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Clark J. Cotton
College of Saint Benedict/Saint John's University, ccotton@csbsju.edu

Henry J. Harlow

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Title

Do hypothermic tissue tolerances limit torpor expression?

Clark J. Cotton[a, b] and Henry J. Harlow[a]

[a] Zoology and Physiology, University of Wyoming, 1000 E. University Ave., Department 3166, Laramie, WY 82070

[b]Corresponding author, ccotton@uwyo.edu, Tel (307)-766-3380, Fax (307)-766-5625

Abstract

1. Arrest temperatures and Q_{10} values for extensor digitorum longus (EDL), soleus, trabecula, and jejunum muscle twitch strength, contraction time, and 0.5 relaxation time were calculated for a deep torpor hibernator, white-tailed prairie dog (*Cynomys leucurus*), a shallow torpor hibernator, black-tailed prairie dog (*Cynomys ludovicianus*), and a non-hibernator, lab rat (*Rattus norvegicus*) to test the hypothesis that tissue temperature tolerances limit the depth of expressed torpor.

2. There were no temperature tolerance differences between the tissues of the two species of hibernators. Both hibernating species had arrest temperatures and Q_{10} values more indicative of cold temperature tolerance than the lab rat in all tissues, with the exception of the soleus muscle.

3. These data imply that a limited cold tolerance of contractile tissue does not preclude a shallow torpor hibernator such as the black-tailed prairie dog from expressing deep torpor patterns. Other mechanisms, such as central neural control, are more likely to be important in determining the torpor strategy utilized by hibernating species.

Keywords: hibernation, contractile performance, thermal biology, skeletal muscle, smooth muscle, cardiac muscle.

1. Introduction

Hibernation allows some small mammals to survive prolonged periods of cold and food scarcity through a marked reduction in metabolic rate with concomitant energy conservation (Nedergaard and Cannon, 1990). Although 7 of the 25 mammalian orders have species that hibernate (Geiser and Ruf, 1995), not all hibernators utilize the same strategies or perhaps do not express genes associated with hibernation to the same extent. Harlow (1995) summarized two different types of torpor strategy in small mammals. One group of hibernators typically express photoperiod driven, circannual onset of winter torpor bouts that are characteristically of regular, long duration and with a low body temperature. For the purposes of this paper, we will refer to these animals as deep torpor hibernators. A second group of hibernators enter sporadic, short duration, mildly hypothermic torpor bouts that can be initiated any time during the winter in association with acute changes in ambient temperature and / or lack of food and shall be referred to as shallow torpor hibernators.

Two species that provide a useful model to study these different hibernation strategies are the white-tailed prairie dog (*Cynomys leucurus*) and the black-tailed prairie dog (*C. ludovicianus*). Both of these prairie dog species have evolved from a spermophiline ancestor likely resembling the Gunnison's prairie dog (*C. gunnisonii*), which is a deep hibernator (Pizzimenti, 1975; Rayor et al., 1987). While the white-tailed prairie dog (WTPD) appears to have retained the ancestral expression of deep hibernation as populations expanded into the great basin of North America (Pizzimenti, 1975; Harlow and Menkens, 1986), the black-tailed prairie dog (BTPD) became more of a shallow hibernator that exhibited reduced expression of torpor (Pizzimenti, 1975; Harlow and

Menkens, 1986; Lehmer et al., 2003) after populations spread into the Great Plains. WTPDs routinely exhibit rhythmic hypothermic torpor bouts with an average body temperature of 7°C for periods of 5-6 days during their hibernation season (Bakko and Nahorniak, 1986; Harlow and Menkens, 1986). In contrast, BTPDs generally lower their body temperature to 32°C – 27°C with torpor bouts lasting 1 – 2 days and occurring much less frequently than WTPDs (Harlow and Menkens, 1986; Lehmer et al., 2001). However, it has been reported that BTPDs can, on rare occasions, undergo regular bouts of torpor with body temperatures approaching 10°C (Lehmer et al., 2003; Lehmer et al., 2006). Harlow and Menkens (1986) showed in laboratory studies with *ad libitum* food and water, total darkness, and 4°C ambient temperature that WTPDs will initiate these deep torpor bouts in early October, while BTPDs engage in their more shallow and sporadic torpor only when completely deprived of food and water.

