The Cross-Bridge Spring: Can Cool Muscles Store Elastic Energy?

N. T. George *et al.*

Science **340**, 1217 (2013);
DOI: 10.1126/science.1229573

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

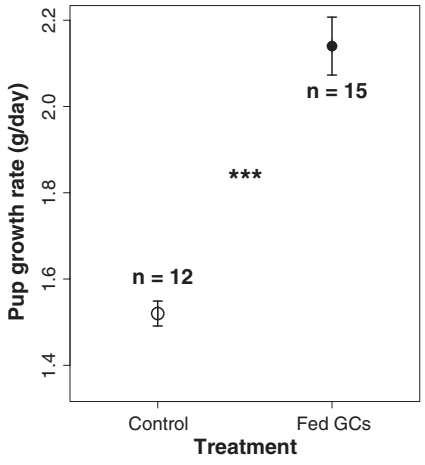

Fig. 4. Offspring produced by female red squirrels provisioned with cortisol grew significantly faster than those from controls. Raw offspring growth rates (mean ± SE) are shown on *y* axis. Sample sizes denote number of pups. Fed GCs corresponds to provisioning with three different cortisol concentrations (fig. S2). ***$P < 0.0001$ (table S6).

period [$t_{98} = 1.94$, $P = 0.028$ (table S5)]. Last, offspring born to females with experimentally increased glucocorticoid levels during pregnancy [fed cortisol (fig. S1)] grew 41% faster than those produced by control females [$t_{26} = 4.98$, $P < 0.0001$ (table S6 and Fig. 4)].

Our results suggest that elevated maternal glucocorticoid levels in response to heightened population density induced an adaptive hormone-mediated maternal effect on offspring growth. In contrast to the widespread assumption that heightened maternal glucocorticoid levels are detrimental to offspring (*22*), our results emphasize that in free-living animals they can instead lead to adaptive adjustments in offspring (*23*, *24*). Under high-density conditions, squirrels spend less time feeding and in the nest (*10*), suggesting that increased offspring growth is not a simple outcome of increased maternal care or milk provisioning. Alternatively, elevated exposure to glucocorticoids early in life (*22*, *25*) could increase offspring growth by directly influencing offspring physiology or behavior (*22*, *26*) and subsequent changes in growth hormone secretion in offspring (*27*).

For nearly 100 years, food availability has been considered to be a universal variable affecting population dynamics and life-history traits (*28*). Increased food availability also increases the population density of consumers, which has made it difficult to distinguish whether the plasticity in life-history traits after periods of high food availability is due to relaxation of food limitation or to adaptive reproductive adjustments to changes in density-mediated selection. Our results provide evidence that female red squirrels can produce faster-growing offspring in the absence of additional resources but only do so when the fitness prospects warrant this increased investment. In fact, offspring produced by females exposed to high-density cues but with no access to additional food grew as fast as those produced by food-supplemented females that were also experiencing increased density [1.79 ± 0.09 squirrels/ha (Fig. 2 and table S2)]. Therefore, some of the plasticity in female life history traits is due to the expected fitness benefits of producing faster-growing offspring under high-density conditions rather than only reflecting a relaxation of food limitation.

Experimental increases in food resources that result in increased reproductive output are typically interpreted as evidence for resource limitations on reproduction (*29*). However, if animals use food abundance as a cue of upcoming density-mediated selection, then reproductive responses to food supplementation might reflect not only relaxation of food limitation but also an adaptive adjustment to an anticipated change in natural selection resulting from an impending increase in density. Cues of population density may be a general signal that animals use to make adaptive reproductive adjustments in anticipation of density-dependent natural selection on offspring phenotypes.

