marker placement, recovery, locomotor kinematics analysis
  - At baseline and monthly for up to ~4 months
- Recovery period of at least 1 day
- Anesthesia and in vivo musculoskeletal imaging (pQCT) followed immediately by bone densitometry scanning (DEXA)
  - At baseline and monthly for up to ~4 months
- Recovery period of at least 1 day
- Anesthesia and in vivo muscle function analysis (ankle torque) of one leg followed by SC injection of buprivacaine and lidocaine (2 separate injections) prior to in vivo bone material property analysis (BioDent) of the other leg
  - At baseline and monthly for up to ~4 months
- 1^{st} IP injection of calcein for dynamic histomorphometric analysis ex vivo ~12 days prior to sacrifice
- 2^{nd} IP injection of calcein for dynamic histomorphometric analysis ex vivo ~4 days prior to sacrifice
- Approximately 24-48 hrs after the final in vivo testing timepoint, mice will be anesthetized for a non-survival surgery to isolate muscles for in vitro testing followed immediately by euthanasia.

Total animals for Experiment 1-2 = 40 C57BL/6 mice (2 groups x 20 mice/group)

Specific Aim 2: To demonstrate the osteogenic and myogenic effects of 2 experimental, continuous-activity enclosures on the adult mouse musculoskeletal system (Fine Branch Arboreal environment and Non-Linear Locomotion environment) compared to their respective controls.

Weanling C57BL/6 mice (~4 weeks) will be raised to ~4 months in control environments (experiment-specific controls as well as control mice raised in standard mouse caging). At approximately 4 months old, when mice are typically considered skeletally mature, animals will be transferred to experimental enclosures (FBA and NLL) and raised until ~8 months. Once they are transferred to experimental enclosures, mice will be subjected to baseline and monthly in vivo measurements of bone structural and material properties (dual-energy x-ray absorptiometry, reference point indentation) and muscle mechanical properties (ankle dorsiflexion and plantarflexion tetanic torque) to longitudinally track osteogenic and myogenic responses to the exercise regimen.

Approximately two weeks prior to euthanasia, in order to label active bone remodeling sites, animals will be injected with calcein (10 mg/kg, subcutaneously or IP, ~0.4 mL volume) using a 1-7-1-3 schedule meaning that label will be injected, approximately seven days will be allowed to pass (at least 5 days, no more than 10), another label will be administered, and then the animals will be euthanized approximately three days later (no less than two, no more than seven). Following euthanasia bones and muscles will be collected for later analyses, including mechanical testing (the primary outcomes of the study) and histomorphometry.

Immediately prior to sacrifice, animals will undergo a non-survival surgery where select calf muscles will be excised under deep anesthesia to permit measurement of their linear mechanical (contractile) properties in vitro.

Major outcomes for experiments of Aim 2: Assessments of bone and muscle structural and material properties in vivo and ex vivo in a mouse model of low-impact, multi-directional loading. Bone outcomes include strength, stiffness and toughness. Muscle function outcomes include length-tension, force-velocity, force-frequency, stiffness, and fatigability.

Experiment 2-1: Examination of the osteogenic and myogenic responses of the Fine Branch Arboreal (FBA) experimental enclosure in the skeletally mature mouse.

The specific objective of this experiment is to examine the longitudinal effects on bone and muscle structural and mechanical properties of an exercise regimen that either prevents (control environment) or encourages grasping with hands and feet while balancing and climbing above narrow substrates (FBA environment) (Fig 1). This exercise regimen results in low-impact, multi-directional mechanical loading on the musculoskeletal system, which may be an important treatment strategy for improving bone and muscle health in individuals with an already elevated fracture risk and who should not engage in high-impact activities.

Groups:

- Weanling mice group housed in the control environment for ~4 months and then transferred to the FBA experimental environment for ~4 months
- Weanling mice housed in standard mouse enclosures for ~4 months and then transferred to the FBA experimental environment for ~4 months.
- ~4 month old mice purchased from LARC approved vendor

Sequential list of procedures:

- Acquisition of weanling or skeletally mature mice from vendor
- Acclimation at IUSM for minimum of 3 days
- Assignment to enclosure
  - Weanling mice: Random assignment to control environment or standard caging groups and raised to ~4 months of age before transfer to the FBA experimental enclosure
  - Skeletally mature mice: Assignment to FBA experimental enclosure after 3 day acclimation period at IUSM
- Animals transferred to FBA experimental enclosure (water flood level in floor of FBA environment < 2 cm).
- Anesthesia for tail vein blood draw and kinematic marker placement, recovery, locomotor kinematics analysis, ear punching/notching for identification
  - At baseline (~4 months) and monthly for up to ~8 months
- Recovery period of at least 1 day
- Anesthesia and in vivo musculoskeletal imaging (pQCT) followed immediately by bone densitometry scanning (DEXA)
  - At baseline (~4 months) and monthly for up to ~8 months
- Recovery period of at least 1 day
- Anesthesia and in vivo muscle function analysis (ankle torque) of one leg followed by SC injection of buprivacaine and lidocaine (2 separate injections) prior to in vivo bone material property analysis (BioDent) of the other leg
  - At baseline (~4 months) and monthly for up to ~8 months
- At the end of Week 1, water flood level in floor of FBA environment raised to ~10 cm
- 1^{st} IP injection of calcein for dynamic histomorphometric analysis ex vivo ~12 days prior to sacrifice
- 2^{nd} IP injection of calcein for dynamic histomorphometric analysis ex vivo ~4 days prior to sacrifice
- Approximately 24-48 hrs after the final in vivo testing timepoint, mice will be anesthetized for a non-survival surgery to isolate muscles for in vitro testing followed immediately by euthanasia.

Total animals for Experiment 2-1 = 60 C57BL/6 mice (3 groups x 20 mice/group). Again, these experiments will be conducted in series (10 mice/group) in order to minimize error associated with building multiple FBA enclosures.

Experiment 2-2: Examination of the osteogenic and myogenic responses of the Non-Linear Locomotion (NLL) experimental enclosure in the skeletally mature mouse.