Several studies have been undertaken to determine if some basic physiological differences exist that explain why the BTPD defends a torpor state with a higher T_b and shorter duration than the closely related WTPD. For example, total body fat content (Harlow, 1997), brown adipose tissue response (Harlow, 1997), polyunsaturated fatty acid profiles (Harlow and Frank, 2001), and renal function (Harlow and Braun, 1995) were not different between the two species. As an alternative, tissue temperature tolerance may influence the expression of deep torpor by these two species. Past studies have consistently shown that hibernators tend to have cardiac tissue (Lyman and Blinks, 1959; Lyman, 1964; Jacobs and South, 1973; Burlington and Darvish, 1988), skeletal muscle (South, 1961; Nelson et al., 1977), and smooth muscle (Kamm et al., 1979; Carey, 1990; Wolowyk et al., 1990; Carey, 1992) that are better adapted to survive and

perform at the low temperatures associated with hibernation than tissue from non-hibernators. The objective of this study was to investigate the performance of these three muscle types: cardiac (trabecula), skeletal (soleus and extensor digitorum longus), and smooth muscle (jejunum) at low temperatures by a representative non-hibernator (lab rat); deep torpor hibernator (WTPD); and shallow torpor hibernator (BTPD).

We hypothesize that there is a gradient of low temperature tolerance and performance of these three muscle types between deep torpor hibernators, shallow torpor hibernators, and non-hibernators. If no such response gradient is observed, tissue tolerance to very cold temperatures may not be a genetically controlled determinant of hibernation ability and it may simply be a result of phenotypic plasticity dictated by other unidentified variables. To answer this question, we measured the arrest temperatures in addition to temperature effects on contraction strength and muscle relaxation time by these three muscle tissues taken from the WTPD, BTPD, and laboratory rat.

2. Methods

Six rats (3 male, 3 female; mean weight = 373.86g, Charles River Albino, 60 days old), six WTPDs (4 male, 2 female; mean weight = 1021.83g), and eight BTPDs (5 males, 3 females; mean weight 709.31g) were collected for the study. All experimental protocols were approved by the University of Wyoming IACUC committee.

In Vitro Set-up

Each animal was anesthetized with ketamine hydrochloride at a dosage of 190mg/Kg body mass in late April. After deep anesthesia was obtained, the right and left EDL, soleus, jejunum, and heart trabecula were removed from each animal followed by immediate euthanasia with an overdose of pentabarbitol, Beuthanasia®-D Special

(Kreeger, 1996). The tissue samples were connected to force transducers and placed into Krebs buffer (NaCl 118.1mM, KCl 3.4mM, KH_2PO_4 1.2mM, $MgSO_4 7H_2O$ 1.0mM, Dextrose 10.8mM, $NaHCO_3$ 25.0mM, and $CaCl_2$ 2.5mM) aerated with a mixture of oxygen and carbon dioxide maintained at a pH of 7.4 at 25°C for the EDL and soleus and 37°C for the jejunum and heart trabecula.

EDL and soleus tissue preparations were stretched to obtain optimal twitch tension and stimulated every 30 seconds with a 2ms 9V stimulus provided by a CB Sciences CK-100 stimulator. The jejunum was also stretched to obtain optimal contractions, but did not receive external stimulation. The heart trabecula was stretched to obtain optimal twitch tension and stimulated at 0.25Hz using a 10ms 50V stimulus. Using a Haake D1 and Fisher Isotemp 1000 circulating water baths, buffer temperatures were gradually lowered to -2°C for the EDL and soleus and 0°C for the jejunum and heart trabecula. Both tissue arrest temperatures and Q_{10} data were utilized as indices of temperature sensitivity. Tissue arrest temperatures were recorded when contraction strength dropped below background noise (typically 25mg). Temperature quotient (Q_{10}) values for contraction strength (CS), contraction time (CT), and 0.5 relaxation time (0.5RT) were recorded for each tissue using the equation $Q_{10} = (R_2 / R_1)^{\wedge 10 / (t2 - t1)}$ where R_2 is the rate or measurement at temperature t_2 and R_1 is the rate or measurement at temperature t_1. Using these criteria, Q_{10} values that deviate from 1.0 indicate tissue that is temperature sensitive, while Q_{10} values that approximate 1.0 indicate tissues that are temperature insensitive for the given range of temperatures. Q_{10} values were calculated for the largest temperature range over which all animals could still elicit a viable tissue response. Temperature ranges of 25°C to 10°C for skeletal muscle, 37°C to 20°C for

jejunum, and 37°C to 10°C for trabecula were used to compare tissue sensitivity to temperature by these three species. No differences between male and female animals were detected for any of the species, as a result, all male and female data were grouped together.