References and Notes

1. M. J. Wade, S. Kalisz, *Evolution* **44**, 1947 (1990).
2. A. D. C. MacColl, *Trends Ecol. Evol.* **26**, 514 (2011).
3. R. Levins, *Evolution in Changing Environments* (Princeton Univ. Press, Princeton, NJ, 1968).
4. N. A. Moran, *Am. Nat.* **139**, 971 (1992).
5. D. Réale, A. G. McAdam, S. Boutin, D. Berteaux, *Proc. Biol. Sci.* **270**, 591 (2003).
6. A. Charmantier *et al.*, *Science* **320**, 800 (2008).
7. T. A. Mousseau, C. A. Fox, Eds., *Maternal Effects as Adaptations* (Oxford Univ. Press, Oxford, 1998).
8. D. J. Marshall, T. Uller, *Oikos* **116**, 1957 (2007).
9. Materials and methods are available as supplementary materials on *Science* Online.
10. B. Dantzer, S. Boutin, M. M. Humphries, A. G. McAdam, *Behav. Ecol. Sociobiol.* **66**, 865 (2012).
11. J. M. LaMontagne, S. Boutin, *J. Ecol.* **95**, 991 (2007).
12. Q. E. Fletcher *et al.*, *Ecology* **91**, 2673 (2010).
13. K. W. Larsen, S. Boutin, *Ecology* **75**, 214 (1994).
14. S. Boutin *et al.*, *Science* **314**, 1928 (2006).
15. C. C. Smith, S. D. Fretwell, *Am. Nat.* **108**, 499 (1974).
16. A. G. McAdam, S. Boutin, *Evolution* **57**, 1689 (2003).
17. A. G. McAdam, S. Boutin, A. K. Sykes, M. M. Humphries, *Écoscience* **14**, 362 (2007).
18. M. M. Humphries, S. Boutin, *Ecology* **81**, 2867 (2000).
19. S. Descamps, S. Boutin, A. G. McAdam, D. Berteaux, J.-M. Gaillard, *Proc. Biol. Sci.* **276**, 1129 (2009).
20. S. Descamps, S. Boutin, D. Berteaux, A. G. McAdam, J.-M. Gaillard, *J. Anim. Ecol.* **77**, 305 (2008).
21. S. Creel, B. Dantzer, W. Goymann, D. R. Rubenstein, *Funct. Ecol.* **27**, 66 (2013).
22. A. Harris, J. Seckl, *Horm. Behav.* **59**, 279 (2011).
23. O. P. Love, T. D. Williams, *Am. Nat.* **172**, E135 (2008).
24. R. Boonstra, *Funct. Ecol.* **27**, 11 (2013).
25. A. Catalani *et al.*, *Neuroscience* **100**, 319 (2000).
26. C. L. Moore, K. L. Power, *Dev. Psychobiol.* **19**, 235 (1986).
27. C. M. Kuhn, J. Pauk, S. M. Schanberg, *Dev. Psychobiol.* **23**, 395 (1990).
28. C. S. Elton, *Br. J. Exp. Biol.* **2**, 119 (1924).
29. S. Boutin, *Can. J. Zool.* **68**, 203 (1990).

Acknowledgments: We thank Agnes Moose and family for access to their traditional trapping area; F. E. Stewart, S. E. Evans, S. E. McFarlane, Q. E. Fletcher, S. Hossain, R. W. Taylor, and all squirrelers for assistance; A. Sykes and E. Anderson for data management; and A. Charmantier, T. Getty, K. E. Holekamp, J. S. Lonstein, C. T. Williams, T. D. Williams, and three anonymous reviewers for incisive comments. Funded by Natural Sciences and Engineering Research Council of Canada, NSF (DEB-0515849 and IOS-1110436), and Ontario Ministry of Research Innovation. Data have been deposited in the Dryad Repository (http://dx.doi.org/10.5061/dryad.b3h4q). This is publication no. 69 of the Kluane Red Squirrel Project.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1235765/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 to S7
References (*30*–*54*)

28 January 2013; accepted 5 April 2013
Published online 18 April 2013;
10.1126/science.1235765

The Cross-Bridge Spring: Can Cool Muscles Store Elastic Energy?

N. T. George,[1] T. C. Irving,[2] C. D. Williams,[1,3] T. L. Daniel[1]*

Muscles not only generate force. They may act as springs, providing energy storage to drive locomotion. Although extensible myofilaments are implicated as sites of energy storage, we show that intramuscular temperature gradients may enable molecular motors (cross-bridges) to store elastic strain energy. By using time-resolved small-angle x-ray diffraction paired with in situ measurements of mechanical energy exchange in flight muscles of *Manduca sexta*, we produced high-speed movies of x-ray equatorial reflections, indicating cross-bridge association with myofilaments. A temperature gradient within the flight muscle leads to lower cross-bridge cycling in the cooler regions. Those cross-bridges could elastically return energy at the extrema of muscle lengthening and shortening, helping drive cyclic wing motions. These results suggest that cross-bridges can perform functions other than contraction, acting as molecular links for elastic energy storage.

