UNIVERSITY OF CALIFORNIA,
IRVINE

Strategies for overcoming thermal constraints on skeletal muscle function in ectotherms

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

by

Jordan P. Balaban

Dissertation Committee:
Associate Professor Emanuel Azizi, Chair
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2018

DEDICATION

To my family for their encouragement,

to Laura for her love and support

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5. **Balaban, J.P.**, Azizi, E. 2017. Lowering metabolic rate mitigates muscle atrophy in western fence lizards. *Journal or Experimental Biology*. 220, 2748-2756.

4. Bizzarro, J.J., Peterson, A.N., Blaine, J.M., **Balaban, J.P.**, Greene, H.G., & Summers, A.P. 2016. Burrowing behavior, habitat, and functional morphology of the Pacific sand lance (*Ammodytes personatus*). *Fishery Bulletin*. 114(4), 445–460.

3. **Balaban, J.P.**, Summers, A.P., & Wilga, C.A. 2015. Mechanical Properties of the Hyomandibula in Four Shark Species. *Journal of Experimental Zoology, Part A*. 323(1), 1–9.

2. Cressman, V.L., **Balaban, J.**, Steinfeld, S., Shemyakin, A., Graham, P., Parisot, N., & Moore, H. 2010. Prefrontal and thalamic inputs to the basal amygdala prune at distinct stages during adolescence in the rat. *Journal of Comparative Neurology*. 518(14), 2693-2709.

1. Kleinhaus, K., Steinfeld, S., **Balaban, J.**, Goodman, L., Craft, T. S., Malaspina, D., Myers, M.M., Moore, H. 2010. Effects of Excessive Glucocorticoid Receptor Early Stimulation during Gestation on Psychomotor and Social Behavior in the Rat. *Developmental Psychobiology*. 52(2), 121–132.

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23. **J Balaban**, E Azizi. Elastic energy storage broadens the thermal performance range of accelerating lizards. Oral. Annual conference for the Society for Integrative and Comparative Biology. San Fransisco, CA. January 2018. *Integrative and Comparative Biology*. 58(suppl 1), e10.

22. A Nguyen, **JP Balaban**, E Azizi, RJ Talmadge, AK Lappin. Fatigue Resistant Jaw Muscles Facilitate Long-lasting Courtship Behavior in the Southern Alligator Lizard (*Elgaria multicarinata*). Oral. Annual conference for the Society for Integrative and Comparative Biology. San Fransisco, CA. January 2018. *Integrative and Comparative Biology*. 58(suppl 1), e164.

21. **J Balaban**, E Azizi. Lizards amplify muscle power to maintain performance at low temperatures. Oral. Southwest Organismal Biology Meeting. Claremont, CA. October 2017.

20. N McMasters[*], **J Balaban.** Temperature effects on muscle biology and locomotion in fence lizards. Poster. Conference for the summer undergraduate research program (**SURF**). University of California, Irvine. Irvine, CA. August 2017.
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19. **J Balaban**[*], E Azizi. Do lizards use springy tendons to maintain running performance at low temperatures? Oral. Winter Ecology and Evolutionary Biology Graduate Student Symposium. University of California, Irvine. Irvine, CA. March 2017.
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18. **J Balaban**, E Azizi. Elastic energy storage and thermal performance in fence lizards. Oral. Annual conference for the Society for Integrative and Comparative Biology. New Orleans, LA. January 2017. *Integrative and Comparative Biology*. 57(suppl 1), e198.

17. **J Balaban**, E Azizi. Temperature effects on muscle-tendon interactions in fence lizards. Oral. Southwest Organismal Biology Meeting. Fullerton, CA. November 2016.

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15. E Azizi, **J Balaban**, N Holt. Contractile and connective tissue interactions in skeletal muscles. Oral. International Congress on Vertebrate Morphology. Washington, DC. July 2016.

14. G Hernandez[*], **J Balaban**, E Azizi. Effects of Metabolic Rate on Muscle Atrophy. Poster. UCI Undergraduate research symposium. 2016.
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13. **J Balaban**, E Azizi. Reduced Metabolic Rate Mitigates Muscle Atrophy in Western Fence Lizards. Oral. Annual conference for the Society for Integrative and Comparative Biology. Portland, OR. January 2016. *Integrative and Comparative Biology*. 56(suppl 1), e10.

12. **J Balaban**, E Azizi. The Effect of Metabolic Rate on Muscle Atrophy in Western Fence Lizards. Oral. Southwest Organismal Biology Meeting. Pomona, CA. September 2015.

11. **J Balaban**, E Azizi. Effects of metabolic rate on muscle atrophy in a hibernating lizard. Oral. Winter Ecology and Evolutionary Biology Graduate Student Symposium. University of California, Irvine. Irvine, CA. March 2015.

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7. I Nowinowski[*], **J Balaban**, CA Wilga. Shape Changes in Hyoid Arch Elements in Four Shark Species. Oral. Annual conference for the Society for Integrative and Comparative Biology. Austin, TX. January 2014. *Integrative and Comparative Biology*. 53(suppl 1), e154.
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6. **J Balaban**, AP Summers, CA Wilga. Mechanical Properties of a Shark Jaw Support Structure. Oral. Annual conference for the Society for Integrative and Comparative Biology. San Fransisco, CA. January 2013. *Integrative and Comparative Biology*. 53(suppl 1), e9.

5. **J Balaban**. A Biomechanical Analysis of Jaw Suspension. Oral. Society for Integrative and Comparative Biology, Northeast Divisions of Vertebrate Morphology and Comparative Biomechanics meeting. Kingston, RI. November 2011.

4. AP Summers, **J Balaban,** N Gidmark, JJ Bizzarro. Integrating Behavior and Functional Constraints in Sand Lance: Why Do They Burrow Where They Burrow. Oral. American Fisheries Society Conference. Seattle, WA. September 2011.

3. **J Balaban**, JJ Bizzarro, AP Summers. Burrowing Preference and Capability in the Pacific Sand Lance. Poster. Society for Integrative and Comparative Biology. Salt Lake City, UT. January 2011. *Integrative and Comparative Biology*. 51(suppl 1), e161.

2. **J Balaban**, JJ Bizzarro, AP Summers. Burrowing Preference and Capability in the Pacific Sand Lance. Oral. Society for Integrative and Comparative Biology, Northeast Divisions of Vertebrate Morphology and Comparative Biomechanics meeting. Cambridge, MA. September 2010.

1. VL Cressman, **J Balaban**, N Parisot, A Chemiakine, S Steinfeld, H Moore. 2007. Region-specific remodeling of prefrontal and thalamic inputs to the amygdala during preadolescence in the rat. Poster. Society for Neuroscience conference. San Diego, CA. 2007

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2014	Best Student Presentation, Southwest Organismal Biology Meeting
2012	University of Rhode Island Graduate Teaching Excellence Award (Biological Sciences)

SERVICE

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ABSTRACT OF THE DISSERTATION

Strategies for overcoming thermal constraints on skeletal muscle function in ectotherms

By

Jordan P. Balaban

Doctor of Philosophy in Ecology and Evolutionary Biology

University of California, Irvine, 2018

Professor Emanuel Azizi, Chair

Daily and seasonal fluctuations in the environmental temperature pose a challenge to ectotherms as they move through their environment. Their skeletal muscles must generate enough power to allow them to accelerate and move quickly enough to catch prey or escape predation, but muscle is highly temperature sensitive. Ectotherms circumvent these problems through behavioral modifications at low temperatures such as hiding and entering brumation, a hibernation-like state which can involve several months of inactivity without eating. A potential drawback to brumation is that long periods of inactivity can lead to skeletal muscle atrophy, which would lower muscle power upon the resumption of activity. Ectotherms have also evolved mechanisms to maintain locomotor performance at moderate temperatures, however the mechanisms used to maintain running performance are heretofore unknown. In my dissertation I use the western fence lizard, *Sceloporus occidentalis*, as a model running ectotherm. In chapter 1, I use *in vitro* muscle preparations and histology to find that the lowered metabolic rate conferred by a low body temperature is sufficient to mitigate muscle atrophy after long periods of muscle disuse. I then use inverse dynamics in chapter 2 to calculate hind-limb joint powers and determine that fence lizards amplify muscle power

using tendons to maintain acceleration performance at moderate temperatures. In chapter 3, I measure EMG activity in an ankle extensor muscle of running fence lizards. I combine that data with an *in silico* muscle model to determine that lizards alter the timing of activation of their muscles to cycle energy through tendons rather than muscles while running, and that this helps them maintain speed at moderate temperatures.

INTRODUCTION

In an evolutionary arms race, predators and prey must continuously evolve improvements to some combination of speed, agility, intelligence, and crypsis to avoid starvation or predation. Factors that affect speed and agility, such as acceleration and running velocity are potentially limited by the power that skeletal muscles can produce. Skeletal muscle is highly temperature sensitive, which poses a problem for ectotherms since they rely on the environmental temperature and sunlight to regulate their body temperature rather than producing their own heat as do endotherms. Operating at low temperatures is not ideal, as they must still quickly escape predation while basking during the cool hours of dawn and dusk or on cold winter days. In my dissertation I investigate the physiological and biomechanical mechanisms ectotherms have evolved to catch prey and avoid predation at low body temperatures.

Skeletal muscle contraction is thermally sensitive because it is enzymatically driven. The sliding filament theory of muscle contraction explains how the interaction of the overlapping filamentous proteins actin and myosin generate muscle force and shortening (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). The enzymatic breakdown of ATP by myosin ATPase generates the cross-bridge cycling that generates force in a skeletal muscle contraction (Rayment et al., 1993) . Enzymatic processes are temperature sensitive because molecular movement slows down as temperature decreases. Q_{10} is a measure of how temperature effects the rate of physiological processes. The maximum shortening velocity and power of skeletal muscle contractions, which are largely enzymatically driven, have a Q_{10} of around 2 (Bennett, 1984), which means that for a decrease in temperature of 10°C, the

maximum muscle shortening velocity and maximum muscle power is reduced by half.

One way animals circumvent the problem of reduced muscle performance at low temperatures is to hide and become inactive during the cold winter months. Many mammals hibernate during winter, and many ectotherms undergo a similar process called brumation. During hibernation, mammals lower their metabolic rate and also activate physiological pathways to prevent protein degradation (Ohta et al., 2006; Xu et al., 2013). Overwintering ectotherms must lower their metabolic rate as temperature decreases, but to our knowledge, they do not activate the same physiological pathways as do mammals. This may cause overwintering ectotherms, which can be inactive for several months on end, to lose substantial muscle mass and reduce their performance when they exit brumation, though this has not been measured in many species.

Rather than entering brumation, some ectotherms can maintain performance as their body temperature starts to decline. They unexpectedly maintain almost the same performance at low temperatures as they do at their optimal temperature (Marsh and Bennett, 1986b; Anderson and Deban, 2010; Deban and Lappin, 2011). In powerful, ballistic movements such as jumping or tongue projection this can be explained by the interaction of skeletal muscle with the elastic elements, such as tendons or aponeuroses, that are in-series with the muscle (Anderson and Deban, 2010; Deban and Lappin, 2011; Olberding and Deban, 2017). In vertebrates, a muscle and it associated tendon (muscle-tendon unit (MTU)) can be tuned to amplify muscle power or reduce muscle work (Roberts and Azizi, 2011). MTUs can amplify muscle power when the MTU is held at a constant length through a physical or dynamic catch mechanism while the muscle undergoes a shortening contraction, which stretches the tendon, storing elastic strain energy. When the catch is released, the energy

stored in the tendon is released as work on the skeleton, and happens at a much faster rate than muscle can do alone. Since power = work/time, the reduced time component amplifies muscle power. MTUs can also reduce muscle work during steady state running. During each stride, the MTUs in the calf of a running or hopping animal must lengthen when the foot hits the ground and then shorten to extend the ankle and propel the animal off the ground again. If the timing of muscle activation is tuned correctly, the muscle can contract isometrically while the ankle or foot tendon stores and releases work as elastic energy. A classic example of MTUs reducing muscle work is seen in hopping kangaroos (Biewener et al., 1998). Muscle shortening velocity and power are temperature sensitive, but maximum muscle force is much less so, and tendon is insensitive to temperature. Since muscle force determines the amount of work that can be stored in the tendon, the proper tuning of MTUs can lead to thermally robust animal movements (Anderson and Deban, 2010; Deban and Lappin, 2011; Olberding and Deban, 2017).

In my dissertation, I investigate the physiological and biomechanical strategies used by ectotherms to overcome the thermal constraints of skeletal muscle using the western fence lizard, *Sceloporus occidentalis* Baird & Girard 1852, as a model organism. Western fence lizards are a species of iguanid lizard in the family *Phrynosomatidae*. They can be found at all elevations and all habitats from Baja, California through parts of Washington state (Davis and Verbeek, 1972), and they feed on a variety of insects. I use fence lizards because they are an important predator and prey species in the western United States and because they use predominately sprint locomotion. Many other studies on the mechanisms animals use to overcome the effects of temperature on skeletal muscle function focus on jumping (Olberding and Deban, 2017), feeding through tongue projection (Anderson and Deban, 2010; Deban

and Lappin, 2011), and swimming (Johnston and Temple, 2002). I aim to shine a light on how running ectotherms cope with temperature fluctuations.

Lizards have different behavioral strategies for coping with environmental temperatures. At and below 15°-20°C, most species of lizard do not run away from a potential threat, instead they hold their ground and become more aggressive (Herrel et al., 2007). This is because they cannot run as quickly at these low temperatures and cannot outrun predators. Instead of actively hunting and exposing themselves to predation, lizards stop eating and go dormant at low temperatures (Navas and Carvalho, 2010). Above body temperatures of 20°-25°C, lizards quickly run away from potential predators and actively hunt for insects (Herrel et al., 2007). This poses a potential challenge because the optimal temperature of western fence lizards is around 35°C (Wilhoft and Anderson, 1960). Lizards should be around half as fast at 25°C since their muscle has a Q_{10} of around 2 (Marsh and Bennett, 1986b). However, the acceleration and sprint performance of fence lizards at 25°C is almost as good as those at 35°C.

Summary of work

The goal of my dissertation is to improve our understanding of the physiological and biomechanical mechanisms that ectotherms use to circumvent the limitations of muscle function at low temperatures.

In chapter 1, we used the western fence lizard, *Sceloporus occidentalis*, to determine whether a reduction in metabolic rate is sufficient to resist muscle atrophy. We induced atrophy through sciatic denervation of the gastrocnemius muscle and housed lizards at either

15°C or 30°C for 6-7 weeks. Following treatment, we used muscle ergometry to measure maximum isometric force, the force-velocity relationship, and contractile dynamics in the gastrocnemius. This approach allowed us to relate changes in the size and morphology to functional metrics of contractile performance. A subset of samples was used to histologically determine muscle fiber types. At 30° C, denervated muscles had a larger reduction in muscle mass, physiological cross-sectional area and maximum isometric force compared to 15°C. Maximum shortening velocity of the muscle decreased slightly in animals housed at 30°C but did not change in those housed at 15°C. Our results suggest that metabolic rate alone can influence the rate of muscle atrophy and that ectothermic vertebrates may have an intrinsic mechanism to resist muscle atrophy during seasonal periods of inactivity.

In chapter 2, we used an inverse dynamics approach to test whether western fence lizards are using stored elastic energy to amplify muscle power and maintain performance at low temperatures. We used three-dimensional kinematic and ground reaction force data to calculate the instantaneous joint powers about the ankle, knee, and hip during acceleration at 15°C, 25°C, and 35°C. Lizards were not maximally activating their hip extensor muscles at 15°C or 35°C, but the muscle power required to generate the calculated joint powers about the ankle and knee was higher than the power that the muscles are capable of generating, so our results indicate that western fence lizards are storing and releasing elastic energy in their ankle tendons during accelerations. This power amplification partially explains the relatively constant performance above 25°C. At 15°C, lizards seem to modify their behavior towards aggression rather than giving maximal effort towards sprint performance. At 35°C, lizards are reducing the energetic cost of locomotion without sacrificing the performance gains generated through power amplification in the distal joints.

In chapter 3, we answer two questions to address how western fence lizards maintain running speed above 25°C: 1) Do lizards alter the timing of muscle activation within a stride at different temperatures? and 2) Do lizards power running entirely through their muscles or do they cycle energy through elastic tendons? To address these questions we collected EMG data from the largest ankle extensor muscle, the medial gastrocnemius to measure the timing of activation within a stride at 15°C, 25°C, and 35°C and then used the muscle activation data in an *in silico* muscle work loop model at different temperatures with and without series elastic compliance. We found that western fence lizards alter the timing of muscle activation within a stride when running at different temperatures. Our work loop model indicates that lizards at all temperatures are activating their muscles to maximize work cycled through the compliant tendons rather than relying on muscle work to power locomotion. Our results provide the first evidence, to our knowledge, of an additional function for compliant MTUs: thermal robustness of running performance.

CHAPTER 1

Lowering metabolic rate mitigates muscle atrophy in western fence lizards

Originally published in the *Journal of Experimental Biology* (Balaban and Azizi, 2017)

Introduction

The contractile and mechanical properties of skeletal muscles are plastic and readily change in response to changes in loading conditions. Extended bed rest, spinal or nerve injury, and extended periods of microgravity often result in a substantial loss of contractile protein (atrophy) in the skeletal muscles of humans and other animals. This loss of muscle mass can decrease the ability of muscles to generate force, mechanical work and mechanical power and in turn compromises locomotor performance, thereby increasing the risk of predation and decreasing prey capture success.

Atrophy does not impact all muscles, or even muscle fibers, equally. In atrophied muscles, slow oxidative fibers have a decreased cross-sectional area (Caiozzo et al., 1994; Ohira et al., 2002), and represent a smaller proportion of the overall fibers compared to healthy muscle (Caiozzo et al., 1996). In contrast, larger, fast glycolytic fibers and the intermediate fast glycolytic/oxidative fibers tend to maintain their cross-sectional area and either maintain or increase as a proportion of fibers within a muscle (Caiozzo et al., 1994, 1996; Ohira et al., 2002). This pattern of remodeling results in an increase in maximal contractile speed along with a decrease in force (Caiozzo, 2002). The rates and patterns of atrophy can vary based on the frequency and pattern of recruitment prior to a disuse signal. This is thought to explain

why slow oxidative fibers, which are recruited more frequently even at submaximal levels of activation, are more prone to atrophy. This pattern can be extended to comparisons of different muscles where muscles with similar fiber type distributions atrophy at different rates if one was activated more frequently prior to disuse (Lieber, 2002). Therefore, a muscle's propensity to atrophy in response to a disuse stimulus depends on a number of factors; the relative decrease in use, fiber type composition, metabolic rate, and the method used to induce atrophy (Caiozzo, 2002; Lieber, 2002; Winiarski et al., 1987; Hudson and Franklin, 2002).