The specific objective of this experiment is to examine the longitudinal effects on bone and muscle structural and mechanical properties of an exercise regimen that encourages only linear locomotion (control environment) or

encourages only non-linear locomotion (NLL environment). This exercise regimen results in low-impact, multi-directional mechanical loading on the musculoskeletal system, which may be an important treatment strategy for improving bone and muscle health in individuals with an already elevated fracture risk and who should not engage in high-impact activities.

Groups:

- Weanling mice individually housed in the control environment for ~4 months and then transferred to the NLL experimental environment for ~4 months
- Weanling mice housed in standard mouse enclosures for ~4 months and then transferred to the NLL experimental environment for ~4 months.
- ~4 month old mice purchased from LARC approved vendor

Sequential list of procedures:

- Acquisition of weanling or skeletally mature mice from vendor
- Acclimation at IUSM for minimum of 3 days
- Assignment to enclosure
  - Weanling mice: Random assignment to control environment or standard caging groups and raised to ~4 months of age before transfer to the NLL experimental enclosure
  - Skeletally mature mice: Assignment to NLL experimental enclosure after 3 day acclimation period at IUSM
- Anesthesia for tail vein blood draw and kinematic marker placement, recovery, locomotor kinematics analysis
  - At baseline (~4 months) and monthly for up to ~8 months
- Recovery period of at least 1 day
- Anesthesia and in vivo musculoskeletal imaging (pQCT) followed immediately by bone densitometry scanning (DEXA)
  - At baseline (~4 months) and monthly for up to ~8 months
- Recovery period of at least 1 day
- Anesthesia and in vivo muscle function analysis (ankle torque) of one leg followed by SC injection of buprivacaine and lidocaine (2 separate injections) prior to in vivo bone material property analysis (BioDent) of the other leg
  - At baseline (~4 months) and monthly for up to ~8 months
- 1st IP injection of calcein for dynamic histomorphometric analysis ex vivo ~12 days prior to sacrifice
- 2nd IP injection of calcein for dynamic histomorphometric analysis ex vivo ~4 days prior to sacrifice
- Approximately 24-48 hrs after the final in vivo testing timepoint, mice will be anesthetized for a non-survival surgery to isolate muscles for in vitro testing followed immediately by euthanasia.

Total animals for Experiment 2-2 = 60 C57BL/6 mice (3 groups x 20 mice/group)

Description of non-surgical procedures

Tail vein blood draws. Animals will undergo blood draws via tail vein (maximum 0.2 ml) to measure serum biomarkers of bone (OC, osteocalcin; BALP, bone alkaline phosphatase) and muscle growth (IGF-1, insulin-like growth factor-1; CK, creatinine kinase). Blood will be drawn under isoflurane anesthesia prior to undergoing in vivo skeletal imaging procedures described below. Blood draws will occur at baseline, and then monthly until ~4 months.

Ear punch or notch for identification of group housed mice. Standard procedure for mouse identification will be performed under isoflurane inhalation anesthesia. For notches, a small wedge-shaped notch (~3mm) will be made with sterilized scissors on the outer edge of the pinna (externa ear). For punches, a sterilized ear punch instrument will be utilized to create a hole or gap at the edge or center of the pinna. The notches or punches may be single or double in either or both ears, consistent with established rodent numbering systems. Scissors and ear punches will be cleaned with 70% ethanol between animals of the same enclosure; fresh sterile instruments will be used for each enclosure.In vivo bone mechanical property testing. Bone material properties will be evaluated by in vivo reference point indentation (Biodent). Animals will be mildly sedated using inhalation isoflurane. The skin overlying the anterior tibia will be shaved and aseptically prepared. A local anesthetic will be injected just below the skin at the site of testing. The reference probe will be inserted through the skin/muscle to contact the bone and a series of indentations (up to 10) will take place on the tibia. The probe is roughly the size of an 18-gauge needle. Based on our experience with dogs and rats, animals show no post-testing complications and ambulate normally upon recovery from anesthesia. We expect this procedure to take ~10-20 minutes per animal.

In vivo muscle function testing. At each testing session, animals will be anesthetized in an induction box hooked up to an

inhalation isoflurane system. Once under anesthesia the animals will be transferred to a nose cone apparatus and placed on a warming pad to maintain body temperature. The skin of the leg will be shaved and aseptically prepared and then transferred, with the warming pad, to the testing system platform. The animal's foot will be secured with tape onto the footplate of the joint torque testing apparatus in order to measure muscle contractile force during ankle plantarflexion and dorsiflexion. Two sterile shielded monopolar stimulated electrodes will be inserted near the common peroneal nerve under the skin in order to stimulate a muscle twitch response. Electrode placement may be adjusted to achieve maximum twitch response and then a series of stimulation protocols will be initiated to measure plantorflexion isometric twitch torque, plantarflexion isometric tetanic torque, dorsiflexion isometric twitch torque, and dorsiflexion isometric tetanic torque. Muscle stimulation will be at frequencies up to 300 Hz. Maximum voltage will not exceed 20V. The testing protocol will take approximately 45 minutes per stimulation protocol (dorsiflexion or plantarflexion). Following testing, animals will 1) be removed from the nose cone apparatus and allowed to recover or 2) undergo non-survival surgery to harvest muscles for in vitro testing.

In vitro muscle function testing. In some animals, in vitro tests of muscle function may be performed to further characterize the muscle adaptations to arterial insufficiency and to determine if the in vivo muscle function is limited by vascular perfusion rather than muscle adaptations.

List the total number of animals requested for all experiments described above. If more than 1 species, list total for each species.	200

Species and Number of Animals

Provide the information requested in the table below.

- **Category B:** Animals that will be bred or purchased for breeding, but not used for experiments. This includes breeders, offspring that cannot be used because of improper genotype or gender and any other animals that will not participate in the research studies.

- **Category C:** Animals used in research, experiments, or tests which involve no pain or distress or only momentary or slight pain or distress that WOULD NOT REQUIRE anesthetic, analgesic or tranquilizing agents (examples: s.c., i.m., i.p. or percutaneous i.v. injection, a brief period of restraint, tissue harvesting after euthanasia has been performed).