Statistical Analysis

Changes in contraction time and 0.5 relaxation time were evaluated using Mann-Whitney rank sum test, while contraction strength for each species was evaluated using a t-test. Arrest temperatures and Q_{10} values were compared using a one-way ANOVA and Tukey post-hoc tests for significant interactions. All statistical analysis was performed using Sigma Stat 3.1 (Systat Software Inc., Point Richmond, CA, USA) with significance accepted at $p < 0.05$.

3. Results

Arrest Temperatures

The arrest temperatures for the isolated heart trabecula muscle were significantly lower for both prairie dog species compared to the rat (WTPD $q = 5.597$, $p = 0.003$; BTPD $q = 4.239$, $p = 0.021$) but there were no differences between the two prairie dogs (Figure 1). The jejunum arrest temperatures were also significantly lower for both the WTPD and BTPD compared to the rat but not between each other (WTPD, $q = 4.845$, $p = 0.009$; BTPD $q = 4.594$, $p = 0.013$; Figure 1). The EDL arrest temperatures for both the WTPDs ($q = 5.067$, $p = 0.006$) and BTPDs ($q = 9.268$, $p < 0.001$, Figure 1) were significantly lower than that of the rat. The EDL arrest temperature was also lower for the BTPDs than the WTPDs ($q = 3.851$, $p = 0.037$, Figure 1). The arrest temperatures for soleus muscles did not differ between the three species (Figure 1).

Q_{10} Values

Contraction and 0.5 relaxation times increased for all tissues as temperature decreased (Table 1). There were no differences in EDL or soleus contraction time Q_{10} values between species. However, EDL Q_{10} values for 0.5 RT were higher in WTPD, but not BTPD, than rats (WTPD $q = 5.052$, $p = 0.006$; BTPD $q = 3.577$, $p = 0.054$; Table 2), as were the soleus 0.5 RT Q_{10} values for both prairie dog species compared to the rat (WTPD $q = 10.543$, $p < 0.001$; BTPD $q = 7.777$, $p < 0.001$, Table 2). There were no differences between species in trabecula and jejunum Q_{10} values for CT and 0.5RT. There were also no differences in Q_{10} values between prairie dog species for contraction or 0.5 relaxation times.

Strength tended to decrease with temperature for all tissues, the exception being prairie dog trabecula muscle, which increased in contraction strength with decreasing temperature (Figure 2). Both prairie dog species had trabecula contraction strength Q_{10} values that were lower than the rat and less than one, indicating a more robust contraction with low temperatures (WTPD $q = 5.508$, $p = 0.003$; BTPD $q = 5.226$, $p = 0.005$; Table 2). The Q_{10} value for EDL strength of both prairie dogs was less than the rat (WTPD $q = 3.611$, $p = 0.005$; BTPD $q = 3.943$, $p = 0.032$; Table 2). However, contraction strength Q_{10} values for the soleus were higher for both species of prairie dogs than the rat (WTPD, $q = 3.025$, $p < 0.05$; BTPD $q = 2.791$, $p < 0.05$). There were no differences in jejunum contraction strength Q_{10} values between the species. There were no differences in the Q_{10} values for strength between prairie dog species for any of three muscle tissues investigated.

4. Discussion

There is much interest in identifying which factors may limit an animal's ability to hibernate and what determines whether an animal expresses deep or shallow torpor. However, no studies have examined if there are differential temperature tolerances for muscles which control blood flow, locomotion, and digestion by closely related mammalian species expressing different depths of torpor. The present investigation compares the functional capacity of cardiac, skeletal, and smooth muscle at cold tissue temperatures in a non-hibernator, shallow torpor, and deep torpor hibernator.