Elastic energy storage is heralded as a critical design characteristic of animal movement, because it promotes efficient locomotion. Canonical examples of elastic energy-storage sites include tendons of mammals and resilin, the rubberlike protein in insect cuticle (*1*, *2*). Elastic energy storage is particularly important to flying insects, reducing the otherwise prohibitive inertial power costs of accelerating and decelerating the wings (*3*, *4*). Two main sites of elastic

energy storage have been proposed for insect flight: resilin (*1*) and elastic myofilament proteins within flight muscles [e.g., thick and thin filaments (*5*, *6*), cross-bridges that are attached or in rigor (*7*–*9*), collagen fibrils (*7*), and titin (*10*)]. We propose that an intramuscular temperature gradient selectively increases cross-bridge attachment time, constraining axial and radial myofilament movement and thus enabling elastic energy storage in both cross-bridges and myofilaments. This temperature gradient is an inevitable consequence of metabolic heat production combined with convective and radiative heat loss (*11*, *12*). Because muscles' activation and deactivation rates depend on temperature, all of the kinetics associated with cross-bridge cycling are likely to vary substantially along a temperature gradient, causing higher rates of cross-bridge cycling in warmer regions of a muscle but reduced turnover and longer attachment times in cooler regions (fig. S1) (*11*, *13*–*15*). Consequently the timing of cross-bridge attachment and detachment in a given length cycle will vary spatially. Thus, at any given moment in the contraction cycle, cross-bridges in the cooler region of a muscle will be less likely to detach from their actin binding sites, forming a lattice increasingly linked by these elastic elements as temperature decreases. This elastic lattice can store energy both (i) at the extrema of the lengthening phase and (ii) at the extrema of the shortening phase. At the very end of the lengthening phase, elastic energy can be stored in the axial deformation of cross-bridges that remain bound because of the delayed activation and deactivation times associated with lower temperatures. The energy imparted to these bound cross-bridges can then be released back into the lattice as they shorten upon initiation of the shortening phase. Conversely, at the very end of the shortening phase, elastic energy can be stored in cross-bridges that remain bound as they are radially extended, orthogonal to the direction of shortening (because muscle cells are isovolumetric, they necessarily undergo radial expansion as they shorten). As the shortening phase ends and the lengthening phase begins, this elastic energy may be released back into muscle's elastic lattice, providing a restoring force to help drive cyclic wing motions (*16*).

We documented these events in *Manduca sexta*, a large moth known to have a significant dorsoventral temperature gradient in its dominant flight muscle, the dorsolongitudinal muscle (DLM_1) (*11*). We used high-speed time-resolved x-ray fiber diffraction techniques to monitor changes in myofilament lattice spacing and in the distribution of mass around the thick and thin filaments' long axes. Changes in mass distribution are due to changes in the radial position of cross-bridges and, by implication, their degree of association with the thin filaments (*17*). By pairing this visualization technique with simultaneous force and length measurements under controlled muscle stimulation, we coupled molecular observations with mechanical measures of whole-muscle performance (*18*).

We cyclically oscillated the DLM_1 at 25 Hz (wingbeat frequency) and periodically stimulated the muscle at *M. sexta*'s in vivo phase of activation while recording force and length, establishing a work-loop that measures the cyclic mechanical energy exchange of activated muscle (*13*, *19*). Specifically, we conducted work-loops at two muscle temperatures, 25° and 35°C, to cover the range of *M. sexta*'s temperature gradient (Fig. 1 and fig. S2) (*11*). Additionally, to control for the regional specialization of contractile dynamics, we positioned the x-ray beam on either a ventral or a dorsal location within the DLM_1. Diffraction patterns were collected five times during each 40-ms contraction cycle. From this diffraction movie, we plotted cyclical changes in contractile dynamics by measuring variations of spacing and intensity in each diffraction pattern (movie S1 and Fig. 2). We tracked the d_{10} lattice spacing, the distance between thick filaments, as in (*20*). In addition, from the intensities of the 2,0; 1,1; and 1,0 equatorial reflections we found the equatorial intensity ratio, an estimate of the association of cross-bridges with the thin filament; higher ratios indicate shifts in cross-bridge mass toward the thin filament and away from the thick filament backbone (supplementary text). We expected the warmer ventral region of the DLM_1 to behave as the main power generator and therefore to have rapid cross-bridge turnover. However, in the cool dorsal region of the muscle, we expected reduced contraction rates to result in longer cross-bridge attachment times, supporting a lattice of springs that can store and return elastic energy from the axial and radial deformation of cross-bridges that remain bound at the