- **Category D:** Animals used in research, experiments, or tests where appropriate anesthetic, analgesic, or tranquilizing agents are used to avoid pain or distress (examples: major and minor surgery, tissue or organ collection prior to euthanasia, retro-orbital blood collection, prolonged restraint accompanied by tranquilizers or sedatives). Animals used in research, experiments, or tests which, if they experience pain or distress cannot be treated with an anesthetic, analgesic or tranquilizer, but the agent or procedure producing the pain/distress is immediately discontinued or the animal is euthanized to prevent pain and/or suffering.

- **Category E:** Animals used in research, experiments, or tests involving pain or distress in which the use of appropriate anesthetic, analgesic or tranquilizing agents would have adversely affected the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests (examples: studies which allow endpoints that are painful or stressful, addictive drug withdrawals without treatment, pain research, noxious stimulation). ***IF YOU LIST ANIMALS IN THIS CATEGORY YOU MUST PROVIDE A DETAILED JUSTIFICATION***

Species	**Strain/ Nomenclature/ genotype**	**Weight or Age**	**Source ("LARC Vendor" is acceptable)**	**Total Number of Animals per Category**			
				B	**C**	**D**	**E***
Mouse	C57BL/6	Weanling (~4 weeks)	LARC Vendor		160		
Mouse	C57BL/6	~4 months	LARC Vendor		40		
			Total Numbers of Animals		200		

NOTE: These totals should match the number of animals needed for experiments and those generated from breeding (used and not used from the breeding table in the breeding section)

FOR CATEGORY E ANIMALS ONLY

Provide a scientific justification to explain why the use of anesthetics, analgesics, sedatives or tranquilizers during and/or following painful or distressing procedures is contraindicated:

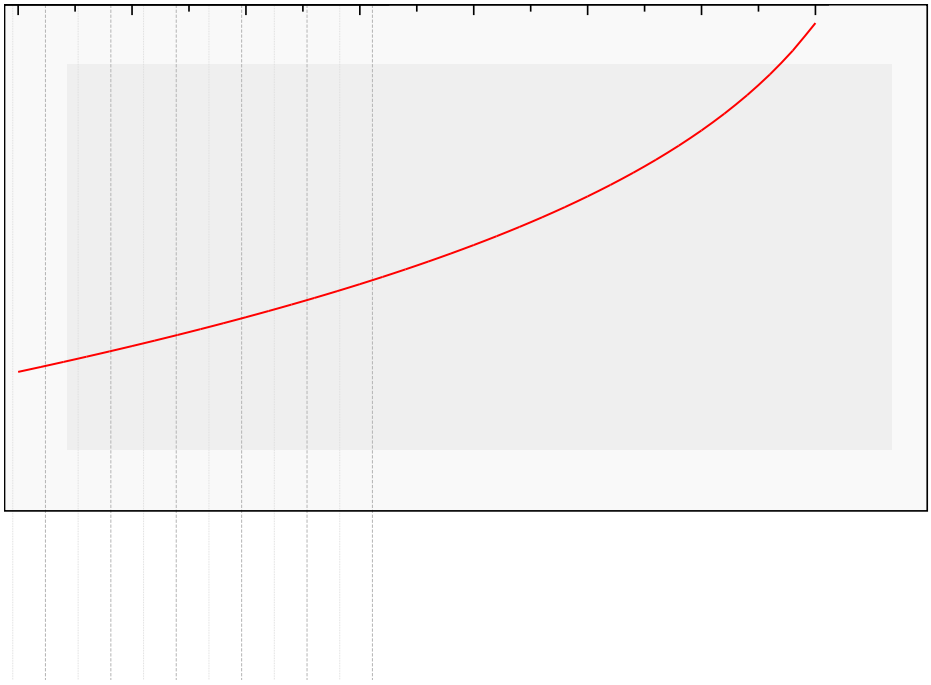
Number Justification (address each species individually by copying/pasting this table)	
Species:	Mouse

The number of animals requested for this protocol is based on the following (select all that apply):

x	A statistical estimate (power analysis) is used to estimate the number of animals and experimental groups. Please provide the justification and details below.

We have not performed these measurements previously in mice, but we assume that measurement variability will be similar to what we observe in rats. Using preliminary in vivo isometric torque data from normal Sprague Dawley rats on protocol 10613, we estimate that a sample size of 16 mice/group for the FBA and NLL experiments (as well as their respective controls) will give us >85% power to detect a 15% difference in plantarflexion peak isometric torque (see figure below) and a 15% difference in dorsiflexion peak isometric torque.

In this protocol we request the use of 20 animals/group in order to account for potential attrition during the experimental timeline.



Outcome being measured: Plantarflexion peak isometric torque
Expected variability (SD): 0.03 Nm
Minimum scientifically meaningful treatment effect: 15%

	The estimated minimum number necessary to achieve the goals of the study in the absence of a statistical estimate. Explain:
	The number necessary to obtain sufficient tissue or other material for testing or analysis, i.e. collection of cells for in vitro experiments. Explain:
	The number required to provide sufficient technical training or practice for the number of trainees expected. Explain:
	Other. Explain:

Go to Beginning of Document | Go to Protocol Summary and AAALAC Procedures Check List

Section C
Research Sites/Use Areas

Where will animals be housed?

x	LARC
	Methodist Research Institute (MRI)
	Other. Please specify the animal facility:

For other institutions, have you submitted an IACUC form to those campuses?

	Yes
	No

Will all live animal procedures or transportation be done inside the designated animal facility?

x	Yes	If Yes, go to the next section
	No	If No, complete this section

If live animals are used outside the designated animal facility: Please identify each location outside the designated animal facility and list the procedure. When listing procedure please only use the terms that are in *Protocol Summary and AAALAC Procedures Check List*

Building and Room Number	Procedure
RR 123	1. Ear punching/notching 2. Kinematic analysis of gait 3. In vivo muscle and bone mechanical property testing 4. Tail vein blood draws 5. Non-survival surgery for in vitro muscle testing 6. Euthanasia/necropsy
RR 213	1. In vivo imaging (DEXA and pQCT)

Lab Housing > 12 hours

If animals used in this protocol will be housed or held outside of any animal facility for more than twelve (12) consecutive hours, please complete the table with the appropriate information

Building and Room Number	Number of Animals at Any One Time	Length of time animals will be housed	Contact Person and Phone Number of person who is responsible for animal care

Justification for housing animals outside the designated animal facility for more than 12 consecutive hours:

If the below listed procedures are in this study, please provide the location for record maintenance.