Cardiac Muscle

The ability of the hibernator's cardiovascular system to function at the low temperatures associated with hibernation is a predominant factor limiting non-hibernators from entering deep torpor. The arrest temperature for the rat heart trabecula muscle was considerably higher (6.60^{o}C) than that of both species of prairie dog. However, contrary to our prediction, the deep and shallow torpor prairie dogs had almost identical arrest temperatures (WTPD = 1.58^{o}C and BTPD = 2.76^{o}C) similar to those reported for other deep torpor hibernators such as ground squirrels (Smith and Katzung, 1966; Burlington and Darvish, 1988) and hamsters (South and Jacobs, 1973).

A most remarkable observation in this study was that while the rat trabecula had a 40% reduction in contraction strength between 37^{o}C and 10^{o}C with a Q_{10} greater than 1.0, both species of prairie dog had a Q_{10} less than 1.0 and exhibited strengths at 10^{o}C that were nearly 150% of euthermic values. The increased strength of the heart at these temperatures may help counteract the effects of increased peripheral vasoconstriction and blood viscosity encountered by hibernators at low temperatures (Zatzman, 1984; Zatzman and Thornhill, 1987), particularly during arousal from deep torpor. Enhanced cardiac

performance by the hearts of hibernators at low temperatures is likely a result of increased action potential length (Marshall and Willis, 1962; Jacobs and South, 1973), heightened intracellular ion regulation (Burlington and Darvish, 1988; Wang et al., 2002), and myofilament sensitivity to calcium at low temperatures (Liu et al., 1993), as well as novel expression of genes regulating cardiac metabolism (Andrews et al., 1998). In combination, these aforementioned adaptations could account for the elevated strength of contraction observed for the trabecula muscle from both species of prairie dogs tested at a cold tissue temperature compared to a 40% cold induced drop in performance by rat hearts.

Skeletal Muscle

Arousing from deep torpor hibernation requires a significant amount of heat generation in the form of shivering and non-shivering thermogenesis. Non-shivering thermogenesis primarily takes place in brown adipose tissue (Hashimoto et al., 2002; Cannon and Nedergaard, 2004), although uncoupling proteins are found in other tissues, such as skeletal muscle (Boyer et al., 1998; Raimbault et al., 2001), and appears to be especially important during the early stages of arousal from torpor (Fons et al., 1997; Ho et al., 2001). However, arousal can take place in the absence of functional BAT in both placental and marsupial hibernators (Lyman and O'Brien, 1986; Geiser and Baudinette, 1990). Since skeletal muscle makes up 30-40% of total body mass (Kim et al., 2002), heat generated from this tissue due to uncoupling proteins and / or shivering muscle contractions could offer a significant contribution to elevating the body temperature from a state of torpor during arousal from deep torpor. In addition to thermogenesis, skeletal muscle from deep torpor hibernators may also have adaptations that help maintain ion

gradients at low temperatures, such as a decreased K^+ leak and increased Na^+ / K^+ pump activity, thereby preventing excessive K^+ loss to the blood (Willis and Li, 1969; Willis et al., 1971; Willis et al., 1980). Once again, the large size of skeletal muscle makes these adaptations particularly significant. Clearly skeletal muscle in these animals must continue to function without impairment at the low temperatures encountered during deep torpor hibernation.

The present study reports lower arrest temperatures and Q_{10} values for strength in both species of prairie dog EDL than in the lab rat EDL, indicating a greater cold tolerance for both species of prairie dog. Overall, the soleus had higher Q_{10} values for strength and higher arrest temperatures than the EDL for all species. This agrees with other studies demonstrating increased temperature sensitivity of predominantly slow oxidative fibers (Johnston and Gleeson, 1984; Ranatunga, 1984; Bottinelli et al., 1996). The soleus was unique in our present study in that arrest temperatures were almost identical for all three species but the Q_{10} values for contraction strength by prairie dogs suggested greater temperature sensitivity for this slow oxidative muscle in prairie dogs. Our results imply that the fast twitch muscles (EDL) are more capable of functioning at low temperatures, even below freezing (Figure 1C), which provide the deep hibernator the capacity to function and arouse from a hypothermic state that renders other muscle tissues inoperable. These observations taken together do not support the hypothesis that a shallow hibernator has less cold tolerant skeletal muscle than a deep hibernator. However, they do suggest that for primarily fast twitch muscle, hibernators have skeletal muscle that is more cold tolerant than non-hibernators.