Some animals have evolved mechanisms to slow the rate of muscle disuse atrophy despite long periods of inactivity. Hibernating animals often remain completely inactive through the winter but suffer little or no loss in muscle performance. Many hibernating mammals are able to maintain a large percentage of their pre-hibernation muscle mass (James et al., 2013; Lin et al., 2012; Rourke, 2004; Wickler et al., 1991) and force (James et al., 2013; Lohuis et al., 2007). In hibernating ground squirrels, the ratios of different fiber types do not follow the typical pattern of mammalian atrophy and remain largely unchanged or are shifted towards a larger proportion of slow oxidative fibers (Rourke, 2004; Xu et al., 2013). Given the impressive ability of hibernating organisms to resist atrophy, there has been an increased focus on investigating the underlying mechanisms used to retain muscle mass and performance during long periods of disuse. Some hibernating mammals can lower their body temperature and suppress their metabolic rate below what would be expected given the Q_{10} of metabolic reactions (Geiser, 2004; Muleme et al., 2006; Staples, 2014). Additionally, hibernating ground squirrels have been shown to activate the PGC-1 α mediated exercise pathway to prevent muscle atrophy and shift muscle fibers towards fatigue resistance (Xu

et al., 2013). Antioxidant production has also been shown to be up-regulated in hamsters during brief periods of arousal from hibernation (Ohta et al., 2006; Okamoto et al., 2006), as well as in Australian burrowing frogs aestivating at high temperatures (Young et al., 2013). The up-regulation of antioxidants can counteract the reactive oxygen species that increase the rate of proteolysis through mechanisms such as the ubiquitin-proteasome system (Powers et al., 2007; Bonaldo and Sandri, 2013; Schiaffino et al., 2013). These diverse strategies of metabolic rate suppression, antioxidant production, and exercise pathway activation appear to provide hibernating organisms the unique ability to mitigate muscle atrophy despite significant periods of inactivity.

The various strategies used by animals to resist muscle atrophy during hibernation are not mutually exclusive and a given animal may combine a number of mechanisms to maintain muscle function. Consequently, it is difficult to parse the individual contribution of any one of these strategies. We aim to functionally decouple the effect of metabolic rate from the effect of other physiological pathways activated during hibernation. To determine whether a reduction in metabolic rate alone is sufficient to mitigate muscle atrophy we use the western fence lizard, *Sceloporus occidentalis*, as an ectothermic model organism to control metabolic rate via ambient temperature, while applying a disuse stimulus by denervating the gastrocnemius muscle. As mammalian muscle from non-hibernators follows a distinct pattern of atrophy, we also use this system to investigate whether the muscles of lizards follow the same pattern of atrophy as mammalian muscle. To address these questions we quantify and compare the size, morphology, contractile properties, and fiber type composition in the gastrocnemius muscle of control animals and denervated animals housed at 15° and 30° C. Hibernating animals can suppress their metabolic rate lower than temperature alone would

predict (Staples, 2014). This yields a Q_{10} much greater than 2, which is the approximate Q_{10} of metabolic rate in animals. Thus, if the lizards housed at 15°C enter a hibernation-like state over the course of this study, then the Q_{10} between 15°C and 30°C is expected to be higher than 2.3, which is the unacclimated Q_{10} of *S. occidentalis* between 20-30°C (Dawson and Bartholomew, 1956). If the Q_{10} remains low, then we assume our temperature perturbation is only affecting metabolic rate. Our intent is to relate metabolic rate to the rate at which skeletal muscle remodels and broaden our understanding of skeletal muscle plasticity in ectothermic organisms. If lizard muscle atrophies similarly to mammalian muscle, we predict that denervated muscles from animals housed at 30° C will have a reduction in muscle mass, muscle physiological cross-sectional area, maximal contractile force, muscle fiber cross-sectional area, and proportion of fast glycolytic fibers when compared to control muscles. We also predict a higher maximal contractile velocity in denervated muscles from lizards housed at 30° C compared to control muscles. If a reduced metabolic rate mitigates muscle atrophy, we hypothesize little to no differences between denervated and control muscles from lizards housed at 15° C.

Materials and Methods

Animals

Thirty six western fence lizards, *Sceloporus occidentalis* Baird & Girard 1852 (10.13 ± 3.15g, mean ± s.d.), were caught on the University of California, Irvine campus using a California Department of Fish and Wildlife scientific collectors permit SC-12906 issued to JPB. Lizards were housed individually in terraria with a sandy substrate, given water *ad libitum* and fed crickets supplemented with calcium. Before entering treatment, lizards were given a UV light and a heating lamp on a 12/12 light/dark cycle to provide a thermal gradient to allow for thermoregulation. In the treatments, lizards were housed at either 15°C or 30°C with a UV light on a 12/12 light/dark cycle, but no heating lamp to ensure constant metabolic rates. This work was carried out at UC Irvine under Institutional Animal Care and Use Committee protocol no. 2013-3110.

Denervation

We used the medial gastrocnemius, a major ankle extensor, for all experimental protocols. To denervate the muscle, lizards were anesthetized with 5% isoflurane for at least ten minutes, and until the self-righting and toe pinch reflexes were no longer present. A small dorsal incision was made on the posterior portion of hindlimb (where the thigh meets trunk). Ceramic-coated forceps were then inserted in between the iliofibularis and ilioischiotibialis muscles to grasp the sciatic nerve, which was isolated and pulled free from surrounding structures (Figure 1A). A section of the sciatic nerve no less than 2mm in length was then

removed to prevent regrowth of the nerve within the six-week experimental period. Sham surgeries were performed on control limbs following the exact same procedure as the denervated group, without extracting and cutting the sciatic nerve. Sutures (6-0 silk) were used to close the incision. These surgical procedures were performed in a semi-sterile field. Lizards were allowed to recover for two days in a cage with a 12/12 light dark cycle with UV broad-spectrum light (Exo Terra Repti Glo 2.0, 24) and a heating bulb (Exo Terra Sun Glo Basking Spot Lamp, 75W) placed above one side of the cage to allow for behavioral thermoregulation. After recovery, lizards were placed in new cages with sandy substrate and the cages were placed randomly into either a 15°C (N=18) or 30°C (N=18) environmental chamber for six weeks. A period of six weeks was chosen as it was long enough to elicit significant muscle atrophy, but not so long as to negatively impact animal health.

Metabolic Rate

To ensure that cold-housed lizards were not hibernating, we measured the metabolic rate in both groups during the fifth week of acclimation using a subsample of lizards from 15°C and 30°C (N=3 and N=5 respectively). We placed the lizards into a 108 ml chamber housed in a temperature controlled cabinet at 17°C for the cold-housed lizards or 30°C for the warm-housed lizards. Metabolic rates were measured at 17°C instead of 15°C since that was the lowest temperature our metabolic chamber could reach. Q_{10} measurements should not be affected by this slight difference in temperature, and less than 24 hours at this slightly elevated temperature should not have an effect on rates of muscle atrophy over six weeks. Room air scrubbed of CO_2 and H_2O was passed through a long coiled copper tube inside

the temperature cabinet to bring the air to the correct temperature. After the coil, the air passed through the chamber housing the lizard at a rate of 100 ml min^{-1}. Once air passed over the lizards it was again scrubbed of H_2O and passed through a CO_2 gas analyzer (model 6262, Li-Cor Inc., Lincoln, NE, USA). Data was collected using ExpeData software (Sable Systems) for at least 12 hours, until the metabolic rate held constant for at least one hour. Data from the constant portion of the curve was used to calculate the standard metabolic rate (SMR) in ml CO_2 g^{-1} h^{-1}. The Q_{10} of the lizards was calculated as the change in SMR over 10°C using the equation:

$$Q_{10} = \left(\frac{SMR_{30}}{SMR_{17}}\right)^{\frac{10}{\Delta T}}$$

In vitro muscle preparation

Lizards were euthanized via an overdose of 5% inhaled isoflurane followed by a double pithing protocol. Medial gastrocnemius muscles were isolated and the distal tendon was separated from the ankle. The femur immediately proximal to the knee and the tibia and fibula immediately distal to the knee were cut, along with all of the muscles aside from the medial gastrocnemius. Kevlar thread was then tied around the origin of the the muscle, immediately distal to the knee. The other end of the thread was attached to a dual-mode servomotor (Aurora Scientific 360C, Aurora Scientific, Cambridge, MA, USA). The distal tendon of the muscle was fixed in place via a small screw clamp mounted to an aluminum post. Sandpaper (150 grit) was affixed to the inside edges of the clamp to prevent the tendon from slipping. Muscles were bathed in a 23°C oxygenated Ringer's solution (100 mM NaCl, 2.5 mM

KCl, 2.5 mM NaHCO3, 1.6 mM CaCl, 10.5 mM Dextrose) for the duration of the experiment.

Contractile data were collected using a 16-bit data acquisition system (National Instruments, TX, USA). Data were collected at 1000 Hz and analyzed using Igor Pro software (V 6.22A, Wavemetrics, Lake Oswego, OR, USA). A series of twitch contractions was used to determine optimal voltage (between 50V and 70V using parallel platinum plate electrodes) to supramaximally stimulate the muscle. Another series of twitch contractions were used to determine the force-length relationship of the muscle. Tetanic contractions were achieved using a stimulation pulse duration of 0.2 ms and a frequency of 80 pulses s^{-1} for 400 ms. A tetanic contraction starting at the optimal length of the twitch force-length curve (L_0) was used to determine maximal isometric force (P_0). A series of isotonic tetanic contractions were used to characterize the force-velocity relationship of each muscle (Figure 1B). After force developed to a preset value it was maintained by the servomotor and the muscles shortened. Force-Velocity curves were characterized for each muscle from seven to ten isotonic contractions ranging from 5% to 90% of P_0. We elicited an isometric contraction in the middle and at the end of each experiment to ensure no drop in P_0. Any muscle where maximum isometric force fell below 85% of the initial P_0 was removed from analysis. Once all contractions were completed, muscle length at L_0 and wet mass were measured. Muscles were then fixed at L_0 in 10% formalin for fiber length and pennation angle measurements.

We obtained velocity measurements from each contraction by calculating the average change in muscle length divided by the change in time during the constant velocity portion of muscle shortening. Force measurements were taken as the average force over the duration of time the velocity measurements were taken from (Figure 1B). To characterize the

force-velocity properties of the gastrocnemius, we plotted the forces and their corresponding shortening velocities and fit the following hyperbolic-linear equation to the data as an alternative to the Hill equation (Marsh and Bennett, 1986a):

$$V = \frac{B(1-F)}{(A+F)} + C(1-F)$$

Where V is velocity in fascicle lengths per second, F is force in Newtons, and A, B, and C are constants that are iteratively adjusted to fit the force-velocity data (Figure 1C).

Morphology

To characterize the degree of muscle atrophy we measured muscle mass and physiological cross-sectional area. Following *in vitro* characterization of contractile properties, gastrocnemius muscles were submerged in 10% formalin solution for 10 minutes to partially fix them at their optimal length for force productions (L_0). Muscles were then patted dry using paper towel until there were no apparent wet marks on the paper. We weighed the muscles to the nearest 0.001 g and then stored them in formalin to continue the fixation process. Fiber length (F_L) was measured by dissecting out fascicles from fixed tissue and measuring them using a set of electronic calipers. Average values of three fibers taken from different regions of the muscle were used as average fiber length. We took images of the muscles using a digital camera attached to a dissecting microscope (AMscope, MD, USA). Pennation angle (θ) was then taken from these images using ImageJ software (NIH, USA). We took the angle as the average of the angles between the central tendon and the fibers to its right and left.

We calculated physiological cross sectional area ($PCSA$), using the equation:

$$PCSA = \frac{M_{muscle} \times cos\theta}{\rho \times F_L}$$

Where ρ is muscle density (1.06 g/cm^2) , a value taken from the literature (Biewener, 2002) and θ is the pennation angle of the muscle measured relative to the line of action.

Histology

We flash froze gastrocnemius muscles in isopentane cooled with liquid nitrogen. Muscles were pinned when the ankle and knee joints were in 90° flexion to ensure consistency of lengths before freezing. Muscle cross-sections were prepared with a cryotome at -24 °C (12 μm). Sections were taken perpendicular to fiber orientation for accurate comparison of fiber cross-sections. These sections were allowed to dry for 12-24 hours. We then stained for both NADH-dehydrogenase and alkali-stable mATPase to visualize the oxidative and glycolytic capacities of the muscle using an established protocol for lizard muscle (Moritz and Schilling, 2013). A histology staining kit was used (Scientific EasyDip Slide staining system, Simport, Mtl, CA) to stain the sections.

Two cross sections per muscle taken at different locations along the muscle were imaged using a high-powered dissecting microscope with a camera attached (Discovery V20, Zeiss). Individual fibers were identified as fast glycolytic (FG), fast oxidative/glycolytic (FOG), and slow oxidative (SO) by the presence of the diffuse brown stain for mATPase (FG), the dark blue stain of NADH-dehydrogenase in the mitochondria (SO), or both stains simultaneously (FOG) (Figure 2). Individual fibers were counted within each section, and the total number

of each fiber type were averaged across sections. The cross-sectional area of a representative subsection from each section of muscle was measured using ImageJ software (NIH, USA). Fiber cross-sectional areas within this subsection were then measured to determine the average cross-sectional area of each fiber type. To indirectly measure the collagen content of the muscles, the proportion of the cross-section that is not composed of muscle fibers was measured as the total cross-sectional area of a subsection minus the sum of the cross-sectional areas of the fibers in that subsection divided by the total cross-sectional area of the subsection. Only clean sections without artifacts due to flash-freezing or cryosectioning were used for the cross-sectional area analyses.

Statistics

All statistical analyses were performed in R (R Core Team, 2015). Using the lme4 (Bates et al., 2015) and car (Fox and Weisberg, 2011) packages, we ran linear mixed effects models on all morphological, contractile, and histological variables to test for differences between denervated and control muscles at 15°C and 30°C. Individual was used as a random variable in all tests to account for individual variation and because some lizards were unilaterally denervated with the contralateral limb as a sham surgery control, and others were bilaterally denervated or bilaterally given a sham surgery. In the analyses of muscle mass and P_0 we included body mass as a covariate, in the $PCSA$ analysis we included the square of snout-vent length as a covariate, and in our analysis of (V_{max}) fascicle length was included as a covariate. We compared metabolic rate data between temperature groups using a student's T-test.

Results

Metabolic Rate

The average $\dot{V}_{CO2}$ was 0.56 ± 0.05 $mlCO_2$ kg^{-1} min^{-1} (mean±s.e., (N=3) for lizards housed at 15°C for six weeks (tested at 17°C), and was 1.47 ± 0.12 $mlCO_2$ kg^{-1} min^{-1} (mean±s.e., (N=5) for lizards housed at 30°C for six weeks. Metabolic rate differed significantly between the two temperature groups (P=0.001, students T-test), with a Q_{10} of 2.08 for metabolic rate across this temperature range. This result is similar to a previous study, which measured a slightly higher Q_{10} of 2.3 for *S. occidentalis* between 20-30°C (Dawson and Bartholomew, 1956) and confirms that experimental animals held at 15°C were not suppressing their metabolic rate as do hibernating mammals. If they were, Q_{10} values would be expected to be higher than the previously recorded value of 2.3. This indicates that fence lizards may not be activating other atrophy prevention pathways and that any effects on atrophy are solely a result of changes in metabolic rate.

Morphology

Gastrocnemius muscle mass was lower in denervated lizards (N=13) than in controls (N=14) at 30°C (P<0.001, LME model with body mass as a covariate and individual as a random effect, Table 1). In lizards housed at 15°C, denervated gastrocnemius muscles (N=9) were smaller than controls (N=14, P=0.031, LME model with body mass as a covariate and individual as a random effect, Table 1). When corrected for body mass, denervated muscles were 14% smaller than controls in lizards housed at 30°C and 3% smaller in lizards housed

at 15°C (Figure 3A). There was a trend showing a possible interaction effect between temperature and denervation for muscle mass (P=0.058).

Physiological cross-sectional area (PCSA) was lower in denervated lizards (N=9) than in controls (N=9) at 30°C (P=0.001, LME model with snout-vent length squared (SVL^2) as a covariate and individual as a random effect, Table 1). PCSA of denervated (N=8 gastrocnemius muscles in lizards housed at 15°C was lower than controls (N=12, P=0.048, LME model with SVL^2 as a covariate and individual as a random effect, Table 1). When corrected for SVL^2, denervated muscles had 11% smaller PCSA than controls in lizards housed at 30°C and 6% smaller PCSA in lizards housed at 15°C (Figure 3B).There was an interaction effect between temperature and denervation for PCSA (P=0.025).

Contractile Properties

Gastrocnemius denervation resulted in lower maximum isometric force (P_0) at 30°C (P=0.004, LME model with body mass as a covariate and individual as a random effect, N=5 & 8 respectively for denervated and control, Table 1) and at 15°C (P=0.045, LME model with body mass as a covariate and individual as a random effect, N=6 & 12 respectively for denervated and control, Table 1). When corrected for body mass, denervated muscles produced 17% lower force than controls in lizards housed at 30°C and 8% lower force in lizards housed at 15°C (Figures 3C, 4B). There was no interaction between temperature and denervation for P_0 (P=0.596).

Maximum shortening velocity (V_{max}) was slower in denervated lizard muscles than in controls at 30°C (N=6,8, P=0.040, LME model with fascicle length as a covariate and individual as a random effect, Table 1). When corrected for fascicle length, this difference is 6% (Figures 3D, 4B). No difference was detected in the V_{max} of gastrocnemius muscles in lizards housed at 15°C (P=0.853, LME model with fascicle length as a covariate and individual as a random effect, Table 1). There was no interaction effect between temperature and denervation for V_{max} (P=0.442).

Histology

The cross-sectional area of fast glycolytic (FG) (control=4121 ± 1332 μm^2, N=8, denervated=3482 ± 1138 μm^2, N=7, P=0.34) and fast oxidative/glycolytic (FOG) (control=2770 ± 916 μm^2, N=8, denervated=2519 ± 914μm^2, N=7, P=0.60) fibers were not different in control and denervated muscles (Figure 5A) in the 30°C group. However, individual slow oxidative (SO) fibers were, on average, 36% larger in the denervated gastrocnemius muscles (655.58 ± 76.09 μm^2, N=7) of lizards housed at 30°C than in controls (483.10 ± 27.54 μm^2, N=8, P=0.04, Figure 5A). We did not detect any differences between denervated and control muscles in the percentage of cross-sectional area not composed of muscle fibers. There was a trend toward a 10% larger proportion of FG fibers in denervated muscles (74.71 ± 3.29%, N=7) than in control muscles (67.85 ± 2.18%, N=8, P=0.098, Figure 5B). The proportion of FOG fibers was lower in denervated (18.03 ± 2.68%) muscles than in control (25.74 ± 2.38%, P=0.05, Figure 5B). The proportion of SO fibers was not different between groups at 30°C (P=0.46, Figure 5B).

Neither FG (P=0.88), FOG (P=0.87), nor SO (P=0.14) fibers were significantly different in cross-sectional area between denervated (N=5) and control (N=4) muscles of lizards housed at 15°C. Likewise, there was no difference between denervated and control muscles in the proportion of cross-sectional area not composed of muscle fibers. Additionally, we found no differences in the proportion of FG(P=0.75), FOG(P=0.79), and SO(P=0.68) between denervated and control muscles at 15°C (Figure 5B).