[1]Department of Biology, University of Washington, Seattle, WA 98195–1800, USA. [2]Center for Synchrotron Radiation Research and Instrumentation and Department of Biological and Chemical Sciences, Illinois Institute of Technology, Chicago, IL 60616, USA. [3]Department of Organismic and Evolutionary Biology, Concord Field Station, Harvard University, Cambridge, MA 01451, USA.

*Corresponding author. E-mail: danielt@uw.edu

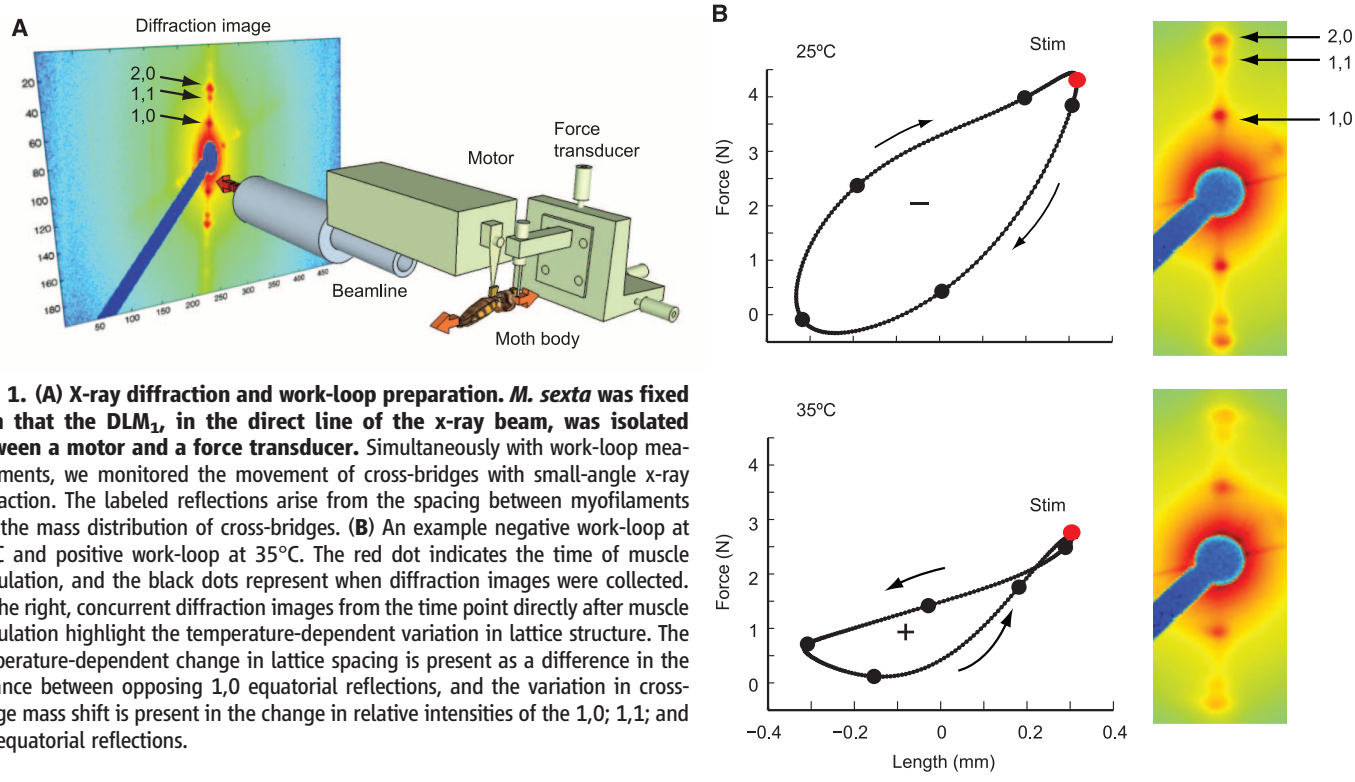

Fig. 1. (A) X-ray diffraction and work-loop preparation. *M. sexta* was fixed such that the DLM_1, in the direct line of the x-ray beam, was isolated between a motor and a force transducer. Simultaneously with work-loop measurements, we monitored the movement of cross-bridges with small-angle x-ray diffraction. The labeled reflections arise from the spacing between myofilaments and the mass distribution of cross-bridges. (**B**) An example negative work-loop at 25°C and positive work-loop at 35°C. The red dot indicates the time of muscle stimulation, and the black dots represent when diffraction images were collected. On the right, concurrent diffraction images from the time point directly after muscle stimulation highlight the temperature-dependent variation in lattice structure. The temperature-dependent change in lattice spacing is present as a difference in the distance between opposing 1,0 equatorial reflections, and the variation in cross-bridge mass shift is present in the change in relative intensities of the 1,0; 1,1; and 2,0 equatorial reflections.

extrema of the lengthening and shortening cycle, respectively.

Mean power output of the DLM_1 depends strongly on temperature. At 35°C, mean mechanical power output was 42.98 ± 1.62 W kg^{-1}. In contrast, power output at 25°C was significantly negative, with a mean of –161.20 ± 3.20 W kg^{-1} (n = 5 moths, mean ± SEM; t test, $P < 0.0001$). These values are consistent with mechanical power output measures from a prior *M. sexta* work-loop study also conducted at the in vivo phase of activation (*13*). Lattice spacing and cross-bridge cycling dynamics were also significantly temperature dependent. For a comparison of the effect of temperature on these two factors, we first highlight results from the biologically relevant condition, the ventral region of the DLM_1 at 35°C versus the dorsal region at 25°C (*11*). The relationship between temperature and myofilament