Smooth Muscle

Data from this study shows, as hypothesized, that both species of hibernating prairie dogs have lower arrest temperatures for jejunum segments than the rat. Indeed, it has been reported in other studies that the GI tract of hibernators maintain functional enzyme activity (Galluser et al., 1988; Carey and Martin, 1996), epithelial transport (Carey, 1990; Carey, 1992), and perhaps even increase digestive efficiency during torpor (Humphries et al., 2001). We hypothesized that the ability to maintain gut function at relatively low temperatures would be greater for WTPD which enters deeper torpor compared to the BTPD which consumes food throughout the hibernation season but does not enter deep hypothermia. However, we found that the BTPD had similar jejunum arrest temperatures and contractile properties as the deep torpor and anorexic WTPD. It could be that gut function is necessary for both species either in deep or shallow torpor to process ingested food, utilize endogenous proteins that are sloughed from the GI tract of these animals (Carey, 1995), and recycle urea nitrogen during torpor and fasting (Nelson, 1973; Riedesel and Steffen, 1980; Harlow, 1987). We believe that, as a result, there is no distinct difference in intestinal smooth muscle function at low temperatures that discriminate between deep and shallow torpor prairie dogs.

Summary

In both species of prairie dog, heart and EDL have the greatest cold temperature tolerance. Cardiac tissue from both prairie dog species appears to be uniquely adapted to cold temperatures with peak contraction strength occurring at temperatures well below that of the non-hibernator which may help to maintain tissue perfusion in the face of peripheral vasoconstriction and high blood viscosity associated with torpor. Unlike the rat, the anaerobic, fast twitch EDL muscle from both species of hibernators can function

below freezing and may act as an emergency heat source to augment BAT nonshivering thermogenesis and prevent the body from falling into the lethal cold range. Results from this study indicate that while muscle tissues of these two hibernators are superior to non-hibernators in many aspects of cold resistance, there does not appear to be any distinctive differences between species utilizing deep or shallow torpor strategies. We believe that while black-tailed prairie dogs have evolved away from a rigid expression of torpor, they have organs which can maintain a functional capacity to operate in deep hibernation, suggesting that the loss of rhythmic bouts of deep hypothermia in their natural history is merely a decrease in the phenotypic expression of this trait and not a loss of its genetic capacity.

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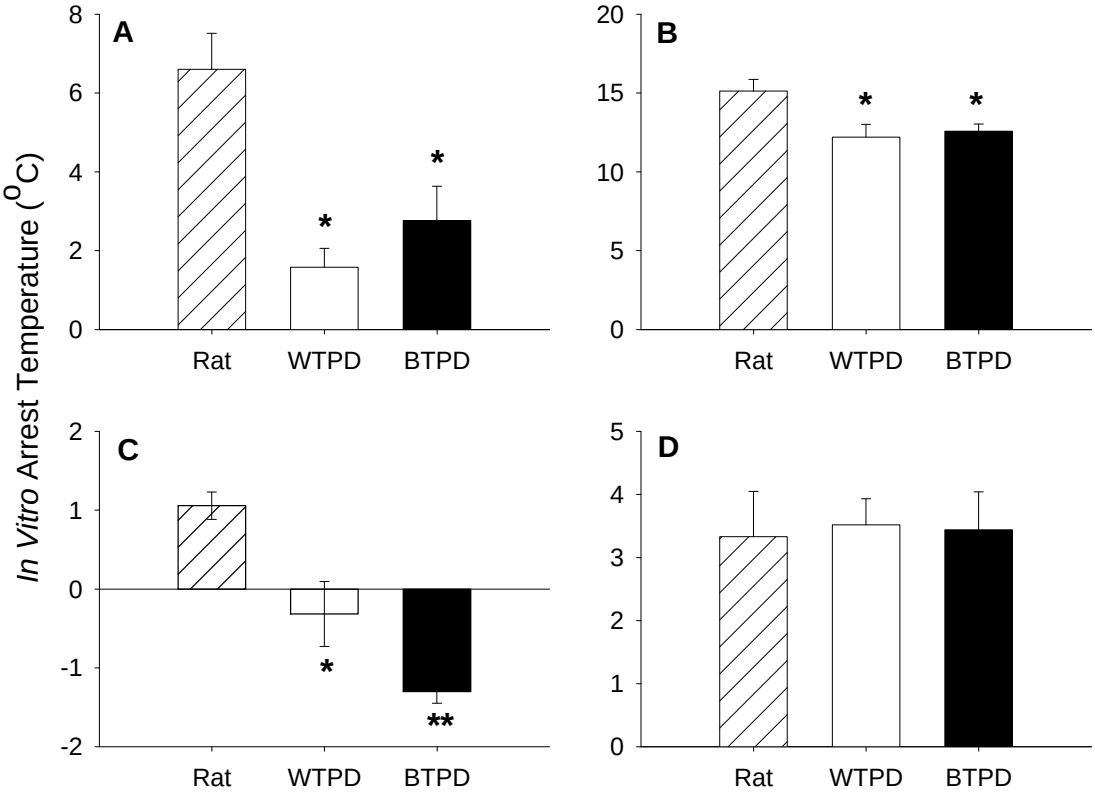