Discussion

Lizards housed at 15°C have a lower metabolic rate than lizards housed at 30°C corresponding to a Q_{10} of 2.08, which is in line with that found previously in western fence lizards (Dawson and Bartholomew, 1956). Though some hibernating animals have additional mechanisms to avoid atrophy, all hibernating and aestivating animals activate mechanisms that supress metabolic rate (Tessier and Storey, 2016; Hudson and Franklin, 2002). Our measured Q_{10} of 2.08 suggests that no animals housed at 15°C were activating any additional hibernation pathways to limit atrophy. After six weeks, denervated gastrocnemius muscles from 30°C housed lizards had a substantial decreases in mass, force, physiological cross-sectional area, and V_{max} compared to control muscles (Figures 3,4). In 15°C lizards we found no differences between groups in muscle cross-sectional area or V_{max}. Muscle mass and force were reduced in the denervated muscles, though not as severely as the 30°C groups. We propose that the different responses to denervation seen in the two groups are due to differential rates of muscle protein degradation in direct response to the lower metabolic rate of the cool lizards.

Much of what we know about muscle atrophy is based on studies of mammals. The typical pattern of muscle atrophy in mammals is described by a loss of contractile proteins, which unsurprisingly results in a lower maximum contractile force (P_0). Less intuitively, the maximal shortening velocity (V_{max}) of atrophied muscles increases, as does muscle fatigability. This shift is partly because slow oxidative fibers are recruited at all levels of activation and therefore face a substantial reduction in their level of activity in response to a disuse

stimulus. In addition, fatigue resistant slow oxidative fibers have an abundance of mitochondria, are highly metabolically active, and produce relatively large levels of reactive oxygen species as a byproduct of oxidative phosphorylation. This likely causes relatively rapid atrophy of fatigue resistant slow muscle fibers in comparison to the fast fibers and, in some cases, transitions from slow to fast fibers. This remodeling leads to the increase in muscle speed and fatigability with atrophy (Caiozzo, 2002; Lieber, 2002). While these findings generally hold true, they are largely based on a few species of endothermic mammals with high and steady metabolic rates, namely rats, cats, and humans (Caiozzo, 2002; McDonagh et al., 2004). In addition, many of the seminal studies using spaceflight, hindlimb suspension or limb immobilization to induce atrophy document the most significant and impressive changes in the soleus muscle, which is predominantly composed of slow twitch fibers (Booth, 1982; Caiozzo, 2002). While these studies have provided important insight into the conditions that induce atrophy and the functional consequences of disuse, it is difficult to extend the generality of the findings to other muscles or other species. When comparing a wide variety of vertebrates, the process of skeletal muscle atrophy and an organism's response to a disuse stimulus can be highly variable. Patterns of atrophy can vary based on muscle fiber type and pattern of muscle use (Caiozzo, 2002; Lieber, 2002; Winiarski et al., 1987), metabolic rate (Hudson and Franklin, 2002), gene expression (Bodine, 2013; Xu et al., 2013), method of inducing muscle atrophy (Fitts et al., 1986; Lieber, 2002; McDonagh et al., 2004), and even the metric used to quantify atrophy (Mantle et al., 2009). Even when comparing individual muscles within a single animal, the patterns and severity of atrophy can vary significantly. For example, in aestivating frogs the response of four hindlimb muscles vary significantly after more than six months of disuse (Mantle et al., 2009). The observed differences between

muscles are made more complicated because the commonly used indicators of atrophy such as fiber cross-sectional area, muscle cross-sectional area, muscle mass, maximal force production, maximal power production, or total protein content do not change at the same rate and often indicate divergent outcomes. In the same study of atrophy in aestivating frogs authors noted that the iliofibularis muscles had significantly less mass, a smaller whole muscle CSA and fiber CSA whereas the sartorius muscles had less mass and whole muscle CSA with no changes in fiber CSA, the gastrocnemius muscles had only a decrease in whole muscle CSA, and the cruralis muscles only had decreases in fiber diameter (Mantle et al., 2009).

The variation in the response of a muscle can also depend on the disuse protocol used in the study. For instance, denervation often leads to an initial period of hypertrophy of muscle fibers due to swelling of the tissue, despite a disorganization of contractile proteins which likely leads to an overall loss of force (Jirmanová and Zelená, 1970; Hikida and Bock, 1972). Though denervation does not always lead to muscle fiber hypertrophy (Bakou et al., 1996; Lin et al., 2012), it could explain the relatively larger drop in force compared to muscle mass observed in our 15° C denervated lizards (Figure 3). In contrast to studies using denervation, experimental manipulations such as hindlimb suspension and microgravity often result in a decrease in force and muscle fiber diameter, though the time course of remodeling may again vary between the two methods (Winiarski et al., 1987; Fitts et al., 1986; Caiozzo et al., 1994). The observed level of variation in response to a disuse stimulus suggests that properties of the muscle, the time course of the response, the protocol used to induce atrophy and the metric used to characterize atrophy can all obscure the general relationship between the phenotypic response and the underlying mechanisms of atrophy.

In this study we have focused on understanding how the differences in metabolic rate

can induce variation in the rate of muscle atrophy. We combine our results with data from previous studies in order to quantify the relationship between mass-specific metabolic rate and the rate of muscle atrophy (Figure 6). This analysis is a modified and updated version of a previously performed analysis (Hudson and Franklin, 2002). To account for the variation between animals, muscles, perturbations, and metrics, we only include the highest rates of atrophy measured in each study. Using the maximum rate of atrophy may allow us to minimize the inherent variation in the response of different muscles from different species using different experimental manipulations to induce atrophy. This analysis allows us to compare hibernating and non-hibernating endotherms and ectotherms with various atrophy inducing perturbations and metrics of atrophy. We find that across vertebrates, there is a significant relationship between mass-specific metabolic rate and rate of muscle atrophy, regardless of disuse condition, the muscle being used or whether the organism was an endotherm or ectotherm (R^2=0.95, P<0.05, Figure 6). This pattern holds true even though our analysis includes a number of aestivating and hibernating species, which may be activating other atrophy resistance pathways. Despite the significant variation observed in studies of muscle atrophy, our analysis indicates that metabolic rate is a strong predictor of muscle atrophy across vertebrates.

Though metabolic rate seems to drive the rate of muscle atrophy, other mechanisms used by hibernating and aestivating animals are likely to alter the patterns of atrophy. Many hibernators and aestivators can suppress their metabolic rate lower than a decrease in body temperature alone would allow, which likely serves to slow the rate of muscle atrophy (Geiser, 2004; Storey, 2015; Staples, 2016). However, there are additional mechanisms that are thought to slow or prevent muscle atrophy in these organisms. Increased production of

antioxidants, such as superoxide dismutase, occurs in many hibernating and aestivating animals, and can prevent reactive oxygen species from disrupting protein synthesis or enabling proteolysis (Hudson et al., 2006; Allan and Storey, 2012; Powers et al., 2011; Vucetic et al., 2013). Activation of the exercise pathway gene, PGC-1α in hibernating 13-lined ground squirrels shifts the composition of muscle fiber types towards a more fatigue resistant profile, though does not by itself prevent atrophy (Rourke et al., 2004; Bodine, 2013; Xu et al., 2013). Activation of the gene SGK-1 in hibernating 13-lined ground squirrels inactivates FoxO3a, which normally mediates the atrophy response and activates mTOR, which stimulates muscle growth (Andres-Mateos et al., 2013). The mechanisms found to resist muscle atrophy in hibernating animals extends well beyond those mentioned here and the full scope of these mechanisms was the focus of a recent review (Tessier and Storey, 2016). These mechanisms have received much attention recently, and have clear potential as therapeutic targets for slowing human muscle atrophy. The presence of these atrophy-resisting mechanisms does not slow the rate of atrophy in hibernators and aestivators beyond what would be predicted by metabolic rate alone, though they may serve to alter the pattern of atrophy.

Different muscles with similar fiber type distributions and similar functions may still atrophy at different rates (Lieber, 2002). Though the molecular underpinnings of this are unclear, the relative activity level in the muscles before the application of a disuse stimulus may provide an explanation. Terrestrial mammals are generally more active than terrestrial ectothermic vertebrates (Bennett and Ruben, 1979). Therefore, mammalian leg muscles are acclimated to a much higher level of activity before they enter hibernation than the muscles of ectotherms. In the absence of the molecular mechanisms aimed at mitigating atrophy, mammalian muscles may incur significant atrophy even at a low metabolic rate because of

the relatively large disuse stimulus. Additionally, antioxidant defenses and the activation of PGC-1α may act to specifically preserve the slow oxidative tissues, which due to their high mitochondrial content produce reactive oxygen species at a higher rate than other muscle fiber types and are thus susceptible to faster rates of atrophy than other fiber types. Sparing of slow fibers would still result in normal patterns of atrophy in predominately fast muscles. Moreover, metabolic rate is suppressed globally, but that does not mean that all tissues are equally affected. In green-striped burrowing frogs, levels of citrate synthase, lactate dehydrogenase, and cytochrome C oxidase were suppressed to different levels in different muscles during six and nine months of aestivation (Mantle et al., 2010). Metabolic rate may therefore correlate with muscle atrophy on a muscle specific level, and muscles that are the most important for locomotion may be preferentially spared. Finally, muscle atrophy may not result from disuse in hibernating mammals. In some cases, there seems to be a "pre-programmed" shift in muscle properties that occurs during hibernation. Golden mantled ground squirrels will hibernate at the same time of year regardless of environmental temperatures, yet the same pattern of atrophy in fast muscle fibers and sparing of slow muscle fibers is observed regardless of temperature (Nowell et al., 2010). Seasonally induced muscle plasticity may be fundamentally different from disuse-induced atrophy and comparisons between these two mechanisms should be viewed with skepticism.

Conclusions

We found that in the ectothermic western fence lizard, *Sceloporus occidentalis*, muscle atrophy, after six weeks of denervation, is mitigated by a low metabolic rate to a similar degree as is seen in other vertebrates. We conclude that a reduced metabolic rate alone is sufficient to spare muscle protein over a long period of disuse. Though there are other mechanisms which may spare certain muscle fiber types or specific muscles, our findings indicate that any mechanism which can lower the metabolic rate of an animal can significantly slow muscle atrophy.

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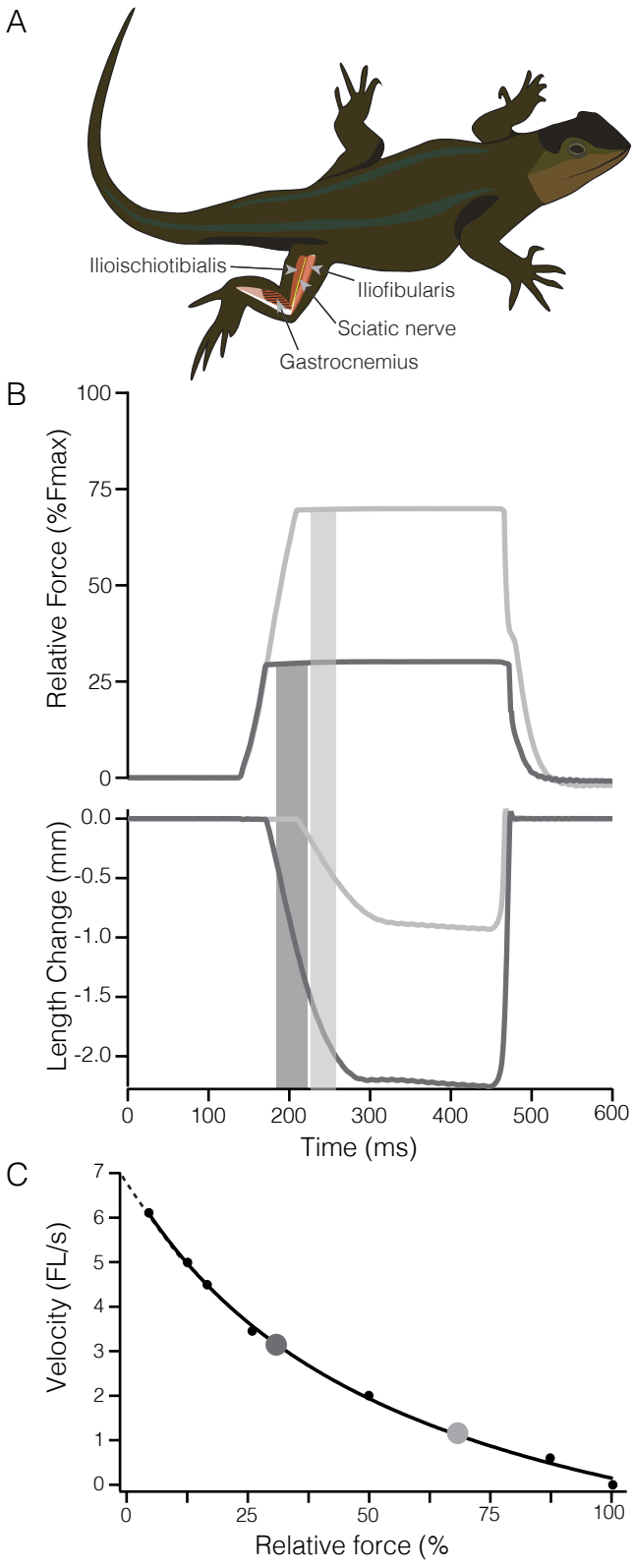

Figure 1. Experimental design. (A) Leg anatomy of the western fence lizard. An incision was made on the dorsal proximal thigh. The sciatic nerve is severed to denervate the medial gastrocnemius muscle. (B) Representative time series traces showing force and length during two isotonic contractions at 30% and 70% of P_0. Gray vertical bars indicate the time at which force and velocity measurements were taken for each contraction. (C) Force-velocity relationship for a representative muscle characterized using a series of isotonic contractions between 5% and 100% of P_0. The contractions shown in (B) are highlighted with gray symbols. The force velocity data are fit with a hyperbolic-linear equation (Marsh and Bennett, 1986a).

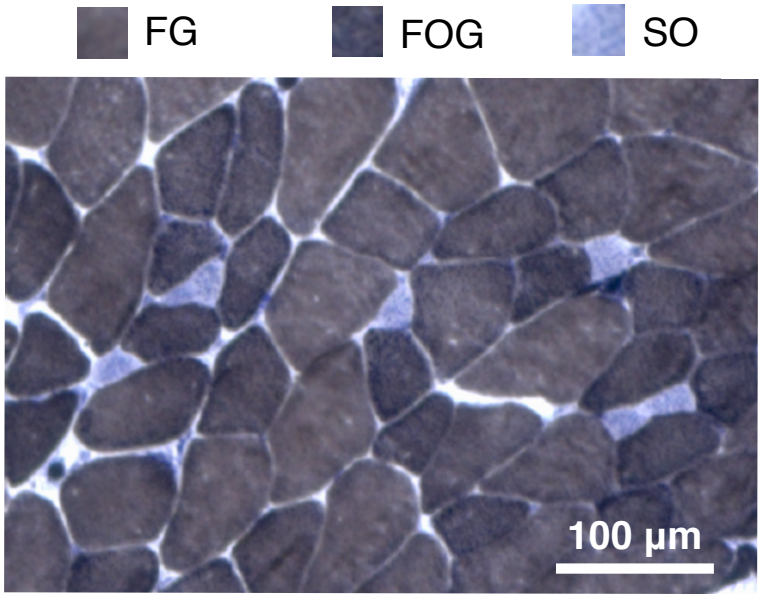

Figure 1.2. Representative histology section from double stained gastrocnemius muscles. Fast glycolytic (FG) fibers stained only for alkali-stable myosin ATPase. Slow oxidative (SO) fibers stained only for NADH-dehydrogenase. The intermediate fast oxidative/glycolytic (FOG) fibers staining for both alkali-stable myosin ATPase and NADH-dehydrogenase.

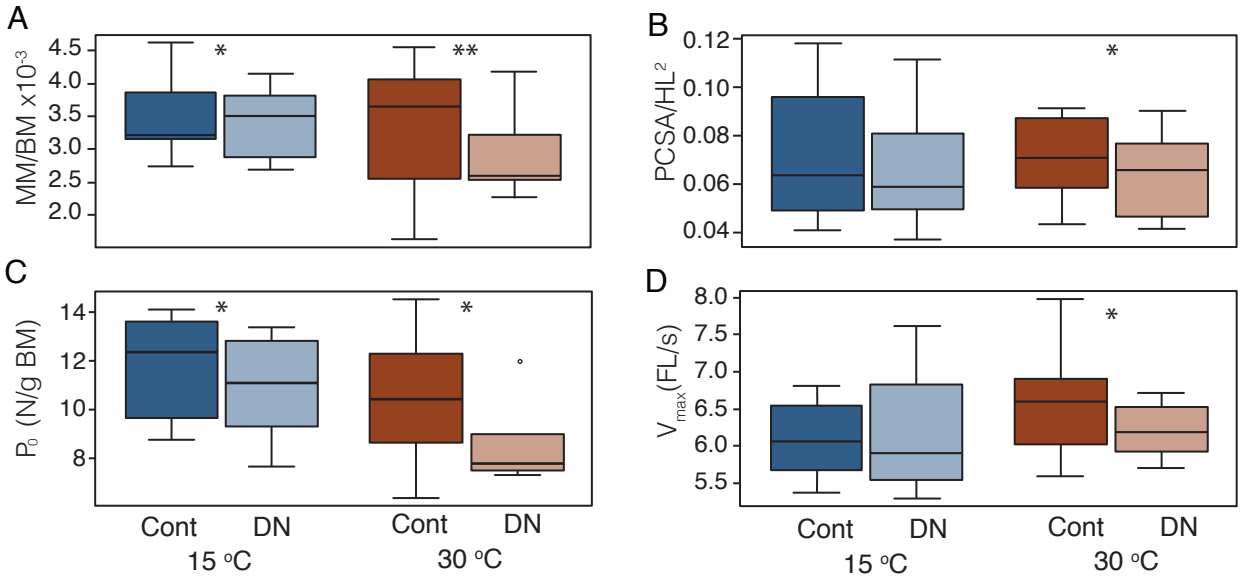

Figure 1.3. Morphological and contractile data from all treatments. Box plots represent the median, minimum, 25th percentile, 75th percentile and maximum data points. Muscle mass/body mass (MM/BM), physiological cross-sectional area/snout-vent length^2 (PCSA/SVL^2), and maximal isometric force (P_0) corrected for body mass are all around 15% lower and V_{max} is 6% lower in denervated muscles from lizards housed at 30° C (N=13, 9, 5, and 6 respectively). P_0 is 8% lower in denervated muscles from the 15° C group (N=5), but MM/BM (N=9), PCSA/SVL^2 (N=8) and V_{max} (N=7) are similar to controls. Cont= control group, DN= denervated group, ○ = outlier, * P<0.05, ** P<0.001