Survival surgery and recovery	RR 123
Anesthesia	RR 123
Food, water and husbandry (if maintained outside animal facility)	

Will any live animals be transported to Veteran's Administration (VA) facilities, or utilized by VA researchers while on VA time?

	Yes	(Approval is required from the Veterans Administration Research & Development Committee)
x	No	

Go to Beginning of Document | Go to Protocol Summary and AAALAC Procedures Check List

Section D
Animal Procedures

Section D 02
Anesthesia, Sedation, Analgesia

If anesthesia is required, list the purpose of the anesthesia and the maximum frequency an animal will be subjected to anesthesia. Then proceed to and complete the remaining sections.

Pre-Anesthetic Regimen – Select One

x	Pre-Anesthetic Regimen from **Appendix 2** will be applied. **Cut and paste** the information from Appendix 2 in the space below.

- Isoflurane inhaled to effect, with waste gas scavenging
- Xylazine (5-10mg/kg) + ketamine (90-150mg/kg) IP
- Xylazine (2.5-5mg/kg) + acepromazine (1.0-2.5mg/kg) + ketamine (90-100mg/kg) IP
- Dexmedetomidine (0.5mg/kg) + ketamine (75mg/kg) IP
- Sodium pentobarbital (50-90mg/kg) IP
- Thiopental (30-100mg/kg) IP, IV
- Tribromoethanol [Avertin®] [for single survival anesthesia only] (125-250mg/kg) IP
- *Atipamezole (0.1-1mg/kg) SC or IP; Reversal agent for xylazine and dexmedetomidine*

	Other Pre-Anesthetic regimen will be followed as specified. List the length of time for withholding food and/or water:

Anesthetic/Sedation Regimen - Induction drug(s) - Select One	
x	Anesthetic/Sedation from **Appendix 2** will be applied. **Cut and paste** the information from Appendix 2 in the space below.

- Isoflurane inhaled to effect, with waste gas scavenging
- Xylazine (5-10mg/kg) + ketamine (90-150mg/kg) IP
- Xylazine (2.5-5mg/kg) + acepromazine (1.0-2.5mg/kg) + ketamine (90-100mg/kg) IP
- Dexmedetomidine (0.5mg/kg) + ketamine (75mg/kg) IP
- Sodium pentobarbital (50-90mg/kg) IP
- Thiopental (30-100mg/kg) IP, IV
- Tribromoethanol [Avertin®] [for single survival anesthesia only] (125-250mg/kg) IP
- *Atipamezole (0.1-1mg/kg) SC or IP; Reversal agent for xylazine and dexmedetomidine*

	Other criteria will be followed as specified. Provide the sedative/tranquilizers (name, dose, & route of administration). The maintenance anesthetic agents(s) (name, dose, & route of administration).

Anesthesia Monitoring	
How will you determine that the plan of anesthesia is adequate. How frequently will you monitor for adequate anesthesia? Also, indicate when additional anesthetic will be administered.	
Visual examination, reflexes	
x	Anesthesia Monitoring from **Appendix 3** will be applied. **Cut and paste** the information from Appendix 3 in the space below.

Signs of adequate anesthesia: Rodents will be unresponsive to surgical or procedural stimulation. They will not have withdrawal reflexes when their rear toes are pinched. Incisions will not be made until loss of reflex responses has occurred. Anesthetized animals have regular respiration rates. Their ears and feet remain pink, indicating that peripheral perfusion is adequate. A source of supplemental heat (recirculating hot water pad, microwaveable gel pack, hot water bottle, or heating pad) will be used to prevent hypothermia.
Criteria for administration of additional anesthetic: Respiration rate increases in response to surgical or procedural stimulation, withdrawal reflexes return. Animals should not be re-dosed with injectable anesthetics more than once. The LARC veterinary staff should be consulted if the selected anesthesia does not appear to be effective with the species or strain.
Monitoring frequency during procedure: Visually monitor every 2-3 minutes during the procedure. Because the surgeon and anesthesiologist are typically the same individual when working with these species, it is not necessary to document monitoring during the procedure. However, the fact that the animal was monitored should be described in a surgery log (e.g. surgery monitoring sheet, notation in lab notebook, etc). Records are kept by the P.I. (Records should be available for review by the IACUC during semi-annual inspections.)

	Other monitoring criteria will be used as specified. Describe Below:

Neuromuscular blocking agents (pancuronium, succinylcholine, other paralytic drugs) - Select One	
x	These agents will not be used
	These agents will be used. If checked, complete the section below
List the drug(s), dosage(s) and route of administration. Describe in detail how you will determine that an adequate level of anesthesia is being maintained while the animal is paralyzed. Paralytic agents cannot be used without anesthetics and assisted ventilation.	

Record keeping parameters (this question is for USDA species only) – Select all parameters which will be monitored and recorded.			
x	Visual Examination		EKG
	Heart Rate		Blood Pressure
	Respiration		Temperature
x	Reflexes		O_2
			EEG

Anesthetic Recovery Monitoring - Select One	
	Not applicable. These criteria do not apply to this study.
x	Anesthetic Recovery from **Appendix 4** will be applied. **Cut and paste** the information from Appendix 4 in the space below.
Monitoring frequency during recovery period: Rodents will be directly observed at least once every 15 minutes until they can respond to gentle manipulation and have regained a righting reflex. They will be kept in a warmed recovery cage that contains a solid substrate. They will not be returned to the animal room until they are fully recovered from anesthesia. This is necessary to ensure that the PI can intervene if there are problems with the surgical site (e.g. incision opens when animal begins moving), to ensure that post-procedural pain has been alleviated, and to minimize communication errors with the LARC staff (e.g. anesthetized animals reported as ill). Anesthetic Monitoring records: The fact that the animal was monitored should be described in a surgery log (e.g. surgery monitoring sheet, notation in lab notebook, etc). Records are kept by the P.I. (Records should be available for review by the IACUC during semi-annual inspections.)	
	Other procedures will be applied
If other criteria will be applied, described them here	