lattice spacing, as indicated by d_{10}, is shown in Fig. 2A. Although there was no significant difference in lattice spacing throughout the contraction cycle for muscle at 25° or 35°C, there was a significant difference because of muscle temperature [repeated-measures analysis of variance (ANOVA): effect of time $F(4,36) = 1.3$, $P = 0.29$; effect of temperature $F(1,36) = 13.1$, $P < 0.001$]. Because there was no effect of time, we combined the results for each temperature and found that lattice spacing was lower on average in cool dorsal muscles, with myofilaments ~0.8 nm closer together than in warm ventral muscle (t test, $P < 0.01$). The reduced lattice spacing in cool muscles indicates that the longer attachment times of these cross-bridges results in a higher portion remaining bound during the cycle, thus acting as molecular linkages and restraining radial expansion and axial stretch. These results are consistent with a prior study on skeletal muscles (*21*).

The intensity ratio, an estimate of cross-bridge association with the thin filaments, was significantly affected by both temperature and time point in the contraction cycle [repeated-measures ANOVA: effect of time $F(4,32) = 3.1$, $P < 0.05$; effect of temperature $F(1,8) = 14.1$, $P < 0.01$]. The intensity ratio across the whole contraction cycle averaged 37% higher in 35°C ventral muscles than in 25°C dorsal muscles (t test, $P < 0.0001$). The higher overall intensity ratio in warm muscles may be ascribed to the elevated cross-bridge activity expected of a power-producing muscle. Figure 2B also demonstrates the cyclical change in cross-bridge mass distribution expected of warm muscles versus the more likely bound cross-bridges in cooler muscles. This is indicated by the larger absolute percent change in the intensity ratio between progressive points in the cycle in warm muscle (mean = 17%, maximum = 29%) compared with that in cool muscle (mean = 7%, maximum = 11%). Care must be taken in interpreting the relationship between lattice spacing and intensity ratio in a muscle whose length is changing and whose temperature is spatially variable. In the isovolumetric case, lattice spacing should change as the inverse square root of length changes. At same time, however, it is possible that cross-bridge binding could influence lattice spacing. Indeed, the cooler muscle data suggest that cross-bridges do restrict lattice motion.