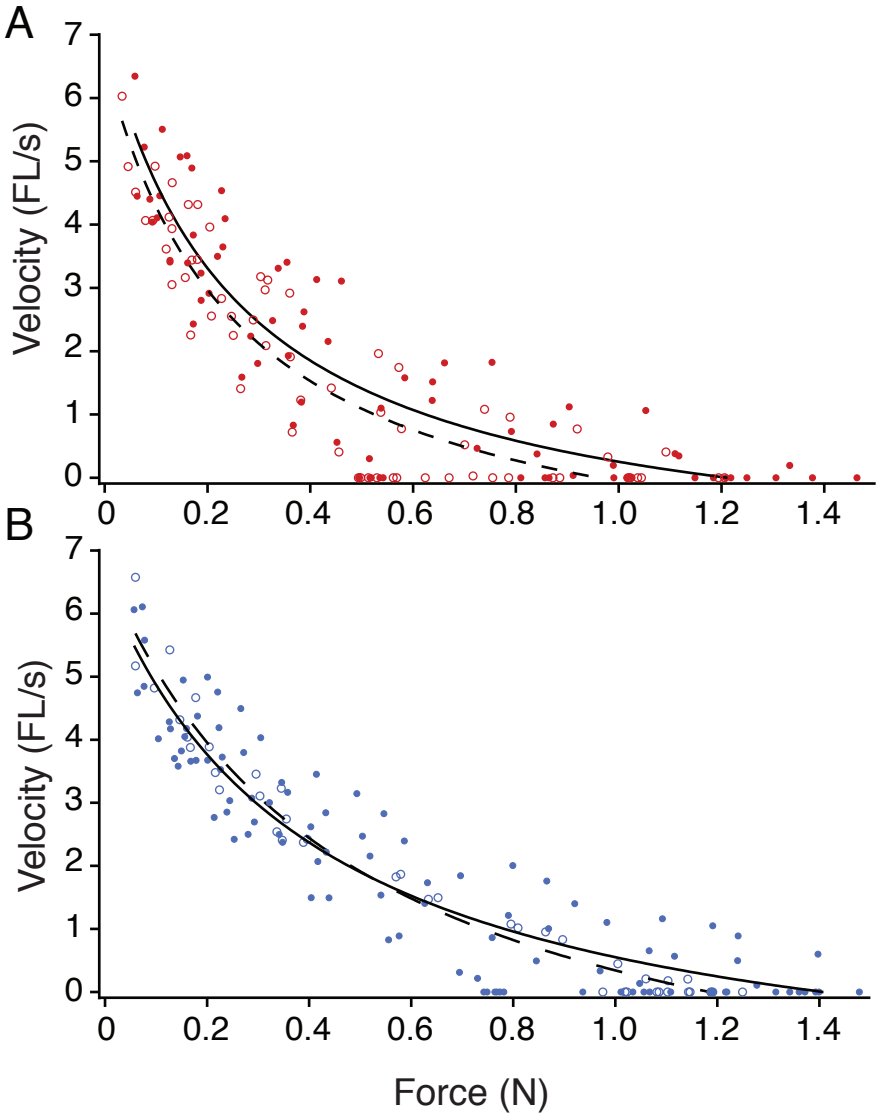

Figure 1.4. Summary of force-velocity data from all lizards housed for six weeks at (A) 30°C and (B) 15°C. Open symbols are from denervated muscles and closed symbols are from control muscles. Dashed lines are the average force-velocity fits for data from denervated muscles, and solid lines are the average force-velocity fits for data from control muscles. Velocities are presented corrected for fascicle length and force is presented as absolute values in newtons. (A) P_0 corrected for body weight is 13% lower in denervated muscles (N=6). Maximum shortening velocity (V_{max}), was 6% slower in denervated muscles. (B) Corrected P_0 was 10% lower in denervated muscles (N=7), but we found no difference in V_{max}.

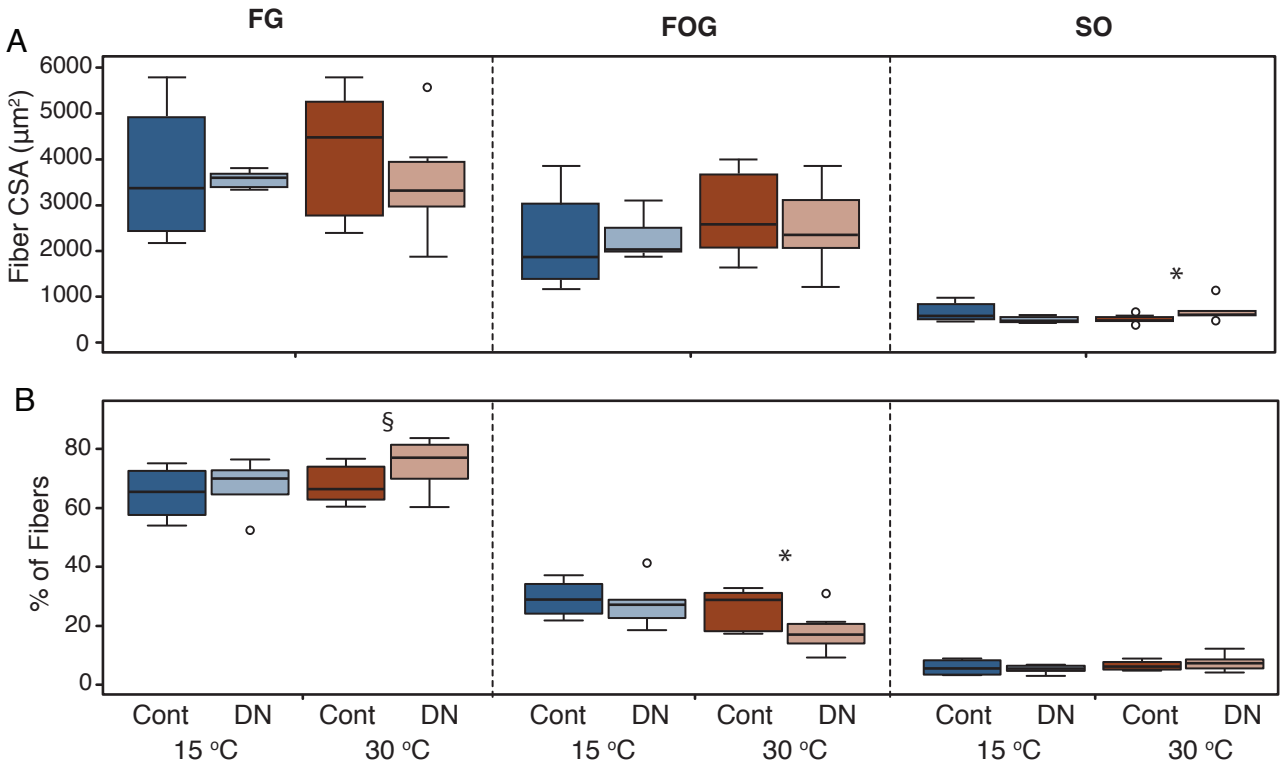

Figure 1.5. Histological data from all treatments. Box plots represent the median, minimum, 25th percentile, 75th percentile and maximum data points. (A) Box plots of fiber cross-sectional area of fast glycolytic (FG), fast oxidative/glycolytic (FOG), and slow oxidative (SO) fibers. SO fibers are 36% larger in denervated muscles from lizards housed at 30° C (P=0.04). No other differences were significant at 15° C (N=4) or 30° C (N=7), but note that the average cross-sectional area of FG fibers are about 15% smaller in denervated muscles from the 30° C group. (B) Proportions of the total fiber population composed of fast glycolytic (FG), fast oxidative/glycolytic (FOG), and slow oxidative (SO) fibers. FOG fibers were a 30% smaller proportion of overall fibers and there was a trend for 10% larger proportion of FG fibers in denervated in denervated muscles from lizards housed at 30°C. Cont= control group, DN= denervated group, ○ = outlier, * P=0.05, § P<0.10

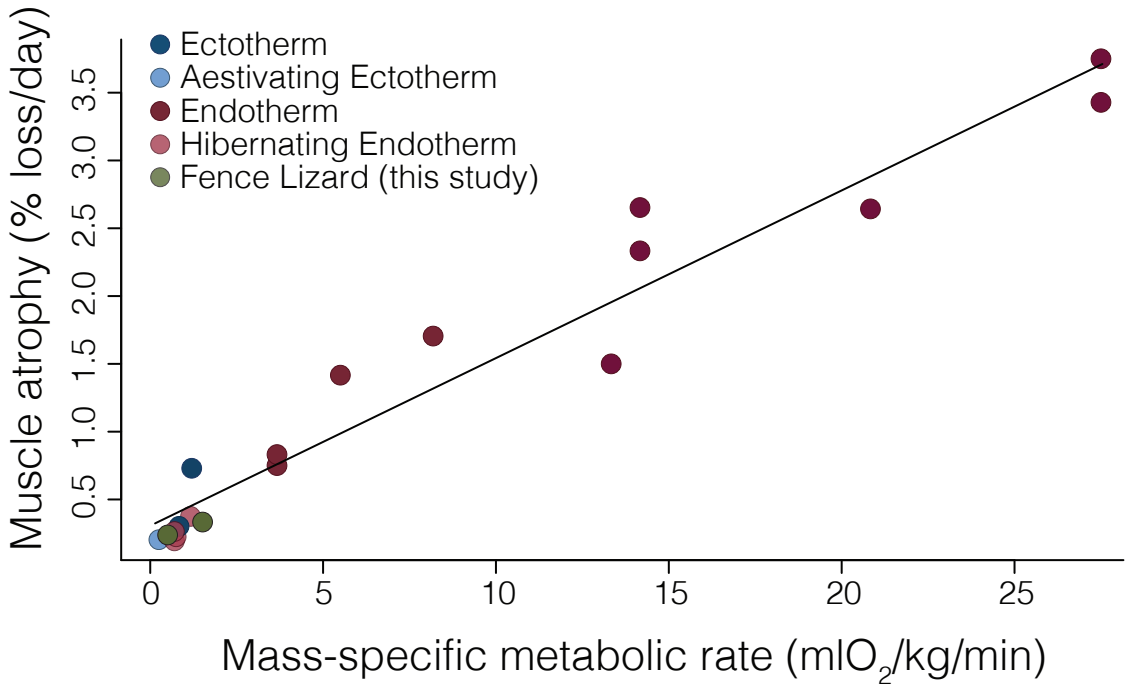

Figure 1.6. Rate of muscle atrophy plotted against mass-specific metabolic rate. Different muscles atrophy at different rates even within the same animal, so only the muscle with the maximal rates of atrophy in each study was used to generate the plot. Disuse conditions include immobilization, aestivation, hibernation, hindlimb unweighting, and denervation. Animals include ectotherms, endotherms, and heterotherms. From left to right are aestivating burrowing frogs (Mantle et al., 2009), denervated fence lizards housed at 15° C (present study), hibernating black bear (Lohuis et al., 2007), hibernating and denervated black bear (Lin et al., 2012), hibernating ground squirrel (Wickler et al., 1991), immobilized turtle (McDonagh et al., 2004), hibernating hamster (Wickler et al., 1987), immobilized savannah monitor lizard (James Hicks and Amanda Szucsik, unpublished data), denervated fence lizards housed at 30° C (present study), immobilized humans (Veldhuizen et al., 1993), denervated black bear(Lin et al., 2012), immobilized dog (Bebout et al., 1993), denervated chicken (Jirmanová and Zelená, 1970), immobilized guinea pig (Maier et al., 1976), immobilized rat (Boyes and Johnston, 1979), hindlimb unweighted rat (Thomason and Booth, 1990), hindlimb unweighted hamster (Thomason and Booth, 1990), immobilized mouse (Soares et al., 1993), and hindlimb unweighted mouse (Thomason and Booth, 1990). There is a significant correlation between mass-specific metabolic rate and % atrophy (% daily atrophy= 0.124*mass-specific metabolic rate + 0.306, R^2=0.95, P<0.05).

Table 1: Morphological and Contractile Data

	15 Control			15 Denervated			30 Control			30 Denervated		
	Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N	Mean	S.E.	N
Muscle Mass (mg)	35.95	4.28	14	32.83	4.53	9	34.53	2.89	14	31.22	3.02	13
Body Mass (g)	10.46	1.06	14	10.13	1.15	10	11.44	0.81	13	11.85	1.05	13
P_0 (N)	1.14	0.11	12	0.93	0.12	6	1.05	0.08	8	0.93	0.10	5
Power (Watts)	4.8×10^{-3}	5.84×10^{-4}	12	3.53×10^{-3}	5.46×10^{-4}	6	4.40×10^{-3}	3.59×10^{-4}	8	3.57×10^{-3}	3.29×10^{-4}	5
PCSA (mm^2)	7.07	0.77	12	6.59	0.91	8	7.02	0.58	9	6.50	0.60	9
SVL (mm)	66.69	1.84	14	66.49	2.07	10	69.66	1.31	14	70.05	1.80	14
V_{max} (mm/s)	28.48	1.06	10	28.99	1.91	4	30.51	1.15	8	28.88	1.29	6
FL (mm)	4.69	0.13	13	4.61	0.14	9	4.61	0.15	10	4.70	0.12	10

Table 1. Morphological and Contractile Data. Mean, standard error (S.E) and sample size (N) for all morphological and contractile measurements. P_0 - Maximum contractile force, PCSA - Physiological Cross-Sectional Area, SVL - Snout-Vent Length, V_{max} - Maximum shortening velocity, FL - Fiber Length.

CHAPTER 2

Muscle power amplification broadens the thermal performance of lizard acceleration

Introduction

To survive and reproduce, animals must be swift enough to escape predation and, if they are predators, to catch prey. Since ectotherms rely on environmental temperatures and sunshine to behaviorally thermoregulate, they have the additional challenge be able to perform well even as temperatures cool down. The muscles that produce work and generate animal movement are temperature sensitive, so this can indeed pose a significant challenge.

Some ectotherms are able to maintain performance as their body temperature decreases. One such ectotherm, the western fence lizard, *Sceloporus occidentalis*, is able maintain running performance down to 25° C even though the optimal temperature of this species is 35° C (Marsh and Bennett, 1986b). Q_{10} is a metric of how the rate of a physiological process changes with respect to a 10° C shift in temperature. Western fence lizard muscle has a Q_{10} of around 2 at all biologically relevant temperatures, meaning that for a decrease in muscle temperature of 10° C, the maximum muscle shortening velocity and power output decreases by 50% (Marsh and Bennett, 1986b,a). Despite this, the running speed of western fence lizards only has a Q_{10} of around 1.2 between 25° C and 35° C. Unpublished data that I have collected confirms that fence lizard acceleration performance also decreases much less than expected based on muscle contractile data as lizards are cooled to 25° C. One explanation for how these lizards are able to maintain performance could be that muscle power is not

a constraint above 25° C, and that something other than muscle power constrains speed and acceleration at high temperatures. However, two other species of lizard, including another *Sceloporus* lizard species, have just enough muscle to power the maximal accelerations measured at their optimal body temperatures (Curtin et al., 2005; McElroy and McBrayer, 2010). So the question remains, if fence lizards have just enough muscle to power acceleration at 35° C, how are they able to maintain acceleration performance at 25° C when their muscles are half as powerful? I hypothesize that lizards are using stored elastic energy to amplify muscle power and maintain acceleration performance at low temperatures.

Many muscles operate in series with spring-like tendons. Tendons or other collagenous elastic elements in-series with muscle, such as aponeuroses, can decouple the length changes that occur in a muscle from the movement of an organism by storing and releasing elastic energy (Roberts and Azizi, 2011). Muscles and their series elastic elements are collectively referred to as a muscle-tendon unit (MTU). If a tendon is used effectively during acceleration, the MTU initially remains the same length while the tendon lengthens against a shortening muscle. The tendon then releases its stored energy into the limb skeleton, like a slingshot, at a much faster rate than muscle alone could perform. The amount of work done by the system does not change, but since tendons allow for a much faster release of that work, power ($work/time$) is amplified. One important difference between muscles and tendons is that muscles contract using enzymatic and biochemical reactions that are temperature sensitive, whereas tendons are passive mechanical structures that are largely temperature insensitive across biologically relevant temperatures (Rigby et al., 1959; Kubo et al., 2005). Therefore, tendons can allow for fast, powerful accelerations as well as mitigate the slowing effects that low temperature has on muscle.

Muscle power amplification through series elastic elements has been measured in many vertebrates across a variety of behaviors. Chameleon, salamander, and toad tongue projection (Anderson and Deban, 2010; Lappin, A Kristopher et al., 2006; Scales, Jeffrey A et al., 2016); frog, bushbaby, and human jumping (Astley and Roberts, 2014; Aerts, 1998; Kurokawa et al., 2003); and turkey acceleration (Roberts and Scales, 2002) have all shown strong evidence for decoupling muscle and tendon work to amplify muscle power. In ectotherms, muscle power amplification has the simultaneous benefit of allowing animals to maintain close to maximal performance at low temperatures (Anderson and Deban, 2010; Lappin, A Kristopher et al., 2006; Scales, Jeffrey A et al., 2016). Some studies have argued that, due to the relatively large diameter of tendon compared to muscle, small running or hopping animals are not capable of storing enough energy in their tendons to effectively use the stored elastic energy and only large animals can effectively use series elasticity during running or hopping (Biewener et al., 1981; Biewener and Blickhan, 1988). However, theoretical work has shown that despite the relatively small amount of work stored in the tendons of small animals, they can still store a significant proportion of the work done by an animal over their relatively shortened stride lengths compared to larger animals (Bullimore and Burn, 2005). Indeed, the long toe tendons of zebra-tailed lizards, which are around the same size as western fence lizards, are capable of storing around 40% of the work done in a stride when biologically relevant forces are applied to them (Li et al., 2012).

In this study, I use an inverse dynamics approach to test whether western fence lizards are using stored elastic energy to amplify muscle power. My analysis uses three-dimensional kinematic and ground reaction force data to calculate the instantaneous joint powers about the ankle, knee, and hip joints during acceleration. This technique is commonly used to

measure joint powers in vertebrates and has been used to provide evidence for power amplification (Carroll et al., 2008; Farris and Sawicki, 2011; Astley and Roberts, 2014). If the muscle power required to generate the calculated joint powers is higher than the power that the muscles are capable of generating, then some mechanism must be responsible for the power amplification. The most likely explanation would be that muscle work is temporarily stored in series-elastic elements and then rapidly released to amplify muscle power. Series elasticity would then explain the relative maintenance of animal acceleration performance down to 25° C.

Materials and Methods

Animals

Nine western fence lizards, *Sceloporus occidentalis* Baird & Girard 1852 (9.18 ± 2.06 g, mean ± s.d.),were caught on the University of California, Irvine campus using a California Department of Fish and Wildlife scientific collectors permit SC-12906 issued to JPB. Lizards were housed individually or in pairs with a 12/12 light dark cycle with UV broad-spectrum light (Exo Terra Repti Glo 2.0, 24) and a heating bulb (Exo Terra Sun Glo Basking Spot Lamp, 75W) placed above one side of the cage to allow for behavioral thermoregulation. They were housed in terraria with a sandy substrate, given water *ad libitum* and fed crickets supplemented with calcium. This work was carried out at UC Irvine under Institutional Animal Care and Use Committee protocol no. 2013-3110.