Analgesia Management/Analgesic drugs - Select One	
	Not applicable. These criteria do not apply to this study
x	Non USDA species- General guidelines and analgesic drugs will apply as outlined in **Appendix 5**. **Cut and paste** the information from Appendix 5 in the space below.
• Buprenorphine 0.05-2.0mg/kg SQ every 6-12 hours SQ, IP, or IM • Buprenorphine 0.05-2.0mg/kg every 6-12 hours SQ or IM + Carprofen 5mg/kg once daily • Butorphanol 2-5mg/kg SQ or IM every 4 hours • Carprofen 5-10mg/kg PO or SQ daily; can be combined with opioids • Meloxicam 1.0-10.0mg/kg SQ, IP daily; can be combined with opioids • Morphine 5-10mg/kg SQ every 2-4 hours • Local: lidocaine, lidocaine/bupivicaine, lidocaine patch, bupivicaine.	
x	Non USDA species - The list for specific analgesia drugs are described in the table below.
	USDA species - The list for specific analgesia drugs are described in the table below. Guidelines for each species can be found in **Appendix 5,** but must be listed individually in this table.

Species	Procedure	Analgesic Name	Dose	Route	Minimum Duration
Mouse	Bone mechanical property testing	Bupivacaine Lidocaine	2.5 mg/ml 10 mg/ml (mixed in 1:1 volume not to exceed more than 1 mg/kg of each)	Local SC dose	Given prior to testing

Scientific Justification if analgesic drugs cannot be given
If analgesic drugs cannot be given, provide scientific reason.

For USDA covered species, you must consult with a LARC Veterinarian on the pain management plan:	
Provide the date(s) you consulted with LARC Veterinarian:	

Name of LARC Veterinarian:	

Compliance with pharmaceutical grade drugs	
Investigators are expected to use pharmaceutical-grade anesthetics and analgesia whenever possible. Please consult the IACUC Policy on Non-Pharmaceutical grade medications to be aware of recent clarifications to this policy.	
x	**Yes**, I have read and understand the IACUC policy on Non-pharmaceutical grade medications
List any non-pharmaceutical grade anesthesia and analgesia proposed.	
For any of the non-pharmaceutical grade anesthesia and analgesia proposed above, will the criteria outlined in the IACUC policy on Non-pharmaceutical grade medications be applied?	
	No. Please provide justification for a deviation from IACUC policy
	Yes. The criteria outlined in the IACUC policy will be used

Section D 03
Surgical Procedures

Select All that Apply	
x	Non-Survival/Terminal Surgery
	Single Survival Surgery
	Multiple Survival Surgeries
Provide scientific justification for multiple survival surgeries:	

Is the surgery taking place outside a LARC facility?	
	Yes (please make sure the room where the surgery is taking place is listed in the core section of the protocol)
	No

x	Pre-surgical preparation of the animal described in **Appendix 6** will be followed. **Cut and paste** the information from Appendix 6 for the species in this study in the space below.
Instruments are re-sterilized between rodents using an autoclave or bead sterilizer. Instruments will not be used on more than 4-5 rodents prior to re-autoclaving. Sterile drapes are used if the abdominal or thoracic cavities are opened. Aseptic technique is used. Surgery Area: The laboratory area is disinfected before use. A sterile field can be prepared through the placement of a sterile drape. Hands are washed prior to donning surgical gloves. Surgeon Prep: The surgeon wears sterile surgical gloves, cap, mask and clean lab coat or gown. Patient Prep: If hair is present over the incision site, it is removed with clippers to include an area twice as large as the intended surgery site. Skin is disinfected using three alternating rounds of surgical disinfectant scrub/solution (betadine, hibiclens, chlorhexadine, etc.) with 70% isopropyl alcohol or sterile saline rinses. Care is taken to avoid over-wetting fur outside of the surgical area as this will increase hypothermia. (Consider application of disinfectant/alcohol using cotton-tip applicators rather than larger gauze squares which may be harder to control application in small areas). Instrument Prep: Instruments, suture, wound clips, and implanted devices are sterilized in an autoclave or ethylene oxide sterilizer prior to surgery and a sterile field is maintained during surgery.	
	Other pre-surgical preparation will be will be followed as described below.

Detailed description of each surgical procedure
Muscle Isolation. Calf muscles will be isolated and harvested under deep anesthesia prior to euthanasia for in vitro muscle testing in order to preserve the integrity of the muscle. A skin incision will be made over the leg and the skin reflected to expose the muscle(s) to be harvested. The muscle(s) will be carefully isolated, beginning with the distal tendon (Achilles tendon), and then excised with euthanasia occurring immediately afterward.

	Post-operative care for the animal described in **Appendix 7** will be followed. **Cut and paste** the information from Appendix 7, for the species in this study, in the space below.
	Other post-operative care for the animals will be will be followed as described below.

If there is an implanted device, provide the approximate dimensions of the implanted device and describe how the device will be sterilized and the method and frequency of monitoring.

Section D 04
Agent Administration

Chemical Agents	
Compliance with pharmaceutical grade agents. Investigators are expected to use pharmaceutical-grade agents whenever possible. Note that per federal guidelines the use of non-pharmaceutical grade agents requires justification, even for acute procedures. Please consult the IACUC Policy on Non-Pharmaceutical grade medications to be aware of recent clarifications to this policy.	
If any non-pharmaceutical grade agent is used, will the criteria outlined in the IACUC policy on non-pharmaceutical grade agents be applied (i.e., justification from "always acceptable" list)?	
	Yes. The criteria outlined in the IACUC policy will be used
x	No. Please provide justification for a deviation from IACUC policy

Pharmaceutical, Non-pharmaceutical grade and Non-hazardous compounds				
Non-Pharmaceutical grade (yes or no)	Agent Name	Route of administration & volume	Max Dose (mg/kg, gm, mL, etc.)	Frequency
Yes	Calcein	IP or subcutaneously	10 mg/kg, dosed in ~0.4 mL	2 times

Please provide justification for using any Non-Pharmaceutial grade computnds listed in the above table.