Controlling for the effect of location, the contractile dynamics of the DLM_1 subregions were not adapted to compensate for or enhance local temperature differences. Diffraction patterns from the ventral and dorsal locations held at the same temperature showed insufficient variation to indicate physiological compensatory mechanisms that could negate the effect of a temperature gradient on regional contractile dynamics. Lattice spacing was not significantly different between dorsal and ventral muscles at 25° or 35°C (two-way ANOVA, $P = 0.63$ at 25°C and $P = 0.45$ at 35°C; Fig. 2A). Myofilament spacing was similarly more restrained in ventral muscle at cold temperatures than at warm temperatures (~0.6 nm less; paired t test, $P < 0.01$). Although there was an effect of location on the intensity ratio at 25° and 35°C (two-way ANOVA, $P < 0.001$ at 25°C and $P < 0.01$ at 35°C), the overall response, cyclic cross-bridge binding at 35°C versus stable cross-bridge activity at 25°C, was comparable between locations (Fig. 2B). Taken together, these data indicate that there is no effective regional specialization in molecular cycling dynamics.

Cyclical changes in the intensities and positions of major reflections in the DLM_1 of *M. sexta* suggest that a temperature gradient likely induces a gradient in cross-bridge cycling dynamics within a single muscle. Furthermore, the spatial variation in cross-bridge turnover rates appears to result in an energy-storing lattice of linked elastic elements within the cooler regions of muscles (fig. S1). Indeed, because the thick and thin filaments must be linked by cross-bridges to store energy in axial stretching, the creation of this stretching in the thick and thin filaments creates an equal store of energy in the stretching of the linked cross-bridges. Additionally, cross-bridges are able to store energy above and beyond that imparted by axial deformation, because their geometry requires them to undergo radial deformation alongside any axial stretching (*16*).

Temperature gradients within a single muscle inevitably result from the balance between metabolic heat production and surface heat loss. Because rates of muscle contraction are temperature dependent, this gradient has substantial implications for muscle power production and function (*11*, *13*–*15*). The DLM_1 of *M. sexta* has been generally presumed to operate solely as an actuator, producing positive power to indirectly accelerate the wings downward. However, we show that, in the presence of a substantial temperature gradient, power output varies regionally from positive values (warm sectors) to negative values (cool sectors) within this single muscle. We found that significant variation in contractile dynamics (lattice spacing and intensity ratio) are associated with this decrease in power production and may provide a mechanism by which cross-bridges contribute stored elastic energy to the overall energy needed for flight. At high temperatures, rapidly cycling cross-bridges drive filament sliding and permit large length changes, but they may not be bound at the extrema of the cycle. At the coldest temperatures, accommodating large length

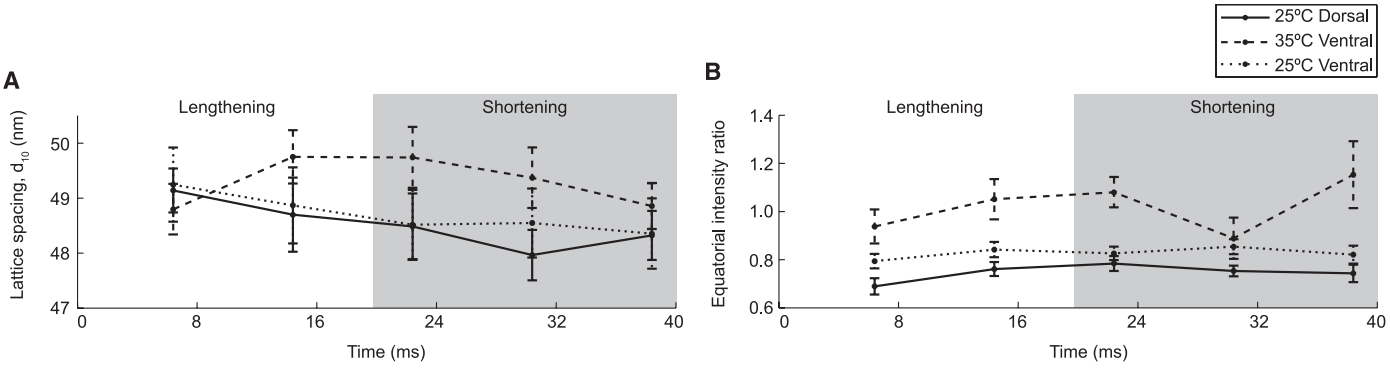

Fig. 2. Variation in lattice structure throughout the contraction cycle (mean ± SEM; n = 5 moths). (**A**) Lattice spacing, determined by d_{10}, plotted as a function of contraction cycle for dorsal muscles at 25°C and ventral muscles at 35° and 25°C. Across the five time points, mean lattice spacing was significantly lower in 25°C muscles than in 35°C muscles, regardless of location and time point in the contraction cycle. (**B**) Equatorial intensity ratio as a function of contraction cycle. Muscles at 35°C showed the expected cyclic response in intensity ratio. In contrast, muscles at 25°C showed a stable intensity ratio. The similar response of both locations at 25°C supports that the dorsal muscle's contractile dynamics are not specialized to operate at lower temperatures.