Acceleration kinematics

We acclimated the lizards for at least two hours in an environmental chamber held at 15°C (N=4), 25°C (N=7), or 35°C (N=9). After acclimation, lizards were placed on a custom track in which we embedded a nano17 force transducer (ATI Industrial Automation, Apex, NC, USA). All running surfaces were covered with 80 grit sandpaper to provide traction. We marked the left hindlimb joints as well as the center of mass of the animals with white-out and a black marker at the center for digitizing (Figure 2.1a). We placed the lizards on the track so that their left hindfoot, but no other body part, was fully in contact with the force transducer. We then encouraged them to accelerate towards a darkened chamber

while filming at 1000 Hz with two Edgertronic high-speed cameras (Model SC1, Sanstreak Corporation, San Jose, CA, USA) placed dorsally and laterally to the force plate. A custom-built 48-point calibration object was used to calibrate the two camera views. Force data were collected using a 16-bit data acquisition system (National Instruments, TX, USA). Data were collected at 1000 Hz and initially analyzed using Igor Pro software (V 6.22A, Wavemetrics, Lake Oswego, OR, USA) before being imported into MATLAB (MathWorks, Natick, MA, USA) for further analysis. Cameras were triggered using an external trigger. The trigger signal was split and simultaneously sent to the cameras and to Igor Pro to synchronize the force and kinematic data.

We used DLT software in MATLAB (Hedrick, 2008) to digitize the 3D motion of the joint centers and the lizard centers of mass. To orient the camera frame of reference to the force plate frame of reference, we digitized four points on the surface of the force transducer corresponding with the positive and negative x and y force vectors, while calculating the z force vector as orthogonal to x and y.

Inverse Dynamics Analysis

We wrote a custom MATLAB code to analyze the kinematics and run the inverse dynamics analysis (Figure 2.2). The center of pressure of the foot was estimated as the x and y position of the joint between the metatarsals and proximal phalanges, along with the z position of the floor. Given that we do not know the relative force on each of the toes in contact with the ground, this is a conservative estimate of the position of the center of pressure and, if anything, leads to an underestimate of joint powers.

We calculated the joint angles using a transformation matrix assuming the knee is a hinge joint. This allowed us to assume that the knee, ankle, and hip joints were in the same plane, which we used to calculate the displacement in the foot and center of mass in an orthogonal plane. We used the output angles and displacements from the transformation matrices to plug into the equations of motion:

$$\sum F_x = ma_x \qquad eq.1$$

$$\sum F_y = ma_y \qquad eq.2$$

$$\sum F_z = ma_z \qquad eq.3$$

$$\sum \tau = I\alpha + ma * d \qquad eq.4$$

Where F = force, m = mass, a = acceleration, τ = torque, I = moment of intertia, and d = displacement. We calculated I for each limb segment from the dice-CT scans (see below). However the limb segment I were low enough to have a negligible effect of joint power, and only served to increase the noise of the signal. For this reason we left the $I\alpha$ term out of our final analysis.

DICE-CT

To measure the distance of the skin markers and the muscle insertions to the joint centers wed visualized the muscle and bony tissue using diffusible iodine contrast-enhanced computed tomography (DICE-CT) (Gignac et al., 2016) (Figure 2.1b). Lizards were euthanized via an

overdose of 5% inhaled isoflurane followed by a double pithing protocol. We then preserved the specimens in 10% formalin solution for at least 3 days. Following this, we cut the hindlimb and portion of the trunk with the center of mass markers from the rest of the body. We inserted an insect pin into the marked areas on each joint of the left hindlimb and the center of mass so that the superficial tip of the insect pin was flush with the skin. We then removed the skin and placed the samples in 100% lugols iodine solution for 5-7 days. Following this, we took μCT scans of the lizards (Xradia VersaXRM 410) with a resolution of 40.231 μm voxel^{-1} at the UC Irvine Materials Research Institute (University of California, Irvine, USA). 3D images were rendered using the software program Horos (v2.4.0), and all structures were assembled and distance measurements taken using MeshLab (v2016.12)(Cignoni et al., 2008).

Statistics

All statistical analyses were performed in R (R Core Team, 2015). Using the lme4 (Bates et al., 2015) and car (Fox and Weisberg, 2011) packages, we ran linear mixed effects models using the maximum values for each lizard at each temperature for joint powers, joint muscle powers, joint angular velocities, and lizard accelerations. For stride duration and the delay between the start of the stride, which we defined as the beginning of the increase in ground reaction force, and the movement of each joint we ran linear mixed effects models using the values for all lizard runs at 15°C, 25°C, and 35°C. Differences between temperatures were determined using a least square means analysis. In all tests, temperature was a fixed effect and individual was used as a random effect to account for individual variation.

Results

Joint Muscle Power

The maximum ankle muscle power in accelerating fence lizards was 180.15 ± 26.11 W/kg SEM (N=4) at 15°C,631.96.15 ± 49.95 W/kg SEM (N=7) at 25°C, and 880.94 ± 78.14 W/kg SEM (N=9) at 35°C. The maximal ankle muscle power produced by each animal was significantly different between temperatures ($P < 0.001$, LME model with individual as random effect, Figure 2.3). There were significant differences between 15°C and 25°C ($P < 0.001$, LSM) and between 25°C and 35°C ($P = 0.001$, LSM, Figure 2.3). The Q_{10} of ankle muscle power between 15°C and 25°C is 3.45 ± 0.25 SEM (N=4) and between 25°C and 35°C is 1.50 ± 0.11 SEM (N=7). Ankle muscle power amplification, based on previous measurements of fence lizards muscle power (Balaban and Azizi, 2017) at 15°C was 2.20 ± 0.32 SEM (N=4). At 25°C power amplification was 3.79 ± 0.30 SEM (N=7). At At 35°C power amplification was 2.68 ± 0.24 SEM (N=9).

Knee muscle power was 127.44 ± 26.53 W/kg SEM (N=4) at 15°C,393.11 ± 51.22 W/kg SEM (N=7) at 25°C, and 432.84 ± 49.82 W/kg SEM (N=9) at 35°C. There were significant differences between temperatures ($P < 0.001$, LME, Figure 2.4). Knee muscle powers at 15°C and 25°C were different ($P < 0.001$, LSM), but no differences were seen between 25°C and 35°C ($P = 0.2$, LSM, Figure 2.4). The Q_{10} of knee muscle power between 15°C and 25°C is 3.20 ± 0.45 SEM (N=4) and between 25°C and 35°C is 1.32 ± 0.19 SEM (N=7). Knee muscle power amplification at 15°C was 1.55 ± 0.32 SEM (N=4). At 25°C power amplification was 2.39 ± 0.31 SEM (N=7). At At 35°C power amplification was 1.31 ± 0.15

SEM (N=9).

Hip muscle power was 40.90 ± 11.20 W/kg SEM (N=4) at 15°C,176.25 ± 41.74 W/kg SEM (N=7) at 25°C, and 193.14 ± 29.55 W/kg SEM (N=9) at 35°C. Hip muscle power was significantly different between temperatures ($P < 0.001$, LME, Figure 2.5). There were significant differences between 15°C and 25°C ($P < 0.001$, LSM), but not between 25°C and 35°C ($P = 0.65$, LSM, Figure 2.5).The Q_{10} of hip muscle power between 15°C and 25°C is 7.18 ± 1.98 SEM (N=4) and between 25°C and 35°C is 1.66 ± 0.61 SEM (N=7). Hip muscle power amplification at 15°C was 0.49 ± 0.14 SEM (N=4). At 25°C power amplification was 1.07 ± 0.25 SEM (N=7). At At 35°C power amplification was 0.58 ± 0.09 SEM (N=9).

Acceleration

The maximal instantaneous acceleration produced by each lizard was 18.20 ± 3.58 m/s at 15°C (N=4), 32.71 ± 3.22 m/s at 25°C (N=7), and 52.01 ± 3.86 m/s at 35°C (N=9). There was a significant difference among temperatures in maximal acceleration ($P < 0.001$, LME model with individual as random effect). There was a significant difference between 15°C and 25°C ($P < 0.001$, LSM) and between 25°C and 35°C ($P < 0.001$, LSM). The Q_{10} of maximal acceleration within individuals was 2.49 ± 0.57 SEM between 15°C and 25°C (N=4) and between 25°C and 35°C the Q_{10} is 1.74 ± 0.19 SEM (N=7).

Discussion

Our results indicate that western fence lizards, *Sceloporus occidentalis* are storing and releasing elastic energy in their ankle tendons during accelerations. At the ankle, we measured an average power 3.79 times higher than predicted by muscle alone at 25°C with the highest power output measured at 4.73 times the capability of the muscles (Figure 2.3). We measured a more modest knee muscle power amplification of 2.39 fold at 25°C (Figure 2.4). Many knee extensor muscles in western fence lizards have large aponeuroses (personal observation), so it is likely that there is some elastically driven muscle power amplification about the knee joint. Alternatively, though we accounted for force transfer from the hip to the knee, there may be a larger degree of power transfer than we assumed. Hip powers are largely within the ranges we expect to see from muscle powered systems (Balaban and Azizi, 2017) (Figure 2.5), indicating that hip extension is driven solely by muscle power. Since temperature has a minimal impact on tendon properties at biologically relevant temperatures (Rigby et al., 1959; Kubo et al., 2005), lizards are able to maintain high accelerations at relatively low temperatures.

To amplify muscle power, fence lizards are likely storing energy in distal tendons and aponeuroses through the dynamic movement of their limbs. Lizards have a proximal to distal limb extension, which is seen in other running and jumping animals (Bels et al., 1992; Astley and Roberts, 2014)(Figure 2.6). This proximal to distal movement of the limbs allows the ankle extensors to contract isometrically or even eccentrically as the proximal muscle activity loads the ankle in flexion (Astley and Roberts, 2014). Isometric and eccentric con-

tractions produce significantly more force in less time than shortening contractions because of the trade-off between force and velocity in contracting muscle (Hill, 1938). These large muscle forces stretch the tendon, storing more work than would be possible during a shortening contraction. Proximally generated inertial loads have been demonstrated to stretch tendons to amplify muscle power output in other organisms (Marsh, 1999; Galantis and Woledge, 2003). In jumping frogs, inertial loading, in combination with a dynamic mechanical advantage of ankle extension is used to amplify muscle power (Roberts and Marsh, 2003; Astley and Roberts, 2014). This dynamic inertial catch mechanism allows frogs to take off with more power than they would be able to do with muscle alone, and also allows them to maintain performance at lower temperatures (Astley and Roberts, 2014; Olberding and Deban, 2017).

The effect of temperature on our measured muscle power output matches the pattern seen in the running performance of fence lizards (Marsh and Bennett, 1986b). We measured a Q_{10} of ankle muscle power to be 3.45 between 15°C and 25°C, which is a very large effect of temperature. The Q_{10} of muscle power around the ankle joint between 25° C and 35°C, by contrast, is only 1.67, which is lower than predicted by *in vivo* muscle experiments (Marsh and Bennett, 1986b). We see a similar pattern at the knee joint, with high Q_{10} values between 15°C and 25°C and low Q_{10} values between 25°C and 35°C. This supports our hypothesis that power amplification around the ankle and knee joints contributes to maintenance of acceleration performance at high temperatures. Since we measured hip extension to be driven by muscle power alone, we expected a Q_{10} around 2, which is similar to that of in vitro muscle power (Marsh and Bennett, 1986a,b), between all temperatures. However, even at the hip, the Q_{10} from 15°C to 25°C is a very high 4.31, while the Q_{10} from 25°C to 35°C

is only 1.23. This is the same trend as we see around the ankle and knee. Our measured hip joint powers at each temperature could explain this unexpected finding.

Lizards may tune hip muscle activation levels at high temperatures to load ankle tendons. At 35°C, hip muscle power is only 58% of its maximal capability, while at 25°C hip muscles are operating maximally (Figure 2.5). Similarly, at 15°C, the hip extensor muscles are producing only 49% of their theoretical maximal power. At high temperatures, lizards may sub-maximally activate their hip extensor muscles but still maintain high performance by tuning the movements to allow for the effective storage of elastic energy in-series with ankle and knee extensor muscles. Any additional hip extensor power at 35°C may inhibit the effective power amplification of the ankle extensor muscles. To maintain the tuning of the ankle extensor muscle-tendon unit as the lizards body temperature decreases, they increase their level of hip activation until at 25°C they must activate their muscles maximally to generate enough force about the ankle to load the ankle extensor and foot tendons. This mechanism would allow lizards to save energy in their hip muscles at high temperatures without sacrificing the performance gains generated through power amplification of the distal joint extensor muscles.

The effects of temperature on muscle activation and timing during locomotion has been understudied in running terrestrial vertebrates, but there is much variability in the timing of muscle activation, muscle activation level, and muscle strain between different gaits in level running (Gillis and Biewener, 2001). This tuning of dynamic muscle properties can effectively offload work production from the muscles to the tendons (Biewener et al., 1998; Roberts, 1997; Gillis and Biewener, 2001). Alternatively, there may be some other physical constraint to hip muscle power at high temperatures, such as a high risk of their foot slipping

during acceleration. However, in previous studies on lizard running, lizards were measured to have just enough muscle to power acceleration at their optimal temperature (Curtin et al., 2005; McElroy and McBrayer, 2010). Since fence lizards are not producing maximal muscle power in their hip extensor muscles at their optimal temperature during acceleration, they are likely using stored elastic energy in their ankle tendons to improve performance while minimizing the energetic cost of acceleration.

The low hip muscle powers measured at 15° C are likely due to lizard behavior. At cold temperatures, many species of lizard hold their ground rather than flee (Hertz et al., 1982; Mautz et al., 1992; Crowley and Pietruszka, 1983). Lizards are not able to run as quickly, but their bite force remains high at low temperatures, so they become aggressive rather than try to escape when they become too cold (Herrel et al., 2007). During our running trials, lizards were much more hesitant to move (personal observation) and we were not able to get many trials of them running at this temperature. The difference in fence lizard behavior at low temperatures is reflected in the kinematics of acceleration. At 25°C and 35°C we see little difference in kinematics with a proximal to distal movement of the limbs. The timing of hip extension, knee extension, and ankle extension are very similar across the top two temperatures (Figure 2.6). At 15°C however, there is much more variability and the average kinematics show the ankle, knee, and hip extending all at once.

Despite the change in kinematics at 15°C, lizards still have some power amplification of their ankle and knee extensor muscles. Since we did not see a consistent proximal to distal limb extension at 15°C, we expected the inertial loading of the ankle and knee tendons to be limited. However, ankle muscle power was amplified 2.2 fold over the expected value at 15° C. This is less than the power amplification of 3.79 at 25° C or 2.68 at 35° C. If lizards

were able to generate the same forces to stretch their tendons during acceleration and match performance at the higher temperatures, we would expect to see a power amplification of at least 7.5. We likely see the modest power amplification measured at 15° C because even with simultaneous limb segment extension, there should still be some inertial loading of the long, thin foot tendons as the foot is bent back during the first half of the acceleration step.

Conclusions

Extreme power-amplified systems are seen across a wide variety of biological systems (Ilton et al., 2018). These systems allow performance capabilities far greater than muscle alone could achieve. In this study, we show that elastically driven muscle power amplification can also act to more subtly impact performance through a broadening of the thermal performance plateau and by reducing energy consumption at high temperatures without losing elastically driven performance gains. We also present the first evidence of muscle power amplification in a small running animal. These results are likely applicable to many other generalist or sprinting lizards and have implications for better understanding the life history of lizards and how they may respond to a changing environment.

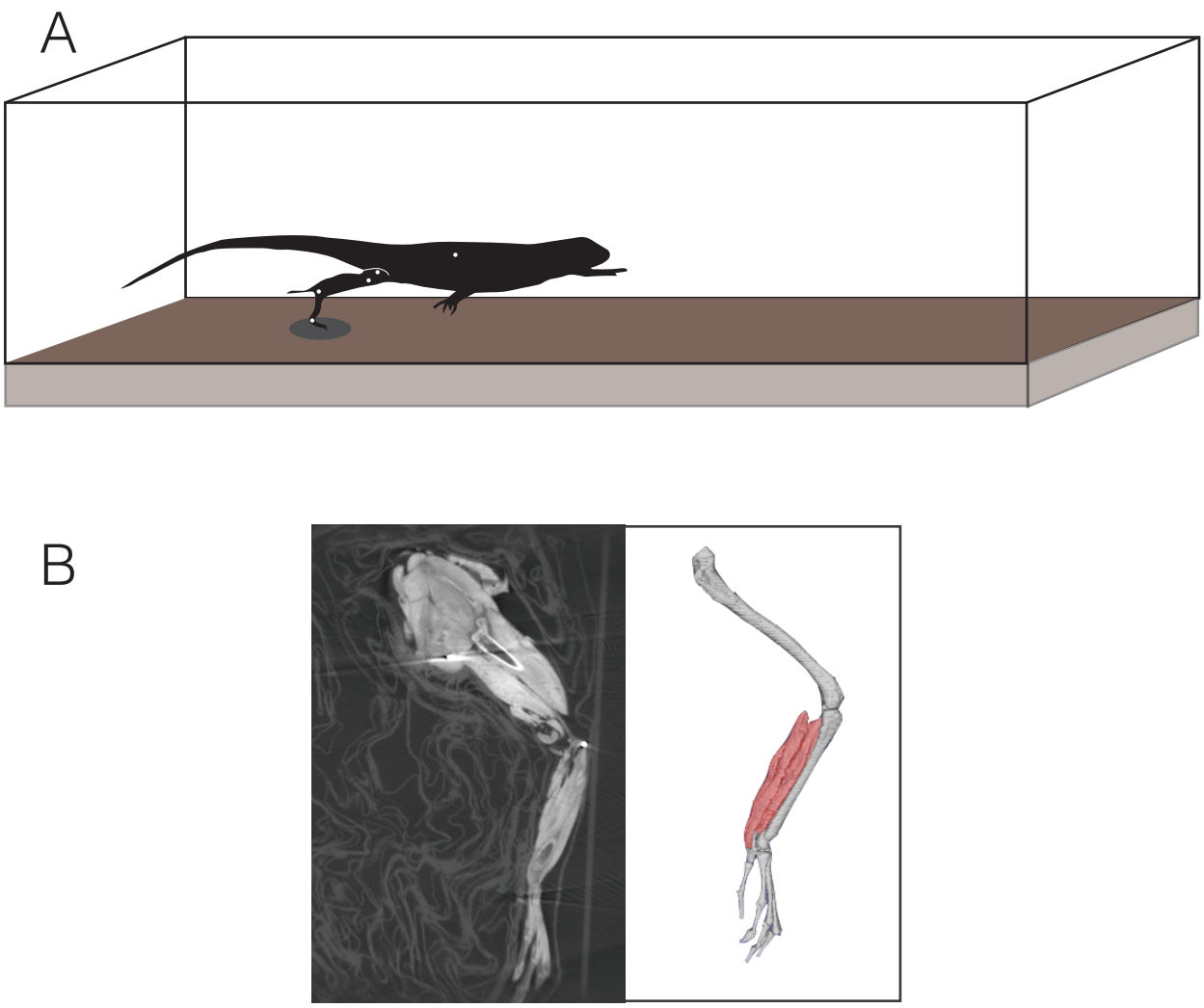

Figure 2.1 A) Lizard run experimental setup. Lizards were marked with whiteout and sharpie at the toes, ankle, knee, hip, and center of mass. They were then filmed with two high speed cameras accelerating off of a six-axis force transducer (gray circle) to measure the kinematics and ground reaction forces in three dimensions. B) Sample output from DICE-CT scan. On the left is a single CT slice. Notice that the musclulature is visable due to the iodine staining. On the right is a reconstruction of the hindlimb bones and ankle extensor muscles. Muscle in-lever distances were taken from these reconstructions.