Hazardous Compounds (including, carcinogens, toxins, teratogens, etc.)						
Agent Name	Type of agent	Route of administration & volume	Max Dose (mg/kg, gm, mL, etc.)	Frequency	Route of excretion	Will LARC or lab workers be exposed?

Biohazard agents (biological toxins, blood, body fluids, normal/neoplastic tissues or cells, recombinant DNA, etc.)					
Protocols using biohazard agents will not receive IACUC approval until the Investigator provides evidence of approval from the Institutional Biosafety Committee (IBC). If you are unsure, please contact the IBC office.					
Do you have an IBC approved protocol for the work described in this study?					
	Yes. Please provide the IBC protocol #				
	No. Please contact the IBC https://research.iu.edu/policies/institutional-biosafety-committee.html				
Agent Name	ABS Level; 1, 2, 2+3 Precautions, or 3	Route of administration & volume	Is the agent infectious to humans?	Is the agent infectious to animals?	Is the agent shed in feces, urine, or body secretions?
Note: It is required that the LARC BSL2 veterinarian be notified prior to any work with biohazard or hazardous agents. See notice in the assurance section.					

Section D 05
Irradiation/Imaging with Ionizing Radiation, and Other Radioisotope Administration

Check all equipment/procedures that will be used in this study. To avoid delay in approval, all procedures checked below must be clearly described in the "Timeline Section". If you are doing imaging that does not involve radiation, please list the details in the experimental procedures.

	***In Vivo* Therapeutics Core Irradiators**
	Whole-body irradiation
You must obtain the Irradiation protocol and SOP for the Irradiators from the In Vivo Therapeutics Core Manager (274-8811) and submit both documents with this submission.	

	Indiana Institute for Biomedical Imaging Sciences (IIBIS) Facilities
	IndyPET II or III small animal PET scanner (R2)
	HR+ PET scanner (R2)
	mCT PET/CT scanner (GH)
	Biograph PET/CT scanner (IUSCC)
	microCT (R2)
	Fluoroscope (R2)
	Other scanner
List imaging outside IIBIS	
Do you have IIBIS approval for these procedures?	
	Yes. Provide IIBIS #
	No. IIBIS must be contacted before the study can begin.

	Machine-Produced Radiation - non-IIBIS Facilities	
	Fluoroscope	
x	X-ray	
x	CT	
	microCT	
	Gamma Knife	
	Proton Beam	
	Other, please list:	
Please provide the name of the facility and/or the approval source(s):		GE Lunar PIXImus2 dual energy x-ray absorptiometer (DEXA); Norland Stratec XCT Research SA+ peripheral quantitative computed tomography (pQCT) scanner

	Injection of long half-life radionuclides into a live animal, e.g., [H3], [C14], [P32], [S35], etc. This does NOT include PET or SPECT tracers.
Does the PI (or a collaborator) have a Radiation Safety permit for possession of the radionuclides?	
	Yes. Provide a copy of the permit with this submission.
	No. You must secure permission to possess the radionuclide before the study can begin.

Section D 06
Blood Sampling

Please use ranges when completing the below table.				
Species	**Method of withdrawal**	**Volume of each withdrawal (e.g. mL)**	**Total number of withdrawals per animal**	**Interval between withdrawals**
Mouse	Tail Vein	0.2 ml	N=5 starting at baseline	~4 weeks

If you are performing longitudinal studies, please list the number of times and time intervals this procedure will be performed. (i.g. – glucose tolerance test and pharmacokinetics)

Section D 07
Behavioral Testing

Describe all behavioral tests used in this protocol and provide scientific justification for the need for conducting these tests, especially for those tests that cause the animal distress (e.g., such as physical or acoustic startle tests.)

Assessment of Locomotor Kinematics. We will evaluate gait parameters in our experimental and control animals in order to assess whether or not kinematic strategies for maintaining balance are different among groups. To collect kinematic variables, 2 separate enclosures will be used: 1) For the FBA experiment, an enclosure that includes only a single slender ~50 cm long "branch" (~2.5 mm in diameter); 2) For the NLL experiment, an enclosure that includes on a single horizontal board ~50 cm long (~10 cm wide). Experimental subjects from treatment and control groups will be mildly sedated with isoflurane and the skin over over the hip, knee, ankle, shoulder, elbow, and wrist will be shaved and marked with reflective tape and/or dots of non-toxic white paint. Subjects will be placed at one end of their appropriate kinematic analysis enclosure and allowed to cross voluntarily. Each crossing of the substrate will be recorded with high-speed video (~100-200 frames/sec), and video will be digitized in order to measure a number of locomotor parameters such as speed, limb phase, duty factor, mean limb support number, hindlimb stride length, hindlimb stride frequency and hindlimb flexion.

Please provide the following information:

a) The number of animals used for each behavioral test.
b) The length of time an animal will be subjected to the testing for a single episode.
c) The total number of times an animal will be tested in this study.
d) The length of time for animal recovery between test episodes.

Approximately 5 animals from each study group (experimental and control) will be assessed for locomotor kinematics at baseline and then monthly until the end of the experiment (4 total kinematic analysis sessions/animal). Each locomotor kinematic testing session is expected to last ~30-45 minutes.

List any special behavioral testing equipment that will be used, and how it will be cleaned or sanitized between uses.

2 separate enclosures will be built: 1) for the FBA experiment, an enclosure that includes only a single slender ~50cm long "branch" (~2.5 mm in diameter); 2) for the NLL experiment, an enclosure that includes only a single flat horizontal board ~50 cm long (~10 cm wide). High speed video cameras will record locomotor patterns from outside the enclosures. The enclosures and substrates within them will be cleaned with 70% ethanol in between subjects.

Section D 08
Special Caging, Husbandry, Food/Water Deprivation/Restriction

Will this protocol require any other special animal husbandry requirements for any species? (Variations in caging size, housing density, cage change frequency, dietary manipulations, etc.) Consult appendix for standard environmental conditions.