changes with bound cross-bridges requires energy to disrupt attachments, resulting in negative power production. However, intermediate temperatures permit some detachment to accommodate length changes in addition to some attachment at the extrema of the length cycle. At these intermediate temperatures, cross-bridges that remain bound at the very end of lengthening or shortening can store energy in their axial or radial extension, respectively. This stored energy may return energy into the lattice when the cross-bridges detach at the start of the subsequent phase. In doing so, the deformed cross-bridges could assist antagonistic muscles. Prior studies have shown that elastic energy storage is indeed crucial for meeting the high inertial power costs of flight (*3*, *4*). If even a portion of these cross-bridges facilitate elastic energy savings via a temperature gradient, they would contribute to the overall energy savings in locomotion. Because temperature gradients are an inevitable consequence of internal energy generation and heat dissipation in both vertebrates and invertebrates, this mechanism of energy storage could be a general phenomenon in locomotor systems (*11*, *12*).

References and Notes

1. M. Jensen, T. Weis-Fogh, *Philos. Trans. R. Soc. London Ser. B* **245**, 137 (1962).
2. R. M. Alexander, *Am. Zool.* **24**, 85 (1984).
3. R. M. Alexander, H. C. Bennet-Clark, *Nature* **265**, 114 (1977).
4. C. P. Ellington, *J. Exp. Biol.* **115**, 293 (1985).
5. H. E. Huxley, A. Stewart, H. Sosa, T. Irving, *Biophys. J.* **67**, 2411 (1994).
6. M. Dickinson *et al*., *Nature* **433**, 330 (2005).
7. J. G. Tidball, T. L. Daniel, *Am. J. Physiol.* **250**, R56 (1986).
8. I. Dobbie *et al*., *Nature* **396**, 383 (1998).
9. A. F. Huxley, R. M. Simmons, *Nature* **233**, 533 (1971).
10. K. C. Nishikawa *et al*., *Proc. R. Soc. B* **279**, 981 (2012).
11. N. T. George, T. L. Daniel, *J. Exp. Biol.* **214**, 894 (2011).
12. F. G. Carey, J. M. Teal, *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1464 (1966).
13. N. T. George, S. Sponberg, T. L. Daniel, *J. Exp. Biol.* **215**, 471 (2012).
14. R. K. Josephson, *J. Exp. Biol.* **108**, 77 (1984).
15. A. F. Bennett, *J. Exp. Biol.* **115**, 333 (1985).
16. C. D. Williams, M. Regnier, T. L. Daniel, *PLOS Comput. Biol.* **8**, e1002770 (2012).
17. A. Miller, R. T. Tregear, *Nature* **226**, 1060 (1970).
18. Information on materials and methods is available on *Science* Online.
19. M. S. Tu, T. L. Daniel, *J. Exp. Biol.* **207**, 2455 (2004).
20. T. C. Irving, in *Nature's Versatile Engine: Insect Flight Muscle Inside and Out*, J. O. Vigoreaux, Ed. (Springer, New York, 2006), pp. 197–213.
21. G. Wang, M. Kawai, *J. Physiol.* **531**, 219 (2001).

Acknowledgments: We thank H.-M. Hsu, E. Carrington, and R. Huey for their contributions. This research was supported by NSF Graduate Research Fellowship no. DGE-0718124 to N.T.G., NSF grant IOS-1022471 to T.L.D and T.C.I., NSF grant EEC 1028725 to T.L.D., and the University of Washington Komen Endowed Chair to T.L.D. Use of the Advanced Photon Source was supported by the U.S. Department of Energy under contract no. DE-AC02-06CH11357. Use of BioCAT was supported by grants from the NIH (2P41RR008630-17 and 9 P41 GM103622-17). The data reported in this paper are deposited in the Dryad Repository (http://dx.doi.org/10.5061/dryad.cg873). For inquiries about materials, please contact N.T.G. (ntgeorge@uw.edu).