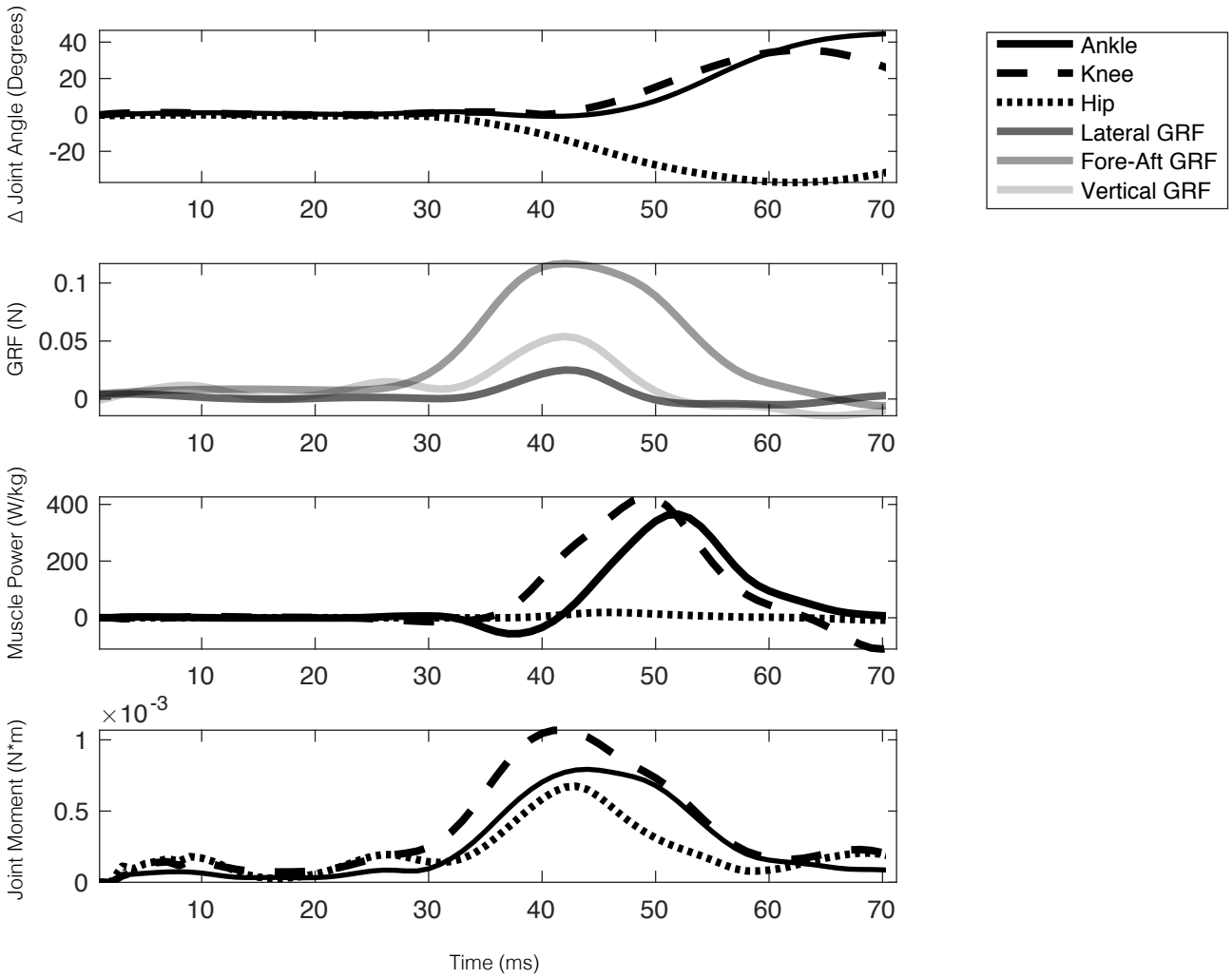

Figure 2.2 Example output from custom code used to analyze kinematics and run inverse dynamics analysis.

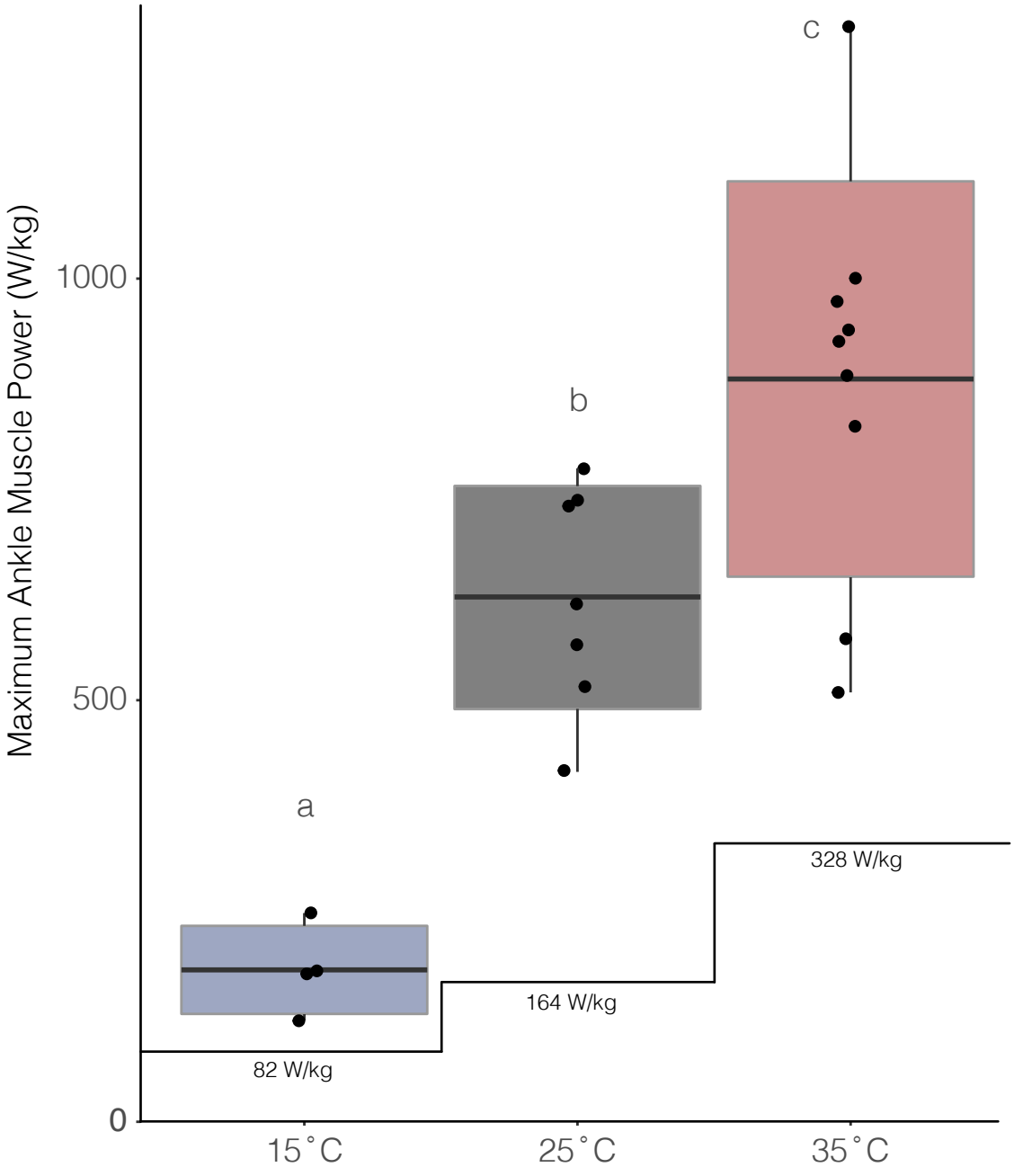

Figure 2.3 Maximal ankle muscle power measured in each individual at 15° C, 25° C, and 35° C. Box plots represent mean, standard deviation, minimum, and maximim values. Indivudual data are plotted with random jitter for visualization. Maximal muscle power for ankle extensor muscles at each temperature is presented as the lines with power values listed next to them. There is a significant effect of temperature on ankle muscle power ($P<0.001$).

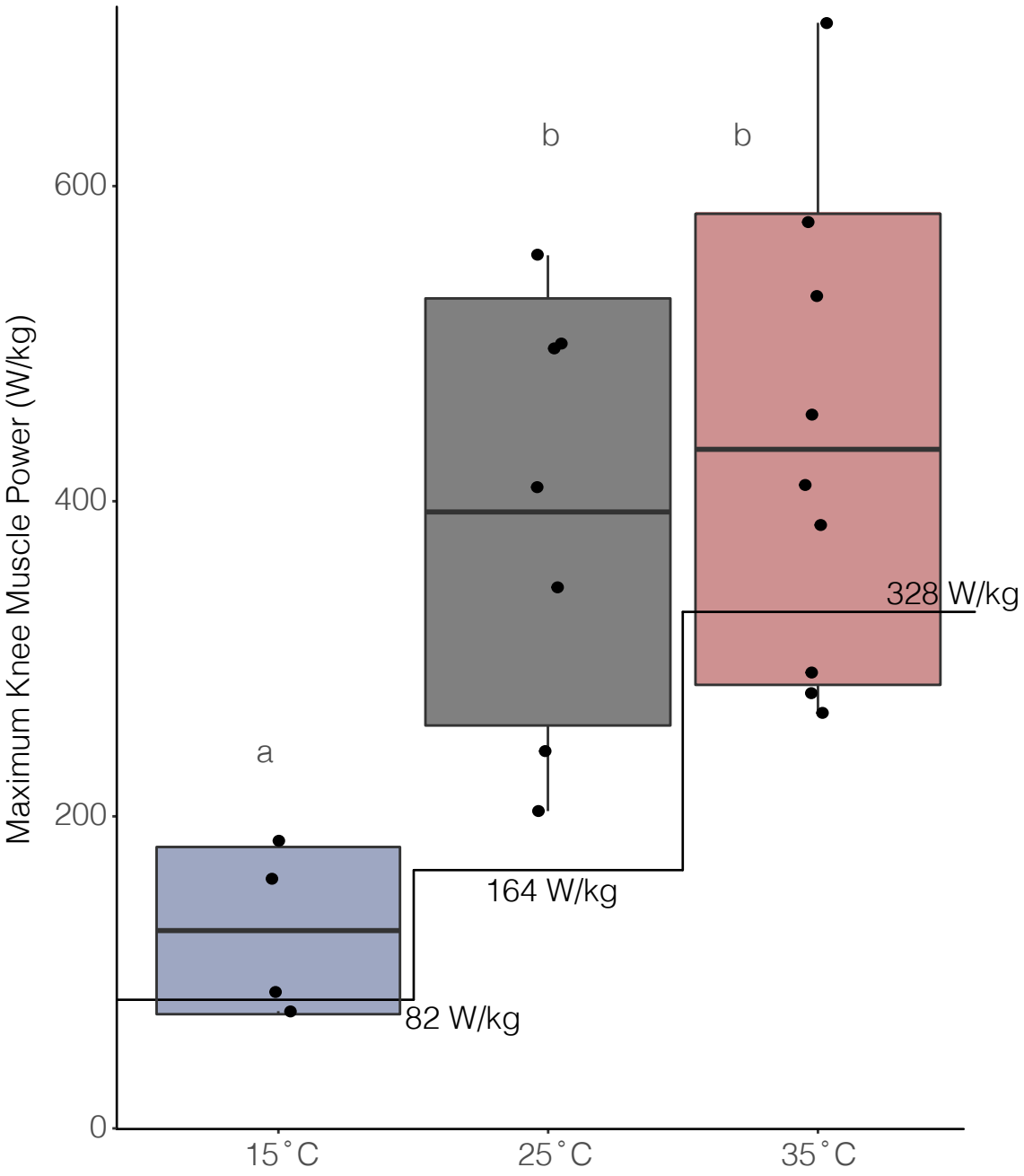

Figure 2.4 Maximal knee muscle power measured in each individual at 15º C, 25º C, and 35º C. Box plots represent mean, standard deviation, minimum, and maximim values. Indivudual data are plotted with random jitter for visualization. Maximal muscle power for knee extensor muscles at each temperature is presented as the lines with power values listed next to them. There is a significant effect of temperature on ankle muscle power ($P<0.001$), but there is no difference between 25º C and 35º C ($P=0.20$).

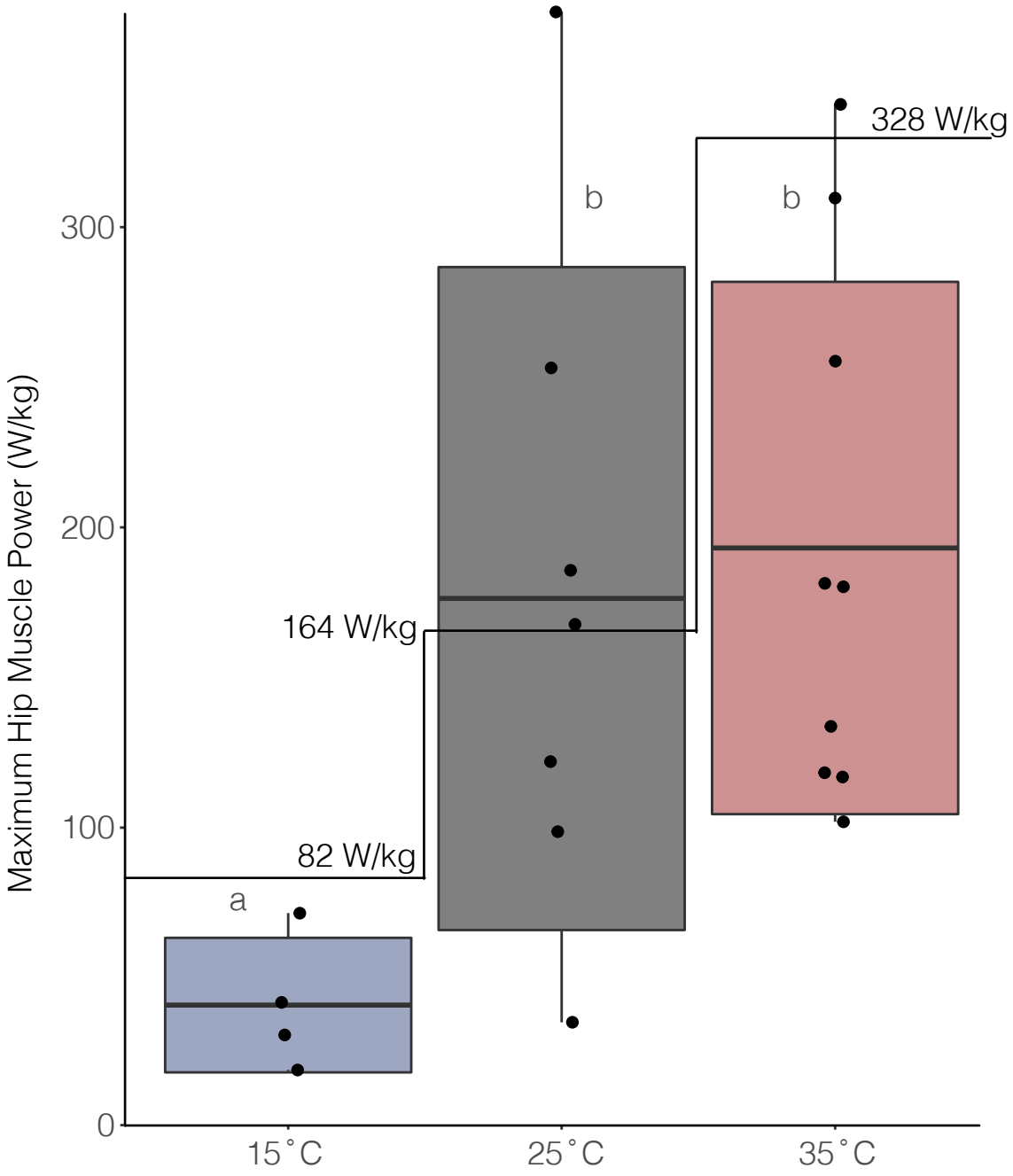

Figure 2.5 Maximal hip muscle power measured in each individual at 15º C, 25º C, and 35º C. Box plots represent mean, standard deviation, minimum, and maximim values. Indivudual data are plotted with random jitter for visualization. Maximal muscle power for hip extensor muscles at each temperature is presented as the lines with power values listed next to them. There is a significant effect of temperature on ankle muscle power ($P<0.001$), but there is no difference between 25º C and 35º C ($P=0.65$).

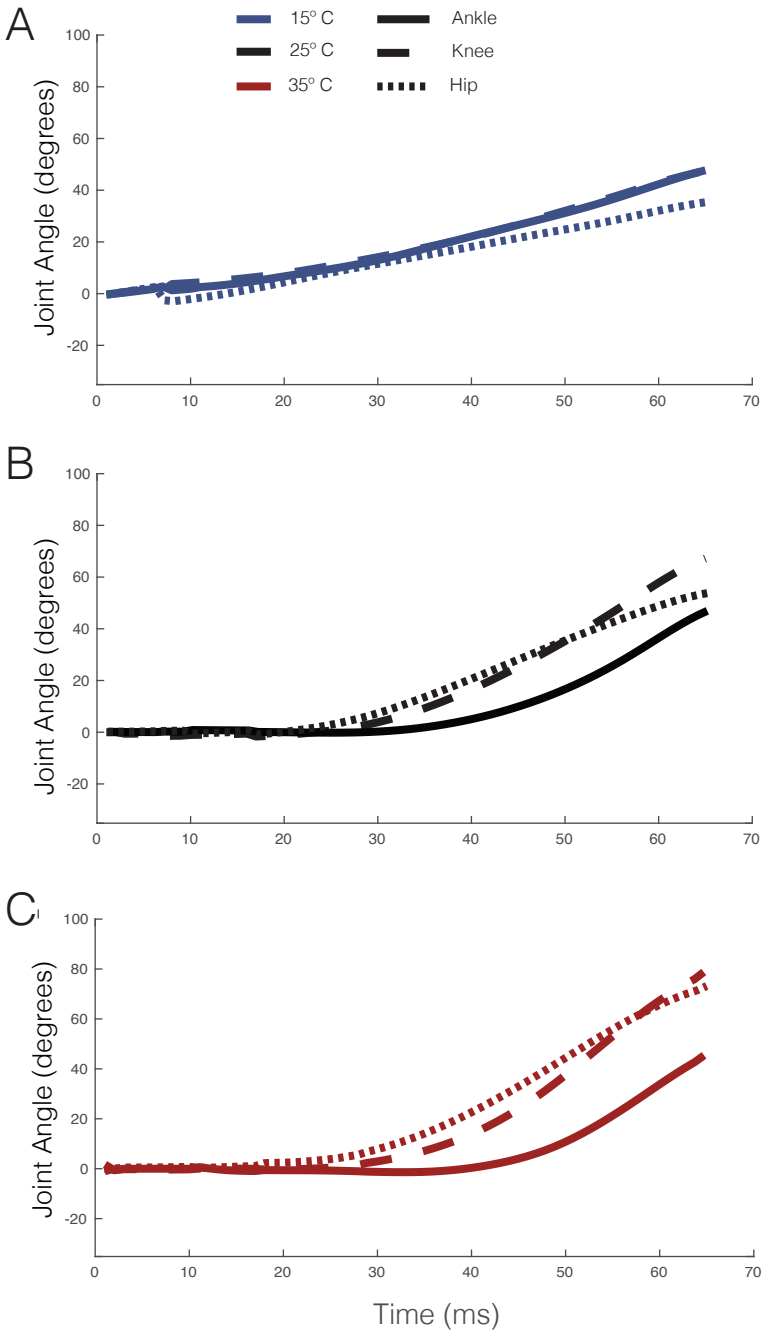

Figure 2.6 Average joint angles during acceleration in fence lizards at 15° C, 25° C, and 35° C. Notice that the kinematics are very similar between 25° C, and 35° C but that at 15° C there is not the same proximal to distal movement of the limbs.