Figure 1.

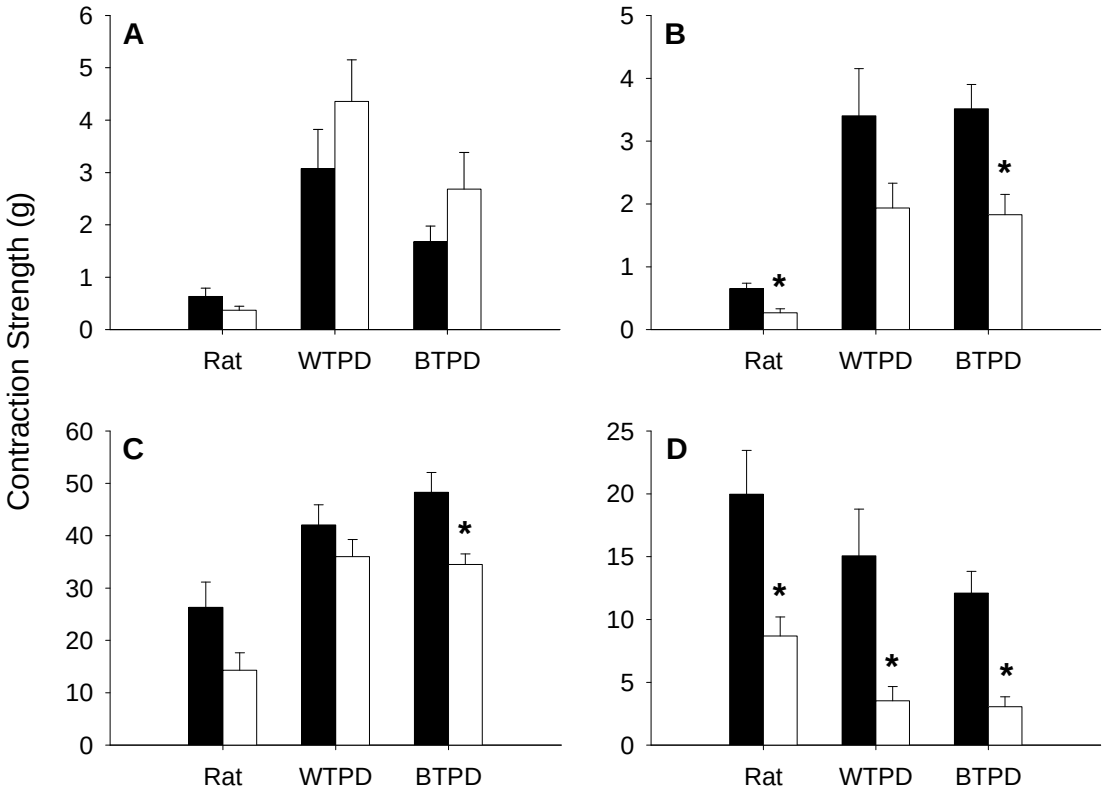

Figure 2.

Figure Captions

Figure 1. Comparisons of A) trabecula, B) jejunum, C) EDL, and D) soleus arrest temperatures for rat (n = 6), WTPD (n = 6), and BTPD (n = 8). Single asterisk depicts a significant difference in arrest temperature ($p < 0.05$) from rat. Double asterisk depicts a significant difference in arrest temperature ($p < 0.05$) from rat and between prairie dog species. Vertical bars depict ± SEM.