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1229573/DC1
Materials and Methods
Figs. S1 and S2
References (*22*, *23*)
Movie S1

31 August 2012; accepted 28 March 2013
Published online 25 April 2013;
10.1126/science.1229573

Structural Systems Biology Evaluation of Metabolic Thermotolerance in *Escherichia coli*

Roger L. Chang,[1] Kathleen Andrews,[2] Donghyuk Kim,[2] Zhanwen Li,[3,4] Adam Godzik,[3,4] Bernhard O. Palsson[1,2,5]*

Genome-scale network reconstruction has enabled predictive modeling of metabolism for many systems. Traditionally, protein structural information has not been represented in such reconstructions. Expansion of a genome-scale model of *Escherichia coli* metabolism by including experimental and predicted protein structures enabled the analysis of protein thermostability in a network context. This analysis allowed the prediction of protein activities that limit network function at superoptimal temperatures and mechanistic interpretations of mutations found in strains adapted to heat. Predicted growth-limiting factors for thermotolerance were validated through nutrient supplementation experiments and defined metabolic sensitivities to heat stress, providing evidence that metabolic enzyme thermostability is rate-limiting at superoptimal temperatures. Inclusion of structural information expanded the content and predictive capability of genome-scale metabolic networks that enable structural systems biology of metabolism.

Cellular thermosensitivity depends on proteome stability. Chaperones and proteases are well-characterized heat shock proteins (HSPs), and chaperones improve survival at superoptimal temperatures (*1*). Protein folding and structural stability required for function are disrupted at high temperatures. Many individual proteins and their mutant variants have been studied to identify structural loci within a protein that are destabilized at high temperatures, leading to denaturation. Replacing heat-sensitive loci with more stabilizing residues has allowed engineering of thermostable proteins (*2*). By analogy, identifying the proteins that confer susceptibility to heat within the cellular system is critical to uncovering mechanisms for cellular thermosensitivity. Strategies for increasing thermotolerance have included introduction of chemical chaperones, overexpression of HSPs, pretreatment with moderate heat, or random mutagenesis to evolve stress tolerance (*3*). Instead, we sought to directly identify the particular proteins that confer thermosensitivity in the system.

The emerging discipline of structural systems biology (*4*) has enabled new insights into topics that include the structure-function relations in metabolism in a hyperthermophile (*5*), identification of causal off-target actions of drugs that cause adverse side effects (*6*), identification of protein-protein interactions (*7*, *8*), and determination of causal mutations for disease susceptibility (*8*, *9*). We used a structural systems biology approach to discover points of thermosensitivity in the mesophilic bacterium *Escherichia coli* K-12 MG1655. Metabolic thermosensitivity, affected by enzyme activity in a genome-scale model (GEM), was assessed as a function of protein thermostability, providing mechanistic explanations for effects of mutations in evolved thermotolerant strains (*10*, *11*) and leading to the discovery of metabolic limitations to thermotolerance.

To assess protein thermostability, we integrated a genome-scale model of *E. coli* metabolism (*i*JO1366) (*12*) with protein structures (GEM-PRO) by associating metabolic reactions with structures of their catalytic enzymes (database S1), thereby enabling parameterization of the network model on the basis of protein structural properties. The main objectives of this reconstruction (Fig. 1A) were to (i) maximally cover amino acid sequence (Fig. 1B), (ii) represent the native structure of each wild-type (WT) protein (Fig. 1C), (iii) map existing amino acid functional annotations to structures (*13*–*16*) (Fig. 1C), and (iv) represent changes in functional conformation or induced fit caused by protein-substrate

[1]Bioinformatics and Systems Biology Graduate Program, University of California San Diego, La Jolla, CA 92093–0412, USA. [2]Department of Bioengineering, University of California San Diego, La Jolla, CA 92093–0412, USA. [3]Bioinformatics and Systems Biology Program, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA. [4]Joint Center for Structural Genomics (JCSG), La Jolla, CA 92037, USA. [5]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2970 Hørsholm, Denmark.

*Corresponding author. E-mail: palsson@ucsd.edu