CHAPTER 3

Lizards broaden the thermal performance of running by tuning muscle activation to cycle work through tendons

Introduction

Ectothermic animals must operate across a wide range of body temperatures as environmental temperature and sunlight fluctuate daily and seasonally. This has important implications for survival, since a cool lizard basking in the morning is at risk for predation. Despite the strong thermal sensitivity of muscle contractions, many ectotherms are able to operate effectively across a broad thermal range. One such ectotherm is the western fence lizard, *Sceloporus occidentalis*, which is able to maintain nearly the same maximum running speed at 25°C as at 35°C, despite having 50% lower muscle shortening velocity and power at the lower temperature (Marsh and Bennett, 1986b).

The maintenance of maximum running velocity in lizards can be partially explained by the twitch kinetics (the time it takes for muscle to develop and lose force) of muscle (Swoap et al., 1993). Above 25°C, lizards are able to contract their muscles quickly enough to maintain a stride frequency of 12 Hz, which is the measured stride frequency of lizards at their optimal temperature of 35°C (Swoap et al., 1993). However, since muscles take longer to develop force and longer to relax at 25°C than at 35°C, lizards must either alter the timing of activation of their muscles to generate sufficient force for the stride or they must produce less force at 25°C. Lower force production could cause a drop in performance since running animals must generate force quickly enough to decelerate and then accelerate their body

weight during each stride (Full and Farley, 2000). Lizards at moderate temperatures could develop more force in their limbs if they activated their muscles earlier in each limb cycle, giving them more time to develop force, but too large a shift in the timing of activation could lead to the muscle generating negative work (Josephson, 1985) and thus lizards would not be able to maintain speed. Alternatively, lizards may take advantage of long foot tendons and ankle extensor muscle aponeuroses to mitigate the problems caused by slow muscles at intermediate temperatures.

Elastic elements, in-series with muscles, such as tendons and aponeuroses, can decouple muscle work from animal movement. Wallabies (Biewener et al., 1998), rheas and turkeys (Cavagna et al., 1977), and many other species (Roberts and Azizi, 2010; Roberts, 2016) cycle energy through their tendons rather than their muscles during locomotion. This minimization of muscle work can reduce the energetic cost of locomotion, reduce the swing weight of legs by moving muscle masses proximally, and allow for a greater excursion of the muscle-tendon unit than the muscle alone would be able to accommodate (Biewener et al., 1998; Roberts and Azizi, 2010; Roberts, 2016; Holt et al., 2014). Despite arguments that small running animals are not able to store enough energy in the tendons of their distal hind limbs to relieve an appreciable amount of muscle work (Biewener and Blickhan, 1988), models of animal locomotion show that small animals do less work per stride than large animals, and the energy they can store in their tendons during a stride is enough to power locomotion (Bullimore and Burn, 2005). Zebra-tailed lizards, which are similar in size to western fence lizards, are able to store up to 40% of the work done in a stride in their long foot tendons (Li et al., 2012).

Muscle-tendon units (MTUs), which include muscles and their associated series elastic

elements, can mitigate performance loss at low temperatures in power amplified movements such as ballistic tongue projection (Anderson and Deban, 2010; Deban and Lappin, 2011) and jumping (Olberding and Deban, 2017). But we do not know how the presence of an elastic tendon impacts a muscle operating cyclically over a range of temperatures. The work loop technique is an *in vitro* method that has been used extensively to study the work output of muscles undergoing biologically relevant stretch/shorten cycles and timing of muscle activation in running, flying, and swimming animals (Josephson, 1985; Johnson et al., 1993; Askew and Marsh, 2001). The work loop technique has also been used to study the effect of temperature on work output (Johnson and Johnston, 1991; Swoap et al., 1993; Swank and Rome, 2000). Recently, this technique has been modified to explain how MTUs are naturally tuned to cycle work through the tendon instead of the muscle when cycling at the resonant frequency of the MTU (Robertson and Sawicki, 2015; Sawicki et al., 2015).

Fence lizards are too small to directly measure the elongation of their foot tendons *in vivo*, and it is not possible to isolate the ankle extensor muscles and the intact foot tendons to do work loop experiments of lizard MTUs *in vitro*. However, modeling muscle work loops *in silico* with and without series elastic compliance can allow us to generate predictions of the timing of muscle activity, which we can measure *in vivo*. In this study, we address two questions to answer how lizards maintain running speed above 25°C: 1) Do lizards alter the timing of muscle activation within a stride at different temperatures? and 2) Do lizards power running entirely through their muscles or do they cycle energy through elastic tendons? To address these questions we collect EMG data from the largest ankle extensor muscle, the medial gastrocnemius to measure the timing of activation within a stride and then use the muscle activation data in an *in silico* muscle work loop model at different temperatures with

and without series elastic compliance. These results may provide evidence for an additional function of series elastic elements: to maintain performance in cyclical movements such as running across a broad temperature range.

Materials and Methods

Animals

Six western fence lizards, *Sceloporus occidentalis* Baird & Girard 1852 (6.41 ± 1.91 g, mean ± s.d.),were caught on the University of California, Irvine campus using a California Department of Fish and Wildlife scientific collectors permit SC-12906 issued to JPB. Lizards were housed in pairs before surgery and individually after surgery with a 12/12 light dark cycle with UV broad-spectrum light (Exo Terra Repti Glo 2.0, 24) and a heating bulb (Exo Terra Sun Glo Basking Spot Lamp, 75W) placed above one side of the cage to allow for behavioral thermoregulation. They were housed in terraria with a sandy substrate, given water *ad libitum* and fed crickets supplemented with calcium. This work was carried out at UC Irvine under Institutional Animal Care and Use Committee protocol no. 2013-3110.

EMG surgery

We measured the electrical activity of the EMG in the medial gastrocnemius, a major ankle extensor, for all lizards. Lizards were anesthetized with 5% isoflurane for at least ten minutes, and until the self-righting and toe pinch reflexes were no longer present.

Incisions were made in the back and in the dorsal skin of the leg, immediately superficial to the medial gastrocnemius muscle. EMG electrodes were inserted under the skin from the back, through the leg under the skin, and into the left medial gastrocnemius muscle. A small incision was made in the muscle into which the electrode held in places via silk suture (6-0). The incisions were sutured closed and the EMG transducer was sutured onto the back

of the lizards to ensure the wires were not pulled out during running trials. These surgical procedures were performed in a semi-sterile field. Lizards were allowed to recover overnight in a cage with a 12/12 light dark cycle with UV broad-spectrum light (Exo Terra Repti Glo 2.0, 24) and a heating bulb (Exo Terra Sun Glo Basking Spot Lamp, 75W) placed above one side of the cage to allow for behavioral thermoregulation. After at least 24 hours of recovery, lizards were used in the running trials.

EMG and stride timing

We acclimated the lizards for at least two hours in an environmental chamber held at 15°C (N=5), 25°C (N=6), or 35°C (N=5). After acclimation, lizards were placed on a 2 meter long, 6 cm wide track, which we covered with 80 grit sandpaper to provide traction. We filmed lizards running at 1000 Hz with two Edgertronic high-speed cameras (Model SC1, Sanstreak Corporation, San Jose, CA, USA) placed dorsally and laterally to the force plate. We used two camera views to ensure that we would be able to visualize the beginning and end of stance phase for each stride in the left foot.

EMG data were collected using a 16-bit data acquisition system (National Instruments, TX, USA). Data were collected at 1000 Hz and initially analyzed using Igor Pro software (V 6.22A, Wavemetrics, Lake Oswego, OR, USA) before being imported into MATLAB (MathWorks, Natick, MA, USA) for further analysis. Cameras were triggered using an external trigger. The trigger signal was split and simultaneously sent to the cameras and to Igor Pro to synchronize the EMG and kinematic data. We used DLT software in MATLAB (Hedrick, 2008) to digitize the beginning and end of the stance phase of each stride. We

measured the start of stance as the first frame in which the foot made contact with the ground and the end of stance as the last frame where the toes were still in contact with the ground (Figure 3.1).

Workloop Muscle Model

We modified a muscle model made in Simulink (MathWorks, Natick, MA, USA) (Robertson and Sawicki, 2014) to simulate western fence lizard gastrocnemius muscle work loops at 15°C, 25°C, and 35°C while varying the timing of activation within the stretch shorten cycle and the compliance of the in-series tendon (Figure 3.2a,b). Model parameters were adjusted to match lizard ankle extensor contractile dynamics and work loop frequency and stimulation duration were altered to match *in vivo* measurements of EMG and stride timing at 15°C, 25°C, and 35°C (Table 3.1). The models were also run at the phase timings measured from our *in vivo* EMG and stride timing measurements. A muscle stimulation phase of 0% is the equivalent of foot touch down, and a stimulation phase of 50% is the equivalent of mid-stance (Figure 3.2a). Any negative stimulation phases are the equivalent of muscles activating before the lizards foot touches the ground.

Statistics

159 strides over the three temperatures were used for analysis. All statistical analyses were performed in R (R Core Team, 2015). Using the lme4 (Bates et al., 2015) and car (Fox and Weisberg, 2011) packages, we ran linear mixed effects models at each temperature for the stride time, stance time, duration of EMG activity within a stride, time between foot touch

down and the start of EMG activity, and time between toe off and the end of EMG activity. Stride time is defined as the moment the foot touches down from in one stride until the foot touches down during the following stride. In all tests, temperature was a fixed effect and individual was included as a random effect to account for individual variation. Differences between temperatures were determined using a least square means analysis.

Results

EMG and Stride Timings

In most variables, there was a significant difference between temperatures in timing (Figure 3.3). The average stride time at 15°C is 276.1 ± 31.78 ms (ave ± sd, N=5), at 25°C it is 154.83 ± 36.47 ms (N=6), and at 35°C it is 134.98 ± 34.11 ms (N=5). There is a significant effect of temperature on stride time ($P < 0.0001$, LME model with individual as random effect). There were significant differences between 15°C and 25°C ($P < 0.0001$, LSM) and 15°C and 35°C ($P < 0.0001$), but not between 25°C and 35°C ($P = 0.439$).

The average stance time at 15°C is 178.88 ± 7.60 ms (ave ± sd, N=5), at 25°C it is 87.55 ± 25.08 ms (N=6), and at 35°C it is 71.31 ± 20.87 ms (N=5). There is a significant effect of temperature on stance time ($P < 0.0001$, LME model with individual as random effect). There were significant differences between 15°C and 25°C ($P < 0.0001$, LSM) and 15°C and 35°C ($P < 0.0001$), but not between 25°C and 35°C ($P = 0.078$).

The average duration of EMG activity at 15°C is 102.47 ± 22.24 ms (ave ± sd, N=5), at 25°C it is 68.08 ± 18.54 ms (N=6), and at 35°C it is 57.67 ± 18.32 ms (N=5). There is a significant effect of temperature on the duration of EMG activity ($P < 0.0001$, LME model with individual as random effect). There were significant differences between 15°C and 25°C ($P < 0.0001$, LSM) and 15°C and 35°C ($P < 0.0001$), but not between 25°C and 35°C ($P = 0.21$).

The average time between foot touch down and the start of EMG activity at 15°C is 22.41 ± 22.21 ms (ave ± sd, N=5), at 25°C it is -11.32 ± 4.58 ms (N=6), and at 35°C it

is 1.55 ± 10.02 ms (N=5). There is a significant effect of temperature on the time between foot touch down and the start of EMG activity ($P < 0.0001$, LME model with individual as random effect). There were significant differences between 15°C and 25°C ($P < 0.0001$, LSM), 15°C and 35°C ($P = 0.008$), and 25°C and 35°C ($P = 0.05$).

The average time between toe off and the end of EMG activity at 15°C is 53.99 ± 4.11 ms (ave ± sd, N=5), at 25°C it is 30.79 ± 3.85 ms (N=6), and at 35°C it is 12.09 ± 8.55 ms (N=5). There is a significant effect of temperature on the time between toe off and the end of EMG activity ($P < 0.0001$, LME model with individual as random effect). There were significant differences between 15°C and 25°C ($P < 0.0001$, LSM), 15°C and 35°C ($P < 0.0001$), and 25°C and 35°C ($P < 0.0001$).

Model Output

We measured net work (J/kg of muscle) from the fourth work loop cycle for each temperature, stimulus phase, and tendon compliance (Table 3.2). The stimulus phase measured *in vivo* was 12.5% at 15°C, -14.5% at 25°C, and -4.5 at 35°C (Figure 3.4). We calculated the net work expressed as a percentage of work done by a lizard during a stride, which we estimated from our data on running western fence lizards. At 15°C, the compliant model predicts that the gastrocnemius produces 2.4% of the work done in a stride while in the stiff condition it produces -18.7%. At 25°C, the muscle model produced -5.8% of stride work when in series with an elastic element and -39.4% of whole animal work with a stiff tendon. At 35°C, the model produced -2.6% of animal with with a compliant tendon, and -35.9% with a stiff tendon.

Discussion

Western fence lizards alter the timing of muscle activation within a stride when running at different temperatures. Lizards had a similar stride frequency and duration of EMG activity at 25°C and 35°C. However, within the stride cycle, lizards activated and deactivated their ankle extensor muscles much earlier at 25°C than they did at 35°C. At all temperatures, the timing of the deactivation of ankle extensor muscles seem to be tuned such that they are still producing force at toe-off due to the relaxation rate of muscle at each temperature, and such that they lose force shortly after toe-off (figure 3.3). Our EMG results at 35°C match the timings measured in another species of *Sceloporus* lizard (Reilly, 1995), and so we expect that our results are applicable to all species of fence lizards, and likely many other sprinting species of lizard.

In our work loop model, lizard ankle extensor muscle-tendon units (MTUs) with a compliant tendon produced minimal net muscle work when we used *in vivo* values of cycle frequency and EMG activity (Figure 3.4). At 15°C muscle work was positive, and at 25°C and 35°C it was negative, but in all cases total muscle work was less than 6% of total animal work. In an animal running at a steady speed, net muscle work is expected to be close to zero as the muscle performs negative work to decelerate the lizard in the first half of stance and then positive work to re-accelerate the lizard in the second half of stance (Full and Farley, 2000). When we modeled MTUs with a stiff tendon, all work loops were negative and net muscle work ranged from -19% to -39% of total animal stride work (Figure 3.4). Therefore, if lizards were running with stiff tendons, the ankle extensor muscles would absorb a

significant amount of work to decelerate the animal and then not produce nearly as much positive work to re-accelerate the animal, which would result in the lizard slowing to a stop. Interestingly, if lizards at 25°C activated their ankle extensor muscles at the same phase in the stride cycle as did lizards at 35°C, they would have produced positive work loops (Table 3.2). Though we might have predicted that lizards would want to produce positive muscle work to drive locomotion, the timing of activation required to do that would lead to less work cycling through their tendons.

At different temperatures, fence lizards may passively alter the timing of muscle activation within a stride to cycle energy through distal tendons rather than through muscles during steady state running. Muscles can naturally tune to in-series elastic elements with no other input aside from stimulation at the resonant frequency of the MTU (Robertson and Sawicki, 2015). In a compliant MTU, muscle work is minimized and much of the work in the system is cycled through the tendon rather than the muscle. The timing of activation within the cycle naturally moves to the place where tendon displacement is maximized without active neural control (Robertson and Sawicki, 2015). This resonance tuning of MTUs may allow for a passive tuning of the timing of muscle activation as muscle properties change at low temperatures. Since the ankle extensor muscles of running lizards, which are temperature sensitive (Marsh and Bennett, 1986a,b), are operating in series with elastic elements, which are temperature insensitive at biologically relevant temperatures (Rigby et al., 1959; Kubo et al., 2005), the system may be self-tuning. The consistent EMG duration and stride frequency coupled with the change of onset and offset timing of EMG activation indicate that lizard ankle extensor MTUs may be operating with the same central pattern generator (CPG) at 25°C and 35°C, but that the changing muscle properties at the lower temperature cause

the system to naturally tune the timing of activation to maximize energy cycled through the tendon at each temperature. Without further investigation, we cannot rule out the possibility that some feedback mechanism from the timing and rate of force production in the ankle extensors may generate a shift in the EMG activation pattern at the level of the CPG, but this feedback would still tune the MTU to cycle energy through the tendon rather than the muscle.

An inability to effectively tune the MTU at low temperatures can explain the large decrement in running speed at 15°C. The onset of muscle activation is much later at 15°C than at the higher temperatures, especially considering how long cold muscles take to generate force (figure 3.3). Steady state running is generally driven by a central pattern generator (CPG) (Grillner and Wallen, 1985). CPGs are used to produce rhythmic motor patterns, such as running. They are controlled through the spinal cord without the need for neural processing in the brain. At 15°C, fence lizard muscles cannot build or lose force quickly enough to maintain the same stride frequency as they achieve at 25°C or 35°C (Marsh and Bennett, 1986b; Swoap et al., 1993), and EMG activity appears to be a reflex response to the foot touching the ground rather than coordinated by a CPG. However, since rates of neural transmission and muscle force development are temperature sensitive, and CPGs patterns are often temperature dependent (Robertson and Money, 2012; Abram et al., 2016; Vicente et al., 2015), fence lizards are likely altering the CPG at low temperatures to ensure the proper force requirements for each phase of stance. This results in more positive work done by the muscle and less energy cycled through the tendon. *In vitro* muscle speed and power output in fence lizards have a Q_{10} of around 2 (Marsh and Bennett, 1986a), meaning that for a decrease in temperature of 10°C, muscle speed and power output are decreased by half.