Figure 2. Comparison of (A) trabecula, (B) jejunum, (C) EDL, and (D) soleus contraction strengths for rat (n = 6), WTPD (n = 6), and BTPD (n = 8). Black bars indicate an *in vitro* temperature of 25°C for EDL and soleus, and an *in vitro* temperature of 37°C for trabecula and jejunum. White bars indicate an *in vitro* temperature of 10°C for trabecula, EDL, and soleus and 20°C for jejunum. Single asterisk depicts a significant difference in contraction strength ($p < 0.05$) between the temperature groups. Vertical bars depict ± SEM.

Table 1. *Comparison of contraction time (CT) and half relaxation time (0.5RT) for skeletal muscle, cardiac muscle, and smooth muscle in WTPDs, BTPDs, and Lab Rats*

	Rat	WTPD	BTPD
EDL			
CT (sec)			
25ºC	0.033 ± 0.001	0.041 ± 0.003	0.046 ± 0.002
10ºC	*0.181 ± 0.008	*0.220 ± 0.008	*0.224 ± 0.008
0.5RT (sec)			
25ºC	0.021 ± 0.001	0.022 ± 0.002	0.024 ± 0.001
10ºC	*0.175 ± 0.010	*0.138 ± 0.008	*0.163 ± 0.007
Soleus			
CT (sec)			
25ºC	0.071 ± 0.003	0.129 ± 0.004	0.124 ± 0.004
10ºC	*0.506 ± 0.047	*0.848 ± 0.045	*0.841 ± 0.065
0.5RT (sec)			
25ºC	0.077 ± 0.004	0.068 ± 0.001	0.073 ± 0.004
10ºC	*1.887 ± 0.141	*0.824 ± 0.035	*0.784 ± 0.067
Trabecula			
CT (sec)			
37ºC	0.081 ± 0.004	0.120 ± 0.008	0.126 ± 0.005
10ºC	*0.716 ± 0.037	*0.907 ± 0.059	*1.052 ± 0.054
0.5RT (sec)			
37ºC	0.074 ± 0.018	0.073 ± 0.006	0.078 ± 0.003
10ºC	*0.576 ± 0.030	*0.551 ± 0.022	*0.550 ± 0.019
Jejunum			
CT (sec)			
37ºC	0.828 ± 0.043	1.655 ± 0.086	1.524 ± 0.050
20ºC	*3.538 ± 0.465	*8.273 ± 0.348	*6.871 ± 0.450
0.5RT (sec)			
37ºC	0.416 ± 0.027	0.933 ± 0.040	0.895 ± 0.042
20ºC	*2.274 ± 0.324	*5.101 ± 0.371	*4.486 ± 0.273

Table 2. *Comparison of Q_{10} values for skeletal muscle, cardiac muscle, and smooth muscle twitch strength, contraction time (CT), and half relaxation time (0.5RT) in WTPDs, BTPDs, and Lab Rats*

	Rat	WTPD	BTPD
EDL[1]			
Strength	1.589 ± 0.157	*1.110 ± 0.012	*1.250 ± 0.046
CT	0.323 ± 0.007	.324 ± 0.013	0.351 ± 0.012
0.5RT	0.245 ± 0.007	*0.293 ± 0.010	0.277 ± 0.009
Soleus[1]			
Strength	1.773 ± 0.113	*3.023 ± 0.291	*3.035 ± 0.386
CT	0.276 ± 0.017	0.286 ± 0.007	0.284 ± 0.010
0.5RT	0.120 ± 0.006	*0.190 ± 0.006	*0.209 ± 0.010
Trabecula[2]			
Strength	1.211 ± 0.086	*0.855 ± 0.033	*0.895 ± 0.055
CT	0.446 ± 0.009	0.473 ± 0.011	0.457 ± 0.010
0.5RT	0.458 ± 0.040	0.472 ± 0.013	0.484 ± 0.007
Jejunum[3]			
Strength	1.808 ± 0.195	1.439 ± 0.110	1.637 ± 0.235
CT	0.440 ± 0.034	0.388 ± 0.007	0.417 ± 0.015
0.5RT	0.390 ± 0.039	0.371 ± 0.012	0.390 ± 0.011

[1]EDL and soleus Q_{10} values were calculated over a temperature range of 25°C to 10°C.
[2]Trabecula Q_{10} values were calculated over a temperature range of 37°C to 10°C.
[3]Jejunum Q_{10} values were calculated over a temperature range of 37°C to 20°C.