	No. Only standard BSL-1 housing conditions will be required	
x	Yes. Please select all that apply:	
	x	Caging type
		Bedding type
		Maximum cage density
		Environmental Enrichment
		Sanitization interval
		Diet (e.g. Wet Food, Treated Food)
		Water (e.g. Treated Water)
		Room temperature
		Light cycle
		Other

Please describe and provide scientific justification for the requested modification(s) from routine husbandry that have been checked.

The FBA enclosure is not technically a wire bottom cage, as the floor of the terrarium is solid and will be flooded with ~10 cm of water. However, because the locomotor substrates in the FBA enclosure are wire, I felt it was important to indicate this here. The justification for using these wire substrates is to encourage animals to develop pedal grasping in combined with increased lateral movements of the tail to facilitate balance in this enclosure, and to prevent falling. By encouraging this grasping and balancing behavior, we are subjecting these animals to low-impact, multi-directional

mechanical loading of their feet, legs, and tails (at least), which is the ultimate goal of this exercise regimen.

FBA Environment: This experimental design differentiates between an environment where climbing is mandatory and an environment where climbing is not possible (Section E, Fig. 1). Experimental mice are group housed (10 mice/enclosure) in an 2 ft x 2 ft x 2 ft enclosure that is traversed by numerous wire "branches" that cross one another at ~45 degree angles. In the FBA enclosure, weanlings will be introduced to the climbing environment and food and water will be placed adjacent to the nesting site. The floor of the terrarium will be flooded with less than 2 cm of water. After subjects acclimate to these conditions for 1 week, the water level will be raised to approximately 10 cm and the nest, food, and water will be placed in random locations within the FBA environment to encourage exploratory climbing behavior. Control mice will be group housed (10 mice/enclosure) in a vertically-stratified enclosure which does not encourage climbing behaviors and prevents manual and pedal grasping, and the weanlings will be introduced in a similar manner with nest, food, and water all provided near to each other before randomizing after 1 week to encourage exploration.

NLL Environment: This experimental design differentiates between non-linear locomotion (diverse-orientation loading) and linear locomotion (stereotypic-orientation loading) (Section E, Fig. 2). Mice are housed individually in experimental and control enclosures, which are modified "shoebox" mouse enclosures. Enclosures are constructed from standard rat cages with modified tunnel apparatuses installed. The wire top of each enclosure is replaced with standard acrylic to prevent climbing behaviors. Food and water sources are placed at opposite ends of the enclosure, encouraging animals to traverse the enclosure numerous times a day.

Our intention is to build these enclosures to be housed in LARC facilities, and we have already discussed this with Dr. Debra Hickman (mid-December 2013).

FBA experiment animals will be group-housed (10 mice/enclosure, experimental and control), and NLL experiment animals will be individually housed (10 experimental enclosures and 10 control enclosures).

Food and/or Water Deprivation/Restriction See **Appendix 1** for Food and Water Restriction or Deprivation
Provide a brief justification for fasting and/or water deprivation. Include a description of how animals will be monitored for distress (i.e., what physiological signs indicate distress; general criteria may be specified), and how frequently animals will be monitored. If water is also to be withheld, provide justification. In rodents, withholding food overnight is considered food deprivation/restriction and must have scientific justification. In other species, fasting for purposes other than surgery preparation must be justified. *This section does not pertain to withholding food as standard preparation for surgery in higher mammals.*

Go to Beginning of Document | Go to Protocol Summary and AAALAC Procedures Check List

Section E
Potential Experimental Complications and Emergency Management Plan
Consult one of the LARC Vets if needed (http://medicine.iu.edu/larc/faculty/faculty/)

Can the animals be euthanized for health reasons before completion of the study?	
	No Please provide a scientific justification of why early euthanasia (humane endpoints) cannot be used on this study.
x	**Yes** Please answer the following questions.
Describe any expected complications/symptoms. Include induced disease condition including animal phenotypes from breeding and/or complications from surgeries.	
The animals could potentially develop infection at the site of electrode insertion during in vivo muscle testing and/or the site of reference probe insertion during in vivo bone mechanical testing.	
Describe how the animals will be monitored for the development of these complications/symptoms. Include the frequency of monitoring.	

If these animals were to develop heath issues, we will work with the LARC veterinarians to assess animal health and if it is determined by the vet that an animal needs to be euthanized we will follow their recommendation.
How will the complications/symptoms be managed/treated prior to euthanasia?
Per LARC veterinary staff advice
Who will be responsible for performing the monitoring and managing the complications?

Name:	Dr. Example		
Campus Phone:	4-1234	Emergency Phone:	123-456-7890

Describe the criteria that will be used in this study to determine if and when animals will be euthanized humanely prior to the planned termination of the experiment.
Place entire animal in fridge for PI.
In the case of emergency veterinary care; please list the classes of drugs that cannot be used.
No
In the case of an animal emergency, is initiating euthanasia by the LARC veterinary staff in **the absence of PI staff** acceptable?

x	**Yes.** Please describe what samples should be collected post-mortem. Please include information about how the samples should be stored (e.g. formalin fixed, blood collected in EDTA, frozen, etc). Include any devices that need to be collected, as well.
Place entire animal in fridge for PI.	
	No. Please explain

Go to Beginning of Document | Go to Protocol Summary and AAALAC Procedures Check List

Section F
Euthanasia and Disposition

Final Disposition of Animal	
x	Euthanasia. If checked, please complete the below questions.
	Return to Colony
	Transfer to a Different Protocol (following IACUC and LARC procedures to transfer animals)
	Other:

The IUSM IACUC and LARC adhere to the approved methods of euthanasia as recommended by the American Veterinary Medical Association Panel on Euthanasia. If animals are to be euthanized under this protocol, even if different procedures within the protocol require separate methods, agents, dosages or routes of administration to accomplish euthanasia, this section should be completed for each euthanasia procedure. Note that a secondary method of euthanasia is required to ensure death.

List your primary method of euthanasia

Examples include: anesthetic overdose, carbon dioxide delivered by a gas cylinder, flow meter, and regulator (the use of dry ice is unacceptable), decapitation with anesthesia, etc.

Species	Agents	Method, Dosages (mg/kg) or Routes (IM, IV, IP) of Administration
Mouse	Carbon dioxide chamber or Isoflurane chamber	

List your secondary method of euthanasia

Examples include: pneumothorax, exsanguination, cervical dislocation, perfusion, decapitation, other.
NOTE: Cervical dislocation cannot be used in rats > 200 g body weight.