However, sprint speed in fence lizard has a Q_{10} of 2.5 between 15°C and 25°C, while it is only 1.25 between 25°C and 35°C (Marsh and Bennett, 1986b). Very slow muscles leading to a decrease in stride frequency (Swoap et al., 1993) combined with the reduction in energy cycled through temperature insensitive tendon can explain the much lower maximal sprint speed seen in lizards at 15°C compared to those at and above 25°C (Marsh and Bennett, 1986b).

Many functions have been proposed for compliant MTUs in cyclic movements such as running. Compliant tendons can reduce the energetic cost of locomotion (Dawson and Taylor, 1973; Biewener et al., 1998; Roberts, 2002), though they may not be necessary to improve the efficiency of locomotion (Holt et al., 2014). Long tendons can allow for large MTU excursions with relatively short, proximally located muscles (Biewener, 1998). MTUs can provide a boost to acceleration performance in running animals by amplifying the power of ankle extensor muscles (Roberts and Scales, 2002) (Balaban, Chapter 2 of this dissertation). And compliant MTUs can also provide running stability in uneven or unpredictable terrain when direct motor control is not sufficiently fast (Jindrich and Full, 2002; Full and Koditschek, 1999; Daley and Biewener, 2006; Daley and Usherwood, 2010). In lizards, long, tendinous feet may be advantageous for lizards to reduce metabolic cost, maintain stability or to have low swing weights in their distal limbs to move quickly (Farley, 1997; Full and Koditschek, 1999; Li et al., 2012). In this study we present the first evidence, to our knowledge, of an additional function for compliant MTUs: the thermal robustness of running performance.

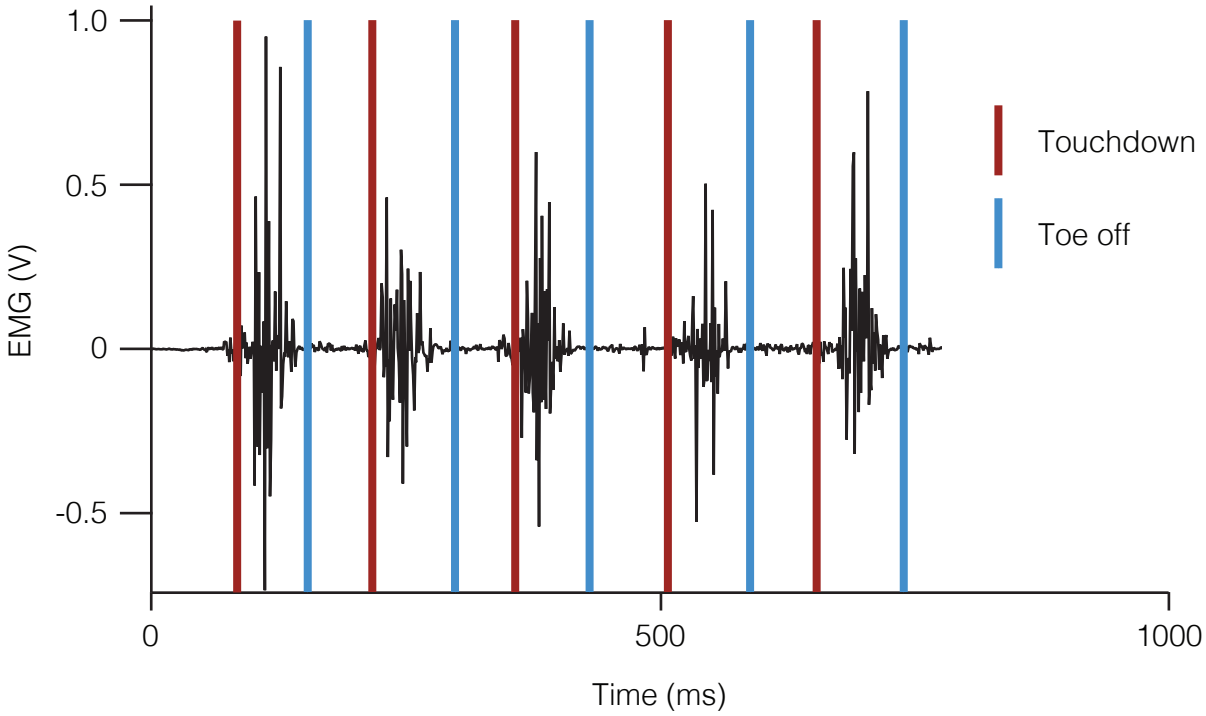

Figure 3.1 Representative raw EMG trace with stride timing overlayed.

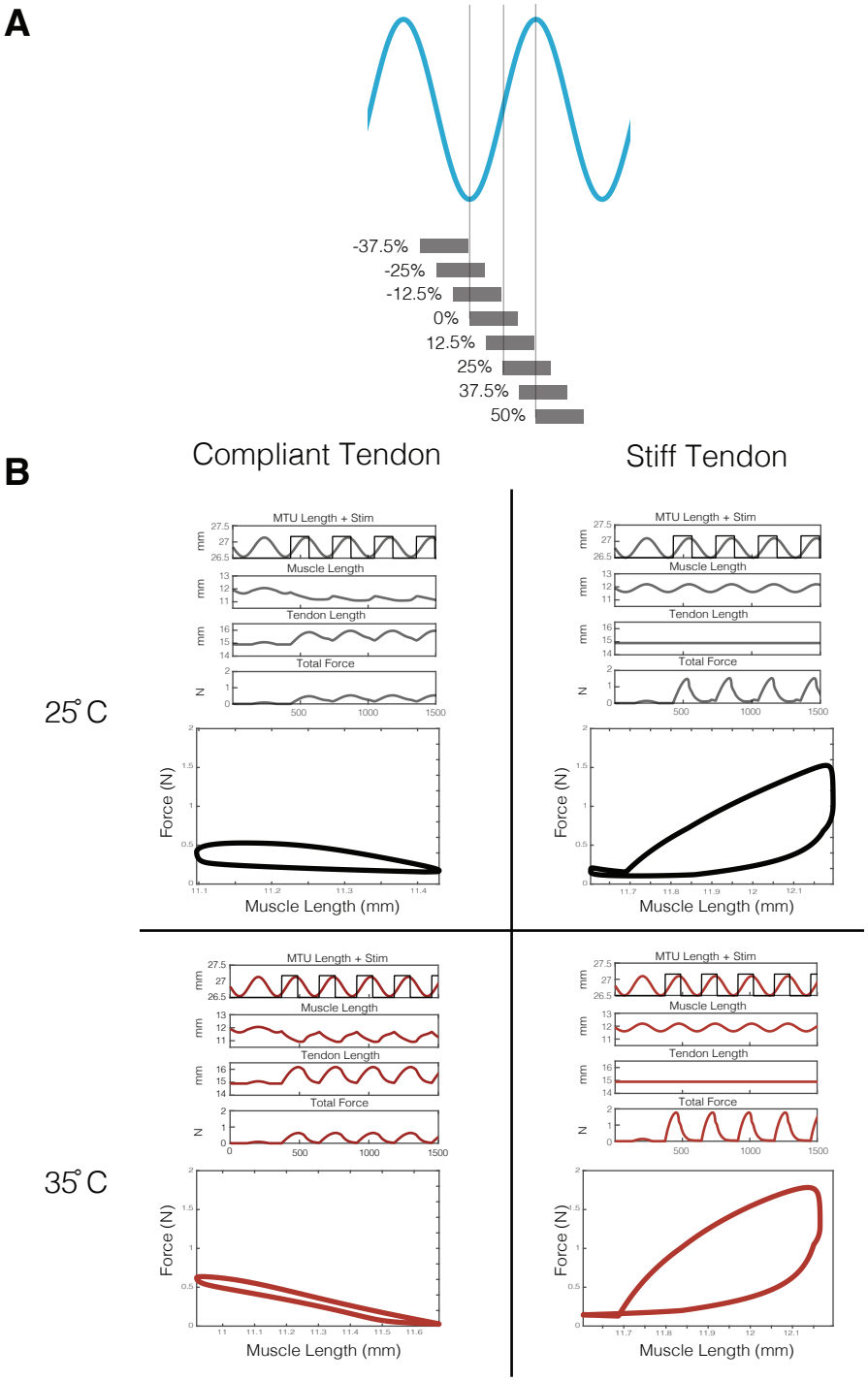

Figure 3.2 Muscle model workloop parameters. A) Lizard gastrocnemius muscle workloops were modelled with a 0.3 mm amplitude sin wave. The model was run with stimulation at multiple phases throughout the sin wave. Sin wave period 276, 155, and 135 ms for 15˚C, 25˚C, and 35˚C respectively and stimulus duration was 102.5 ms, 68 ms, and 57.5 ms respectively. B) Model output data for 12.5% stimulus phase for 25˚C and 35˚C with a compliant and stiff tendon.

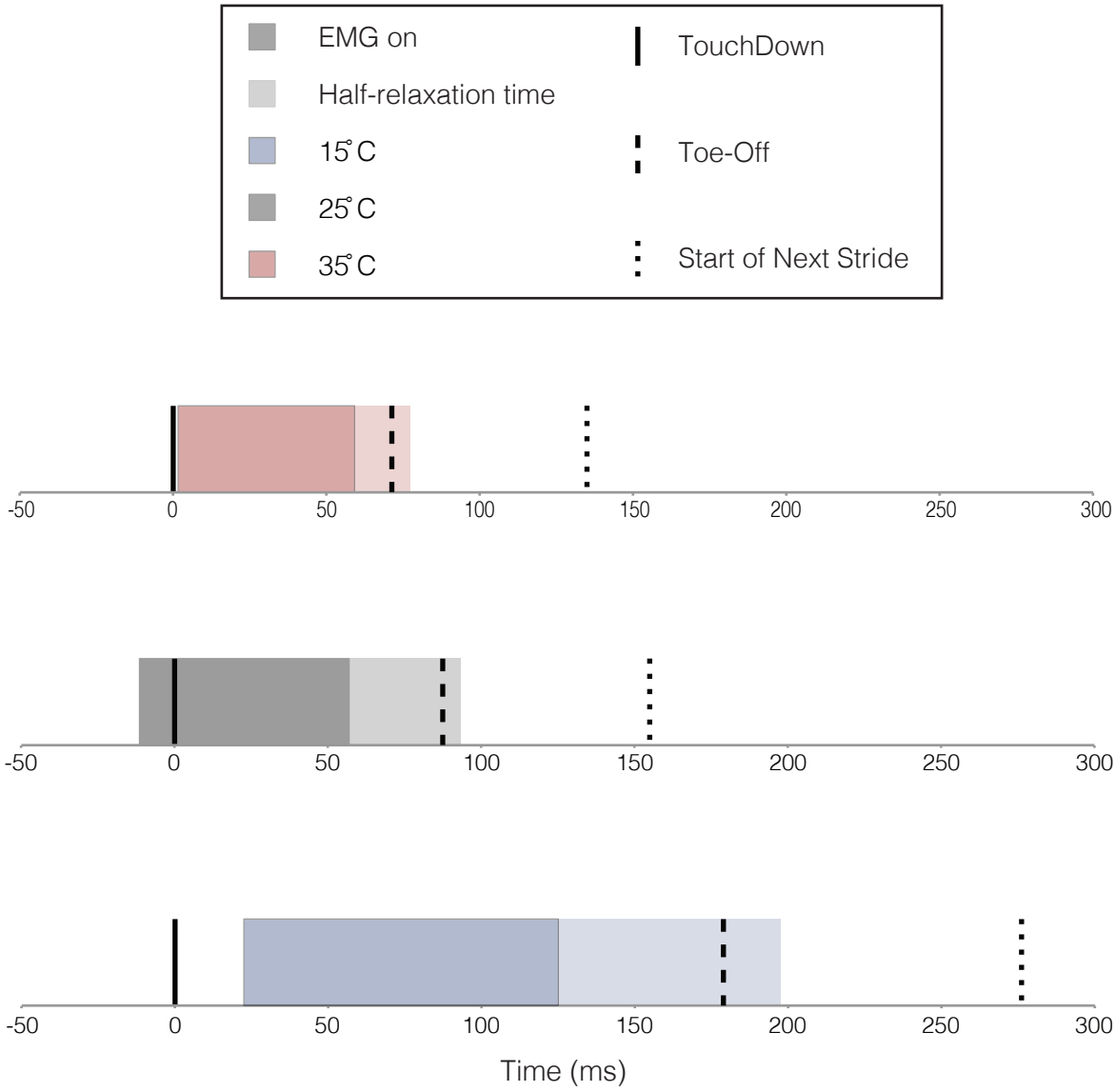

Figure 3.3 Average timing of EMG activity relative to stride at 15˚C, 25˚C, and 35˚C. Traces were aligned so that the foot touchdown is time zero for all temperatures. The half relaxation time for fence lizard ankle extensor muscles was added after EMG activity turned off to show the relative force still produced by the muscles at toe-off.

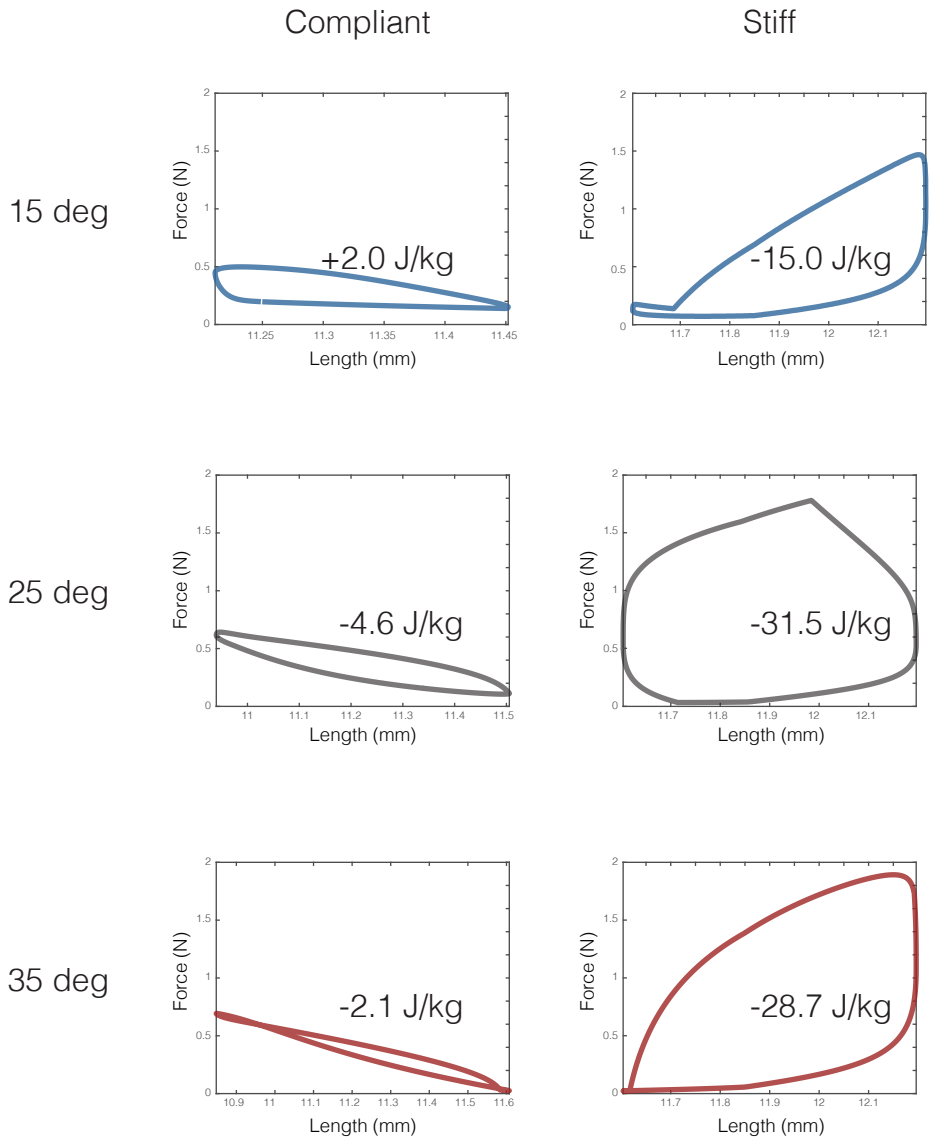

Figure 3.4 Workloop outputs using measured in vivo timing and duration of activation with compliant and stiff tendons. At 15˚C the workloop phase is 12.5%, at 25˚C the workloop phase is -14.5%, and at 35˚C the workloop phase is 4.5%. The values in the boxes indicate the net work done in the fourth workloop cycle in Joules/kilogram.

	15°C	25°C	35°C
T_{act}(s)	0.083	0.415	0.02075
T_{deact}(s)	0.0725	0.03625	0.018125
F_{init}(N)	0.02	0.02	0.02
L_0(m)	0.012	0.012	0.012
V_{max}(m/s)	-0.0185	-0.037	-0.074
F_{max}(N)	1.13	1.13	1.13
K_m (N/m)	400	400	400
$K_{t,c}$ (N/m)	500	500	500
$K_{t,s}$ (N/m)	5000000	5000000	5000000
L_t (m)	0.0149	0.0149	0.0149
Workloop Amplitude (m)	2.96×10^{-4}	2.96×10^{-4}	2.96×10^{-4}
Period (s)	0.276	0.155	0.058
Frequency (Hz)	3.62	6.46	7.41
Stimulus duration (s)	0.103	0.068	0.058

Table 3.1 Model parameters

Parameters used in the workloop model for muscle at 15°C, 25°C, and 35°C. T_{act} - describes speed of force rise, T_{deact} - describes speed of force loss when muscle is deactivated, F_{init} - initial force on muscle-tendon unit, L_0 - initial length of muscle, V_{max} - maximum muscle shortening velocity, F_{max} - maximum force production of muscle, K_m - spring constant of muscle, $K_{t,c}$ - spring constant of compliant tendon, $K_{t,s}$ - spring constant of stiff tendon, L_t - initial length of tendon. All muscle data were used from previous

invitro experiments on lizard gastrocnemius muscles at 25° C (Balaban and Azizi, 2017). Values at 15°C and 35°C were extrapolated assuming a Q_{10} of 2. Workloop amplitude was estimated from the joint angle excursions and the distance from the muscle insertion to the joint centers. Workloop period and frequency and stimulus duration were taken from the EMG and stride analysis from this study.

Stim phase (%)	15°C comp	15°C stiff	25°C comp	25°C stiff	35°C comp	35°C stiff
-37.5	-5.95	-10.95	-7.20	-16.01	-4.84	-6.21
-25	-6.95	-20.30	-7.36	-26.95	-10.26	-23.40
-12.5	-4.70	-26.70	-3.90	-31.58	-11.14	-35.28
0	-0.46	-24.88	0.71	-26.12	-5.16	-33.13
12.5	1.95	-14.98	2.84	-14.25	3.07	-18.26
25	2.36	-5.07	2.87	-3.87	7.57	-1.14
37.5	0.42	-1.15	0.11	-0.72	6.45	8.14
50	2.84	-3.90	-3.99	-5.78	1.54	5.72

Table 3.2 Model work output
Net work output presented as J/kg of muscle. Comp - compliant, Stim phase - phase of workloop cycle at which muscle stimulation begins (Figure 3.2a).

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