Species	Method
Mouse	Bilateral pneumothorax with scalpel as secondary method

Other Methods of Euthanasia: Physical Method Alone
A physical method of euthanasia such as cervical dislocation or decapitation without sedation or anesthesia requires a scientific justification. Please provide a detailed description of the proposed method(s) and the justification for this method(s) be used

Please note: If LARC Staff are requested to perform euthanasia, a written request specifying the animal(s) and date to perform such euthanasia must be signed by the principal investigator, co-investigator, faculty sponsor or responsible technician.

Go to Beginning of Document | Go to Protocol Summary and AAALAC Procedures Check List

Section G
Justification, Duplication & Painful Procedures

Animal Justification	
The justification for using live vertebrate animals rather than alternative means of achieving the research goal is: (check all that apply)	
x	The complexity of the processes being studied cannot be duplicated or modeled in simpler systems because:
Alternatives to studying mechanical properties of bone and muscle are available in vitro through tissue/cell cultures. However, these methods do not allow one to assess longitudinal changes in mechanical properties after intervention. In order to study the effect of low-impact multidirectional loading on the growing and adult skeleton in vivo and longitudinally, it is necessary to use live animals. The in vivo imaging procedures and muscle testing procedures are common methods for assessing anatomy and physiology. The in vivo bone testing procedures described here are relatively new to the bone research community and there is very little literature (outside of our own publications) where reference point indentation is used in vivo – other studies have used this procedure ex vivo with success in the mouse.	
	There is not enough information known about all the processes being studied to design nonliving models. (explain):
	Other (explain):

Species Justification	
Address each species individually. (if there is more than one species, copy and paste this table for each species)	
Species:	Mouse
This species was selected for the study because of the following attributes (select all that apply):	
	A large database exists allowing comparisons with previous data. (explain):
x	The anatomy or physiology is uniquely suited to the study proposed. (explain):
Studying mechanical properties of the musculoskeletal system requires the use of vertebrate species. We have chosen to use a rodent model because there is a vast literature on skeletal biology to which we can compare our data. While much of the classic skeletal muscle literature centers on frog thigh musculature, the applicability to human health and disease is not as direct as it is with mammalian models. There are no suitable alternatives to study how bone and muscle mechanical properties adapt together to low-impact loading environments in vivo with a non-animal approach such as tissue culture.	
	This is the lowest species on the phylogenetic scale that is suitable for the proposed study. (explain):
	Other attributes (details required):

Duplication	
x	I certify that I have determined that the research proposed is not unnecessarily duplicative.

Painful Procedures	
Note: The IACUC recommends the use of this site if you need assistance: http://libguides.library.umkc.edu/alternatives	
	For all pain category D (anesthesia / analgesia provided to relieve potential pain) and pain category E (pain not relieved by anesthesia / analgesia) animals use procedures, **by checking this box,** I certify that I have reviewed the pertinent scientific literature and the sources and/or databases noted in this application and found no scientifically acceptable alternative to any of those procedures that would result in less pain or distress.

Literature Search for Alternative to Painful Procedures (Maintain a copy of the search, but do not submit it to the IACUC office)									
Place an X in the checkboxes that apply to indicate which databases were used:									
	Ovid Medline		EMBASE		OneSearch	x	PubMed Medline		Other
Date(s) the database search was performed:	1/28/2014								
Years covered by the search *(e.g., 1985 to present):*	January 1950-Present								
Keywords used in the search:									
Did the literature search reveal less painful alternatives to the potentially painful procedures that were proposed?									
	No alternatives were found								
	Yes, but they cannot replace the procedures that were proposed for the following reason(s):								

Potentially Painful Procedures in this Protocol (match to keywords used)	Write a BRIEF explanation why the alternatives found to this potentially painful procedure were not acceptable alternatives.
Non-animal alternative bone muscle mechanical loading mouse exercise bone muscle in vivo	Alternatives to studying mechanical properties of bone and muscle are available in vitro through tissue/cell cultures. However, these methods do not allow one to assess longitudinal changes in mechanical properties after intervention.

Go to Beginning of Document | Go to Protocol Summary and AAALAC Procedures Check List

Section H
Investigator Assurance

Review each statement and check each box to indicate agreement. Completion of the checkboxes and the signing of this form are the responsibility of the principal investigator. Completion of the approval process will fulfill Public Health Service and USDA requirements under the federal Animal Welfare Act, and will serve as documentation for users and the public of Indiana University School of Medicine's commitment to humane care and use of animals.

I certify that:	
x	These studies will be conducted in compliance with Public Health Service (PHS) policy, the Animal Welfare Act, “Guide for the Care and Use of Laboratory Animals", and other applicable University policies and procedures.
x	All individuals listed on the protocol will read and understand the appropriate sections of the protocol, will enrolled in the occupational health program, and will received appropriate training in the procedures that they will be conducting prior to participating in the study.
x	The IACUC will be notified regarding any unexpected study results that impact the welfare of the animals and any unanticipated pain or distress, morbidity, or mortality will be reported to the appropriate veterinary staff and the IACUC as soon as possible.
x	All procedures, treatments, anesthetic and analgesic regiments will be adhered to as outlined in this protocol and any changes to these studies will be submitted to the IACUC via an amendment form and not initiated until approved by the IACUC.
x	The proposed work will utilize pharmaceutical grade compounds whenever possible, as is consistent with PHS policy, and the use of non-pharmaceutical grade materials, when necessary will be carried out in accordance with policies of the Indiana School of Medicine IACUC.
x	The proposed work is congruent with the scope of any grants or external funding arrangements listed in the funding section of this protocol.
x	I will alert the LARC BSL2 veterinarian (http://medicine.iu.edu/larc/faculty/faculty/) before starting any animal work with a biohazardous agent. This ensures that appropriate signage can be generated and posted, and any other pertinent SOPs or paperwork can be reviewed and/or completed

I acknowledge responsibility for this protocol.	
	1/30/2014

Dr. Example	
Typed Name or Electronic Signature of PI	**Date**